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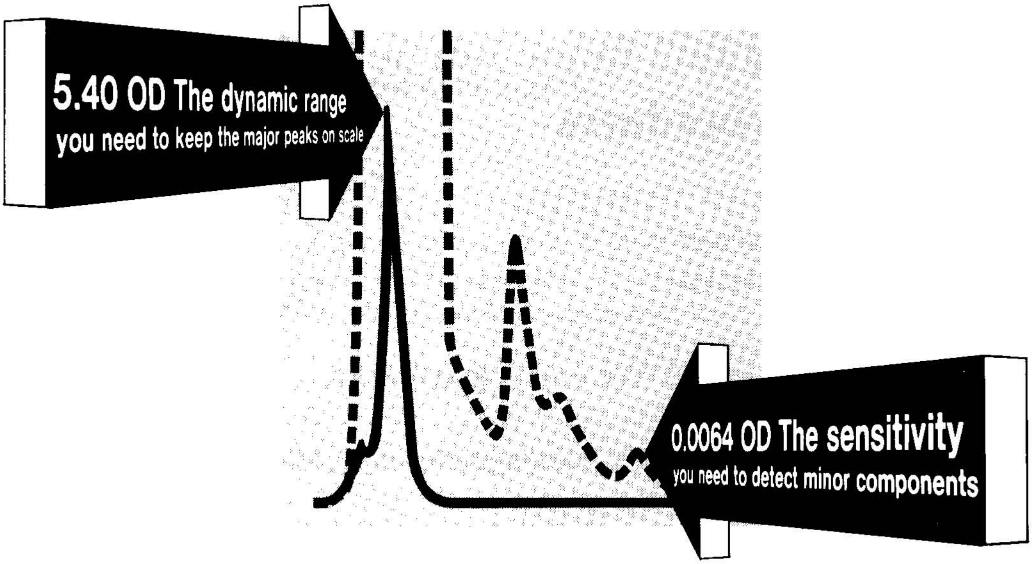
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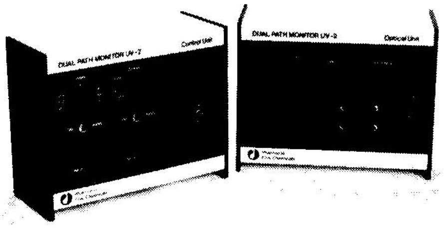
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ISOTACHOPHORESIS: THE CONCEPTS OF RESOLUTION, LOAD CAPACITY AND SEPARATION EFFICIENCY

I. THEORY

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Department of Instrumental Analysis, Eindhoven University of Technology, Eindhoven (The Netherlands)
(First received February 14th, 1978; revised manuscript received June 15th, 1978)

SUMMARY

The fundamental definitions of resolution and separability in isotachophoresis are given and extensively discussed. The resolution of a constituent is given as its fractional separated amount and can vary between zero and unity. The steady-state configuration is characterized by resolution values of unity and/or zero and is determined by both the leading electrolyte and the sample. The separability of two constituents depends largely on their physico-chemical characteristics and the time allowed for resolution.

The isotachophoretic separation process is elucidated using a transient-state model for monovalent, weakly ionic constituents. The influence of operational parameters, *i.e.*, pH, electrical driving current, sample load and counter constituent, on the separation process is described in terms of resolution time, detection time and load capacity. The efficiency of the separation process is given by the dimensionless separation number.

It is shown that optimization procedures are governed by three rationales: the electrical driving current, the common counter constituent and the pH. Of these, only the electrical driving current has no influence on the separation efficiency and load capacity. For anionic separations a low pH of both sample and leading electrolyte favours resolution. When dealing with cationic separations a high pH is preferable. The counter constituent should have a low mobility and the electrical driving current should be maximized.

INTRODUCTION

In isotachophoresis a steady-state configuration is obtained as the result of a separation process that proceeds according to the moving boundary principle[1,2]. Although this separation process is a transient state, it is governed by the same regulating function concept as the steady state[2]. A quantitative and qualitative descrip-

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tion of the transient state provides information on the time needed for an isotachophoretic separation[3]. Moreover, such a description requires the definition of resolution and separability in isotachophoresis and shows the results that can be expected from optimization procedures.

In this paper we present a relatively simple model, dealing with the theoretical concepts of the separation process, resolution, separability and separation efficiency. The practical implications concerning resolution time, detection time and load capacity are deduced. Optimization procedures by means of operational conditions and electrolyte systems are given. In a forthcoming paper the practical evaluation will be presented.

GENERAL EQUATIONS AND DEFINITIONS

In electrophoresis the migration velocity, v, of a constituent i is given by the product of effective mobility $\bar{m}_i$ and the local electrical field strength, E:

$$v_i = \bar{m}_i E \tag{1}$$

The electrical field strength is vectorial so the effective mobilities can be taken as signed quantities, positive for constituents that migrate in a cathodic direction and negative for those migrating anodically. As a constituent may consist of several forms of sub-species in rapid equilibrium, the effective mobility represents an average ensemble. Not dealing with constituents consisting of both positively and negatively charged subspecies in equilibrium, we can take concentrations with a sign corresponding to the charge of the sub-species. Thus the total constituent concentration, $\bar{c}_i$, is given by the summation of all of the sub-species concentrations, c_n:

$$\bar{c}_i = \sum_n c_n \tag{2}$$

Following the mobility concept of Tiselius[4], the effective mobility is given by

$$\bar{m}_i = \sum_n \frac{c_n m_n}{\bar{c}_i} \tag{3}$$

where m_n is the ionic mobility of the sub-species. In dissociation equilibria the effective mobility can be evaluated using the degree of dissociation, α:

$$\bar{m}_i = \sum_n \alpha_n m_n \tag{4}$$

The degree of dissociation can be calculated once the equilibrium constant, K, for the sub-species and the pH of the solution are known. For a restricted pH range a very useful relationship has been given by Hasselbalch[5]:

$$\text{pH} = \text{p}K \pm \log\left(\frac{1}{\alpha} - 1\right) \tag{5}$$

where pK is the negative logarithm of the protolysis constant; the positive sign holds for cationic sub-species and the negative sign for anionic sub-species.

All electrophoretic processes are essentially charge-transport processes that obey Ohm's law. In electrophoresis this law is most conveniently expressed in terms of electrical current density, J, specific conductance, κ, and electrical field strength:

$$J = \kappa E \tag{6}$$

The specific conductance is given by the individual constituent contributions:

$$\kappa = F \sum_i \bar{c}_i \bar{m}_i \tag{7}$$

where F is the the Faraday constant.

The equation of continuity states for any electrophoretic process[6] that

$$\frac{\partial}{\partial t} \cdot \bar{c}_i = - \frac{\partial}{\partial x} \left(\frac{\partial}{\partial x} D_i \bar{c}_i - v_i \bar{c}_i \right) \tag{8}$$

where t and x are time and place coordinates, respectively, and D is the diffusion coefficient. Neglecting diffusional dispersion we can apply eqn. 8 for each constituent and the overall summation of the constituents gives

$$\frac{\partial}{\partial t} \sum_i \bar{c}_i = \frac{\partial}{\partial x} \cdot E \sum_i \bar{m}_i \bar{c}_i \tag{9}$$

In combination with the specific conductance (eqn. 7) and the modified Ohm's law (eqn. 6), it follows that

$$\frac{\partial}{\partial t} \sum_i \bar{c}_i = 0 \quad \text{or} \quad \sum_i \bar{c}_i = \text{constant} \tag{10}$$

For monovalent weakly ionic constituents, eqn. 8 can be written as

$$\frac{\partial}{\partial t} \cdot \bar{c}_i = \frac{\partial}{\partial x} \cdot E m_i c_i \tag{11}$$

where m_i and c_i are the mobility and the concentration of the charged species i. Division by m_i and application of the resulting relationship for each constituent and overall summation gives

$$\frac{\partial}{\partial t} \sum_i \frac{\bar{c}_i}{m_i} = \frac{\partial}{\partial x} \cdot E \sum_i c_i \tag{12}$$

Electroneutrality, however, demands $\sum_i c_i = 0$, so

$$\frac{\partial}{\partial t} \sum_i \frac{\bar{c}_i}{m_i} = 0 \quad \text{or} \quad \sum_i \frac{\bar{c}_i}{m_i} = \text{constant} \tag{13}$$

Eqn. 13 is well known as the Kohlrausch regulating function[7].

In an electrophoretic system different zones can be present, in which a zone is defined[8] as a homogeneous solution demarcated by moving and/or stationary boundaries. We can apply the continuity principle (eqn. 8) to a boundary (Fig. 1) and derive the general form of the moving boundary equation[9]:

$$\bar{m}_i^K \bar{c}_i^K E^K - \bar{m}_i^{K+1} \bar{c}_i^{K+1} E^{K+1} = \nu^{K/K+1} \left(\bar{c}_i^K - \bar{c}_i^{K+1} \right) \tag{14}$$

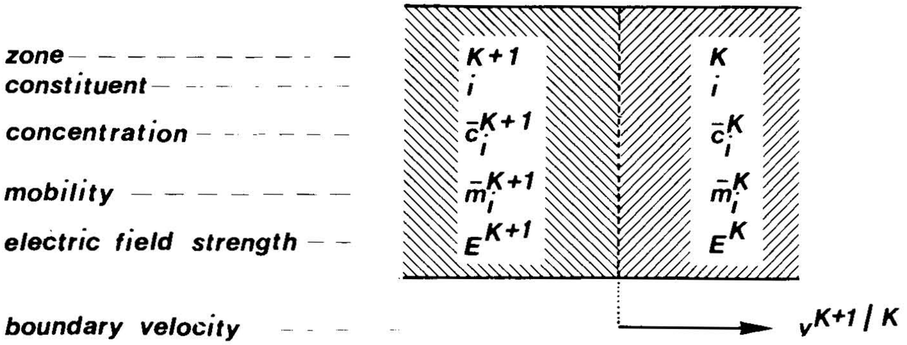

Fig. 1. A moving boundary.

where $v^{K/K+1}$ represents the drift velocity of the separating boundary between the zones K and $K + 1$. In the case of a stationary boundary, the boundary velocity is zero and eqn. 14 reduces to

$$\frac{\bar{m}_i^{K+1}\bar{c}_i^{K+1}}{\bar{m}_i^{K}\bar{c}_i^{K}} = \frac{E^K}{E^{K+1}} = \text{constant} \tag{15}$$

From eqn. 15 it follows directly that for monovalent weak and strong electrolytes all ionic subspecies are diluted or concentrated over a stationary boundary to the same extent, because

$$\frac{c_i^{K+1}}{c_i^K} = \text{constant} \tag{16}$$

In isotachophoresis sample constituents migrate in a stacked configuration, steady state, between a leading ionic constituent of high effective mobility and a terminating constituent of low effective mobility. From the moving boundary equation (eqn. 9) it follows directly that, in a separation compartment of uniform dimensions at constant electrical driving current, all boundary velocities within the isotachophoretic framework are equal and constant. Accordingly to Joule's law, heat generation will occur, resulting in different temperature regimes that are moving or stationary. In order to reduce the effects of temperature, relative mobilities, r, can be introduced. Obviously the leading constituent, L, provides the best reference mobility:

$$r_i = \frac{m_i}{m_L} \tag{17}$$

Moreover, as in most isotachophoretic separations, only one counter constituent, C, will be present, the reduced mobility, k, can be introduced:

$$k_i = \frac{1 - r_C}{r_i - r_C} \tag{18}$$

Using the derived equations it is possible to calculate all dynamic parameters of analytical importance. Moreover, model considerations can be extended to moving boundary electrophoresis as well as to zone electrophoresis.

The criterion for separation

As in all differential migration methods, the criterion for separation in isotachophoresis depends simply on the fact that two ionogenic constituents will separate whenever their migration rates in the mixed state are different. For two constituents i and j, this means that according to eqn. 1 their effective mobilities in the mixed state must be different:

$$\frac{\bar{m}_i}{\bar{m}_j} \neq 1 \tag{19}$$

When the effective mobility of i is higher than that of j the latter constituent will migrate behind the former. Consequently, two monovalent weakly anionic constituents will fail to separate when the pH of the mixed state, pH^{MO}, is given by

$$\mathrm{pH}^{M} = \mathrm{pH}^{MO} = \mathrm{pK}_j + \log \left(\frac{1 - \dfrac{r_j K_j}{r_i K_i}}{\dfrac{r_j}{r_i} - 1} \right) \tag{20}$$

where K_i and K_j are the protolysis constants for the sub-species of the constituents i and j. When the more mobile constituent has a higher protolysis constant, we are dealing with a "straight" pair of constituents; when the more mobile constituent has a lower protolysis constant, we have a "reversed" pair of constituents, for which the separation configuration is a function of the pH. Possible configurations are illustrated in Fig. 2. For cationic species equivalent relationships can be obtained.

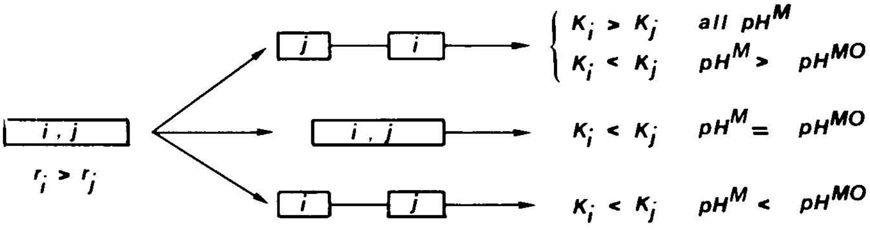

Fig. 2. Possible migration configurations for anionic constituents.

RESOLUTION

Once the criterion for separation has been satisfied, the time needed for resolution becomes important. When a constituent zone contains all of the sampled amount, resolution has been obtained for that constituent. We therefore define the resolution, R, as the separated fractional amount of the constituent:

$$R_i = \frac{\text{separated amount of } i}{\text{sampled amount of } i} \tag{21}$$

From this definition, it follows that during the separation process the resolution increases from zero to its maximal value, unity. Constituents that fail to separate remain at zero resolution and can be termed ideally mixed zones[10].

Complete separation of a sample requires the resolution values of all constituents of interest to be unity. Maximal speed of separation is obtained whenever the resolution rate, $\partial/\partial t \cdot R_i$, is optimized during the separation process. As expected, the resolution and its time derivatives are complex functions of the constituents involved and the driving forces applied. Moreover, the mathematical intricacy involved in calculating optimal process variables increases rapidly with increasing number and complexity of the sample constituents. For strong electrolytes relevant mathematical formulations have been published[3,9], but most separations nowadays concern weak electrolytes. In this case dissociation equilibria, and therefore a proper choice of pH, are tools in the control and optimization of the separation process[2]. When dealing with complex formation, association equilibria should be involved. Others[11] have suggested that the difference in migration rates, *e.g.*, $v_i - v_j$, is of decisive importance in separation. However, in isotachophoresis and moving boundary electrophoresis this does not apply, and in these instances it is more beneficial to optimize the ratio of the migration rates, *e.g.*, v_i/v_j. Whereas the velocity difference will reach a maximal value as a function of pH[11], the ratio shows no such optimum[12]. As the local electrical field strength for both constituents will be the same, it follows directly that eqn. 19 must be maximized or minimized, depending on the migration configuration (Fig. 2). Introducing equilibrium constants and ionic mobilities it follows that in anionic separations the lowest pH will give the better mobility ratio, and *vice versa* for cationic separations[12,13]. It should be emphasized, however, that pH extremes have only limited experimental applicability and that practical considerations often govern the proper choice of pH. Moreover, a low numerical value of the effective mobility will induce a high electrical field strength in order to obtain an appreciable migration rate and other elektrokinetic effects may then prevail.

Steady state

A unique feature of isotachophoresis is that, once the separation process has been completed, all electrophoretic parameters remain constant with time. Assuming a uniform current density, all sample constituents within the leading–terminating framework will migrate at identical speed. Moreover, at constant current density local migration rates will be constant. In this steady state, resolution values of stacked constituents will be either unity or zero.

The basic features of steady-state configurations have been extensively discussed[2].

THE SEPARATION PROCESS

The applicability of the above equations and definitions and the resulting implications are best illustrated by using a relatively simple two-component sample. We shall deal with the case where all constituents involved are monovalent weak electrolytes. Although essentially immaterial, we shall consider a separation compartment of uniform dimensions at a constant electrical driving current and a constant temperature. The separation process and some relevant information are given in Fig. 3.

It should be emphasized that within the separator three different regions are present and each has its own regulating behaviour. The regulating functions (eqns.

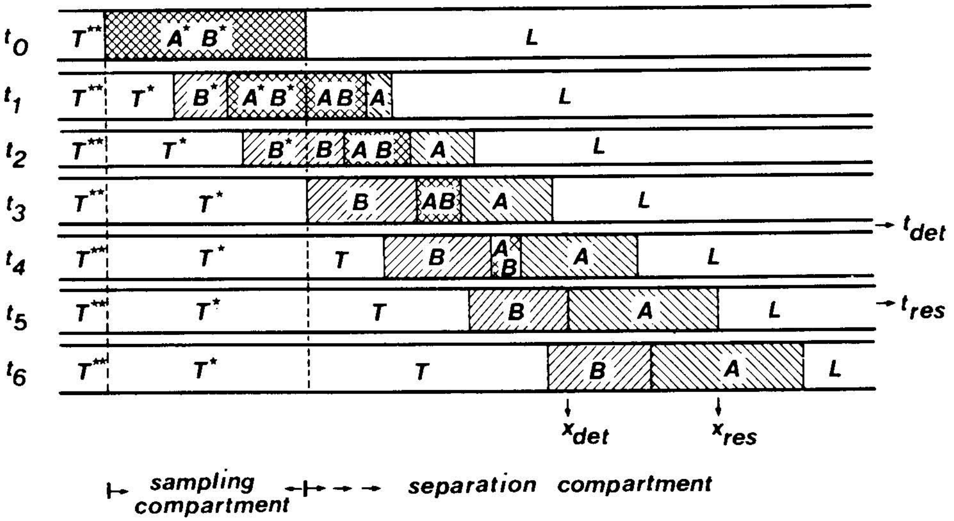

Fig. 3. Process of separation of two constituents. In the initial situation, t_0, the sampling compartment has been filled with a homogeneous mixture of the two sample constituents A and B. The separation compartment contains the leading constituent L and the terminating compartment is filled with the terminating constituent T. A counter constituent C, to preserve electroneutrality, is common to both sample constituents, the leading and the terminating constituents. Electrolyte changes in the electrode compartments, temperature and activity effects are neglected. Each compartment may have its own regulating function, due to the initial composition of the electrolytes. Starting from t_0 the separation of the sample will occur according to the moving boundary principle. All zone characteristics are, as long as they exist, constant with time. At different times several moving boundaries can be present: A/L, AB/A, B/AB, B/A, T/B, B^*/AB^*, T^*/B^*. Boundary velocities are given by local conditions. The sampling compartment causes the stationary boundaries: AB^*/AB, B^*/B, T^*/T, T^{**}/T. At t_3 the sample is leaving the sampling compartment and from this time on the total zone length of the sample zone will be constant. The properties of the mixed zone in the separation compartment will be in agreement with the local regulating function and the nature of the sample. At t_5 resolution is obtained and from this moment the individual zone lengths will be constant. It follows that both of the constituents have been concentrated. Resolution was obtained at t_{res} with a resolution length of x_{res}. Detection could have been started at t_{det} with the detection system located at x_{det}.

10 and 13) are the mathematical expression of this regulating behaviour and locally they cannot be overruled by the electrophoretic process[7]. All changes in electrophoretic parameters, *e.g.*, concentration, pH and conductance, will be in agreement with the local regulating function. Applying eqn. 16 to the stationary boundary between the separation and the sampling compartment it follows that

$$\varphi = \frac{c_B^{M*}}{c_A^{M*}} = \frac{c_B^{M}}{c_A^{M}} = \text{constant} \qquad (22)$$

Hence, the sampling ratio, φ, for the charged sub-species is invariable. Taking the leading electrolyte as a frame of reference, the regulating functions (eqns. 10 and 13) will result in

$$\bar{c}_L^L + \bar{c}_C^L = \bar{c}_A^M + \bar{c}_B^M + \bar{c}_C^M \qquad (23)$$

and

$$\frac{\bar{c}_L^L}{r_L} + \frac{\bar{c}_C^L}{r_C} = \frac{\bar{c}_A^M}{r_A} + \frac{\bar{c}_B^M}{r_B} + \frac{\bar{c}_C^M}{r_C} \tag{24}$$

where C is the counter constituent common to all constituents to be separated. The equilibrium relationships (eqn. 5) and electroneutrality imply that

$$\begin{aligned} \bar{c}_A^M &= c_A^M\,[1 + 10^{\pm(\mathrm{p}K_A - \mathrm{pH}^M)}] \\ \bar{c}_B^M &= \varphi c_A^M\,[1 + 10^{\pm(\mathrm{p}K_B - \mathrm{pH}^M)}] \\ \bar{c}_C^M &= -\,(1 + \varphi)\,c_A^M\,[1 + 10^{\pm(\mathrm{pH}^M - \mathrm{p}K_C)}] \end{aligned} \tag{25}$$

Combining eqns. 23, 24 and 25 we obtain

$$\bar{c}_L^L + \bar{c}_C^L = c_A^M\left[\frac{1}{\alpha_A^M} + \frac{\varphi}{\alpha_B^M} - \frac{(1 + \varphi)}{\alpha_C^M}\right] \tag{26}$$

and

$$c_L^L(1 - r_C) = c_A^M\left[\frac{r_A - r_C}{r_A\alpha_A^M} + \frac{\varphi(r_B - r_C)}{r_B\alpha_B^M}\right] \tag{27}$$

We now introduce the relative leading concentration

$$\varrho = \frac{\bar{c}_C^L}{\bar{c}_L^L}$$

and the reduced mobility. Elimination of c_A^M gives a quadratic equation for the proton concentration in the mixed zone. Only one root will have physical significance.

$$a \cdot 10^{2\mathrm{pH}^M} + b \cdot 10^{\mathrm{pH}^M} + c = 0 \tag{28}$$

The constants for the equation are given in Table I.

TABLE I

DYNAMIC CONSTANTS FOR THE pH OF THE MIXED ZONE (EQN. 28)

Anionic constituents	*Cationic constituents*
$a = 10^{-\mathrm{p}K_C}(1 + \varphi)$	$a = 10^{-\mathrm{p}K_A}\left(\frac{1 + \varrho}{r_A k_A} - 1\right) + \varphi \cdot 10^{-\mathrm{p}K_B}\left(\frac{1 + \varrho}{r_B k_B} - 1\right)$
$b = (1 + \varrho)\left(\frac{1}{r_A k_A} + \frac{\varphi}{r_B k_B}\right)$	$b = (1 + \varrho)\left(\frac{1}{r_A k_A} + \frac{\varphi}{r_B k_B}\right)$
$c = 10^{\mathrm{p}K_A}\left(\frac{1 + \varrho}{r_A k_A} - 1\right) + \varphi \cdot 10^{\mathrm{p}K_B}\left(\frac{1 + \varrho}{r_B k_B} - 1\right)$	$c = 10^{\mathrm{p}K_C}(1 + \varphi)$

Once the pH in the mixed zone has been calculated, all dynamic process variables can be calculated by using eqns. 1–27. Moreover, steady-state configurations are obtained by the introduction of zero or infinite sampling ratios. Moving boundary experiments can be simulated by introduction of a high load of sample. Computerization allows multiple calculations of all dynamic process variables[13].

The pH of the mixed zone

As the criterion for separation has to be satisfied and the ratio of effective constituent mobilities must be optimized, the pH of the mixed zone is of decisive importance. According to eqn. 28, this pH is influenced by the physico-chemical characteristics of the species to be separated and the counter constituent, by the sampling ratio, φ, and the relative leading concentration, ϱ. The last parameter is closely related to the pH of the leading electrolyte and the former to the pH of the sample. We shall consider anionic separations, but equivalent relationships and conclusions can be made for cationic separations.

In isotachophoresis the leading constituent must have a high effective mobility, so strong ionic species like chloride are commonly used[2]. In this instance it follows that

$$-\infty < \varrho = -\frac{1}{\alpha_C^L} < -1 \tag{29}$$

At $\varrho = -1$ the counter constituent is used far below its pK value and it behaves like a strongly ionic species. In this event the leading electrolyte has no buffering capacity. At $\varrho = -2$ the counter constituent is used at its pK value, $\text{pH}^L = \text{p}K_C$, and therefore it exhibits its full buffering capabilities. High negative values for the relative leading concentration again imply low buffering. Moreover, the concentration of the counter constituent will be high in comparison with that of the leading constituent, which can be favourable in complex formation. It is easily shown that for increasing $\text{pH}^L - \text{p}K_{i,j}$ *i.e.*, the constituents to be separated are only partially dissociated at the pH of the leading electrolyte, $\text{pH}^M - \text{pH}^L$ will increase. Constituents that are completely ionized at the pH of the leading electrolyte will induce only a slight elevation of pH^M and therefore will be separated as strong electrolytes. Counter constituents with a low pK value in comparison with the pH of the leading electrolyte show a tendency to diminish this increase in pH^M. When the leading constituent is a strongly ionic species the pH of any following zone will be higher than the pH of the leading zone. If, however, a weak constituent is chosen as the leading constituent negative pH steps can occur under appropriate conditions[2].

Obviously, problems in separation generally occur when both the pK values and the ionic mobilities of the constituents show only slight differences. An example of such a pair is given in Fig. 4. When the more mobile constituent has the higher dissociation constant (a straight pair), the criterion for separation will always be satisfied (Fig. 4a). Optimization in this instance is straightforward: low pH^L and low $\text{p}K_C$. However, when the more mobile constituent has the lower equilibrium constant (a reversed pair), the criterion for separation need not always be satisfied. It will depend on the proper choice of pH^L and $\text{p}K_C$ whether the critical pH, pH^{MO} (as indicated in Fig. 4a with an asterisk) will be obtained.

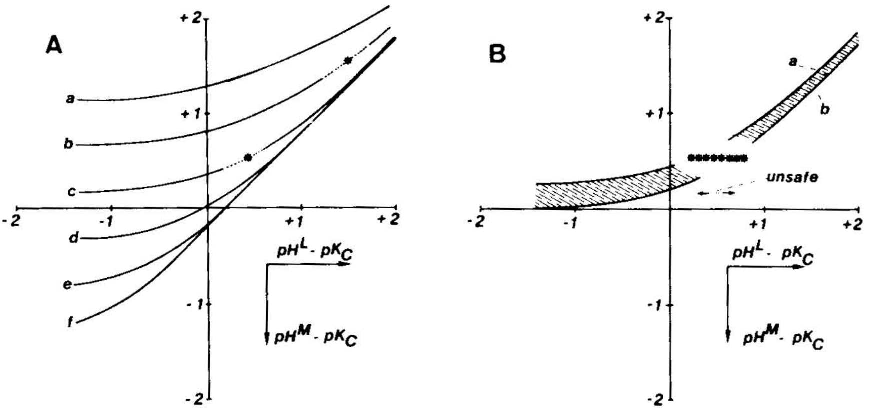

Fig. 4. (A) Influence of the pH of the leading electrolyte on the pH of the mixed zone. Leading electrolyte: $m_L = -77 \cdot 10^{-5}$, pH^L ordinate, pK_C = variable. Sample: $m_A = -45 \cdot 10^{-5}$, pH^M abscissa; $m_B = -30 \cdot 10^{-5}$, $\varphi = 1$; straight pair, $pK_A = 4.00$ and $pK_B = 4.50$; reversed pair, $pK_A = 4.50$ and $pK_B = 4.00$. Variable: (a) $pK_C = 2$; (b) $pK_C = 3$; (c) $pK_C = 4$; (d) $pK_C = 5$; (e) $pK_C = 6$; (d) $pK_C = 7$. (B) Influence of the sampling ratio on the pH of the mixed zone. Data: as in (A), with $pK_C = 4$. (a) $\varphi = \infty$ for the straight pair and $\varphi = 0$ for the reversed pair; (b) $\varphi = \infty$ for the reversed pair and $\varphi = 0$ for the straight pair.

From eqn. 20, it follows that the given pair will not separate at $pH^{MO} = 4.52$. If a counter constituent is chosen with $pK_C = 4$, there will be no separation at $pH^L = 4.40$. Above this pH^L, constituent B will migrate behind A, whereas the order will be reversed at low pH^L. It is easily shown that a low pH of the leading electrolyte will give a better effective mobility ratio. It should be emphasized that the influence of the mobilities of the constituents is only marginal owing to their limited numerical extension.

The influence of the sampling ratio is shown in Fig. 4b, where limiting values of φ are given. At zero sampling ratio the pH of the "mixed" zone will be that of the isotachophoretic A zone, whereas at infinite sampling ratio the pH^M will be governed by the constituent B. Hence, whatever the pH of the sample or its molar concentration ratio, the pH of the mixed zone will always lie between the pH values of the completely resolved zones. In common practice sampling ratios can show appreciable fluctuations due to the sample pH or the molar concentration ratio. Fig. 4b therefore gives an indication of the "unsafe" margin, which in this particular instance extends over 0.4 pH unit. It is obvious that the pH of the leading electrolyte must be chosen well out of this "unsafe" region. Sampling ratios can show an even larger influence, when the pK values of the constituents show more distinct differences.

Time of resolution and length of resolution

Resolution has been defined as the separated fractional amount of the constituent under investigation. Maximal resolution, $R = 1$, is obtained whenever the constituent zone contains all of the sampled amount n. From Fig. 3 it can be concluded that the time for resolution of the constituent A can be expressed as a function of the boundary velocities $v_{L/A}$ and $v_{A/A+B}$:

$$t_{\text{res}} = \frac{l_A}{v_{L/A} - v_{A/A+B}} \tag{30}$$

Using the appropriate relationships, we obtain

$$t_{\mathrm{res}} = \frac{n_A F}{I} \left(\frac{1 + \varphi \cdot \dfrac{k_A}{k_B}}{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}} \right) \left(1 - \frac{r_C}{r_A} \right) \tag{31}$$

Hence it follows that the time of resolution is a complex function of the concentration and the pH of both the leading electrolyte and the sample, of the sampled amount, the sampling ratio, the electric driving current and all ionic mobilities and dissociation constants involved. It should be noted that in eqn. 31 it is the ratio of the effective mobilities and not their difference that is important. Further, this equation emphasizes the importance of the pH of the mixed zone.

For the length of separation compartment needed to contain the completely resolved state, x_{res}, it follows that

$$x_{\mathrm{res}} = \frac{n_A F}{O \bar{c}_L^L} \left(\frac{1 + \varphi \cdot \dfrac{k_A}{k_B}}{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}} \right) \left(\frac{1}{k_A r_A} \right) = l_A \left(\frac{1 + \varphi \cdot \dfrac{k_A}{k_B}}{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}} \right) \tag{32}$$

where O is the area of the separation compartment and l_A is the zone length of the resolved constituent A.

For a given sample and electrolyte system, the resolution length is independent of the applied current density or electrical field strength, whereas the time of resolution is inversely related to the electrical driving current. From the resolution length the load capacity of the column can be deduced. Obviously, a high load capacity is always favoured by a low resolution time, so we shall confine our considerations to the resolution time.

Taking limiting values for eqn. 31, it follows that

$$\frac{n_A F}{I} < t_{\mathrm{res}} < \infty \tag{33}$$

The relationship between the sampled amount and the time of resolution is obviously linear. Moreover, for a two-constituent sample, resolution for both constituents will be obtained simultaneously. From eqns. 28 and 31 it can be concluded that both the time of resolution and the pH of the mixed zone are affected by the mobility of the common counter constituent. Fig. 5 shows the variation of the time of resolution as a function of the relative mobility of the counter constituent. It follows that a low r_C value favours the time of resolution, partly because of its influence on the pH of the mixed zone and partly because it increases the efficiency of the current transport. The influence of the mobility of the counter constituent on the pH of the mixed zone is, however, marginal. For the lower limiting value of zero it follows that the pH of the mixed zone becomes independent of the constituent mobilities.

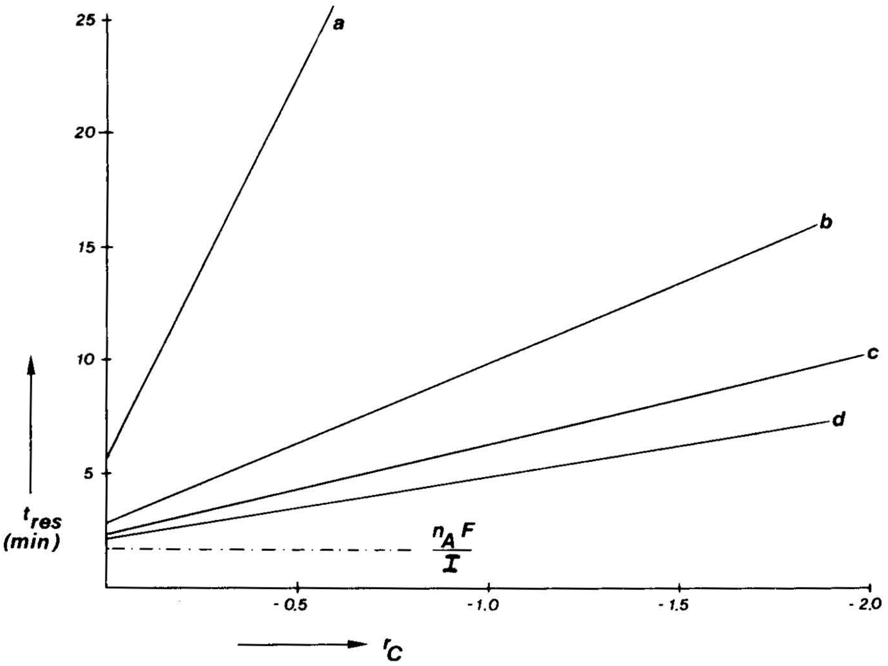

Fig. 5. Time for resolution as a function of the counter constituent mobility. Leading electrolyte: $m_L = -70 \cdot 10^{-5}$, $pH^L = 4.00$, $pK_L = 0$ m_C ordinate, $pK_C = 4.00$, $\bar{c}_L^L = -0.01\ M$. Sample: $m_A =$ variable, $pK_A = 4.00$, $m_B = -30 \cdot 10^{-5}$, $pK_B = 5.00$, $\bar{c}_A^{M*} = \bar{c}_B^{M**} = 0.05\ M$, $pH^{M*} = 4.00$; sample load, $n_A = n_B = 10^{-7}$ mole. Driving current: $I = 100\ \mu A$, $O = 0.002\ cm^2$. Variable: $r_A/r_B =$ (a) 0.5, (b) 1, (c) 1.5, (d) 2.0.

As eqn. 31 is a function of the effective constituent mobilities in the mixed zone, the pH of the zone is very important. Recognizing that all mixed zone characteristics are determined by the leading electrolyte as well as by the sample, it is obvious that the relative leading concentration, ϱ, and the sampling ratio, φ, can be used in optimization procedures. Both φ and ϱ are functions of pH and can be chosen arbitrarily within practical limitations. In Fig. 6, the influence of the pH of the leading electrolyte for ionic mobilities of different species on the time of resolution is shown. The counter constituent has been chosen for its maximal buffering capacity at the pH of the leading electrolyte. Dealing with monovalent anionic constituents, it follows that, whenever the more mobile constituent has the higher dissociation constant (the straight pair in Fig. 6a), resolution and therefore load capacity are favoured by a low pH of the leading electrolyte, and *vice versa* for cationic constituents.

It should be noted that the effect on the time of resolution is appreciable. When the ionic mobilities are almost equal, it follows that a low pH^L must be chosen in order to obtain an acceptable time of resolution. For species that already have large differences in their ionic mobilities, the effect of decreasing the pH of the leading electrolyte is less pronounced. Greater differences in equilibrium constants give even higher results. The flattening of the sigmoidal curves at high pH indicates that the sample constituents are being separated as monovalent strong ions, in which event

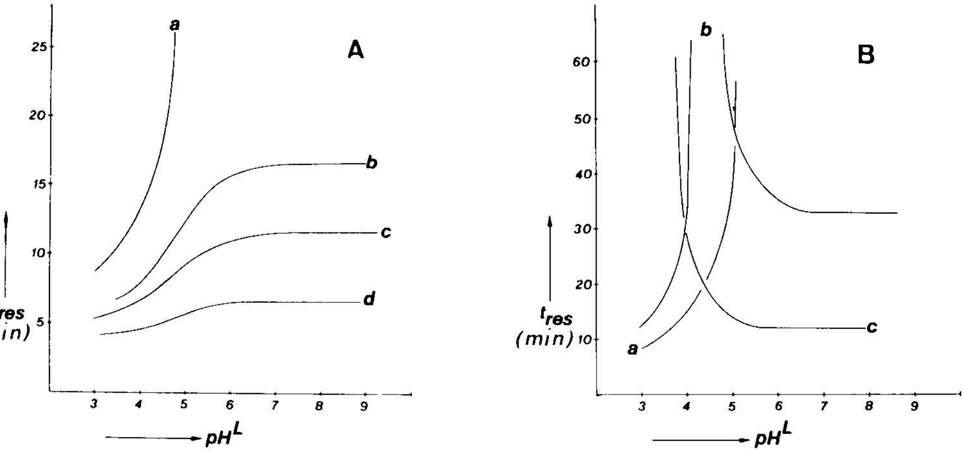

Fig. 6. Time for resolution as a function of the pH of the leading electrolyte. Leading electrolyte: $m_L = -77\cdot10^{-5}$, pH^L ordinate, $pK_L = 0$, $m_C = 30\cdot10^{-5}$, $pK_C = pH^L$, $\bar{c}_L^L = -0.01\ M$. Sample: m_A = variable, $m_B = -30\cdot10^{-5}$, $\bar{c}_A^{M*} = \bar{c}_B^{M*} = -0.05\ M$, $pH^{M*} = 4.00$; sample load, $n_A = n_B = 10^{-7}$ mole. (A) Straight pair; $pK_A = 4.00$ and $pK_B = 4.50$. (B) Reversed pair; $pK_A = 4.50$ and $pK_B = 4.00$. Driving current: $I = 100\ \mu A$, $O = 0.002\ cm^2$. Variable: (A) r_A/r_B = (a) 1, (b) 1.5, (c) 1.67, (d) 2.0; (B) r_A/r_B = (a) 1, (b) 1.33, (c) 2.0.

there is, of course, no influence of pH^L. If the more mobile constituent has the lower dissociation constant (the reversed pair in Fig. 6b) the situation becomes more complex. The pH of the mixed zone at which no separation will occur and its relation to the pH of the leading electrolyte have already been discussed. From Fig. 6 it follows that the pH of the leading electrolyte must be at least one pH unit different from the critical pH^L in order to obtain an acceptable time of resolution. The question of whether a high or a low pH must be chosen depends on the physico-chemical characteristics of the constituents to be separated. Nevertheless, the tendency that a low pH^L is favourable still holds. For example, when the mobility ratio is 1.33 resolution will be given at pH^L = 7, but a higher resolution rate will be obtained at pH^L = 3. At high pH the constituents will migrate in order of ionic mobility, whereas at low pH they will migrate in order of dissociation constants.

Although in practice the pH of the sample will show only a low degree of freedom, its influence can nevertheless be substantial. Fig. 7 shows this influence on the time of resolution as a function of the pH of the leading electrolyte. Again, resolution is favoured by a low sample pH for the separation of a straight pair. Therefore, in this instance a low pH of the leading electrolyte and the sample promotes the fastest separation. From Fig. 7b, where $r_A > r_B$ and $K_A < K_B$, the guidances for reversed pairs can be deduced. When running such a sample at a high pH of the leading electrolyte it is also preferable to use a sample with a high pH. Optimal conditions are obtained, however, at a low pH of both the sample and the leading electrolyte. In both instances the arrangement of the constituents in the sampling compartment and the separation compartment will be the same. If, however, a low pH of the leading electrolyte is combined with a high pH of the sample, B will separate in the sampling compartment and in the separation compartment[12]. This phenomenon (illustrated in Fig. 8), although remarkable, has no other influence on the separation

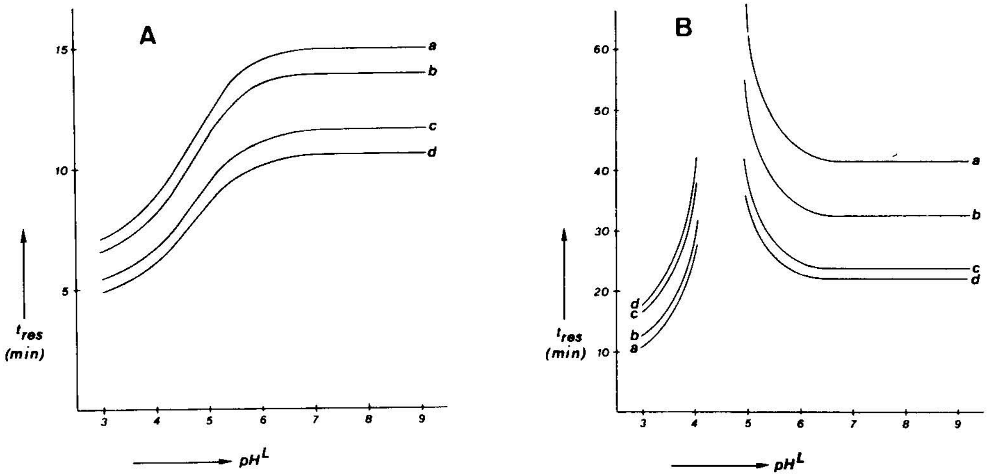

Fig. 7. Influence of sample pH on the resolution time. Leading electrolyte: $m_L = -70 \cdot 10^{-5}$, pH^L ordinate, $pK_L = 0$, $m_C = 30 \cdot 10^{-5}$, $pK_C = pH^L$, $\bar{c}_L^L = -0.01$ M. Sample: $m_A = -40 \cdot 10^{-5}$, $m_B = -30 \cdot 10^{-5}$, $\bar{c}_A^{M*} = \bar{c}_B^{M*} = -0.05$ M, pH^{M*} = variable; sample load, $n_A = n_B = 10^{-7}$ mole. (A) Straight pair; $pK_A = 4.00$ and $pK_B = 4.50$. (B) Reversed pair; $pK_A = 4.50$ and $pK_B = 4.00$. Driving current: $I = 100$ μA, $O = 0.002$ cm². Variable: (A) pH^{M*} = (a) 8, (b) 5, (c) 4, (d) 3; (B) pH^{M*} = (a) 3, (b) 4, (c) 5, (d) 8.

process, as has been discussed already. Its typical behaviour will be discussed in a later paper.

From Fig. 7b, it follows that in the given example the pH of the sample has almost no influence on the critical pH^L at which a reversal of order occurs, although this need not always be the case.

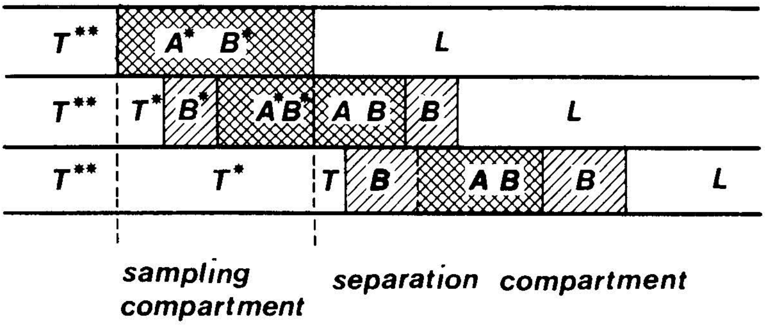

Fig. 8. The dual separation phenomenon.

Resolution and resolution rate

It follows directly from eqn. 31 that for a given sample and electrolyte system the amount of constituent A resolved into its proper zone is given by

$$n_A^{\text{separated}} = \frac{tI}{F} \left(\frac{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}}{1 + \varphi \cdot \dfrac{k_A}{k_B}} \right) \left(1 - \frac{r_C}{r_A}\right)^{-1} \tag{34}$$

Thus, for the effective resolution $\bar{R}_A$ and its time derivative, the effective resolution rate, it follows that

$$\bar{R}_A = \frac{t}{t_{\mathrm{res}}} \quad \text{and} \quad \frac{\partial}{\partial t} \cdot \bar{R}_A = \frac{1}{t_{\mathrm{res}}} \tag{35}$$

It must be emphasized that, owing to eqn. 30, this resolution is an average. The actual resolution, according to eqn. 21, can be different from the effective one, due to discontinuities in the separation process. A separation configuration causing such discontinuities is shown in Fig. 8 and the actual and effective resolution of this reversed pair is given in Fig. 9.

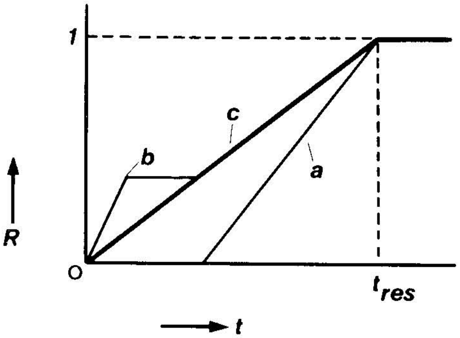

Fig. 9. The effective and the actual resolution. The separation configuration is given in Fig. 8. During some time the sample constituent A will not separate at all. After a definite time, however, this constituent will separate accordingly to (a); its effective resolution, however, is given by (c). The sample constituent B will start to separate at a high resolution rate (b). When the sample has left the sampling compartment the resolution of the constituent B will remain constant until a zone of pure A is formed. The effective resolution for the constituent B again is given by (c).

As a high resolution rate is always desirable, all conclusions regarding the time of resolution will apply. Eqn. 34, however, offers a unique possibility for deriving the dimensionless separation number S. Differentiation of the separated amount with respect to time and multiplication by F/I gives

$$S_A = \frac{F}{I} \cdot \frac{\partial}{\partial t} \cdot n_A = \left(\frac{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}}{1 + \varphi \cdot \dfrac{k_B}{k_A}} \right) \left(\frac{r_A}{r_A - r_C} \right) \tag{36}$$

The advantage of this separation number is that it is essentially independent of the amount of sample, column geometry and electrical driving current. The physical significance of the dimensionless separation number is that it gives the efficiency of the separation process. Taking limiting values, it follows that

$$0 < S_A < 1 \tag{37}$$

The separation number for the constituent B is closely related to that of A, as

$$S_B = \chi S_A \tag{38}$$

where χ is the molar concentration ratio, $\bar{c}_B/\bar{c}_A$, in the sample. The relationship between χ, φ and the pH of the sample is straightforward[13]. From eqn. 36 it follows that the sampling ratio, φ, may have a decisive influence on the separation number. Fig. 10 shows this influence for different ionic constituents. The counter constituent has again been chosen for its maximal buffering capacity in the leading electrolyte.

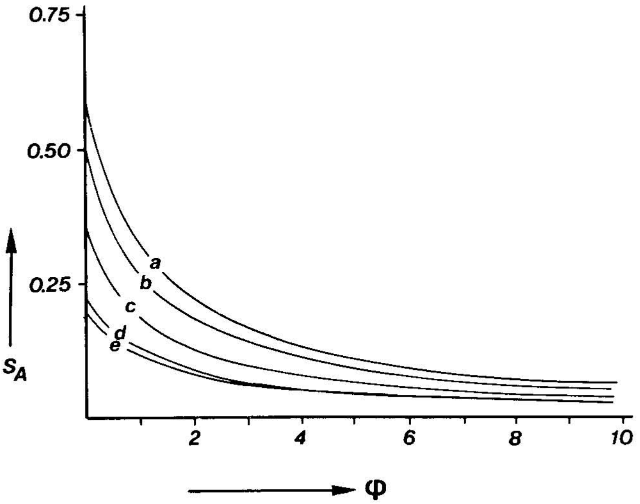

Fig. 10. Influence of the sampling ratio on the dimensionless separation number. φ = Variable, $\varrho = -2$, $r_A = 0.6$, $r_B = 0.4$, $r_C = -0.4$. (a) $pK_A = 4$, $pK_B = 9$, $pK_C = 6$; or $pK_A = pK_B = 6$, $pK_C = 4$. (b) $pK_A = 4$, $pK_B = 7$, $pK_C = 6$; or $pK_A = pK_B = 5$, $pK_C = 4$. (c) $pK_A = 4$, $pK_B = 6$, $pK_C = 6$; or $pK_A = pK_B = 4$, $pK_C = 4$. (d) $pK_A = 4$, $pK_B = 5$, $pK_C = 6$; or $pK_A = pK_B = 5$, $pK_C = 6$. (e) $pK_A = 4$, $pK_B = 4$, $pK_C = 6$.

It follows that the separation number decreases rapidly with increased sampling ratio. Introducing limiting values, the transport numbers[14] for the constituents in their resolved zones are obtained: $S_A = 0.6$ and $S_B = 0.5$. Numerical calculations[13] show many of the curves that are obtained when the physical parameters pK and m are varied show congruent behaviour (Fig. 10). At low sampling ratios a large difference in pK values will induce a high separation number for the more mobile constituent. At high sampling ratios this effect is much less pronounced. Moreover, from curves d and e in Fig. 10 it follows that for the separation of a straight species pair a low pH of the leading electrolyte is favourable. It should be recognized that exact data for constituents are generally not known and therefore an appreciable variation in the input data has to be taken into consideration. Reasons for these variations are obvious: lack of data, unreliable data, temperature effects, activity effects, etc. The broadening effect due to the parameter fluctuations, however, is marginal[13], as many of these are counter active.

Time of detection and load capacity

Eqn. 32 suggests that a fixed-point detector must be located at x_{res} from the sampling compartment. From Fig. 3, however, it follows that this is not always the case, as detection can already have commenced before the sample has been completely resolved. As the criterion for detection, only resolved constituents must be detected, *i.e.*, the mixed zone should resolve the moment it reaches the detection system. Hence, for the minimal length at which the detector must be located, x_{det}, it follows that

$$x_{\text{det}} = t_{\text{res}} v_{A/AB} \tag{39}$$

and, for the moment at which detection must be started, t_{det},

$$t_{\text{det}} = x_{\text{det}}/v_L \tag{40}$$

It follows directly that the time of resolution will be greater than or equal to the time of detection, as holds for the resolution distance and the detection distance. Using the appropriate relationships we obtain

$$\frac{x_{\text{det}}}{x_{\text{res}}} = \frac{t_{\text{det}}}{t_{\text{res}}} = \frac{\varphi \cdot \dfrac{k_B}{k_A} + \dfrac{\alpha_B^M r_B}{\alpha_B^M r_A}}{1 + \varphi \cdot \dfrac{k_B}{k_A}} \tag{41}$$

For a non-scanning detector it is important to minimize both x_{det} and t_{det}, and optimization procedures are analogous to the minimization of the time of resolution. Fig. 11 shows the influence of the sampling ratio on the ratio of detection time to resolution time. As might be expected, the effect is considerable. For a low sampling ratio and low mobility ratio the time of detection will be very small compared with the time of resolution. In practical terms, this means that, whenever the more mobile sub-species has a high concentration compared with that of the less mobile sub-species, detection can be started early and only a short separation compartment is needed. At high sampling ratios, the time of detection will be equal to the time of resolution.

In common practice, however, the detector will be located at a fixed position in the separation compartment, x_{detfix}, so it is impossible to choose the actual length of the separation compartment. For the maximal load capacity, n^{max}, for the column we obtain

$$n_A^{\text{max}} = n_L^{\text{load}} r_A k_A \cdot \frac{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}}{\varphi \cdot \dfrac{k_A}{k_B} + \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}} \tag{42}$$

where n_L^{load} is the amount of the leading constituent filling the separation compartment from the sampling compartment to the detector. The maximal load capacity

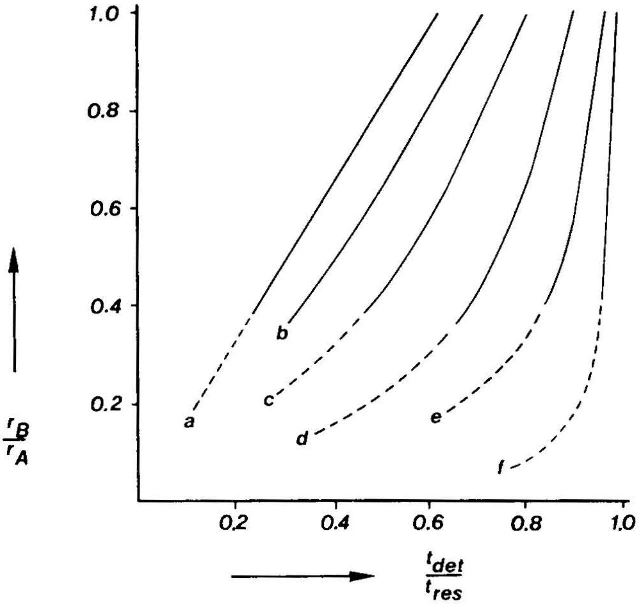

Fig. 11. Relationship between the time of detection and the time for resolution. Leading electrolyte: $m_L = -77\cdot10^{-5}$, $pH^L = 4$, $pK_L = 0$, $m_C = 30\cdot10^{-5}$, $pK_C = 4$. Sample: m_A abscissa, $pK_A = 4$, $m_B = -30\cdot10^{-5}$, $pK_B = 4.5$, $pH^{M*} = 4$, φ = variable; sample load, $n_A = 10^{-7}$ mole, thus n_B = variable. Driving current: $I = 100\,\mu A$, $O = 0.002\ cm^2$. Variable: sampling ratio; φ = (a) 0.01, (b) 0.26, (c) 0.78, (d) 2.33, (e) 7.00, (f) 21.0.

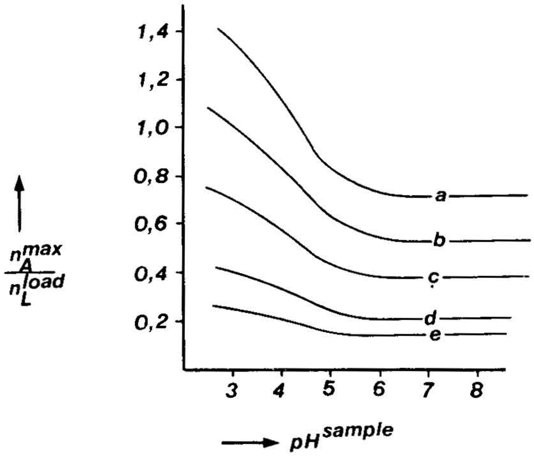

Fig. 12. Influence of the sample pH on the load capacity. Leading electrolyte: $m_L = -77\cdot10^{-5}$, $pH^L = 4$, $pK_L = 0$, $m_C = 30\cdot10^{-5}$, $pK_C = 4$. Sample: m_A = variable, $pK_A = 4$, $m_B = -30\cdot10^{-5}$, $pK_B = 4.5$, pH^{M*} ordinate, $n_A = n_B$. Variable: r_A/r_B = (a) 2.17, (b) 1.83, (c) 1.50, (d) 1.33, (e) 1.00.

for the second constituent follows directly from the given definitions. Moreover, eqn. 42 can be transformed directly into a time-based or distance-based form, using

$$n_L^{\text{load}} = Ox_{\text{detfix}}\bar{c}_L^L = t_{\text{detfix}}\cdot\frac{I}{F(1-r_C)} \qquad (43)$$

A maximal load capacity is obtained by minimizing the time for resolution. Fig. 12 shows the influence of the pH of a sample on the load capacity of a column. For this straight pair of constituents it follows that by introducing a sample at a low pH a substantial increase in load capacity can be obtained.

DISCUSSION AND CONCLUSIONS

In the transient-state model we neglected several secondary effects, *e.g.*, temperature distribution and activity effects. Although these effects are not always marginal, they will generally not imply other guidances. With regard to uneven temperature distributions, either longitudinal or transverse[15], it should be emphasized that their effect will be deleterious only under extreme operating conditions. Working at moderate current densities, without excessive cooling, convective disturbances are negligible and temperature differences can be well controlled. In special cases, temperature effects can have a favourable influence on separation but so far temperature programming has not been studied.

A fundamental question concerns the applicability of the transient-state model under extreme pH values of the electrolytes. The hydroxyl and/or proton concentration can be introduced into the specific conductance without difficulty. It has been suggested, however, that it is not necessary to incorporate the hydrogen constituent into the moving boundary equation[8,16]. Neglecting the solvent effect at low or high pH gives differences in the zone characteristics compared with those of steady-state models[2,8,17–19]. The differences, however, are small[13] and their experimental significance is still under investigation.

The applicability of the transient-state model and the resulting implications have been considered for a sample containing two monovalent weakly ionic constituents. The relative simplicity of the model allows a fundamental understanding of the isotachophoretic separation process and provides a realistic view of optimization procedures. It is obvious that the model could be extended to multivalent weak electrolytes. The efficiency of such considerations will be poor as no other guidances will be found. Concerning multi-component samples, it has already been mentioned that the mathematical intricacy increases rapidly with increase in the number of constituents. For strong electrolytes extension of the model is not difficult but is laborious and monastic[20] for practical purposes. The model given already indicates clearly the importance of physico-chemical and operational parameters. For multicomponent samples optimization procedures will generally be difficult, as their success depends largely on the constituents involved.

Optimal separation has been identified with a resolution of unity for the constituent of interest. As the separation boundary between two resolved zones will always have a finite interfacial thickness, in which major concentration changes will occur, the ideal resolution of unity can never be obtained. On most occasions, however, when the sharpening effect of the applied electrical field and the dispersion by diffusional and convective forces are optimized, the interfacial thickness is so small that it cannot be detected in these instances. Therefore, deviations of the resolution from unity can be neglected. For very small zone lengths (<0.05 mm), the interfacial thickness has a deleterious effect on resolution. Moreover, the presence of a zone profile, parabolic or otherwise, makes reliable detection of very small zones difficult, as not the actual zone length but the zone profile will be measured. Therefore, for accurate quantitative determinations the zone length should be reasonable.

The lower limit of resolution represents the case in which the constituent of interest does not separate at all and therefore forms a mixed zone with another constituent. In an ideal mixed zone[10] a homogeneous concentration distribution should be present. Experimental steady-state mixed zones will have zero resolution but generally with a non-uniform concentration distribution[21].

For a completely resolved sample, the resolution of all components should be unity. In practice, however, it is sufficient to obtain maximal resolution for the constituent of interest. It has been shown that for a two-constituent sample, the resolution will generally increase linearly with time. The exceptional behaviour of reversed pairs with respect to continuity and linearity has been indicated. With multicomponent systems discontinuities and non-linear behaviour will be encountered more frequently.

A maximal resolution rate is obtained when the ratio of the effective mobilities of the constituents is minimized or maximized. Very low numerical values for the effective mobilities imply high electrical gradients. The resulting temperature effects and non-electrophoretic phenomena in this instance may have deleterious effects on resolution and resolution rate. Minimization or maximization procedures naturally must fit within the constraints of the leading–terminating electrolytes. It must be emphasized that the ratio of effective mobilities from the completely resolved, *i.e.*, steady-state, configuration gives only indirect information about the separability and separation efficiency of two constituents. Hence constituents showing virtually no difference in steady-state effective mobilities can nevertheless sometimes be separated efficiently[12]. In such instances the transient-state model shows that the pH of the mixed zone is the separation-determining parameter.

In the steady state, constituents will generally migrate in order of effective mobilities, *i.e.*, the effective mobilities decrease from leading to terminating electrolyte. In special cases, however, a constituent with low effective mobility can migrate isotachophoretically in front of a constituent with a relatively high effective mobility. Such separation configurations have been called "enforced isotachophoresis" and are stable with respect to time[2]. It follows that measurement of the step heights of single constituents gives only an indication of the separation configuration for a mixture of constituents. Moreover, it has been shown that, depending on pH, constituents can migrate in a different steady-state configuration. The importance of the pH of the leading electrolyte in this respect has been extensively discussed. It is generally assumed that the nature of the sample, especially its pH and concentration, has no influence on the steady state. The transient-state model, however, reveals the importance of the pH of the sample. Dealing with a reversed pair of sample constituents near the critical pH of the leading electrolyte, at which a reversal of order can occur, the pH of the sample may, theoretically, be the deciding factor. Hence it must be concluded that the steady-state characteristics of the zone are not influenced by the pH of the sample, but this can affect the separation configuration. However, in practice this will not generally occur. It is obvious that, for constituents separating very slowly, it will be difficult to conclude whether the steady state has been reached or not. This holds especially for complex mixtures such as natural protein mixtures, in which the numerous constituents, each with possible microheterogeneity, may give rise to a continuous mobility spectrum. Such complex mixtures require a relatively long separation time. Obviously the use of spacers for such samples, whether ampholines or discrete substances, will decrease the efficiency of the separation process, but can increase the interpretability[22]. Whenever possible the use of discrete spacers at low concentration is to be preferred.

The critical point of separability has been expressed in the criterion for separation, *i.e.*, the ratio of effective mobilities in the mixed state should be different from unity. It should be recognized that this criterion gives only an academic answer to the question of whether constituents can be separated or not. Dealing with separability in its limiting case, it is obvious that dispersive factors become important and should be incorporated into the equation of continuity and its resulting relationships. Relevant mathematical formulations have already been given for calculating the structure of separation boundaries in isotachophoresis[23–25].

Dispersion, however, may have several causes, *e.g.*, temperature distribution, osmotic and hydrodynamic flow and density gradients, and may exceed diffusional dispersion by several orders of magnitude[27]. This overall dispersion is closely related to the chosen operating conditions and the design of the equipment. Allowance can be made for such dispersive factors, but the resulting uncertainty in the criterion for separation causes this to remain academic. The model presented clearly indicates that in dealing with practical separability, other parameters are important, such as resolution, time for resolution, time of detection and load capacity. It has been shown that in addition to the physico-chemical characteristics of the constituents, the sample load, the sample ratio, the pH of the leading electrolyte and of the sample and the applied electrical driving current determine in practice whether resolution can be obtained within an acceptable time. For separations in which a long analysis time is needed, ultrapure electrolyte systems must be used in order to prevent a progressive decay of the steady-state configuration[26]. In optimization procedures three rationales can be recognized, which of course, are not completely independent:

(i) The electrical driving current acts directly on the time of analysis. As the time for resolution is inversely related to the electrical driving current, it is obvious that this operational parameter must be maximized. In practice this will mean that a compromise must be found between the quantitative and qualitative accuracy required and the allowable driving current. The electrical driving current, if temperature effects are reglected, has no influence on the efficiency of the separation process, so the length of resolution, the location of the detection system and the load capacity are all independent of it. This is consistent with the fact that only the current–time integral is important[7]. In order to separate a given sample a definite number of coulombs are necessary and the time interval in which this amount must be delivered is immaterial.

When performing isotachophoretic analyses, it is therefore not necessary to work at a constant electrical driving current. Using a fixed point detector, however, a constant electrical driving current greatly facilitates the interpretation of the isotachopherograms obtained. Further, the operating conditions are more easily standarized and better controlled.

(ii) The efficiency of the current transport is directly influenced by the mobility of the common counter constituent. The favourable effect of a counter constituent with a low ionic mobility is directly reflected in the time for resolution, time for detection, separation number and load capacity. In practice, however, only few substances will satisfy all requirements[2]: low mobility, low buffering capacity and no UV absorption.

(iii) The efficiency of the separation process is determined by the properties of the mixed zone. The transient-state model shows that these properties are also governed by the nature of the leading electrolyte as well as the nature of the sample. Considering the ratio of effective constituent mobilities in the mixed state, it follows that, owing to the limited numerical extension of ionic mobilities, pH or complex formation provides the best optimization parameter. In anionic separations a low pH of both the leading electrolyte and the sample will favour a high resolution rate and a high separation number. For cationic separations a high pH will be preferable.

The presence of reversed pairs of constituents may complicate the optimization procedure. In general, it can be taken that the pH values of the leading electrolyte and the sample should not differ too much. For known species the critical pH values at which separation will not occur can easily be calculated and hence can be avoided.

LIST OF SYMBOLS

α	degree of dissociation
A	constituent to be separated
B	constituent to be separated
$\bar{c}$	constituent concentration (mole/cm^3)
c	sub-species concentration (equiv./cm^3)
C	counter constituent
D	diffusional coefficient (cm^2/sec)
E	electrical field strength (V/cm)
F	Faraday constant (C/equiv.)
i, j	constituent, sub-species
J	electrical current density (A/cm^2)
κ	electrical specific conductance ($\Omega^{-1} \cdot$cm^{-1})
k	reduced mobility
K	dissociation constant
L	leading constituent
l	zone length (cm)
m	mobility (cm^2/V$\cdot$sec)
$\bar{m}$	effective mobility (cm^2/V$\cdot$sec)
n	sub-species, A, B, C, L, T
n	amount of constituent (mole)
O	area (cm^2)
pH	pH
pK	negative logarithmic transform of K
φ	sampling ratio
χ	molar sampling ratio
r	relative mobility
R	resolution
ϱ	relative leading concentration
S	separation number

T	terminating constituent
t	time coordinate (sec)
t_{res}	time of resolution (sec)
t_{det}	time of detection (sec)
t_{detfix}	running time (sec)
v	linear velocity (cm/sec)
x	place coordinate (cm)
x_{res}	length of resolution (cm)
x_{det}	length of detection (cm)
x_{detfix}	running length (cm)

Subscripts

i, j, n	A, B, L, T, C constituent, species indicator

Superscripts

K	A, B, L, T, M (mixed) zone indicators
$**$	terminating compartment
$*$	sampling compartment
	separation compartment

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ISOTACHOPHORESIS: THE CONCEPTS OF RESOLUTION, LOAD CAPACITY AND SEPARATION EFFICIENCY

II. EXPERIMENTAL EVALUATION

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SUMMARY

Resolution, load capacity and separation efficiency in isotachophoresis are experimentally evaluated and the results are compared with the theoretically expected values. The theoretical and experimental results show good agreement and confirm the reliability of the transient-state model. The importance of the dimensionless separation number and load capacity for the determination and standardization of experimental performance conforms with theory. It is shown that, in view of these two parameters, the pH of the leading electrolyte is the best rationale for optimization, whereas the pH of the sample has only restricted possibilities. Steady-state configurations in which constituents are not migrating in order of decreasing effective mobilities are shown and discussed.

INTRODUCTION

Resolution in isotachophoresis has been defined as the fractional separated amount of the constituent under consideration[1]. According to this definition, its numerical value may vary between the limiting values of unity and zero. At zero resolution no separation has occurred and the constituent forms an ideally mixed zone with at least one other constituent. Obviously the maximal resolution value of unity should be reached in the shortest time possible and with the most convenient experimental conditions. It follows that the resolution rate must be maximized.

In Part I[1] three rationales and their theoretical background were considered. It was shown that any optimization procedure must act directly on the transient state, in which the sample constituents are separating according to the moving boundary principle. The mixed zones being resolved during the transient state have well defined characteristics governed by the Kohlrausch regulating function concept[2]. By a proper

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choice of operating conditions and operational systems, the mixed zone characteristics that influence the separating power can be well controlled. The result is a steady state in which constituents are migrating in a well defined order with a definite velocity towards a detection system. Using a constant electrical driving current, constituents will migrate with equal velocity through the detector, allowing an easy qualitative and quantitative evaluation[3].

In Part I[1] the three optimization rationales were given as the electrical driving current, the mobility of the counter constituent and the chemical equilibria of the electrolytes. The electrical driving current acts directly on the time needed to resolve a sample. In order to separate a given sample a definite number of coulombs are necessary, but the time interval in which this amount must be delivered is immaterial[2], *i.e.*, the time interval is restricted within its limiting values by diffusional and convective forces.

The time for resolution and the electrical driving current are inversely related, so the latter must be maximized. Neglecting temperature effects, the driving current has no influence on the efficiency of the separation process. It follows that for a given sample the length of the separation compartment is independent of the driving current.

The mobility of the counter constituent acts directly on the transport efficiency. When performing isotachophoretic analyses it is disadvantageous to transport the counter constituent, in which there is no interest, at a relatively high migration rate. In order to obtain a high transport efficiency the ionic mobility of the counter constituent should be as low as possible[3].

The properties of dissociation and complex formation can be used to optimize the separation efficiency. It must be recognized however, that the mobility of the counter constituent has some (marginal) influence on this efficiency. For an optimal separation it has been shown that the ratio of effective mobilities in the mixed state is of decisive importance[1]. Whenever this ratio is unity no separation will occur, as the migration rates, given by the product of the electrical field strength and the effective mobility, in this instance will be identical. Obviously, the mobility ratio must be minimized or maximized depending on the separation configuration[1]. A well known mechanism influencing effective mobilities selectively is given by the dissociation and complex formation equilibria[3,4]. In the former instance the pH can be used in optimization procedures, and until now it has been the most frequently used parameter[3]. For anionic separations optimal conditions will generally be found at low pH, whereas for cationic separations a high pH is preferable.

The direct result of optimal separation efficiency will be a favourable time for resolution and optimal load capacity. The separation efficiency is best expressed by the dimensionless separation number[1], which has its maximal value at unity. This separation number is, neglecting temperature effects, independent of various operating conditions such as the electrical driving current and column geometry. It is, however, strongly affected by the nature of the applied electrolytes and therefore can be used to compare the efficiency of analyses. The load capacity gives direct information on the amount of constituent that can be sampled in a given operational system. Again, this parameter is independent of various operating conditions.

The load capacity can be optimized by following the same rationales as for the separation efficiency[5]. Both parameters can be used to evaluate the experimental performance of isotachophoretic separations.

EXPERIMENTAL

All experiments were performed using the isotachophoretic equipment developed by Everaerts *et al.*[3]. The separation compartment consisted of PTFE narrow-bore tubing with I.D. 0.45, 0.2 or 0.15 mm and corresponding O.D. 0.75, 0.4 and 0.3 mm. The direct constant electrical driving current was obtained from a modified Brandenburg (Thornton Heath, Great Britain) high-voltage power supply. Potential gradient detectors, used in either the potential gradient or the conductance mode, were used for the determination of transient-state and steady-state characteristics. All chemicals used were of pro analisi grade or additionally purified by conventional methods. Operational systems are summarized in Table I. Theoretical calculations were performed with the computerized transient-state model[5] and physico-chemical data were taken from refs. 6, 7 and 8.

TABLE I

OPERATIONAL SYSTEMS

Parameter	*System No.*				
	1	*2*	*3*	*4*	*5*
pH of leading electrolyte	3.60	4.03	4.60	5.04	6.02
Leading constituent	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-
Concentration (*M*)	0.01	0.01	0.01	0.01	0.01
Counter constituent*	BALA	GABA	EACA	CREAT	HIST
Terminating constituent	C_2H_5COOH	C_2H_5COOH	C_2H_5COOH	C_2H_5COOH	MES**
Concentration (*M*)	0.005	0.005	0.005	0.005	0.005
Additive to leading electrolyte	0.05% MOWIOL***	0.05% MOWIOL	0.05% MOWIOL	0.05% MOWIOL	0.05% MOWIOL
Temperature	Ambient	Ambient	Ambient	Ambient	Ambient
Electrical driving current (A/cm^2):					
d_t = 0.45 mm	0.0503	0.0503	0.0503	0.0503	0.0503
d_t = 0.20 mm	0.0796	0.0796	0.0796	0.0796	0.0796
d_t = 0.15 mm	0.1415	0.1415	0.1415	0.1415	0.1415

* BALA = β-alanine; GABA = γ-aminobutyric acid; EACA = ε-aminocaproic acid; CREAT = creatinine; HIST = histidine.

** MES = 2-(N-morpholino)ethanesulphonic acid.

*** MOWIOL = polyvinyl alcohol, N-88, Hoechst, Frankfurt, G.F.R.

RESULTS AND DISCUSSION*

Transient-state characteristics can be easily obtained experimentally and several important parameters can be evaluated directly as most are interrelated[1]. For isotachophoretic analyses it is most convenient to use a separation compartment of well defined and constant volume and to apply a constant electrical driving current. Using a fixed point detector and a given operational electrolyte system, all characteristics can be evaluated by injection of known amounts of sample and accurate measurement of all electrical gradient and time events. Because, under these con-

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* For symbols and abbreviations, see Part I[1].

ditions, the amount of the leading constituent filling the separation compartment, n_L^{load}, is constant, the first boundary that reaches the detector will always be registered after the same time interval, t_{detfix}:

$$t_{\mathrm{detfix}} = n_L^{\mathrm{load}} \cdot \frac{F}{I} (1 - r_C) \tag{1}$$

where F is the Faraday constant, I the applied electrical driving current and r_C the ionic mobility of the counter constituent, relative to the leading constituent. Experimentally, the amount of leading constituent can be determined by the injection of a known amount of leading constituent, Δn_L, and measurement of the resulting time delay, Δt, with respect to t_{detfix}:

$$n_L^{\mathrm{load}} = t_{\mathrm{detfix}} \cdot \frac{\Delta n_L}{\Delta t} \tag{2}$$

Moreover, because for a one-constituent zone the dimensionless separation number, S, is identical with the transport number[9], T, the experimental and theoretical transport efficiency can be compared:

$$T = \frac{F}{I} \cdot \frac{n_L^{\mathrm{load}}}{t_{\mathrm{detfix}}} = \frac{m_i}{\sum\limits_i m_i} \tag{3}$$

where m_i is the ionic mobility of the constituent i. It should be noted that the transport number of monovalent weakly ionic constituents does not contain effective mobilities, but rather ionic mobilities, because, owing to electroneutrality, the degree of dissociation cancels out. Some experimental results are given in Table II.

TABLE II

CHARACTERISTICS OF THE LEADING ELECTROLYTE

Parameter	*Value*	
Leading constituent, chloride	m_{Cl^-}	$= -77 \cdot 10^{-5}$ cm²/V · sec
Concentration	$\bar{c}_L^L$	$= 0.01$ M
Counter constituent, γ-aminobutyric acid	m_{GABA}	$= 30 \cdot 10^{-5}$ cm²/V · sec
	$\mathrm{p}K_{\mathrm{GABA}}$	$= 4.03$
Electrical driving current	I	= 80 μA
Diameter of separation compartment	d_i	= 0.45 mm
Appearance of the first boundary	t_{detfix}	= 1112 sec
Amount of leading constituent sampled	Δn_L	= 100 nmole
Time delay to amount sampled	Δt	= 59.2 sec
Response	$\frac{\Delta n_L}{\Delta t}$	= 0.592 nmole/sec
Load of leading constituent	n_L^{load}	= 658 nmole
Transport number: experimental	T_{exp}	= 0.714
theoretical	T_{theor}	= 0.720
Transport efficiency: experimental	$\varepsilon_{\mathrm{exp}}$	= 71%
theoretical	$\varepsilon_{\mathrm{theor}}$	= 72%

Eqn. 3 is useful, as it provides a practical procedure for the determination of the amount of the leading constituent, once the ionic mobilities and t_{detfix} are known. It also provides a method for the determination of counter-constituent mobilities from experimental results, as

$$r_C = 1 - \frac{1}{T_{\mathrm{exp}}} \tag{4}$$

Obviously, a high transport efficiency, due to a low ionic mobility of the counter constituent, is always favourable as it guarantees efficient use of the power applied. For the operational systems given in Table I we can expect transport efficiences between 70 and 75%. The characteristics of a separation process can be evaluated by the injection of known amounts of sample[5]. An example of such a procedure is given in Fig. 1 and relevant parameters are summarized in Table III. The fact that, at a constant load of leading constituent, the first boundary will always be detected at the same time interval, t_{detfix}, is illustrated in Fig. 1 by the resolution line L/A. The low coefficient of variation confirms the excellent performance of the equipment. Injection of a small amount of sample will cause two zones, stacked between the leading constituent L and the terminating constituent T. A sample load of 1.3 μl of the constituent mixture (Fig. 1) where $n_A = 65$ will give a time-based zone length of 124.2 sec for constituent A and detection must be started 1112 sec after injection. The zone length of the second constituent, B, will be 148.1 sec. Other sample loads give

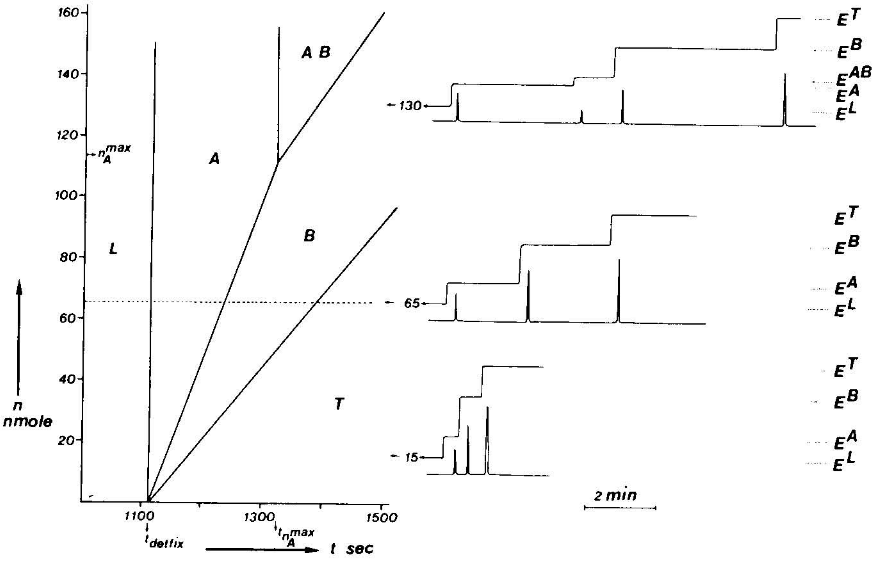

Fig. 1. Resolution lines for a two-constituent mixture. Operational system: Table I, system No. 2, and Table II. L = chloride; A = formic acid; B = glycolic acid; T = propionic acid; $E^{L,A,B,T}$ = electrical field strength; n = amount sampled.
Sample: $\bar{c}^{\mathrm{sample}}_{\mathrm{formate}} = 0.05\ M$; $\bar{c}^{\mathrm{sample}}_{\mathrm{glycolate}} = 0.05\ M$; $\mathrm{pH}^{\mathrm{sample}} = 3.00$.

TABLE III

RESOLUTION DATA

For operational system see Fig. 1 and Table II. Resolution line: $n = at + b$ (nmole).

Boundary	No. of determinations	*a*		*b*		Coefficient of variation or Correlation coefficient
		Experimental	*Theoretical*	*Experimental*	*Theoretical*	
L/A	53	0	0	−1112	−1112	0.8%
A/B	13	0.525	0.530	584	590	1.000
B/T	45	0.242	0.251	270	279	1.000
A/AB	6	0	0	−1328	−1317	0.4%
AB/B	6	0.321	0.316	314	312	0.998

Parameter	*Experimental*	*Theoretical*
Load of leading constituent (n_L^{load})	658	647
Maximal sample load ($n_A^{\max}$)	113	108
Separation number (S_A)	0.103	0.099
(S_B)	0.103	0.099
Load capacity (C_{load})	0.172	0.167
Separation efficiency (ε, %)	21	20

proportional zone lengths. The characteristics of these steady-state zones have already been discussed extensively and the close agreement of the calculated and experimental resolution lines, L/A, A/B and B/T, indicates the reliability of the calculations.

As the separation compartment has a limited load capacity, at a high load a mixed zone will be detected. The characteristics of these mixed zones are determined by both the leading electrolyte and the sample and are constant with time, as long as they exist. The time interval, $t_{n_A}{}^{\max}$, at which the mixed zone will be detected, is again constant, as illustrated in Fig. 1 by the resolution line A/AB:

$$t_{n_A}^{\max} = t_{\text{detfix}} \frac{\bar{m}_L^L E^L}{\bar{m}_B^M E^M} \tag{5}$$

The maximal zone length for the resolved constituent A, on a time base, is given by $t_{n_A}{}^{\max} - t_{\text{detfix}}$. The maximal sample load, $n_A^{\max}$, is given by the intercept of the resolution lines A/B, A/AB and AB/B. For the given pair of constituents, formate and glycolate, the maximal sample load was 113 nmole, which was close to the theoretical value (Table III). From the maximal sample load the load capacity[1], C_{load}, and the dimensionless separation number[1], S, can be calculated directly. Optimal column dimensions can be obtained from the load capacity, whereas the dimensionless separation number gives the relationship between amount sampled and electrical driving current or time for resolution. The appropriate procedure is given in Fig. 2.

From the load capacity, C_{load}, of 0.172, it follows that for a sample that contains 5 nmole of both constituents, 29.1 nmole of chloride are necessary. The available volume can now be calculated once the concentration of the leading constituent has been chosen, $\bar{c}_L^L = 0.01$ mole/l. If the inside diameter of the separation compartment is 0.2 mm, the length must be 9.25 cm. Obviously, if we had chosen a higher concen-

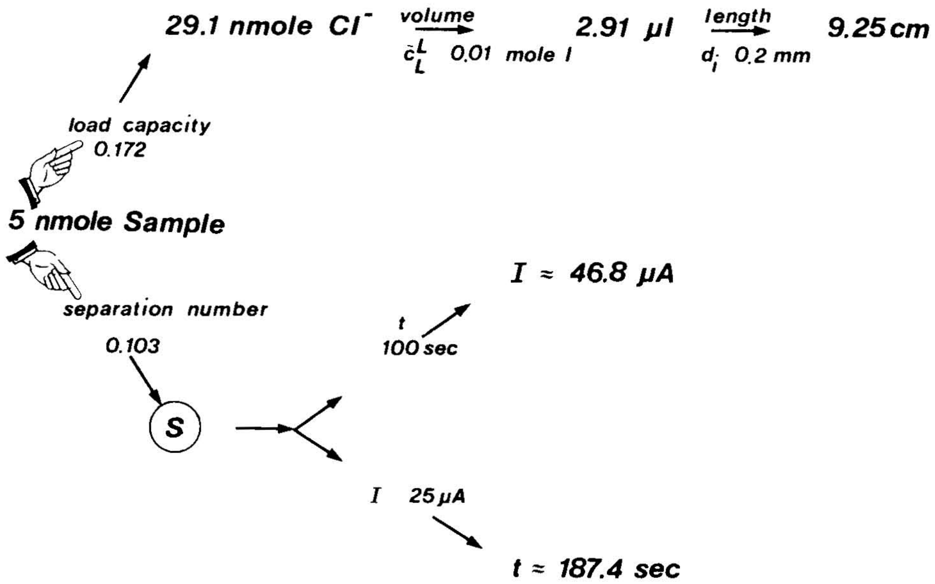

Fig. 2. Column evaluation.

tration of the leading constituent for the same dimensions of the separation compartment, the maximal amount of sample would have been proportionally higher. From the dimensionless separation number, $S = \frac{F}{I} \cdot \frac{n}{t_{res}}$ it follows that for a resolution time of 100 sec, an electrical driving current of 46.8 µA must be applied. At a driving current of 25 µA we can expect a resolution time of 187.4 sec. Although all resolution lines were determined experimentally, the load capacity and the separation number can be obtained with sufficient accuracy from only a few experiments, in which a mixed zone is present. The number of necessary determinations depends largely on the performance of the equipment. A high performance implies that the coefficients of variation in both t_{detfix} and $t_{n_A}{}^{\text{max}}$ are low. It should be emphasized that the column evaluation in Fig. 2 applies to only one specific sample. In general, a sample will show fluctuations in composition and an appropriate safety margin should be considered.

In analytical practice the fluctuations in composition may be due to concentration and/or pH. Once the extreme values of these fluctuations are known, the safety margin can easily be calculated.

In the experimental determinations, not only time events are being registered but also potential gradients. As eqn. 5 contains only one unknown quantity, $\bar{m}^M_B$, the effective mobility of the trailing constituent in the mixed zone can be obtained from the experimental results. For the glycolate constituent in the mixed zone of Fig. 1, it follows that

$$\frac{\bar{m}^{\text{Mixed}}_{\text{glycolate}}}{\bar{m}^{\text{Leading}}_{\text{chloride}}} = \frac{1112}{1328} \cdot 0.481 = 0.403$$

Provided that the ionic mobility of glycolic acid is known, the pH of the mixed zone can be derived. Using the appropriate data and relationships it follows that $\text{pH}^M =$

4.28, which is very close to the theoretically expected value of 4.30. From this we can evaluate how much the ratio of effective mobilities in the mixed zone differs from the critical value of unity at which no separation can occur:

$$\frac{\bar{m}^{\text{Mixed}}_{\text{formate}}}{\bar{m}^{\text{Mixed}}_{\text{glycolate}}} = 1.37$$

It follows that this 37% deviation from unity is responsible for the separation of the two sample constituents.

From Fig. 1 and Table III we conclude that even in the presence of a mixed zone, the relationship between the total zone length of the sample and the amount sampled is still linear. Deviations[10,11] from this rule are the result of experimental inaccuracies such as hydrodynamic flow, improper injection and mixing of leading electrolyte with sample and/or terminating electrolyte.

From the separation number in Table III it follows that the separation efficiency, ε, is about 20%. As the separation process is strongly dependent on the pH of both the sample and the leading electrolyte, optimization by choosing suitable electrolytes should be possible. The effect of the pH of the sample has been given in Fig. 3. Owing to the small difference in the dissociation constants of the sample constituents glycolic acid and formic acid ($\Delta pK \approx 0.08$), the effect on the separation efficiency of the pH of the sample is minimal. For the given pair of constituents the theoretical and experimental results show good agreement. When the difference in the

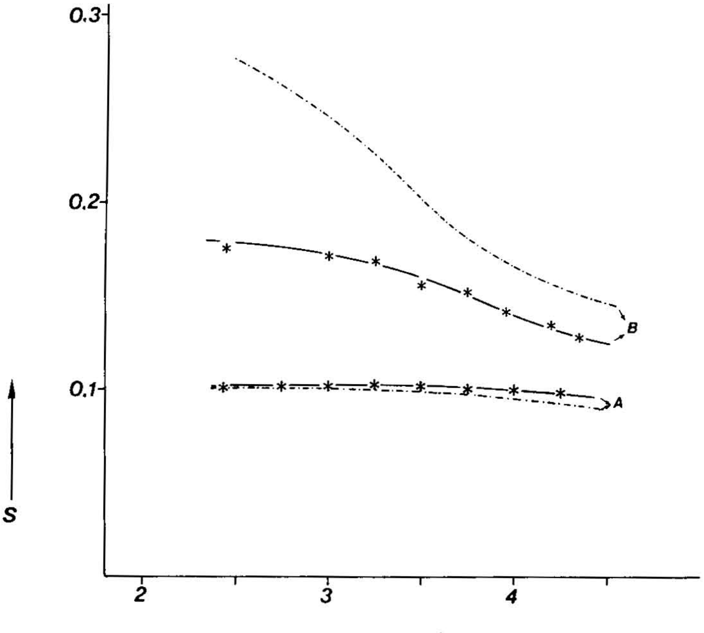

Fig. 3. Influence of the sample pH on the dimensionless separation number. Operational system: Table I, system No. 2. Sample: A = formate–glycolate, equimolar; B = chlorate–formate, equimolar. Solid line and points, experimental values; broken line, theoretical values.

dissociation constants is larger, the effect of the sample pH is much more pronounced, as confirmed by the constituent pair chlorate–formate. In this instance a low pH of the sample clearly favours resolution. Although theoretical considerations suggest a rapid, continuous increase in efficiency, decreasing the pH of the sample, the experimental curve indicates only a moderate increase. In the transient-state model[1] we made no allowance for the influence of a relatively high proton concentration at low pH. Functioning as a mobile counter constituent, protons, at a relatively high concentration, will decrease the efficiency of the separation process. When the constituents have only a small difference in their dissociation constants (formic acid–glycolic acid), the theoretical and experimental results will show only small differences.

When the pK values of the constituents differ substantially, higher pH shifts can be expected and larger deviations result, as confirmed by the constituent pair chlorate–formate.

Irrespective of the numerical discrepancy, for anionic separations a low pH of the sample favours resolution. Appropriate incorporation of the hydrogen and/or hydroxyl constituent into the relevant mathematical formulations is still under investigation, but seems complicated.

In common practice the pH of the sample shows only a small degree of freedom and the most useful optimization parameter is the pH of the leading electrolyte. In most instances a low pH of the leading electrolyte will increase the efficiency of the separation process, dealing with anionic separations. Table IV gives some experimental and theoretical results for the constituents in Fig. 3. From both the theoretical and experimental results it follows that for constituents that have only a small difference in their pK values, the pH of the leading electrolyte is not very useful for optimization. In our theoretical considerations we showed that the ratio of effective constituent mobilities is of importance when considering separability and separation efficiency.

Obviously, the pH of the leading electrolyte has only a minor influence on the mobility ratio when the pK differences are small. For the two samples in Table IV, the ratio of effective mobilities is given in Fig. 4 as a function of the pH of the mixed zone. It can easily be shown that for the limiting values of the mobility ratio it is valid that:

$$10^{(\mathrm{p}K_A - \mathrm{p}K_B)} \cdot \frac{m_B}{m_A} < \frac{\bar{m}_B}{\bar{m}_A} < \frac{m_B}{m_A} \tag{6}$$

TABLE IV

INFLUENCE OF THE pH OF THE LEADING ELECTROLYTE ON THE DIMENSIONLESS SEPARATION NUMBER

Parameter		*Constituents*			
		Chlorate–formate		*formate–glycolate*	
Concentration (M)		-0.05,	0.05	-0.05,	-0.05
$\mathrm{pH}^{\mathrm{sample}}$		2.41		2.51	
pH^L	*Counter constituent*	S_{exp}	S_{theor}	S_{exp}	S_{theor}
3.60	BALA	0.259	0.368	0.095	0.099
4.03	GABA	0.179	0.275	0.098	0.100
6.02	HIST	0.075	0.105	0.101	0.092

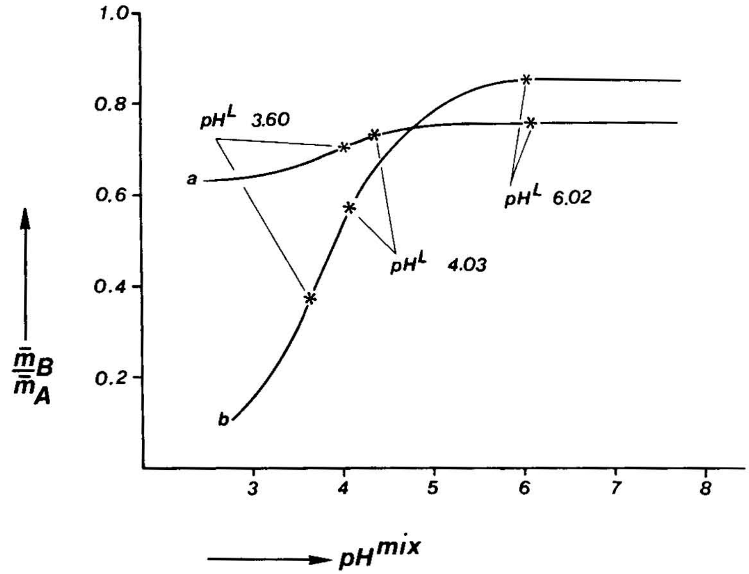

Fig. 4. Influence of the pH of the mixed zone on the ratio of effective constituent mobilities. Operational systems: Table I. Sample: (a) formate (*A*)-glycolate (B); (b) chlorate (*A*)-formate (*B*).

Therefore, for the constituent pair chlorate–formate the ratio of effective mobilities can vary between its maximal value of 0.764 at high pH and its minimal value of 0.635 at low pH. Hence any pH shift, due to either the leading electrolyte or the sample, has hardly any influence on the separation efficiency. Owing to the relatively low mobility of the counter constituent histidine, the separation at $pH^L = 6.02$ has the greater efficiency, although the differences are slight. When differences in pK_A values are more substantial, the rationale for optimization is clearer, as can be seen from the second constituent pair, chlorate–formate, in Table IV and Fig. 4. In this instance the ratio of effective constituent mobilities can vary between zero at low pH and 0.846 at high pH, and therefore the pH can be of great importance. A high pH of the leading electrolyte will cause a high pH of the mixed zone, resulting in an unfavourable ratio of effective constituent mobilities. A low pH of both the leading electrolyte and the sample will result in an optimal ratio and therefore optimal separation efficiency.

From Fig. 4 it can also be seen that for small differences in pK values, the difference in the pH values of the mixed zone and the leading zone is relatively high. When the sample contains stronger acids, this difference is considerably smaller, resulting in a relatively low pH of the mixed zone. Whenever the pH of the leading electrolyte is substantially higher than the pK values of the constituents to be separated, the difference will be small and the constituents will be resolved as ionic species. From the examples given it follows directly that a separation based on pK values is generally more efficient than one based on ionic mobilities.

For anionic straight pairs of constituents, where $m_B < m_A$ and p$K_A <$ pK_B, the rationale for optimization is straightforward: low pH of the leading electrolyte and the sample. With anionic reversed pairs[1], where $m_B < m_A$ and p$K_A >$ pK_B, this

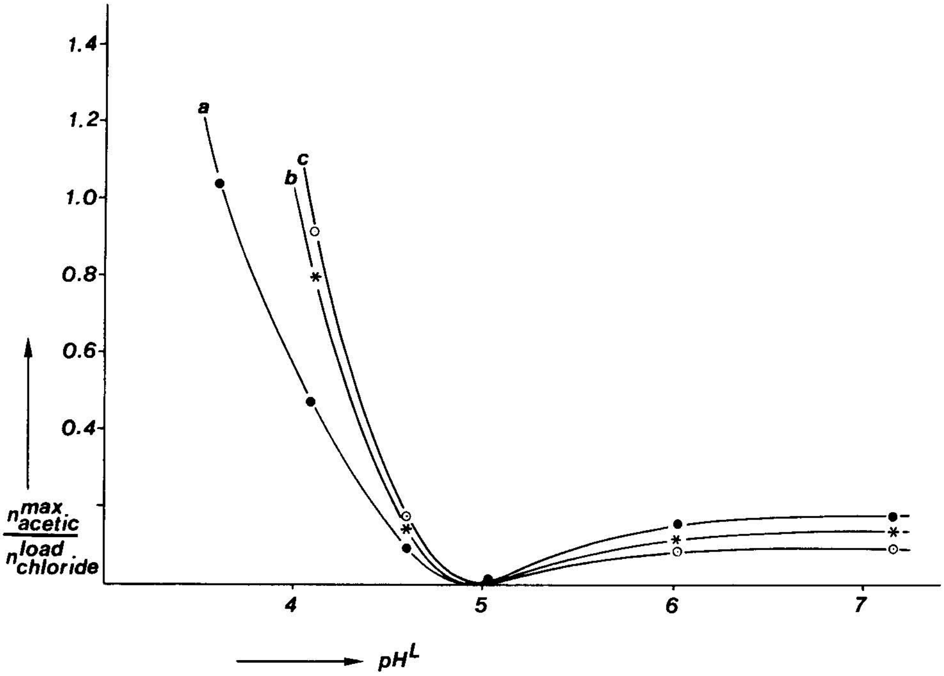

Fig. 5. Influence of the pH of the leading electrolyte on the load capacity for a reversed pair of constituents. Operational systems: Table I. Constituent data: Table V. Sample: acetate–naphthalene-5-sulphonate. (a) $pH^{sample} = 7.0$; (b) $pH^{sample} = 4.75$; (c) $pH^{sample} = 3.00$.

rationale is more complicated. It has been shown that for such pairs a pH will exist, pH^{MO}, at which no separation occurs[1]. Of course, this pH will cause an infinite time for resolution, zero separation number and zero load capacity. Moreover, at this critical pH the order in which the constituents migrate will be reversed. Experimental results concerning the load capacity for a reversed pair are given in Fig. 5 as a function of the pH of the leading electrolyte and the sample. The experimental curves confirm the theoretically predicted behaviour. Using the appropriate data (Table V) and relevant mathematical formulations[1], it follows that the criterion for separation[1] will not be satisfied at a mixed zone pH of 5.19. Obviously, this pH can be generated by numerous combinations of leading electrolytes and sample compositions. Working at the maximal buffering capacity of the common counter constituent, *i.e.*, $\varrho = -2$

TABLE V

DATA FOR A REVERSED PAIR OF SAMPLE CONSTITUENTS

Constituent	*Mobility* ($cm^2/V \cdot sec$)	*pK*	*Concentration (M)*	pH^{sample}
Acetate	$41 \cdot 10^{-5}$	4.75	−0.005	4.75
Naphthalene-2-sulphonate	$-30 \cdot 10^{-5}$	0	−0.005	4.75

No separation at $pH^{MO} = 5.19$
No separation at $pH^{L} = 4.98$ ($\varrho = -2$, $m_C = 30 \cdot 10^{-5}$ cm²/V · sec)

or $pH^L = pK_C$, and introducing an acceptable ionic mobility for the counter constituent, $m_C = 30 \cdot 10^{-5}$ cm²/V·sec, the critical pH of the leading electrolyte, at which no separation occurs, is 4.98.

This was confirmed experimentally by the separation at $pH^L = 5.04$, at which hardly any load capacity was present. At a pH^L higher than the critical value, sample constituents migrate in order of ionic mobilities, and separations can be performed with only moderate efficiency. At low pH^L, however, constituents are migrating in order of their pK values and a much greater efficiency can be obtained, resulting in a high load capacity. For example, the resolution of a 1.5-nmole sample (an absolute amount that can be detected without difficulty) would take about 18 sec, $S = 0.26$, at $pH^L = 4.10$ and $pH^S = 3.00$, whereas the same sample can be resolved in 105 sec, $S = 0.045$, at $pH^L = 7.10$.

The required length of the separation compartment in the former instance is 5.8 times shorter than in the latter. Obviously, for specific samples rigid optimization procedures can be followed, resulting in very short analysis times, small dimensions of the separation compartment and efficient use of the power applied. It must be emphasized, however, that the success of optimization procedures depends largely on the physico-chemical characteristics of the species to be separated and the performance of the equipment. When there are only small differences in ionic mobilities and dissociation constants, optimization procedures are elaborate and result in only a small increase in efficiency. The results from Fig. 5 confirm the predicted behaviour[1] that for anionic reversed pairs a high pH of the leading electrolyte is best, combined with a high pH of the sample, whereas at low pH^L the separation efficiency is favoured by a low sample pH. The theoretical background for this exceptional behaviour has already been extensively discussed. An evaluation of the dual separation phenomenon[1] will be given in a later paper.

Another important conclusion can be drawn from the results shown in Fig. 5. It follows that efficient separations can be achieved whenever the sampling ratio, φ (i.e., the concentration of the charged trailing species divided by the concentration of the charged leading species), is small. Biochemical samples often contain substantial amounts of very mobile species such as chloride or perchlorate. Such samples represent typical low "φ cases", which can be separated on a relatively short column. The time of analysis, however, will increase substantially in the presence of these mobile species. In very special cases lowering of the sample pH by the addition of, for example, hydrochloric acid will increase the load capacity by the suggested mechanism.

The dimensionless separation number seems to be a reliable quantity for describing the separation performance, as it is independent of operating conditions such as the electrical driving current and column geometry. Table VI gives the observed experimental efficiencies for the same sample under different operating conditions. From these results it follows that, although differences in the separation numbers occur, the overall efficiency is not significantly different. In our theoretical considerations we introduced relative mobilities to suppress the influence of temperature effects. Comparing literature data on ionic mobilities[6-8], it must be concluded that temperature effects are eliminated only partially in this way, as many non-linear effects occur. Moreover, when considering temperature effects, dissociation constants should also be corrected. Mathematical iteration procedures to involve temperature

TABLE VI

INFLUENCE OF THE INSIDE DIAMETER OF THE NARROW BORE TUBE ON THE DIMENSIONLESS SEPARATION NUMBER

Sample: A, formate (= 0.05 *M*); B, glycolate (= 0.05 *M*); pH^{sample} = 2.41. For operational system, see Table I, system No. 2.

Parameter	*Inside diameter* (d_i, *mm*)		
	0.45	*0.20*	*0.15*
Driving current (I, μA)	80	25	25
Sample load (n_A^{max}, nmole)	87.5	23.5	16.6
(n_B^{max}, nmole)	87.5	23.5	16.6
Time for resolution (t_{res}, sec)	1081	1030	703.7
Separation number (S_{theor})	0.100	0.100	0.100
($S_{A,exp}$)	0.098	0.088	0.091
($S_{max,theor}$)	1.000	1.000	1.000
Efficiency (ε, %)	19.6	17.6	18.2

corrections for various physico-chemical parameters can be introduced into the transient-state model with a probable consequent increase in accuracy. There is, however, a lack of reliable data on physico-chemical parameters and the present model is sufficiently reliable, the predicted parameters being confirmed experimentally.

From the theoretical formulations, it follows that the measurement of steady-state effective mobilities can only be used as an indication for experimental separability[1]. Constituents that have equal steady-state effective mobilities can sometimes be separated efficiently. Moreover, it has been shown that enforced isotachophoretic configurations[12] in which a more mobile is migrating behind a less mobile constituent are stable with respect to time. An example is given in Fig. 6.

Several sample constituents confirm the general principle that constituents in isotachophoresis are migrating at equal velocity in order of decreasing effective mobilities. The constituents lactic and mandelic acid (constituents 3 and 4), however, show virtually no difference in effective mobilities, as for their isotachophoretic migration the same electrical gradient seems to be necessary (Fig. 6 and Table VII). From the linear conductance trace it appears that this pair has not resolved during the separation process. The UV trace, however, indicates clearly that the mandelic acid (3) has been resolved from the lactic acid (4) and that the former migrates in front of the latter. The transient-state model[1] reveals that the pH of the mixed zone, from which the pure zones are formed, is just below the critical pH of 4.32 at which no separation occurs. As this is a reversed pair, the mandelic acid will be resolved in front of the lactic acid.

From the data in Table VII it follows that the experimental and theoretical zone characteristics are in good agreement. The minor difference between the transient-state and the steady-state results, X3 (ref. 12), has already been mentioned. The occurrence of a lactic acid ion in the resolved mandelic acid zone will cause a 2.6% deviation from the critical value of unity for the ratio of effective constituent mobilities. Mandelic acid ions, in the resolved lactate zone, would lead to a 1% deviation. From the UV trace it follows that these deviations are large enough to guarantee a sharp separation boundary. The theoretical calculations show a greater difference and for the ratio of effective mobilities in the mixed zone a 2.6% deviation from unity

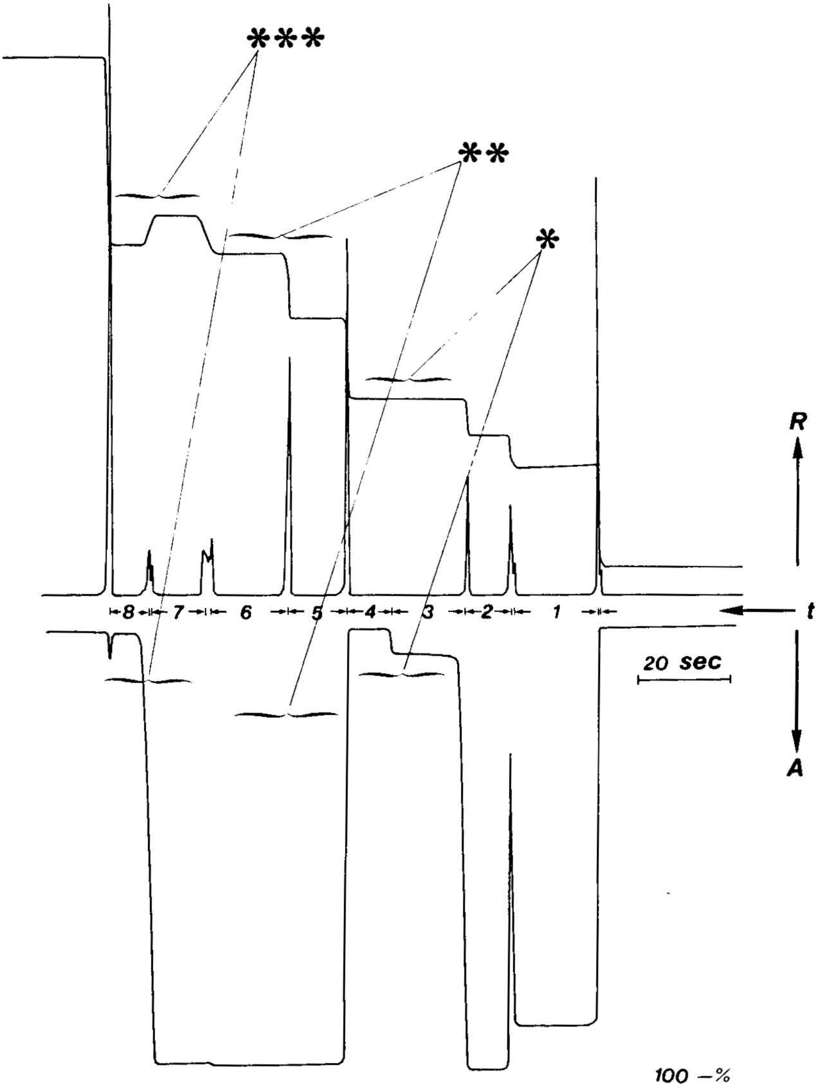

Fig. 6. Isotachophoretic steady-state configurations. Operational system: Table I, system No. 2, $pH^L = 3.95$. R = resistance; A = UV absorption at 254 nm; t = time. 1 = Adenosine-5′-triphosphoric acid (A5TP); 2 = sulphanilic acid; 3 = DL-mandelic acid; 4 = DL-lactic acid; 5 = guanosine-5′-monophosphoric acid (G5MP); 6 = adenosine-3′-monophosphoric acid (A3MP); 7 = adenosine-5′-monophosphoric acid (A5MP); 8 = acetic acid. * A pair of constituents for which conductometric detection indicates no resolution whereas UV detection does; ** a pair of constituents for which UV detection indicates no resolution whereas conductometric detection does; *** a pair of constituents in an enforced isotachophoretic configuration.

was calculated. The experimental separation confirms that this deviation is sufficient to obtain resolution. It must be emphasized, however, that the small deviation results in a low separation efficiency and column overloading can easily occur. Fig. 6 nevertheless indicates clearly that isotachopherograms in which only one detection system

TABLE VII

COMPARISON OF ZONE CHARACTERISTICS

Trans = computerized transient-state model[5]; X3 = computerized steady-state model[12].

Parameter	*Chloride zone, experimental*	*Mandelate zone*			*Lactate zone*		
		Trans	*X3*	*Experimental*	*Trans*	*X3*	*Experimental*
p*K*	2	3.37			3.86		
m (cm²/V · sec)	$-77 \cdot 10^{-5}$	$-28 \cdot 10^{-5}$			$-33 \cdot 10^{-5}$		
pH	3.95	4.21	4.22	4.25	4.27	4.28	4.29
E^L/E^X	1.00	0.319	0.320	0.322	0.318	0.319	0.322
c_X^X (mM)	−10.00	−6.47	−6.34		−7.16	−7.06	
$\dfrac{\bar{m}^{\text{mandelate zone}}_{\text{lactate}}}{\bar{m}^{\text{mandelate zone}}_{\text{mandelate}}}$		0.956	0.960	0.974			
$\dfrac{\bar{m}^{\text{lactate zone}}_{\text{mandelate}}}{\bar{m}^{\text{lactate zone}}_{\text{lacate}}}$					1.027	1.017	1.010
$\dfrac{\bar{m}^{\text{mixed zone}}_{\text{mandelate}}}{\bar{m}^{\text{mixed zone}}_{\text{lactate}}}$	1.027						

is used must be interpreted with great care. The same applies, of course, when only UV detection is used. From the UV trace in Fig. 6 it would be concluded that the nucleotides G5MP and A5MP have not been resolved. The conductance trace, however, clearly confirms the separation of these two constituents. On most occasions small amounts of impurities, with either UV-absorbing or non-UV-adsorbing properties, will indicate the separation boundary. Moreover, in this particular instance, a difference is visible when the UV results are being traced in the absorbance mode.

The sample constituents A5MP and acetate (constituents 7 and 8) are migrating in an enforced isotachophoretic configuration. The effective mobility of the acetate constituent in its proper zone is higher than that of the nucleotide A5MP in its proper zone, as indicated in Fig. 6 by the lower conductance of zone 7 in comparison with zone 8. For the relative effective mobilities it follows that $\bar{m}^{\text{Acetate}}_{\text{Acetate}}/\bar{m}^{\text{Chloride}}_{\text{Chloride}} = 0.212$ and $\bar{m}^{\text{A5MP}}_{\text{A5MP}}/\bar{m}^{\text{Chloride}}_{\text{Chloride}} = 0.198$.

The 7% deviation from unity of the mobility ratio ($\bar{m}^{\text{A5MP}}_{\text{A5MP}}/\bar{m}^{\text{Acetate}}_{\text{Acetate}} = 0.93$) allows a satisfactory sharpness of the separation boundary between the two constituents. The reason for the stability can be found in the difference in the pH values in the two resolved zones. Using the appropriate relationship it follows that the pH of the acetate zone is 4.57. A nucleotide ion, lost owing to convection or diffusion from its proper zone (7) into the acetate zone, will migrate with an higher effective mobility than that of the acetate constituent. In the nucleotide zone the pH is 4.32, so any acetate ion in the nucleotide zone will migrate with a considerably lower velocity than the nucleotide $\bar{m}^{\text{A5MP}}_{\text{A5MP}}/\bar{m}^{\text{A5MP}}_{\text{Acetate}} = 1.37$.

Hence the self-restoring capabilities of the separation boundary allow the enforced isotachophoretic configuration to be stable with respect to time. It should be noted, however, that enforced isotachophoretic configurations will not be encountered frequently in practice.

From both our previous theoretical considerations[1] and the experimental evaluation presented here, it follows that through optimization a considerable increase in separation efficiency and load capacity and a decrease in time for resolution can be obtained. It must be emphasized that the success of such a procedure depends largely on the nature of the sample. We restricted our theoretical and experimental studies mainly to two constituent samples but the same optimization rationales hold, to a lesser extent, for multi-constituent samples[5]. For very complex mixtures, in which multi-component information must be obtained, optimization can sometimes be elaborate and difficult. Analyses in more than one operational system are inevitable. Moreover, in one-component analyses of multi-constituent samples a considerable amount of effort is put into the separation of constituents of little interest. Column-switching techniques[13] may prove useful here.

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RETENTION DATA OF CHROMIUM CHELATES WITH DIFFERENT β-DIKETONES AS LIGANDS

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SUMMARY

Relative retention data and relative heats of solution were determined for chromium chelates with five different ligands on eleven non-polar and moderately polar stationary phases in order to find the minimum retention times. It was found that minimum retention was observed for the second or third members of the homologous series of fluorinated chromium chelates. Partial fluorination of the ligands decreased the retention times of chromium chelates, particulary on stationary phases with a large number of phenyl groups in the molecule. The relative molar entropy of solution is the main factor that decreases the retention times of the fluorinated chelates.

INTRODUCTION

Mixtures of some metals can be analysed as chelates with extremely high sensitivity by gas chromatography (GC). Although the GC analysis of 46 metal chelates has been reported[1–7], quantitative results have been given for only nine metals on a level of 10^{-8}–10^{-14} g. Poor reproducibility, chemical reactions occurring in the column and high interphase adsorption limit the quantitative analysis of chelates[6–9]. The appearance of many peaks due to decomposition products has been reported, and some attempts have been made to minimize the retention times of the chelates in the column.

The retention time can be decreased by (a) increasing the flow-rate of the carrier gas, (b) decreasing the amount of the stationary phase in the column, w_L, (c) increasing the column temperature, (d) choosing an appropriate stationary phase and (e) choosing appropriate ligands for the chelates. The carrier gas flow-rate is of limited use in achieving an acceptable column performance; decreasing w_L seems to be the most promising technique[1,6]. However, interphase adsorption takes part in such changes and it is not desirable to use packings with $w_L < 3\%$[9]. When the column temperature is increased, the decomposition rate is also increased. Hence, factors (d) and (e) seem to be the most promising for resolving the problem of chelate analysis.

Partial fluorination is a technique commonly used to increase the volatility of chelates[1–4,7,10,11]; a decrease in the Van der Waals forces has been reported as the reason for this effect. However, the stability of the fluorinated chelates is lower than that of the corresponding non-fluorinated chelates[12,13]. Trifluoroacetylacetone (HTFA) and hexafluoroacetylacetone (HHFA) are the most commonly used ligands (Table I); the latter is used less often because of its higher reactivity. It should be noted that the choice of the fluorinated ligands for metal chelates in previous work was arbitrary and no systematic reports have been published about the effect of the fluorination of different acetylacetone homologues on retention time. Moreover, some workers[14–16] have reported that in some instances the fluorination of the ligands does not increase the volatility of the chelates obtained. The question of the choice of stationary phase has not been studied adequately: only a few examples of the reaction of chelates with polar stationary phases have been reported[17]. No attention has been paid to the thermodynamic properties of the stationary phases, selectivity or chelates with different ligands.

The aim of this work was to compare different fluorinated and non-fluorinated ligands and some non-polar and low-polarity stationary phases in order to determine conditions for obtaining minimal retention times of the chelates. Stable chromium chelates were chosen.

EXPERIMENTAL

Retention data were measured with Tsvet-104 gas chromatograph with a flame-ionization detector and a flow-rate of carrier gas (argon) of 25 ml/min. A glass-lined injector (heated to 250 °C) and glass columns (1 m × 3 mm I.D.) were used. The column temperature was maintained to within 0.15 °C. The contact time of the chelates with the stainless-steel capillary connecting the column exit with the detector was about 0.1 sec.

In order to prevent irreversible chelate adsorption on the glass-wool plug, the latter was treated with phosphorous acid and the amount of it was kept to the minimum.

Stationary phases (5%) were loaded on the support (Chromaton N AW HMDS, 0.125–0.16 mm; Lachema, Brno, Czechoslovakia) from chloroform solution. The following stationary phases were used (in order of increasing polarity): polyisobutylene (synthesized, average molecular weight 2000, polar impurities removed with silica gel), silicones E-301, OV-1, OV-101 (methylsilicones), SE-52 (5% of phenyl groups), SE-54 (5% of phenyl, 1% of vinyl groups), OV-17 (50% of phenyl groups), PPMS-6 (50% of phenyl groups, manufactured in the U.S.S.R.), 6-ring polyphenyl ether (PPE, manufactured in the U.S.S.R.), silicone elastomer SKTFT-50 (fluorosilicone, manufactured in the U.S.S.R.) and silicone OV-225 (25% of phenyl, 25% of cyanopropyl groups).

To investigate the dependence of the retention data on fluorine in the molecule different ligands were synthesized (Table I). To compare the retention data of fluorinated and non-fluorinated ligands, ligand 3 (Table I) was also prepared. The

TABLE I

SOME β-DIKETONES USED AS LIGANDS FOR CHELATES

No.	*Formula*	*Name*	*Abbreviation*
1	CF_3–CO–CH_2–CO–CH_3	1,1,1-Trifluoropentane-2,4-dione	HTFA
2	CF_3–CO–CH_2–CO–CF_3	1,1,1,5,5,5-Hexafluoropentane-2,4-dione	HHFA
3	CH_3–CO–CH_2–CO–$C(CH_3)_3$	2,2-Dimethylhexane-3,5-dione	HPM
4	CF_3–CO–CH_2–CO–$C(CH_3)_3$	1,1,1-Trifluoro-5,5-dimethylhexane-2,4-dione	HTPM
5	C_2F_5–CO–CH_2–CO–$C(CH_3)_3$	1,1,1,2,2-Pentafluoro-6,6-dimethylheptane-3,5-dione	HPPM
6	C_3F_7–CO–CH_2–CO–$C(CH_3)_3$	1,1,1,2,2,3,3-Heptafluoro-7,7-dimethyloctane-4,6-dione	HHPM
7	C_4F_9–CO–CH_2–CO–$C(CH_3)_3$	1,1,1,2,2,3,3,4,4-Nonafluoro-8,8-dimethylnonane-5,7-dione	HNPM

ligands were prepared by condensation of pinacolone with methyl esters of different acids in the presence of sodium methylate. Diketones were extracted as copper salts, which were then decomposed with sulphuric acid; free diketones were extracted with diethyl ether and distilled. GC analysis showed that the amount of impurities in the ligands was 1.5%. The identities of the ligands were confirmed by nuclear magnetic resonance spectroscopy.

Chromium(III) chelates were prepared by adding stoichiometric amounts of β-diketones to a boiling methanolic solution of chromium nitrate at *ca.* pH 6. Chromium chelates with HPM, HTPM and HNPM (for abbreviations see Table I) are dark-green greases; after distillation *in vacuo* they become dark red, slowly crystallized substances. The chromium chelate with HPPM is a dark-red grease, and that with HHPM a dark-brown solid.

The retention data were measured as the relative retentions, r, anthracene being the standard. In some instances when the retention time of anthracene was relatively large, naphthalene was used as the standard and then all relative retentions were re-calculated to anthracene as the standard. Relative retention data were determined at 3–5 different temperatures. Relative molar heats of solution, ΔH_s^0, were calculated using the relationship log r *versus* $1/T$. Relative mean standard deviations were 0.2 and 5% for r and ΔH_s^0, respectively.

To determine reproducible retention data, the dependence of retention time, t_N (the difference between the retention time of the solute and that of a non-sorbed gas, methane) on peak height, h, was established for the solutes. In many instances no such dependence was found; when a dependence of t_N on h was observed, the retention data were measured at a constant h value (so-called isobaric retention data). To calculate t_N at constant h the following empirical equation[18] was used:

$$t_N = \frac{A_1}{\log h} + B \qquad (1)$$

where A_1 and B are constants. This equation is valid for a restricted range of h (about 2–3 orders of magnitude) for the "adsorption" region of the relationship between t_N and h; increasing t_N with decreasing h is observed in this region.

When a dependence of t_N on h was observed, the isobaric retention data were calculated at $1/\log h = 0.4$ (h is measured in millimetres, using a 250-mm recorder chart, re-calculated to full-scale 10^{-11} A).

To evaluate the retention times of the chelates under the same conditions we used the t_N/t_0 ratio (where t_0 is dead retention volume) as the value proportional to retention volume because $1/t_0$ is proportional to flow-rate. If w_L, the column temperature and the flow-rate are kept constant, the t_N/t_0 ratio is the relative value of the retention time for the solute on different packings.

RESULTS AND DISCUSSION

Dependence of retention time on peak height

The dependence of t_N on h is a curve with a minimum[18]. Let us restrict the discussion to only the "adsorption" section of the relationship between t_N and h because large samples are required for other parts of the curve. To evaluate the non-linearity of the sorption isotherm one can use the slope of the relationship between t_N and $1/\log h$. A relative value of this slope can be calculated by using the retention times, t_N, measured at $1/\log h$ equal to 0.4 and 0.5 ($t_{N\,0.4}$ and $t_{N\,0.5}$, respectively):

$$A = \frac{t_{N0.5} - t_{N0.4}}{t_{N0.4}} \qquad (2)$$

Table II shows that the dependence of t_N on h is observed only at low column temperatures and for $CrPM_3$ and $CrNPM_3$. When using column temperatures of 469–487 °K the A values are negligibly small on most of the stationary phases.

A marked effect of h on r is seen only when using the non-polar stationary phase polyisobutylene. The A value increases with increasing length of the fluorinated functional group in the ligand. A similar effect is seen for the packings with PPE and OV-225, but in this instance interphase adsorption acts on the solid surface.

TABLE II

A VALUES OF CHROMIUM(III) CHELATES AT DIFFERENT COLUMN TEMPERATURES ON VARIOUS PACKINGS

Chelate	*OV-225, 420° K*	*PIB*			*PPE, 420° K*
		416° K	*424° K*	*431° K*	
$Cr(PM)_3$	Decomp.	0.026	0.015	0.009	Decomp.
$Cr(TPM)_3$	0.000	0.000	0.000	0.000	0.000
$Cr(PPM)_3$	0.000	0.000	0.000	0.000	0.002
$Cr(HPM)_3$	0.009	0.006	0.008	0.000	0.100
$Cr(NPM)_3$	0.029	0.022	0.018	0.011	0.120

When using low column temperatures (about 140 °C) partial decomposition of the non-fluorinated chelates on PPMS-6, PPE, OV-225 and SKTFT-50 was observed: four peaks appeared on the chromatogram instead of only one. This is additional proof of chelate decomposition with long contact times with the packing.

Taking into account these effects, the following determinations of retention data were carried out under conditions such that t_N does not depend on h and no decomposition is observed.

Retention data for homologeous series of fluorinated ligands

Ligands 3–7 (Table I) differ in the number of CF_2 groups in the molecule, and therefore the chromium chelates with these ligands differ in the number of these groups (C_F). A linear relationship between log r and the number of carbon atoms in homologeous molecules has often been reported, except for the first members of the series; only in rare instances have some minima been observed (for example, n-alcohols on glycerol[19]). Fig. 1 shows the non-linear relationship between log r and C_F and the minimum is observed for the chelates with $C_F = 6$–9. A marked minimum is noted for the stationary phases with a large number of phenyl groups in the molecule. The polarity of the stationary phase does not seem to be an important factor for the depth of the minimum.

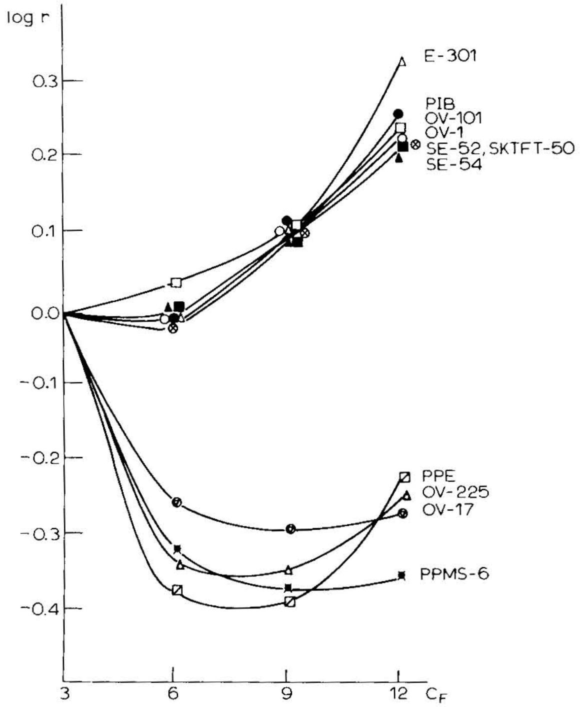

Fig. 1. Relationship between log r and number of fluorinated carbon groups in molecule of chelate, C_F.

The marked decrease in the relative retentions for PPE, OV-225, OV-17 and PPMS-6 for $C_F \approx 6$–9 is of great interest in attempts to reduce the retention times of the chelates, and the reason for this effect therefore has to be established. The dependence of ΔH_s^0 for chromium chelates on C_F is shown in Fig. 2. An almost linear

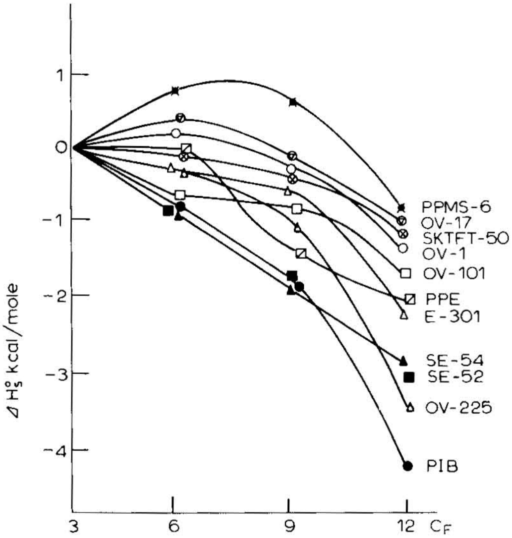

Fig. 2. Relationship between relative molar heats of solution, ΔH_s^0, and number of fluorinated carbon groups, C_F.

relationship between ΔH_s^0 and C_F is observed for PIB, E-301, SE-52 and SE-54; a slight maximum at $C_F = 6$ is found for PPMS-6 and OV-1.

The non-linear relationship between ΔH_s^0 and C_F may be explained as follows. It was reported[20] that for aromatic hydrocarbons and olefins hyperconjugation of methyl groups with π-electrons increases the intermolecular forces for the solutes. When a methylene group is inserted between a methyl group and a system with double bonds (for example, in passing from toluene to ethylbenzene), the effect of hyperconjugation disappears. By analogy with alkylbenzenes it is reasonable to assume that the same effect will occur for the fluorinated ligands where, in passing from $-CF_3$ to $-CF_2-CF_3$, the intermolecular interaction decreases.

On comparing Figs. 1 and 2 it can be seen that only in a few instances do the minima in Fig. 1 coincide with the maxima in Fig. 2; therefore, some other factor must determine the minimum value for chromium chelates. The only remaining term is the relative molar entropy of solution, ΔS_s^0. Using the basic thermodynamic relationships we can write the following equation for the distribution coefficient, K:

$$-RT \ln K = \Delta H_s - T\Delta S_s \tag{3}$$

where ΔS_s is the molar entropy of solution.

Taking into account that $r = K_x/K_{st}$ (the subscripts x and st refer to the solute and the standard solute, respectively), eqn. 3 can be rewritten in terms of relative molar heat of solution and relative molar entropy of solution:

$$-RT \ln r = \Delta H_s^0 - T\Delta S_s^0 \tag{4}$$

Because of the compensation effect between ΔH_s and ΔS_s, the entropic selectivity term, F^0, has been proposed[21], which relates to the loss of rotational entropy during solution:

$$F^0 = \Delta S_s^0 - \frac{0.435}{T} \cdot \Delta H_s^0 \qquad (5)$$

where ΔH_s^0 is measured in calories per mole.

The variation of F^0 with C_F is shown in Fig. 3. The stationary phases with a large decrease in F^0 in the homologous series (PPE, OV-17, PPMS-6 and OV-225), which is not compensated for by the corresponding variation in ΔH_s^0, show a marked minimum in the relationship between log r and C_F.

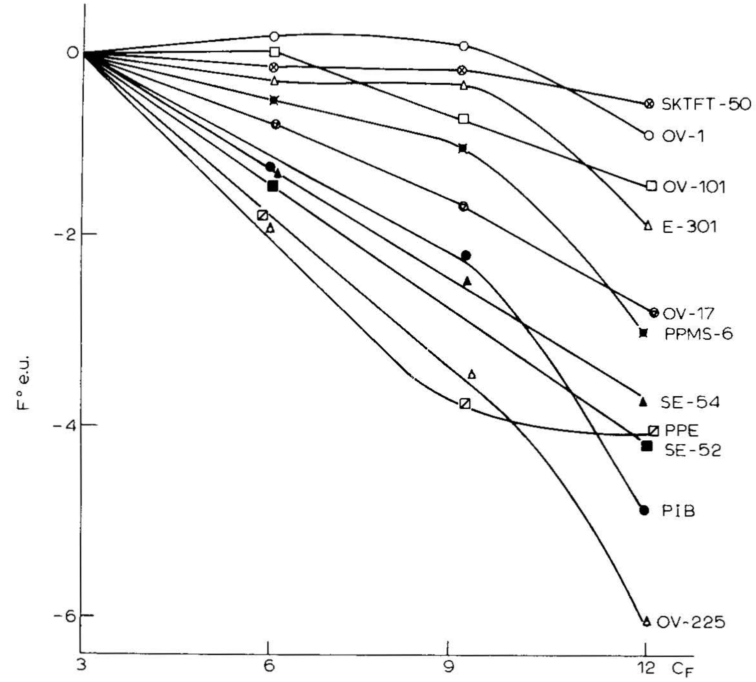

Fig. 3. Relationship between entropic selectivity of stationary phase, F^0, and number of fluorinated carbon groups, C_F.

Comparing the results in Figs. 1–3, one can conclude that the appearance of a minimum in the relationship between log r and C_F relates mainly to an entropic factor of selectivity of the stationary phases. The presence of phenyl groups in the stationary phase molecules is the main reason for achieving a minimum in the retention time for members of homologous series with $C_F \approx 6$–9.

Effect of fluorination of methyl group on retention time of chalates

As a rule, the vapour pressure of a compound increases on fluorination. In spite of this rule, the ratio of the relative retention of a fluorinated chelate (ligand 4, Table I) to the corresponding non-fluorinated chelate (ligand 3, Table I), r^*, is greater than unity for many stationary phases (Table III). This shows that fluorination of the methyl group reduces the retention times of the chelates in only a few instances (stationary phases OV-225 and PIB). The determination of ΔH_s^0 shows that after fluorination the relative molar heats of solution increase from 1.6 to 3.5 kcal/mole, which leads to an increase in the relative retentions of the fluorinated chelates. These results are in good agreement with the increase in the polarity of substances after partial fluorination: solutes with a CF_3 group are more polar than the corresponding hydrocarbon chelates.

TABLE III

RETENTION PARAMETERS OF CHROMIUM(III) CHELATES

The first three columns refer to comparison of non-fluorinated and fluorinated chelates.

Stationary	r^*	ΔH_s^0 *(kcal/mole)*	F^0 *(e.u.)*	r_1	t_N/t_0	*Minimum relative retention time*
PIB-2000	0.95	−1.6	−4.08	0.92	21.0	2.15
E-301	2.95	2.16	−1.46	2.95	22.0	13.6
OV-1	3.03	3.45	−2.12	2.98	21.8	13.2
OV-101	2.98	−2.4	−1.13	2.93	15.7	9.9
SE-52	2.85	2.95	−1.96	2.81	26.1	13.2
SE-54	2.65	−2.2	−1.06	2.58	24.5	11.9
OV-17	1.47	−2.35	−2.42	0.74	64.4	4.9
OV-225	0.78	−3.5	−14.55	0.36	59.8	3.1
PPMS-6	1.31	−3.5	−12.9	0.55	66.6	59.2
SKTFT-50	2.70	−2.35	−1.31	2.59	17.5	13.4
PPE					7.14	1.7

On only two stationary phases (PIB and OV-225) is the relative retention of the fluorinated chelate lower than that of the corresponding non-fluorinated chelate, in accordance with the relative molar entropies of solution.

To make the effect of fluorination on relative retention more evident, let us compare the minimum values of the relative retentions of fluorinated chromium chelates (see Fig. 1) with the relative retentions of the corresponding non-fluorinated chelates; this ratio is represented by r_1 (Table III). The r_1 data show that the relative retentions of the fluorinated chelates are lower than those of the non-fluorinated chelates on four stationary phases (OV-225, PIB, OV-17 and PPMS-6). Two other stationary phases (OV-17 and PPMS-6) can be added because of the large decrease in the relative retentions for homologous series of fluorinated chelates.

The calculated minimum retention times for fluorinated chelates on different stationary phases are listed in the last column in Table III, and have a relative mean standard deviation of about 4%. On comparing the minimum retention times, one can conclude that these values depend markedly on the choice of the stationary phase, for example, the retention time of the chelate on PPMS-6 is 35 times greater than that on PPE. There are four stationary phases on which the retention times of the chelates have minimum values, PPE, PIB, OV-225 and OV-17; three of these have phenyl groups in the molecules. On comparing the retention data for fluorinated and non-fluorinated chelates, it can be seen that fluorination does not always decrease the retention times of chelates.

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RETENTION BEHAVIOR OF PTEROYL-OLIGO-γ-L-GLUTAMATES IN REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

The effect of eluent pH on the retention of pteroyl-oligo-γ-L-glutamates containing up to eight glutamyl residues is investigated in reversed-phase chromatography with octadecyl-silica column. When the carboxylic groups of the solutes are largely undissociated, at pH 2, the retention of oligoglutamates increases with the number of glutamyl residues and the elution order parallels that in anion-exchange chromatography. At sufficiently high eluent pH the carboxylic groups are dissociated and the elution order is reversed so that solute molecules having smaller number of charges, *i.e.* less glutamyl residues, are retained stronger. The logarithm of capacity factor, with the exception of folic acid, is linearly dependent on the number of glutamyl residues over a wide range of eluent pH. The dependence of the capacity factor on pH for oligoglutamates is quantitatively interpreted considering the different dissociation constants for the α- and γ-carboxyls. The results suggest that in reversed-phase chromatography the selectivity of separation for polyionogenic compounds can be drastically modulated by changing the pH of the eluent.

INTRODUCTION

Derivatives of folic acid (FA) are coenzymes essential in the synthesis of proteins and nucleic acids[1]. The FA molecule consists of a pteridine moiety linked to *p*-aminobenzoylglutamic acid. Numerous derivatives of FA, which differ in the substitution at N^5 and N^{10} positions and the state of oxidation, have been identified. In many cases more than one glutamic acid is attached via γ-carboxylic group to the rest of the molecule[2]. The general formula of such folate–oligo-γ-L-glutamates, which usually contain 2 to 9 glutamyl residues, is shown in Fig. 1 and we can see that each of such molecules contains one γ-carboxylic group and n α-carboxylic groups.

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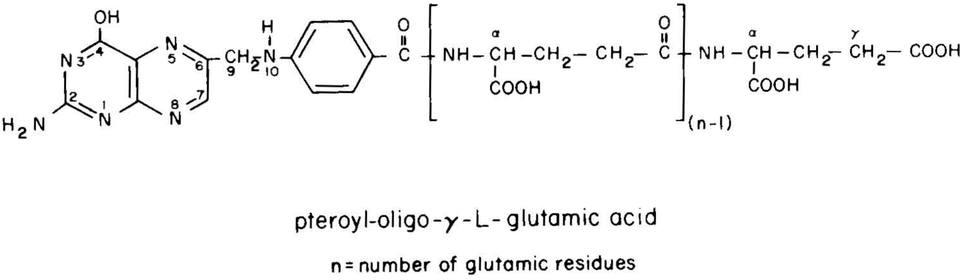

Fig. 1. Structure of pteroyl-oligo-γ-L-glutamic acids, $PtGlu_n$, where *n* is the number of glutamyl residues. For the parent compound, FA, *n* equals one.

It was suggested that the derivatives of tetrahydrofolate–polyglutamates are biologically active coenzymes in microorganisms[3]. The reduced forms of pteroyl-tri-γ-glutamate ($PtGlu_3$) and pteroyl-hepta-γ-glutamate ($PtGlu_7$) are the principal folate–oligo-γ-glutamates found in bacteria and yeast, respectively. Recently, oligo-γ-glutamates were also isolated from mammalian cells and found to be active as co-enzymes in several folate-dependent "one carbon" enzymic reactions[4,5]. The presence of biologically active oligo-γ-L-glutamates of *p*-aminobenzoic acid (PABA) in mammalian cells in culture has also been postulated[5]. The study of folate metabolism has been hampered by the large number and low physiological concentrations of active folate derivatives and the lack of sufficiently efficient separation methods and sensitive analytical tools.

Column chromatography was first used in 1957 to fractionate a complex mixture of folates[6]. Thereafter the technique was refined and ion-exchange columns packed with DEAE-cellulose and DEAE-Sephadex have been used widely for the separation of folate coenzymes[7,8].

Recently, high-performance liquid chromatography was introduced to afford rapid analysis of various FA derivatives. Initially, columns packed with micro-particulate bonded anion exchangers were used[9,10] for the separation of reduced and oxidized mono- and polyglutamates. It has been shown, however, that reversed-phase chromatography with microparticulate octadecyl-silica as the stationary phase also yields high resolution and fast analysis[11]. In fact, reversed-phase chromatography offers an excellent alternative to ion-exchange chromatography for microanalysis of folate derivatives.

In this study the effect of pH on the retention of oligoglutamates in reversed-phase chromatography is examined. In this technique hydrophobic interactions between the eluites and the non-polar stationary phase play an important role in determining the retention behavior[12]. Therefore results obtained in reversed-phase chromatography can shed light on the hydrophobic properties of the sample components.

EXPERIMENTAL

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 601 liquid chromatograph with a Rheodyne (Berkeley, Calif., U.S.A.) Model 7010 sampling valve and a variable wavelength UV detector Model SF-770 (Schoeffel, Westwood, N.J., U.S.A.) were used. In some experiments, a Schoeffel fluorimetric detector, Model FS-970 was also used. One experiment was carried out using a DuPont Model 830 liquid chromatograph with UV detector in the gradient elution mode.

Octadecyl-silica columns were prepared from 10 in. × 0.25 in. O.D. × 4.6 mm I.D., No. 316 stainless-steel tubing by slurry packing. Two kinds of material were used: 5-μm Partisil ODS 2 with 16% carbon load was donated by Whatman (Clifton, N.J., U.S.A.); 5-μm Spherisorb ODS with 6% carbon load was purchased from Phase Sep (Hauppage, N.Y., U.S.A.).

FA was obtained from Sigma (St. Louis, Mo., U.S.A.) and the oligo-γ-glutamates of FA and *p*-aminobenzoic acid were synthesized as described[11,13]. Acetonitrile was obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.) and the other chemicals were reagent-grade and purchased from Fisher (Pittsburgh, Pa., U.S.A.).

Isocratic elution was carried out with a 0.1 *M* phosphate buffer containing a few per cent (v/v) of acetonitrile. In experiments with gradient elution the starting eluent was neat aqueous phosphate buffer and acetonitrile was used as the gradient former. Column temperature was held at 45° by using a water jacket connected to a thermostated circulator bath (Lauda Messgeräte, Lauda, G.F.R.).

Solute retention has been expressed by the dimensionless retardation factor, k, which is calculated from the formula $k = (t_R - t_0)/t_0$, where t_R is the retention time of the eluite and t_0 is the hold-up time for an unretained solute. In this study t_0 was evaluated from the retention time of sodium nitrate.

The analysis of the data was performed on a PDP 11/10 minicomputer (Digital Equipment, Maynard, Mass., U.S.A.) equipped with a floppy disk unit, a VT-55 CRT and a Decwriter. The computer program used for parameter estimation was written in BASIC language.

RESULTS AND DISCUSSION

Ionized biological substances such as FA derivatives are traditionally separated by ion-exchange chromatography. Recent results, however, clearly indicate that reversed-phase chromatography is eminently suitable for the separation of peptides[14] and has a great potential for the assay of folate–oligoglutamates[11]. The physicochemical phenomena which govern solute retention in the two chromatographic techniques are often quite different. In ion-exchange chromatography coulombic interactions between the fixed charges on the stationary phase and the oppositely charged solute molecules play an important role in determining the magnitude of retention. Consequently, the retention of the polyglutamates of FA on an anion-exchange column, which is customarily employed for their separation, increases with increasing number of glutamic acid residues, *i.e.*, with increasing number of ionized carboxylic groups in the molecules[9,11].

On the other hand, in reversed-phase chromatography the stationary phase is non-polar and it has been shown[15,16] that an enhancement of electrostatic interactions between the solute and solvent, *e.g.*, that which occurs upon solute ionization in aqueous buffer solutions, leads to a decrease in retention. In other words, the hydrophobic effect which largely affects the magnitude of retention in reversed-phase chromatography is attenuated by increasing the charge on the solute molecule. It follows then that the elution order of the oligo-γ-glutamates in reversed-phase chromatography, when the pH of the eluent is sufficiently high for the carboxylic groups to be ionized, should be the opposite of that observed in anion-exchange chromatography. Indeed the chromatogram of pteroyl-γ-L-glutamates in Fig. 2 demonstrates that retention decreases with increasing number of glutamyl residues at pH 6 where the carboxylic groups are almost completely dissociated. As the retention order of oligo-γ-glutamates in reversed-phase chromatography under such conditions is just the opposite of that observed in anion-exchange chromatography, the two methods complement each other. The identification of polyglutamate peaks can be greatly facilitated by using both techniques concomitantly[11].

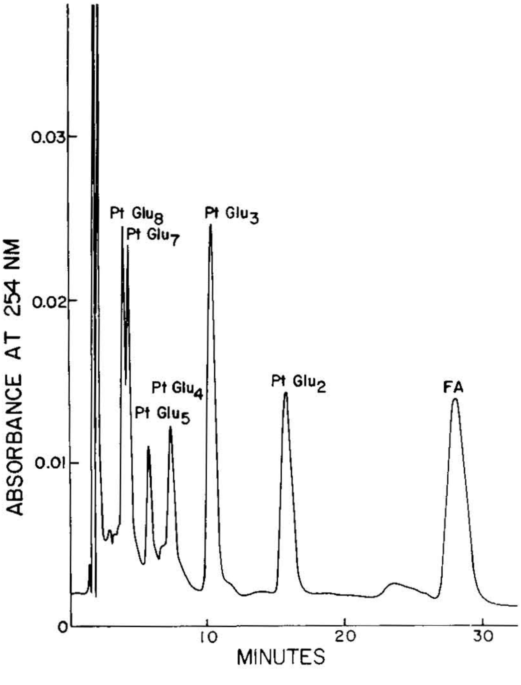

Fig. 2. Reversed-phase chromatogram of pteroyl-oligo-γ-L-glutamates obtained by isocratic elution at pH 6. Column, 5-μm Spherisorb ODS, 250 × 4.6 mm; eluent, 0.1 *M* phosphate buffer, pH 6.0, containing 1% (v/v) acetonitrile; flow-rate, 1.5 ml/min; inlet pressure, 11.42 MN/m^2; temperature, 45°; sample size, approx. 10 μg of each component.

As expected from the preceding discussion when oligoglutamates of *p*-aminobenzoic acid are chromatographed at sufficiently high eluent pH on an octadecyl-silica column retention also decreases with increasing number of glutamyl residues. How-

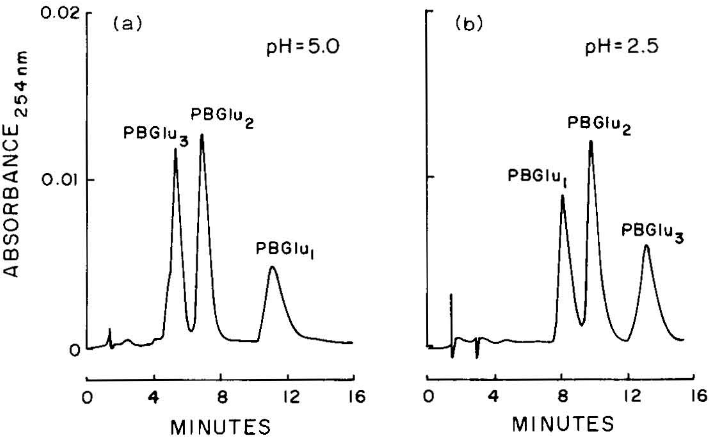

Fig. 3. Reversal of elution order for *p*-aminobenzoyl-oligo-γ-L-glutamates upon changing the pH of the eluent in reversed-phase chromatography. Column, 5-μm Partisil ODS-2, 250 × 4.6 mm; eluent (a) 0.1 *M* plain aqueous phosphate solution, pH 5.0, (b) 0.1 *M* phosphate buffer, pH 2.5, containing 3% (v/v) acetonitrile; flow-rate, 1.5 ml/min; inlet pressure, 11.5 MN/m^2; temperature, 45°; sample size, approx. 10 μg of each component.

ever, a dramatic reversal of the retention order is observed when the dissociation of the carboxylic acids is suppressed by lowering the pH of the eluent. Similar effects have been observed with folate–oligoglutamates as well. The comparison of the chromatograms obtained at pH 5.0 and 2.5 as shown in Fig. 3, clearly demonstrates this phenomenon.

Therefore, we investigated the effect of eluent pH and the number of glutamyl residues in pteroyl-oligo-γ-L-glutamates on retention in reversed-phase chromatography under a wide range of conditions. The pH was varied between 2.2 and 6.0 and the retention of eluites having 1, 3, 5 and 7 glutamyl residues was measured. The results of this study are illustrated in Fig. 4. The graph shows the capacity factor (on a logarithmic scale) as a function of the number of glutamyl residues with the eluent pH as the parameter. The straight lines in Fig. 4 demonstrate that log k is proportional to the number of glutamyl residues. This behavior is in agreement with that postulated by the solvophobic theory[15] for solute retention in reversed-phase chromatography. FA ($n = 1$) shows "irregular" behavior in the pH range 3.5–5.0 where it is partially protonated.

Changes in the elution order manifest themselves in the changing slopes on the graph in Fig. 4. At low pH the slope is positive indicating that retention increases with the number of glutamyl residues. Then the line becomes horizontal between pH 3.0 and 3.8 so that all sample components elute together and no separation occurs. At high pH the slope is negative and the capacity factor decreases with increasing number of glutamyl residues. In reversed-phase chromatography, therefore, the elution order of polyglutamates can be the same as in ion-exchange chromatography when the ionization of the carboxylic groups is suppressed at low eluent pH. However, the elution order of the polyglutamates with dissociated carboxylic groups, *i.e.*, at sufficiently high eluent pH, is the reverse of that observed in ion-exchange chromatography.

The dependence of the capacity factor on the hydrogen ion concentration, [H⁺], in the pH domain investigated is evidently related to the degree of dissociation of the carboxylic groups in the glutamyl moiety. For a diprotic acid, such as FA, it has been shown[15] that the capacity factor can be expressed by

$$k = \frac{k_0 + k_1 \dfrac{K_1}{[H^+]} + k_2 \dfrac{K_\gamma K_1}{[H^+]^2}}{1 + \dfrac{K_1}{[H^+]} + \dfrac{K_\gamma K_1}{[H^+]^2}} \qquad (1)$$

where k_0, k_1 and k_2 are the capacity factors of FA when none, one and both of the carboxylic groups are dissociated, respectively. The dissociation constants of the α-

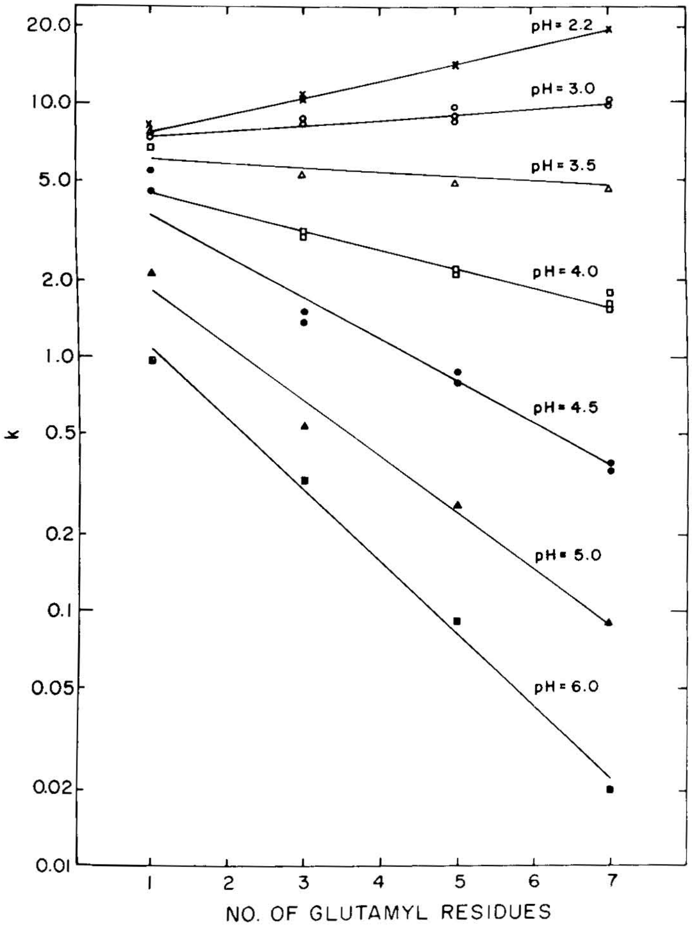

Fig. 4. Plots of the capacity factor (on a logarithmic scale) against the number of the glutamyl residues in pteroyl-oligo-γ-glutamates at different pH of the eluent. Column, 5-μm Partisil ODS-2; eluent, 0.1 *M* phosphate solution containing 6% (v/v) acetonitrile; temperature, 45°.

and γ-carboxylic groups in the glutamyl residue are given by K_1 and K_γ, respectively.

The α- and γ-carboxylic groups in FA are expected to have significantly different pK_a values although a literature search failed to reveal any pK_a values. The low solubility of FA in aqueous solutions at pH below 6 makes the determination of the pK_a values difficult. Nevertheless for tetrahydrofolic acid the pK_a values of the α- and γ-carboxylic groups have been reported[17] as 3.5 and 4.8, respectively, and we may assume that the corresponding pK_a values are similar for FA. Therefore the degree of dissociation of the carboxylic group will change in the pH domain from 2 to 6. On the other hand, the amino functions in the pteridine moiety are largely ionized under such conditions.

With the pteroyl-oligo-γ-glutamates the situation is similar except that the number of α-carboxylic groups, which is equal to n, increases with the number of glutamyl residues. For these substances eqn. 1 can be generalized as

$$k = \frac{k_0 + \sum\limits_{i=1}^{n} k_i \frac{\prod\limits_{j=1}^{i} K_j}{[\mathrm{H}^+]^i} + k_{n+1} \frac{K_\gamma \prod\limits_{i=1}^{n} K_i}{[\mathrm{H}^+]^{n+1}}}{1 + \sum\limits_{i=1}^{n} \frac{\prod\limits_{j=1}^{i} K_j}{[\mathrm{H}^+]} + \frac{K_\gamma \prod\limits_{i=1}^{n} K_i}{[\mathrm{H}^+]^{n+1}}} \tag{2}$$

where k_i and K_j are the capacity factor of the molecule containing i dissociated α-carboxylic groups and the acid dissociation constant of the ith α-carboxylic groups, respectively. On the other hand, k_{n+1} and K_γ are the capacity factors of the fully dissociated species and the dissociation constant for the γ-carboxylic group, respectively.

It is seen from eqn. 2 that the calculation of the capacity factors as the function of pH for these compounds necessitates a large number of parameters whose evaluation would require a large amount of very precise data obtained at narrow pH intervals. The number of parameters can be drastically reduced, however, by using certain assumptions. First, the capacity factor ratio of the homologues differing in one ionized α-carboxylic group can be considered constant, so that

$$C = \frac{k_{i+1}}{k_i} \tag{3}$$

This assumption can be justified by the results of a previous study on the pH dependence of the retention of ionogenic substances in reversed-phase chromatography[16]. Substituting eqn. 3 into eqn. 2 we obtain

$$k = \frac{k_0 + \sum\limits_{i=1}^{n} k_0 C^i \frac{\prod\limits_{j=1}^{i} K_j}{[\mathrm{H}^+]^i} + k_0 C^{n+1} \frac{K_\gamma \prod\limits_{i=1}^{n} K_i}{[\mathrm{H}^+]^{n+1}}}{1 + \sum\limits_{i=1}^{n} \frac{\prod\limits_{j=1}^{i} K_j}{[\mathrm{H}^+]} + \frac{K_\gamma \prod\limits_{i=1}^{n} K_i}{[\mathrm{H}^+]^{n+1}}} \tag{4}$$

In order to evaluate the K_i's, another assumption is made. It is based on widely accepted thermodynamic consideration that the energy required for the ionization of a carboxylic group increases with the number of already dissociated carboxyl functions in the molecule. In the simplest case the energy is proportional to the number of ionized groups and the dissociation constant of the ith α-carboxylic group in the oligo-γ-glutamate moiety, K_i, can be expressed by

$$K_i = K_1 \exp\left(-f \sum_{j=1}^{i} j\right) \tag{5}$$

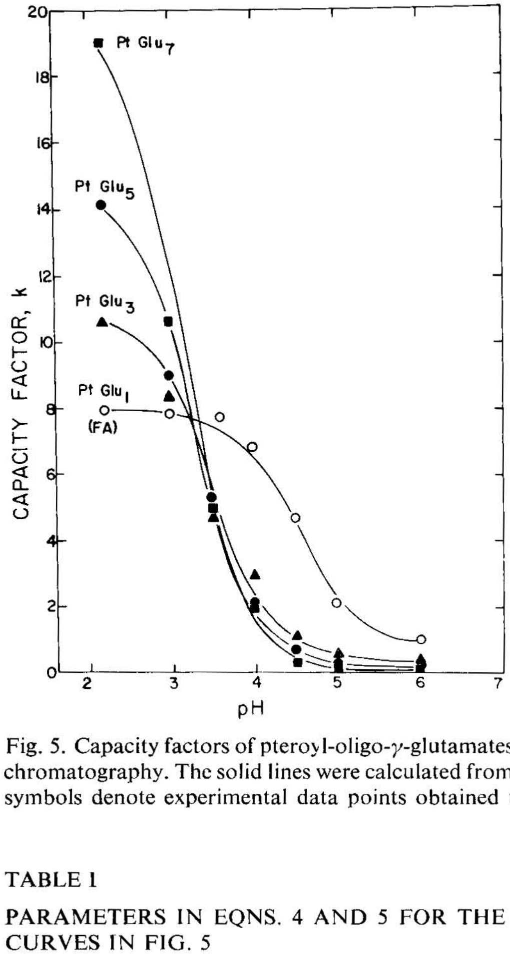

Fig. 5. Capacity factors of pteroyl-oligo-γ-glutamates as a function of the eluent pH in reversed-phase chromatography. The solid lines were calculated from eqn. 4 with the parameters given in Table 1. The symbols denote experimental data points obtained under conditions stated in Fig. 4.

TABLE 1

PARAMETERS IN EQNS. 4 AND 5 FOR THE CALCULATION OF THE THEORETICAL CURVES IN FIG. 5

The value of pK_γ was taken as 4.8.

Eluite	*pK_1*	*C*	*f*	*n*
PtGlu$_1$(FA)	4.5	0.40	0.3	1
PtGlu$_3$	3.5	0.41	0.3	3
PtGlu$_5$	3.1	0.48	0.3	5
PtGlu$_7$	3.0	0.52	0.25	7

where K_1 is the dissociation constant of the α-carboxylic group in FA and f is the above mentioned proportionality factor. Knowing the values of k_0, k, K_γ and K_1, we can evaluate C and f from the experimental data by using eqns. 4 and 5. The parameters used to obtain the theoretical curves shown for the pH dependence of the pteroyl-oligo-γ-glutamates in Fig. 5 were evaluated by using this approach and are listed in Table I.

It is seen from Fig. 5 that this approach offers a quantitative interpretation for the observed pH dependence of retention for oligo-γ-glutamates. The results demonstrate again that the behavior of FA is different from that of higher oligoglutamates. The difference is due to the effect of the α-carboxylic groups whose pK_a is relatively low. The physiological significance of this difference is not known, however. In fact, the physiological role of oligo-γ-glutamates has not yet been fully elucidated.

The results depicted in Figs. 4 and 5 can be interpreted by considering the effect of the number of glutamyl residues on the size of, and, at sufficiently high pH, on the charge carried by the oligoglutamate molecules. The conjugate bases, present at low pH, are retarded in the order of increasing molecular size in agreement with the prediction of the solvophobic theory that the free energy of the reversible association process between the solute and the hydrocarbonaceous ligands of the stationary phase is linear in the contact area between the two species provided all other factors remain the same[15]. This process of separation by size is contrasted with separation by charge which occurs when the carboxylic groups are dissociated. Under such conditions an increase in the number of glutamyl residues is tantamount to an increase in the number of charges in the solute molecules. According to the theory[16] the energy of the electrostatic interaction between the solute and eluent increases with the number of charges and results in a concomitant decrease in chromatographic retention on non-polar stationary phases. Consequently, the dissociated species elute in the order of decreasing n in reversed-phase chromatography.

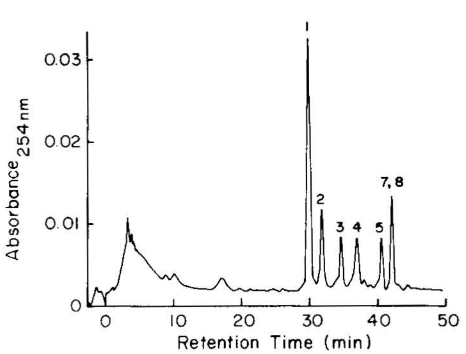

Fig. 6. Reversed-phase chromatogram of pteroyl-oligo-γ-L-glutamates at low pH and with gradient elution. Column, 5-μm Spherisorb ODS, 250 × 4.6 mm; starting eluent, 0.1 M phosphate buffer, pH 2.2, containing 4% (v/v) acetonitrile; gradient former, the same buffer with 10% (v/v) acetonitrile at No. 4 concave setting on DuPont 830; inlet pressure, 11.72 MN/m^2; temperature, 45°; sample size, 3–10 μg of each component. The peak numbers correspond to the number of glutamyl residues, n, in the eluite molecules (see Fig. 1).

In contradistinction, retention in anion-exchange chromatography is due to the interaction between the negatively charged oligo-γ-glutamates and the fixed positive charges on the stationary phase[9]. Consequently, the elution order for separation by charge is the opposite of that observed with the dissociated oligo-γ-glutamates in reversed-phase chromatography and corresponds to that observed with separation by size.

In general the efficiency of ion-exchange chromatography with bonded phases such as Partisil SAX (Whatman) appears to be higher than that of reversed-phase chromatography at low pH on octadecyl-silica at the same particle size and column dimensions. It is recommended, therefore, that ion-exchange chromatography be used when an elution order in the increasing number of glutamyl residues is desired. On the other hand, reversed-phase chromatography is eminently suitable for separations with reversed elution order as at high eluent pH adequate resolution can be obtained by using isocratic (see Fig. 2) or in the case of complex mixtures, gradient elution. Experiments with a variety of octadecyl-silica columns gave very similar results and demonstrated the analytical usefulness of this approach.

Reversed-phase chromatography at low pH however, also can give satisfactory results when gradient elution is used. It is shown in Fig. 6 by the chromatogram of FA-oligoglutamates obtained at pH 2.2 by using gradient elution with exponentially increasing acetonitrile concentration. A caveat is necessary when working at low eluent pH because the elution order of the first three members of the oligoglutamate series was found to be $PtGlu_2$, $PtGlu_3$ and $PtGlu_1$ with certain other types of octadecyl-silica columns when the concentration of acetonitrile in the eluent was greater than 6% (v/v). The observed increase in the retention of FA with the concentration of the organic modifier in the eluent is likely to arise from changes in the dielectric properties of the eluent.

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PYROLYSIS GAS CHROMATOGRAPHY APPLIED TO COAL TAR- AND PETROLEUM-PITCHES

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SUMMARY

Pyrolysis gas chromatography has been used to study 12 coal tar- and petroleum-pitches used as binders and impregnants, respectively, in the production of thermic graphite electrodes. Parameters such as coking value, softening point and quinoline insolubles, which are normally used to characterize these pitches, have been related to changes in some of the peaks in the chromatograms. Computerized data collection and a nitrogen-specific detector, combined with a flame ionization detector, allowed for easy comparisons of different pitches and aided in the identifications of some of the pyrolysis products. The use of this technique for possible screening of unknown samples is suggested.

INTRODUCTION

The combination of pyrolysis and gas chromatography (PGC) was first demonstrated by Davison *et al.*[1] in 1954 for the study of polymers. In subsequent years, this technique has grown in importance for the fingerprinting and identification of polymers and paints, particularly in forensic applications[2–4]. Recently, PGC has been extended to microbiological samples and enzymes[5–8].

Coal tar-pitches are "obtained as a high aromatic, thermoplastic residue by the distillation of coal tar", and their properties and uses have been recently summarized by Collin and Kohler[9]. Petroleum-pitches are similarly obtained from the distillation of petroleum products, namely, decant oils and thermal tars. Both types of pitches find a wide variety of uses, including road building, production of technical carbon products, and, in combination with plastics, production of insulating and anti-corrosive materials[9]. One of the primary uses, however, is in the graphite industry as binders and impregnants in the production of thermic electrodes. King and Robertson[10] have compared coal tar-pitches with petroleum-pitches with respect to their

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usefulness as electrode binders. In addition, some of the pitches used in this study have been examined elsewhere using thermogravimetric analysis[11] and viscosity–temperature relationships[12].

Coal and petroleum samples have been studied using PGC by a number of authors, especially in the mid-1960s[13–18]. However, recent advances in pyrolysis units (allowing rise times of up to 20°/msec and final temperature of up to 1400°) and specific detectors for GC, in particular a nitrogen-specific detector, should allow substantial improvement of these earlier results. Computerized data acquisition was also available in the present work to provide easy comparisons of the complex chromatograms.

We have identified a number of the major peaks obtained from PGC and have correlated them to physically significant parameters. In particular, we have been able to relate our results to the coking value, softening point, and quinoline insolubles. The coking value is the most crucial since trends in the latter two parameters are often similar to those of the coking value. As a result, the use of this technique for the screening of pitch samples for the production of thermic graphite electrodes appears to be feasible.

EXPERIMENTAL

Materials

Ten coal tar-pitches and two petroleum-pitches were typical samples used in the graphite industry. The pitch numbering scheme corresponds to the one used elsewhere[11,12].

The column packing for the analysis was 3% OV-11 on 80–100 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). A second column of 3% Dexsil 300 on 100–120 mesh Chromosorb W AW DMCS (Johns Manville, Denver, Colo., U.S.A.) was used for peak identification. Both columns were packed in stainless-steel tubing, 5 m × 2 mm I.D., which had been suitably washed in 50% nitric acid, water, chloroform, isopropanol and pentane.

Nitrogen was used as the carrier gas, and air and hydrogen as the detector gases. All three gases were purified by passage through silica gel and molecular sieve 5A (Linde).

Apparatus

The gas chromatograph was a Perkin-Elmer 3920 (Norwalk, Conn., U.S.A.) equipped with a flame ionization detector (FID) and a nitrogen–phosphorus detector (NPD). A Perkin-Elmer Dial-a-Flow controller was used to maintain the carrier gas flow. The pyrolysis unit was a Chemical Data Systems 190 Pyroprobe (Oxford, Pa., U.S.A.) equipped with a platinum coil probe.

Data collection and analysis were performed with a PDP 11/20 minicomputer (Digital Equipment, Maynard, Mass., U.S.A.) programmed in real-time BASIC using an Anscan analog-to-digital converter (Beckman Instruments, Fullerton, Calif., U.S.A.).

Procedures

Each pitch was ground to a 20-mesh (U.S. Series) particle size and pyrolyzed in the solid form. A 2.4-mg sample was placed inside a 26 mm × 2 mm I.D. quartz tube with a glass wool plug in the back end of the tube to hold the solid. The tube was placed in the coils of the Pyroprobe which was then inserted into the injection port of the chromatograph. The sample was pyrolyzed from ambient temperature to 850° with a temperature rise of 20°/msec and held at the final temperature for 20 sec. The injector temperatures were 250 and 350°, respectively. Using the OV-11 column, the temperature program was: 4 min at 90°, a 4°/min ramp from 90 to 280° and a hold time of 32 min at the final temperature. The pyrolysis products were chromatographed using a carrier gas flow-rate of 35 ml/min. An air flow-rate of 90 ml/min was used with both detectors while the hydrogen flow-rates were 100 and 4 ml/min for the FID and NPD, respectively. The background bead current of the NPD was 20 pA.

Each pitch was run at least twice, with certain pitches run several times to determine reproducibility. Data were collected at one point/sec on both detectors, and the chromatograms were stored on DECTape for subsequent analysis.

A similar pyrolysis procedure was used with Dexsil 300 column for peak identification. Standard solutions of polynuclear aromatic hydrocarbons (PAH) in benzene were injected on both columns.

The pitches were characterized using the standard methods listed in Table I[19].

TABLE I

AMERICAN SOCIETY FOR TESTING OF MATERIALS STANDARD TESTS FOR PITCHES

Test	*ASTM No.*[*]
Softening point, SP (°C)	D-36
Sulfur (wt. %)	D-271
Benzene insolubles, BI (wt. %)	D-2317
Quinoline insolubles, QI (wt. %)	D-2318
Dimethylformamide insolubles, DMFI (wt. %)	D-2764
Coking value (wt. %)	D-2416

[*] See ref. 19.

RESULTS

Pitch characterization

The results of the standard methods of characterization for the 12 pitches are listed in Table II. The coking value is a measure of the usefulness of the pitch in the process of fabricating thermic electrodes. In general, the higher the coking value, the more useful the pitch in manufacturing the electrode. The softening point is an indicator of the ease of using the pitch in the process. However, because coking value increases as softening point increases, some balance between the desired carbon residue and thermal capability of the mixing equipment must be made before a given pitch is chosen for a particular graphite product. For obvious environmental reasons, a low sulfur content is desirable in any pitch.

The pitches used in the present study exhibited a wide range of values for the various standard tests, but they can be divided into three groups (labelled A, B, and C). This division follows the general pattern of high values for coking, softening

TABLE II

RESULTS OF ASTM TESTS FOR PITCHES

Group	*Pitch*	*Coking value (wt. %)*	*Softening point (°C)*	*Sulfur (wt. %)*	*Benzene insolubles (wt. %)*	*Quinoline insolubles (wt. %)*	*Dimethylformamide insolubles (wt. %)*
A	6	74.2	156.0	0.50	45.2	22.6	53.9
	6A	73.8	154.1	0.46	44.7	21.7	50.9
	5	69.5	136.2	0.50	41.7	21.7	46.9
	4	67.6	130.0	0.49	40.0	17.6	44.2
B	2	58.8	103.5	0.65	31.5	14.3	34.3
	2B	58.7	103.2	—	31.4	12.6	—
	2A	57.5	103.8	0.61	33.6	12.7	33.3
	1A	57.1	100.8	0.53	24.7	11.1	31.0
C	2C	53.4	105.1	0.34	34.0	5.4	34.7
	PP1	52.5	114.0	3.09	8.4	0.42	14.9
	0	51.6	79.5	0.51	28.2	12.2	31.0
	PP2	50.7	104.2	4.2	23.8	4.3	30.7

point, quinoline insolubles (QI), benzene insolubles (BI), and dimethylformamide insolubles (DMFI) for group A; low values of these five parameters for group C; and intermediate values for group B. These groupings also correspond roughly to the use of the pitches in the process of electrode fabrication, with those in group A generally being the most desirable and those in group C the least desirable. Indeed, the pitches in group C are generally used as impregnants, while the other two groups are used as binders. A more detailed discussion of the importance of each of the parameters in electrode manufacturing is contained in two recent articles[11,12].

PGC

A typical chromatogram for PGC using both the FID and NPD results is shown in Fig. 1 for pitch 6A. The FID plot shows a number of major peaks, eight of which were used in the specific analyses to be discussed later. The NPD plot is less complex, but still exhibits a few peaks which occur in all of the samples. Since the FID was more reproducible than the NPD, the latter detector was used only qualitatively and as an indicator for peak identification, while most of the final correlations were done using the FID data. We were able to identify, using the OV-11 and Dexsil columns and the standard PAH solutions, three peaks in the NPD chromatograms and nine peaks in the FID chromatograms.

Although many of the same peaks occurred in all 12 pitches, an examination of three selected FID plots in Fig. 2 (one from each group A, B, and C) revealed that the general appearance of the chromatogram changed, particularly between pitches that were unlike using other standard characteristics (see Table II). The fact that these discrepancies were due to the differences in pitch composition can be seen by examining Fig. 3, where pitches 2B and 2 have very similar physical characteristics and almost identical chromatograms.

In our general approach to the analysis of these chromatograms, we found that no comparison based on a single peak or group of peaks was sufficient by itself

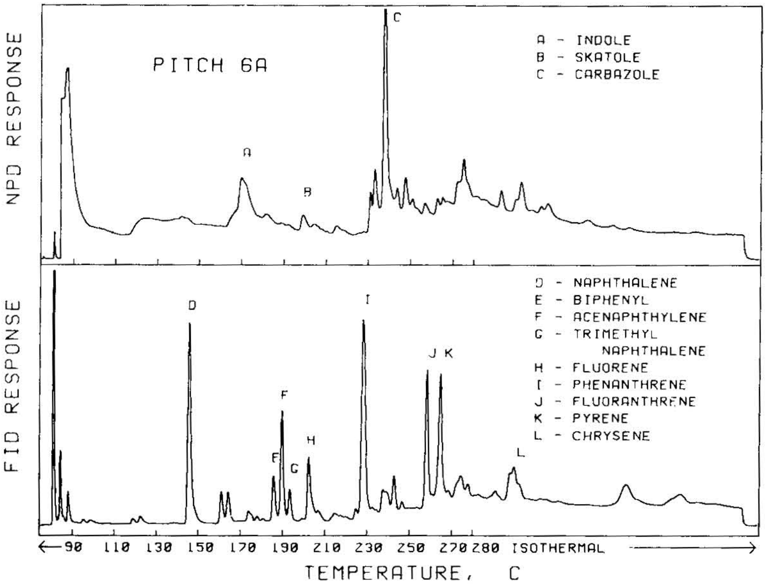

Fig. 1. PGC chromatogram of coal tar-pitch 6A with major peak identification.

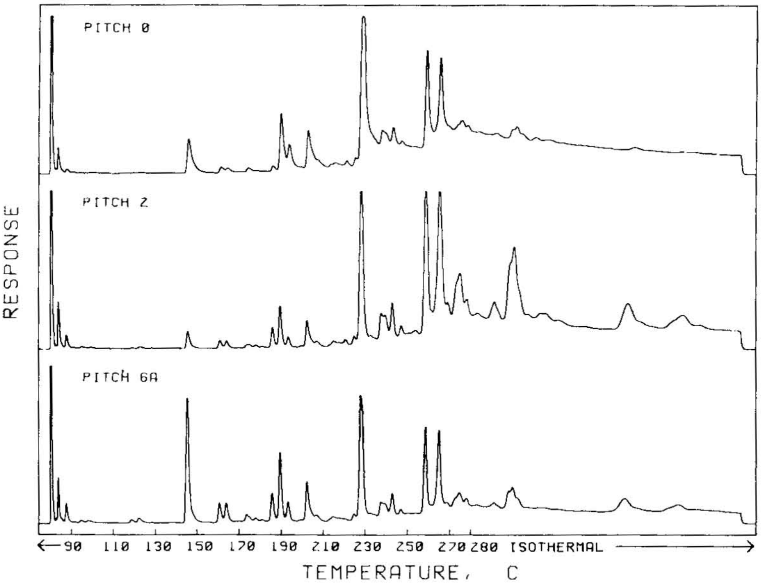

Fig. 2. Comparison of three coal tar-pitches. Pitch ø from group C, pitch 2 from group B, and pitch 6A from group A. Flame ionization detector.

to distinguish and segregate them into groups similar to those in Table II. However, four independent comparisons could be used successfully.

Seven main peaks selected for study are labelled in Fig. 1 and were later identified as follows: D, naphthalene; E, biphenyl; F, acenaphthylene; H, fluorene;

Fig. 3. Comparison of two similar coal tar-pitches, 2B and 2. Flame ionization detector.

I, phenanthrene; J, fluoranthene; and K, pyrene. The heights of these seven peaks were first summed and then the height of each peak was expressed as a percentage of the total. The results of this analysis are listed in Table III.

The most important observation is that for the more useful pitches, group A, all seven peaks were present in amounts of at least 4% and no single peak or group of peaks dominated the chromatogram. In contrast, for the group C pitches, the phenanthrene, fluoranthene and pyrene peaks dominated the chromatogram (see Fig. 2,

TABLE III

COMPARISON OF SEVEN MAIN PEAKS

Values are percent of the sum of the total peak heights of the seven peaks.

Group	*Pitch*	*Coking value (wt. %)*	*Naphthalene*	*Biphenyl*	*Acenaphthylene*	*Fluorene*	*Phenanthrene*	*Fluoranthene*	*Pyrene*
A	6	74.2	9	9	15	4	14	23	26
	6A	73.8	21	5	12	7	22	16	16
	5	69.5	8	8	12	4	17	25	26
	4	67.6	6	4	6	4	24	28	27
B	2	58.8	3	4	7	5	27	27	27
	2B	58.7	4	5	7	7	26	26	26
	2A	57.5	3	2	4	5	29	29	29
	1A	57.1	3	5	17	4	23	23	24
C	2C	53.4	1	—	—	2	31	33	33
	PP1	52.5	6	1	—	4	32	17	39
	0	51.6	6	2	11	8	29	23	21
	PP2	50.7	3	—	—	—	10	25	62

pitch Ø, where the latter portion of the plot was substantially greater in area than the earlier portion). Indeed, for many of the group C pitches, some peaks were totally absent, acenaphthylene being a notable example. As expected, the group B pitches appeared to exhibit behavior between the other two groups.

Although these initial observations gave some correlation to the physical properties of the pitches, these results were not conclusive. For example, pitch Ø resembled the group A pitches in this initial comparison, and only secondary characterizations given below could differentiate it from the more useful pitches. Therefore, we decided to supplement this primary characterization with three secondary characteristics listed as I, II, and III in Table IV.

TABLE IV

SECONDARY CHARACTERIZATION

Group	*Pitch*	*Coking value (wt. %)*	*I*	*II*	*III*		
			Phenanthrene/ naphthalene	*Subareas 2/1*	*Biphenyl*	*Triplet acenaphthylene**	*Trimethyl-naphthalene*
A	6	74.2	1.7	1.48	34	59	7
	6A	73.8	1.0	1.27	25	57	18
	5	69.5	2.0	1.81	37	53	10
	4	67.6	3.9	2.66	36	50	14
B	2	58.8	8.9	3.82	28	56	16
	2B	58.7	7.1	3.41	31	48	21
	2A	57.5	10	5.31	24	52	24
	1A	57.1	7.5	2.40	21	72	7
C	2C	53.4	35	9.93	—	—	—
	PP1	52.5	5.4	18.13	50	—	50
	ø	51.6	4.5	2.68	8	62	30
	PP2	50.7	2.9	3.24	—	—	—

* Triplet numbers are percent of sum of the three peak heights.

The first of these new characteristics is that a low (<2.5) ratio of the heights of the phenanthrene to naphthalene peaks (I and D in Fig. 1) is an excellent indication of a group A pitch. The ratio of 3.9 for pitch 4 is not as low as others, but it is still a clue that it is a more useful pitch. Petroleum-pitch 2 also has a relatively low ratio of 2.9, but it should be noted that the other characterizations will place this pitch in group C.

It was further noted that the chromatograms could be divided into three subareas that gave another secondary characterization. In the pitches in group B and C, the later regions of the chromatograms dominated the total area, but in the group A pitches the subareas of the three regions (labelled 1, 2, and 3 in Fig. 4) were approximately the same. These observations are shown quantitatively in Table IV where the subarea ratios of regions 2 to 1 are listed. A low ratio indicated a group A pitch, while a high ratio was indicative of a group B or C pitch. Again, the application of this one test was not conclusive (for example, pitches Ø and PP2).

Although most of our correlations were with the coking value (and thus only

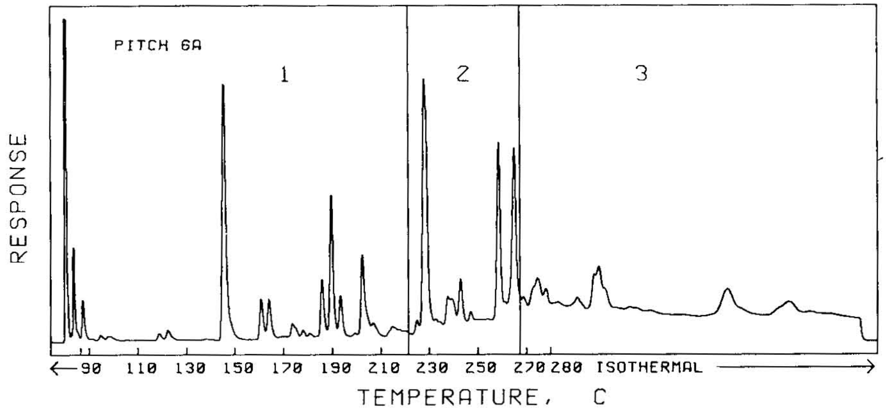

Fig. 4. Three subareas of FID chromatogram used for secondary characterization.

indirectly to the other physical characteristics), we did note one correlation of potential import with respect to the quinoline insolubles. The three pitches we tested that had a low QI (2C, PP1, and PP2) gave no acenaphthylene peak. This result could be coincidental, except that a significant amount of QI is normally necessary to produce a good final electrode, and acenaphthylene itself exhibits a high degree of graphitization[20,21]. Hence, there may be some connection between these two observations.

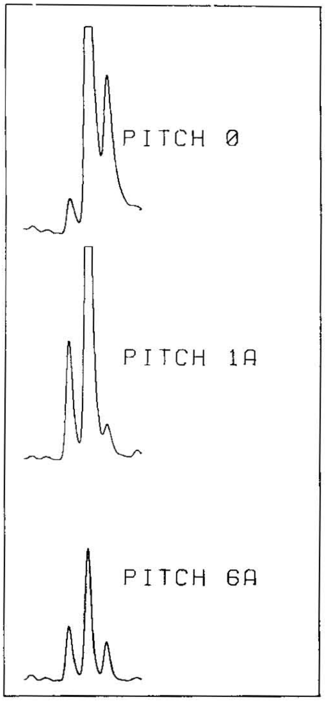

Fig. 5. Comparison of triplet peaks centered around acenaphthylene for FID plots of three coal tar-pitches.

DISCUSSION

The use of PGC for the study and characterization of such complex samples as coal tar- and petroleum-pitches appears to be promising, particularly for preliminary screening. Among the many potential advantages of this technique are the small sample (<5 mg), the rapid analysis (<2 h total time) and the ability to acquire all of the useful data in one run, as opposed to getting only one physical characteristic from each of several standard tests.

The need for more extensive testing of a variety of pitches is evident, even though we appear to have good correlations for the pitches we have studied. For example, the relationship between the presence of an acenaphthylene peak to a moderate-to-high QI value would have practical utility in screening pitches.

We have examined the use of the nitrogen detector from a qualitative viewpoint, but more extensive studies with both this and other specific detectors (in particular, a sulfur detector) could be of considerable interest with these samples. Finally, the use of pyrolysis units at higher final temperatures and various heating rates, corresponding more closely to actual graphitizing conditions, could be useful for studying the mechanisms involved in a process such as graphitization.

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CHROM. 11,370

APPLICATION OF GAS CHROMATOGRAPHY USING NITROGEN-SELECTIVE DETECTION TO OIL SPILL IDENTIFICATION

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SUMMARY

The use of a gas chromatograph employing a 36-m SE-30 glass SCOT column with dual flame-ionization–nitrogen–phosphorus detection (NPD) for oil identification is described. A rapid extraction and concentration procedure for increasing the size of NPD peak patterns from oils of low N-content and eliminating spurious peaks due to excess aliphatic hydrocarbons is presented. Studies of artificially "weathered" oils indicate that the gas chromatographic–NPD fingerprint of light distillate oils is lost, but that of heavier oils is unchanged. The capabilities and limitations of the gas chromatographic–NPD fingerprints for oil identification are illustrated with reference to real spill case samples.

INTRODUCTION

Gas chromatography (GC), with its unparalleled separation efficiency, is especially suitable for forming a fingerprint of the complex mixtures of compounds which make up petroleum oils. Such fingerprints can be used to match an oil spilled on water to its source in order to assist in establishing legal responsibility for the consequences of the spill. By splitting the effluent from the GC column to a flame-ionization detector (FID) and a flame-photometric detector (FPD) tuned to respond to organic sulfur-containing compounds, we have been able to acquire two independent GC fingerprints simultaneously[1]. Another element often found in some oils is nitrogen[2,3], and thus a thermionic nitrogen-selective GC detector would provide yet another oil fingerprint from a GC effluent. The operation and applications of the newer forms of such nitrogen–phosphorus detectors (NPD), which employ independently heated rubidium glass beads, are described by Kolb and co-workers[4,5].

Lee *et al.*[6] described the use of the NPD with glass capillary columns for the forensic analysis of used engine oils. They observed many NPD peaks which appeared to result from nitrogen compounds produced in the oil by the operation of the engine and whose patterns depended on the particular engine and its operating conditions. Glajch *et al.*[7] have employed the NPD for the characterization of coal tar and petroleum pitches by pyrolysis GC. We have previously described our preliminary evaluation of the NPD for GC oil fingerprinting[8,9]. Using packed OV-101 columns we found that one must use dual FID–NPD detection to assess the contribution of spurious FID response from the NPD due to high concentrations of simultaneously eluting hydrocarbons. There was some question of the value of the NPD fingerprints for oil identification since oils of similar geological origin displayed very similar NPD patterns. To address this latter problem we surveyed over 70 different crudes, distillates, lube oils and synthetic oils from both similar and different geological sources, using dual FID–NPD detection with a 15.75-m Dexsil-300 SCOT column operating under conditions specified for the U.S. Coast Guard's oil spill identification system[1]. The chromatograms of this survey have been published along with an alumina adsorption column procedure for isolating and concentrating the compounds producing the NPD patterns[10]. The data in that report supported the conclusion that there is sufficient variability among high resolution GC–NPD patterns of oils to warrant the use of the NPD for oil fingerprinting.

The purpose of this paper is to present NPD fingerprint data obtained from artificially weathered oils and real spill cases and to assess the usefulness of such fingerprints in oil identification. We also present a new extraction procedure to concentrate the N-containing oil fraction to enhance the GC–NPD patterns of oils of low nitrogen content, and describe some of the problems inherent with the application of the NPD to oil identification.

EXPERIMENTAL

Chromatography parameters

Chromatograms were obtained with a Perkin-Elmer 990 GC equipped with an AS-41 automatic, solvent-free, capsule injection system. The column was a 36 m × 0.5 mm SE-30 glass SCOT manufactured by Scientific Glass Engineering (North Melbourne, Australia). For the period covered in this work we calculated a theoretical plate value of 35,000 for hexadecane run at 180° and a resolution value of 21 for the hexadecane–octadecane peaks. The column was pressure controlled to give a flow-rate of 4 ml/min at room temperature for helium. The column oven temperature was programmed from 75 to 300° at 8°/min and held for 16 to 32 min for light and heavy oils, respectively. These flow and temperature conditions sacrificed some resolution to attain more rapid sample throughput of the heavier oils. The injector temperature was 250° and the manifold and detector blocks were at 325°. Helium carrier make-up gas was supplied at 40 ml/min to the column effluent and this was then split in a ratio of 1:1 to the FID and NPD.

Detector parameters

The FID was operated to produce a maximum response with a hydrogen flow-rate of 21.6 ml/min and an air flow-rate of 353 ml/min. Note that FID curves are displayed inverted above the NPD curves in this paper.

The response of the Perkin-Elmer NPD was a complex function of carrier, hydrogen and air flow-rates as well as the heating current supplied to the rubidium silicate bead. We optimized the response according to the manufacturer's instructions to give a selectivity ratio of 10^5:1 for N,N-dimethylaniline *vs.* light *n*-alkanes. This was achieved with an air flow-rate of 100 ml/min and a hydrogen flow-rate of 2.8 ml/min. Some sensitivity was sacrificed by using a power supply potentiometer setting of 560 corresponding to a heating current of 2.8 A through the bead. This setting was used to prolong the bead life, and it kept the NPD sensitivity relatively constant over a period of six months. Lubkowitz *et al.*[11] have recently discussed the effects of these parameters on NPD response reproducibility.

In our previous work with this NPD, we reported that an electrode polarity setting of 2 on the detector power supply module gave much better selectivity for nitrogen compounds at the cost of some sensitivity. Consequently, all work in this report was done at the same setting. It was later discovered that with the SCOT column flow parameters used in this work, the higher sensitivity polarity setting 3 would produce the more selective response. This behavior is consistent with the change in carrier flow parameters when switching from packed column to SCOT column operation[12]. More recent NPD designs have dispensed with the polarity variation as an unnecessary complexity.

Oil sample preparation

Heavy oils were pentane-deasphalted, dried over anhydrous $MgSO_4$ and stripped of pentane (DDS), according to the procedure in ref. 1. With heating of the sample when necessary, quantities of less than 1.0 μl were dispensed from a syringe into the injection capsules. Lighter oils were loaded into the capsules without treatment (NEAT).

Methanol extraction procedure

Weigh 0.2 g of DDS or NEAT oil into a 16 × 125 mm disposable culture tube and add 1.0 ml of pentane and 5.0 ml of a solution 10% (v/v) of water in methanol. Vortex 30 sec, centrifuge if necessary to separate phases, remove the pentane layer and transfer the methanol–water layer to a 10-ml conical-bottom centrifuge tube. Evaporate the solution to dryness under nitrogen flow at 100°, reconstitute the dried sample with 100 μl of toluene and transfer 5.0 μl of this solution to a GC injection capsule and allow the toluene to evaporate under a gentle stream of nitrogen before sealing the capsule. The NPD pattern of such a sample is approximately equivalent to that from a 12-μl injection of the NEAT or DDS oil with almost complete elimination of spurious FID response peaks.

RESULTS AND DISCUSSION

The FID and NPD curves labeled A in Fig. 1 are from the injection of 0.2 μl of NEAT API No. 2 fuel oil. The NPD curve illustrates several general features of NPD oil fingerprints. The majority of the small peaks appearing before 20 min result from the spurious FID response of the NPD to the much higher concentration of aliphatic hydrocarbons eluting during this time. Note the apparent reflection of the upper, inverted, highly attenuated FID curve above the NPD curve.

The peaks labeled X and Y are frequently seen contaminants in our oil samples which are not of petroleum origin. We suspect that they originate from containers in which the oils are shipped as their concentrations are erratic and variable among samples of the same oil and often increase with time. Such contaminant artifacts are often unavoidable with detectors of such great sensitivity as the NPD and the analyst must become familiar with their appearance in order not to be confused in

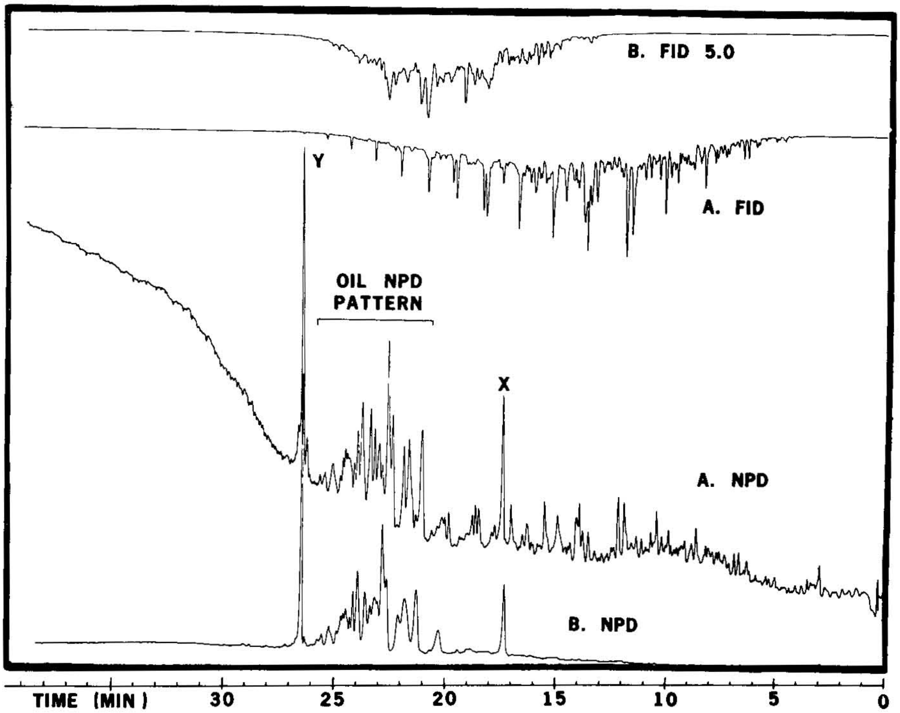

Fig. 1. Dual FID–NPD GC curves of NEAT and methanol extracted API No. 2 oil. (A) 0.25 μl NEAT oil, FID ×3200, NPD ×2; (B) 5.0 μl of toluene-reconstituted methanol extract, FID ×3200, NPD ×32.

the interpretation of the fingerprint. Another source of NPD contaminant patterns (illustrated in ref. 10) is the Apiezon M grease used as a lubricant in the AS-41 capsule injection system. These latter patterns can be eliminated by careful cleaning of the system and the use of Dow Corning silicone grease on the capsule holding magazines. Chromatographers using syringe injection techniques will need to investigate the contribution of septum bleed to the NPD peak patterns.

The apparent column bleed in the NPD curve, for times greater than 26 min, is often observed at high sensitivity. It does not result from decomposition of the stationary phase or from the light oil injected, but is the prolonged tail of the unresolved envelope of nitrogen compounds from the preceding GC analysis of a very heavy residual oil with a high nitrogen content. Such an artifact is not noticed when many such heavy oils are run in succession, but it will be evident in the NPD curves of immediately succeeding light oils. It can be eliminated by cycling the GC through several temperature programs before examining a series of light oils.

The resolution of the NPD peaks displayed in Fig. 1 is not as complete as that seen in earlier work (*cf.* ref. 10, Fig. 6). It is adequate, however, to produce a distinctive NPD fingerprint, and most importantly, this fingerprint will display the same pattern if the oil is repeatedly analyzed over a period of one to two weeks. As is the case with other GC oil fingerprinting methods, we recommend that oils to be matched by GC–NPD patterns be analyzed in a continuous sequential series on the same column.

We have observed[10] that many oils contain such small NPD patterns when analyzed NEAT or DDS that a concentration procedure for the nitrogen compounds which also removes the bulk of the hydrocarbons producing the spurious FID response is required. We previously described[9,10] such a procedure employing adsorption of the oil on a 5% water-deactivated alumina column followed by elution with pentane and benzene and concentration of the benzene effluent. While it is effective, it is cumbersome and time consuming to apply it uniformly to all the samples of a large spill case. Simple extraction procedures involving vortexing and pipet transfers between tubes are more suitable for large sample loads. Lee *et al.*[6] recommended nitromethane to extract the NPD fingerprints of used engine oils, but this solvent presents problems of toxicity and flammability. Saner *et al.*[13] have demonstrated that methanol successfully extracts most of the heavier aromatic compounds from oils for HPLC–fluorescence analysis. As we expected that most, if not all, of the NPD peaks we observe in the characteristic oil patterns result from large N-heterocycle compounds, we developed the simple methanol extraction and concentration procedure described in the experimental section. The combined effects of the variables of each solvent concentration as well as the percentage of water in the methanol solvent were optimized using a three dimensional, two level, factorial design. The optimized procedure was then tested by triplicate extractions for each of four different oils. The NPD fingerprints of the replicated extracts were indistinguishable. Fig. 2 shows the NPD patterns of 3 successive extractions of both

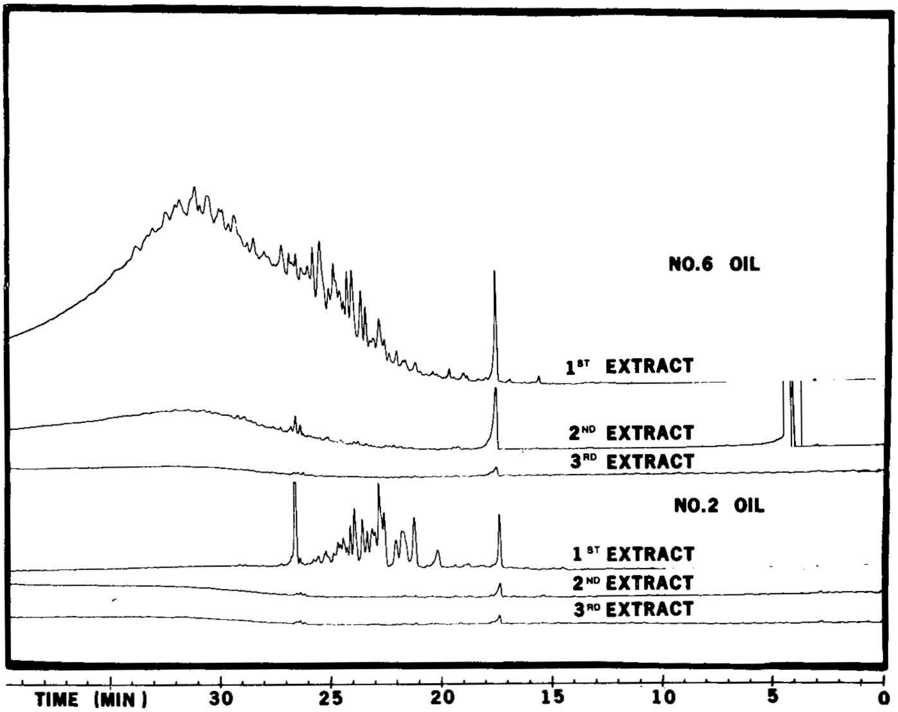

Fig. 2. GC–NPD curves of 5.0 μl of toluene-reconstituted successive methanol extractions of No. 6 and No. 2 oils. NPD × 32.

heavy and light distillate oils. These data as well as identical experiments on crudes indicate that the single step extraction recovers at least 90% of the compounds producing the NPD pattern.

Curves B of Fig. 1 display FID and NPD patterns of the methanol extracts of the same API No. 2 oil as the NEAT curves A. The FID pattern illustrates the loss of *n*-alkanes and is likely to be composed mostly of the more polar compounds extracted into the methanol. The NPD pattern is similar to that of the NEAT sample of curve A, but the larger attenuation used illustrates the concentration factor attained. Note the degraded resolution of the early NPD peaks and the shift in peak retention times of the extract curve relative to the NEAT curve. This behaviour was consistently observed with the methanol extracts of light oils, and this chromatogram displays the worst instance of the phenomenon we have observed. This may be associated with an unusually large amount of co-eluting aromatic compounds which are seen in the FID curve to have been extracted from this light distillate. However, the altered NPD patterns observed from the extracts of such oils were consistently repeatable, and this is the prime criterion of an identification method. On the other hand, the NPD patterns of the extracts of heavier oils and crudes were indistinguishable from the NPD patterns of DDS samples except in magnitude.

The NPD fingerprint pattern of an oil spill may differ from the original source oil if some of the nitrogen compounds evaporate, dissolve into the water column, biodegrade or are photochemically altered. Such weathering (WX) effects distort the fingerprints observed with other analytical methods as well. To study this problem, a series of 4 different oils were subjected to controlled simulated weathering conditions in tanks of sea water at the Research Institute of the Gulf of Maine (TRIGOM), and the changes in the GC–FID oil fingerprints resulting from this treatment have been described[14]. We used the same TRIGOM weathering series sample set to evaluate the effects of weathering on GC–NPD fingerprints.

Fig. 3 displays the effect of weathering on two light oils. Curves A and B indicate that the NPD pattern of a No. 2 oil is degraded immediately. and after 4 days of weathering it had disappeared completely. The pattern of a No. 4 oil was stable for one day, but 4 days weathering also eliminated its NPD fingerprint. From these data and other cases we conclude that GC–NPD patterns are unsuitable for fingerprinting spilled light distillate oils, after weathering has occurred.

Fig. 4, curve A displays the NPD curve of a DDS sample of the unweathered TRIGOM crude. This oil is a Louisiana crude with a very low content of nitrogen compounds, and the peaks seen in curve A are either contaminants or spurious FID response. This oil requires the use of the methanol extraction procedure to produce usable NPD fingerprints, as illustrated in curves B through D. These NPD curves show that the patterns of this crude are unaffected by weathering. The NPD pattern of the methanol extract of a different Louisiana crude is displayed in curve E. Because of its similar geological origin, this oil has an NPD pattern very similar to that of the TRIGOM crude, but the enhancement of the patterns produced by the extraction and concentration procedure can allow the possibility of distinguishing the oils.

Fig. 5 displays the NPD patterns of the weathering series of a heavy residual oil, and the curves illustrate the excellent stability of the nitrogen fingerprint with respect to weathering for such oils. The compounds forming many of the peaks of

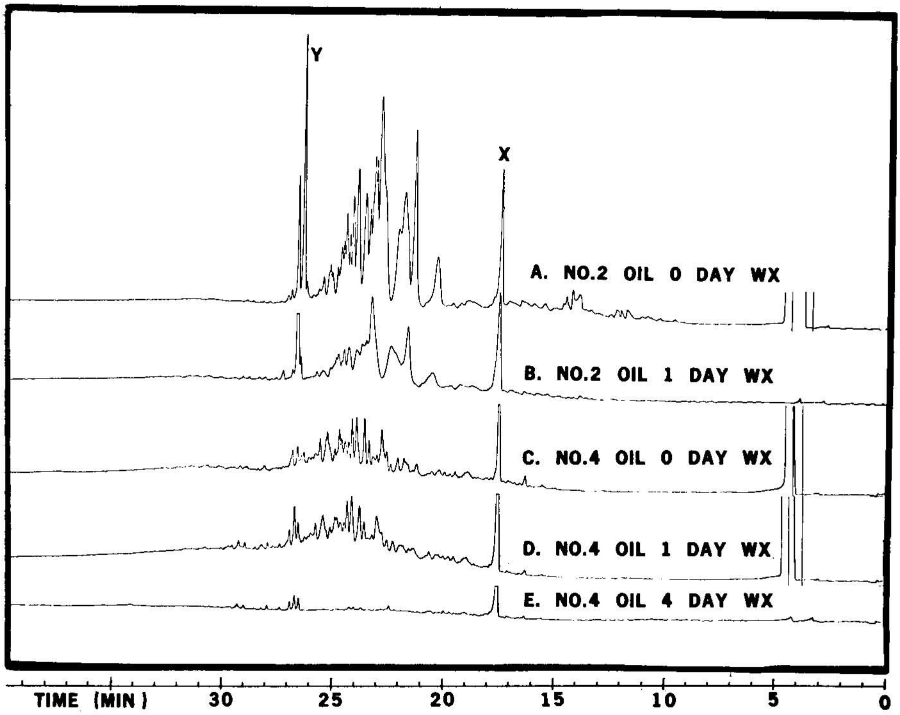

Fig. 3. GC–NPD curves of 5.0 μl of toluene-reconstituted methanol extracts of TRIGOM light distillate oils weathering series. NPD ×32.

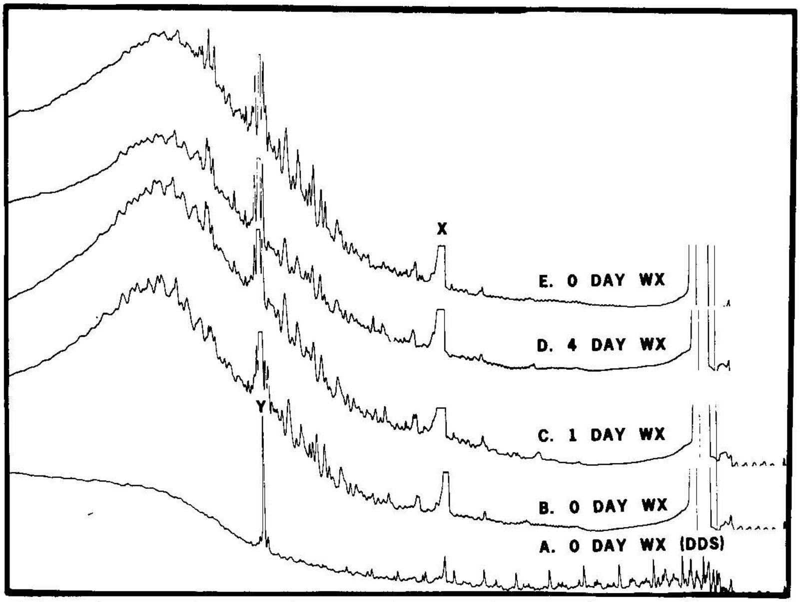

Fig. 4. GC–NPD curves of weathered TRIGOM crudes, NPD ×4. (A) 0.5 μl DDS unweathered; (B) 5.0 μl methanol concentrate unweathered; (C) 5.0 μl methanol concentrate 1 day WX; (D) 5.0 μl methanol concentrate 4 days WX; (E) 5.0 μl methanol concentrate unweathered S. La. crude.

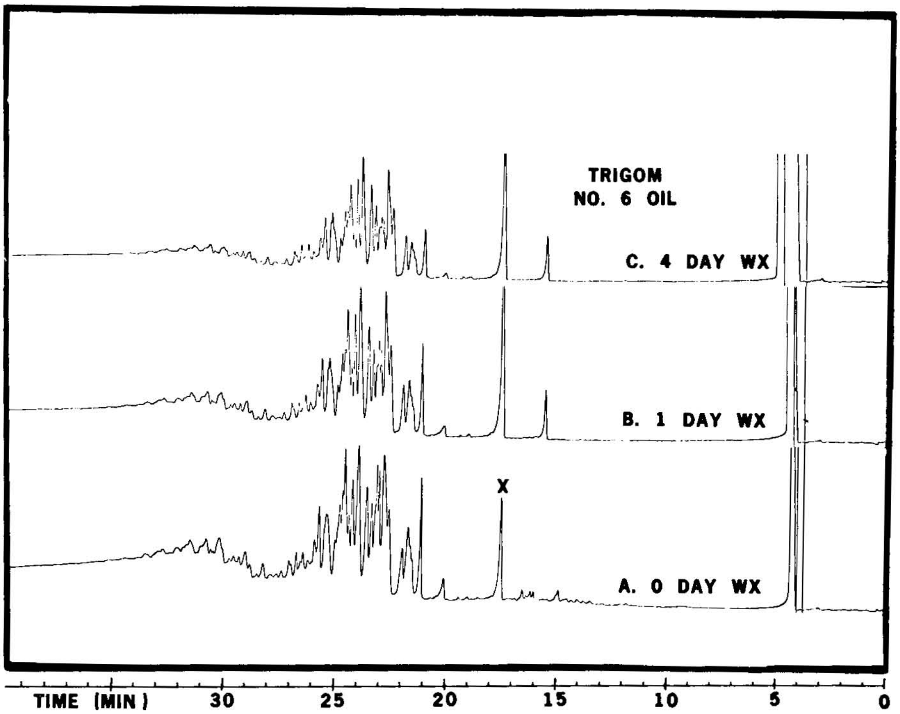

Fig. 5. GC–NPD curves of 5.0 μl of toluene-reconstituted methanol extracts of TRIGOM No. 6 oil weathering series. NPD ×32.

the NPD patterns are likely to be the same in both heavy and light oils, since there are often similarities in the patterns and retention times which are observed[10]. In view of the long retention times of characteristic NPD peaks seen in petroleum oils, the compounds forming them probably have limited volatility at ambient temperature and are polar in nature. It is therefore likely that the primary mechanism for the weathering losses of most of these compounds is dissolution into the underlying water, and this process is likely to proceed less efficiency from the more viscous heavy oils.

Fig. 6 displays the NPD chromatograms of spill and source oils from two spill cases in which positive matches had been previously obtained by the U.S. Coast Guard's four-method (GC, thin-layer chromatography, IR and UV fluorescence) oil identification system[1]. The methanol extract GC–NPD patterns of case 1 also match excellently. The peak patterns of the DDS samples of case 2 also tend to confirm the match in that case. The spill samples display variable quantities of some NPD peaks in the latter parts of the chromatograms. This behavior indicates that the spill samples may have been slightly contaminated with small variable amounts of NPD-responsive compounds, which, however had no effect on the fingerprints of the other methods. The appearance of this type of contamination lessens the usefulness of the NPD fingerprint.

Such small contaminant NPD peaks have been seen in other spill samples, and we believe then to be due to compounds of biological origin which have been

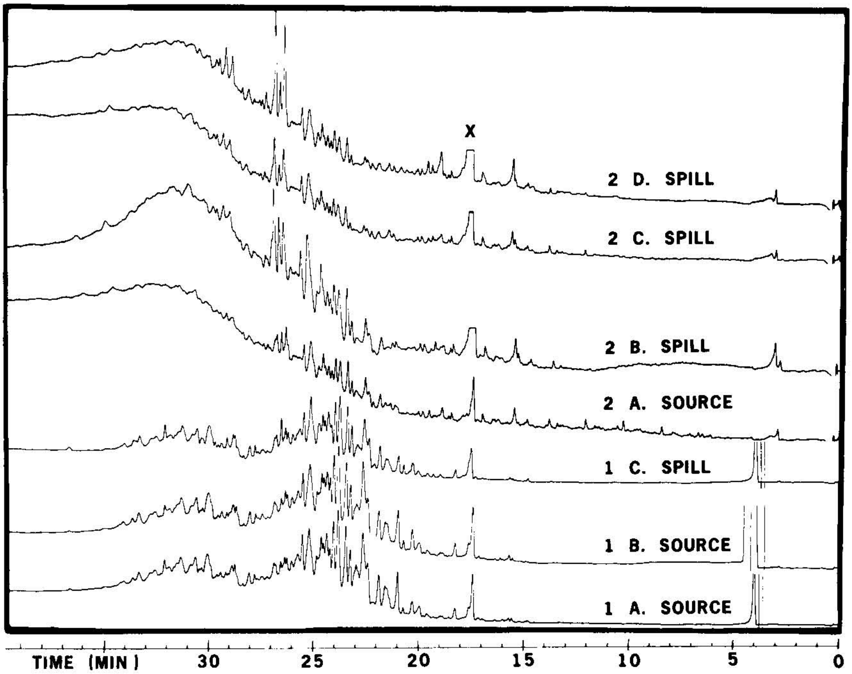

Fig. 6. GC–NPD curves of matched source and spill samples from two real spill cases. Case 1, 5.0 μl methanol concentrates NPD ×32; case 2, 0.5 μl DDS NPD ×4.

picked up by the spilled oil during its movement on the water. Our more time consuming alumina adsorption concentration procedure[9,10] has often succeeded in removing such contaminant peaks without affecting the NPD peak pattern of the oil fingerprint. Our experience with a KOH–methanol saponification and extraction procedure for removing contaminants of this type[15] indicates that the methanol extraction procedure for NPD pattern concentration would have carried along these contaminants.

There is no evidence of weathering degradation of the NPD fingerprints in either case, despite the fact that the FID curves showed that weathering occurred on the case 2 spill samples.

Fig. 7 displays an unusual dual FID–NPD trace from a real spill sample. The FID pattern is characteristic of a lube oil, and unused lube oils are generally found to be free of NPD peaks[10]. The very large, complex NPD pattern displayed in this sample bears no relation to any patterns we have seen which originate from petroleum products[10]. The peaks may have been generated during engine operation in the manner described by Lee *et al.*[6]. The source sample was matched by the four method technique[1], and while the relative NPD peak heights differed substantially between the source and the spill, the same unusual series of peaks appeared in both samples. Thus it is possible that the NPD can be used to advantage for fingerprinting spilled lube oils if a sufficiently unique series of NPD peaks appears in both the source and spill oils, even though weathering effects may change the relative peak heights.

Fig. 8 illustrates both favorable points and disadvantages of the use of GC–

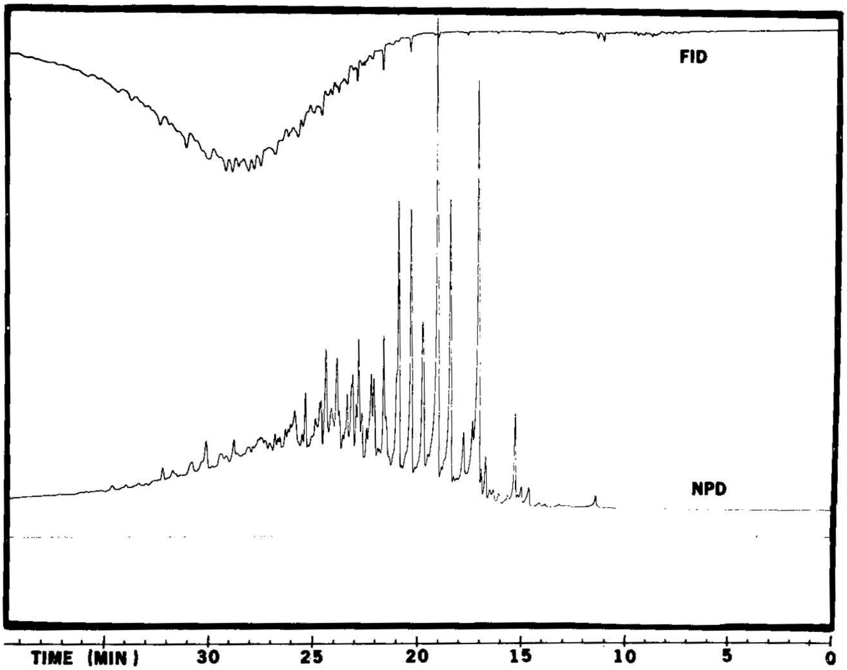

Fig. 7. Dual FID–NPD GC curves of 0.5 μl DDS lube oil spill sample. FID ×1600, NPD ×128.

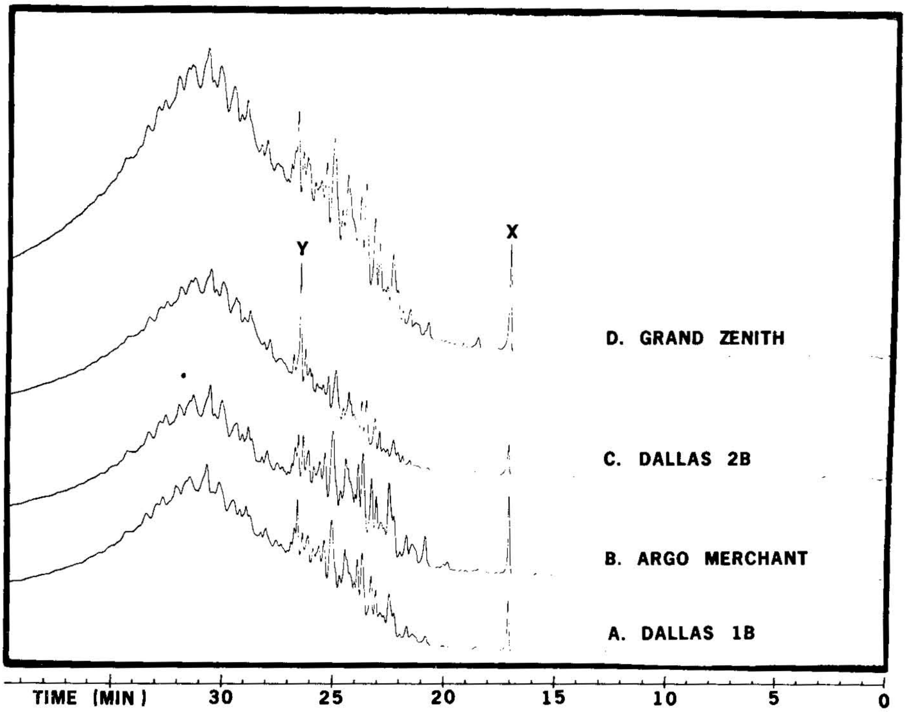

Fig. 8. GC–NPD curves of 5.0 μl of toluene-reconstituted methanol extracts of two matched source–spill pairs. NPD ×32. (A) 1st spill sample taken by Cutter *Dallas*; (B) *Argo Merchant* cargo sample; (C) 2nd spill sample taken by Cutter *Dallas*; (D) *Grand Zenith* cargo sample.

NPD fingerprints of heavy residual oils. Curves B and D are from samples of the cargos of the grounded tanker *Argo Merchant* and the missing tanker *Grand Zenith*, respectively. Curve A is from an oil slick picked up by the USCG Cutter *Dallas* three weeks after the *Argo Merchant* grounding over 320 nautical miles distant. The four-method oil identification system positively matched samples A and B, and it is thus satisfying to see how well the NPD fingerprints match even after the long exposure of the spill sample to weathering conditions. Curve C is from another oil slick sampled by the *Dallas* which was positively matched by the four-method system to sample D. Again the NPD patterns are also matches except for some easily recognized contaminants. The problem arises from the fact that all 4 NPD fingerprints are sufficiently similar that the spills could not be distinguished from the two possible sources or from each other. However, when the other 4 fingerprinting methods were applied to these pairs of matching spills and sources, there was no problem distinguishing them from each other. This demonstrates the advantages of using multiple identification techniques. Our atlas of dual FID–NPD chromatograms of oils[10] contains additional instances of dissimilar oils which possess very similar NPD fingerprints, and this circumstance increases the probability of obtaining an indeterminate result or a false identification with GC–NPD fingerprints unless other fingerprinting methods are also applied to the samples.

CONCLUSION

The GC–NPD fingerprints of oils can provide useful additional information for the identification of oils spilled on water subject to the following qualifications.

(1) The NPD patterns of light distillate oils are likely to be distorted or removed by weathering effects and must be used with caution for fingerprinting.

(2) Additional fingerprinting methods must be applied to the oils to allow for the possibility that the NPD patterns of some different oils may appear identical. Thus the GC–NPD fingerprint serves, at best, as a confirming analysis, not as a primary identification method.

(3) Oils of low nitrogen content require the use of an extraction and concentration procedure to produce NPD patterns of a size adequate for fingerprinting. The NPD patterns of such extracts are sufficiently reproducible for this purpose.

(4) The high sensitivity of the NPD detector, combined with the small quantity of nitrogen compounds forming the NPD pattern of an oil, renders the GC–NPD fingerprint more susceptible than the GC–FID fingerprint to contaminants and other artifacts. Our experience shows that these can be recognized by the analyst or eliminated by additional sample preparation.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF PEPTIDES AND PROTEINS

XI. THE USE OF CATIONIC REAGENTS FOR THE ANALYSIS OF PEPTIDES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

This report describes the effect of different cationic reagents (tetraalkylammonium, alkylammonium and inorganic salts) on the retention times of di- to pentapeptides chromatographed on a reversed-phase support (*i.e.* a μBondapak-alkylphenyl column). Several trends are apparent with these reagents which can be explained on the basis of either ion-pairing or ion-exchange interaction of the reagent with the peptide sample. Reagents which generate in solution small highly solvated cations, *e.g.* Li, Na or Mg salts, give retention times similar to those obtained for ammonium salts. Tetraethylammonium salts give a modest increase in retention times relative to ammonium salts. By contrast, hydrophobic cations with long or bulky carbon chains, *e.g.* tetrabutylammonium or dodecylammonium ions, cause substantial decreases in retention times, resulting in very rapid elution of all peptides examined from the reversed-phase column. These observations are consistent with the composite interplay of ion-pair partition and dynamic ion-exchange effects for the cationic reagents. The use of a mixture of dodecylammonium acetate and sodium dodecylsulphate for the analysis of peptides and proteins is described. It is anticipated that such a chromatographic system will be useful for the analysis of proteins which readily aggregate.

INTRODUCTION

In earlier reports[1,2] we have demonstrated that hydrophobic ion-pairing of unprotected amino groups is a useful technique for the analysis and isolation of

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peptides by high-pressure liquid chromatography (HPLC). The association of a peptide sample with a hydrophobic anion in the eluent leads to a less polar complex which has an increased retention time on a reversed-phase column. By contrast, the use of a hydrophilic anion such as dihydrogen phosphate results in a decreased retention time for the peptide on the same column[3,4]. Thus, depending on the amino acid composition and sequence of the peptide, selectivity can be achieved by a suitable choice of negatively charged counter ions.

In a similar manner it was expected that ion-pairing of a cationic reagent with the carboxyl groups of a peptide sample would lead to useful modifications of its chromatographic behaviour. Previous studies have used tetraalkylammonium ions in the analysis of polar pharmaceuticals[5,6] and organic carboxylates[7]. In this report the effect of a series of tetraalkylammonium, alkylammonium and inorganic reagents on a variety of peptides is described. The results indicate that, depending on the cationic species involved, the retention time of the peptide can either be significantly increased (*e.g.* tetraethylammonium ion) or decreased (*e.g.* dodecylammonium ion) on a μBondapak-alkylphenyl reversed-phase column.

MATERIALS

High-pressure liquid chromatography

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which included two M6000 solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatograph injector, coupled to a Cecil 212 (Cecil, Cambridge, Great Britain) variable wavelength UV monitor with an 8-μl flow-through cell and a Linear Instruments (Costa Mesa, Calif., U.S.A.) double-channel chart recorder.

The μBondapak-alkylphenyl column (10 μm, 30 cm × 4 mm I.D.) was obtained from Waters Assoc. Sample injections were made with a Pressure-Lok liquid syringe, Series B-110 from Precision Sampling (Baton Rouge, La., U.S.A.). Filtration of solvents was carried out using a pyrex filter holder (Millipore, Bedford, Mass., U.S.A.) while peptide samples were filtered using a Swinney Filter (Millipore).

Reagents

All solvents were Analar grade. The methanol was used as supplied by Mallinckrodt (St. Louis, Mo., U.S.A.). The acetonitrile, supplied by Fisher Scientific (Pittsburgh, Pa., U.S.A.) was further purified as described previously[3]. Orthophosphoric acid was from May & Baker (Dagenham, Great Britain). Water was glass-distilled and deionised. The peptides were purchased from Research Specialties (Richmond, Calif., U.S.A.). All amino acids were of the L-configuration. The tetraalkylammonium ions were obtained from the following suppliers: tetramethyl (BDH, Poole, Great Britain), tetraethyl (Merck, Munich, G.F.R.), tetrapropyl (Pfaltz Bauer, Flushing, N.Y., U.S.A.) and tetrabutyl (BDH). All other cationic reagents were obtained from BDH and were of reagent grade.

METHODS

A flow-rate of 1.5 ml/min was used which was maintained by a pressure of

2000 p.s.i. All tests were at room temperature (*ca.* 22°). Sample sizes varied between 1 and 50 μg of peptide material injected in volumes of 1–50 μl. All peptides were taken up in deionised, distilled water and, prior to injection, made up in the eluting solvent. Water was degassed for at least 30 min, the solvents were mixed in the required volumes, filtered through a 0.45-μm Millipore filter and then equilibrated to room temperature. All solvents were stirred magnetically during equilibration and elution. All columns were equilibrated to new solvents for at least 30 min.

Unless otherwise indicated, the same mobile phase conditions were used, *i.e.* methanol–water (1:1), a 2 m*M* solution of the reagent, acetate as the anion and adjusted to pH 4. A high concentration of methanol was chosen so that a wide range of samples and solute concentrations could be examined with the same chromatographic system. All retention times were reproducible and represent at least two determinations.

RESULTS

The results from the analysis of seven different peptides on a μBondapak-alkylphenyl column with a series of tetraalkylammonium reagents are shown in Table I. The acetate salts were chosen for this study because we had previously shown[3] that the presence of acetate anions caused minimal shifts in the retention of peptides and proteins on reversed-phase columns. As can be seen from Table I there is a small but progressive increase in retention time as the alkyl chain length of the reagent increases. A similar trend has previously been observed[2] with anionic ion-pairing reagents. The tetrabutylammonium reagent, on the other hand, caused decreased retention in each case. The addition of tetrapropylammonium ions to the mobile phase gave rise to double peaks for several of the peptidic samples. It is unlikely that the emergence of a second peak is due to contaminants since these

TABLE I

COMPARISON OF THE EFFECT OF TETRAALKYLAMMONIUM SALTS ON THE RETENTION TIME OF PEPTIDES

The other eluent parameters are described under Methods.

*Peptide**	*Retention time (min)*				
	Ammonium salt ($R_4\overset{+}{N}\overset{-}{OAc}$)				
	$R = H$	$R = CH_3$	$R = CH_2CH_3$	$R = (CH_2)_2CH_3$	$R = (CH_2)_3CH_3$
L–W–M–R	2.8	3.0	3.05	3.5	2.7
L–W–M–R–F	5.7	6.8	6.5	8.7	4.0
G–F	2.4	2.4	2.6	2.7	2.05
G–G–Y	2.0	2.0	2.2	1.8**, 2.05	1.7
M–R–F	2.6	2.7	2.8	3.0	1.7
G–L–Y	2.4	2.5	2.8	2.7	2.1
R–F–A	2.3	2.3	2.6	2.1**, 2.7	1.8

* The code for amino acids of M. O. Dayhoff[14] is used. A = alanine, D = aspartic acid, F = phenylalanine, G = glycine, K = lysine, L = leucine, M = methionine, R = arginine, W = tryptophan, Y = tyrosine.

** Two peaks were observed.

samples had previously been shown to be homogeneous by several chromatographic procedures and by compositional data. Rather, the presence of two chromatographic species suggests the involvement of two discrete mechanisms operating via different partition effects. It is possible that a transition from ion-pair formation to a dynamic ion-exchange mode occurs with the twelve-carbon tetrapropyl group. Certainly, the shorter retentions observed with the tetrabutylammonium cation are consistent with a different separation mode operating with this reagent compared to the lower homologues.

The alkylammonium salts also show a similar trend with the lower homologues again causing increased retention (Table II). In general, the observed retention follows the order $NH_4^+ < \overset{+}{N}H_3CH_3 \approx \overset{+}{N}H_3CH_2CH_2OH > \overset{+}{N}H_3(CH_2)_3CH_3 > \overset{+}{N}H_3(CH_2)_5CH_3 \gg \overset{+}{N}H_3(CH_2)_{11}CH_3$ (common anion acetate). With the elution system containing dodecylammonium acetate the peaks obtained with the various peptides had very high plate efficiencies even allowing for the short retention times observed.

TABLE II

COMPARISON OF THE EFFECT OF DIFFERENT ALKYLAMINE SALTS ON THE RETENTION TIME OF PEPTIDES

The other eluent parameters are described under Methods.

Peptide	*Retention time of amine salt ($R\overset{+}{N}H_3$)*					
	$R = H$	*$R = CH_3$*	*$R = CH_2CH_2OH$*	*$R = (CH_2)_3CH_3$*	*$R = (CH_2)_5CH_3$*	*$R = (CH_2)_{11}CH_3$*
L-W-M-R	2.7	3.05	3.1	2.9	2.4	1.6
L-W-M-R-F	5.7	7.0	6.1	6.8	4.4	2.1
G-F	2.4	2.5	2.4	2.3	2.25	2.0
G-G-Y	2.0	2.2	2.3	2.0	1.8	1.4
M-R-F	2.6	2.75	2.7	2.5	2.2	1.45
G-L-Y	2.4	2.6	2.6	2.45	2.25	2.0
R-F-A	2.3	2.3	2.45	2.1	2.0	1.3

These results contrast with the small effects noted with alkaline metal salts on the retention times of the peptides. In this case (Table III) the observed retention times follow the apparent order Li $\approx$ Na $<$ K $<$ Cs and with the divalent cations, intermediate between Li and Cs, Mg $<$ Ca (again common anion acetate).

In view of the significant decrease in the retention of these peptides with dodecylammonium ion, reflecting an apparent increase in polarity of the peptidic molecules, it was attractive to use this reagent in conjunction with the anionic detergents which are known[2,8] to involve dynamic ion exchange. Table IV shows the comparative effects of dodecylammonium acetate, sodium dodecylsulphate and an equimolar mixture of these two reagents on the retention times observed for the series of peptides. As we have noted[2] before the eluent containing sodium dodecylsulphate resulted in increased retention times irrespective of whether the reagent was complexing with the amino terminus or side chain cationic group. Eluents containing dodecylammonium acetate, in contrast, showed the opposite effect with invariant increases in apparent polarity of the peptidic samples. Significantly, equimolar mixtures of the anionic and cationic reagents resulted in the rapid elution of peptide (Table IV and Fig. 1) and protein (Fig. 2) samples.

TABLE III

COMPARISON OF THE EFFECT OF METAL IONS ON THE RETENTION TIME OF A SERIES OF PEPTIDES

The other eluent parameters are described under Methods.

Peptide	*Cationic reagent*					
	Li^+	Na^+	K^+	Cs^+	Mg^{2+}	Ca^{2+}
L-W-M-R	2.9	2.9	3.0	3.4	2.8	3.2
L-W-M-R-F	4.7	4.7	4.8	4.8	4.5	4.9
G-F	2.2	2.2	2.35	2.5	2.2	2.5
G-G-Y	2.1	2.1	2.3	2.4	2.2	2.3
M-R-F	2.6	2.6	2.7	2.8	2.6	3.0
G-L-Y	2.3	2.3	2.5	2.7	2.3	2.5
R-F-A	2.3	2.2	2.45	2.8	2.3	2.6

TABLE IV

COMPARISON OF THE EFFECT OF DODECYLAMINE ACETATE AND SODIUM DODECYLSULPHATE ON THE RETENTION TIME OF PEPTIDES

Peptide	*Retention time (min)*		
	$CH_3(CH_2)_{11}SO_3^-Na^+$ *(A)**	$CH_3(CH_2)_{11}NH_3^{+-}OAc$ *(B)***	*(A) + (B)***
L-W-M-R	16.2	1.6	2.3
L-W-M-R-F	40.5	2.1	4.45
G-F	2.5	2.0	2.2
G-G-Y	6.2	1.4	1.6
M-R-F	>58	1.45	2.1
G-L-Y	2.7	2.0	2.2
R-F-A	33.2	1.3	1.9

* A 2 m*M* solution of this reagent was used in methanol–water (1:1) (pH 6.5).

** The eluent parameters are described under Methods.

DISCUSSION

Peptides and proteins show a wide range of polarities and it would be surprising to find a single ion-pairing reagent suitable for the analysis of such an extensive diversity. However, selectivity can be achieved by taking advantage of free amino or carboxyl groups in peptides or proteins. We recently demonstrated[1-4] that hydrophilic, hydrophobic ion-pairing and dynamic ion exchange of peptides and proteins via amino-group interactions is a valuable aid in the analysis and isolation of these materials. It is the purpose of this study to examine the potential of cationic reagents for ion-pairing of carboxyl groups of peptides and proteins. Such reagents have already been applied to a variety of separations, *e.g.* tetrabutylammonium ions have been used in the analysis of polar pharmaceuticals[5,6], vitamins[7,8] and recently nucleotides[9].

In Tables I, II and III, the effect of a series of tetraalkylammonium, alkylammonium and inorganic salts on the retention time for a number of peptides is described. It should be noted that these changes in retention time were quite reproducible. At lower concentrations of the organic components in the mobile phase larger differences could be observed between the various cationic reagents. A relatively high methanol concentration was used in these studies so that a wide range of eluant conditions and sample polarities could be examined with the same mobile phase. From these results it can be seen that the retention time of peptides can be significantly modified by the addition of various cationic reagents to the mobile phase. For example, the addition of small polar cations, *e.g.* Li^+, Na^+, Mg^{2+}, caused a decrease in retention time while less polar cations, *e.g.* Cs^+, Ca^{2+}, $\overset{+}{N}(CH_2CH_3)_4$ caused an increase in retention time relative to $\overset{+}{N}H_4$. It is likely, therefore, that these cationic reagents will provide a useful addition to the conditions available for the analysis of complex mixtures of peptides and proteins. The results obtained, however, were less dramatic than those obtained for ion-pairing of the amino groups of peptides and proteins and, presumably, reflects the dominant effect of the alkylammonium ions on the polarity of these molecules[3].

The changes in retention time observed for tetramethyl- and tetra-ethylamine, ethylamine, ethanolamine and inorganic salts can be explained on the basis of ion-pairing between carboxyl groups of the peptide sample and the cation added to the mobile phase. For example, if a more polar cation forms an ion pair with the peptide, the resulting complex would be expected to show decreased retention on a non-polar reversed-phase column. This suggestion is supported by a recent observation[10] that the use of trimethylammonium phosphate in the eluent gives excellent resolution and recoveries of peptide and protein mixtures on reversed-phase HPLC. It is possible that both the polar trimethylammonium and phosphate ions associate with the carboxylate and amino functional groups of peptides and proteins respectively and thus allow their facile chromatography.

Several authors have demonstrated, however, that ion-pair reagents can also partion between the stationary and mobile phase[8,9,11 13]. Under such conditions, the ion-pair reagent can be retained and form a dynamic surface coating on the stationary phase. These solvent-generated stationary-phase modifications can permit ionic interactions between the peptide and the stationary phase. This effect appears to be the dominant mechanism with anionic detergents like sodium dodecylsulphate and probably also for cationic detergents like dodecylammonium acetate. At pH 4 a peptide would be predominantly protonated and thus show minimal interaction with a cationic stationary phase. This will be reflected in short retention times as can be seen, for example with dodecylammonium acetates in Tables II and IV and the decrease in retention time observed for alkylamines of increasing C-chain length (see Table II).

The increase and then decrease in retention time for the tetraalkylammonium ion series (Table I) could be explained by considering the possible balance between the ion-pairing and ion-exchange effects of the reagents added to the mobile phase. It is possible that the tetramethyl- and tetraethylammonium ions act predominantly by ion-pairing effects while the tetrabutylammonium ion undergoes little ion-pairing with the solute due to the steric bulk of the cation but acts by forming a surface coating on the stationary phase. Several of the peptides showed two peaks with the intermediate case of the tetrapropylammonium ion (see Table I), an observation which could be explained on the basis of these competing effects. The data in Tables I and II also suggest that an alkyl chain of a significant number of C-atoms and surface bulk is required for an effective coating of the stationary phase.

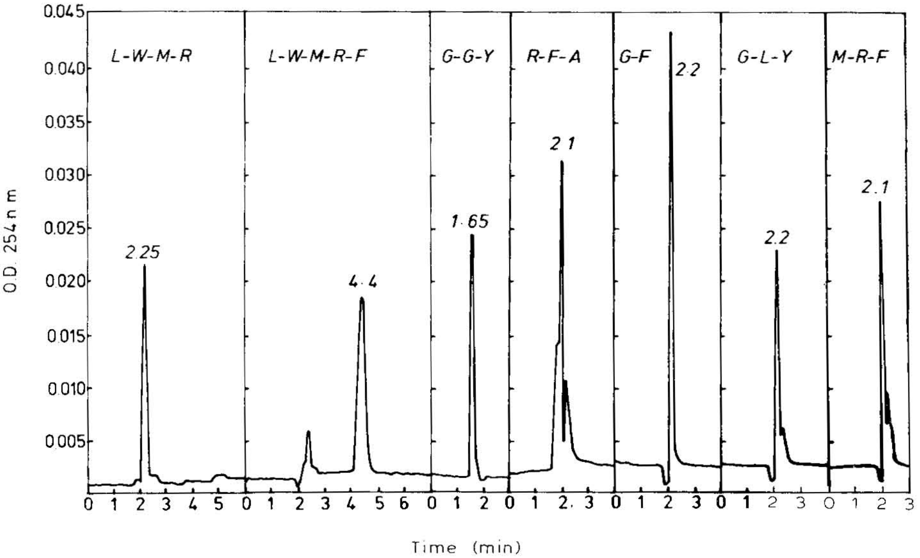

Fig. 1. The analysis of a series of peptides on a μBondapak-alkylphenyl column with an eluent which consisted of 2 m*M* sodium dodecylsulphate and 2 m*M* dodecylammonium acetate dissolved in methanol–water (1:1) at pH 4.

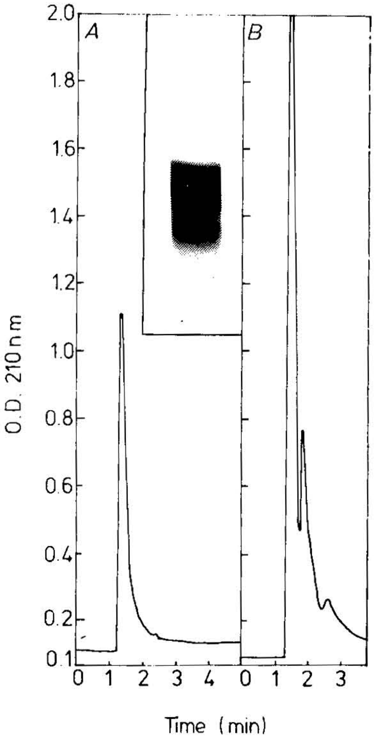

Fig. 2. The elution profile of a sample of sheep liver aldehyde dehydrogenase which was chromatographed on a μBondapak-C_{18} column with an eluent which consisted of isopropanol–water (1:4) 2 m*M* dodecylammonium phosphate, pH 2.6. (A) 2 μl of a dialysed sample of enzyme; (B) 10 μl of a dialysed sample of enzyme. The inset shows the electrophoretic pattern obtained for the analysis of 100 μg of the protein on a 7% polyacrylamide gel at pH 8.9 in the presence of 0.1% sodium dodecylsulphate.

In Table IV the opposing effects of dodecylamine acetate and sodium dodecylsulphate is shown. Together the two reagents offer a powerful method of increasing or decreasing the retention of peptides on reversed-phase systems. As is shown in Fig. 1 a mixture of the two reagents leads to a useful analytical system in which peptides can be rapidly analysed under dissociating conditions. The use of this system for the analysis of proteins which are sensitive to aggregation, such as membraneous proteins and lipoproteins, is currently under investigation. Fig. 2 shows the analysis of the enzyme sheep liver aldehyde dehydrogenase with an eluent which contained dodecylammonium acetate, pH 4. A minor impurity observed by polyacrylamide gel electrophoresis was also observed in the elution profile shown in Fig. 2 and presumably is caused by a small amount of a contaminating protein. In the absence of dodecylamine the protein sample was indefinitely retained on a reversed-phase column even in the presence of 0.1% H_3PO_4 and high concentrations of organic solvents.

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SEPARATION OF UNCONJUGATED PTERIDINES BY HIGH-PRESSURE CATION-EXCHANGE LIQUID CHROMATOGRAPHY

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SUMMARY

In the course of determining the levels of unconjugated pteridines occurring in various biological fluids, such as urines, plasma and tissue culture media, a method has been developed for the separation and quantitative determination in the picomole range of ten 2-amino-4-hydroxy substituted pteridines. This method involves separation by high-pressure cation-exchange liquid chromatography and fluorescence detection of the eluted compounds at 450 nm. Optimal separation was obtained by isocratic elution with 3 mM phosphoric acid–7% methanol–1% acetonitrile at a flow-rate of 2 ml/min or with 1 mM ammonium dihydrogenphosphate pH 2.8–7% methanol–5% acetonitrile at a flow-rate of 1.5 ml/min. With either solvent, the order of elution of the compounds is: isoxanthopterin, pterin-6-carboxylic acid, xanthopterin, pterin-6-carboxaldehyde, D-*erythro*-neopterin, L-*threo*-neopterin, biopterin, 6-hydroxymethylpterin, pterin, 6-methylpterin. In addition, a systematic investigation of the effects of ammonium ion concentration and pH of the solvent as well as column temperature on the separation of these compounds was also conducted.

INTRODUCTION

Separation of pteridines has been accomplished by a variety of chromatographic techniques: paper chromatography and monodimensional thin-layer chromatography (TLC)[1,2] have widely been used, but two-dimensional TLC[3], ion-exchange chromatography[4], gas chromatography–mass spectrometry[5], and finally, high-pressure liquid chromatography (HPLC)[6] have also found application in this field. The quantitative determination of pteridines in biological materials, however, has usually been accomplished by laborious and time-consuming techniques involving a combination of ion-exchange chromatography and paper or TLC[3,4,7–9]. Only recently, rapid methods for such determinations have been developed using gas chromatography–mass spectrometry[10] and a combination of HPLC and gas chromatography[11]. These methods, however, were not chosen for determination of all the pteridines known to occur in biological materials, but only applied to the quantitation of two of them, biopterin and neopterin.

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During our attempts to determine the levels of unconjugated pterins (2-amino-4-hydroxy substituted pteridines) occurring in urines of normal individuals as well as in urines of cancer patients[12], it became necessary to adopt a chromatographic system that would separate and quantitate all the pterins that were likely to be found in biological fluids. HPLC became the method of choice when speed, reproducibility and cost were considered.

In the present communication, we report the results of a systematic investigation on the separation of most of the biologically occurring pterins by cation-exchange HPLC. The aims of our study are two-fold: (a) The method should separate closely related pterins and have a wide applicability, *i.e.*, it should be useful for the separation of pterins from a wide variety of biological sources. (b) By making use of their natural fluorescence, the method should lend itself to the quantitation of pterins in the picomole range.

MATERIALS AND METHODS

Isoxanthopterin, xanthopterin, pterin and pterin-6-carboxylic acid (Pt-6-COOH) were purchased from Sigma (St. Louis, Mo., U.S.A.); biopterin was obtained from Calbiochem (San Diego, Calif., U.S.A.); pterin-6-carboxaldehyde (Pt-6-CHO) and 6-hydroxymethylpterin (Pt-6-CH_2OH) were prepared by the method of Thijssen[13] with minor modifications as described previously[14]. Neopterin was prepared by the method of Rembold and Eder[15] and 6-methylpterin (Pt-6-CH_3) by reductive cleavage of folic acid[16]. Organic solvents used to prepare buffers for the chromatographic separations were of the "liquid chromatography" grade. HPLC was performed with an Altex Model 100 pump equipped with an Altex model 905-42 sample injector (Altex Scientific, Berkeley, Calif., U.S.A.). A 25 cm × 4.6 mm Partisil-10 SCX column (Whatman, Clifton, N.J., U.S.A.) equipped with a 5 cm × 4.6 mm precolumn filled with the same kind of packing as the analytical column was used for all the separations described. Detection of the eluant was performed with a Farrand A_4 fluorimeter (Farrand Optical, Valhalla, N.Y., U.S.A.) equipped with a 7-60 excitation filter (band center at 360 nm) and a narrow band interference filter with peak transmittance at 450 nm. The aperture of the fluorimeter was set at 5 and the range at 1. In some experiments, the column temperature was varied by means of a water bath. The compounds were injected in various volumes; varying the injection volume between 5 and 200 μl did not significantly affect peak shapes nor retention times. The solvents used throughout were filtered with a 0.4-μm millipore filter and degassed under vacuum just prior to use. When solutions of ammonium dihydrogenphosphate and organic solvents were prepared, we first adjusted the pH to the required end point, and then we mixed the salt solution with the required volume of organic solvent.

RESULTS

In our continuing study of folates and pterins metabolism, we became interested in the separation and quantitation of pterins present in biological samples such as body fluids, enzymatic reactions, tissue culture media, etc. In previous investigations[12,14], we have made use of thin-layer and paper chromatography, but these techniques do not have enough resolving power and are not easily amenable to quantitation.

Thus, we became interested in ion-exchange HPLC. Although HPLC systems have been developed for the separation of many diverse pteridine derivatives[6], these systems could not separate the pterins in which we are interested, such as the pterins known to occur in urines.

Our approach to the separation of such pterins is based on the consideration that the p*K* for many of these compounds ranges between the limits of pH 1–3 (ref. 17); thus, we tested various concentrations of phosphoric acid for their ability to separate the compounds of interest on a strong cation-exchange column. We found that solutions of 1–10 m*M* phosphoric acid were able to separate most of the pterins studied except for xanthopterin and Pt-6-CHO which cochromatographed under those conditions. However, the simple addition of 5–10% methanol to the phosphoric acid buffer made this separation possible. Fig. 1 shows the separation of nine pterins on a Partisil-10 SCX column when eluted isocratically with 3 m*M* phosphoric acid–7% methanol–1% acetonitrile. The addition of 1% acetonitrile was rendered necessary by the very long elution time of Pt-6-CH$_3$ when the 3 m*M* phosphoric acid–7% methanol system was used. Addition of a small amount of acetonitrile probably helps to overcome hydrophobic interactions of the methyl group on the pteridine with the column backbone. This explanation is supported by the observation that while 6-methylpterin can be eluted with a solvent containing only 1% acetonitrile, 6,7-dimethylpterin requires a greater proportion of acetonitrile in the solvent in order to be eluted from the same column (results not shown).

Although the 3 m*M* phosphoric acid–7% methanol–1% acetonitrile system gives reproducible retention times, it is very sensitive to the presence of cations in the

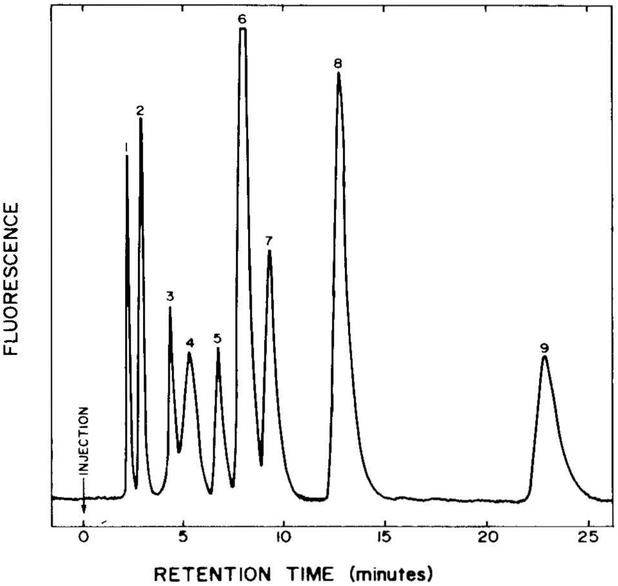

Fig. 1. Separation of pterins on a Partisil-10 SCX column. Solvent, 3 m*M* phosphoric acid–7% methanol–1% acetonitrile; flow-rate, 2 ml/min; column temperature, 20°. Peaks: 1 = isoxanthopterin; 2 = Pt-6-COOH; 3 = xanthopterin; 4 = Pt-6-CHO; 5, D-*erythro*-neopterin; 6 = biopterin; 7 = Pt-6-CH$_2$OH; 8 = pterin; 9 = Pt-6-CH$_3$.

solutions injected. Thus, if, for instance, a solution of pterins in ammonium hydroxide is injected, retention times will be somewhat changed depending on the injection volume and concentration of the cation. For this reason, we studied the chromatographic behavior of some pterins when eluted from a cation-exchange column with ammonium dihydrogen phosphate solutions rather than phosphoric acid. Fig. 2 shows how the mobility of some pterins changes as a function of the NH_4^+ concentration in the eluting buffer, while the pH was kept constant to a value of 2.6 with phosphoric acid. Clearly, the higher the NH_4^+ concentration, the poorer the separation obtained. A good compromise between buffering capacity and separation was chosen at a value of 1 m*M* NH_4^+. We also studied the mobility of pterins as a function of the pH of the eluting buffer (1 m*M* ammonium dihydrogenphosphate). The pH was adjusted to the indicated values (Fig. 3) by additions of phosphoric acid. A pH optimum for separation was obtained at a value between 2.6 and 2.8, thus indicating that separation was probably being achieved on the the basis of the basic p*K* of these compounds. Separation decreases on either side of the pH optimum, probably because the positive charge on the pterin is lost at higher pH values and because at lower pH values the exchanger groups start to get protonated. It is interesting to note that

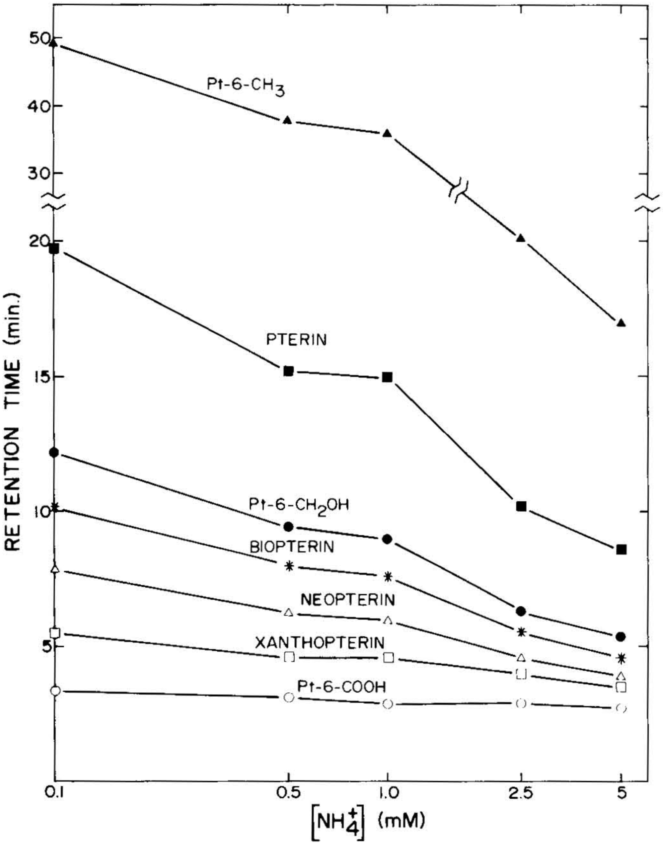

Fig. 2. Effect of NH_4^+ concentration of the solvent on retention times of pterins on Partisil-10 SCX. Solvent, different concentrations of ammonium dihydrogenphosphate adjusted to pH 2.6 with phosphoric acid; flow-rate, 2 ml/min; column temperature, 23°.

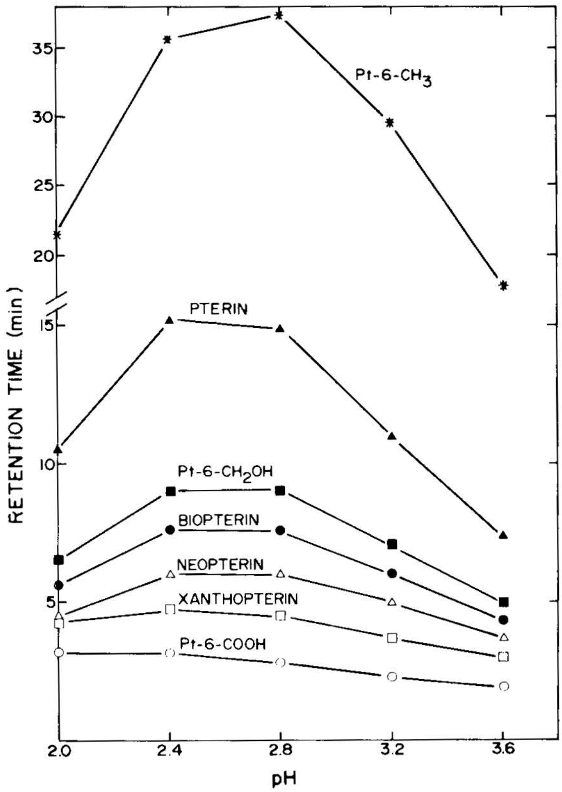

Fig. 3. Effect of pH of the solvent on retention times of pterins on Partisil-10 SCX. Solvent, 1 m*M* ammonium dihydrogenphosphate adjusted to the indicated pH with phosphoric acid; flow-rate, 2 ml/min; column temperature, 23°.

between the pH values tested (2.0–3.6), the pterins studied retain the same relative position with respect to each other.

Mobility and therefore resolution is also affected by the column temperature. Fig. 4 shows the change in retention times as a function of the column temperature for the same compounds tested in the studies of Fig. 2 and 3. The pterin most affected by the temperature change is Pt-6-CH$_3$, which shows a dramatic decrease in retention time as the column temperature is increased. Peak shapes are also affected by the temperature; that is, in general, the peaks get sharper with increasing temperature. This phenomenon can be attributed to the earlier elution, as it is known that the number of theoretical plates (a measure of column efficiency) is directly proportional to the square of the retention time. When the parameter N (number of theoretical plates) calculated for Pt-6-CH$_3$ is plotted against the elution temperature, an optimum temperature of elution for Pt-6-CH$_3$ is reached around 45° (results not shown). However, for maximum resolution, zone separation must be considered in addition to zone width. An interesting phenomenon occurs only with the parent compound, pterin, at a temperature of 30°. At this specific temperature pterin chromatographs as a double peak, that is, as a peak with a shoulder, but at any other temperature it gives only a single peak (Fig. 5). One possible explanation for this phenomenon is offered by the

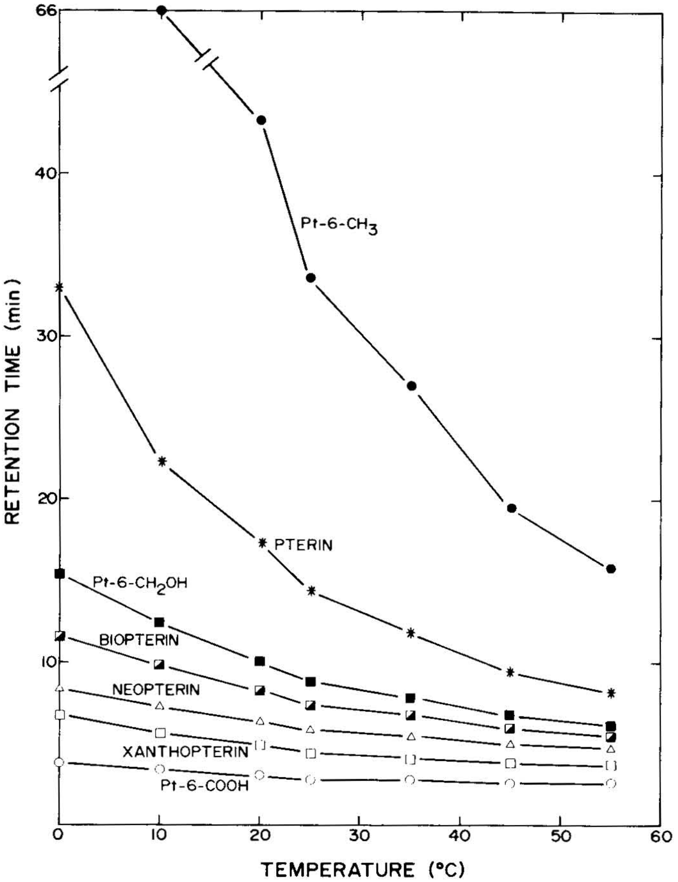

Fig. 4. Effect of column temperature on retention times of pterins on Partisil-10 SCX. Solvent, 1 m*M* ammonium dihydrogen phosphate, pH 2.6; flow-rate, 2 ml/min.

existence of an hydrated form of the compound in rapid equilibrium with the non-hydrated form[17]. It is possible that during chromatography at 30° the conditions are not favorable for the equilibration of the two forms, thus resulting in the appearance of a new entity.

Thus, column temperature, pH and counter-ion concentration of the solvent as well as flow rate, can all be optimized to obtain the best resolution. However, separation between Pt-6-CHO and xanthopterin can only be achieved by addition of 7% methanol to the salt solution, as in the case of the phosphoric acid solution. Fig. 6 shows the separation of ten closely related pterins obtained with a 1 m*M* ammonium dihydrogenphosphate, pH 2.8–7% methanol–5% acetonitrile system at 25°. In this case, the small amount of acetonitrile is needed to decrease the "tailing" effect observed with the ammonium dihydrogenphosphate-containing buffers, as well as to decrease the elution time of Pt-6-CH$_3$. The resolving power of this technique is well evidenced by the ability to separate two optical isomers of neopterin: the D-*erythro*- from the L-*threo*-neopterin, both of which have been reported to occur in human

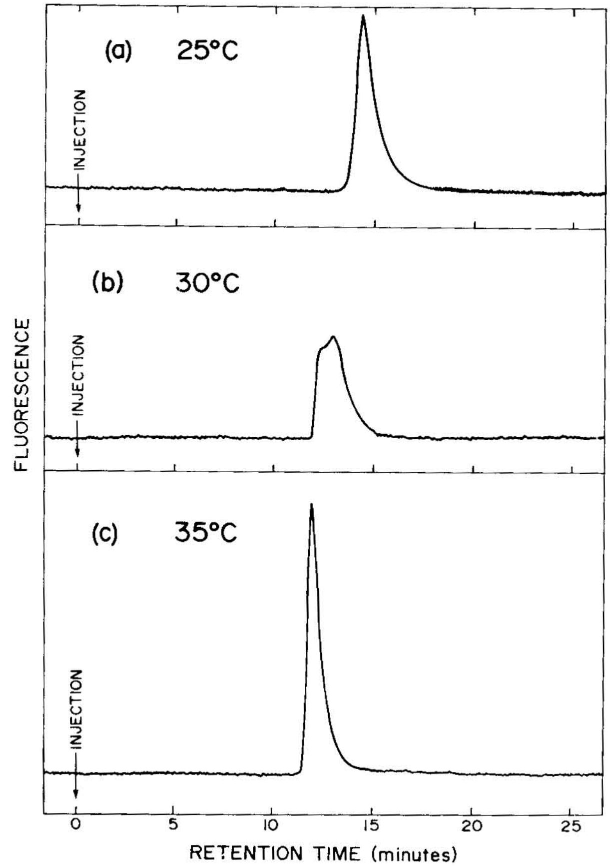

Fig. 5. Effect of column temperature on the shape of the pterin peak. Solvent, 1 m*M* ammonium dihydrogenphosphate, pH 2.6; flow-rate = 2 ml/min; column temperature, (a) 25°; (b) 30°; (c) 35°.

urine[9]. The method is also extremely sensitive; the peaks reproduced in Fig. 6 represent 20 pmoles of isoxanthopterin and Pt-6-COOH, and 100 pmoles of all the other compounds. The sensitivity of the fluorimeter can still be increased by a factor of four with a good signal-to-noise ratio, thus it is possible to detect sub-picomole amounts of isoxanthopterin and Pt-6-COOH and at least 5–10 pmoles of other pterins.

DISCUSSION

The method that we have developed allows a very rapid and sensitive determination of many biologically occurring pterins in a single chromatographic run, which can be accomplished in a time-span of about 40 min. We included Pt-6-CHO and Pt-6-CH_2OH among the pterins that we wanted to separate, because the former has been reported to occur in urines from cancer patients[12] when they were chromatographed by TLC, and the latter in tissue culture media of malignant cells[14]. Pt-6-CH_3, though it has not been found in biological materials, was included because of its potential use as

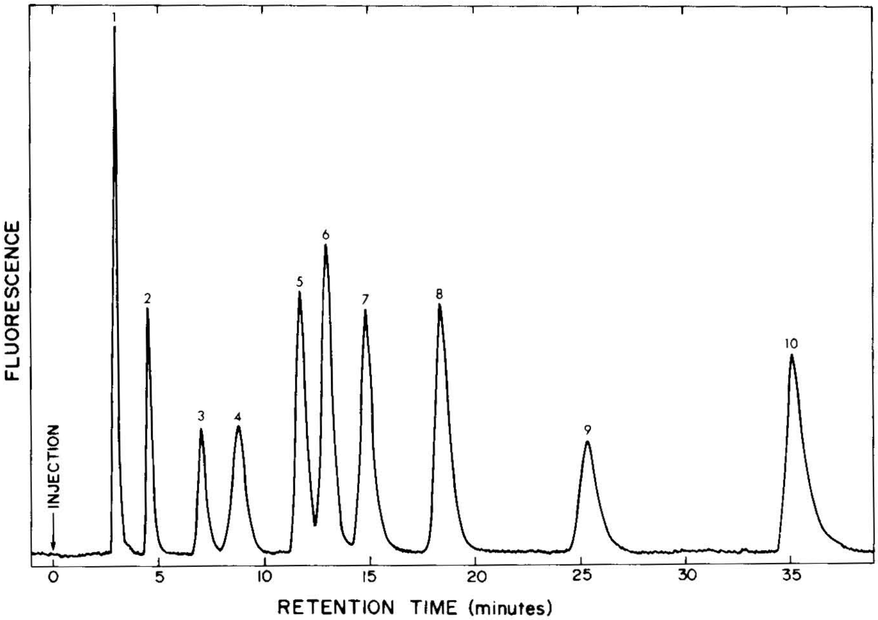

Fig. 6. Separation of pterins on Partisil-10 SCX. Solvent, 1 m*M* ammonium dihydrogenphosphate, pH 2.8–7% methanol–5% acetonitrile; flow-rate, 1.5 ml/min; column temperature, 25°. Peaks: 1 = isoxanthopterin; 2 = Pt-6-COOH; 3 = xanthopterin; 4 = Pt-6-CHO; 5 = D-*erythro*-neopterin; 6 = L-*threo*-neopterin; 7 = biopterin; 8 = Pt-6-CH_2OH; 9 = pterin; 10 = Pt-6-CH_3.

internal standard, as in many cases a preliminary purification of the biologically occurring pterins is necessary before separation and quantitation by HPLC can be accomplished[10,11]. We have successfully applied this technique in (i) the quantitation of the metabolic products of Pt-6-CHO by normal and malignant cells grown in culture[15]; (ii) the measurement of the levels of enzyme(s) involved in the metabolism of Pt-6-CHO[18]; (iii) monitoring purification of the 6-isomer from the 7-isomer produced during synthesis of some of the pterins utilized in this study (results not shown); and finally, (iv) the quantitative determination of pterins in human urine[19]. Because of its versatility, reproducibility and sensitivity, this procedure lends itself to a wide range of applications.

ACKNOWLEDGEMENTS

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CHROM. 11,352

FRACTIONATION OF MANNOSE-LABELED NEUTRAL GLYCOPEPTIDES BY QAE-SEPHADEX CHROMATOGRAPHY

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SUMMARY

Mannose-labeled cellular glycopeptides derived from human diploid fibroblasts (KL-2) were separated into two classes by QAE-Sephadex chromatography. High-voltage paper electrophoresis and Sephadex G-50 chromatography were used to characterize the glycopeptides further. At least five distinct neutral fractions were isolated with molecular weights ranging from 1050 to 2000 daltons. A linear gradient of ammonium acetate eluted a heterogeneous population of acidic glycopeptides. The use of QAE-Sephadex enables a single-step fractionation of both neutral and acidic glycopeptides on one column. The technique appears to be sensitive enough to distinguish growth-dependent alterations between growing and non-growing cells.

INTRODUCTION

Cellular glycoproteins constitute a very heterogeneous population of molecules[1,2]. Extensive proteolytic digestion has been used to reduce this material to oligosaccharides with relatively few amino acids[3]. Among the techniques used to isolate and characterize glycopeptides have been high-voltage paper electrophoresis (HVPE)[4,5], gel filtration[6,7], affinity chromatography[8], and ion-exchange chromatography. Anionic exchangers have been used to separate net negatively charged molecules containing sialic acid[9–11] or, possibly, charged amino acids. Cation exchangers have been used to separate neutral mannose-rich glycopeptides[12,13]. This communication describes an alternative method of fractionating both acidic and neutral species of glycopeptide with one column whereas, otherwise, a combination of the above-mentioned techniques would be required. The technique permits the large-scale purification of glycopeptides with essentially 100% recovery.

MATERIALS AND METHODS

Cell labeling and partial glycopeptide purification

Human diploid fibroblasts (KL-2) were cultured in Eagle's minimum essential media supplemented with 5% calf serum and 5% fetal calf serum, and buffered to maintain pH at optimal growth conditions[14]. The cells were tested for mycoplasmal infection and found to be negative[15]. Cells were labeled for 20–24 h either during exponential growth or in the non-growing state with D-[2-^{3}H]mannose (5 μCi/ml, specific activity 1 Ci/mmol, Amersham–Searle Radiochemical Centre, Arlington Heights, Ill., U.S.A.) or D-[^{14}C]mannose (0.5 μCi/ml, specific activity 240 mCi/mmol, Schwartz-Bioresearch, Orangeburg, N.Y., U.S.A.). Cell surface material was removed by mild Pronase digestion, and the remaining material was designated as cellular glycopeptides. The cell material was extensively digested with Pronase and partially purified by Sephadex G-50 chromatography[14].

Characterization of sugars in labeled glycopeptides

Hydrolysis of any glycopeptide sample under conditions to hydrolyze mannose and galactose (1 *N* HCl for 4 h at 100°) released radioactivity migrating with authentic mannose. Descending paper chromatography using Whatman 3MM paper was carried out with butanol–pyridine–water (6:4:3), developed for 15 h. Less than 10% radioactivity was found to migrate with authentic fucose when hydrolyzed with 0.1 *N* HCl at 80° for 45 min.

High-voltage paper electrophoresis

Cellular glycopeptides were lyophilized either as partially purified from Sephadex G-50 chromatography or fractionated on QAE-Sephadex, redissolved in 1–2 ml of water and loaded (approximately 160 μl) onto 1.5-in.-wide strips of Whatman 1MM paper. Electrophoresis was carried out at 4000 V for 3.5 h in pyridine–acetic acid–water (10:0.4:89.6), pH 6.5. Strips were dried, cut into 1-cm fractions, eluted with 0.5 ml of water for 30 min and counted aqueously in a liquid scintillation counter.

QAE-Sephadex chromatography

QAE-Sephadex A-25 (Pharmacia, Piscataway, N.J., U.S.A.), 3.0 ± 0.4 mequiv./g, was charged with 1 *M* ammonium acetate brought to pH 8.5 (at room temperature) with ammonium hydroxide and washed repeatedly with 5 m*M* ammonium acetate pH 8.5 until conductivity readings were stable. Care should be taken to avoid excessive stirring of the beads as this will affect the flow-rate. A column (42 × 1.5 cm I.D.) was prepared and thereafter all operations were done at 4°. Glycopeptides partially purified by Sephadex G-50 chromatography were initially eluted with 5 m*M* ammonium acetate pH 8.5 until only background radioactivity was detected. Five-milliliter samples were collected at a flow-rate of 20 min per tube. Thereafter a linear ammonium acetate gradient, pH 8.5, from 5 m*M* to 300 m*M* was used to elute the material remaining on the column. At completion of the gradient, elution by 1 *M* ammonium acetate recovered no significant radioactivity.

Mannose-containing glycopeptides were routinely checked for the presence of free mannose before application to QAE-Sephadex by descending paper chromatography[14].

RESULTS AND DISCUSSION

Mannose-containing cellular glycopeptides partially purified by Sephadex G-50 chromatography contain a mixture of acidic species (whose carbohydrate portion terminates with sialic acid) and neutral species (terminating with neutral sugars)[4]. As shown below, when a mixture of these glycopeptides is placed on QAE-Sephadex, neutral species have a weak affinity, since they rapidly elute at low salt concentration, while acidic species (carrying a net negative charge) are retained but elute with a linear gradient of higher salt concentrations.

Glycopeptides collected by Sephadex G-50 chromatography were lyophilized, redissolved in a minimal amount of water, and applied to a column of QAE-Sephadex. The mannose-labeled glycopeptides were initially eluted from the QAE-Sephadex with 5 m*M* ammonium acetate pH 8.5. As shown in Fig. 1a, they separated into at least five distinct peaks. The molecular weight of each peak was estimated by Sephadex G-50 chromatography based on their mobilities compared to known standards.

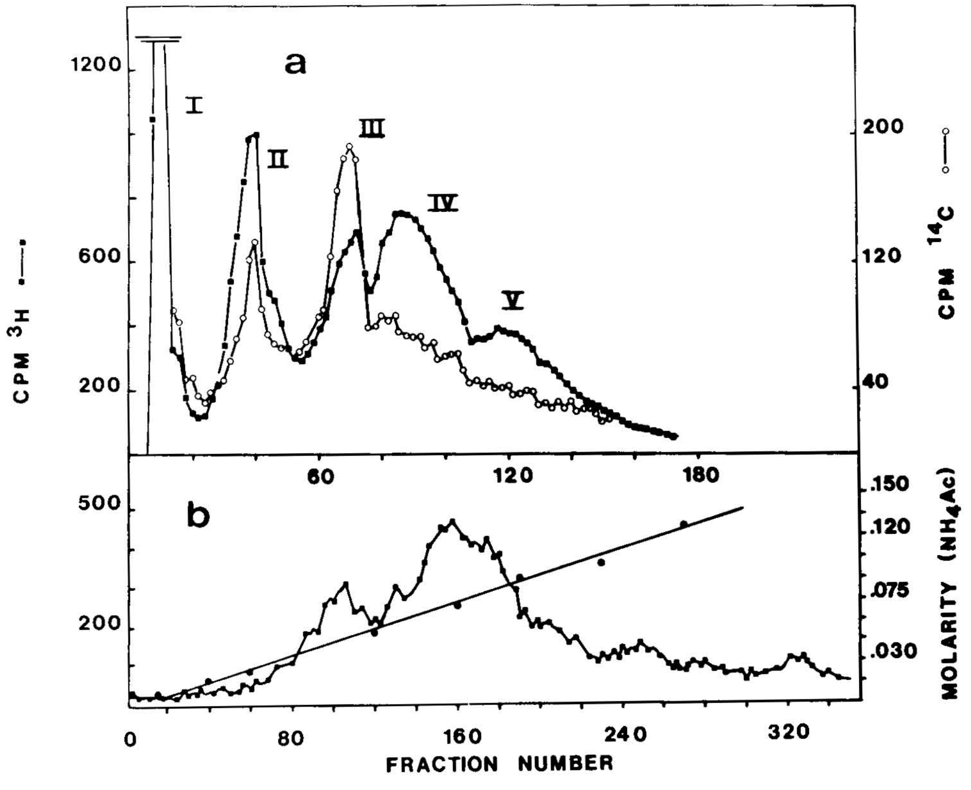

Fig. 1. QAE-Sephadex fractionation of cell material. Exponentially growing cells were labeled with D-[2-^{3}H]mannose (5 μCi/ml, 1 Ci/mmol) and non-growing cells with D-[^{14}C]mannose (0.5 μCi/ml, 240 mCi/mmol) as described under Materials and methods. The mannose-containing glycopeptides eluted and pooled from Sephadex G-50 columns were lyophilized and further chromatographed by QAE-Sephadex. (a) The sample (1.1 × 10^6 ^{3}H cpm and 3.0 × 10^5 ^{14}C cpm) was applied to a column (42 × 1.5 cm I.D.) and eluted with 5 m*M* ammonium acetate, pH 8.5. Five-milliliter fractions were collected at a flow-rate of 20 min per tube. Elution was continued until only background radioactivity was detected. (b) Glycopeptides eluted with a linear gradient, 5 m*M* to 300 m*M* ammonium acetate, pH 8.5. 1500 ml of 5 m*M* ammonium acetate in the mixing chamber and 1500 ml of 300 m*M* ammonium acetate in the reservoir. No significant radioactivity was recovered by subsequent elution with 1 *M* ammonium acetate. For simplicity, only the growing material is plotted in 1b, but is similar for the non-growing.

The standards used were two [^{14}C]acetylated ovalbumin glycopeptides of known structure[16], namely [^{14}C]acetylAsn(GlcNAc)$_2$(Man)$_5$ (molecular weight 1393) and [^{14}C]acetylAsn(GlcNAc)$_2$(Man)$_6$ (molecular weight 1555); and calf thyroglobulin unit A (molecular weight about 2000). All peaks eluted within the glycopeptide region and no radioactivity was recovered in the region of free mannose. The apparent molecular weight for the components ranged from approximately 1050 to 2000 daltons; they were neutral (see below). In general, neutral glycopeptides contain a simple oligosaccharide chain, (Man)$_n$(GlcNAc)$_2$, linked to asparagine[17,18]. The difference in molecular weight between each peak reported here (*cf.* Fig. 1a) can be accounted for by one or two monosaccharide residues. These results are consistent with the "oligomannosyl cores", ranging from 3–7 or 8 mannose residues, proposed by Muramatsu *et al.*[19] to exist in neutral glycopeptides obtained from diploid fibroblasts. However, recent studies on peak I (Fig. 1a) suggest that it may contain both glycopeptide and oligosaccharide material.

When a linear gradient of 5–300 m*M* ammonium acetate was applied to the QAE-Sephadex column, a heterogeneous mixture of acidic mannose-labeled material was obtained between 30 m*M* and 150 m*M* salt (Fig. 1b). With the present material it was not possible to detect distinct species in the eluted material. However, a similar analysis of cell surface material from exponentially growing and non-growing cells, to be reported separately, gave three distinct major peaks and several minor ones[20].

Glycopeptides partially purified by Sephadex G-50 chromatography can also be separated into acidic and neutral populations by HVPE (Fig. 2a). Peaks I–V (Fig. 1a) were also analyzed by HVPE. All five migrated as neutral glycopeptides (Fig. 2b–f). Of particular interest is the fact that the five species, while differing significantly in molecular weight, migrated only 3–4 fractions apart, and thus would be virtually indistinguishable from each other by HVPE. It was noted that the cellular glycopeptide profile (Fig. 2a) contains an apparent basic mannose-labeled species, comprising about 3–4% of the total material. Only peak I (Fig. 2b) was found to contain this component. With respect to the order of elution from the column, the neutral peaks appear to become progressively more contaminated with acidic material, ranging from no contamination in peak I to 33% contamination in peak V.

The heterogeneous peak of mannose-labeled glycopeptides eluted from the column between 30 m*M* and 90 m*M* salt concentration was collected (fractions 80–116, 120–140, 150–160 and 170–190) and an aliquot of each fraction was analyzed by HVPE; 83–98% of the radioactivity migrated as acidic glycopeptides. In general it was noted that each of the four fractions contained decreasing amounts of neutral material ranging from 17% in fractions 80–116 to 2% in fractions 170–190. The apparent cross contamination of neutral and acidic glycopeptides observed is not likely to be due to overlapping peaks on the QAE-Sephadex column since elution of the neutral glycopeptides is routinely continued to fraction 240 (only background radioactivity is recovered beyond fraction 180) before the linear gradient is applied.

A comparison of the neutral glycopeptides derived from non-growing and growing cellular material suggested growth-dependent alterations (Fig. 1a). The neutral glycopeptides derived from non-growing cellular material were markedly reduced in the high-molecular-weight species (IV and V) relative to those derived from growing cellular material. A similar result has previously been reported by Muramatsu *et al.*[19]. They used two specific endo-β-N-acetylglucosaminidases[21,22] to

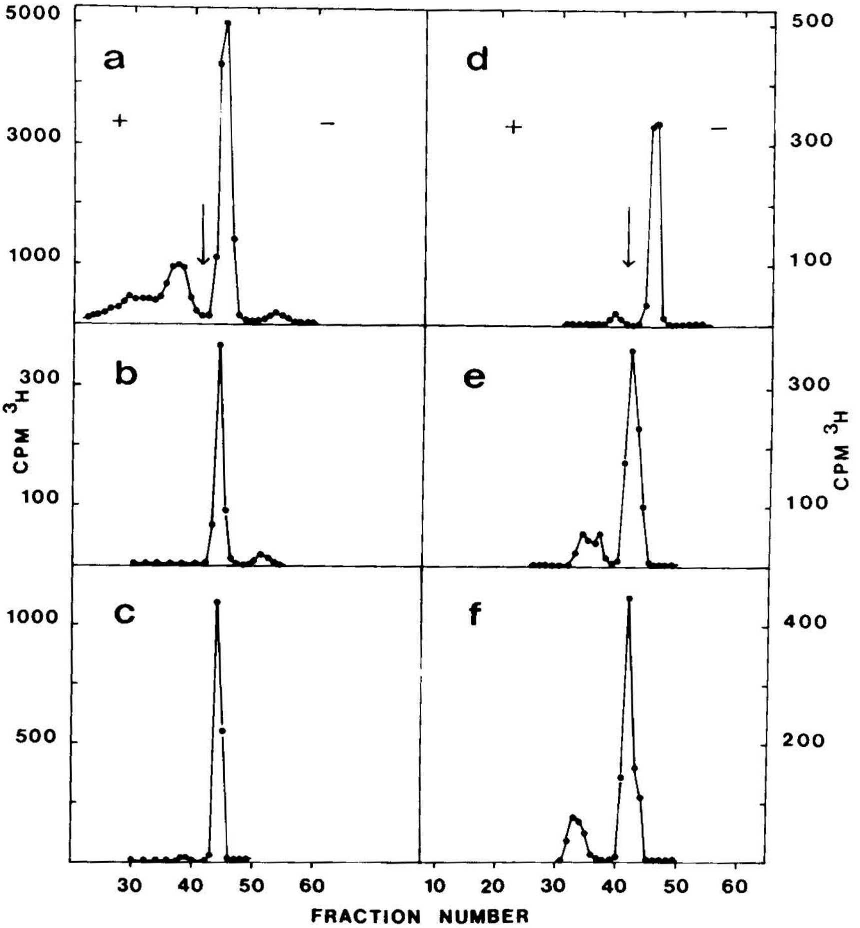

Fig. 2. Electrophoretic analysis of neutral glycopeptides. Mannose-labeled glycopeptides were prepared as outlined in the legend of Fig. 1 and further characterized by HVPE at pH 6.5.(a) Cell material was partially purified by Sephadex G-50 chromatography and analyzed by HVPE, at 4000 Volts for 3.5 h. Acidic glycopeptides migrate to the positive pole (fractions 20–40) and neutral glycopeptides move slightly to the negative pole (fractions 41–46). Neutrality is defined by the characteristic migration of neutral sugars under identical conditions. The arrow indicates the origin (fraction 41). (b) Species I eluted with 5 mM ammonium acetate; (c) species II; (d) species III; (e) species IV; and (f) species V. Apparent acidic contamination for each peak was as follows (measured as percent acidic cpm of total radioactivity recovered from paper): Species I, 0%; species II, 0%; species III, 5%; species IV, 22%; and species V, 33%. For simplicity, only the ^{3}H-labeled glycopeptides from growing cellular material are shown. The ^{14}C-labeled non-growing cellular material gave similar results.

convert the mixture of cellular neutral glycopeptides isolated by HVPE into an array of oligosaccharides, and paper chromatography to identify different neutral species of glycopeptides. HVPE separates cellular glycopeptides into two heterogeneous classes based on charge differences[4] (*cf.* Fig. 2a). It should be stressed that the present technique, however, permits the separation of cellular glycopeptides into acidic material and at least five distinct neutral peaks whose presence could not be readily predicted without prior glycosidase digestion. Thus the technique has the advantage of making direct comparisons of intact glycopeptides derived from two different cell populations. Thereafter, fractions of interest may be further studied using specific enzymatic probes and the digested products analyzed by paper chromatography.

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CHROM. 11,331

GEL CHROMATOGRAPHIC STUDY OF THE POLYMERIZATION OF SILICIC ACID

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SUMMARY

The polymerization of silic acid in the range from pH 9.5 to 1 *N* hydrochloric acid has been studied by gel chromatography. A mechanism for the growth of particles of polysilicic acids is proposed. It is concluded that the growth of particles of polymers in the pH range 9.5–9 and below 2 is due mainly to the polymerization between monosilicic and polysilicic acids, and in the pH range 7–4 mainly to the random condensation with each other of all of the polymer species. The growth rate of particles of polymers is maximal at pH 6.5–7.

INTRODUCTION

The kinetics of the polymerization of silicic acid have previously been studied by colorimetry[1–3], because monosilicic acid reacts with molybdate reagent in acidic solution to give a yellow colour, whereas polysilicic acid does not react with molybdate reagent.

Other methods for studying the polymerization of silicic acid have been used. Alexander[4] studied the kinetics of the polymerization by freezing-point depression measurements. Audsley and Aveston[5] used light scattering, ultracentrifugation, and viscosity measurements. However, the change in the molecular weight of silicic acids in solutions with a wide pH range has not been studied.

Gel chromatography is a method for separating solute molecules according to their size, larger molecules being eluted faster than smaller molecules. The elution volumes of molecules are correlated with their molecular weight (size). Tarutani[6] suggested that gel chromatography is useful for the study of the polymerization of silicic acid. Kojima and Tarutani[7] studied the formation of polysilicic acid from monosilicic acid adsorbed on iron(III) hydroxide by gel chromatography.

In this work, the effect of pH on the polymerization of silicic acid was studied by gel chromatography and the mechanism of the growth of particles of polysilicic acids is discussed here.

EXPERIMENTAL

Sample solutions and reagents

All reagents used were of analytical-reagent grade.

Monosilicic acid solution was prepared by fusing 0.500 g of anhydrous silica with 2.5 g of sodium carbonate, dissolving the melt in distilled water and diluting to 1000 ml.

Blue Dextran 2000 solution of concentration 0.4% was used.

The eluent was 0.1 *M* sodium chloride solution of pH 2.

Preparation of Sephadex column

Sephadex G-100 (Pharmacia, Uppsala, Sweden) was suspended in the solution to be used as the eluent and allowed to swell for 3 days. The column was a 45 × 1.0 cm glass tube with a porous polystyrene disc at the bottom. The Sephadex G-100 column was prepared as described in the literature[8]. The total volume of the gel bed was about 30 ml.

Procedure

Monosilicic acid solution (500 ppm of SiO_2) was adjusted to the desired pH with hydrochloric acid. At different times, the pH of an aliquot of the sample solution was adjusted to 2 with hydrochloric acid or sodium carbonate solution, because polymerization and depolymerization do not occur for a short period at pH 2 (ref. 6). Five millilitres of sample solution were used for the determination of monosilicic acid by colorimetry. Two millilitres of sample solution were placed on the gel bed just as the last layer of eluent soaked into the bed and, when the last portion of the sample solution had vanished into the bed, the eluent was applied. Using an automatic fraction collector, the effluent was collected in fractions of 1 ml, with a flow-rate of 4–6 ml/h. The amounts of silicic acid in the fractions were measured by colorimetry after decomposition of polysilicic acid with alkali or atomic-absorption spectrometry using a Nippon–Jarrell Ash Model AA 781 instrument.

Blue Dextran 2000 was eluted by using the same procedure.

All experiments were carried out at 20 ± 1°.

RESULTS AND DISCUSSION

The rate of polymerization of silicic acid differs with pH. In Fig. 1, the concentrations of monosilicic acid measured by colorimetry after 24 and 75 h are plotted against pH. The monosilicic acid concentration decreased rapidly in slightly alkaline solutions but did not decrease within 24 h in the pH range 1–3. The decrease in the concentration of monosilicic acid with time is due to polymerization between monomers and between monomer and polymer. However, no information is available on the change in particle (molecular) size of the polymers with time.

The elution curves for silicic acid in solutions of various pH are shown in Figs. 2–4. The position shown as BD is the elution volume of Blue Dextran 2000, which is considered not to be able to diffuse in the gel phase. It can be assumed that the elution volume of Blue Dextran 2000 is equal to the void volume of the bed. The peaks on the right are due to monosilicic acid and those on the left to polysilicic acid. The concentration of monosilicic acid at a given time is shown in parentheses.

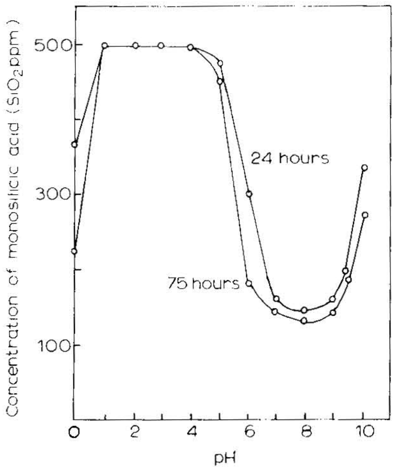


Fig. 1. Variation of the concentration of monosilicic acid with pH.

At pH 9.5 (Fig. 2), the elution curves for the polymers indicate a symmetrical distribution of particle sizes. The distribution of particle sizes of the polymers obtained after 250 h was almost the same as that obtained after 100 h. This suggests that when the concentration of monosilicic acid is close to the solubility of amorphous silica, the growth rate of the particles of the polymers becomes very slow. It is reasonable to assume that the growth of the particles of the polymers is due mainly to the polymerization between monomer and polymer, and the polymerization between polymer species hardly proceeds. Although the rate of polymerization at pH 9 was rapid compared with that at pH 9.5, similar results were obtained.

At pH 6 (Fig. 3), the elution curve for the polymers after 24 h is different from that at pH 9.5 and indicates a broad and random distribution of particle sizes. The

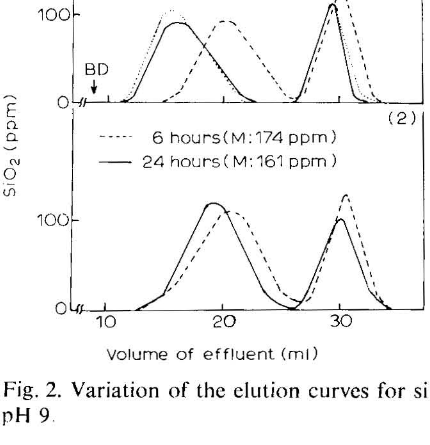
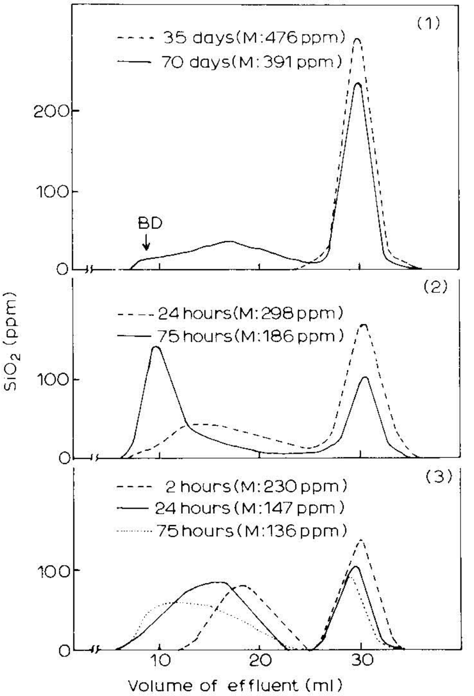


Fig. 2. Variation of the elution curves for silicic acid in alkaline solutions with time. 1, pH 9.5; 2, pH 9.



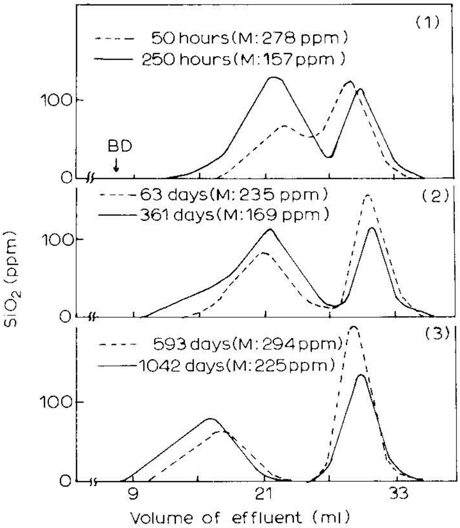


Fig. 3. Variation of the elution curves for silicic acid in solutions of pH 4, 6 and 8 with time. 1, pH 4; 2, pH 6; 3, pH 8.

Fig. 4. Variation of the elution curves for silicic acid in acidic solutions with time. 1, 1 *N* HCl; 2, pH 1; 3, pH 2.

highest peak was obtained at the elution position of BD after 75 h. This suggests that the particles of the polymers rapidly grew with time, because polymers that have larger sizes than the possible separation range for Sephadex G-100 are eluted together at the elution position of BD. Therefore, it is reasonable to conclude that a broad and random distribution of particle sizes of the polymers arises from a random condensation with each other of all of the polymer species. Although the rates of polymerization at pH 4 and 7 were different from that at pH 6, similar results were obtained.

At pH 8, the elution curve for the polymers after 2 h was symmetrical. Although the concentration of monosilicic acid after 75 h was not very different from that after 24 h, the growth of the particles of the polymers still continued and the elution curves became asymmetrical with time. However, the rate of random condensation with each other of all of the polymer species was slower than that at pH 6.

Variations in the elution curves for silicic acid in acidic solutions with time are shown in Fig. 4. The size distribution of the polymers is symmetrical. Therefore, although the rate of polymerization is very slow, it is suggested that the mechanism of the growth of the particles of the polymers is polymerization between monomer and polymer.

If the change in the particle size of polysilicic acids with pH and time is measured, the pH at which the growth rate of the particles of the polymers is maximal can be obtained. The pH of monosilicic acid sample solutions was adjusted to 5–9.5 with hydrochloric acid and, after standing for 24 and 75 h, silicic acid in the sample solutions was chromatographed. As an example, elution curves for silicic acid after 24 h are shown in Fig. 5. The particle sizes of polysilicic acids formed in each pH solutions are randomly distributed.

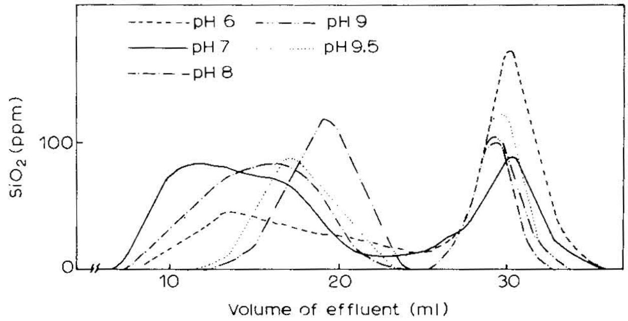


Fig. 5. Variation of the elution curves for silicic acid with pH.

Laurent and Killander[9] defined a constant, K_{av}, for a given gel in gel chromatography:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \tag{1}$$

where V_0 is the void volume, V_t the total bed volume and V_e elution volume. Here V_0, V_t and V_e are easily measured. As the K_{av} value is correlated to V_e, it is a measure of molecular size and decreases with increasing particle size. As the elution volumes of polymers cannot be obtained directly from the elution curves shown in Fig. 5, mean elution volumes must be calculated. The mean elution volume, V_e, is given by

$$V_e = \frac{\Sigma C_i V_i}{\Sigma C_i} \tag{2}$$

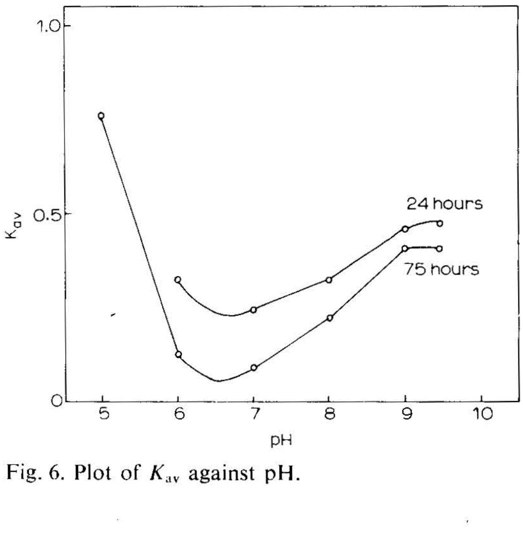


Fig. 6. Plot of K_{av} against pH.

where C_i is the concentration in the ith fraction and V_i is the volume of the ith fraction. In Fig. 6, K_{av} values, which were calculated from eqns. 2 and 1, are plotted against pH. K_{av} is minimal at pH 6.5–7, which means that the growth rate of the particles of the polymers is maximal at this pH.

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ELECTROPHORESIS OF HALIDES AND PHOSPHATE ON DEAE-CELLULOSE

APPLICATION TO ELEMENTAL ANALYSIS OF ORGANIC COMPOUNDS BY SODIUM FUSION

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SUMMARY

Electrophoresis on strips of the weakly basic anion exchanger, diethylaminoethyl-cellulose, is used to separate the halides and phosphate in any of four electrolytes spanning a wide range of pH but most effectively in an electrolyte at pH 2 containing a mixture of formic and acetic acids. The anions on pherograms treated with silver nitrate and exposed to ultraviolet light react in different ways depending on the nature of the electrolyte remaining on dried strips and on the concentration of silver ions in the reagent applied.

The method is adapted to the detection and identification of halogens and phosphorus present in organic compounds following sodium fusion on a micro-scale.

The separation also facilitates quantitative estimation of the individual anions present in mixtures, and an example is given of the estimation of residual inorganic bromide present in a chicken feed following fumigation with methyl bromide.

INTRODUCTION

The halides are separable in a variety of ways by chromatographic methods, and reliable procedures using paper[1–3], thin-layer[4–7] and column[7–10] techniques are now available, but it appears that the paper electrophoresis of these ions, with special reference to their separation, has not been as well explored. The results of several electrophoretic studies indicate that the mobilities of chloride, bromide and iodide are too similar to permit their convenient separation from mixtures when ordinary filter paper is used as the supporting medium[11–16], but Tokutomi and Kamiya[17] have shown that, by substituting paper impregnated with anion-exchange resins, such as Amberlite WB-2 paper, satisfactory electrophoretic separations of the halides are possible, using common buffer systems as electrolytes.

It is now shown that strips of the weakly basic exchanger, diethylaminoethyl (DEAE)-cellulose in which the ion-exchange properties are realized by chemical modification of the cellulose itself, may also be used for the rapid separation of mixtures of halides using electrolytes spanning a wide range of pH values. The work included an examination of the electrophoretic properties of phosphate on DEAE paper and the results have been adapted to the detection and identification of halogens and phosphorus present in organic compounds following sodium fusion on a microscale.

EXPERIMENTAL

Materials and reagents

Whatman chromatography paper DE-81 (DEAE-cellulose paper) was obtained from H. Reeve Angel (London, Great Britain).

Sodium dihydrogen phosphate and sodium or potassium salts of the halides were commercial samples of analytical-reagent grade, as were the organic compounds used for the sodium fusion tests, with the exception of chloromycetin (chloramphenicol) and betamethasone disodium phosphate, which were pharmaceutical preparations supplied, respectively, by Parke, Davis and Co. (Sydney, Australia) and Glaxo Australia (Melbourne, Australia).

Zirconium(IV) oxychloride and sodium alizarinsulphonate were supplied by E. Merck (Darmstadt, G.F.R.).

Poultry Layer Ration was manufactured by KMM (Melbourne, Australia).

Electrolytes

(1) The electrolyte of choice for the separations was an aqueous solution of approximately pH 2, 1 *M* with respect to acetic acid and 0.75 *M* with respect to formic acid[18]. It is hereinafter referred to as the "acid (pH 2) electrolyte".

The following electrolytes were used for comparative purposes:

(2) Acetate buffer (pH 4.6) containing 6.39 g $CH_3COONa \cdot 3H_2O$ and 3.2 g glacial acetic acid in 1 l of water. The solution was 0.1 *M* with respect to total acetate.

(3) Sodium phosphate buffer (pH 7.0) containing 6.24 g $NaH_2PO_4 \cdot 2H_2O$ and 10.68 g $Na_2HPO_4 \cdot 2H_2O$ per l. The solution was 0.1 *M* with respect to total phosphate.

(4) Sodium borate buffer (pH 9.2) containing 0.2 g-atom of boron per l (ref. 19).

Reagents used for the detection of the anions on pherograms

(1) Silver nitrate (0.3 %) in water, (2) silver nitrate (2 %) in water, (3) zirconium (IV) oxychloride (0.05 g) and sodium alizarinsulphonate (0.05 g) dissolved in 2 *N* hydrochloric acid (100 ml); *cf.* ref. 4. The reagent deteriorates on storage, even at 5°, and for best results was freshly prepared each 3–4 days.

Apparatus

Paper electrophoresis was conducted in the enclosed strip apparatus described previously[20] using Whatman DE-81 paper or Whatman No. 4 (filter) paper in strips 13.5 × 57 cm with 45 cm under pressure and cooled by the circulation of water at about 20° through the coils of the cooling-plate.

Procedure

DEAE-cellulose paper is especially fragile when wet with the acid (pH 2) electrolyte, but strips were conveniently dipped, blotted free of excess electrolyte and placed in the apparatus by leaving a few cm on each end dry for handling. Impregnation of the strips was rapidly completed by capillary attraction of electrolyte from the electrode compartments at each end.

The strips were equilibrated under pressure for 10 min and samples (0.7 μl) of solutions of the halides and phosphate (each of various concentrations in the range 10^{-1}–10^{-4} M), then transferred to the starting lines by means of a platinum loop. Caffeine was applied as a marker for zero migration.

Electrophoresis on DEAE paper strips was generally allowed to proceed at about 22 V/cm for 20 min and on filter paper strips at 25 V/cm for 10 min, after which the strips were dried in the oven at 100°. When it was required to detect fluoride on acid papers, they were heated no longer than was necessary to just dry them. Traces of fluoride were best detected by applying the appropriate spray reagent to papers still slightly damp with electrolyte.

Caffeine was located on dried papers as a dark blue spot in the light of a "Chromato-vue" UV lamp (Ultraviolet Products, Calif., U.S.A.). DEAE-cellulose should be fully ionised in the acid (pH 2) electrolyte and, as expected for DEAE pherograms run under these conditions, the electroendosmotic flow, as indicated by the movement of caffeine, was strongly anionic — of the order of 4 cm/h·kV of applied potential.

Detection of the anions

Reagents (1) and (2) (silver nitrate solutions) were used for the detection of chloride, bromide, iodide and phosphate. Dried papers were uniformly sprayed with either reagent and exposed, while still wet, to light of the UV lamp (wavelength 2540 Å) for 5 min.

Reagent (3) [zirconium(IV) oxychloride–alizarinsulphonate–hydrochloric acid] was used for the detection of fluoride and phosphate. Sprayed papers were heated in the oven for about 1 min to develop yellow spots for these anions against a purple background.

The mobilities of the anions were calculated in absolute terms as cm/h·kV of applied potential.

Sodium fusion tests

Chloromycetin, α-bromocamphor-π-sulphonic acid (ammonium salt), *p*-bromomandelic acid, α,β-dibromopropionic acid, iodoacetic acid, 2-chloro-6-fluorobenzaldehyde, *p*-bromoiodobenzene, glucose-6-phosphate (disodium salt) and betamethasone disodium phosphate were tested. Each compound (3–4 mg) was heated in a Pyrex ignition tube with metallic sodium (*ca.* 10 mg) at red heat for 1 min. The tubes were allowed to cool and water (0.5 ml) added to the contents with which it was triturated with warming. The mixtures were applied without further treatment to DEAE papers impregnated with the acid (pH 2) electrolyte and subjected to electrophoresis under the standard conditions.

Dried pherograms were treated with spray reagents and the results interpreted as described below.

Estimation of inorganic bromide in chicken feed fumigated with methyl bromide

Poultry Layer Ration was treated in bulk with methyl bromide[21] and a sample (10 g) defatted with light petroleum. The pellets were crushed and extracted with 3 N

ammonium hydroxide in three batches of 50 ml each, centrifuging between treatments. The clear supernatants were combined and evaporated to small volume (5–10 ml), an equal volume of ethanol added, and the mixture warmed with stirring. The fine precipitate which formed on cooling was removed by filtration through Celite and the clear filtrate evaporated almost to dryness. The residue was made up to 2 ml with water and this stock solution serially diluted for application to a DEAE-paper impregnated with the acid (pH 2) electrolyte. After electrophoresis under the standard conditions, the pherogram was dried and treated with the 2% silver nitrate reagent. Comparison of size and intensity of the series of "unknown" spots with those of standard bromide solutions applied to the same paper enabled quantitative estimation of the bromide present in the treated feed sample (*cf.* ref. 22).

RESULTS AND DISCUSSION

Effects of pH and supporting medium on mobilities

Absolute mobilities of the anions on both DEAE and Whatman No. 4 (filter) papers are shown in Table I at four levels of pH.

TABLE I

MOBILITIES OF HALIDES AND PHOSPHATE IN FOUR ELECTROLYTES ON DEAE AND FILTER PAPERS

For description of electrolytes 1–4 and conditions of electrophoresis, see Experimental. Mobilities are expressed in cm/h·kV of applied potential.

Anion	*Electrolyte*							
	1, pH 2		*2, pH 4.6*		*3, pH 7*		*4, pH 9.2*	
	DEAE paper	*Filter paper*	*DEAE paper*	*Filter paper*	*DEAE paper*	*Filter paper*	*DEAE paper*	*Filter paper*
Chloride	8.2	25.6	16.6	29.0	25.2	28.6	25.9	29.0
Bromide	5.8	25.8	13.5	30.2	22.0	29.1	22.5	29.0
Iodide	2.2	26.4	9.0	29.5	16.6	28.0	17.4	28.4
Fluoride	4.8	2.4	15.3	20.0	20.3	20.1	20.9	18.8
Fluorosilicate	−0.5	—	—	—	—	—	—	—
Phosphate	2.6	4.4	8.6	11.1	—	—	15.5	16.0

On filter paper at pH 2, chloride, bromide and iodide are almost as highly mobile as at higher pH levels, reflecting the fact that these anions correspond to strongly dissociated acids. Tests using the acid (pH 2) electrolyte as eluent have shown Whatman No. 4 paper to have only a weak adsorptive capacity for iodide and little or none for chloride and bromide (*cf.* ref. 16), hence the finding that these ions are not electrophoretically separable on this supports in spite of large differences in atomic weight is probably almost solely due to counterbalancing effects of differences in their degrees of hydration. Fluoride, however, is easily separable from the other halides as a less mobile ion at all levels of pH on filter paper, apparently because it is especially strongly hydrated. The most rapid separation of fluoride occurs at pH 2 where, as the anion of a relatively weak acid, its ionisation is largely suppressed, augmenting the effects of hydration and resulting in a sharp decline in its mobility.

On DEAE paper, under standard conditions of electrophoresis, mixtures of the halides are resolved cleanly as small, circular or oval spots at all levels of pH, but most effectively at pH 2, where the relative differences in their mobilities are greatest. DEAE-cellulose should be fully protonated at this pH and the fixed centres of positive charge thus created on pherograms most strongly retard the movement of anions by electrostatic attraction. Comparison of the mobilities of the halides at pH 2 with those at higher levels on DEAE paper shows this expectation to be fully realized. With the exception of fluoride, mobilities on DEAE paper are, of course, much lower than those on filter paper in the acid electrolytes, but they are also appreciably lower even in the neutral and alkaline electrolytes, and it is puzzling in this respect that Tokutomi and Kamiya[17] report, conversely, that the migration of these and other anions is larger on Amberlite anion-exchange papers than on filter paper.

The retardation of the halides on DEAE pherograms is partially selective resulting in the separation of chloride, bromide and iodide in that order of decreasing mobilities, and it is possibly significant that this is also the order of numerical decrease of the enthalpies of hydration of the ions[23]. Electrophoresis on DEAE paper thus provides a separation which is complementary to that of most chromatographic systems in that the order in which the ions separate is inverted[1-7].

Under standard conditions on DEAE paper, phosphate migrating as the ion $H_2PO_4^-$ is not completely separated from iodide in the acid (pH 2) electrolyte, but the two ions are easily distinguished within a composite spot using the dilute (0.3%) silver nitrate reagent as described below. Longer runs of an hour or more are necessary to completely separate the ions on DEAE paper, but, if required, complete separation of phosphate from iodide and other halides may be achieved more rapidly using other systems such as the acid (pH 2) or acetate (pH 4.6) electrolytes on filter paper.

Detection of the anions

Chloride, bromide, iodide and phosphate. Halides on pherograms treated with silver nitrate each respond in different ways on exposure to ultraviolet light, depending on several factors which include the nature of the medium itself (whether DEAE or filter paper), the nature of the electrolyte remaining on the dried papers and the concentration of silver ions in the spray reagent applied. Reactions occurring on pherograms run in the acid (pH 2) electrolyte are summarised in Table II.

When DEAE pherograms are treated with the more concentrated (2%) silver nitrate reagent after runs in the acid electrolyte, chloride, bromide, iodide and phosphate are revealed as intense spots (deep brown for chloride and bromide, and blue–black for iodide and phosphate) when more than about 0.5–1 μg of each is present, although smaller quantities down to a limit of about one-tenth of this amount are still visible as weaker spots. The dilute (0.3%) silver nitrate reagent gives the same intense blue–black reaction with phosphate as the more concentrated reagent, but in sharp contrast, iodide is revealed with the dilute reagent as a white or cream-coloured spot against a pale brown background. It seems that iodide, present on pherograms even in traces, protects superimposed silver ions against reduction to metallic silver when these are present in low concentrations. This "protective" action is very powerful and operates even in the presence of large excesses of other anions like phosphate, which normally promote reduction, but the action is then incomplete and results in spots of

TABLE II

REACTIONS OF ANIONS TO SPRAY REAGENTS AFTER ELECTROPHORESIS IN THE ACID (pH 2) ELECTROLYTE ON TWO SUPPORTS

B = brown to black; BlB = blue–black; C = creamy-white; Y = yellow; w = weak; m = medium; s = strong; — = nil.

Anion	*DEAE paper*			*Filter paper*		
	0.3% $AgNO_3$	*2% $AgNO_3$*	*Zirconium–alizarin*	*0.3% $AgNO_3$*	*2% $AgNO_3$*	*Zirconium–alizarin*
Chloride	wB	sB	—	mB	mB	—
Bromide	wC	sB	—	sC	wC	—
Iodide	sC	sBlB	—	sC	mC	—
Fluoride	—	—	Y	—	—	Y
Fluorosilicate	—	—	Y	—	—	Y
Phosphate	sBlB	sBlB	Y	mBlB	mBlB	Y

an intermediate pale blue–grey shade. The effect is usefully exploited in the detection and identification of iodide and phosphate present together in the partially resolved spots obtained by electrophoresis under standard conditions on DEAE paper in the acid (pH 2) electrolyte. Three distinct regions are clearly visible within the oval spots; heads consisting solely of phosphate as it begins to separate from mixtures and which therefore give a characteristic deep blue–black reaction, fully "protective" white tails due to iodide alone, and sharply-defined, lens-shaped intermediate zones consisting of mixtures of the anions which give the mutually modified pale blue–grey reaction.

The dilute silver reagent lacks sensitivity in the detection of chloride and bromide on acid DEAE papers, but it is interesting that bromide also acts protectively with this reagent, though much more weakly than iodide. It is noteworthy that the protective action of iodide (and bromide) is fully and consistently expressed on acid pherograms only. Residues of alkaline electrolytes on pherograms tend to obscure the effect and only higher concentrations of the ions are then detected as pale spots using the dilute silver reagent. Lower concentrations give brown to black reactions using either of the silver reagents.

Iodide and bromide also exert their protective action on acid filter papers when either of the silver reagents is used, but the effect is especially sensitive with the dilute reagent, the limit of detection for iodide then being 22 ng/cm^2 of spot area. When spots remain small, as they do during runs of 10 min under the standard conditions on filter paper, this corresponds to 11 ng of iodide actually applied (0.7 μl of $1.25 \cdot 10^{-4}$ M KI solution). Although not specific for iodide, the method therefore compares favourably in terms of sensitivity with the micro-tests of Robbins[24] and of Feigl[25].

If more than about 2 μl of pure water (or any aqueous solution containing other than the halides) is applied to a starting point of a filter paper strip an artefact is likely to appear on the developed pherogram at the position otherwise occupied by the halides. This "water artefact" mimics the protective action of iodide and could therefore be mistaken for a trace reaction due to that ion. Analyses performed during these studies have shown that different batches of No. 4 paper contain, among other background impurities, 6–10 mg/m^2 of surface area of water-soluble chloride. Water applied to starting points on the paper strips radially displaces this soluble chloride leaving circular spots depleted of their normal content of impurity, and when electrophoresis is subsequently conducted for short periods, the depleted spots are preserved as "holes" in the background of chloride as this moves *en masse* toward the anode with its usual mobility. Background chloride is at least partly responsible for the pale brown ground which develops on pherograms treated with silver reagents and the "holes", necessarily moving at the same rate as chloride, then appear as unreactive paler spots against the ground at a position corresponding with this ion.

The artefact is eliminated by pre-washing the papers with water to remove all soluble chloride, when they prove to have an added advantage in the detection of iodide in that small spots due to trace quantities are even more sharply defined.

Fluoride and phosphate. Fluoride separates narrowly from phosphate as a faster moving ion inder the standard conditions on DEAE paper in the acid (pH 2) electrolyte and the two ions are further distinguished by differences in their reaction to the zirconium–alizarin reagent. Fluoride gives an immediate strong yellow reaction with quantities in excess of about 1 μg, but the spot fades and, within a few hours, weaker ones are no longer visible. Conversely, the yellow spots due to phosphate are relatively weak at first, even for quantities of the order of 10 μg but, on acid papers, they slowly intensify and remain strong for several weeks, at least.

In addition, the ions are well separated on filter paper at pH levels 4.6 and 9.2, but apart from the differences in mobility phosphate is yet further distinguished by its strong reaction with silver nitrate, fluoride giving little or no visible response to this reagent at all.

It was found that fluoride present in sodium fusion mixtures prepared according to directions given in the Experimental section was (very slightly) cationic on acid (pH 2) DEAE papers, and that any phosphate also present in a mixture then separated easily as the leading spot. The effect was traced to the presence of silicate in the fusion mixtures, formed by attack of sodium on the ignition tubes, and to the mixtures being acidified immediately on application to the papers, liberating silicic acid and forming the species HF and HF_2^-. The latter are known to react with silica, even at room temperature, to form fluorosilicic acids, the well-known H_2SiF_6 probably being a major product[26]. The conditions being favourable, it is probable that these complex acids form on pherograms at the moment of application of the fusion mixtures, incorporating all of the free fluoride the latter contain.

In view of the probability that all fluorosilicic acids are strong acids[27], it is surprising that they do not display considerable anionic mobility, even on acid DEAE paper, but their apparent cationic mobility may provide the clue; perhaps they are retarded by partial reversible adsorption on the paper support (see Table I).

Mixtures of sodium fluoride and sodium silicate give rise to the same or similar retarded species when run on acid (pH 2) pherograms, but neither these simple solutions nor appropriate sodium fusion mixtures show the effect at levels of pH 4.6 and higher, probably because of an increasing preponderance of the free F^- ion which is known to be unreactive with silica[26].

Sodium fusion tests

Of the anions commonly present in sodium fusion mixtures of organic compounds[28], only the halides and phosphate are detectable under the standard conditions of electrophoresis on acid (pH 2) papers. Two other common ions, cyanide and sulphide, are too volatile to be retained on acid pherograms even while heating to dry them partially[29].

The alkaline fusion mixtures are applied directly to papers without pre-acidification because this may result in the formation of gels from the silicate present and make the transfer of samples difficult. The acid electrolyte is relatively concentrated and ensures the required acidification of applied mixtures before electrophoresis begins.

The separated anions corresponding to elements present in the compounds tested were selectively identified by treating each with all three spray reagents, these being applied separately to different pherograms or to different strips of the same pherograms. Except for the mixture from the β-methasone derivative, only one loopful (0.7 μl) of the fusion mixtures, prepared as directed, sufficed for satisfactory results. Fluorine constitutes less than 4% of the disodium phosphate of β-methasone (9α-fluoro-11β-methylpregnisolone) and 3 μl or more of the fusion mixture was required for unequivocal identification of fluoride (as fluorosilicate). The expected strong spot for phosphate separated cleanly as the leading spot.

Inorganic bromide in chicken feed

A further example of the utility of the method is provided by the estimation of ionisable bromide present in a commercially produced chicken feed which is routinely fumigated with methyl bromide by the Division's SPF (specific pathogen free) Poultry Unit (Maribyrnong, Australia) to destroy micro-organisms, insects and mites which may infest it prior to it being fed to experimental birds. Methyl bromide is readily dispelled from the feed by airing it for a few hours, but by its reactions with protein and other feed components, the fumigant leaves a stable and persistent residue in the form of inorganic bromide[21]. An estimate of this potentially toxic ion in treated feeds is required for the assessment of safety limits, and so help define the conditions of fumigation[30]. Residual inorganic bromide following the present fumigation was found by the method outlined in the Experimental section to constitute 0.03% of the feed, the only other soluble constituents detected with the silver nitrate reagent being chloride and phosphate. No iodide was detected. Although chloride and phosphate were present in considerable excess, they separated cleanly from bromide on the DEAE pherogram and so did not interfere with visual comparison of spots of the serially diluted "unknown" sample with those of the standard bromide solutions.

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IDENTIFICATION OF DRUGS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH DUAL WAVELENGTH ULTRAVIOLET DETECTION

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SUMMARY

Using three solvent–column systems, 101 drugs of forensic interest were characterized by their high-pressure liquid chromatographic relative retention times and by the ratio of their absorbances at 254 and 280 nm. Using relative retention times alone, only 9% of the drugs could be distinguished; while when both the retention times and absorbance ratios were used, 95% of the drugs could be distinguished. The compounds were also characterized by comparisons of their retention times on an adsorption column and a reversed-phase column, however this pair of discriminators were less useful than the former techniques.

INTRODUCTION

Since the early 1970's, there has been a very rapid increase in the use of high-pressure liquid chromatography (HPLC) in drug analysis. In fact, HPLC methods are often used more frequently than gas–liquid chromatographic (GLC) methods in the quality control sections of most pharmaceutical firms. However, the routine use of HPLC for the identification of drugs in forensic and toxicology laboratories is much more limited at this time. In part, this may be due to the lower precision of HPLC relative retention times compared to GLC relative retention times and in part due to the difficulty in corroborating the peak identifications by HPLC. A third reason for the lack of utilization of the method in this area is the paucity of HPLC data on narcotics, amphetamines, barbiturates, tranquilizers, and other drugs of interest.

Recently the use of a octadecylsilane reversed-phase HPLC system for the identification of 30 drugs was reported[1]. In addition, a micro-particulate silica system was reported for the identification of a larger list of drugs[2]. Though these were excellent chromatographic systems, only very few drugs could be uniquely identified because of the overlap of retention times.

There are numerous reports of the use of variable wavelength ultraviolet (UV) detectors as an aid in the identification of compounds and this method was recently applied to a small number of drugs and other molecules of biological interest[3,4]. There were two major limitations of this techniques to the problems of routine identification. In most cases a stopped-flow method must be used to obtain the UV spectra which made it difficult to obtain precise retention times to serve as the primary means of identification. Secondly, it would have been very difficult to obtain quality UV spectra at the low concentrations encountered in forensic and toxicological applications because of the difficulty in obtaining flat UV spectral baselines at high sensitivity.

The use of two UV detectors in series operating at different wavelengths has long been used as a qualitative aid in the identification of compounds in complex mixtures. Recently it was reported that polynuclear aromatic hydrocarbons could be detected and identified in environmental samples at low levels using dual UV detectors in series[5]. In this report, the initial identifications made on the basis of retention times were corroborated through precise measurements of the peak's absorbance ratio at 254 and 280 nm. The same workers have also applied the technique to the identification of nucleosides in serum samples and it has been shown that the absorbance ratio is very reproducible (1.7% relative standard deviation)[6]. This same basic technique has also been used to identify compounds of biological interests in urine samples[7]. In each of these earlier studies it was found that the absorbance ratio could be easily reproduced and it could be satisfactorily measured in complex samples at low concentrations.

The major objectives of the work reported here were to develop a small number of isocratic HPLC systems that would be useful for the majority of the "drugs of abuse" and to evaluate the absorbance ratio technique as an additional identification discrimination.

EXPERIMENTAL

Instrumentation and calibration

A Waters Assoc. Model 202 chromatograph equipped with a U6K injector and Model M6000 pump was used for the study. The column was connected with 0.009-in. I.D. tubing to a 254-nm detector then in series to a 280-nm detector, both of which were connected to a dual pen strip-chart recorder. The absorbance reading of the two detectors were calibrated in a relative manner by adjusting the gain on the 280-nm recorder channel so that an A_{254}/A_{280} peak height ratio of 1.09 was obtained for a morphine reference standard when chromatographed on system B.

Drug standard and chemicals

The majority of the drugs were obtained from the Theta Corp. (Media, Pa., U.S.A.) or from U.S.P. Reference Standards (Rockville, Md, U.S.A.). A small number of the compounds were obtained directly from various pharmaceutical firms. Methanol and methylene chloride used in the mobile phase was freshly distilled before use. All other chemicals were of reagent grade and used without further purification.

Chromatographic system A

A Waters Assoc. 3.9 × 300 mm μBondapak C_{18} column and a mobile phase flow-rate of 2.0 ml/min were used. The mobile phase was prepared by adjusting a 0.025 *M* NaH_2PO_4 in methanol–water (2:3) solution to a pH of 7.0 using 5% aqueous sodium hydroxide solution. Retention times were measured relative to that of phenacetin (6.8 min).

Chromatographic system B

A Waters Assoc. 3.9 × 300 mm μPorasil column and a mobile phase flow-rate of 2.0 ml/min were used. The mobile phase was methanol–2 *N* ammonia–1 *N* ammonium nitrate (27:2:1). Retention times were measured relative to morphine sulfate (3.5 min).

Chromatographic system C

The column and flow-rate used were the same as system B. The mobile phase was prepared by adding 2.0 ml of concentrated ammonia to a closed flask containing 1.0 l of dichloromethane and stirring overnight. Retention times were measured relative to diazepam (4.5 min).

RESULTS AND DISCUSSION

In order for the measurement of any physical parameter of a drug to be useful in its identification, one should have a reasonable estimate of both the short term and long term precision of the measurement. To this end ,a number of drugs were selected on the bases of the diversity of their polarity, acid–base character and UV spectra, and were used as test compounds. The short term relative standard deviation of the HPLC relative retention times (Table I) was found to have an average value of 3.3% which was fairly typical when relative retention times are used. This value was typical of most HPLC studies, but was considerably larger than the relative standard deviation for GLC retention times [0.6% (ref. 8)]. The short term precision of the absorbance ratio measurements was found to be slightly better than the relative retention times measurements. The average value of the relative standard deviation of the absorbance ratio was 1.9%. It was also observed that the relative standard deviation was smallest when the two peaks were of nearly the same size ($A_{254}/A_{280} \approx 1$). If the ratio was very high or very low, the precision of the measurements were satisfactory, but slightly lower.

TABLE I

PRECISION OF RELATIVE RETENTION TIME AND ABSORBANCE RATIO MEASUREMENTS

Drug	*Relative retention time*	A_{254}/A_{280}
Amphetamine	0.519* ± 4.4%**	20.4 ± 3.5%
Phenobarbital	0.862 ± 3.5%	19.7 ± 2.5%
Phenacetin	1.00***	8.42 ± 0.68%
Methaqualone	3.94 ± 1.9%	2.51 ± 0.87%
Average	3.3%	1.9%

* Relative retention time using system A.

** Relative standard deviation.

*** Retention time standard for system A.

The long term reproducibility of the relative retention times and of the absorbance ratio was estimated by repeating the measurements for the drugs listed in Table I over a two month period. It was found that the relative retention times varied by an average of 3.2% and the absorbance ratio values varied by an average of 21%. In retrospect, it is felt that the variation in the long term reproducibility of the absorbance ratio could be improved by more frequent calibration. Though the long term precision of the absorbance ratio values appeared rather poor, the value was still useful in identifying drugs because of the extremely wide variation of the ratio from drug to drug (Tables II–IV).

TABLE II

DATA FOR DRUGS CHROMATOGRAPHED ON SYSTEM A

Drug	*Relative retention time*	A_{254}/A_{280}	A_{254}***	N§
Barbituric acid	0.21*	19.1	2.9	—
Sulfanilamide	0.23	5.25	2.7	4,520
Phenylephrine	0.27	1.73	0.049	2,680
Theobromine	0.28	2.25	0.23	3,190
Acetaminophen	0.30	8.23	1.8	3,750
Aspirin	0.31	0.32	0.029	3,750
Hydroxyamphetamine	0.32	0.94	0.043	3,750
Phenylpropanolamine	0.34	16.0	0.0088	1,420
Theophylline	0.34	3.24	0.46	4,660
Barbital	0.35	6.93	0.0078	416
Dimenhydrinate	0.38	3.67	0.16	5,320
Oxymorphone	0.41	2.80	0.046	7,580
Ephedrine	0.41	17.1	0.0083	1,150
Mescaline	0.43	5.41	—	1,600
Caffeine	0.48	2.15	1.3	2,330
Ectylurea	0.50	17.9	0.013	260
Procaine	0.51	3.0	0.61	1,250
Amphetamine	0.52	20.4	0.019	630
Salicylamide	0.53	0.83	0.094	2,680
Nikethamide	0.58	16.5	0.15	1,480
Phenacemide	0.59	33.5	0.0055	110
Oxycodone	0.60	2.1	0.0026	230
Morphine	0.62	1.67	0.011	950
Dichloralphenazone	0.66	6.0	0.026	240
Mephenoxalone	0.67	1.79	0.0035	240
Allobarbital	0.70	12.0	0.0049	260
Methocarbamol	0.72	1.43	0.0035	190
Dimethyltryptamine	0.75	1.89	—	750
Methylamphetamine	0.75	6.50	0.0021	230
Metharbital	0.77	9.75	0.0032	340
Tetrahydrozoline	0.77	9.46	0.0054	360
Phenmetrazine	0.80	13.3	0.0021	670
Dihydrocodeine	0.81	1.08	0.0043	270
Hydromorphone	0.84	2.0	0.0070	460
Nicotine	0.85	14.4	0.056	990
Phenobarbital	0.86	19.7	0.026	1,950
Aprobarbital	0.87	10.4	0.042	550
Mephentermine	0.88	7.67	0.0020	450
Codeine	0.90	2.47	—	940
Bromural	0.95	14.4	0.0059	710
Naloxone	0.96	2.21	0.0077	600
Hexobarbital	0.97	9.35	0.015	1,140

TABLE II (*continued*)

Drug	*Relative retention time*	A_{254}/A_{280}	A_{254}***	N§
Phenacetin	1.00**	8.42	0.49	2,620
Heroin	1.11	1.75	—	1,190
Lobeline	1.11	8.52	0.0028	1,920
Mephenesin	1.11	3.23	0.0092	880
Naphazoline	1.11	1.14	0.019	500
Butabarbital	1.14	9.67	0.0023	1,840
Fluorescein	1.15	2.12	0.21	2,220
Cyclobarbital	1.22	9.58	0.019	1,920
Butalbital	1.41	11.3	0.0037	1,110
Methylphenidate	1.46	17.0	0.0018	320
Mephobarbital	1.56	10.1	0.013	1,800
Nylidrine	1.59	1.76	0.0025	480
Hydrocodone	1.66	1.94	0.0029	400
Ethylmorphine	1.69	2.18	0.0057	660
Levorphanol	1.78	0.35	0.0006	370
Chlordiazepoxide	2.02	3.69	0.045	1,180
Pentazocine	2.03	0.46	0.0009	360
Diphenylhydantoin	2.07	16.4	0.0091	630
Glutethimide	2.12	12.0	0.0039	1,350
Pentobarbital	2.16	9.33	0.0023	1,660
Phencyclidine	2.16	19.3	—	250
Amobarbital	2.25	11.5	0.0019	1,250
Levallorphan	2.35	0.44	0.0007	650
Phenaglycodol	2.90	16.5	0.0026	860
Doxylamine	2.95	11.4	0.0093	340
Flurazepam	3.21	3.76	0.016	690
Secobarbital	3.28	10.0	0.0008	1,580
Thiopental	3.55	0.33	0.013	3,150
Oxymethazoline	3.62	1.00	0.0014	650
Methaqualone	3.94	2.51	0.049	920
Phenazocine	4.05	0.72	0.0011	780
Oxazepam	4.05	6.16	0.061	1,510
Thiamylal	4.58	0.31	0.016	2,370
Methohexital	4.80	9.0	0.0015	2,600
Papaverine	7.06	3.0	0.029	2,440
Diazepam	9.56	6.04	0.10	2,650

* The column void volume was slightly less than 0.21.

** Phenacetin used as standard, retention time 6.8 min.

*** Absorbance of a 10-μl injection of a 1.0 mg/ml solution of the drug.

§ Number of theoretical plates.

The retention times of the drugs using system A (Table II) were found to correlate very well with those reported for a much more limited series of drugs chromatographed with a similar system[1]. Because of minor impurities in the samples, the correct chromatograph peak assignments were occasionally in doubt. In an effort to verify the chromatographic peak assignments, the A_{254}/A_{280} values of each drug was compared with the UV spectral data of the drug obtained with similar solvents[9,10]. In a more limited number of cases where the correct assignment of the drug was still in question, the fractions of individual peaks were collected, and their UV spectra and thin-layer chromatograms were compared to those obtained for the original drug.

TABLE III

DATA FOR DRUGS CHROMATOGRAPHED ON SYSTEM B

Drug	*Relative retention time*	A_{254}/A_{280}	A_{254}***	N§
Noscapine	0.53*	0.61	0.035	—
Phenacetin	0.53	0.84	0.051	—
Naloxone	0.56	0.82	0.025	—
Papaverine	0.56	1.06	0.070	—
Benzphetamine	0.58	2.10	0.069	—
Piminodine	0.58	3.02	0.16	—
Cocaine	0.61	0.86	—	—
Phenazocine	0.61	0.24	0.0081	—
Procaine	0.61	0.44	0.048	—
Nylidrin	0.61	0.75	0.013	—
Levallorphan	0.64	0.12	0.0026	—
Methylphenidate	0.67	9.50	0.0062	—
Pentazocine	0.67	0.16	0.0049	—
Phendimetrazine	0.67	8.00	0.010	—
Ethinamate	0.70	1.00	0.0020	—
Phenmetrazine	0.72	31.0	0.040	—
Meperidine	0.75	30.7	0.0030	710
Quinine	0.75	0.62	0.0042	1,700
Promethazine	0.76	2.21	0.14	710
Diphenhydramine	0.77	90.0	0.059	1,600
Methapyrilene	0.77	1.89	0.13	750
Phenylpropanolamine	0.78	65.0	0.0021	—
Heroin	0.80	0.64	—	1,800
Methadone	0.83	1.57	0.072	1,140
Phencyclidine	0.83	22.0	—	1,900
Thioridazine	0.83	2.08	0.20	840
Amphetamine	0.86	60.0	0.0039	940
Oxymorphone	0.86	1.17	0.0044	110
Doxylamine	0.89	17.7	0.0017	2,330
Ethylmorphine	0.92	1.15	0.020	2,440
Hydroxyamphetamine	0.92	0.38	0.011	1,080
Propylhexedrine	0.92	4.0	0.0005	290
Oxycodone	0.92	1.13	0.0029	100
Codeine	1.00	0.88	—	450
Morphine	1.00**	1.09	0.016	470
Dimethyltriptamine	1.09	0.77		3,190
Methamphetamine	1.19	31.0	0.0034	1,862
Ephedrine	1.20	52.0	0.0034	1,050
Phenylephrine	1.22	0.50	0.0045	466
Hydrocodone	1.28	0.93	0.0085	1,170
Ethoheptazine	1.31	27.4	0.0022	1,510
Mescaline	1.31	2.93		2,140
Xylometazoline	1.33	8.67	0.0034	2,290
Mephenteramine	1.36	36.3	0.0038	1,370
Dihydrocodeine	1.36	0.53	0.0065	1,290
Oxymetazoline	1.36	0.31	0.012	1,370
Tetrahydrozoline	1.42	16.1	0.038	1,460
Hydromorphone	1.43	1.09	0.0091	970
Strychnine	1.54	3.22	0.0045	370
Dextromethorphan	1.56	0.14	0.0022	1,750
Naphazoline	1.61	0.49	0.062	1,210
Levorphanol	1.64	0.12	0.0013	1,210

* The column void volume was slightly less than 0.53.

** Morphine was used as standard, retention time 3.5 min.

*** Absorbance of a 10-μl injection of a 1.0 mg/ml solution.

§ Number of theoretical plates.

TABLE IV

DATA FOR DRUGS CHROMATOGRAPHED ON SYSTEM C

Drug	*Relative retention time*	A_{254}/A_{280}	A_{254}***	N§
Disulfiram	0.39	1.21	0.29	3,750
Phenaglycodol	0.55	2.90	0.0059	1,160
Benzphetamine	0.57	8.11	0.047	6,400
Propoxyphene	0.61	5.36	0.012	2,220
Methaqualone	0.74	1.40	0.055	2,330
Chlordiazepoxide	0.76	1.80	0.042	2,680
Piminodine	0.76	2.27	0.022	870
Glutethimide	0.79	7.70	0.013	2,440
Diphenoxylate	0.88	7.00	0.0065	2,140
Naloxone	0.89	3.75	0.0049	3,750
Phenylpropanolamine	0.89	2.50	—	2,600
Flurazepam	0.91	2.00	0.0010	1,660
Phenazocine	0.96	2.00	0.029	1,330
Diazepam	1.00**	3.90	0.075	4,340
Noscapine	1.02	0.88	0.054	4,820
Papavarine	1.04	1.81	0.059	420
Fentanyl	1.06	6.00	0.0052	1,020
Procaine	1.61	0.87	0.047	2,330
Promethazine	1.89	5.10	0.12	2,590
Phenacetin	2.71	4.61	0.088	400
Salicylamide	3.20	0.37	0.0078	1,100

* The column void volume was slightly less than 0.39.
** Absorbance of a 10-μl injection of a 10 mg/ml solution.
§ Number of theoretical plates.

The correlation between the lipophilic nature of the barbitutates and their retention time on system A was very high (Fig. 1). The octanol–water partition coefficients were not experimentally obtained, but were calculated using the methods developed by Hansch and co-workers[11,12]. It should also be noted that these partition coefficients were calculated for the non-ionized form while actually 20 to 40% of the

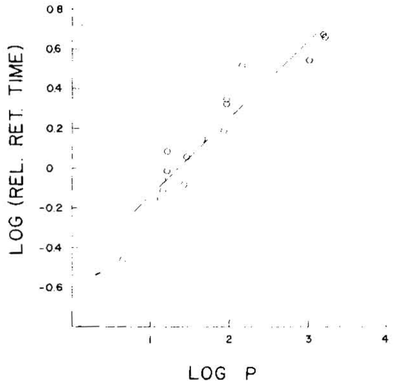


Fig. 1. Correlation of the retention time of barbiturates on system A and their calculated octanol-water partition coefficients.

barbiturate would be in the ionic form at the pH of 7.0 used for system A. Considering that the slight variations in the pK_a values of the barbiturates were not compensated for, the correlation coefficient of 0.95 that was observed was rather high. The slope of the curve in Fig. 1 was found to be 0.39 which indicated that the stationary mobile phase partition coefficient does not increase nearly as rapidly as the octanol–water partition coefficient in response to an increase in the lipophilic character of the drug. Recently very extensive studies on hydrophobic interactions in the μBondapak C_{18} column have been reported[13]. These studies showed that the slope of the long k' *vs.* carbon number (of a series of homologous alkanes or carboxylic acids) decreased markedly as the mole fraction of methanol in water increased. If one were to extrapolate these findings to conditions used for system A, one would have expected a slope of 0.47 for Fig. 1. Thus it would appear that the low value observed for the slope in this study was not related to the partial ionization of the barbiturates or any other property unique to the barbiturates, but was simply the result of decreased hydrophobic interactions in the mobile phase because of the methanol content.

Other examples of correlations between drug lipophilicity and retention time on system A were observed. For example, the retention times of the amphetamine series: methamphetamine > amphetamine > ephedrine > phenylpropanolamine. A similar ordering was also observed for the opiates: naloxone > codeine > hydromorphone > dihydrocodeine > morphine > oxycodone > oxymorphine. In the case of systems B and C (both μPorasil columns), it was generally observed that the retention times increased with the polarity of the drug in a general manner, but they were not nearly as well correlated with calculated partition coefficients as wes the reverse phase column.

The A_{254}/A_{280} values were found to vary over a very wide range (Tables II–IV). The primary value of this parameter was in the identification of individual drugs in a purely empirical manner, but it was also useful in the identification of various classes of drugs. For example, the majority of the morphine analogs run on system A were observed to have A_{254}/A_{280} values in the 1.7 to 2.5 range; most amphetamines, 36 to 56; and most barbiturates, 7 to 12.

In the extensive study of similar reverse phase systems by Twitchett and Moffat[1], it was noted that basic drugs exhibited lower theoretical plate counts than acidic or neutral drugs. In the present study that was made with a larger number of drugs (Table II), it was also observed that basic drugs did tend to have a lower column efficiency. However, this was only a general trend and numerous examples of the converse relationship could also be cited. Other than the trend for basic drugs to show a low column efficiency on system A, no other common structural characteristic could be discerned among the compounds showing a low plate count.

Chromatographic system B was found to have a much higher column efficiency for the basic drugs than system C. In a direct comparison of basic drugs that had been run on both systems, the theoretical plate count was on the average 196% higher on system B than on system A . Chromatographic system C was also found to have satisfactory column efficiency for most of the basic drugs and the values were fairly typical of what would be obtained for neutral compounds.

From a forensic or toxicological applications viewpoint, a major consideration in the evaluation of a method is the usefulness of the technique in uniquely identifying a drug in relatively complex mixtures. The primary objective of the present study was to evaluate the usefulness of accurate absorbance ratio measurements as an additional discrimination in the identification of drugs by relative retention times. As would be expected, there was very little correlation between the A_{254}/A_{280} value and the retention time of a given drug (Fig. 2). It is not an uncommon practice to identify a drug on the basis of GLC retention times on two different liquid phases. In such applications, there is usually a very high correlation between the retention time of a given compound on the two phases, thus the addition of the second discriminator adds little to one's ability to identify a given drug. When HPLC system A was used (Table II), a fairly large number of drugs had relative retention times between 0.7 and 1.0 which made it impossible to identify even one drug using retention times alone. However, the differences in the absorbance ratio values in this group was large enough to permit most of the drugs to be uniquely identified from other members of the group.

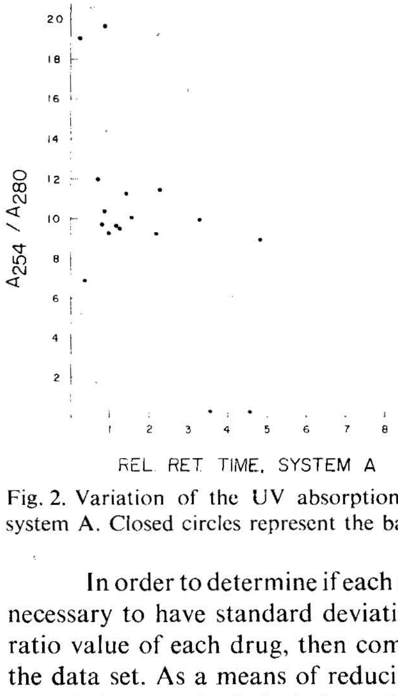


Fig. 2. Variation of the UV absorption ratio with the relative retention time of the drugs on system A. Closed circles represent the barbiturates.

In order to determine if each drug could be uniquely identified it would have been necessary to have standard deviations of the relative retention time and absorbance ratio value of each drug, then compare the two means to each of the other drugs in the data set. As a means of reducing this task to a more reasonable scale, it was assumed that standard deviation of the two parameters was the same as the average values that had been determined from a smaller group of drugs and that have been discussed in the first part of this section (3.3% for the relative retention times, 1.9% for the absorbance ratios). A given drug was then considered identifiable using only the retention time parameter if the difference between the retention time of the drug in question and all other drugs in the data set was greater than the sum of the standard deviation of the drug and the standard deviation of each of the other drugs in the data set. Thus when only the retention time of the drugs run on system A were used, only 9% of the drugs could be uniquely identified (Table V). A drug was considered to be identifiable using both parameters if either the value for the retention time or the absorbance ratio of the given drug differed by more than the sum of the two standard deviations of each of the other drugs in the data set. Thus when the retention time and

TABLE V

IDENTIFICATION OF DRUGS USING MULTIPLE PARAMETERS

First parameter	*Second parameter*	*Number of compounds*	*Identifications using first parameter*	*Identifications using both parameters*
Retention time, system A	A_{254}/A_{280}	78	9%*	95%**
GLC retention time***	Response index***	71	41%	85%
Retention time, system A	GLC retention time***	51	12%	100%
Retention time, system A	Retention time, system B	35	23%	83%

* Percent of drugs uniquely identifiable in the group using only the first parameter. See text for computation method.

** Percent of drugs uniquely identifiable in the group using both of the parameters.

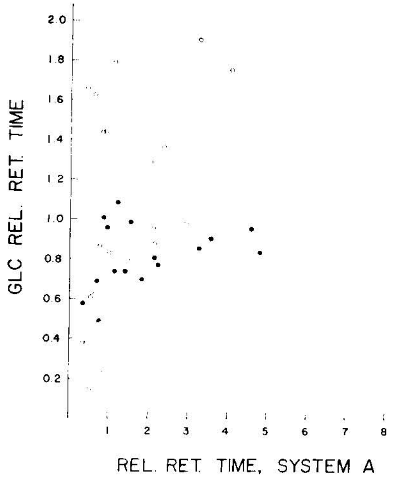
*** Data taken from ref. 8.

absorbance ratio values were used, 95% of the drugs run on system A could be identified.

The HPLC retention time–A_{254}/A_{280} system was at least on a par or slightly better than a GLC dual detector system in the identification of the drugs (Table V). If the HPLC retention times on system A were paired with the GLC retention times of the drugs, the identifiability of the drugs appeared to be even greater, but the increase in the value was largely due to the reduction in the number of drugs in the sample. Only a limited number of drugs were run on both HPLC system A (reversed-phase) and system B (normal adsorption), however, it was clear that the use of these two columns was inferior to the use of one column and the absorbance ratio (Table V, Fig. 4).

In a homologous series of compounds such as the barbiturates, it is often extremely difficult to identify each individual member of the series because of isomeric relationships. Because of the similarity of the UV spectra of all of the barbiturates[9,10] and because of the similarity of the lipophilicities of the isomeric compounds, one would have anticipated that HPLC retention times on a reversed-phase system paired with UV absorbance ratio measurements would not have been very useful in the identification of the compounds (Fig. 2). However, if the barbiturates were considered as a separate group, each of the compounds could be identified. If HPLC retention time was used as the first discriminator and the absorbance ratio as the second (Fig. 2), there was a higher dispersion of the data points for the barbiturates than when GLC retention time was used as the second discriminator (Fig. 3). Since the lipophilicity of the barbiturate and its vapor pressure would closely relate to the length of the side chain of the barbiturate, one might then expect a high covariance between the retention time on HPLC system A and the GLC retention times.

In conclusion, it was found that the use of HPLC relative retention times paired with accurate measurements of the A_{254}/A_{280} value was slightly more useful in the identification of drugs than other commonly paired techniques. This is not to suggest that the other techniques should be abandoned however, but that the method would be a relatively simple and inexpensive way of adding to the certainty of the identification of a specific drug that may have a retention time similar to other drugs. Even in cases where the chromatographic peak was well resolved from other drugs, the absorbance ratio technique would be useful in distinguishing the drug from other compounds present in the sample.



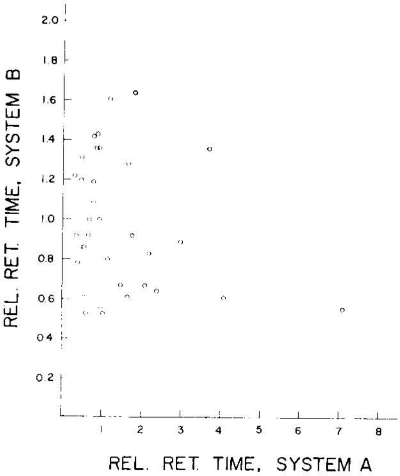


Fig. 3. Variation of the relative retention time of the drug on an OV-17 GLC column (ref. 8) with the relative retention time of the drug on system A. Closed circles represent the barbiturates.

Fig. 4. Variation of the relative retention time of the drug on system B with the relative retention time on system A.

ACKNOWLEDGEMENT

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CHROM. 11,338

MIKROPRÄPARATION WICHTIGER FLUNITRAZEPAM-METABOLITEN DURCH REAKTIONEN AUF DER DÜNNSCHICHTPLATTE

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SUMMARY

Micro-preparation of some important flunitrazepam metabolites by reaction on thin-layer plates

A micro-method is described for the synthesis of 7-aminoflunitrazepam, 7-acetamidoflunitrazepam and N-1-desalkylflunitrazepam on the sorbent of thin-layer chromatograms. By means of the method reported, also many other important benzodiazepine derivatives may be prepared.

EINLEITUNG

In der vorliegenden Arbeit wird die Mikropräparation von drei wichtigen Flunitrazepam-Metaboliten auf der Sorbensschicht der dünnschichtchromatographischen (DC) Platte beschrieben. Die aufgezeigte Technik eignet sich jedoch in analoger Weise auch zur Synthese von 7-Aminonitrazepam, 7-Acetamidonitrazepam, 7-Aminoclonazepam, 7-Acetamidoclonazepam sowie zur Desalkylierung der N-alkylierten Benzodiazepine Diazepam, Flurazepam und Prazepam.

Im Rahmen dieser Mitteilung soll der Schwerpunkt auf der präparativen Seite liegen. Bezüglich qualitativer Aspekte der Methode siehe Lit. 1. Weiterhin wurden analytische Daten der Metaboliten von Clonazepam und Nitrazepam[2] bzw. Flunitrazepam[3] von uns bereits publiziert.

EXPERIMENTELLES

Darstellung von 7-Aminoflunitrazepam und 7-Acetamidoflunitrazepam

Zur Biotransformation von Flunitrazepam. Nach vorangegangenen Untersuchungen[3,4] ist für die Biotransformation von Flunitrazepam das in Fig. 1 wiedergegebene Schema gültig, das in vielen Einzelheiten Analogien zum Stoffwechsel von Clonazepam bzw. Nitrazepam aufweist[5,6].

Wichtige Metabolisierungsschritte sind wiederum (1) Desalkylierung in N-1-Stellung; (2) Reduktion der 7-Nitrogruppe mit anschliessender Acetylierung; (3) Hydroxylierung in 3-Stellung (incl. Konjugatbildung).

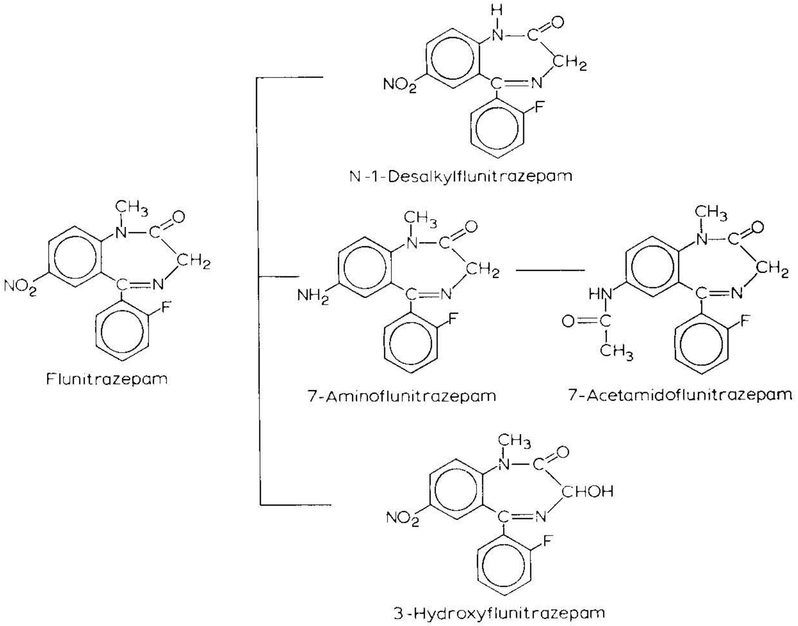


Fig. 1. Biotransformation von Flunitrazepam (Rohypnol).

Flunitrazepam wird, wie alle anderen in 7-Position nitro-substituierten Benzodiazepine, praktisch quantitativ metabolisiert, dass heisst renal werden allenfalls Spuren des unveränderten Pharmakons ausgeschieden. Bei der Aufarbeitung biologischen Materials ist somit nahezu ausschliesslich mit der Gewinnung von Stoffwechselprodukten zu rechnen. Die analytische Erfassung dieser Derivate steht daher im Rahmen der chemisch-toxikologischen Analyse im Vordergrund.

3-Hydroxyflunitrazepam kann ebenfalls auf der DC-Platte dargestellt werden. Im Hinblick auf die geringe Bedeutung dieses Metaboliten im Rahmen der Analytik wurde auf die Wiedergabe der ausführlichen Präparationsvorschrift verzichtet. Zur Darstellung von 3-Hydroxydiazepam bzw. 3-Hydroxyprazepam, von denen insbesondere das zuletzt genannte Derivat eine wesentlich wichtigere Rolle als Biotransformationsprodukt spielt, vgl. Lit. 8.

Darstellung von 7-Aminoflunitrazepam auf der DC-Platte. Auf der Startlinie einer üblichen DC-Fertigplatte Kieselgel $60F_{254}$ (20 × 20 cm; z.B. Art. Nr. 5715; Merck, Darmstadt, B.R.D.), ohne besondere Aktivierung, werden 1–2 mg Flunitrazepam durch Auftragen einer verdünnten Lösung (in 1 ml Essigsäureäthylester) mit Hilfe eines Chromatochargers oder ähnlichen Gerätes aufgebracht. Nach dem Verflüchtigen des Lösungsmittels wird die DC-Platte mit Hilfe von Streifen so abgedeckt, dass lediglich die Auftragezone frei liegt. Anschliessend erfolgt das Aufsprühen des Reagenzes [Titan(III)-chloridlösung, 15 %ige wässrige Lösung; Art. Nr. 808307; Merck]. Die Lösung soll die Sorbensschicht voll durchdringen, durch Betrachten der Platte von der Rückseite kann bequem kontrolliert werden, ob dieser für einen möglichst quantitativen Umsatz erforderliche Zustand erreicht ist.

Die DC-Platte wird nun mit warmer (keineswegs heisser) Luft so lange getrocknet, bis die graublaue Färbung des Titan(III)-chloridsprays verschwindet. Bei höheren Temperaturen muss mit einer Hydrolyse zu Aminobenzophenonen gerechnet werden, da die wässrige Titan(III)-chloridlösung infolge Hydrolyse stark sauer reagiert. Aus diesem Grund liegen die bei der Reduktion entstandenen Amine als Hydrochloride vor und müssen vor der nachfolgenden dünnschichtchromatographischen Trennung bzw. Reinigung in die entsprechenden Basen überführt werden, da nur in dieser Form ein befriedigendes chromatographisches Trennverhalten gegeben ist. Dies geschieht durch etwa 5 minütiges Einstellen der Platte in eine geschlossene Glaskammer (leerer Chromatographiertank), die Ammoniakdämpfe enthält (Becherglas mit etwa 25% Ammoniak auf dem Boden der Kammer).

Zur Entwicklung der Platte eignet sich insbesondere das Fliessmittel Essigsäureäthylester–Methanol–konz. Ammoniak (85:10:5, v/v/v), da sich mit diesem Gemisch einerseits aussergewöhnlich scharfe Substanzzonen auch bei höheren R_F-Werten bilden und andererseits evtl. noch vorhandene Hydrochloridreste durch den Ammoniakanteil quantitativ in die entsprechenden Basen überführt werden. 7-Aminoflunitrazepam kann im R_F-Wert-Bereich 0.85 mit Hilfe der Fluoreszenzlöschung indiziert werden. Anschliessend schabt man die entsprechende Zone des Chromatogrammes aus und saugt den mit 7-Aminoflunitrazepam versetzten Kieselgelanteil in einer Mikrofritte wiederholt mit Essigsäureäthylester ab. Nach dem Einengen des Extraktes im Vakuumrotationsverdampfer sollte die Substanz möglichst rasch chromatographisch bzw. spektroskopisch untersucht werden, da sie als primäres Amin leicht den gewohnten Umwandlungen und Zersetzungen unterliegt.

Beim Einsatz von Diäthyläther als Elutionsmittel ist unbedingt zu berücksichtigen, dass auch die handelsüblichen p.a.-Reinheitsgrade vielfach mit Jonol [typische m/e Werte: $205 = M^+ - 15$; $57 = (CH_3)_3C^+$] zur Stabilisierung versetzt sind.

Darstellung von 7-Acetamidoflunitrazepam auf der DC-Platte. 7-Acetamidoflunitrazepam bildet sich auf der DC-Platte mit nahezu 100%iger Ausbeute, wenn man das Chromatogramm, auf dessen Startlinie sich 7-Aminoflunitrazepam befindet (Darstellung siehe unter *Darstellung von 7-Aminoflunitrazepam auf der DC-Platte*), nach Freisetzen der Base aus eventuellen Salzverbindungen mit dem Fliessmittel Essigsäureäthylester–Essigsäureanhydrid (95:5, v/v) entwickelt. Die Elution (R_F-Wert-Bereich 0.20–0.30) erfolgt in der oben näher beschriebenen Weise. Insbesondere vor dem Messen der Spektren muss die Acetamidoverbindung gründlich von allen Lösungsmittelresten (Schwerflüchtigkeit von Essigsäureanhydrid) befreit werden.

Darstellung von N-1-Desalkylflunitrazepam (Nor-Flunitrazepam)

Allgemeine Ausführungen. Während Desalkylierungsreaktionen *in vivo* eine grosse Rolle spielen, da Fremdstoffe (z.B. Pharmaka) auf diese Weise polarer und damit renal leichter exkretierbar werden, existieren so gut wie keine einfach und mit höherer Ausbeute durchführbaren präparativen Methoden zur Abspaltung von N-Alkylgruppen. Alkylierungsverfahren (z.B. mit Dialkylsulfat oder Diazomethan) sind dagegen vielfältig bekannt und in Anwendung.

Bereits im Rahmen qualitativer Untersuchungen[1] konnte nachgewiesen werden, dass Desalkylierungsreaktionen auf photochemischem Weg möglich sind. Durch eine Optimierung des Verfahrens (vgl. nachstehende Ausführungen) gelang es auch, photolytische Desalkylierungsreaktionen in befriedigender Ausbeute für präparative Zwecke im Milligramm-Massstab einzusetzen. Es muss allerdings ausdrücklich darauf hingewiesen werden, dass die Ausbeuten (nahezu 100%), der unter *Darstellung von 7-Aminoflunitrazepam und 7-Acetamidoflunitrazepam* beschriebenen Reaktionen bei der präparativen Gewinnung der Nor-Verbindungen nicht erreicht wurden. Nach umfangreicher Ausarbeitung der nachstehend beschriebenen Technik lagen die Reaktionsausbeuten bei *ca.* 50%. Unter Berücksichtigung der Tatsache, dass sich, wie dargelegt, Nor-Verbindungen mittels konventioneller chemischer Methoden sonst nur durch eine komplette Synthese mit entsprechenden desalkylierten Ausgangsverbindungen erhalten lassen, erschien jedoch auch die Erarbeitung einer Methode mit geringeren Ausbeuten interessant und vertretbar.

Versuche zur photolytischen Desalkylierung als Lösungsreaktion. Bestrahlungsversuche in Quarzküvetten ergaben trotz Verwendung unterschiedlicher Lösungsmittel und Konzentrationen keine Hinweise auf das Entstehen erwähnenswerter Mengen an Nor-Verbindungen, obwohl sich insbesondere bei langen Belichtungszeiten ausserordentlich viele andere Substanzen bildeten. Eine Erklärungsmöglichkeit könnte darin gesehen werden, dass es sich bei der photolytischen N-Desalkylierung offensichtlich um eine Reaktion handelt, die bereits durch Hydratation bzw. Solvation unterbunden wird. Für diese Modellvorstellung sprechen auch folgende Beobachtungen bei der qualitativen Erprobung der Methode: Offensichtlich fand die Desalkylierung nur bei denjenigen Molekülen statt, die sich unmittelbar auf der Sorbensoberfläche befanden. Wurde eine DC-Platte nämlich nach Bestrahlung mit UV-Licht nochmals mit einem geeigneten Fliessmittel entwickelt, so traten aus tieferen Schichten des Sorbens offensichtlich noch nicht desalkylierte Substanzanteile an die Oberfläche, die bei einer nachfolgenden Bestrahlung wiederum primäre aromatische Amine (Nachweis mit Hilfe der Reaktion nach Bratton und Marshall[7]) lieferten.

Eine Optimierung der Methode musste demnach das Ziel verfolgen, mit Hilfe einer geeigneten Technik möglichst hohe Substanzanteile an die Oberfläche der DC-Platte zu bringen.

Photolytische Desalkylierung von Flunitrazepam unter präparativen Aspekten auf der DC-Platte. In Vorversuchen konnte gezeigt werden, dass sowohl aufgesprühte als auch mit dem Chromatocharger aufgetragene Substanzlösungen tief in die Sorbensschicht eindrangen und so der Einwirkung der UV-Strahlung entzogen wurden. Mit Hilfe der nachstehend beschriebenen Technik gelang es, die zu desalkylierende Substanz im wesentlichen auf der Plattenoberfläche zu konzentrieren: Hierzu legt man eine präparative Dünnschichtplatte (z.B. Art. Nr. 5717; Merck) in Aceton oder Essigsäureäthylester, bis sie ein Maximum an Lösungsmittel aufgesaugt hat. Unmittelbar danach wird die noch tropfnasse Platte mit der Lösung des zu desalkylierenden Benzodiazepins besprüht. Hierbei dringen allenfalls unwesentliche Substanzmengen in das Sorbensinnere, da dieses bereits mit Lösungsmitteln abgesättigt ist. Beim anschliessenden Ablüften der Platte mit Warmluft treten die im unteren Teil der Trägerschicht enthaltenen Lösungsmittelanteile wieder an die Oberfläche wobei Substanzreste, die vorher geringfügig in die Schicht diffundierten, wiederum an die Plattenoberfläche gelangen. Auf diese Weise wird erreicht, dass wesentlich höhere Anteile der Probesubstanz bei der UV-Bestrahlung exponiert sind.

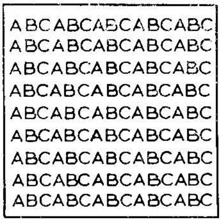
Eine Belichtung der Platte darf erst nach dem Verflüchtigen aller Lösungsmittelreste erfolgen. Auch bei den präparativen Untersuchungen konnte bei Belichtungszeiten über 30 min keine Steigerung der Ausbeute mehr festgestellt werden. Die Anzahl der Nebenprodukte nahm bei längeren Exponierungen beträchtlich zu.

Der Abstand zwischen Strahlungsquelle und DC-Platte betrug etwa 40 cm. Die Platte wurde unter dem Abzug (Ozonbildung) mit Hilfe einer Stativklammer fixiert. Als Strahler wurden sowohl kommerzielle Geräte für Photoreaktionen (z.B. TQ 150; Hanau) als auch eine Höhensonne (allerdings ohne zusätzliche zwangsweise IR-Strahlung, wie sie neuerdings zur Vermeidung von UV-Überdosierungen vorgeschrieben ist) eingesetzt.

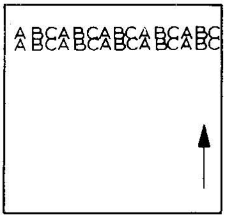
Nach der Bestrahlung befinden sich auf der Plattenoberfläche zahlreiche Substanzen gleichmässig verteilt (z.B. unumgesetztes Flunitrazepam, Nor-Flunitrazepam, zahlreiche Nebenprodukte). Beim Entwickeln der Platte mit einem äusserst polaren Fliessmittel (z.B. Aceton) gelingt es, diese Reaktionsprodukte auf einem Streifen am oberen Ende der Platte (bei aufsteigender Technik) zu konzentrieren, da alle Derivate bei Verwendung von Aceton als Fliessmittel praktisch bis zum R_F-Wert-Bereich 1.0 wandern.

Zur Trennung der verschiedenen Substanzen wird die Platte nach gründlichem Ablüften um 180° gedreht und mit dem Fliessmittelgemisch Benzol–Isopropanol–konz. Ammoniak (80:20:1, v/v/v) entwickelt.

Die geschilderte Methodik ist in der nachstehenden schematischen Darstellung nochmals zusammenfassend wiedergegeben: (a) Nach der Bestrahlung liegen die Substanzen A, B und C verteilt auf der gesamten Plattenfläche vor:



(b) Nach dem Entwickeln mit Aceton wurden alle Substanzen in einem Band am oberen Rand der Platte konzentriert:



(c) Durch Drehen der Platte um 180° und Entwickeln mit Benzol–Isopropanol–konz. Ammoniak werden die einzelnen Komponenten getrennt.

Die einzelnen Derivate können anhand der Fluoreszenzlöschung bei folgenden R_F-Werten indiziert werden:

R_F-Wert	*Substanz*
0.85	Flunitrazepam
0.72	Nor-Flunitrazepam
0.62 und 0.57	Nicht näher identifizierte Nebenprodukte

Die Elution erfolgt zweckmässigerweise mit Essigsäureäthylester (vgl. Ausführungen hierzu unter *Darstellung von 7-Aminoflunitrazepam auf der DC-Platte*).

ERGEBNISSE

7-Aminoflunitrazepam und 7-Acetamidoflunitrazepam lassen sich in nahezu quantitativer Ausbeute mit Hilfe von Reaktionen auf der DC-Platte darstellen. Hierbei entfallen zeitraubende Zwischenextraktionen und Reinigungsschritte. Ausserdem tritt bei der Synthese auf der Platte die sonst im sauren Reaktionsmedium stark ausgeprägte Hydrolyse zu den Aminobenzophenonderivaten in den Hintergrund. Dies dürfte nicht zuletzt auch darauf zurückzuführen sein, dass sich der Reaktionsablauf auf der Platte praktisch bei Zimmertemperatur abspielt, was wiederum als Indiz für die erhöhte Reaktionsfähigkeit vieler Substanzen im absorbierten Zustand auf der Platte anzusehen sein dürfte.

Aufgrund der Untersuchungen kann weiterhin davon ausgegangen werden, dass photolytische Desalkylierungsreaktionen in Lösungen nicht ohne weiteres durchführbar sind. Auf der DC-Platte konnten hingegen nach Entwicklung einer speziellen Arbeitstechnik Umsetzungen mit befriedigenden Ausbeuten erzielt werden.

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Wir danken der Fa. Hoffmann-La Roche für die ständige Unterstützung unserer Arbeiten.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wird eine Mikromethode zur Darstellung von 7-Aminoflunitrazepam, 7-Acetamidoflunitrazepam und N-1-Desalkylflunitrazepam mit Hilfe von Synthesen auf der Sorbensschicht von DC-Platten beschrieben. Die Methode eignet sich auch zur Präparation zahlreicher weiterer wichtiger Benzodiazepinderivate.

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CHROM. 11,381

GAS CHROMATOGRAPHIC AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC CHARACTERISATION OF SOME THIOSULPHONATES AND POLYMETHYLENE DIMETHANE THIOSULPHONATES

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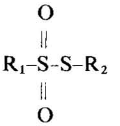
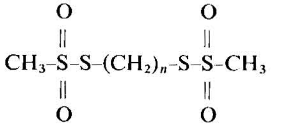
SUMMARY

The characterisation by gas chromatography (GC) and GC–mass spectrometry (MS) of thiosulphonates of the types $R_1 \cdot SO_2S \cdot R_2$ (type I) and $CH_3 \cdot SO_2S \cdot (CH_2)_n S \cdot SO_2CH_3$ (type II) is described. Type I thiosulphonates showed suitable GC–MS properties as the intact molecules. Type II could be characterised by GC–MS only after reduction to the dithiol; direct probe spectra were necessary to characterise all the intact type II compounds by MS.

INTRODUCTION

Thiosulphonates (sulphonate thioesters) have recently found application as thiol reagents in enzyme mechanism studies[1–3] and polymethylene dimethane thiosulphonates similarly are useful in cross-linking studies[4]. The thiosulphonates under discussion can be classified into two types:

Type I:

$$R_1\text{–}\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}\text{–}S\text{–}R_2$$
(where R_1 is usually methyl)

Type II:

$$CH_3\text{–}\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}\text{–}S\text{–}(CH_2)_n\text{–}S\text{–}\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}\text{–}CH_3$$
(n = 5 to 12)

Because these compounds are potentially useful protein thiol reagents, we required a convenient method for characterisation and assessment of purity. The use of gas chromatography–mass spectrometry (GC–MS) was suggested by a brief

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report[5] that several dialkyl thiosulphonates had been gas-chromatographed on Triton X-305 and that molecular ions were present in their mass spectra (direct probe). Similarly, the purification by preparative GC of the analogous polymethylene glycol dimethane sulphonates [$CH_3-SO_2-O-(CH_2)_n-OSO_2-CH_3$, $n = 2$ to 9] on Carbowax 20M has been reported[6]. The mass spectra of these latter types of sulphonates[6] and some of the type I thiosulphonates have been well documented[5,7].

In this paper we report on the characterisation by GC, MS and GC–MS of some type I and type II thiosulphonates and briefly describe the mass spectra of three new type I thiosulphonates and also the mass spectra of the type II series.

EXPERIMENTAL

The synthesis of and enzymic studies utilising the thiosulphonates will be published elsewhere. For GC and GC–MS, samples were dissolved in dry acetone at 5 mg/ml; fresh solutions were prepared for each analysis.

Reduction of type II compounds

Sodium borohydride, 10 μl of 10 mg/ml solution in methanol was added to 100 μl of 10 mg/ml methanol solutions of type II thiosulphonates. When the evolution of hydrogen had ceased (1 to 2 min), aliquots were assayed by GC or GC–MS. This procedure gave type II reduced (IIR) series.

Gas chromatography

Retention data were measured on a 2.1 m × 4 mm I.D. glass column packed with 3% OV-1 on Diatomite CQ, 100–120 mesh, fitted to a Pye 104 chromatograph with flame ionisation detector. The flow-rate of the nitrogen carrier gas was 40 ml/min, type I series were run at 100° column, injector 130°; type II at column temperatures between 220° and 290° (Table I), injector 280°; and type IIR at 130° to 240° (Table I), injector 150° to 250°.

MS and combined GC–MS

Spectra were obtained on a Kratos-AEI MS 30, to which a Pye 104 chromatograph was linked via a membrane separator. All GC–MS samples were run on a 1.5-m glass column packed with the OV-1 phase described above. The flow-rate of the helium carrier gas was 40 ml/min, injector 150°, column temperature 110° to 130° (to give retention times of 3 to 4 min) for type I series, and 150° to 225° for type IIR. MS conditions were source 130°, separator 180°, 24 eV ionising voltage (EI), 300 μA current. Confirmatory analyses of type I liquids were done by direct probe (gas) at 75°. Type II compounds could not all be run directly by GC–MS so the spectra of the "intact" compounds of this series were obtained by direct probe (solid) at temperatures between 120° and 250°. Spectra of probe samples were taken at 70 eV. All spectra were low resolution (1000).

RESULTS

Gas chromatography

The retention times of type I, type II and type IIR compounds are summarised

TABLE I

RETENTION TIMES ON OV-1 OF ACYL THIOSULPHONATES, POLYMETHYLENE DIMETHANE THIOSULPHONATES AND DITHIOL PRODUCTS FROM REDUCTION OF POLYMETHYLENE THIOSULPHONATES

Compound	R_1	R_2	*n*	*Temperature (°C)*	*Retention time (min)*
Type I: R_1–SO_2S–R_2					
A	CH_3	CH_3		100°	3.0
B	CH_3	CH_2CH_3		100°	4.5
E	CH_3	CH_2CH_2F		100°	5.6
F	CH_3	CH_2CF_3		100°	1.8
C	CH_3	$CH(CH_3)_2$		100°	5.9
D	$(CH_3)_2CH$	$CH(CH_3)_2$		100°	9.4
Type II: $CH_3SO_2S\,(CH_2)_n\,SSO_2CH_3$					
			5	220°	9.3
			6	240°	6.4
			8	260°	5.9
			10	290°	3.75 (some decomposition)
			12	(decomposes, does not run)	
Type IIR: $HS(CH_2)_n\,SH$ *and* $\overline{S(CH_2)_nS}$					
Dithiol			5	130°	2.1
Disulphide			5	130°	2.7
Dithiol			6	140°	2.55
Disulphide			6	140°	3.4
Disulphide			8	170°	6.4
Disulphide			10	170°	7.25
Disulphide			12	240°	2.1

in Table I. The longer chain homologues of the type II series showed a progressive decomposition such that for $n = 12$ the parent compound did not elute. Some decomposition is evident from the appearance in all type II samples of one or two rapidly eluting peaks (Fig. 1). GC–MS analysis showed that these peaks were the dithiol and

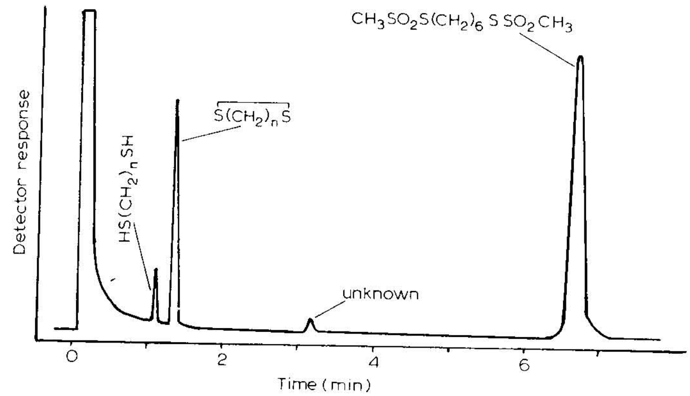

Fig. 1. GC trace of type II thiosulphonate $CH_3SO_2S\,(CH_2)_6S\,SO_2CH_3$ on OV-1 at 240° showing parent compound and thiol decomposition products.

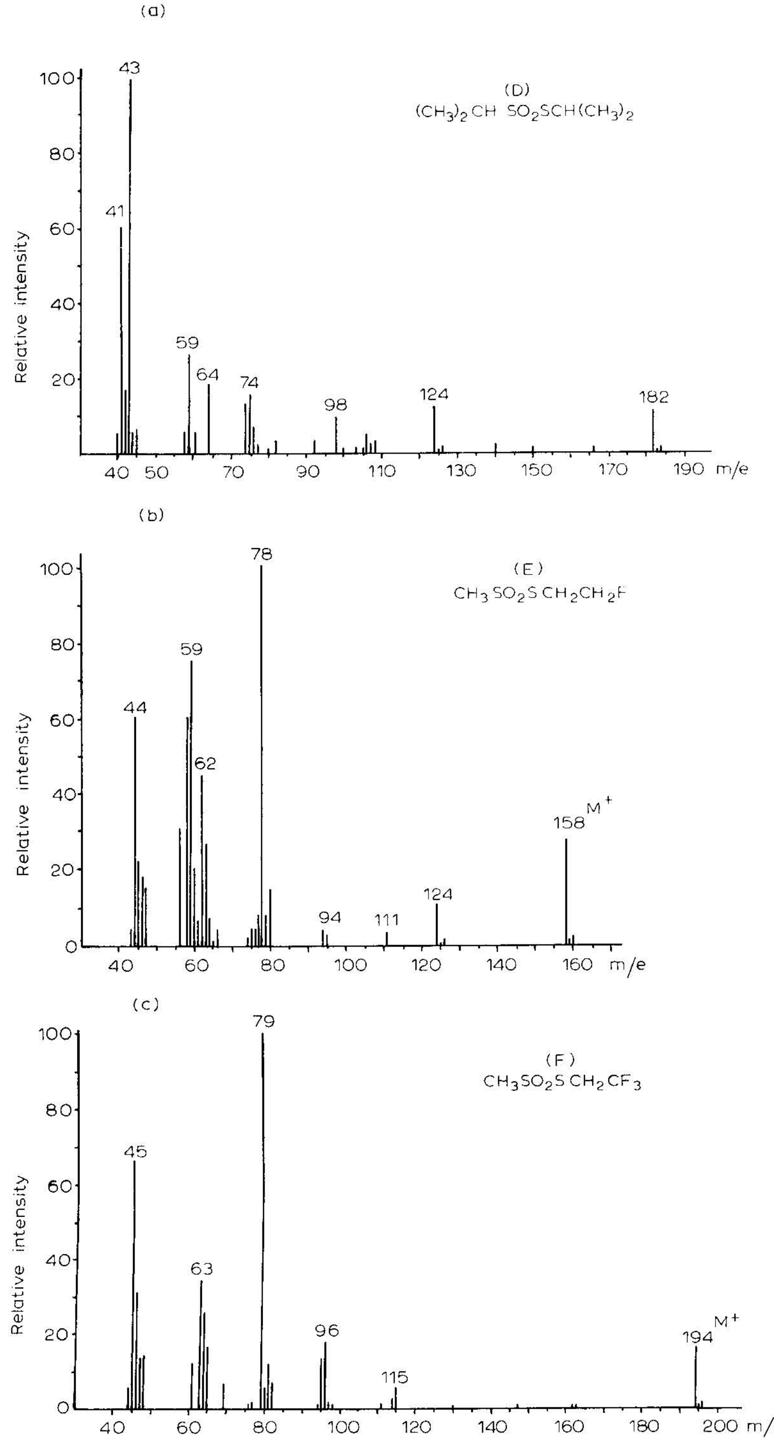

Fig. 2. Normalised mass spectra of type I thiosulphonates: isopropyl isopropane (D), monofluoroethyl methane (E) and trifluoroethyl methane (F) taken using GC–MS.

cyclic disulphide formed by (pyrolytic) decomposition. Confirmation of this identity was established by GC–MS of the type IIR series and this identification has been used in Table I.

GC–MS and MS

In the type I series, the mass spectra of A, B, and C (Table I), have been documented previously[7]. Thiosulphonates D, E and F are new compounds, and their normalised spectra obtained by GC–MS are given in Fig. 2 and briefly discussed below. There was agreement between the GC–MS and direct probe spectra, showing that there was no decomposition during chromatography.

Two representative spectra of type II (direct probe) and the corresponding type IIR (GC–MS) compounds $n = 6$ and 12, are given in Fig. 3. Both type II and IIR series gave characteristic fragmentation patterns; some aspects of these spectra are discussed below and shown in Fig. 5.

DISCUSSION

Many chemical and detailed mass spectral studies[5–7] have been described for dialkyl thiosulphonates (type I) and polymethylene glycol dimethanesulphonates (oxygen ester analogues of type II). However, a simple analytical procedure to characterise these compounds by GC and GC–MS is desirable for the routine assessment of identity and purity where these compounds are to be used in active site and inhibitor studies on enzymes.

Thiosulphonates of the type I series all showed good GC and GC–MS properties (Table I, Fig. 2) and can be characterised readily. The short-chain members, $n = 5$ and 6, of type II series gave main peaks on GC due to the intact molecule, and two fast-eluting peaks, whereas $n = 8$ to 12 compounds showed a progressive loss of GC mobility of the parent compound and one main fast eluting peak. These peaks were shown to be due to a "double de-methanesulphonylation", presumably by pyrolysis on injection, to produce the corresponding open chain disulphides and cyclic dithiols.

This essentially reductive process was intentionally carried out using the facile reduction[8] of thioesters with borohydride, and resulted in products having the same GC retention times and mass spectra as the decomposition products. A full GC characterisation of the type II series is thus possible after reduction to type IIR. For $n = 5$, 6 and 8, the first eluting peak is the straight chain dimercaptan, $HS(CH_2)_nSH$, the second peak the cyclic dithiol $\overline{S{-}R{-}S}$, the re-oxidised form. For $n = 10$ and 12, only the cyclic dithiol form was observed, and after prolonged standing the shorter chain dimercaptans slowly oxidised to the cyclic form, indicating that cyclisation probably is independent of the GC process.

The normalised spectra of the three thiosulphonates, isopropyl isopropane (D), monofluoroethyl methane (E) and trifluoroethyl methane (F) are given in Fig. 2. Fragmentation patterns are similar to those of other thiosulphonates[7]. The major peaks were the molecular ions, and the cleavage of the S–S bond (with H transfer in E) to give a peak at m/e 115 $[SCH_2CF_3]^+$ in F, and base peaks at m/e 78 $[SCHCH_2F]^+$ for E and m/e 79 $[SO_2CH_3]^+$ for F. The base peak in D is m/e 43 $[CH(CH_3)_2]^+$. These and other fragments are outlined in Fig. 4a and b.

Intact type II compounds did not give molecular ions, the first major fragment occurs at $M^+ - 79$ by loss of CH_3SO_2, similar to the oxygen analogues[6]. However, unlike the latter, the corresponding transsulphonylation does not occur except perhaps for $n=8$ where m/e 191 may be the thio analogue $[CH_3SO_2H \cdot S \cdot SO_2\ CH_3]^+$ of the protonated methane sulphonic acid anhydride previously observed[6]. The replacement of oxygen by sulphur presumably promotes charge retention on the thiol fragment such that the principal high-molecular-weight fragments arise by the loss of methane sulphonyl and methane thiosulphonate groups (Fig. 5), and no base peak at 79 is observed. In the absence of high resolution measurements, the identities of the lower mass fragments were assumed to be similar to those in the type IIR series discussed below, in which the oxygens are absent. Facile loss of both methane sulphonyl groups gives a group of peaks (H)S–R–S(H); this, and the resulting fragment series C_nH_{2n-1} and $C_nH_{2n-1}S$, are therefore similar to the type IIR spectra.

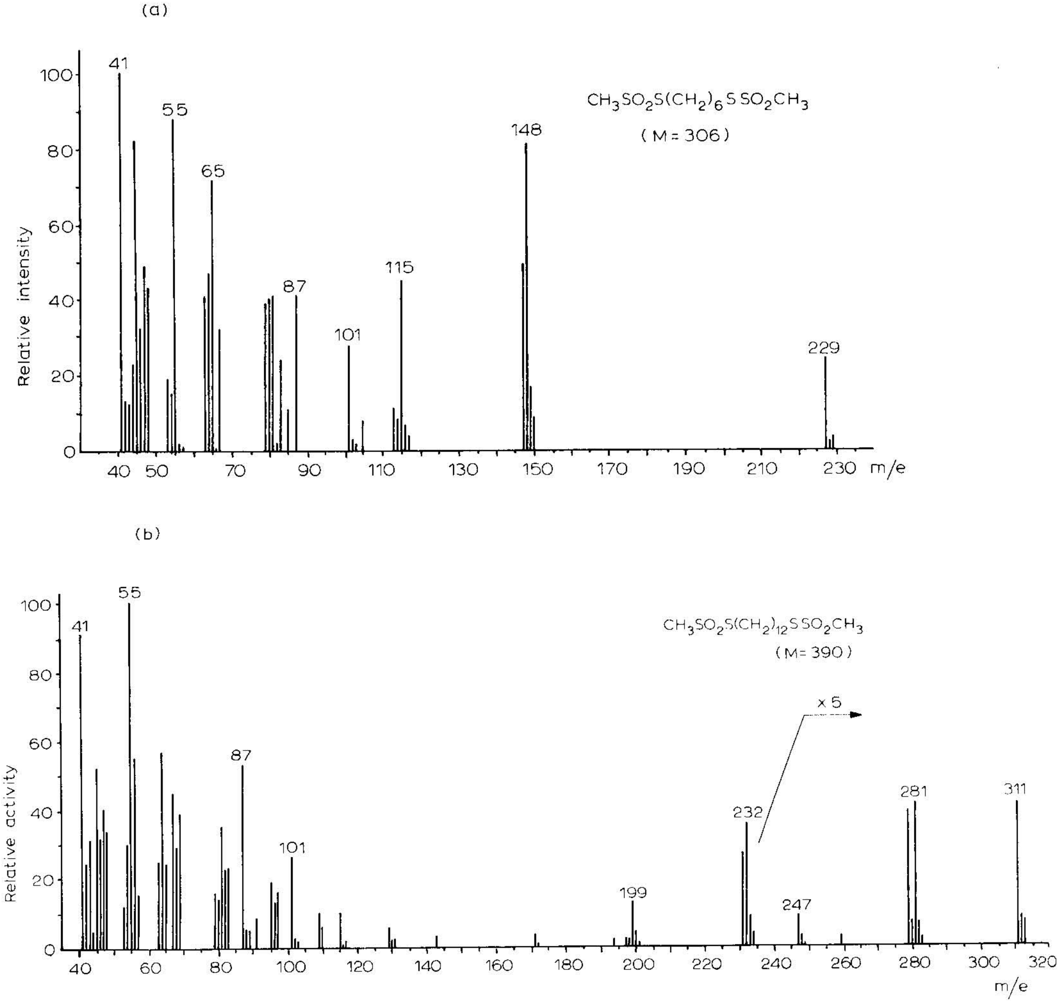

Fig. 3.

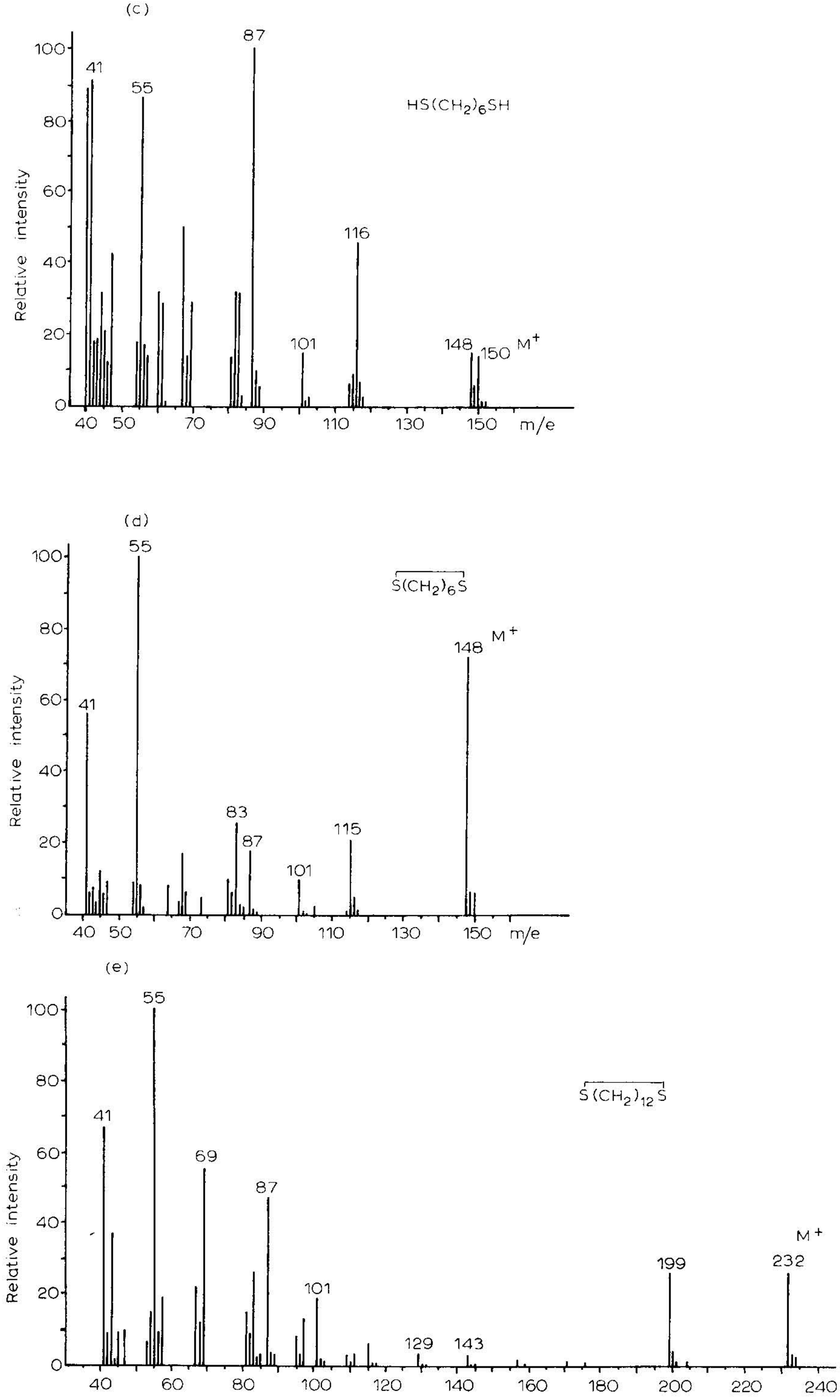

Fig. 3. Normalised mass spectra of type II and type IIR thiosulphonates; (a) and (b) by direct probe, (c), (d) and (e) by GC-MS.

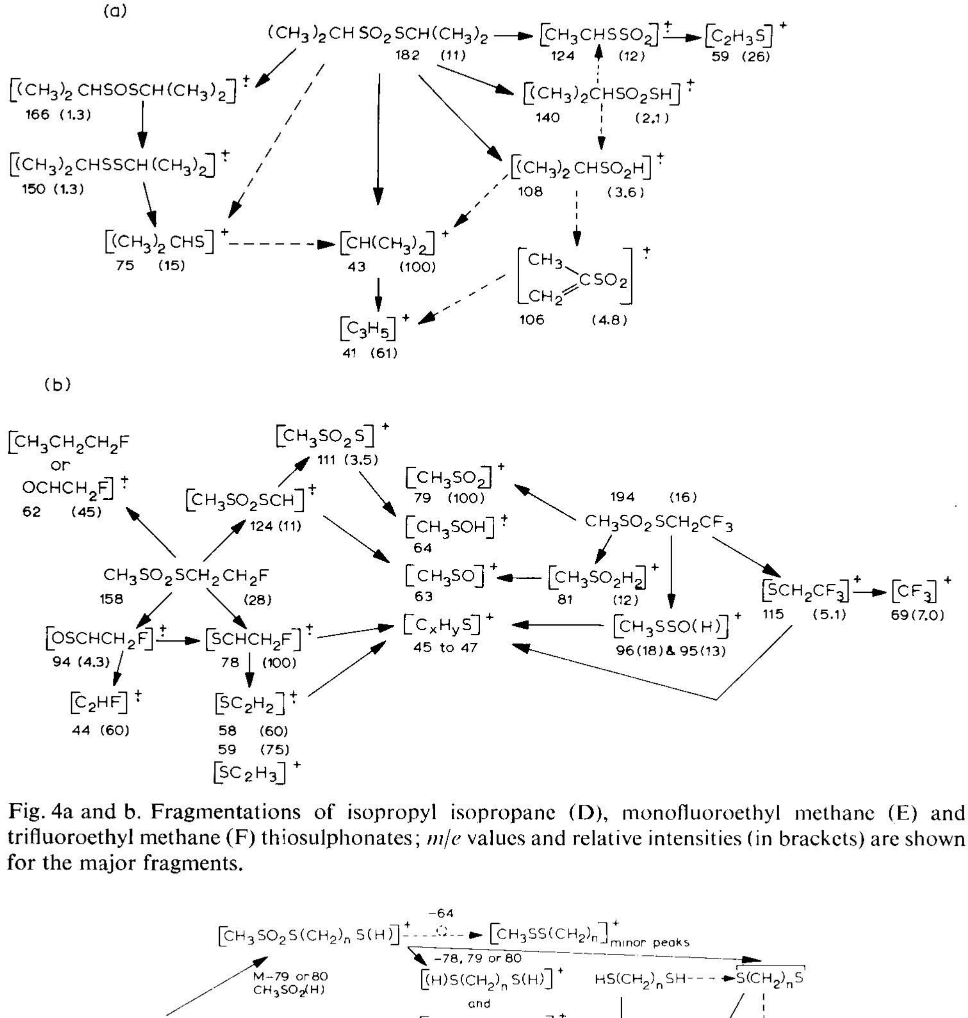

Fig. 4a and b. Fragmentations of isopropyl isopropane (D), monofluoroethyl methane (E) and trifluoroethyl methane (F) thiosulphonates; *m/e* values and relative intensities (in brackets) are shown for the major fragments.

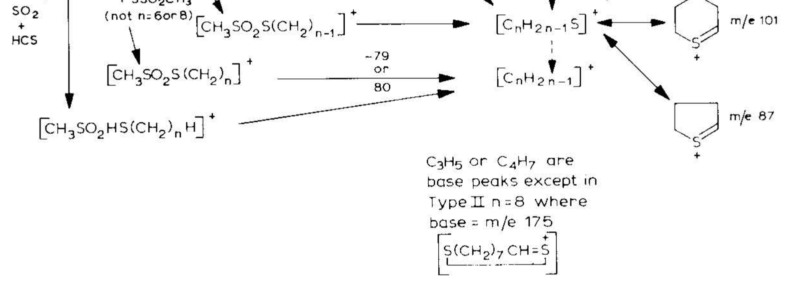

Fig. 5. Generalised fragmentation patterns of $CH_3SO_2S(CH_2)_nS\ SO_2CH_3$ (type II) and reduction products HS $(CH_2)_nSH$ and $\overline{S(CH_2)_nS}$ (type IIR) thiosulphonates.

Type IIR series gave prominent molecular ions on GC–MS, with the disulphides tending to lose 2H (possibly due to some formation of the cyclic form in the MS source) and H_2S, whereas the cyclic dithiols lose SH. The main series of fragments were (H)S–R–S(H), C_nH_{2n-1} and $C_nH_{2n-1}S$, with the sulphur containing ions of higher relative intensity in the disulphide series. A fragment at *m/e* 101 in both series may be due to the formation of the cyclic ion C_5H_9S (Fig. 5) by expulsion of $(CH_2)_{n-4}SH$ from the molecular ion (*cf.* alkyl tetrahydro thiophenes[9]). The spectra of the four carbon[10] and five carbon[11] cyclic dithiols have been described; base peaks at *m/e* 55 or 69 are common to all these cyclic dithiols.

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IDENTIFICATION OF ACETYL DERIVATIVE ISOMERS OF 1,5-ANHYDRO-D-XYLITOL BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

High-resolution gas chromatography and mass spectrometry have been employed to separate selectively acetylated 1,5-anhydro-D-xylitol derivatives and to study their chemical structures.

Complete separation of reaction mixtures was achieved on a capillary column coated with an SE-30 liquid phase. The combination of gas chromatography–mass spectrometry with oxidation reactions allowed the assignment of the chemical structures of all the derivatives, obtained.

INTRODUCTION

The relative reactivities of hydroxyl groups present a fundamental problem in the synthesis and stereochemical study of carbohydrates[1]. Analytical methods have been considerably improved in recent years to allow more accurate measurements of product distribution. Relative reactivity has been found to be influenced by electronic and steric factors.

The selective behaviour of anhydroalditols in catalytic oxidation, acylation and acetylation has been studied[2]. The present paper deals with the study of the selective acetylation reaction of 1,5-anhydro-D-xylitol by means of high-resolution gas chromatography and mass spectrometry (GC–MS).

We used capillary column GC–MS because we had obtained good results in the separation of alditols[3] and anhydroalditols[4] on a capillary column.

This paper is the first report of our studies of the thermodynamic characteristics of the relative reactivities of hydroxyl groups in carbohydrate derivatives.

EXPERIMENTAL

Reaction 1

To 2.68 mg of 1,5-anhydro-D-xylitol (0.02 mM) dissolved in 200 μl of dry pyridine was added 1.9 μl (0.02 mM) of acetic anhydride. The solution was heated for 1 h at 100°, evaporated to dryness in a nitrogen stream, and treated with 200 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). After this mixture had been heated for 15 min at 100°, it was used for GC and GC–MS analyses.

Reaction 2

The above procedure was repeated using 2.7 mg (0.02 m*M*) of anhydroxylitol and 3.8 μl (0.04 m*M*) of acetic anhydride.

Reaction 3

The above procedure was repeated using 2.7 mg (0.02 m*M*) of anhydroxylitol and 5.6 μl (0.06 m*M*) of acetic anhydride.

The three reactions were repeated using labelled acetic anhydride (d_6) to generate derivatives selectively labelled in the acetyl groups.

Reaction 4

To 5 mg (0.056 m*M*) of anhydroxylitol dissolved in 400 μl of pyridine was added 5.3 μl (0.056 m*M*) of acetic anhydride. The solution was heated and divided into two equal samples. The first was derivatized and analysed. The second was oxidized with 5.8 mg (0.027 m*M*) of sodium periodate in water (400 μl) at room temperature for 24 h. Then the solution was evaporated to dryness and the residue treated with 200 μl of BSTFA.

GAS CHROMATOGRAPHY

Column preparation

Borosilicate (Pyrex) glass capillaries (80 m × 0.7 mm O.D. × 0.3 mm I.D.) were drawn from 1.5-m tubes (6.2 mm O.D. × 3.0 mm I.D.) using a Hewlett-Packard glass drawing and coiling apparatus. Prior to drawing, the tubes were rinsed successively with acetone, methylene chloride, 1% aqueous potassium hydroxide and methanol, and dried under vacuum[5]. The diameter of the capillary coil was 10 cm[6].

The glass capillaries were silanized with dimethyldichlorosilane (DCMS) and coated with SE-30 containing Silanox 101 (Cabot Corp., Boston, Mass., U.S.A.) by the German and Horning[7] method and prepared by the dynamic method. In the first step, a plug consisting of Silanox dispersed in a dilute solution of the SE-30 phase in carbon tetrachloride was forced through a column immersed in an ultrasonic bath. After the second coating, the last 20 m of the capillary column was broken off.

The columns were conditioned in a flow of carrier gas (argon) by temperature programming at 1°/min from 40° to 280°, keeping the temperature at 280° for 2 h, then lowering it to 200°, programming it again at 1°/min to 300°, and keeping it at 300° for 1 h. The theoretical plate efficiencies of the resulting columns (for *n*-tetracosane at 250°) exceed 130 000.

Instrumental conditions

The compounds studied were separated with a Varian Aerograph series 1400 gas chromatograph modified to include a glass inlet system with splitter and 60 m glass capillary columns. The inlet system (see Fig. 1) was designed as a very simple, inexpensive, home-made unit to fit all GC injection ports. It contains 2.2 mm I.D. glass tubing in the form of a precolumn packed with glass wool and 3% SE-30 on

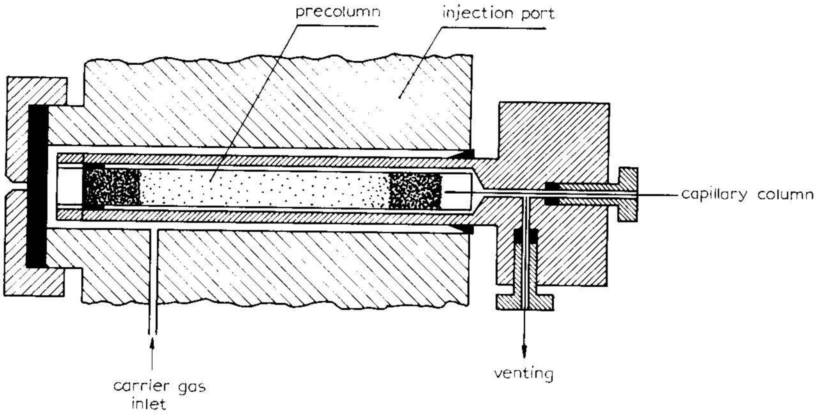


Fig. 1. A modified inlet system.

Gas-Chrom Q. Two capillary columns (60 m and 12 m), were connected to the inlet system. The 12-m column was used as restrictor for venting the carrier gas flow at a splitting ratio of 1:5. The end of the 60-m column was connected to a make-up system (Fig. 2) which in turn fed a flame ionization detector. This system, with an additional argon flow of 20 ml/min, assures maximum column efficiency.

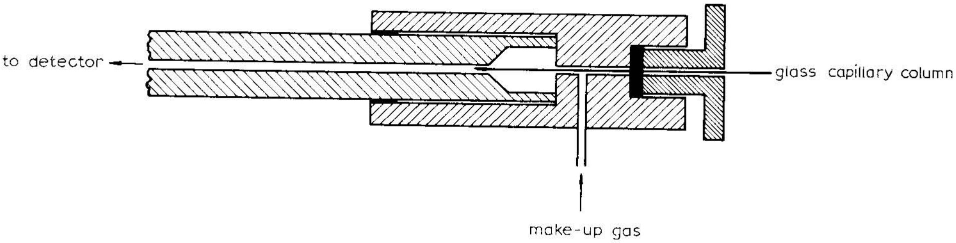


Fig. 2. Make-up system.

A Philips PM 8220 recorder was used. Further GC conditions adopted were: GC oven, 150° (isothermal); precolumn inlet splitter, 250°; detector block, 260°; column inlet pressure, 1.5 kg/cm^2, resulting in a carrier gas (argon) linear velocity of 16–17 cm/sec (200°); hydrogen flow-rate, 30 ml/min; air flow-rate, 250 ml/min.

Quantitative analysis

Calculations were performed by hand, and area measurements by multiplying the height of the peak by its width at half height. The weight response factors were assumed to be equal for all isomers studied.

MASS SPECTROMETRY

The mass spectra were recorded on a LKB 2091 mass spectrometer linked to a mini-computer PDP-11. The mass spectrometer was equipped with a 25-m capillary column coated with an SE-30 liquid phase. The spectrometer was operated at a source temperature of 260°, the molecular separator at 250°; the ionizing voltage was 70 V; a scan from *m/e* 10 to 680 took 2 sec.

RESULTS AND DISCUSSION

1,5-Anhydro-D-xylitol has a plane of symmetry that decreases the number of products because positions 2 and 4 are equivalent. All the hydroxyl groups are equatorial.

The reaction of 1,5-anhydro-D-xylitol with one, two or three equivalents of acetic anhydride (the third reaction corresponds to a molar ratio of one anhydride molecule to one hydroxyl group) was studied. The mixture shows five GC peaks (Fig. 3) in a unimolar reaction (reaction 1). The GC peak labelled F was assigned to per-O-silyl derivative on the grounds of the mass spectrum (Fig. 4) and of the retention time.

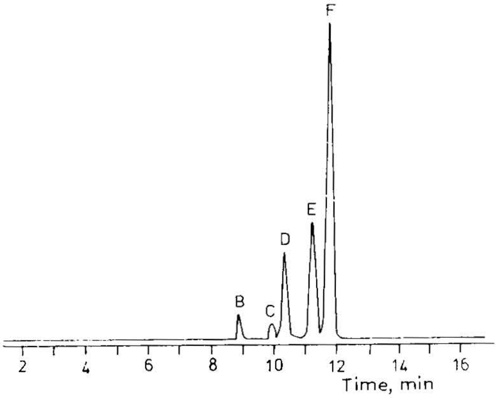


Fig. 3. GLC of the mixture 1.

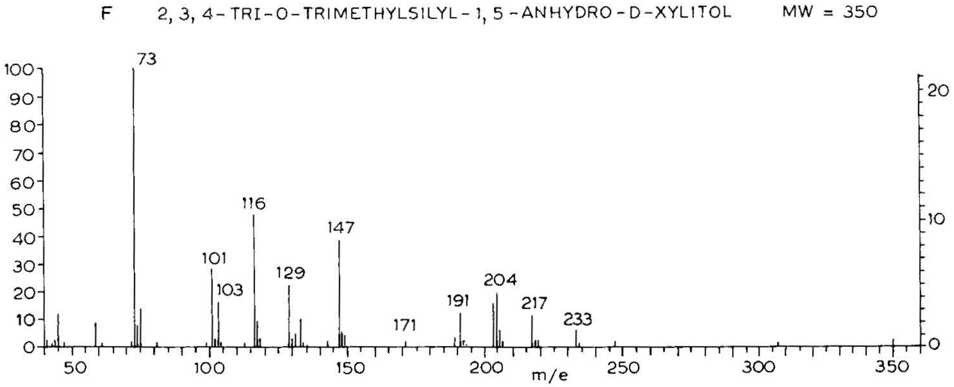


Fig. 4. Mass spectrum of GC peak F.

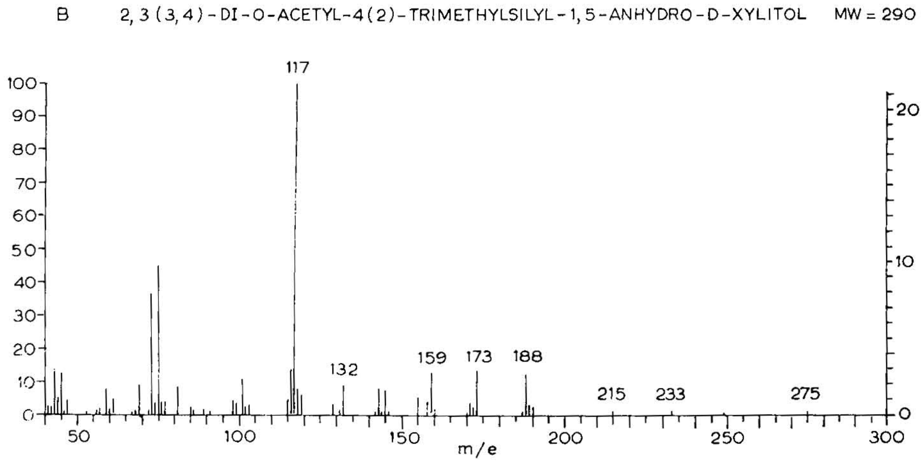


Fig. 5. Mass spectrum of GC peak B.

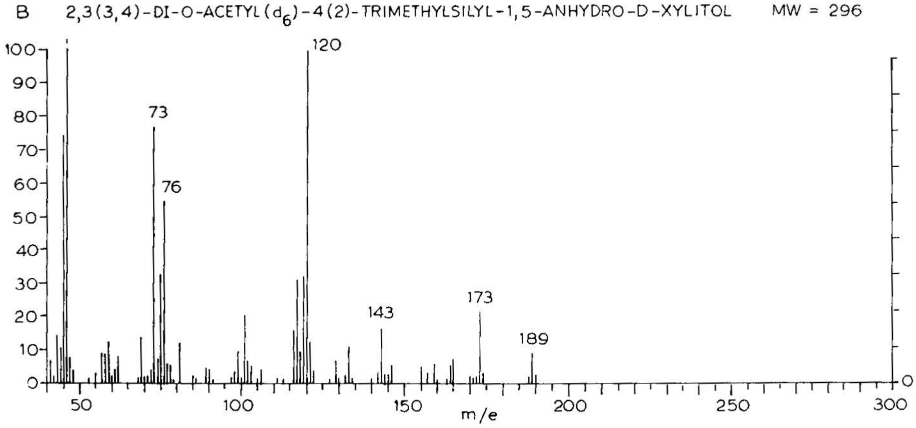


Fig. 6. Mass spectrum of GC peak B (labelled isomer).

The peak labelled B is 2,3(3,4)-di-O-acetyl-4(2)-O-trimethylsilyl-1,5-anhydro-D-xylitol. This structure has been confirmed by mass spectrometry (Figs. 5 and 6). The *m/e* 188/189 ion corresponds to loss of acetic acid (*m/e* 230/233) followed by ketene elimination. This sequence is only possible for an acetyl derivative with two acetoxyl groups on adjacent carbon atoms.

The next GC peak (C) can be assumed to be 2,4-di-O-acetyl-3-O-trimethylsilyl-1,5-anhydro-D-xylitol. The mass spectra (Figs. 7 and 8) contain the ions *m/e* 275/281 ($M-15$) and *m/e* 170/170 ($M-2 \times 60$). Owing to the presence of the plane of symmetry there are only two possible structures for the di-O-acetyl derivatives of 1,5-anhydro-D-xylitol, namely 2,3- and 2,4-. Comparison of the spectra (Figs. 5 and 6 and 6 and 7) eliminated the first structure (2,3-di-O-acetyl) because the GC peak B had previously been shown to be of this derivative.

Thus, the monoacetylsilyl derivatives (peaks D and E) appear in the gas chromatogram before the per-O-silyl derivative (peak F). The mass spectra of both

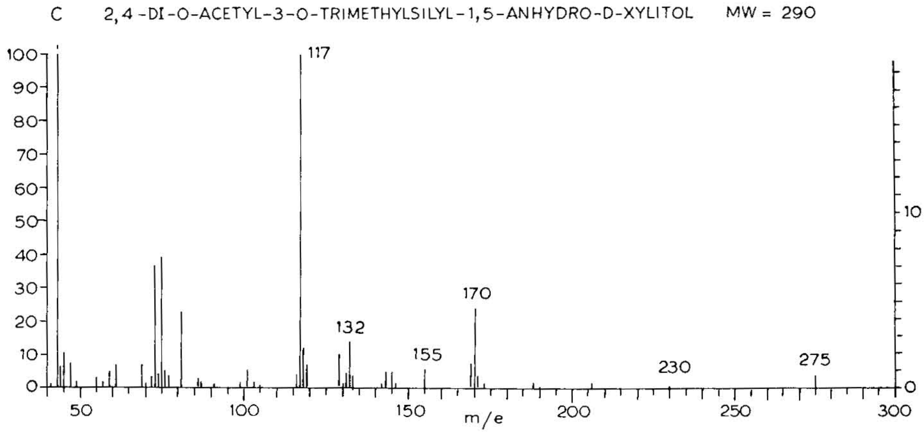


Fig. 7. Mass spectrum of GC peak C.

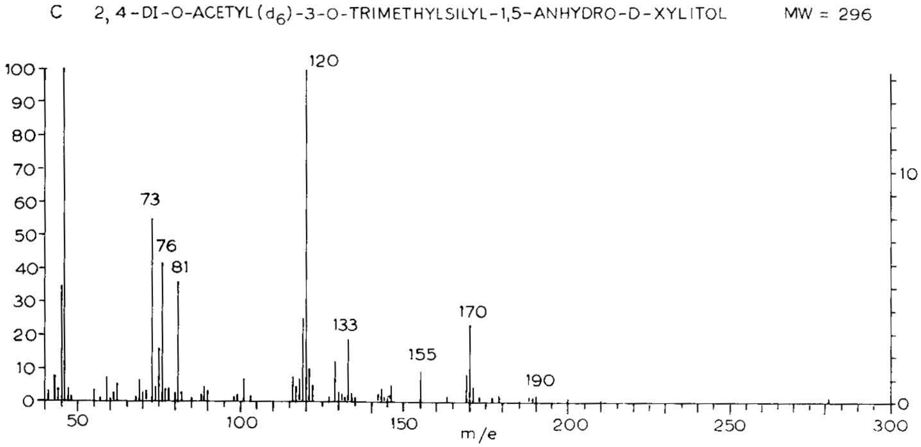


Fig.8. Mass spectrum of GC peak C (labelled isomer).

compounds (Figs. 9–12) reveal significant differences. The second (GC peak E) is assumed to be the 2(4)-O-acetyl-per-O-silyl derivative because its mass spectra (unlabelled and labelled derivatives) exhibit abundant *m/e* 147 ion. The *m/e* 147 ion in the mass spectra of trimethylsilylated sugar ethers has been shown[8] to arise from the ($M-15$) ion by means of a cyclic structure. Havlicek *et al.*[9] have shown by determining the number and type of cyclic processes which provide the TMSiO $=$ SiMe$_2$ ion that it is possible to predict which of an isomeric pair will show a stronger *m/e* 147 ion. 1,2-Diequatorial derivatives (1,2-trans) have been assigned the value 0.75, and 1,3-diaxial (1,3-*cis*) derivatives the value 1.50. The cyclic structure of the 1,3-diaxial group involves a change of ring conformation. As the mass spectra did not differentiate between the two possible monoacetyl isomers, we decided to discover the position of the acetyl group by a chemical method. 1,2-Glycols are known to be oxidized much more easily than 1,3-glycols[10]. A gas chromatogram run for a sample following

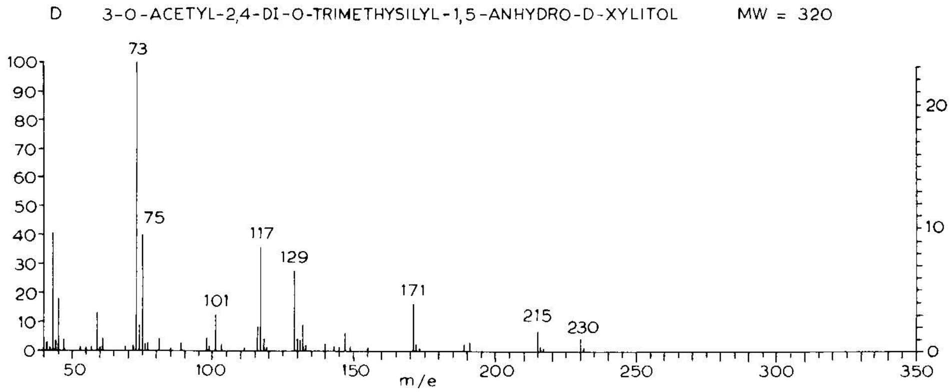


Fig. 9. Mass spectrum of GC peak D.

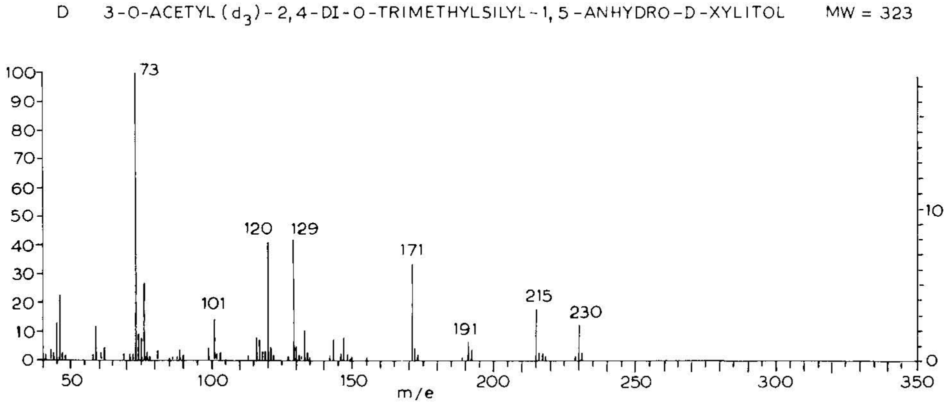


Fig. 10. Mass spectrum of GC peak D (labelled isomer).

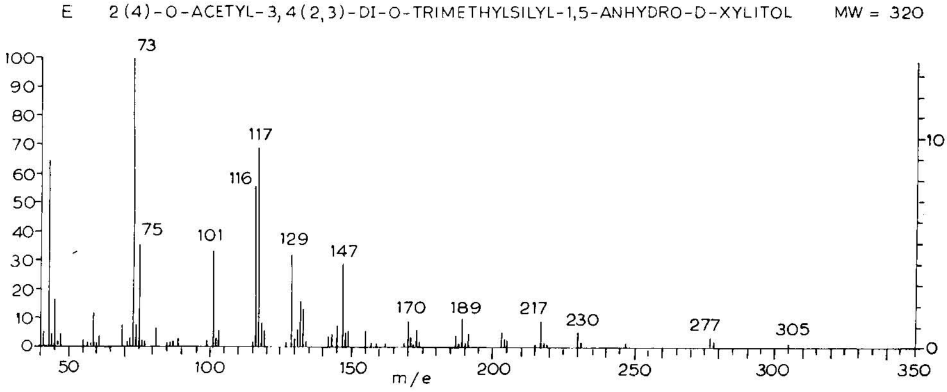


Fig. 11. Mass spectrum of GC peak E.

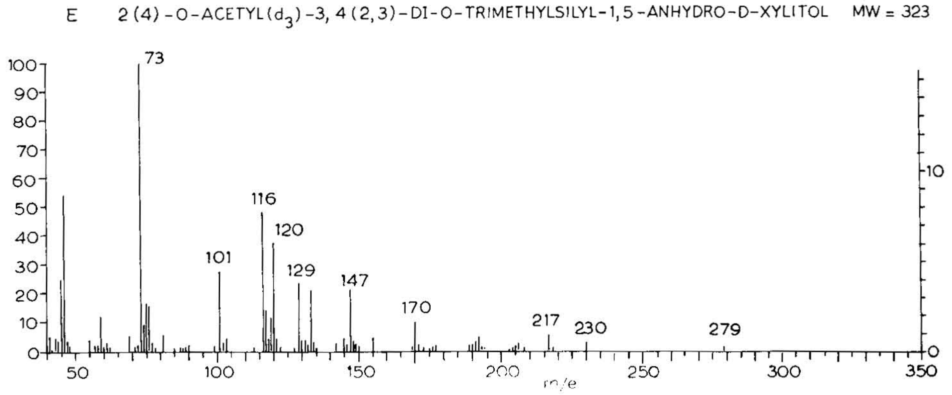


Fig. 12. Mass spectrum of GC peak E (labelled isomer).

sodium periodate oxidation is shown in Fig. 13. In this chromatogram, the GC peaks E and F disappeared completely. This showed that the 2(4)-monoacetyl structure may be assigned to the GC peak E, as peak F has previously been assigned as silyl derivative of 1,5-anhydro-D-xylitol.

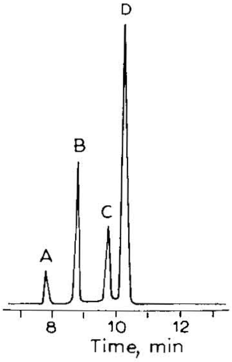


Fig. 13. GLC of the mixture 4.

TABLE I

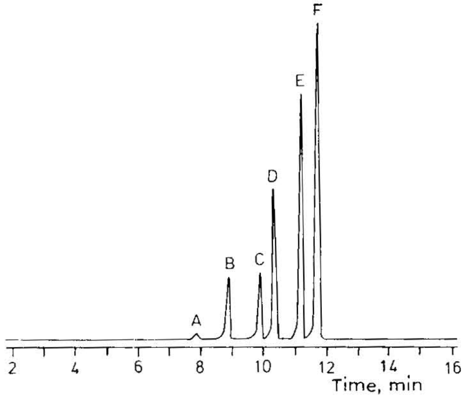
THE COMPOSITION OF REACTION MIXTURES 1, 2 AND 3

GC peak	*Silyl derivative of*	*Reaction mixture*					
		1		*2*		*3*	
		Yield (%)*	*Molar yields*	*Yield* (%)*	*Molar yields*	*Yield* (%)*	*Molar yields*
A	Per-O-acetyl-1,5-anhydro-D-xylitol**	–	–	1.0	0.5	22.5	8.5
B	2,3(3,4)-Di-O-acetyl-1,5-anhydro-D-xylitol	3.0	1.0	7.0	2.5	24.5	8.5
C	2,4-Di-O-acetyl-1,5-anhydro-D-xylitol	2.0	0.7	7.0	2.5	22.5	8.0
D	3-O-Acetyl-1,5-anhydro-D-xylitol	14.0	4.5	17.5	5.5	17.0	5.0
E	2(4)-O-Acetyl-1,5-anhydro-D-xylitol	20.5	6.5	29.0	9.0	10.0	3.0
F	1,5-Anhydro-D-xylitol	60.5	17.5	38.5	11.0	3.5	1.0

* Weight yields from GC peaks area.

** Simple acetyl derivative of 1,5-anhydro-D-xylitol.

The product distributions in samples 2 and 3 are shown in Figs. 14 and 15. If more acetic anhydride is used then more of the per-O-acetyl and di-O-acetyl derivatives is obtained. This confirms the previous structural assignemnts.



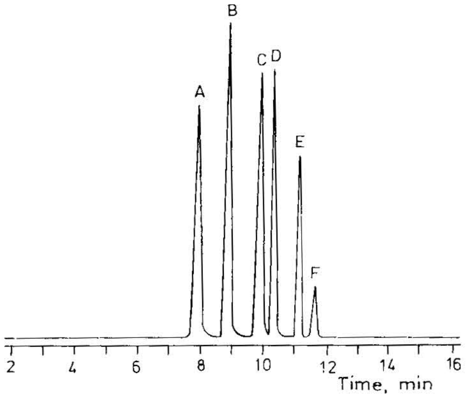


Fig. 14. GLC of the mixture 2.

Fig. 15. GLC of the mixture 3.

Study of the reaction of 1,5-anhydro-D-xylitol with one molar equivalent of acetic anhydride (reaction 1) in pyridine for 50 min at 100° showed that a 48% reaction occurred with a degree of substitution at O-3 and O-2(4) of 1.38 and 1.0, respectively (Table I). A similar ratio of reactivities has been obtained for a 1:2 molar ratio (1.22:1). The degrees of substitution were computed as molar yield ratios, keeping in mind that positions 2 and 4 are equivalent.

We shall publish further conclusions in a paper reporting kinetic studies.

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CHROM. 11,366

DETERMINATION OF *s*-TRIAZINE DERIVATIVES AT THE NANOGRAM LEVEL BY GAS–LIQUID CHROMATOGRAPHY

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(Received July 10th, 1978)

SUMMARY

A gas–liquid chromatographic method for the determination of the triazines cyanuric acid, ammelide, ammeline and melamine at the nanogram level, using trifluoroacetic acid as solvent for the triazines and N-methyl-N-trimethylsilyltrifluoroacetamide as the silylation reagent, is described. Optimal derivatization and chromatographic conditions have been determined. The relative molar response with respect to phenanthrene as the internal standard was found to remain constant for amounts of sample in the range 0.025–10 μg and the minimum detectable amount for quantitative analyses was found to be 2.5 ng (*ca.* 20 pmole) of each triazine.

INTRODUCTION

Because of the importance of derivatives of *s*-triazine possessing herbicidal activity a number of papers have been published on their separation and detection, involving paper[1,2], thin-layer[3,4], gas–liquid[5,6] and high-performance liquid chromatography[7]. In contrast, only a few reports have dealt with the analysis of the parent *s*-triazines, which include the hydroxy and amino derivatives cyanuric acid, ammelide, ammeline and melamine.

Two methods have been developed[8,9] for the separation and detection of these triazines by thin-layer chromatography but, although these methods give satisfactory results for qualitative analyses, they are not entirely suitable for quantitative purposes. In the course of our investigations into the occurrence of N-heterocyclic compounds in carbonaceous meteorites, a technique was needed for the quantitative detection of sub-microgram amounts of these triazines.

We therefore investigated the possibility of using gas–liquid chromatography, which implies the use of a derivatization agent to increase volatility and reduce polarity. It was decided to use trimethylsilylation for this purpose, as this technique has been reported[10] to be especially suitable for the derivatization of compounds containing amino and hydroxy groups. N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was selected because of its high volatility and, consequently, narrower solvent peaks on gas chromatograms compared with other silylation reagents.

The technique presented here has the advantage over existing methods[8,9] of being a quantitative, highly sensitive and rapid technique for the simultaneous determination of cyanuric acid, ammelide, ammeline and melamine.

EXPERIMENTAL

Apparatus

Optimal gas chromatographic conditions were determined by using a Varian Model 1400 gas chromatograph equipped with a flame-ionization detector. Combined gas chromatographic–mass spectrometric (GC–MS) analyses were performed with a Finnigan Model 9500 gas chromatograph coupled to a Finnigan Model 3100 mass spectrometer through an all-glass jet separator. Both gas chromatographs were equipped with constant-flow controllers in the carrier gas lines. Peak areas and retention times were determined by using a Hewlett-Packard Model 3370 B integrator.

Reagents

MSTFA was purchased from Pierce (Rockford, Ill., U.S.A.), hexamethyldisilazane (HMDS) from Aldrich Europe (Beerse, Belgium) and trifluoroacetic acid (TFA), melamine (Mn), ammeline (An) and cyanuric acid (CA) from Fluka (Buchs, Switzerland). Ammelide (Ad) was synthesized via a literature method[11] and was shown to be homogeneous by mass spectrometry. All commercially obtained chemicals were of the highest purity available.

The systematic names of the triazines used in this study are 2,4,6-triamino-, 2-hydroxy-4,6-diamino-, 2,4-dihydroxy-6-amino- and 2,4,6-trihydroxy-*s*-triazine for melamine, ammeline, ammelide and cyanuric acid, respectively; their structures are shown in Fig. 1.

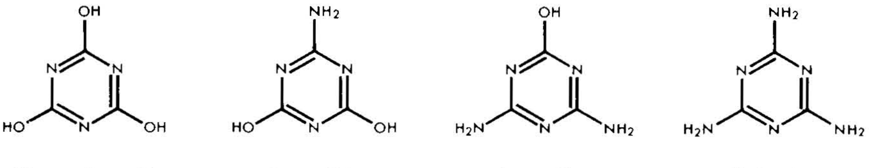


Fig. 1. Structures of the four *s*-triazines.

Chromatographic conditions

For both gas chromatographs 1.5 m × 3 mm I.D. glass columns were used, packed with 3% OV-17 on Chromosorb G AW (100–120 mesh). Helium was used as the carrier gas at a flow-rate of 40 ml/min. The injection block temperatures were 220° for both instruments; the detector temperature on the Varian gas chromatograph (hydrogen, 32 ml/min; air, 350 ml/min) and the jet separator oven temperature on the Finnigan instrument were both maintained at 280°.

Conditioning of both columns was performed by programmed heating (0.5°/min) to 270° while four 5-μl volumes of HMDS at 15-min intervals were injected in the temperature range 165–195°. Heating was continued at 270° for 10 days.

Sample preparation

A stock solution of the triazines was prepared by dissolving 5 mg of each triazine in 10 ml of TFA. This stock solution was stored at 4° and was prepared freshly twice a week.

Derivatization was achieved in the following manner. A 1-μl aliquot of the stock solution was transferred to the bottom of a capillary tube (3 cm × 2 mm I.D.), MSTFA (9 μl) was added and the capillary was immediately sealed with a flame. The contents were then thoroughly mixed and the capillary was immersed in an oil-bath kept at 150 ± 1°.

Routinely, 0.5 μl of the resulting clear solution was injected into the gas chromatograph. Relative molar responses (RMR) were determined with respect to phenanthrene (Ph) as the internal standard.

RESULTS AND DISCUSSION

Gas chromatographic conditions

Optimal separations were obtained by using temperature programming at 4°/min, with an initial column oven temperature of 140°. Under these conditions the triazine derivatives were eluted between 140° and 190°.

Stability of triazines

In order to determine accurately the amount of triazine to be silylated, a solvent was needed that would dissolve the triazines and from which aliquots could be taken for derivatization. TFA has been reported[12] to be a suitable solvent for silylation, especially for polar compounds, and it was tested for its properties with respect to the four triazines used in this work.

Separate stock solutions of each triazine were prepared with phenanthrene as the internal standard (5 mg of each in 10 ml of TFA) and aliquots were derivatized after storage for 0, 3, 4 and 5 days at 4°.

Gas chromatographic analysis of the silylated derivatives showed the triazines to be stable towards acid hydrolysis for 3 days. RMRs calculated for successive analyses after 3 and 4 days did not change significantly in comparison with the initial values. However, a slight hydrolysis resulted in a trace of ammelide appearing on the gas chromatogram of ammeline after 4 days (Fig. 2). Therefore, stock solutions were prepared freshly twice a week.

Silylation of triazines

For the determination of optimal derivatization conditions, the influence of reaction time at a specific temperature on the RMR was studied.

Initial investigation revealed that heating was necessary, because without heating the responses were low and not reproducible. The responses of the various triazines as a function of time at two different temperatures are presented in Table I.

The results demonstrate that, although at both temperatures prolonged heating results in a decrease in RMR, which is most prominent for melamine at 150°, the overall responses are higher on heating at 150°, especially for short periods of time. Therefore, heating for 5 min at 150° was selected for derivatization of the triazine samples. A typical gas chromatogram of the silylated triazines is depicted in Fig. 3.

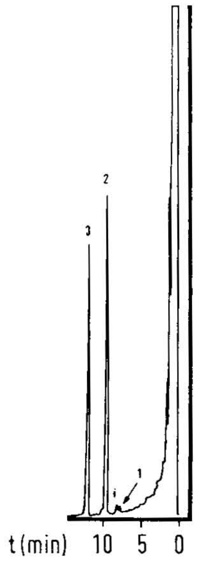


Fig. 2. Gas chromatogram of ammeline, silylated after storage for 4 days at 4°. $8 \cdot 10^{-11}$ A.f.s.d.: 1 μl injected (50 ng); 140–200° at 4°/min. Peaks: 1 = Ad (172°); 2 = An (179°); 3 = Ph (192°); i = impurity.

TABLE I

EFFECT OF REACTION TIME AND TEMPERATURE ON RMR

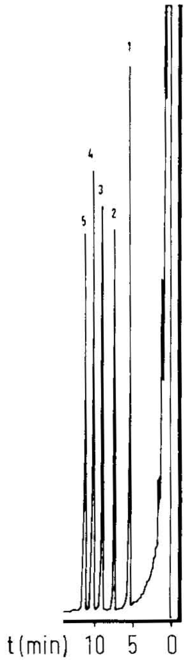
Compound	*RMR at 100°*				*RMR at 150°*			
	5 min	*15 min*	*30 min*	*60 min*	*5 min*	*15 min*	*30 min*	*60 min*
Mn	0.94	0.87	0.79	0.77	1.04	0.75	0.66	0.41
An	0.85	0.80	0.77	0.72	0.88	0.85	0.82	0.79
Ad	0.78	0.77	0.74	0.70	0.84	0.80	0.79	0.72
CA	1.17	1.17	1.13	1.01	1.25	1.20	1.20	1.18

Replacement of one, two or all hydroxyl groups on the triazine nucleus with amino groups (from cyanuric acid through ammelide and ammeline to melamine) results in an increased retention time, which can be explained by interaction of the remaining amino hydrogen atoms with the moderately polar stationary phase OV-17. That the amino groups are indeed monosilylated, leaving one hydrogen atom available for interaction, was confirmed by combined GC–MS analysis; the 70-eV mass spectra of the TMS derivatives of the four triazines showed intense peaks at *m/e* 345, 344, 343 and 342, corresponding to the molecular ions of O,O′,O″-tris(trimethylsilyl)-cyanuric acid, N,O,O′-tris(trimethylsilyl)ammelide, N,N′,O-tris(trimethylsilyl)ammeline and N,N′,N″-tris(trimethylsilyl)melamine, respectively. No evidence was found for N,N-bis(trimethylsilyl) derivatization. A similar observation has been made[13] with the pyrimidines uracil and cytosine and the purines hypoxanthine, adenine, xanthine and guanine.

Minimum detectable amount of triazines

Several dilutions of the triazine stock solution were made and 1-μl aliquots of these solutions were silylated as described above.

At an instrumental setting of $2 \cdot 10^{-11}$ A/mV, 2.5 ng of triazine injected appeared to be the minimum detectable amount for which RMR values could still be determined reliably (Fig. 4). For qualitative analyses the minimum detectable amount at the same instrumental setting was 0.5–0.7 ng at a signal-to-noise ratio of 5, with a 0.5-μl injection.



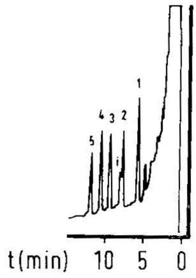


Fig. 3. Gas chromatogram of silylated triazines. $4 \cdot 10^{-11}$ A.f.s.d.; 0.5 μl injected; 140–200° at 4°/min; each peak represents 25 ng. Peaks: 1 = CA (164°); 2 = Ad (172°); 3 = An (179°); 4 = Mn (184°); 5 = Ph (192°).

Fig. 4. Gas chromatogram of silylated triazines. $2 \cdot 10^{-11}$ A.f.s.d.; 0.5 μl injected; each peak represents 2.5 ng. Peaks as in Fig. 3.

Impurities in the silylation reagent

In initial studies the MSTFA was found to contain impurities that were eluted in the temperature range 190–210°. A final column oven temperature of 220° therefore had to be used with this MSTFA. In the course of the investigation another sample of MSTFA became available (purchased from Macherey, Nagel & Co., Düren, G.F.R.) that did not contain these impurities. However, once the protective Teflon seal had been penetrated, the MSTFA attacked the septum material, resulting in an impurity peak on the gas chromatograms eluting at 173° (Figs. 2 and 4).

Effect of triazine concentration on the RMR

The capillaries used for the silylation of nanogram amounts of triazine could not be used for amounts larger than 1 μg, owing to the limited solubility of the triazines in TFA. Therefore, 10-μl aliquots of triazine solutions were silylated with 90 μl of MSTFA in sealed 1-ml glass ampoules. The silylation conditions were the same as described for the capillaries. The RMR was found to remain constant over the sample range 25 ng–10 μg.

Stability of the silylated triazines

Although many trimethylsilylated compounds are thermally stable[10], the decrease in RMR on prolonged heating even at 100° indicated that the TMS derivatives of the triazines used in this work are not thermally stable. Therefore, prolonged heating should be avoided. After heating at 150° for 5 min the sealed capillaries could be stored at 4° for 1 week without significant changes in the RMR. However, once a capillary had been opened a 10–20% decrease in RMR in less than 15 min was observed for samples containing $\leq$ 10 ng of triazine. The susceptibility towards hydrolysis by atmospheric humidity must therefore be greater for the TMS triazines than for MSTFA itself.

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CHROM. 11,393

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PIRIMIPHOS METHYL AND FIVE METABOLITES

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SUMMARY

Pirimiphos methyl is an organophosphorus insecticide which is rapidly metabolised by plants and animals to several modified triesters and free hydroxypyrimidines. A method is described for the determination by reversed-phase high-performance liquid chromatography of pirimiphos methyl and its five major metabolites in plasma and urine. Separations were performed by isocratic and gradient elutions from short columns packed with SAS-Hypersil, a relatively new column packing.

INTRODUCTION

Pirimiphos methyl (N,N-diethylamino-6-methylpyrimidin-4-yl dimethylphosphorothionate, Actellic®, PP 511) is an organophosphorus insecticide used in agriculture and public health, and for the control of pests of stored products. The compound (I in Table I) is extensively metabolised in rats and dogs to other phosphorus-containing triesters, N,N-diethyl-6-methylpyrimidin-4-yl dimethylphosphate (II in Table I), and N-ethylamino-6-methylpyrimidin-4-yl dimethylphosphorothionate (III in Table I). The major excretory products are the parent and modified hydroxypyrimidines: the diethylamino-hydroxypyrimidine (N,N-diethylamino-4-hydroxy-6-methylpyrimidine) (IV in Table I) and the corresponding mono- and di-desethylated hydroxypyrimidines (V and VI, respectively, in Table I)[1].

Gas–liquid chromatography (GLC) determinations of pirimiphos methyl and its phosphorus-containing metabolites have been reported[1,2] and a high-performance liquid chromatography (HPLC) method for the estimation of pirimiphos methyl in formulations has been described[3]. However, no method has been reported for the quantitative estimation of the polar hydroxypyrimidines (IV, V and VI). Assay of these compounds in animal tissues and plant materials has been attempted in this laboratory by GLC of derivatives[4]. This method necessitates extraction of the material with methanol, removal of phosphorus-containing compounds from the extract by

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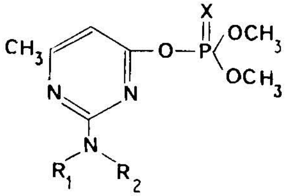
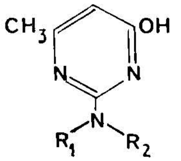
TABLE I

STRUCTURE, ABSORBANCE MAXIMA AND LIMIT OF DETECTION OF PIRIMIPHOS METHYL (I) AND FIVE METABOLITES

The limits of detection were determined for gradient elution of a 25-μl sample of a mixture of compounds I–VI at 235 nm and 0.01 a.u.f.s. (concentrations and conditions as in Fig. 3).

Compound	*Structure*	$\lambda_{max.}$ *(nm)*	*Limit of detection (ng)*
			
I	R_1 and R_2 = C_2H_5, X = S	247	10
II	R_1 and R_2 = C_2H_5, X = O	242	15
III	R_1 = C_2H_5, R_2 = H, X = S	234	20
			
IV	R_1 and R_2 = C_2H_5	227	1–2
V	R_1 = C_2H_5, R_2 = H	215	1–2
VI	R_1 and R_2 = H	206	1–2

diethyl ether extraction at low pH followed by extraction of the hydroxypyrimidines at elevated pH and derivatization with either diazomethane, diazoethane or heptafluorobutyrylimidazole. A complex misture of derivatives may result from this procedure and recoveries are below 60%.

Reversed-phase chromatography has been demonstrated as a very powerful method for the separation of complex mixtures of polar compounds in body fluids[5] by hydrophobic interactions with the apolar bonded phase[6]. As an alternative to the octadecylsilane-bonded silica often used in reversed-phase separations the method described here here uses a bonded-phase packing of mixed chain side-groups which allows polar interactions between solutes and the silica microparticles in addition to the hydrophobic interactions characteristic of reversed phases.

A simple method is described for quantitative estimation of the six compounds listed above by reversed-phase HPLC with gradient elution, and for less-complex mixtures with isocratic elution. An example of the measurement of compounds in the blood plasma of several species is described as is the determination of residues in human urine.

EXPERIMENTAL

Apparatus and chromatography

Chromatography was performed on a component system consisting of a Perkin-Elmer Model 601 high-performance liquid chromatograph and a Perkin-Elmer

Model LC-55 variable-wavelength UV detector (Perkin-Elmer, Norwalk, Conn., U.S.A.). The sample was introduced by on-column injection via a septum injector providing flow of solvent coaxial with the injection guide (HETP, Macclesfield, Great Britain), a modification which maximises the "infinite wall effect", of the smooth-bore stainless-steel column, significantly improving resolution[7]. Injections of a 10-μl volume were made for isocratic elutions, but this was increased to 25 μl (combined with reduction in recorder sensitivity from 0.04 to 0.1 a.u.f.s.) for gradient elutions to minimise baseline drift with increasing methanol concentration. 10-μl and 25-μl microsyringes were used for all injections (Hamilton, Bonaduz, Switzerland).

Elutions were performed isocratically with different mixtures of methanol and phosphate buffer (0.1 *M*, pH 4.5) for elution of simple mixtures and plasma samples. Complex mixtures were eluted with gradients formed from 20% methanol–buffer (v/v) and 55% methanol–buffer (v/v) so that the starting and finishing mixtures would be 20 and 55% methanol, respectively, irrespective of other gradient characteristics. For gradient elution, peak shape was improved by the inclusion of *ca.* 0.1% perchloric acid in both reservoirs (pH readjusted to 4.5). In all experiments a flow-rate of 1.5 ml/min at a pressure of *ca.* 800 p.s.i. was used and the column was maintained at room temperature (22°) throughout.

Columns

Short, smooth-bore stainless-steel columns (10 cm × 4.6 mm I.D.) with zero-dead-volume end-fittings (HETP) were slurry-packed with SAS-Hypersil, a mixed short-alkyl-chain bonded reversed-phase microparticulate silica (5 μm) (Shandon Southern Products, Runcorn, Great Britain). A sonicated mixture of 1.7 g SAS-Hypersil in 10 ml methanol–0.1% (w/v) aqueous sodium acetate trihydrate (80%, v/v) was packed into the column by pumping approx 200 ml of methanol–0.1% (w/v) aqueous sodium acetate trihydrate (50%, v/v) through the packing reservoir (HETP) at 5,000 p.s.i. using a pneumatic amplification pump (HS Chromatography, Bourne End, Great Britain). The top of the column was protected by a stainless-steel gauze and plug of quartz wool arranged so that the injected sample was delivered immediately above the centre of the column packing.

Samples

Standard solutions of pirimiphos methyl and related compounds were prepared at a 100 μg/ml (0.01%, w/v) in 50% (v/v) methanol–phosphate buffer (0.1 *M*, pH 4.5) from analytical standards (ICI Plant Protection Division, Jealott's Hill, Bracknell, Great Britain). Phosphorus-containing compounds were checked for hydrolysis products by thin-layer chromatography [Merck K_{428} precoated plates; diethyl ether–hexane–ethanol (60:30:10) as solvent].

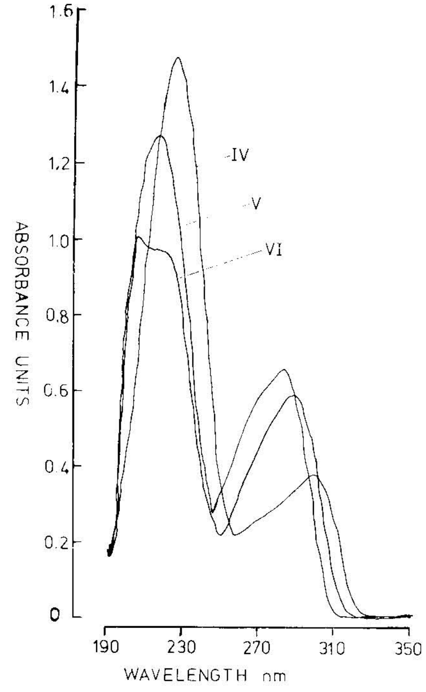
UV absorption spectra of stock solutions of compounds I–VI diluted 1:6 in 50% (v/v) methanol–phosphate buffer (0.1 *M*, pH 4.5) were measured on a Pye SP1800 scanning spectrophotometer between 190 and 350 nm.

Blood plasma samples from individuals of several species containing residues of compounds I, II and IV were diluted 5 times in phosphate buffer (0.1 *M*, pH 7.0) and 0.2-ml samples added to 0.1 ml ice-cold methanol, shaken and centrifuged at 10,000 *g* for 5 min in a Burkard Koolspin bench centrifuge to remove precipitated protein. Samples of 10 μl of this mixture were injected directly on to the column.

Samples of 100 ml of human urine were shell-frozen in 250-ml round-bottom flasks, freeze-dried and extracted with 80 ml of methanol. The methanol extracts were filtered (Whatman No. 1 filter paper) and the filtrate made up to 100 ml with washings. Samples of 10 μl of these extracts were injected directly on to the column and eluted with a solution of 10% methanol–0.1 *M* phosphate buffer (pH 4.5) (v/v) at *ca.* 800 p.s.i.

RESULTS AND DISCUSSION

Previous experience in the authors' laboratories had shown that the quantitative estimation by GLC of the three hydroxypyrimidine degradation products of pirimiphos methyl (IV, V and VI) was an unreliable and time-consuming method since an elaborate clean-up procedure was required before derivatization and even so a complex mixture of derivatives results in methylation or ethylation of the free hydroxypyrimidines. HPLC on reversed phase offered a solution to this problem with the added possibility that by selection of an appropriate reversed phase all the metabolites from the most polar aminohydroxypyrimidine to the least polar fully alkylated parent triester could be resolved in one mixture. UV absorption spectra of the six compounds were measured; the spectra of compound I, II and III are shown in Fig. 1 and those of compounds IV, V and VI in Fig. 2. The wavelength of maximum



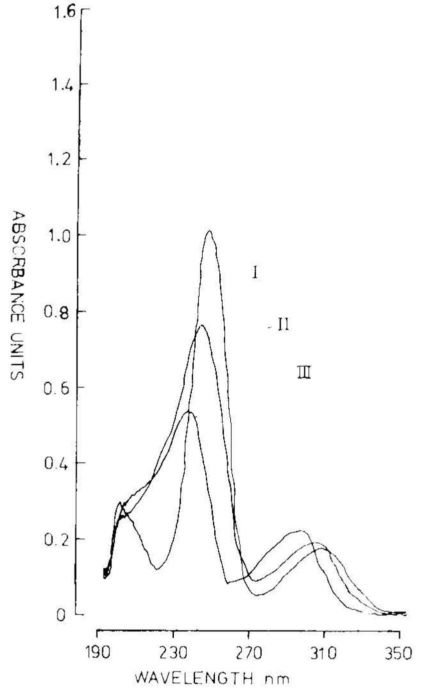


Fig. 1. UV absorption spectra of free hydroxypyrimidines (compounds IV–VI). Concentrations: 14.3 μg/ml in *ca.* 7% (v/v) methanol–0.1 *M* phosphate buffer (pH 4.5); temperature, 22°. Pye-Unicam SP1800; 10 sec/cm; 2 nm/sec; 2.0 a.u.f.s.

Fig. 2. UV spectra of phosphorus triesters (compounds I–III). Concentrations and conditions as in Fig. 1.

absorbance of the parent hydroxypyrimidine is changed by the modifying groups from 207 nm for VI to 248 nm for I: 235 nm was therefore chosen as the best compromise for single-wavelength detection. However the sensitivity towards any particular compound could be significantly increased by measuring the UV absorbance at its own maximum, *e.g.* in the case of compound IV at 227 nm was more sensitive by a factor of 6.3 compared to determination at 254 nm.

Gradient elution

Preliminary chromatography of mixtures of compounds I–VI indicated that isocratic elution was incapable of resolving all six compounds in a reasonable time or of resolving the most polar hydroxypyrimidines and yet preserving good peak shape for pirimiphos methyl. Fig. 3 shows a typical chromatogram for the elution under the best conditions obtained of a 25-μl injection of a mixture of the six compounds. The gradient was run at maximum convexity (1000 arbitrary units) and reached 100% with respect to 55% methanol–phosphate buffer in 16 min. The small, unlabelled peaks observed in gradient-elution chromatograms are considered to be as yet unidentified trace impurities in the standards of compounds II and III. These previously undetected compounds testify to the power of separation of this method.

Isocratic elution

As demonstrated above it was possible to resolve compounds of widely differing polarities as well as closely related pairs of compounds by gradient elution on a reversed-phase system. However in order to enable rapid assay of samples without the necessity of reequilibrating the column to the initial solvent composition between samples the isocratic elution of mixtures was examined. Fig. 4 shows a chromatogram for the elution of compounds IV, V and VI isocratically with a 20% methanol–buffer mixture, and Fig. 5 shows a chromatogram for the elution of the complete mixture with a 55% methanol–buffer mixture. The elution time of pirimiphos methyl was reduced to 16 min at this methanol concentration and the five metabolites were eluted within 7 min, these values being comparable with the time taken for analysis by GLC.

A standard curve of concentration against peak height obtained by isocratic elution at 20% methanol–phosphate buffer of 10-μl samples of dilutions of the parent hydroxypyrimidine stock solution was linear over the range 10–100 ppm. A 10-μl volume of a solution containing 73 ng/μl produced a peak of 50% fullscale deflection (0.1 a.u.f.s.). The limit of detection for this compound was therefore approx. 1 ng (= 0.1 ppm for a 10-μl injection) (see Table I).

Determination of metabolites in plasma samples

Diluted plasma samples (5 times in phosphate buffer) from several species including rat, rabbit, sheep, hen, quail and man were obtained containing residues of pirimiphos methyl and pirimiphos methyl oxon (II) (with which they had been spiked) and also varying levels of compound IV resulting from hydrolysis of II in the plasma. It was possible to determine quantitatively the levels of these compounds in the plasma by injection of a 10-μl sample of the plasma diluted 2:1 in methanol. A typical chromatogram for the determination of compounds II and IV in rat plasma is shown in Fig. 6.

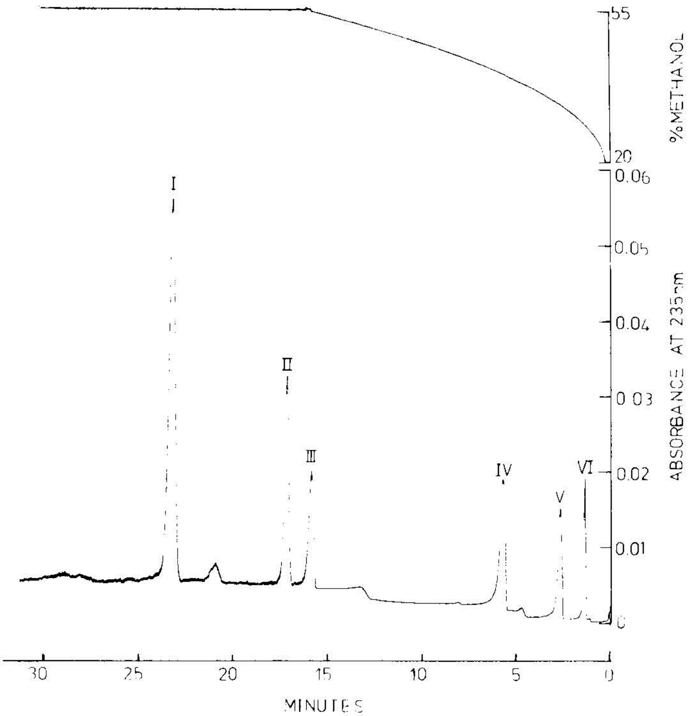


Fig. 3. Chromatography of a mixture of compounds I–VI. Column: 5 μm SAS-Hypersil, 10 cm × 4.6 mm I.D.; flow-rate 1.5 ml/min; temperature, 22°. Gradient elution from 0 to 100%, 55% (v/v) methanol–0.1 M phosphate buffer in 16 min at max. convexity. Standard mixture (25 μl) containing: 1.05 μg I; 0.65 μg II; 0.575 μg III; 0.175 μg each of IV, V and VI. Wavelength, 235 nm, sensitivity, 0.1 a.u.f.s.

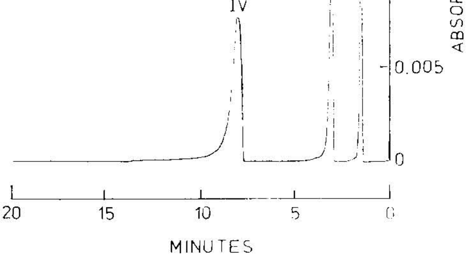
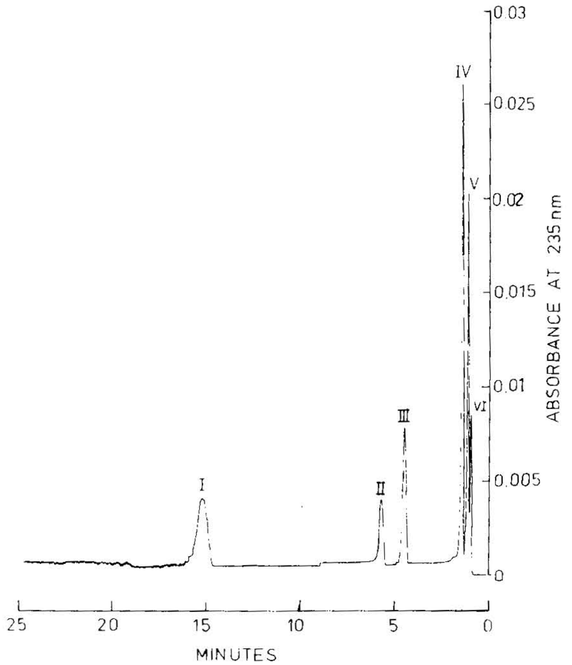


Fig. 4. Chromatography of free hydroxypyrimidines. Column, temperature and flow-rate as in Fig. 3. Isocratic elution at 20% (v/v) methanol–0.1 M phosphate buffer (pH 4.5). A 10-μl mixture containing: 0.33 μg each of IV, V and VI. Wavelength, 235 nm; sensitivity, 0.04 a.u.f.s.



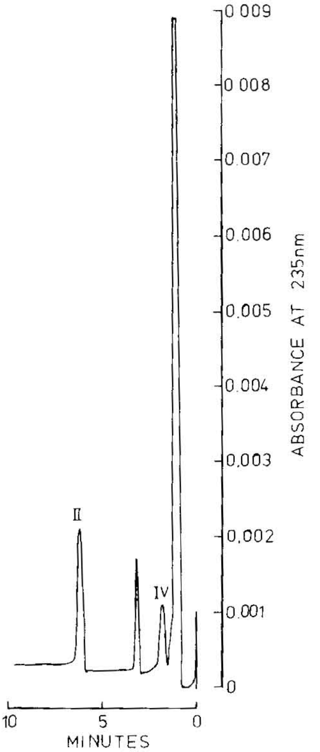


Fig. 5. Chromatography of compounds I–III by isocratic elution. Column, temperature and flow-rate as in Fig. 3. Isocratic elution at 55% (v/v) methanol–0.1 *M* phosphate buffer (pH 4.5). A 10-μl mixture containing: 0.42 μg I; 0.26 μg II; 0.34 μg III; 0.07 μg eazch of IV, V and VI. Wavelength, 235 nm; sensitivity, 0.04 a.u.f.s.

Fig. 6. Chromatography of rat plasma. Column, temperature and flow-rate as in Fig. 3. Isocratic elution at 50% (v/v) methanol–0.1 *M* phosphate buffer (pH 4.5). Sample: 10 μl diluted rat plasma (see text) containing compounds II and IV. Wavelength, 235 nm; sensitivity, 0.01 a.u.f.s. The unlabelled peaks correspond to compounds present in control plasma.

Repeated injections of samples containing large amounts of protein eventually caused deterioration of column performance evidenced by poor peak shape, especially at larger retention times. Excess protein may be removed from samples in methanol by cooling to −4° and centrifugation of the precipitated protein but even without this precaution the column retains good performance for injection of over 200 10-μl samples. Where determination of hydroxypyrimidines only was required this was less of a problem since the column retained good performance for the resolution of these compounds well in excess of 200 samples. Columns which have deteriorated may be partially restored by repacking the initial 1 or 2 cm of the column with fresh packing.

Determination of metabolites in human urine

Determination by HPLC on SAS-Hypersil of metabolite V in human urine was attempted in order to assess the usefulness of this technique in measuring the rate of excretion of pirimiphos methyl metabolites in cases of potential accidental poisoning with the technical insecticide. Samples of urine spiked at different concentrations with pirimiphos methyl were extracted and chromatographed as described above: chromatograms for urine samples obtained from two different individuals are shown in Fig. 7. At a concentration of 10% methanol–buffer (v/v) in the eluting solvent the peak corresponding to metabolite V is well resolved from peaks detected in the control urine sample. Furthermore the method appears to offer a limit of detection of *ca.* 2 ppm in the extract (here equivalent to the same concentration in the urine) as seen in the chromatograms where the peak corresponding to metabolite V represents 36.6 and 2.3 ppm in the extracts of urine from individuals X and Y, respectively. It is noteworthy that interfering substances in the urine varied considerably between individuals and time of collection, more dilute urine presenting less interfering peaks. The analysis of more concentrated urine samples would benefit from an extended clean-up procedure.

HPLC on reversed phase thus appears to offer a rapid and reliable method for the estimation of pirimiphos methyl and its metabolites. The method is especially useful for the determination of hydroxypyrimidine residues in solution in plasma and

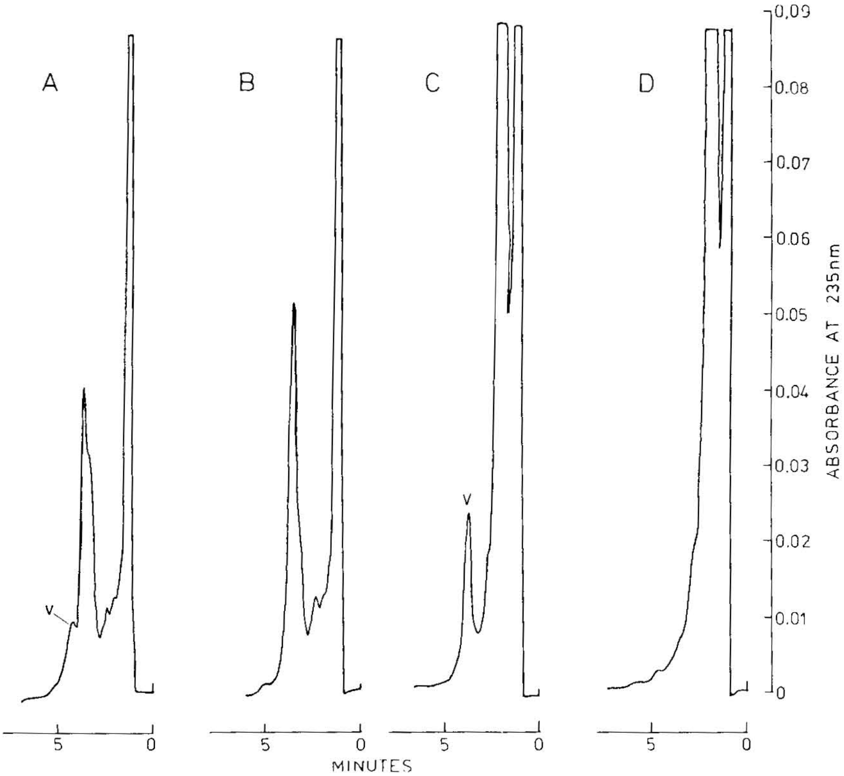


Fig. 7. Chromatography of human urine spiked with compound V. Column, temperature and flow-rate as in Fig. 3. Isocratic elution at 10% (v/v) methanol–0.1 *M* phosphate buffer (pH 4.5). Wavelength, 235 nm; sensitivity, 0.1 a.u.f.s. (A) A 10-μl urine extract from individual X containing compound V at 2.3 ppm. (C) A 10-μl urine extract from individual Y containing compound V at 36.6 ppm. (B) and (D) 10-μl control urine samples from individuals X and Y, respectively.

urine but similar residues in solid plant and animal tissues are easily extractable in methanol[1] and should prove straightforward to assay by direct injection of methanol extracts.

ACKNOWLEDGEMENTS

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CHROM. 11,333

ANALYSIS OF THE ANTI-COCCIDIAL DRUG, HALOFUGINONE, IN CHICKEN FEED USING GAS–LIQUID CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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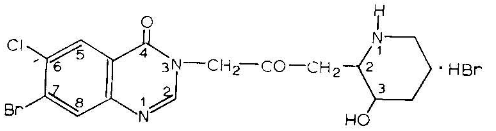
SUMMARY

Methods are described for the analysis of the anti-coccidial drug, halofuginone, at concentrations of 3 ppm in chicken feed, using gas–liquid chromatography and high-performance liquid chromatography. Both methods are based on ethyl acetate extraction, partition into hydrochloric acid and purification and concentration using XAD-2 column chromatography. The precision and accuracy of both methods is given.

INTRODUCTION

Halofuginone (*dl-trans*-7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidyl)acetonyl]-4(3H)-quinazolinone hydrobromide) (I) is an anti-coccidial drug proposed for incorporation in poultry feed to permit continuous administration to broiler chickens. For the purposes of registration, it became essential to develop a sensitive, and precise, method of analysis for halofuginone in chicken feed, at the inclusion concentration of 3 ppm, and in concentrate at 6000 ppm. This paper describes and compares a gas–liquid chromatographic (GLC) and a high-performance liquid chromatographic (HPLC) method of analysis for halofuginone in finished feed (3 ppm). Application of the HPLC procedure to the analysis of mineral supplement (300 ppm halofuginone) and concentrate (6000 ppm halofuginone) is also described.



(I)

EXPERIMENTAL

Apparatus

GLC. A Hewlett-Packard 5710A gas–liquid chromatograph, fitted with a ^{63}Ni electron-capture detector, was used. Glass columns (90 cm × 3 mm I.D.) were packed with 0.5% (w/w) FFAP on Diatomite CLQ (80–100 mesh; J.J.'s Chromatography, King's Lynn, Great Britain). The output from the detector was measured using a Honeywell Electronik 194 chart recorder.

HPLC. A Siemens S100 high-performance liquid chromatograph fitted with a Partisil^{TM} 10 ODS (250 mm × 4.6 mm I.D. × 6.35 mm O.D.) prepacked column (Whatman, Maidstone, Great Britain) was used. Injection was achieved by means of a Rheodyne syringe loading injection valve Model 7120 (Rheodyne, Berkeley, Calif., U.S.A.) fitted with a 100-μl loop. The eluate from the column was monitored by means of a Zeiss PM2DLC variable wavelength UV detector fitted with an 8-μl flow cuvette, path length 10 mm. Output from the detector was measured on a Siemens Kompensograph III chart recorder.

General. The UV spectra of halofuginone were recorded on a Unicam SP1800 double beam spectrophotometer (Pye Unicam, Cambridge, Great Britain) using 10-mm path length quartz cells. Sample maceration was achieved using Ultra-Turrax, Model TP18/2N homogenisers. Glass columns, 30 cm × 1 cm I.D. with sintered filters, were used for macroreticular resin separations. A Beckman Model 3500 digital meter with combination electrodes was used for pH adjustments. Centrifugation was accomplished using an MSE "super medium" centrifuge capable of accepting 200-ml glass centrifuge bottles. Bucchi Rotavapor R rotary film evaporators (RFE; Orme Scientific, Middleton, Great Britain), were used for all evaporations.

Materials

Analytical grade halofuginone (micronised) was obtained from Roussel Uclaf (Paris, France). The macroreticular resin, Amberlite XAD-2 standard grade (BDH Chemicals, Poole, Great Britain), was freed from chloride ions, by water washing, and purified by Soxhlet extraction with methanol for 8 h. The purified resin was stored, under methanol, in stoppered glass bottles. Immediately prior to use, a slurry of the purified resin (10 g), in methanol, was transferred to a glass chromatography column and, after draining of the methanol, washed thoroughly with distilled water.

Acetate buffer was prepared by adding glacial acetic acid (30 ml/l) to ammonium acetate and dilution to produce a 0.25 *M* solution. All other chemicals used were of analytical-reagent grade. Unmedicated (control) poultry diet, "Chickmash", was obtained from Flowers and Sons (Ramsey, Great Britain). Mineral supplement was obtained from Coopers (Witham, Great Britain). Halofuginone concentrate (6000 ppm halofuginone nominal, in Blanc OMYA) was supplied by Roussel Uclaf.

Chromatography

GLC. The GLC column oven was operated isothermally at 235° with the injection port and detector at 250 and 300°, respectively. The carrier gas, argon–methane (95:5), flow-rate was 60 ml/min. Sample injection volume was 5 μl. Strip chart recordings were made at a chart speed of 12 in./h using a detector attenuation of 64. Under these conditions the retention time of halofuginone was approximately 8 min.

HPLC. The HPLC mobile phase consisted of methanol–acetate buffer (70:30, v/v). Sample injection volume was 100 μl. Chromatography was carried out, at ambient temperature, using a solvent flow-rate of 1 ml/min. The eluate from the column was monitored at a wavelength of 243 nm. Strip chart recordings were made at a chart speed of 120 mm/h using a detector attenuation of 0.04 a.u.f.s. Under these conditions the retention time of halofuginone was approximately 7 min.

Procedures

Finished feeds (approximately 3 ppm halofuginone). A representative sample of feed (10 g) was macerated with sodium carbonate solution (10%, w/v; 10 ml) and ethyl acetate (100 ml). The phases were separated by centrifugation and the ethyl acetate phase decanted. The residue was re-extracted with ethyl acetate (100 ml). The combined ethyl acetate extracts were washed with sodium carbonate solution (5%, w/v; 50 ml), previously saturated with sodium chloride. The ethyl acetate phase was separated, extracted with 0.1 *M* hydrochloric acid (2 × 50 ml) and the combined hydrochloric acid extracts backwashed with ethyl acetate (10 ml). Any remaining ethyl acetate was removed from the acid solution (RFE at 38°). The acid solution was passed through an Amberlite XAD-2 macroreticular resin column (prepared as under *Materials*) and the column washed with 0.1 *M* hydrochloric acid (20 ml). Any residual acid solution was removed from the column using low-pressure compressed air. The column was equilibrated with methanol for 10 min and eluted with methanol (100 ml). The solvent was evaporated (RFE at 38°) to residual moisture, acetonitrile (10 ml) added, and the evaporation continued to dryness. The resultant residue was dissolved in either methanol (25 ml) for HPLC, or acetonitrile (25 ml) for GLC (halofuginone concentration typically 1.2 μg/ml).

Mineral supplements (approximately 300 ppm halofuginone). A representative sample (10 g) of supplement was macerated with methanol (200 ml) for 15 min. The solution was filtered and the residue washed with methanol. The filtrate and washings were collected in a volumetric flask (250 ml) and diluted to volume with methanol. For HPLC analysis, this solution was diluted ten-fold using methanol (halofuginone concentration typically 1.2 μg/ml).

Concentrates (approximately 6000 ppm halofuginone). A representative sample (1 g) of concentrate was dissolved in acetate buffer (approximately 100 ml). When effervescence had ceased, the solution was filtered and the filtrate collected in a volumetric flask (250 ml). The residue was washed with acetate buffer, the washings added to the volumetric flask and the volume adjusted to 250 ml with acetate buffer. For HPLC analysis, the solution was diluted ten or twenty fold with methanol (halofuginone concentration, typically 1.2 to 2.4 μg/ml).

Standard solutions

GLC. Halofuginone (50 mg) was dissolved in acetate buffer (250 ml) and an aliquot further diluted with distilled water to provide a concentration of halofuginone of 10 μg/ml.

To produce standard solutions, suitable for injection on to the GLC, portions of the 10 μl/ml standard (3 ml) were added to 10% sodium carbonate solution (10 ml) and processed as described under *Finished feeds (approximately 3 ppm halofuginone).* The final acetonitrile extract was suitably diluted to obtain concentrations of halofuginone (as the hydrochloride) in the range 0–2 μg/ml. Extracts of some feeds were found to enhance the response of the GLC electron-capture detector to halofuginone. Under these circumstances, the final standard solutions were prepared in control diet extracts.

HPLC. Halofuginone (50 mg) was dissolved in acetate buffer (250 ml) and aliquots of the resulting solution diluted with methanol to provide concentrations of halofuginone in the range of 0–3 μg/ml.

Procedural recoveries

Aliquots (typically 1–3 ml) of a standard solution of halofuginone in acetate buffer were added to representative samples of control feed (10 g), feed supplement (10 g), or calcium carbonate* (1 g) to produce concentrations of 3 ppm, 300 ppm and 6.000 ppm, respectively. The resulting mixtures were allowed to remain at room temperature for 30 min before analysing as described under *Procedures.*

Calibration and calculation

Calibration curves of chromatographic peak height *versus* concentration of halofuginone were constructed using the data obtained by injection of aliquots of the standard solutions prepared as described under *Standard solutions.*

Concentrations of halofuginone in samples were determined by measurement of the chromatographic peak height at the characteristic retention time for halofuginone, and, after interpolation to the appropriate calibration curve, application of the necessary dilution factors.

RESULTS AND DISCUSSION

The GLC method of analysis was adapted from a procedure developed jointly in our laboratories and in the Laboratoire Municipal (Bordeaux, France)[1]. One of the major modifications was the use of macroreticular resin[2] which permitted further purification and concentration of the extracts without additional operations on the labile free base form of halofuginone.

Typical chromatograms, obtained when this modified method was applied to finished feed, are shown in Fig. 1, with a calibration curve illustrated graphically in Fig 2. The precision and accuracy of the method is indicated in Table I.

Although suitable for routine quality control, the GLC procedure has the disadvantage that occasional enhancement of detector response by feed extracts necessitates the availability of control feed. Moreover, a polar molecule of high molecular weight, such as halofuginone (495.6 as the hydrobromide), would not be expected to elute easily from a GLC column, even at an oven temperature of 235°, and there is evidence to suggest that the compound eluting is an on-column decomposition product of halofuginone. Because of the possibility that the same decomposition product could also be formed, as a result of storage of halofuginone in chicken feed, a stability-indicating HPLC method was developed.

Typical HPLC chromatograms are shown in Fig. 3, with a calibration curve illustrated in Fig. 4, and the precision and accuracy of the method indicated in Table

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* Simulating Blanc OMYA.

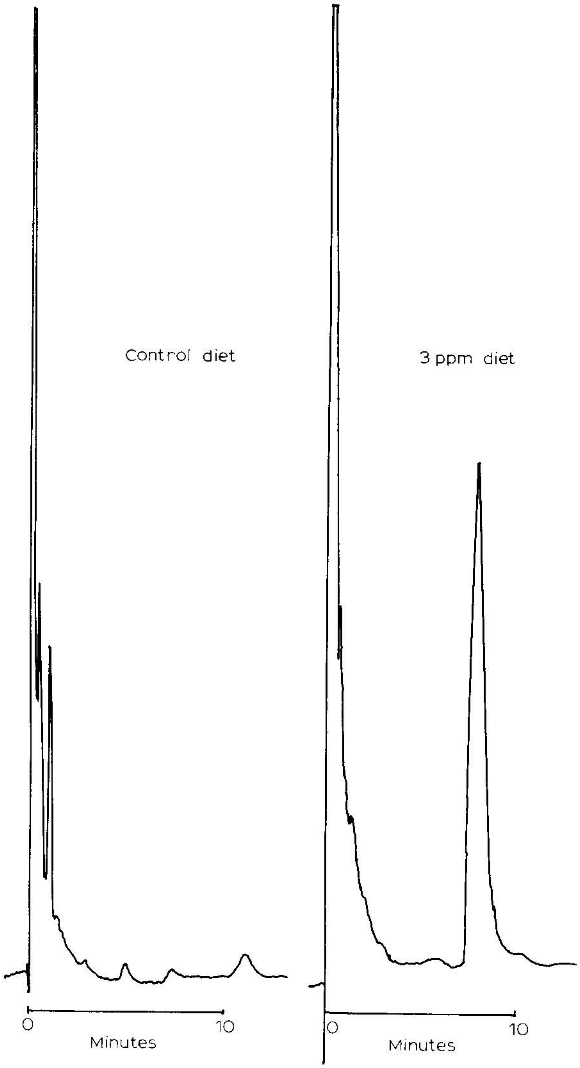


Fig. 1. Chromatograms obtained, using GLC, for extracts from control feed and feed containing halofuginone at 3 ppm.

TABLE I

PRECISION AND ACCURACY (FINISHED FEEDS)

The following procedural recovery data illustrate the precision and accuracy of the HPLC and GLC methods as applied to finished feed.

	HPLC	*GLC*
Concentration (nominal)	3 ppm	3 ppm
Number of determinations	19	15
Mean recovery	95.6%	98.7%
Standard deviation	±7.0%	±12.4%

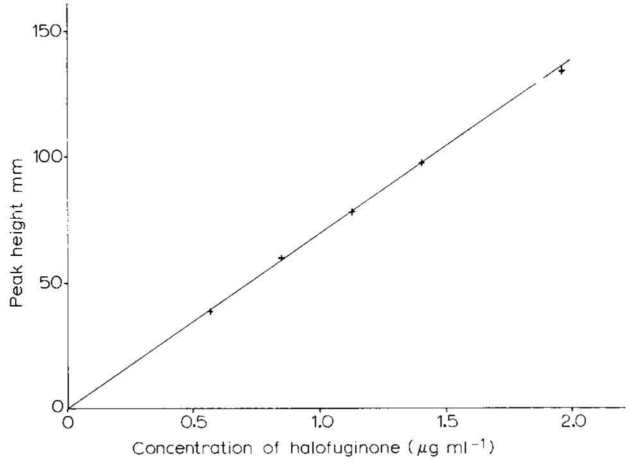
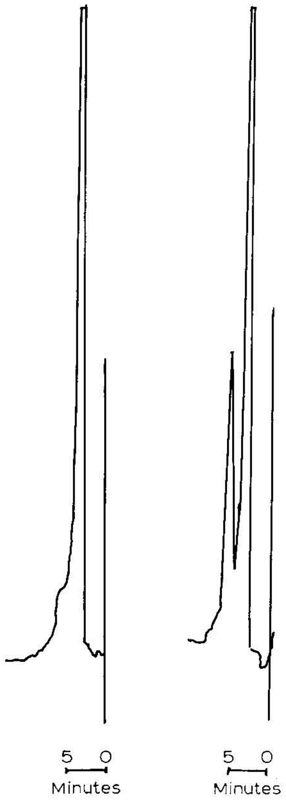


Fig. 2. Calibration curve obtained using GLC.



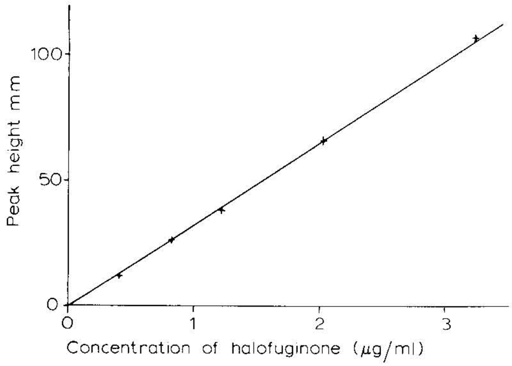


Fig. 3. Chromatograms obtained, using HPLC, for extracts from control feed and feed containing halofuginone at 3 ppm.

Fig. 4. Calibration curve obtained using HPLC.

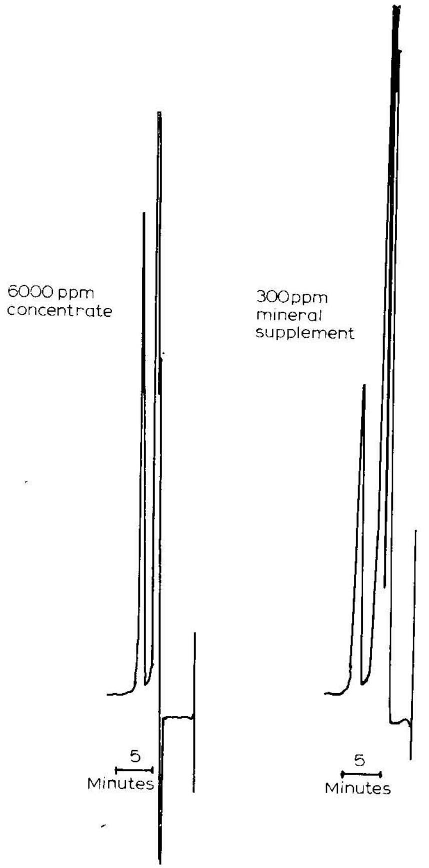
TABLE II

PRECISION AND ACCURACY (MINERAL SUPPLEMENT AND CONCENTRATE)

The following procedural recovery data illustrates the precision and accuracy of HPLC method as applied to mineral supplement and concentrate.

	Mineral supplement	*Concentrate*
Concentration	300 ppm	6.000 ppm
Number of determinations	10	8
Mean recovery	91.6%	95.0%
Standard deviation	±2.08%	±1.7%

I. The method was extended to mineral supplement and concentrate and the relevant chromatographic data are shown in Fig. 5, with precision and accuracy in Table II. The retention times of possible degradation products and the *cis* isomer of halofuginone are shown in Fig. 6. None of these components has the same retention time as



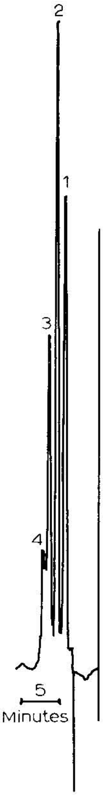


Fig. 5. Chromatograms obtained, using HPLC, for extracts from feed supplement and concentrate.

Fig. 6. Separation using HPLC, of possible degradation products of halofuginone. Peaks: 1 = 5-chloro-4-bromo-2-aminobenzoic acid + 2-hydroxy-6-chloro-7-bromo-quinazolinone; 2 = 6-chloro-7-bromo-quinazolinone; 3 = *trans*-halofuginone; 4 = *cis*-halofuginone.

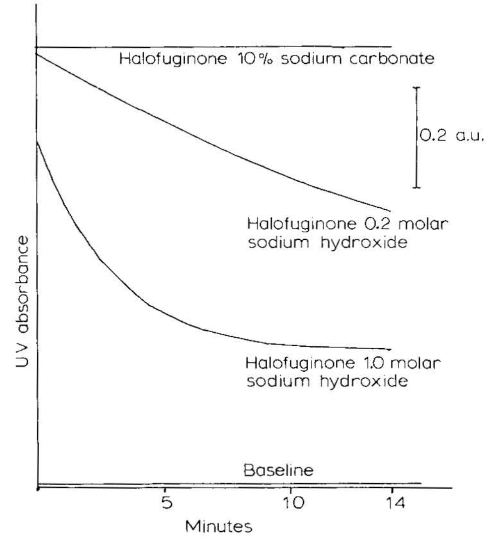


Fig. 7. Rate of degradation of halofuginone in alkaline solution.

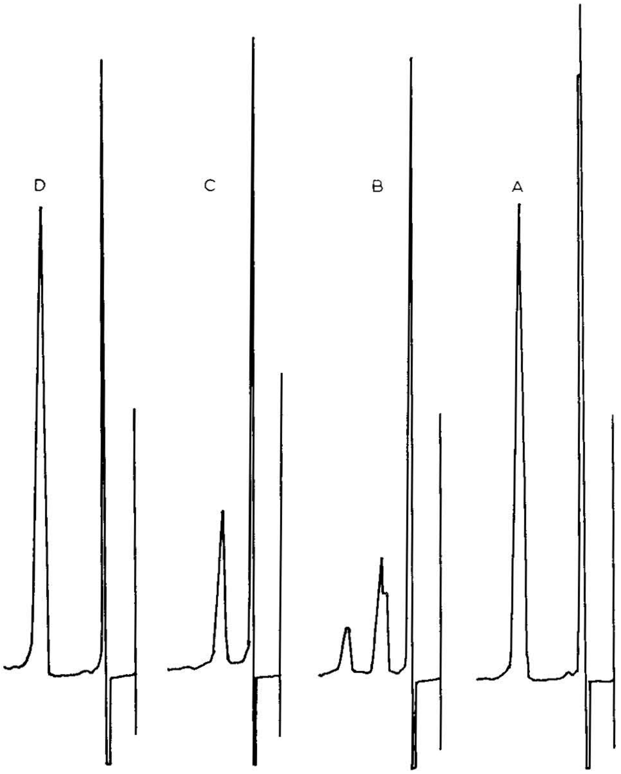


Fig. 8. Chromatograms obtained during the alkaline decomposition of halofuginone. Conditions: methanol–acetate buffer (50:50, v/v). A = 0 min; B = 10 min 1.0 *M* sodium hydroxide; C = 30 min 1.0 *M* sodium hydroxide; D = 25 min 10% (w/v) sodium carbonate.

halofuginone, although reduction of the methanol content of the mobile phase is necessary to achieve complete separation of cis and trans halofuginone. It was shown[3] that the major product of strong alkaline treatment is 5-chloro-4-bromo-2-aminobenzoic acid and Figs. 7 and 8 illustrate how rapid this decomposition is in 1.0 and 0.2 *M* sodium hydroxide. Since the extraction of halofuginone from feed is accomplished under mildly alkaline conditions the stability of the compound was investigated in sodium carbonate solution (0.94 *M*). No decomposition was evident ovei 5 h at laboratory temperature. During routine analysis, halofuginone remains in mild alkaline solution for no more than 15 min.

Both the GLC and HPLC methods of analysis have been used routinely in this laboratory to analyse samples of chicken feed containing halofuginone. The GLC method has proved to be less precise than the HPLC method and for this, and the other reasons detailed above, we have preferred to use HPLC. The HPLC method has

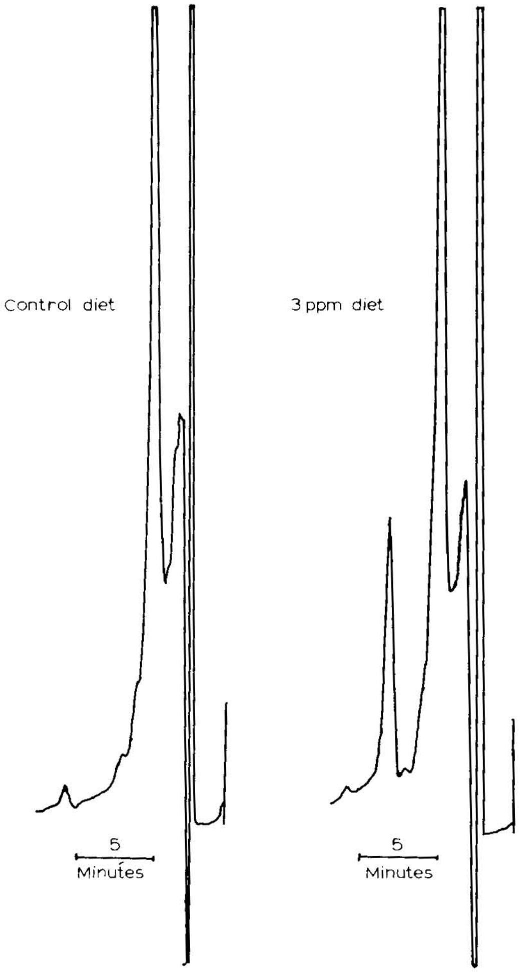


Fig. 9. Separation of halofuginone in feed extracts using a μBondapak C_{18} column. Conditions: methanol–0.125 *M* acetate buffer (60:40, v/v), 1.5 ml/min.

proved versatile and has been used to analyse halofuginone in a variety of chicken feeds. Diets from Argentina, South Africa, Bulgaria, Mexico, Denmark, France, Holland, Germany and Ireland have been analysed in this laboratory. The method has also proved sufficiently sensitive for the analysis of experimental rodent and dog diets containing halofuginone at a concentration of 0.4 ppm and 1.25 ppm, respectively.

The HPLC method has the additional advantage that, because of the excellent purification procedure, it is easily adapted to a variety of reversed-phase HPLC and ion-exchange columns. The separation of halofuginone in feed extracts has been demonstrated using LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.)[4], Partisil 10 SCX (Whatman) and μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.). This laboratory is now routinely analysing halofuginone using a μBondapak C_{18} column and Fig. 9 illustrates typical chromatograms obtained.

ACKNOWLEDGEMENT

The authors would like to thank Roussel Uclaf for sponsoring this work.

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FLUOROMETRIC DETERMINATION OF α-OXOMETHYLENE COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING N^1-METHYLNICOTINAMIDE CHLORIDE*

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(Received July 11th, 1978)

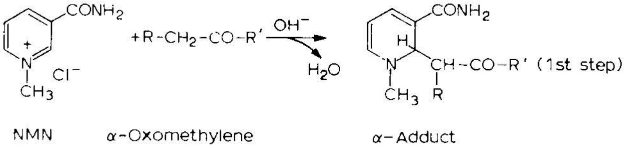
SUMMARY

Before or after the high-performance liquid chromatographic separation, α-oxomethylene compounds are subjected to reaction with N^1-methylnicotinamide chloride in the presence of alkali followed by heating with acid to produce fluorophores, permitting their determination at the picomole level.

INTRODUCTION

We have recently developed a fluorometric assay of α-oxomethylene compounds ($R{-}CH_2{-}CO{-}R'$) at the picomole level by using N^1-methylnicotinamide chloride (NMN)[1]. The reaction probably proceeds as shown below. In the first step, α-oxomethylene compounds react with NMN in the presence of alkali to form α-adducts of NMN. In the second step, the resulting α-adducts are converted by brief heating with excess of acid into fluorophores via non-fluorescent cyclic α-adducts. The fluorophores are stable to acids and alkalis, and therefore the fluorogenic reaction seems to be promising as a method for pre- or post-column derivatization of α-oxomethylene compounds in their high-performance liquid chromatography (HPLC).

In this investigation, the applicability of NMN was examined.



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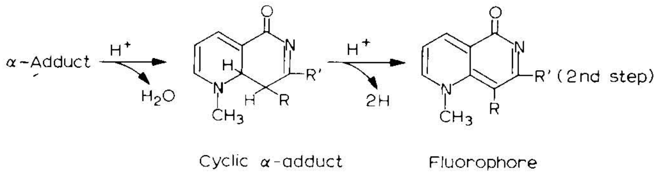

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[*] Presented in part at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April 3–5, 1978.

EXPERIMENTAL

Reagents and materials

N^1-Methylnicotinamide chloride (NMN) was purchased from Tokyo Kasei (Tokyo, Japan). The following α-oxomethylene compounds were purchased from commercial sources: methyl phenyl ketone (acetophenone), ethyl phenyl ketone (propiophenone), *n*-propyl phenyl ketone, cyclopentanone, cyclohexanone, cycloheptanone, sodium pyruvate and oxalacetic acid (*cis*-form) (guaranteed reagents), *n*-butyl phenyl ketone, cyclooctanone (extra-pure reagents) from Tokyo Kasei; acetone, methyl ethyl ketone (guaranteed reagents) from Kanto (Tokyo, Japan); and α-ketoglutaric acid monosodium salt from Sigma (St. Louis, Mo., U.S.A.). The α-oxomethylene compounds were dissolved in ethanol to make 10 m*M* stock solutions, except for the keto acids, which were dissolved in distilled water just before use. Other chemicals and solvents used were of analytical-reagent grade. The following chromatographic resins were used: Wako Gel LC-5K (5 μm) from Wako (Osaka, Japan), Iatrobeads 6CP-2020 (20 μm, styrene–divinylbenzene porous polymer) from Iatron Laboratories (Tokyo, Japan) and Co:Pell ODS (30 μm, C_{18} bonded pellicular vitreous beads) and Partisil-10 SAX (10 μm, microparticulate silica-bonded strong anion exchanger) from Whatman (Clifton, N.J., U.S.A.).

High-performance liquid chromatography

System for pre-column derivatization HPLC. The previous assay procedure[1] was modified as follows for microanalysis of α-oxomethylene compounds. A 10-μl volume of 6 *N* sodium hydroxide solution was transferred into a polyethylene microtest-tube (capacity 1.5 ml; Eppendorf, Hamburg, G.F.R.) and 10 μl each of the sample solution (containing 5 pmole–20 nmole of α-oxomethylene) and 50 m*M* NMN in 10^{-4} *M* hydrochloric acid were added in that order. The tube was allowed to stand for 10 min, 150 μl of 70.4% formic acid were added and the mixture was heated at 92° for 3 min. After cooling, an aliquot (0.5–20 μl) was injected into the chromatographic system. All of the procedures prior to the heating were performed in an ice-bath at 0°.

The jacketed column was maintained at 50° with a water-bath circulator (Type BT-35; Yamato Scientific, Tokyo, Japan). The eluent was delivered through stainless-steel tubing (0.25 mm I.D.) with a Mini-micro pump (Type KHD-16; Kyowa Seimitsu, Tokyo, Japan). On-column syringe injection of samples was performed through a loop injector (20 μl) using dual 4-way valves (Type KMM-4V-2, Kyowa Seimitsu) which operated up to 70 kg/cm^2. A 10-cm pulse-damping column filled with 40-μm glass beads (1 mm I.D.; Durrum, Palo Alto, Calif., U.S.A.) was placed between the pump and the injector. The outlet of the column was connected to a 14-μl quartz flow cell in a Shimadzu fluorescence detector (Type FLD-1; Shimadzu Seisakusho, Kyoto, Japan) equipped with a coated low-pressure mercury lamp emitting continuous light at 300–400 nm (maximum intensity at 360 nm) and an EM-3 secondary filter which cuts off light shorter than 405 nm. The outlet of the flow cell was connected to a 10-m back-pressure coil made of Teflon tubing (0.25 mm I.D.). The fluorescence intensity was recorded with a Shimadzu recorder (Type R-12; Shimadzu Seisakusho).

System for post-column derivatization HPLC. The chromatographic system described above was additionally modified as shown in Fig. 1. The column outlet was

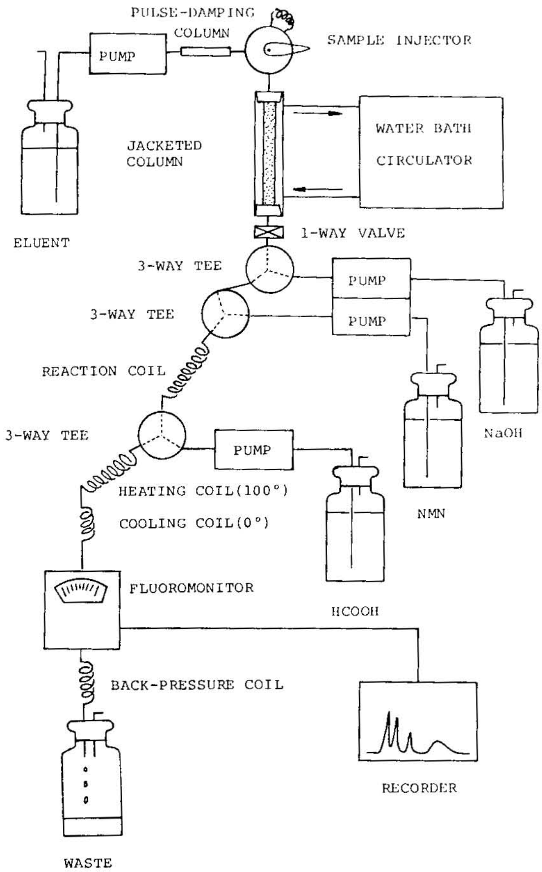

Fig. 1. Flow diagram for the HPLC post-column derivatization of α-oxomethylene compounds.

fitted with a one-way valve to prevent sodium hydroxide solution from entering the column and the eluate was mixed in a Teflon 3-way tee-piece (Kyowa Seimitsu) with 10 *N* sodium hydroxide solution, delivered through a 30-cm length of TFE tubing (0.5 mm I.D.) with a double plunger type Mini-micro pump (Type KHU-W-52; Kyowa Seimitsu). The outlet of the tee-piece was connected to a 3-cm length of Teflon tubing (0.5 mm I.D.), which was connected to the second Teflon 3-way tee-piece (Kyowa Seitmitsu). The alkaline eluate was mixed in the second tee-piece with 100 m*M* NMN in 10^{-4} *M* hydrochloric acid delivered with the other arm of the double plunger pump. The outlet of the second tee-piece was connected to a 10-m reaction coil made of Teflon tubing (0.25 mm I.D.). The latter was connected to the third 3-way tee-piece to which 88% formic acid was delivered with a Mini-micro pump (Týpe KSD-16). The outlet of the third tee-piece was connected to a 5-m heating coil made of stainless-steel tubing (0.5 mm I.D.), which was heated at 100° in a water-bath and was connected to a 30-cm cooling coil made of stainless-steel tubing (0.5 mm I.D.). The cooling coil was immersed in an ice-bath and its end was connected to the flow cell in the fluorescence detector.

The detailed chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

HPLC of fluorophores

The fluorescence of the NMN derivatives of *α*-oxomethylene compounds was generally most intense in acidic media, and the excitation and emission maxima were at 356–420 and at 420–472 nm, respectively[1]. Elution with alkaline eluents caused strong adsorption of fluorophores on the columns tested because of insolubility both in polar and non-polar solvents. Neutral eluents made the peaks rather broad. Acidic solvents in which the fluorophores were in the dicationic form gave the best separation with higher sensitivity. Fig. 2 illustrates an example of the separation of fluorescent NMN derivatives of some *α*-oxomethylene compounds on the column of Iatrobeads 6CP-2020 using acetic acid–methanol–water (6:1:21). Although unsymmetrical ketones ($R–CH_2–CO–CH_2–R'$) may give isomeric fluorophores in theory, methyl ethyl ketone and methyl isobutyl ketone gave single peaks on the styrene–divinylbenzene column and a silica column (Wako Gel LC-5K). The sensitivity of the fluorescence detection was markedly dependent on the compounds, as shown in Fig. 2. Generally, compounds having an *α*-oxomethylene group adjacent to an aromatic ring were detected with much higher sensitivity than aliphatic *α*-oxomethylenes. Acetophenone, one of the compounds giving the most intense fluorescence, could be determined in amounts as low as 0.5 pmole (Fig. 3).

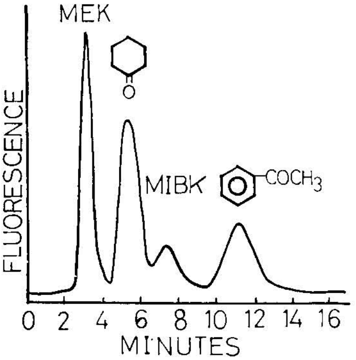

Fig. 2. HPLC separation of some *α*-oxomethylene compounds as their fluorescent NMN derivatives. Sample: mixture containing NMN derivatives of methyl ethyl ketone (400 pmole), cyclohexanone (500 pmole), methyl isobutyl ketone (2 nmole) and acetophenone (25 pmole). Column: Iatrobeads 6CP-2020 (20 μm; 30 cm × 3 mm I.D.) at 50°. Eluent: acetic acid–methanol–water (6:1:21). Eluent flow-rate: 0.5 ml/min.

HPLC of native α-oxomethylene compounds

The formation of *α*-adducts in alkaline media (the first step) is the rate-limiting step in the fluorogenic reaction, and the production of fluorophores increased with increasing concentrations of sodium hydroxide and NMN. The combination of 10 *N* sodium hydroxide solution and 50–100 m*M* NMN in 10^{-4} *M* hydrochloric acid gave satisfactory results in the first step. Fig. 4 shows the separation of C_5–C_8 cyclic ketones on a column of the pellicular reversed phase Co:Pell ODS by isocratic elution with 5% methanol.

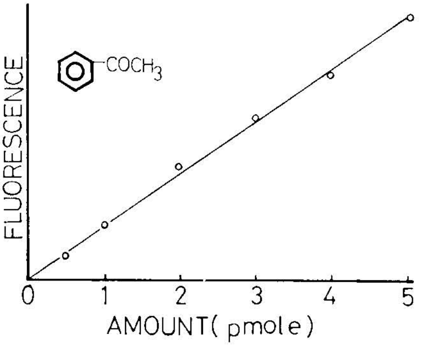

Fig. 3. Working curve for acetophenone. Acetophenone in various concentrations was converted into its fluorophore as described in the text. An aliquot (10 μl) of the reaction mixture was analysed under the same conditions as in Fig. 2. The peak height was plotted against the amount of acetophenone.

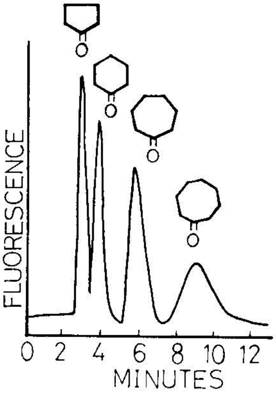

Fig. 4. HPLC separation of some native cyclic ketones. Sample: mixture containing cyclopentanone (200 pmole), cyclohexanone (200 pmole), cycloheptanone (2 nmole) and cyclooctanone (4 nmole). Column: Co:Pell ODS (30 μm; 50 cm × 3 mm I.D.). Eluent: 5% methanol. Flow-rates: eluent, 1.36 ml/min; 10 *N* NaOH, 0.17 ml/min; 100 m*M* NMN in 10^{-4} *M* HCl, 0.68 ml/min; 88% formic acid, 0.68 ml/min. Temperatures: column, 50°; reaction coil, 40°; heating coil, 100°; cooling coil, 0°.

In general, aliphatic α-oxomethylene compounds required heating of the reaction coil in order to accelerate the addition reaction, although the heating also accelerated the alkaline hydrolysis of amide groups of α-adducts and NMN. By heating the reaction coil at 40°, as little as 5 pmole of cyclopentanone and cyclohexanone were determined (Fig. 5). However, cycloheptanone and cyclooctanone were much less fluorescent in the system.

Some phenones were also analysed on the same column with 25% methanol as the eluent (Fig. 6). When the reaction coil was maintained at 0°, acetophenone and propiophenone gave intense fluorescence. Working curves for acetophenone and propiophenone are linear in the range 25–250 pmole of compound (Fig. 7). Heating the reaction coil at 40–50° increased the sensitivities of *n*-propyl phenyl ketone and *n*-butyl phenyl ketone, whereas it markedly decreased the sensitivities of acetophenone and propiophenone. Pyruvate, α-ketoglutarate and oxalacetate were separated on a Partisil-10 SAX column with isocratic elution of 0.1 *M* potassium phosphate solution

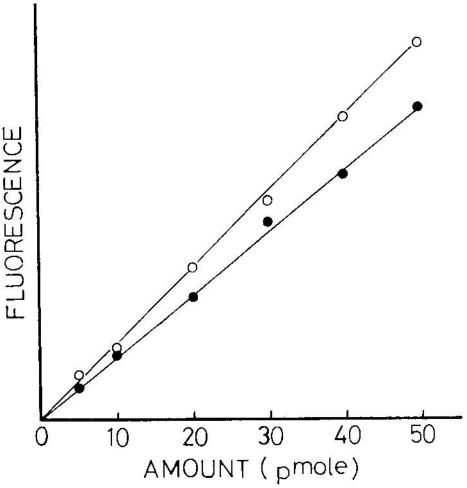

Fig. 5. Working curves for cyclopentanone (○) and cyclohexanone (●). Conditions as in Fig. 4. Peak-height ratios of these compounds to 500 pmole of cycloheptanone (internal standard) were plotted on the ordinate.

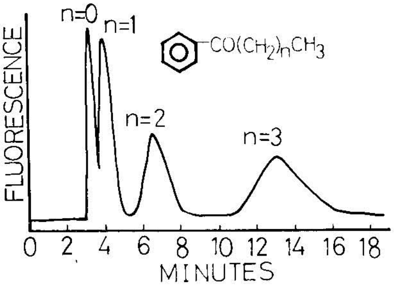

Fig. 6. HPLC separation of some native phenones. Sample: mixture containing acetophenone (200 pmole), propiophenone (200 pmole), *n*-propyl phenyl ketone (25 nmole) and *n*-butyl phenyl ketone (50 nmole). Eluent: 25% methanol. Temperature of reaction coil: 0°. Other conditions as in Fig. 4.

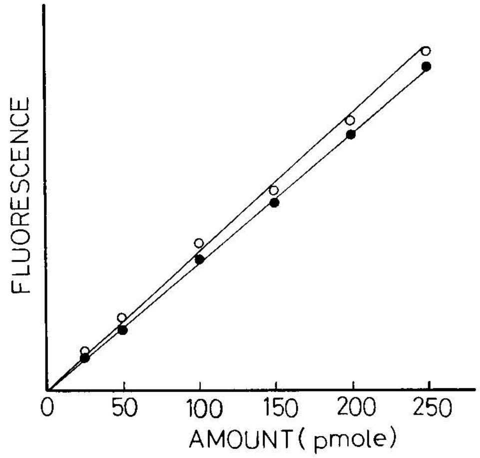

Fig. 7. Working curves for acetophenone (○) and propiophenone (●). Conditions as in Fig. 6. Peak-height ratios of these compounds to 25 nmole of *n*-propyl phenyl ketone (internal standard) were plotted on the ordinate.

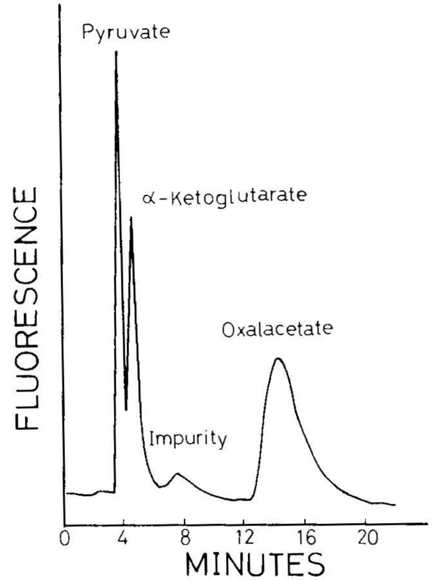

Fig. 8. HPLC separation of native α-keto acids. Sample: mixture containing sodium pyruvate (200 pmole), α-ketoglutaric acid monosodium salt (2 nmole) and oxalacetic acid (2 nmole). Column: Partisil-10 SAX (10 μm; 15 cm × 3 mm I.D.) at ambient temperature. Eluent: 0.1 *M* potassium phosphate (pH 5.5). Other conditions as in Fig. 4.

(pH 5.5), as shown in Fig. 8. Approximately 25 pmole of pyruvate was determined. The ion-exchanger method was demonstrated to be directly applicable to the analysis of urinary α-keto acids without any pre-treatment of the samples (10 μl).

In this investigation, NMN has been successfully applied to the fluorometric analysis of α-oxomethylene compounds by HPLC in both pre-column and post-column derivatization methods. The fluorogenic reaction is specific for the α-oxomethylene group as both the carbonyl and the α-methylene groups are simultaneously required for the formation of fluorophores. The sensitivity of the reaction permits the determination of some reactive α-oxomethylene compounds at the picomole level under mild conditions. The insensitive response of cycloheptanone, cyclooctanone, *n*-propyl phenyl ketone and *n*-butyl phenyl ketone is due primarily to steric hindrance of the α-oxomethylene groups in the first and/or second step of the reaction. At present, the pre-column derivatization method surpasses the post-column derivatization method in sensitivity. This is obviously due to the difference in the period of the addition reaction of α-oxomethylenes with NMN in alkaline media (10 min in the former and less than 2 min in the latter). Therefore, by using a longer reaction coil the sensitivity of the post-column derivatization method would be increased, with some loss of separation.

ACKNOWLEDGEMENT

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CHROM. 11,356

DETERMINATION OF 6-METHYLADENINE IN DNA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received July 10th, 1978)

SUMMARY

A method for the determination of 6-methyladenine (6MA) by high-performance liquid chromatography (HPLC) has been developed. DNA bases were separated by using the strong cation-exchange resin Zipax SCX. Purine bases were obtained by hydrolysis and dialysis of DNA and analysed by HPLC. 6MA in DNA from *Escherichia coli* was determined by the proposed method. It is suggested that the method could be applicable to analyses of 6MA from other biological sources.

INTRODUCTION

It has been suggested that a relationship exists between the formation of methylated bases in nucleic acids by carcinogenic methylating agents and the subsequent neoplastic transformation[1]. In this connection, we initiated studies on the determination of minor bases in nucleic acids and have already developed methods for the determination of 5-methylcytosine, 3-methylcytosine[2] and 7-methylguanine[3] by high-performance liquuid chromatography (HPLC).

6-Methyladenine (6MA), one of minor bases found in deoxyribonucleic acid (DNA), has been determined by a radioisotope method[4] that involves labelling of the base with [$^{14}CH_3$]methionine and by a UV method[5] after its separation by paper chromatography. The application of the former method, however, is limited because it requires *in vivo* labelling prior to analysis, and in the latter method it is difficult to separate minor bases from major bases that are present in large amounts. On the other hand, HPLC provides a superior method for application to nucleic acid bases in terms of resolution, reproducibility and sensitivity. This paper deals with the development of a method for the determination of 6MA by HPLC.

EXPERIMENTAL

Chemicals and reagents

All nucleic acid bases were purchased from Sigma (St. Louis, Mo., U.S.A.). Deionized distilled water was used and hydrochloric acid was distilled after diluting reagent-grade concentrated hydrochloric acid to 6 *N*. All other chemicals were of reagent grade and were obtained from commercial sources. Sources of the DNA used are given in Table III.

Quantitative analysis

HPLC was carried out in a Shimadzu–DuPont 840 liquid chromatograph equipped with a column (1 m × 2.1 mm) packed with Zipax SCX. The column was eluted with 0.1 *M* potassium dihydrogen orthophosphate (KH_2PO_4)–dipotassium hydrogen orthophosphate (K_2HPO_4) solution at a pressure of 40 kg/cm^2 at room temperature. The eluate was monitored with a UV detector (254 nm) at 0.01 a.u.f.s.

Determination of 6-methyladenine in DNA

Fifty milligrams of DNA were hydrolysed under Chargaff's conditions[6], *i.e.*, the DNA was dissolved in 15 ml of water, the pH was adjusted to 1.6 with 0.1 *N* hydrochloric acid and the total volume was adjusted to 20 ml with water previously adjusted to pH 1.6 with hydrochloric acid. The resulting mixture was dialysed against 500 ml of dilute hydrochloric acid (pH 1.6) at 37° for 26 h and 300 ml of outside fluids* containing purine bases were evaporated to dryness under reduced pressure. The residue was dissolved by addition of a small volume of dilute hydrochloric acid and the volume was adjusted to 2 ml with water. Aliquots (5 μl) of the solution were analysed by HPLC. Adenine and 6MA were determined after identifying 6MA by co-chromatography with an authentic specimen.

RESULTS AND DISCUSSION

Conditions for HPLC analysis

Nucleic acid bases are retained on cation-exchange resins to extents that depend on the pK_a values of each base and the hydrogen ion and buffer concentrations of the mobile phase. In this investigation, the strong cation-exchange resin Zipax SCX was employed and various buffer concentrations and pH values were tested using potassium phosphate buffer solution. Optimal conditions for the separation of 6MA from other bases were established by measuring the retention times of major nucleic acid bases and their derivative minor bases under various conditions. It was found that the best resolution was achieved at a buffer concentration of 0.1 *M* and pH 5.0. As shown in Fig. 1, 6MA was separated completely from other bases. As the content of adenine and guanine in the hydrolysate of DNA exceeded that of 6MA, a mixture of authentic adenine, guanine and 6MA in the proportions 100:100:1 was employed as a model hydrolysate for HPLC analysis. The results showed that the separation of 6MA from the other two bases even under such conditions was satisfactory.

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* The fluids present outside the dialysis bag after dialysis.

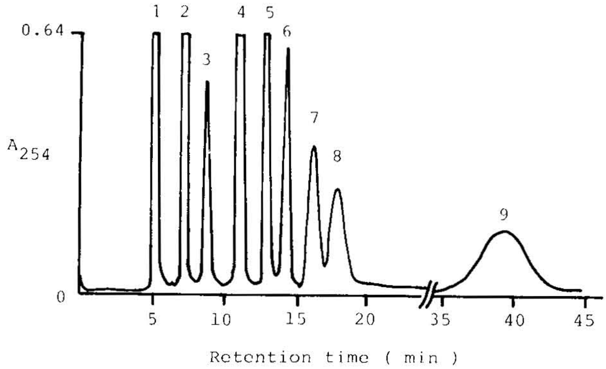

Fig. 1. Chromatogram of nucleic acid bases. Chromatograph, DuPont LC 840; column, Zipax SCX (1 m); mobile phase, 0.1 *M* KH_2PO_4–K_2HPO_4, pH 5.0; temperature, ambient; pressure, 40 kg/cm^2; detector, UV (254 nm); sensitivity, 0.64 a.u.f.s. Peaks: 1 — adenine; 2 — cytosine; 3 — O^6-methylguanine; 4 — N^6-methyladenine; 5 — 2-methyladenine; 6 — 5-methylcytosine; 7 — 1-methyladenine; 8 — 3-methylcytosine; 9 — N^6,N^6-dimethyladenine.

Calibration graphs

Average peak heights in HPLC for various concentrations of 6MA were obtained by injecting five 5-μl volumes each of solutions containing 60, 50, 40, 25, 8 and 0.8 ng of 6MA. As shown in Fig. 2, a straight line was observed for these six concentrations. 6MA was determined by using the least-squares line derived from the calibration graph. For the quantitation of adenine, the integrator count method was employed because of the different scales involved due to the presence of a large amount of this compound in DNA in comparison with 6MA. Five microlitres of solutions containing 750, 500, 250, 100 and 20 ng of adenine were subjected to HPLC in the same manner as for 6MA and a linear calibration graph was obtained.

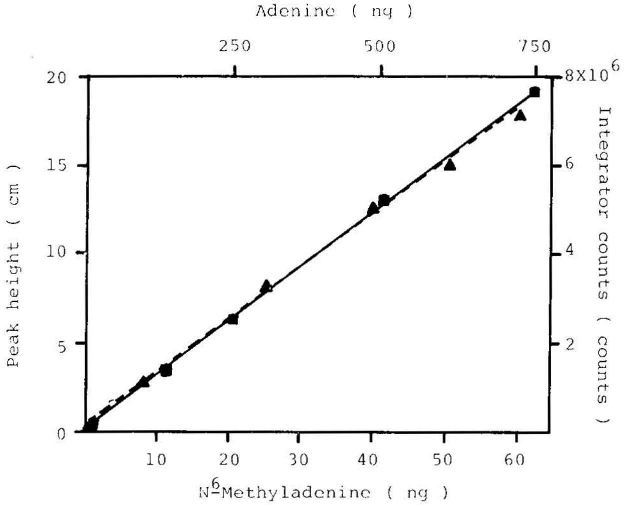

Fig 2. Calibration graph for 6MA (▲---▲) and adenine (■—■). 6MA was measured by the peak-height method and adenine by the integrator method.

Recovery of 6MA

Amounts of 5 mg each of 6MA and adenine were subjected to the entire process and their recoveries are given in Table I.

TABLE I

RECOVERY OF 6-METHYLADENINE AND ADENINE

Experiment No.	*Recovery (%)*	
	6MA	*Adenine*
1	99.2	95.1
2	90.2	100.1
3	92.4	85.5
4	87.1	96.7
5	97.1	93.9
Mean	93.2	94.3
Standard deviation	4.43	4.85
Coefficient of variation	4.75	5.14

Recovery of 6MA added to DNA

Known amounts of 6MA were added to 50 mg of calf thymus DNA and the mixture was subjected to the proposed procedure. A satisfactory recovery of 6MA (94.5%) was obtained, as shown in Table II, and the procedure was therefore applied to the determination of natural 6MA in DNA.

TABLE II

RECOVERY OF 6-METHYLADENINE ADDED TO DNA

6MA added (μg)	*6MA found (μg)*		*Recovery (%)*
0	—		—
92.3	82.5		89.4
96.0	96.0		100.0
96.0	94.7		98.6
96.0	94.7		98.6
96.0	82.3		85.7
		Mean:	94.5
		Standard deviation:	5.78
		Coefficient of variation:	6.12

Determination of 6MA in various DNA sources

Fifty milligrams of calf thymus DNA gave two large peaks for guanine and adenine in HPLC after hydrolysis. No peak corresponding to pyrimidines was observed under the condition employed. For *Escherichia coli* DNA, a peak corresponding to 6MA was observed (Fig. 3) and it was identified as 6MA by co-chromatography with an authentic sample. The amount of 6MA was determined to be 1.4% of that of adenine. No conversion of 1-methyladenine into 6MA was expected as the analysis was carried out under acidic conditions. It was also found that under the

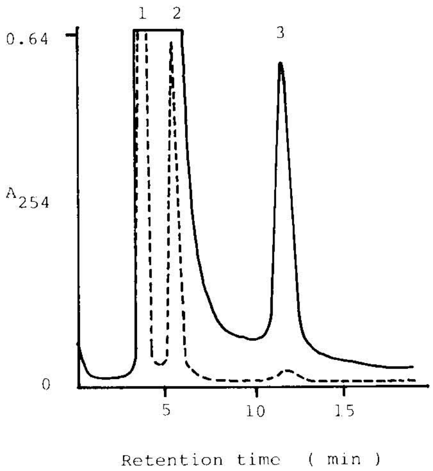

Fig. 3. Chromatogram of *E. coli* DNA purine bases. Chromatographic conditions as in Fig. 1, except sensitivity of detector: solid line, 0.01 a.u.f.s.; broken line, 0.64 a.u.f.s. Peaks: 1 = guanine; 2 = adenine; 3 = 6MA.

conditions employed 6MA was not detectabele in DNAs from the following sources: normal rat liver; rat liver administered intraperitoneally with carcinogenic methylating agents, dimethylnitrosamine (DMN) or N-methyl-N′-nitro-N-nitrosoguanidine (MNNG); rat hepatoma induced by 3′-dimethylaminoazobenzene (3′-DAB); and Ehrlich solid tumour (Table III). DNA from these sources was obtained by Kirby's method[7].

TABLE III

6-METHYLADENINE CONTENT IN VARIOUS DNAs

Origin of DNA	*6MA (% relative to adenine)*
Calf thymus (Sigma)	ND***
DMN-treated rat liver*	ND
MNNG-treated rat liver*	ND
Herring sperm (Sigma)	ND
Normal rat liver	ND
E. coli (Sigma)	1.4
Ehrlich solid tumour	ND
3′-DAB-induced rat hepatoma**	ND

* 30 mg/kg of DMN and MNNG were administered intraperitonealy to five male rats. After 8 and 3 h, respectively, the rats were killed.

** A diet containing 0.06% of 3′-DAB was fed to rats for 6 months.

*** ND = not detected (the detection limit of 6MA was 0.03% relative to adenine).

CONCLUSION

The determination of 6MA in DNA by HPLC has advantages over conventional methods in terms of high sensitivity, simplicity and rapidity. In addition, studies are in progress concerning the possibility of reducing the amount of samples further and also of applying the HPLC analysis to other minor bases.

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CHROM. 11,332

RESOLUTION OF ENZYMES THAT HYDROLYSE PHOSPHATE ESTERS FROM *AGAVE CANTALA* ON AMBERLITE IR-120 (Al^{3+})

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(Received July 17th, 1978)

SUMMARY

A technique for the resolution of functionally similar enzymes is described. Enzymes that hydrolyse phosphate esters, *viz.*, 3′-nucleotidase, pyrophosphatase, phosphatase, and ribonuclease, from *Agave cantala* have been resolved on an IR-120 (Al^{3+}) column free from each other, and their eventual purification thereby obtained is described.

INTRODUCTION

Although several chromatographic procedures exist for the separation of enzymes and proteins, often they do not provide adequate resolution of complex mixtures of functionally similar proteins and enzymes necessary for their precise individual study. In addition, they are not free from operational limitations such as clogging of the column, slow flow-rates leading to irreversible adsorption and consequent loss of biological activity, denaturation and low recoveries.

Proteins, being macromolecules with an infinite variety of molecular species that are very closely related, can be visualised theoretically. When any single criterion of separation is applied, groups of molecules will be segregated. Such an apparently homogeneous material can still be a mixture of very closely related molecules, which may differ marginally with respect to one applied criterion but widely by another. It is evident, therefore, that a number of distinct separation procedures based on different properties of the molecules must be applied in order to achieve their ultimate resolution. For this reason, new methods for the fractionation of proteins and other macromolecules have some value beyond whatever advantages and convenience they may offer.

A method of column chromatography on Amberlite IR-120 (Na^+) equilibrated with aluminium ions developed in this laboratory for the fractionation of DNA[1,2] and RNA[3–6] has already been reported. The separation of chymopapain from papaya latex[7] on IR-120 (Hg^{2+}) has been described.

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This paper reports the resolution of enzymes (3′-nucleotidase, pyrophosphatase, phosphatase and ribonuclease) from *Agave cantala* using Amberlite IR-120 (Al^{3+}).

EXPERIMENTAL

Preparation of Amberlite IR-120 (Al^{3+}) column

Ten grams of dry regenerated Amberlite IR-120 (Na^+) were converted into the Al^{3+} form as described earlier[3] and equilibrated to pH 4.8 with 0.05 *M* acetate buffer. The amounts of aluminium in the influent, effluent and buffer washings were determined colorimetrically by the 8-hydroxyquinoline method[8]. It was observed that 1 mequiv. of Al^{3+} per gram of the resin was retained under these conditions.

Extraction of the enzyme

Leaves of *Agave cantala* preserved at −10° were cut and homogenized in ice-cold saline in a Waring blender for 5 min. A 90-ml volume of 0.14 *M* saline was added per 100 g of *Agave cantala* leaves taken for extraction. The extract was allowed to stand for 1 h at 0° for the insoluble matter to settle.

The supernatant was passed through cheese-cloth and the filtrate was centrifuged at 5000 *g* for 10 min. The resultant pale yellow-green extract was used as the source of enzyme.

Activity and protein determination

Enzyme activities were determined by determining the inorganic phosphorus[9] (P_i) liberated from the specific substrates and conditions as shown in Table I.

After terminating the reaction with trichloroacetic acid (TCA), aliquots were centrifuged at 5000 *g* and P_i determined by Fiske and Subbarow's method[9].

TABLE I

CONDITIONS FOR ENZYME ASSAY

Enzyme	*Samples (suitably diluted) (ml)*	*Substrate*	*Acetate buffer, pH 4.8, 0.05 M (ml)*	*Incubation time*	*10% TCA (ml)*
Phosphatase	1.0	Sodium β-glycerophosphate (0.01 *M*), 1.0 ml	1.0	15 min	2.0
Pyrophosphatase	1.0	Sodium pyrophosphate (0.01 *M*), 1.0 ml	1.0	15 min	2.0
3′-Nucleotidase	0.5	Adenylic acid (0.04 *M*), 1.0 ml	5.0	1 h	1.0
Ribonuclease	1.0	RNA (1 mg/ml), 2.0 ml	2.0 + 3.0 ml of water* or 3′-nucleotidase	24 h	2.0

* Except for the crude extract which contains adequate 3′-nucleotidase, external addition of *Agave cantala* 3′-nucleotidase (free from RNAse) ensured P_i liberation from the nucleotides formed by RNAse action. The P_i liberated wzs taken as a measure of RNAse.

Definition of enzyme unit

One unit of phosphatase, pyrophosphase, 3′-nucleotidase and ribonuclease is defined as the activity that liberates 0.1 mg of P_i under the above special conditions at 37° and with corresponding substrates.

Protein contents of various fractions were determined by the Folin–Ciocalteu method[10]. Specific activities are expressed as units of enzymes per milligram of protein in the fraction.

Chromatographic studies

A 25.0-ml volume of the above extract was loaded on the column of Amberlite IR-120 (Al^{3+}) buffered at pH 4.8. A flow-rate of 2.0 ml/min was maintained throughout the operation. After complete passage of the extract, the column was flushed with 0.05 *M* acetate buffer (pH 4.8) to remove tailing material. Elution of adsorbed enzymes was achieved with a discontinuous gradient of ammonium acetate. The influent, effluent, subsequent buffer washings and the different fractions obtained were assayed for phosphatase, pyrophosphatase, 3′-nucleotidase and ribonuclease activities and protein content.

RESULTS

A typical elution profile, showing the clear separation of the four enzymes phosphatase, pyrophosphatase, 3′-nucleotidase and ribonuclease, is presented in Fig. 1. The enzymes were eluted in the order pyrophosphatase, phosphatase and ribonuclease with recoveries of 98.7, 89.9 and 76.5% and purifications of 3.97-, 14.5- and 4.38-fold, respectively. 3′-Nucleotidase was not adsorbed and was contained in the effluent free from pyrophosphatase, phosphatase and ribonuclease activities. Its recovery was 97.1% with 4.5-fold purification (see Table II).

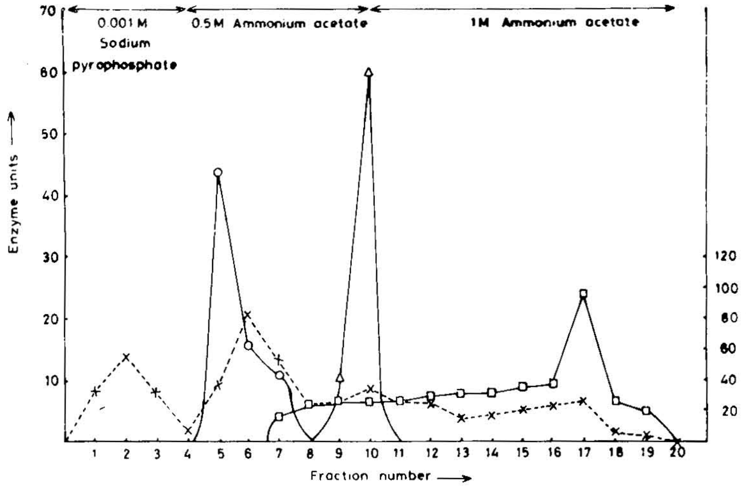

Fig. 1. Elution pattern of enzymes that hydrolyse phosphate esters from *Agave cantala* adsorbed on an IR-120 (Al^{3+}) column. ○, Pyrophosphatase; △, phosphatase; □, ribonuclease; ×, protein (mg).

TABLE II

PURIFICATION OF *AGAVE CANTALA* ENZYMES ON AN IR-120 (Al^{3+}) COLUMN

Enzyme	*Fraction*	*Volume (ml)*	*Total enzyme (units)*	*Total protein (mg)*	*Specific activity*	*Enrichment ratio*	*Recovery (%)*
3′-Nucleotidase	(1) Original extract	25	87.5	950	0.092	—	100
	(2) Effluent and first washing	50	84.88	205	0.414	4.5	97.1
	(3) Ammonium sulphate precipitation	5.0*	79	7.5	10.53	114	90.2
Pyrophosphatase	(1) Original extract	25	71.57	950	0.075	—	100
	(2) Combined eluate (5–7)	75	70.67	176	0.401	5.3	98.74
	(3) Ammonium sulphate precipitation	5.0*	50	20.5	2.439	32.4	69.87
Phosphatase	(1) Original extract	25	78.4	950	0.082	—	100
	(2) Combined eluate (9–10)	50	59	1.196	14.5	14.5	90
	(3) Ammonium sulphate precipitation	5.0*	60.33	5.5	10.96	132.9	76.95
Ribonuclease	(1) Original extract	25	109.1	950	0.114	—	100
	(2) Combined eluate (11–19)	225	83.59	165.57	0.503	4.38	76.58
	(3) Ammonium sulphate precipitation	7.0*	71.2	16.5	4.315	37.6	65.31

* Precipitate of the protein obtained with 2.3 *M* ammonium sulphate concentration was dissolved in acetate buffer (pH 4.8, 0.05 *M*).

The method gives a substantial increase in the specific activities of these enzymes and the pooled fractions can be further purified by ammonium sulphate precipitation. The pooled fractions of the different enzymes were fairly free from each other. With the application of only two general methods of purification, *viz*., passage through an IR-120 (Al^{3+}) column and ammonium sulphate precipitation, one would not expect all of the above enzymes to separate into discrete pure fractions. The enzymes are, in fact, only semi-purified and have not reached a homogeneous state of purity. The significant point is that they are fairly free from each other.

DISCUSSION

The results indicate that an IR-120 (Al^{3+}) column can clearly resolve the enzymes pyrophosphatase, phosphatase, ribonuclease and 3′-nucleotidase from *Agave cantala* leaves. A close examination of the recoveries indicates that there are no significant losses of these enzymes on the IR-120 (Al^{3+}) column under the conditions used.

There are certain inherent advantages that Al^{3+}-equilibrated resins offer. Flow-rates through the IR-120 (Al^{3+}) column are fast and no choking of the column results. This makes the operation very easy and the whole column operation can be finished in a short period with little danger of inactivation of the enzymes.

It should be further noted that Amberlite IR-120 cannot be used in either the H^+ form for enzyme fractionation. With the highly acidic H^+ form, the enzymes are denatured and inactivated. Further, proteins have very little affinity towards the Na^+ form of the resin. Therefore, it is interesting that IR-120 (Al^{3+}) columns can be very useful for enzyme fractionation; they retain 75 mg of *Agave cantala* proteins per gram of the Al^{3+} form of the resin.

Although the affinity of heavy metal ions such as Pb^{2+}, Hg^{2+}, Ag^{2+}, Zn^{2+} and Cu^{2+} towards active groups such as COOH, NH_2, SH, imidazole, phenolic OH and indole in proteins is known, these metal ions cannot be applied with enzymes as many of them are inhibitors. In this respect, Al^{3+} is safe.

It must be noted, however, that aluminium ions are eluted together with the eluting agents used and are distributed in all of the fractions, but they do not interfere in the activity determinations.

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CHROM. 11,415

NOVEL THIN-LAYER CHROMATOGRAPHIC SEPARATION AND SPECTROFLUOROMETRIC QUANTITATION OF LITHOCHOLIC ACID

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SUMMARY

Chromatographic separation of lithocholic acid from a mixture of four unconjugated bile acids was achieved in 5 sec. The system consisted of Gelman ITLC type SG chromatography sheets, Seprachrom miniature chromatography chambers, an isooctane–isopropyl ether–acetic acid (2:1:0.04, v/v/v) solvent system and a 5% H_2SO_4 in methanol detection spray. The fluorescent spots were quantitated by direct scanning of the chromatograms with a Farrand Mark I spectrofluorometer. Maximum excitation and emission wavelengths were 375 and 436 nm, respectively. Accurate measurement of lithocholic acid was performed within the range of 25 to 175 ng.

INTRODUCTION

Lithocholic acid (LC) (3α-hydroxy-5β-cholanic acid) has been isolated in free and conjugated forms from human bile[1,2], feces[3,4], serum[1,5–9] and urine[10]. Sophisticated multi-step purification techniques have been developed for quantitative separation of LC and other bile acids from biological material[1–6,9,10]. Thin-layer chromatography (TLC) has frequently been employed as a preliminary separation technique[2,3,5,9,11–14]. However, present procedures for the quantitative measurement of free and/or conjugated LC and other bile acids are laborious, time consuming, and involve highly sophisticated instrumentation, *e.g.* liquid scintillation counter[7,8], gas–liquid chromatograph[1–3,5,15], gas–liquid chromatograph–mass spectrometer[13,14] and computerized gas–liquid chromatograph–mass spectrometer combination[6,10].

Many TLC procedures[2,11,12,15–23] have separated free and/or conjugated forms of LC from a bile acid mixture. Although some procedures have achieved separation on silica gel impregnated glass micro-fiber sheets, direct quantitative measurement of the separated bile acids was not reported[20–23].

In this paper, a novel 5-sec TLC separation of LC is reported. Sensitive, quantitative measurement of the fluorescent spots is performed by direct scanning of the chromatograms with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment.

EXPERIMENTAL

Materials

Chenodeoxycholic (CDC), deoxycholic (DOC) and lithocholic (LC) acids were purchased from Supelco (Bellefonte, Pa., U.S.A.). Cholic acid (C) (purity 99–100%) and 5β-cholanic acid were obtained from Sigma (St. Louis, Mo., U.S.A.)

Certified grades of 2,2,4-trimethylpentane (isooctane) and diisopropyl ether (isopropyl ether) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Glacial acetic acid and concentrated sulphuric acid were obtained from Canadian Industries (St. Boniface, Canada). Absolute ethanol was purchased from Standard Chemical Co. (St. Boniface, Canada). Reagent-grade methanol was from Canadian Chemical Supplies (Winnipeg, Canada).

Seprachrom miniature chromatography chambers and 20 × 20 cm Instant TLC sheets type SG (ITLC type SG) were supplied by Gelman (Ann Arbor, Mich., U.S.A.). A Gem hand-punch (McGill, Marengo, Ill., U.S.A.) containing a round 3.0-mm die was employed to excise chromatography discs. Bile acids were applied to chromatographic discs with a 10-μl Hamilton syringe from Hamilton (Reno, Nev., U.S.A.).

Chromatography sheets were heated in a Thelco Model 18 oven from Precision Scientific (Chicago, Ill., U.S.A.). The developed chromatograms were scanned with a Farrand Mark I spectrofluorometer (Farrand Optical, Mount Vernon, N.Y., U.S.A.) equipped with a thin-layer scanning attachment and a magnetic xenon arc stabilizer. The instrument was modified by shielding the lamp housing and the photomultiplier with Nu-metal. A Topaz Series 73000 Model L1, a.c. line regulator (Topaz Electronics, San Diego, Calif., U.S.A.) was employed to regulate the line voltage. The intensity of the lamp was reduced by inserting a 60-mesh wire gauze brass screen (Canadian Laboratory Supplies, Winnipeg, Canada) in front of the quartz window at the exit port of the lamp housing. The reduced light intensity prevented the otherwise frequent breakage of the excitation filter. The Color Specification numbers for the excitation and emission filters were 7-54 and 3-73, respectively. The excitation slit widths were 5 nm and the emission slit widths were 10 nm. The intensity of the reflected fluorescence was recorded with a Farrand Model 100 strip-chart recorder (Model SR-204, Heath, Benton Harbor, Mich., U.S.A.). Area measurements were performed with a Koizumi compensating polar planimeter, purchased from Reliable Drafting Supplies (Regina, Canada).

Methods

Preparation of chromatography materials and bile acid standards. ITLC type SG chromatography sheets were cut to size (6.5 × 9.9 cm) to fit the Seprachrom chromatography chambers. The miniature chromatography sheets were heated in a 100° oven for 1 h. Activated sheets were stored above silica gel in a desiccator.

A stock solution containing C, CDC, DOC and LC was prepared by adding 25.0 mg of each bile acid to a 50-ml volumetric flask which was filled to volume with absolute ethanol. A working standard was prepared by adding an appropriate volume of the stock solution to a 10-ml volumetric flask, to produce a standard containing 200 ng of each bile acid per μl of absolute ethanol. Sample application was performed by the procedure of Popowicz[24]. Blank chromatography discs were excised from an activated miniature chromatography sheet with a Gem hand-punch. Holes were punched 7 mm from the bottom and at 13-mm intervals from the left edge of the sheet. A 1 μl volume of the 200-ng bile acid standard was applied with a Hamilton 10-μl syringe to each of four blank chromatography discs. The prepared discs were reinserted into the chromatography sheet.

The chromatography solvent system was prepared fresh prior to use. A 2-ml volume of isooctane, 1.0 ml of isopropyl ether and 0.04 ml of glacial acetic acid were pipetted into a test tube. The solvent system was mixed and transferred into a Seprachrom chromatography trough. A chromatography sheet containing bile acid standard discs was inserted into the chromatography chamber and the solvent was allowed to migrate for 5 sec. The chromatogram was immediately removed, air dried for 5 min, and oven dried for an additional 5 min. The chromatogram was sprayed with 5% H_2SO_4 in methanol[25] and placed in a 95° oven for 20 min. Fluorescent bile acid spots were observed under long-wave UV light. The fluorescence intensity of the LC spots was measured with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment. Maximum excitation and emission wavelengths were determined. The chromatogram was scanned across the origin, the LC spot and the solvent front. The above procedure was performed repeatedly to evaluate reproducibility. The procedure was similarly performed for 10-, 20- and 30-sec solvent migration times. For the 30-sec. migration, the origin was located 13 mm from the bottom of the chromatography sheet.

Lithocholic acid concentration study. A bile acid stock solution was prepared by adding 25.0 mg of each bile acid to a 25-ml volumetric flask which was brought to volume with absolute ethanol. A stock solution of internal standard was prepared by adding 62.5 mg of 5β-cholanic acid to a 50-ml volumetric flask which was brought to volume with absolute ethanol. Working standards were prepared by adding appropriate volumes of the bile acid stock solution and the internal standard stock solution to a series of 5-ml volumetric flasks to produce eight bile acid standards ranging from 25 to 200 ng/μl of absolute ethanol, each standard containing 5β-cholanic acid at a concentration of 500 ng/μl. A 1-μl volume of each of the standards was applied to blank chromatography discs. Chromatographic separation and visualization were performed as described above. A 30-sec solvent migration time was employed to accommodate introduction of the internal standard. Chromatograms were scanned with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment. The LC spot was employed to maximize the excitation and emission monochromators. The intensity of the reflected fluorescence was recorded on a Farrand strip-chart recorder. Peak areas were measured by planimeter. A duplicate set of analyses was performed with freshly prepared stock standard solutions.

RESULTS AND DISCUSSION

The Kelsey and Sexton[23] chromatography solvent system was tested and modified to achieve optimal separation of LC from a mixture containing four unconjugated bile acids. The new solvent system consisted of isooctane–isopropyl ether–acetic acid (2:1:0.04, v/v/v). Rapid chromatographic separation was obtained by positioning the bile acid standard discs within 7 mm from the bottom end of the ITLC type SG miniature chromatography sheet. Excellent chromatographic separation of

LC was achieved in as little as 5 sec (see Fig. 1). It should be noted that splashing of the solvent system must be avoided. However, to attain greater separation distances and improved reproducibility, the standard discs were positioned within 13 mm of the bottom edge of the sheet and a 30-sec solvent migration time was employed. Solvent front migration was approximately 23 mm from the origin. Under the experimental conditions of this laboratory, the R_F value for LC was 0.49. The unseparated CDC, DOC and C bile acid fraction remained near the origin, below R_F 0.13. Each of the aforementioned R_F values represents an average of twenty results.

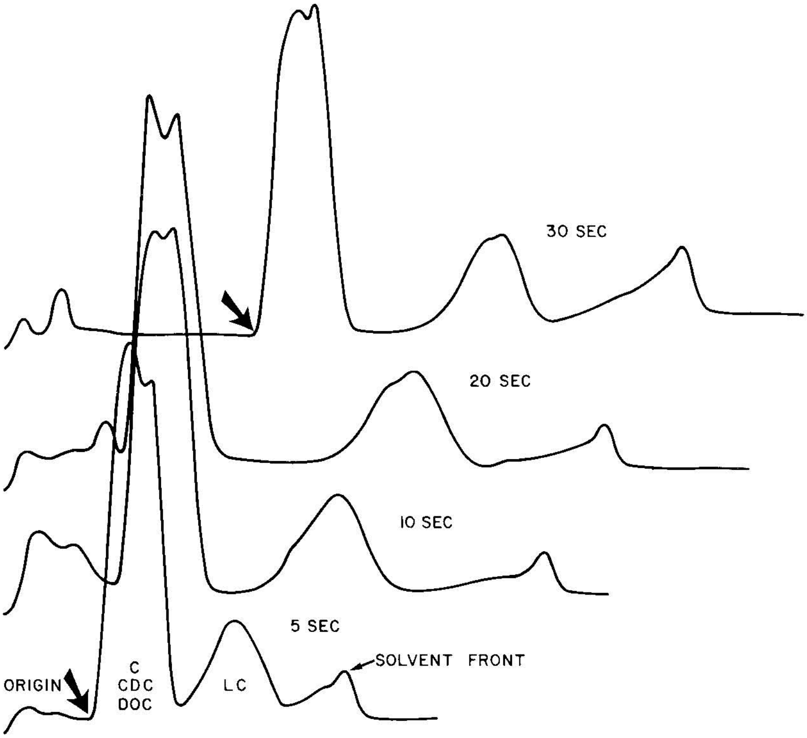

Fig. 1. Spectrofluorometer scans depicting the reflected fluorescence intensity of the bile acid spots. Chromatogram solvent migration times were 5, 10, 20 and 30 sec. Refer to text for complete details.

Heating of the ITLC type SG chromatography sheets was not essential for optimal separation of LC. However, heat-activated chromatograms were observed to produce more uniformly shaped LC spots. The chromatograms were scanned with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment. The damping mode was not employed. The excitation and emission maxima for the LC fluorophore were 375 and 436 nm, respectively. The wavelength accuracy of the Mark I spectrofluorometer was better than 0.5 nm, as determined with a Farrand wavelength calibration unit. Although the ITLC type SG chromatography sheets have an irregular surface appearance, a very high signal-to-noise ratio was observed when the intensity of the reflected fluorescence of the chromatogram was recorded (Fig. 1). Peak area was plotted *versus* quantity of LC (Fig. 2). A linear relationship was observed for LC within the range of 25 to 175 ng.

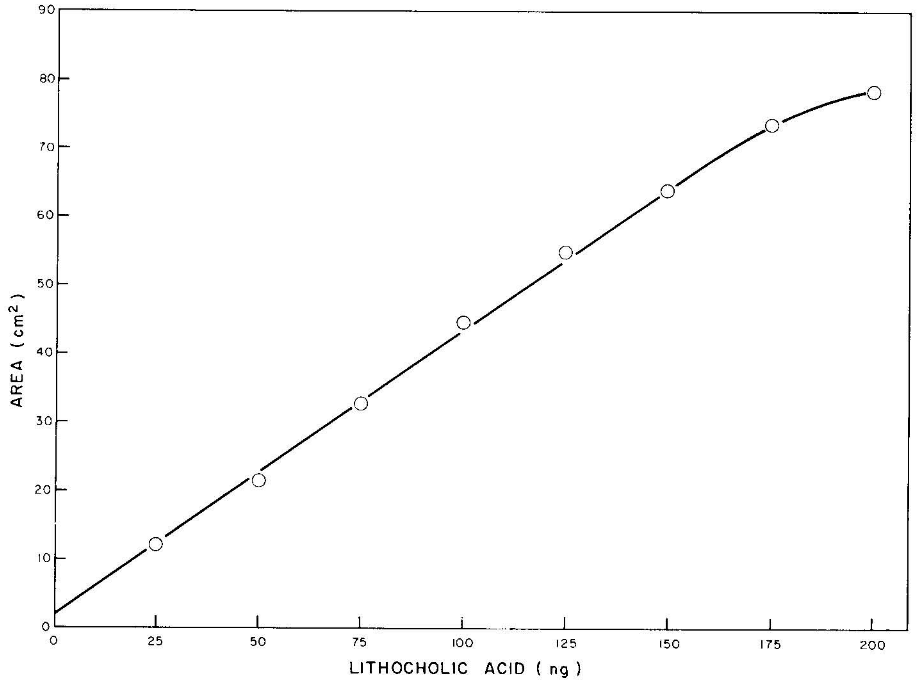

Fig. 2. Lithocholic acid standard curve. The results presented represent an average of two samples at each concentration studied. Refer to text for complete details.

A value of 4.2% was calculated for the coefficient of variation of the 150-ng standard. The average variation calculated from the duplicate results of the linear portion of the LC calibration curve (Fig. 2) was 4.2%. However, it should be emphasized that variations in sample application, detection spraying and oven temperature must be minimized. In general, improved reproducibility has been achieved with less effort for systems employing a ratio procedure, *e.g.* tri-/di-hydroxy bile acids ratio[26] and lecithin/sphingomyelin ratio[27].

The internal standard, 5β-cholanic acid, has been employed for the analysis of bile acids by gas–liquid chromatography[28]. However, attempts to employ 5β-cholanic acid as an internal standard in the presently developed ITLC separation were only partially successful. The 5β-cholanic acid was observed to migrate near the solvent front, R_F 0.88. Solvent front variation was observed to interfere with accurate measurement of the 5β-cholanic acid spot. Although very reproducible results have been achieved (Fig. 3), sheet-to-sheet solvent front variations have also been observed.

The xenon arc lamp of the Mark I spectrofluorometer was stabilized with a magnetic arc stabilizer. The spectrofluorometer was further modified by shielding

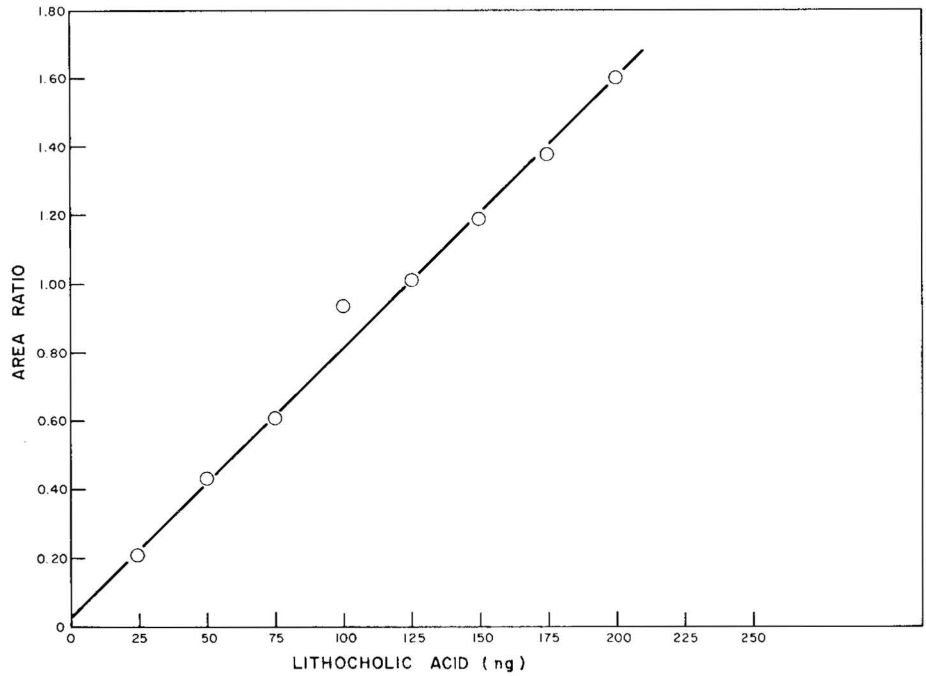

Fig. 3. Lithocholic acid standard curve obtained by area ratio (peak area for lithocholic acid/peak area for 5β-cholanic acid). Refer to text for complete details.

the xenon arc lamp and the photomultiplier tube with Nu-metal. A Stabiline voltage stabilizer was employed to overcome voltage line fluctuations. Under our operating conditions, spectrofluorometer drift was approximately ±1% per 12 h. Furthermore, day-to-day variation of the xenon arc lamp intensity was compensated by adjusting the spectrofluorometer to a Pyrex standard.

The fluorescence intensity of the LC spots was observed to be stable for over 6 h. A gradual decrease of 3.5% was calculated for measurements taken at 12 and 24 h for the 150-ng LC spot. On occasion, diffuse spot formation and decreases in fluorescence intensity have been observed. Similar results have been observed after splashing the solvent system. As such, it is recommended that splashing be avoided and chromatography discs not be placed closer than 1.0 cm from the bottom edge of the chromatography sheet.

ITLC coupled with direct spectrofluorometric quantitation has made possible the development of a rapid, simple, and accurate procedure for the measurement of nanogram quantities of lithocholic acid.

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CHROM. 11,410

Note

Apparatus for the isolation of microgram amounts of compounds from thin layers by elution and direct Millipore filtration

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Thin-layer chromatography is commonly used for the isolation of a particular compound, the layer of sorbent containing that compound being scraped off the chromatogram and eluted with a suitable solvent. If, *e.g.*, mass spectrometry, is subsequently to be applied, it is necessary to have an eluate that is sufficiently concentrated. Therefore, when eluting microgram amounts, the total volume of the eluate should not exceed about 30 μl. When concentrating the eluate by evaporation, impurities in the solvent will also be concentrated and decomposition of the compound may also occur.

In order to avoid the need for evaporation after elution of microgram amounts, an apparatus has been developed that makes it possible to scrape off and collect the sorbent layer, elute the compound in a small volume and filter the resulting solution through a paper filter and a Millipore filter. In this way, microgram amounts of a compound can be isolated in a volume of 15–30 μl. In comparison with other techniques for the transfer of small amounts[1–6], this apparatus has the advantage of being specially constructed for filtration through a Millipore filter, instead of glass-wool[1–5] or cotton-wool[6]. With glass-wool or cotton-wool asfilters, the eluate is not free from particles from the thin layer. Filtration through the Millipore filter results in a particle-free eluate, which can be used directly for high-performance liquid chromatography and mass spectrometry.

APPARATUS

The device consists of three parts, the filtration section, the collector section and the conical tip (Fig. 1). The apparatus is made of PTFE and the diameter of the bore is 1.2 mm. The conical part of the tip fits into the collector section at side 1 (Fig. 1).

Part of the filtration section is milled, in order to simplify its connection with the collector section. The screw-thread used for connecting these two sections and the measure are defined by M8 × 1. The end of the filtration section is round, with a radius of 2.5 mm (R 2.5). As only a small part (1.2 mm^2) of the filters is used for filtration, it is necessary for the end of the collector section to be round, with a radius of 20 mm (R 20), in order to achieve a good connection. At side 1 the inner diameter

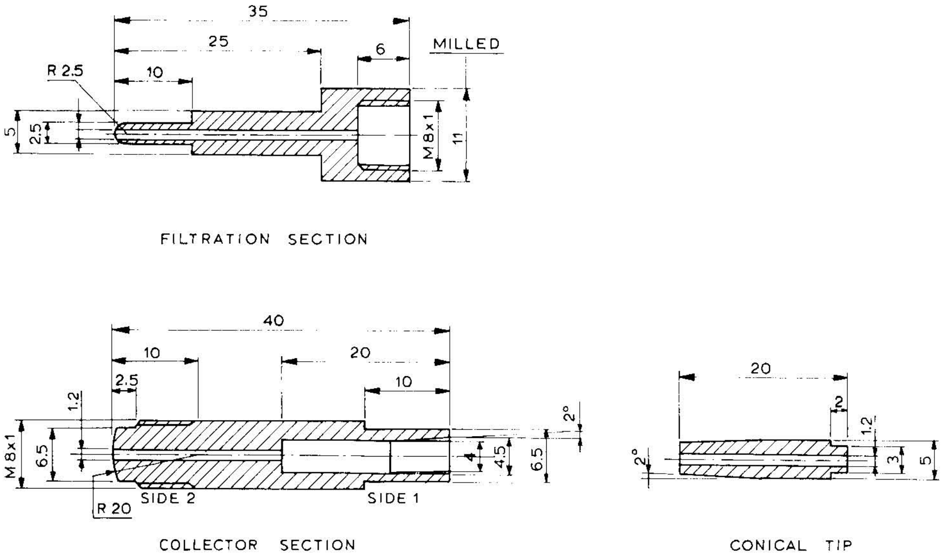

Fig. 1. Components of the apparatus. Measurements in millimeters.

is conical (2°) in order to obtain a sufficiently tight connection with the conical tip of the syringe, which is also conical. The diameter of the non-conical end of the conical tip is reduced from 5 mm to 3 mm over a distance of 2 mm in order to be able to scrape off small areas of sorbent.

Fig. 2 illustrates the collector sectionfitted with the conical tip, used as a scraper and sorbent collector.

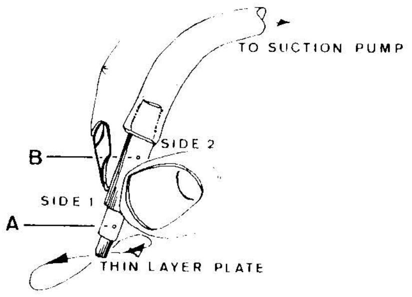

Fig. 2. Collection of sorbent from a thin-layer plate. A – Conical tip; B – collector section.

Fig. 3 illustrates the device for elution and filtration. The sorbent has been sucked into the collector section (B) (as illustrated in Fig. 2). The conical tip at side 1 has been disconnected, and also the tube at side 2, the side with the screw-thread leading to the suction pump.

Three kinds of filters are used: a Millipore filter, a paper filter and a collector filter. The Millipore filter (Sartorius-Membranfilter; Sartorius, Utrecht, The Netherlands; *e.g.*, S.M. 116 for organic solvents) (F, Fig. 2) and the paper filter (Munktell's

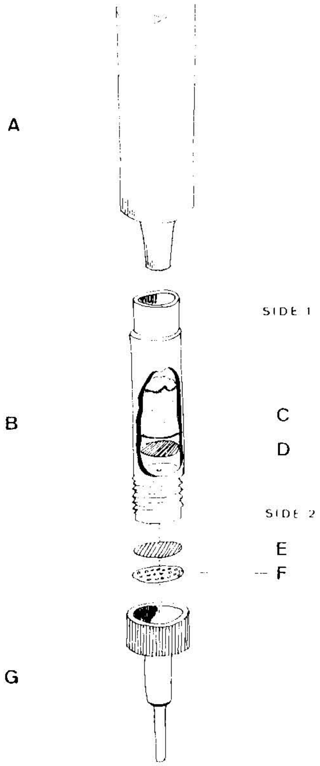

Fig. 3. Illustration of the apparatus set up for filtration after collection of the sorbent. A = Syringe; B = collector section; C = collected sorbent; D = collector filter; E = paper filter; F = Millipore filter; G = filtration section.

Swedish filter-paper; Grycksbo Pappersbruk, Grycksbo, Sweden; No. 2; A2-80-150) (E, Fig. 2), both with a diameter of 5.5 mm, are obtained by cutting the materials with a paper perforator. The collector filter (D, Fig. 2) stops the sorbent in the collector section; it has a diameter of 4 mm and is obtained by perforating a tissue of Kleenex Medical Wipes with a leather perforator.

OPERATION

After deciding which component on a thin layer has to be isolated, the operator brings a collector filter to side 1 of the collector section and pushes it to the bottom of the collector section with the aid of a small PTFE bar of diameter 3.8 mm and length 5 cm, then presses the narrow end of the conical tip into the collector section, also at side 1. Side 2 of the collector section is connected with a suction pump and the conical tip is moved over the thin-layer plate (Fig. 2). After the sorbent has been transferred into the collector section, the suction pump is disconnected and the conical tip is removed. The Millipore filter and the paper filter are placed in the filtration section and the collector section is connected with the filtration section (Fig. 3).

About 50–100 μl of a suitable eluting solvent are placed in the collector section at side 1 (Fig. 3); finally, the fluid is pressed through the device with the help of a syringe (*e.g.*, Inaltera Supra, Henke-Sass Wolf, Tuttlingen, G.F.R., Luer Lok system; a 20-ml syringe is adequate) connected at side 1 (Fig. 3). The clear solution of the compound under investigation thus obtained can, without further purification and concentration steps, be injected into a high-pressure chromatograph or subjected to mass spectrometry.

ACKNOWLEDGEMENTS

I thank Dr. A. Hulshoff for his critical reading of the manuscript, the technical staff for the technical drawings and construction of the device and Mr. P. M. C. Parel for the illustrations.

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Note

Extraction and determination of o-phenylphenol and biphenyl in citrus fruits and apples

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The fungicides biphenyl and *o*-phenylphenol are widely used for the post-harvest protection of citrus fruits and apples. Dipping of citrus fruits in *o*-phenylphenol and the maintenance of a certain biphenyl concentration in the surrounding air, by means of wrappings saturated with biphenyl, for instance prevents spoilage during shipment and storage. Biphenyl and *o*-phenylphenol are commonly separated from citrus fruits and apples by steam distillation and subsequent extraction with an organic solvent. The final quantitative determinations are made by UV or visible-light spectrometry[1–6] or gas–liquid chromatography (GLC)[4,7–10]. A liquid chromatographic procedure has also been described[11–13]. Clean-up of the extract is often necessary before the spectrometric determinations and sometimes before the GLC determinations also.

In this paper we describe a simple and rapid procedure for the simultaneous steam distillation and extraction of *o*-phenylphenol and biphenyl with a modified Likens–Nickerson instrument[14]; the compounds are detected with high sensitivity by high-resolution glass capillary column GLC.

EXPERIMENTAL

Reagents

Cyclohexane was of pro analysi grade. Biphenyl and *o*-phenylphenol were purchased from Fluka (Buchs, Switzerland).

Fruit materials

Fruits free of *o*-phenylphenol and biphenyl were obtained from the Citrus Marketing Board of Israel and used within a few days of harvesting.

Steam distillation and extraction

The Likens–Nickerson instrument (Fig. 1), which has been described in detail elsewhere[14], was modified slightly: the inner diameter of the U-tube was 11 mm

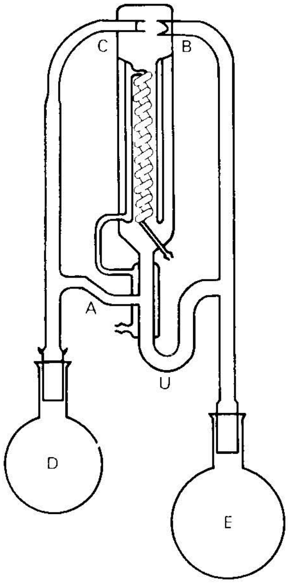

Fig. 1. The Likens–Nickerson instrument.

instead of 9 mm and there was a difference of 10 mm in the heights of the outlets of tube A and the U-tube. It is essential[14] that the outlets of tubes B and C are located so that the vapours are completely mixed.

For the extractions 100 ml of cyclohexane were placed in flask D and 50 g of fruit peel plus 250 ml of distilled water and 20 ml of orthophosphoric acid were added to flask E. A cold trap of dry-ice was used to prevent the loss of biphenyl and *o*-phenylphenol. The contents of the flasks were boiled, the boiling being adjusted so that 2–3 drops of water fell into the U-tube every 10 sec.

After the extraction, a few grams of dry sodium sulphate were added in order to dry the cyclohexane. The GLC determinations were carried out after drying for 15 min. When the concentrations of *o*-phenylphenol and biphenyl were very low, the cyclohexane was concentrated to 1 ml under vacuum.

Gas chromatography

The GLC glass capillary columns were constructed from soda-glass and had an inner diameter of 0.3 mm and lengths of 25–100 m. The inner surfaces of the columns were deactivated with Carbowax 20M as described by Blomberg[15] and the method of Schomburg *et al.*[16] was used for coating the columns with FFAP liquid phase. The non-polar liquid phases OV-101 and OV-1 and a semi-polar phase OV-17 were also used successfully to separate biphenyl and *o*-phenylphenol. The OV columns were constructed as described by Grob and co-workers[17,18].

Carlo-Erba 2300 and Hewlett-Packard 5700 instruments were used. The injections were made by the splitless technique. Hydrogen or helium was used as the carrier gas and the chromatographs were programmed from 50 to 270° at 5–10°/min. A flame-ionization detector was used, but mass spectrometry was used to confirm that the compounds eluted from the column were undecomposed.

Quantitative analyses were bases on the peak areas on the chromatograms obtained from the samples and from a reference solution containing known concentrations of *o*-phenylphenol and biphenyl. For very high accuracy an internal standard can be added to the cyclohexane after the extraction procedure. *m*-Phenylphenol is suitable for this purpose, although the different GLC responses have to be taken into account.

RESULTS AND DISCUSSION

Fig. 2 shows the recoveries obtained when 70 or 20 mg/kg of biphenyl and 10 or 2.5 mg/kg of *o*-phenylphenol were added to fresh oranges (A), grapefruit (B), lemons (C) and apples (D) and allowed to stand for 2 days before the extraction procedure.

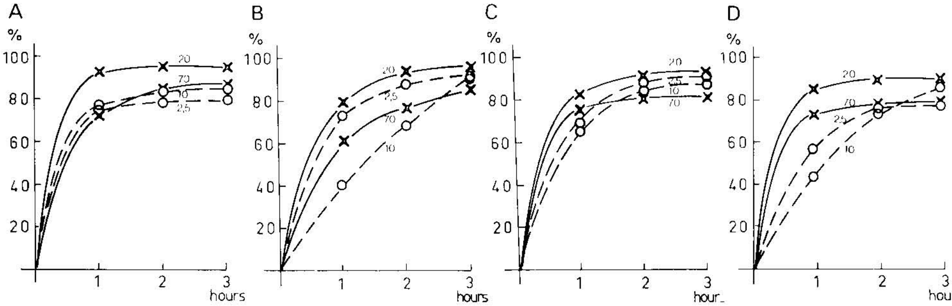

Fig. 2. Recoveries obtained in extracting biphenyl (solid lines) and *o*-phenylphenol (broken lines) from oranges (A), grapefruit (B), lemons (C) and apples (D). The numbers on the curves refer to concentrations of biphenyl and *o*-phenylphenol in parts per million.

Similar experiments were also carried out with 40 ppm of biphenyl and 5 ppm of *o*-phenylphenol and the results were analogous. In most instances 1 h is sufficient for the extraction of biphenyl with a high recovery, whereas 2–3 h are normally needed for the extraction of *o*-phenylphenol.

Fig. 3 shows gas chromatograms obtained from the cyclohexane extracts, without concentration, from grapefruit containing 5 ppm of biphenyl and 5 ppm of *o*-phenylphenol (A), oranges containing 5 ppm of *o*-phenylphenol and 0.1 ppm of biphenyl (B) and apples containing 5 ppm of *o*-phenylphenol and 50 ppm of biphenyl. The samples were steam distilled and extracted for 60 min.

There are a number of methods for determining *o*-phenylphenol and biphenyl in citrus fruits[1-7]. However, the method described here has several advantages, as follows.

The use of the Likens–Nickerson instrument permits the simultaneous steam distillation and extraction of *o*-phenylphenol and biphenyl in a procedure that takes only a few hours. In practice, after treatment with *o*-phenylphenol the citrus fruits are sprayed with wax. This makes it difficult to extract *o*-phenylphenol with a high recovery. It seems, however, that the Likens–Nickerson instrument provides conditions that are severe enough for recoveries of more than 90% to be obtained, even

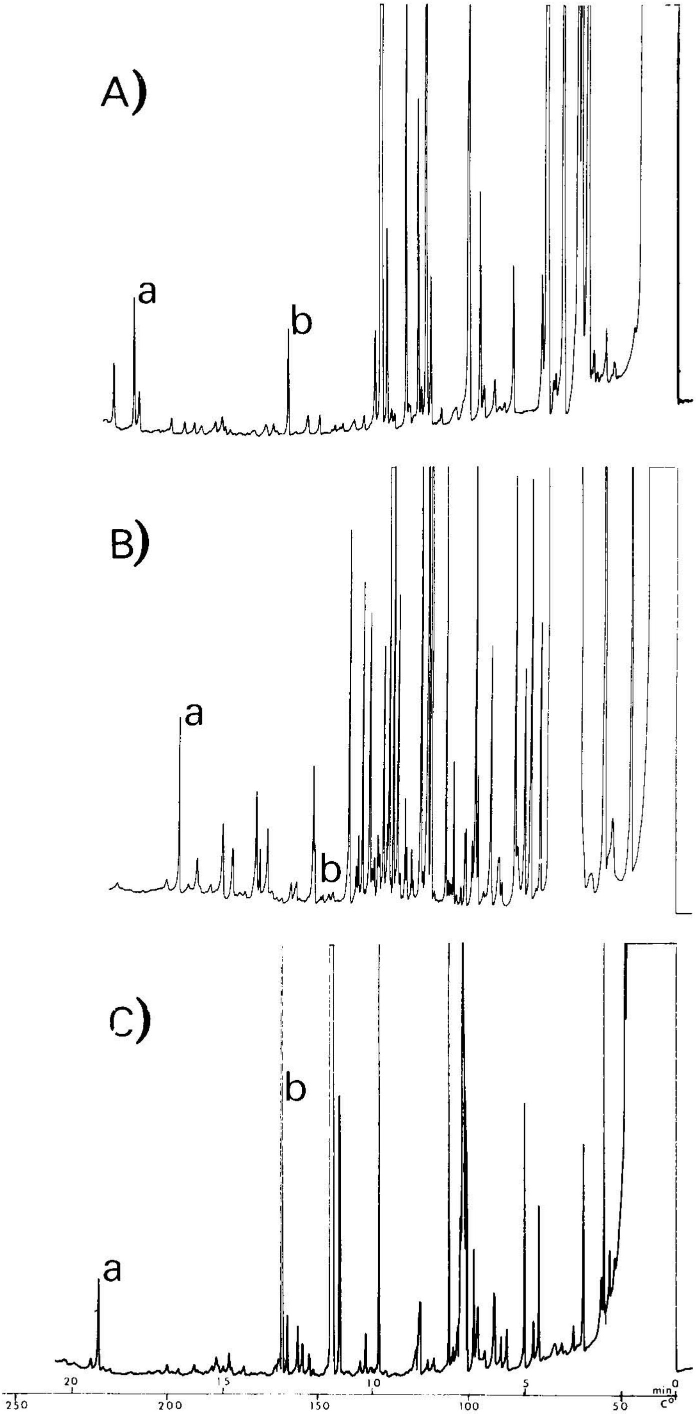

Fig. 3. Gas chromatograms obtained from (A) grapefruit containing 5 ppm of biphenyl and 5 ppm of *o*-phenylphenol, (B) oranges containing 5 ppm of *o*-phenylphenol and 0.1 ppm of biphenyl and (C) apples containing 5 ppm of *o*-phenylphenol and 50 ppm of biphenyl. Column: 30-m FFAP glass capillary. Carrier gas (hydrogen) flow-rate: 2 ml/min.

from samples that have been stored for several months. Thus, when classical steam distillation and extraction with cyclohexane were first used and the samples were then re-treated by the present method, we regularly found an additional 20–100% of *o*-phenylphenol.

The Likens–Nickerson instrument permits vacuum conditions to be used and hence a low temperature, which can be considered advantageous when wórking with unstable compounds.

With high-resolution glass capillary-column GLC, no concentration of the cyclohexane extract is needed. Conventional GLC with packed columns can also be used, but the risk of error increases with the lower separation ability of such columns. Moreover, compared with glass capillary columns the packed columns give a considerably lower sensitivity and greater concentration of the cyclohexane extract may be needed.

Using the method described here, a trained technician can work with several instruments at the same time, and with the proper equipment ten or more analyses can be carried out per day.

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The authors thank Mr. M. Davidson of the Citrus Fruits Marketing Board of Israel for providing fresh fruits free of biphenyl and *o*-phenylphenol and for valuable discussions.

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Note

Separation of chlorophenols and chlorocresols by high-performance liquid chromatographic and gas–liquid chromatographic techniques

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Separation of chlorophenols and chlorocresols using paper chromatographic[1], pH–chromatographic[2], thin-layer chromatographic (TLC)[3,4], gas–liquid chromatographic (GLC)[5] and high-performance liquid chromatographic (HPLC)[6] techniques has been reported. However, none of these methods is satisfactory for the complete analysis of chlorophenols and chlorocresols. Chlorophenols have been recently determined as derivatives in effluent samples by capillary GLC[7]. Separation of chlorophenols and chlorocresols using GLC and HPLC techniques is described here.

EXPERIMENTAL

Chlorophenols and chlorocresols (99% pure) were obtained from Fluka (Faliric) (Buchs, Switzerland) and Aldrich (Milwaukee, Wisc., U.S.A.) and used as such. Cyclohexane and chloroform were pure and did not show any impurities in the 250–400 nm range.

High-performance liquid chromatography

A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC/GPC/244 high-performance liquid chromatograph equipped with Model 6000 A solvent delivery system, Model U6K septumless injector and Model 440 dual channel absorbance detector at 254 nm was used. The pumps are capable of operation at pressures up to 400 bar. A prepacked column of μPorasil of 10 μm (30 cm × 4 mm I.D.) obtained from Waters Assoc. was used. The operating conditions are given in Figs. 3 and 4. Solutions (0.05%) of chlorophenols and chlorocresols were prepared in chloroform and 2.0-μl samples were injected.

Gas–liquid chromatography

An F & M Model 720 dual column programmed temperature gas chromatograph equipped with a thermal conductivity detector and hydrogen as carrier gas was used. The following stationary phases were employed: silicone elastomer-30 (SE-30), diethylene glycol succinate (DEGS) and Carbowax 20M (CW 20M). The preparation and packing of the stationary phase liquid material were carried out in accordance with methods reported in the literature[8]. The operating conditions are given in Table I.

TABLE I

CONDITIONS FOR GLC SEPARATION OF CHLOROPHENOLS AND CHLOROCRESOLS

	SE-30	*DEGS*	*Carbowax 20M*
Support and content of stationary liquid phase	5% (w/w) on Chromosorb P, 45–60 mesh	15% (w/w) on Chromosorb W, 45–60 mesh	25% (w/w) on Chromosorb P, 45–60 mesh
Column length (ft.)	8	8	8
Carrier gas	hydrogen	hydrogen	hydrogen
Flow rate (l/h)	7.2	3.6	7.2
Column temperature (°C)	200 ± 2	200 ± 2	200 ± 2
Bridge current (A)	150	150	150

RESULTS AND DISCUSSION

The retention times relative to phenol of chlorophenols are given in Table II. These chlorophenols were poorly resolved on SE-30: DEGS gave better results. It can be seen from Table II that all the chlorophenols except 2,4-dichlorophenol and 2,5-dichlorophenol were separated on CW 20M. Further, certain pairs of these compounds (*e.g.*, 2,6-dichlorophenol and *p*-chlorophenol, and 2,4,5-trichlorophenol and 2,4,6-trichlorophenol), which have nearly the same same vapour pressures, are easily separated on CW 20M. Compounds having more *ortho*-substituted chlorine atoms in the benzene nucleus, despite their higher boiling points, emerged earlier than those having lower boiling points but with chlorine atoms in *para*- or *meta*-positions (Table II). The earlier elution of these compounds can be explained in terms of the *ortho*-effect[9]. Typical separation of chlorophenols on CW 20M is shown in Fig. 1. It can be seen that *ortho*-chlorine substituted compounds give sharp peaks whereas broad peaks are obtained for those compounds having chlorine atoms in *meta*- and *para*-positions.

TABLE II

RELATIVE RETENTION TIMES OF CHLOROPHENOLS IN GLC

Peak No. (Figs. 1 and 3)	*Compound*	*Boiling point (°C)*	*Relative retention time (relative to phenol)*		
			SE-30	*DEGS*	*CW 20M*
1	*o*-Chlorophenol	175	1.00	0.84	0.60
2	Phenol	180	1.00	1.00	1.00
3	2,6-Dichlorophenol	219	2.50	2.12	1.43
4	2,4-Dichlorophenol	210	1.87	2.08	1.65
5	2,5-Dichlorophenol	211	1.87	2.08	1.65
6	2,4,6-Trichlorophenol	246	2.67	3.73	2.82
7	*p*-Chlorophenol	219	3.12	3.45	3.73
8	2,4,5-Trichlorophenol	244	3.00	–	5.04
9	2,3,4,5-Tetrachlorophenol	–	5.00	–	7.17
10	3,5-Dichlorophenol	233	4.00	–	10.69

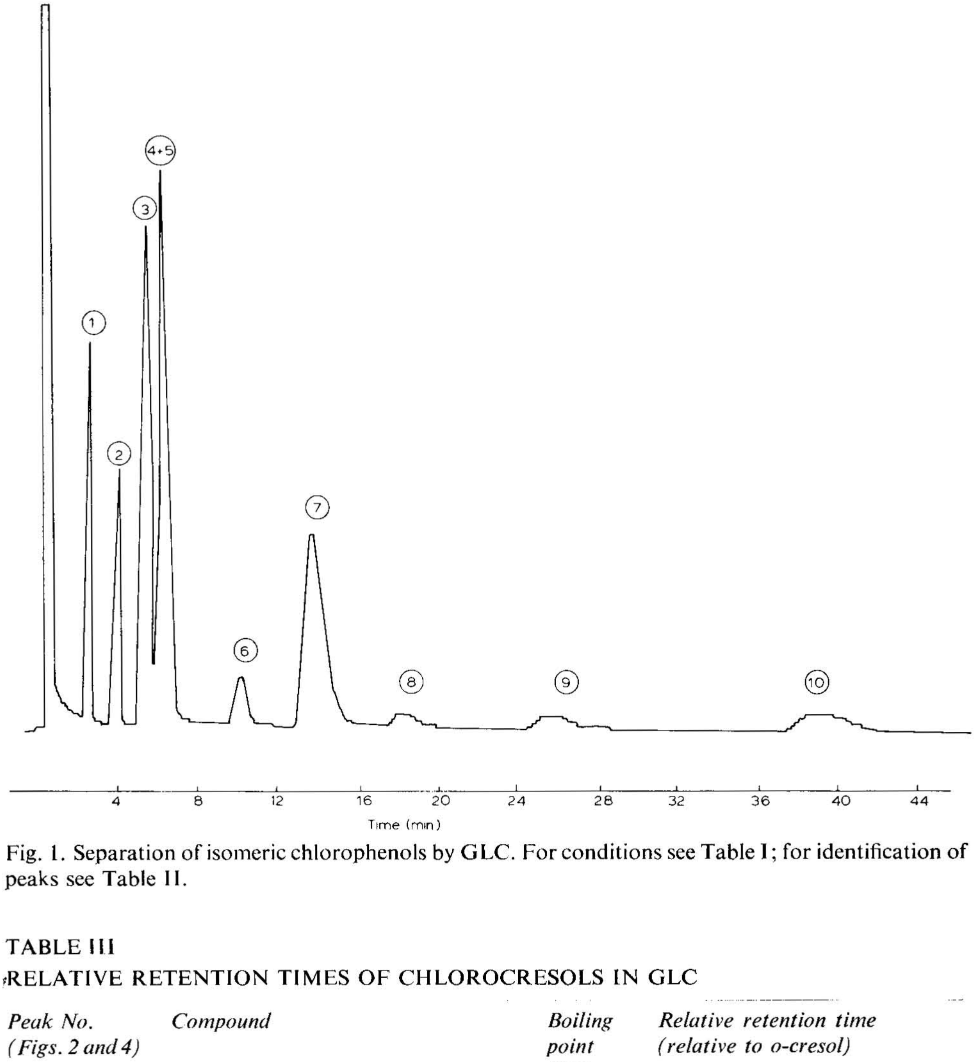

Fig. 1. Separation of isomeric chlorophenols by GLC. For conditions see Table I; for identification of peaks see Table II.

TABLE III

RELATIVE RETENTION TIMES OF CHLOROCRESOLS IN GLC

Peak No. (Figs. 2 and 4)	*Compound*	*Boiling point (°C)*	*Relative retention time (relative to o-cresol)*		
			SE-30	*DEGS*	*CW 20M*
1	6-Chloro-*o*-cresol	188	0.62	0.87	0.63
2	*o*-Cresol	191	1.00	1.00	1.00
3	*m*-Cresol	202	2.00	1.56	1.18
4	*p*-Cresol	202	2.00	1.56	1.18
5	6-Chloro-*m*-cresol	197	2.16	—	1.50
6	2,4-Dichloro-*m*-cresol	235	3.00	—	2.40
7	4-Chloro-*o*-cresol	220	3.00	3.45	3.90
8	4-Chloro-*m*-cresol	235	3.00	—	4.06

Table III gives the relative retention times (relative to *o*-cresol) of chlorocresols. The general trend for the order of elution of these chlorocresols (Table III) and chlorophenols (Table II) was found to be similar on all the liquid stationary phases used. Almost all the chlorocresols except *m*- and *p*-cresol were separated on CW 20M. A typical chromatogram showing the separation of a mixture of chlorocresols on CW 20M is shown in Fig. 2.

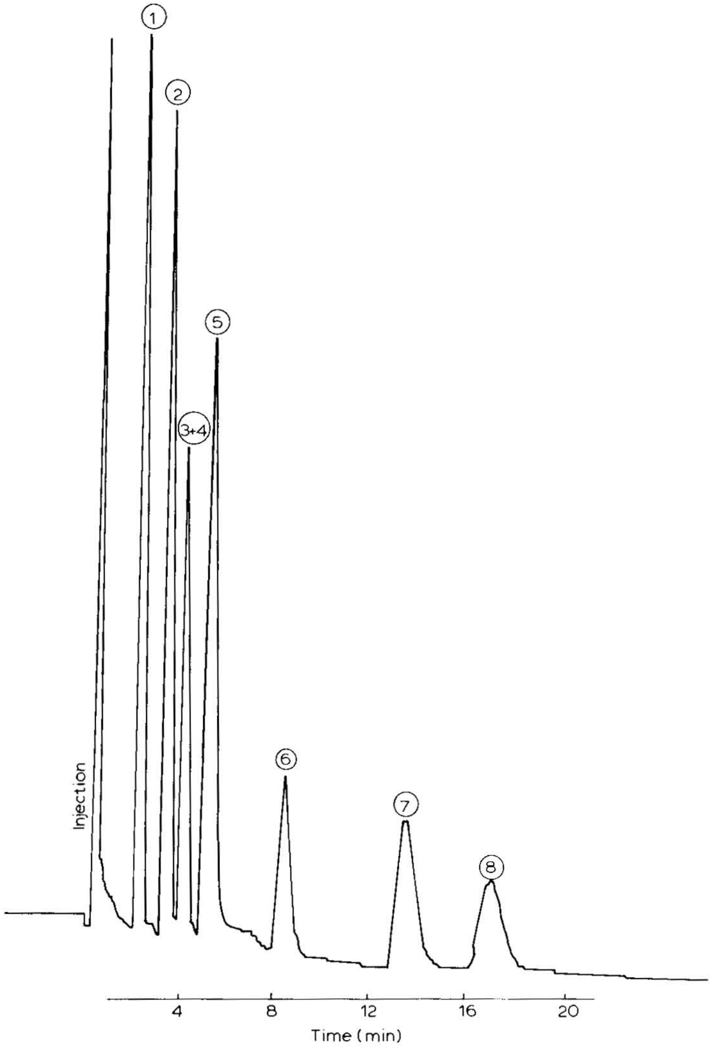

Fig. 2. Separation of isomeric chlorocresols by GLC. For conditions see Table I; for identification of peaks see Table III.

Separation of chlorophenols by HPLC is shown in Fig. 3. Almost all the chlorophenols were separated by HPLC. However, the separation of 2,3,4,5-tetrachlorophenol was not satisfactory as it appeared only as a shoulder of *o*-chlorophenol. Certain compounds such as 2,4-dichlorophenol and 2,5-dichlorophenol which could not be separated by GLC (Fig. 2) were separated by HPLC.

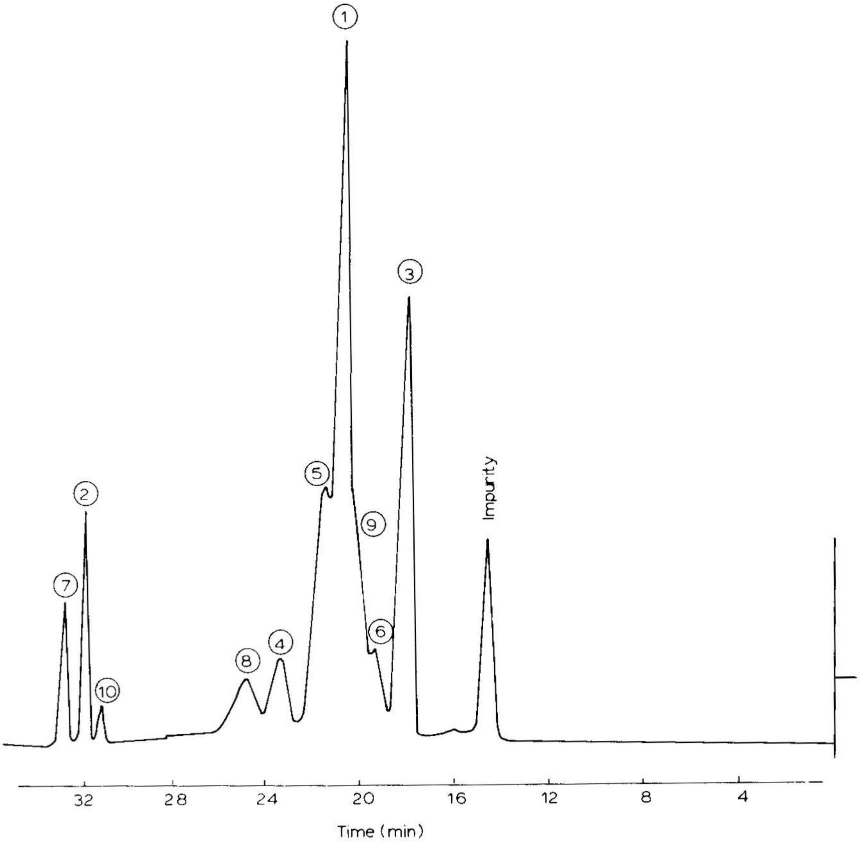

Fig. 3. Separation of chlorophenols by HPLC. For identification of peaks see Table II. Conditions: 300 × 4 mm I.D. column of μPorasil; pressure, 250 p.s.i.; eluent, chloroform–cyclohexane (7:3); flow-rate, 0.3 ml/min.

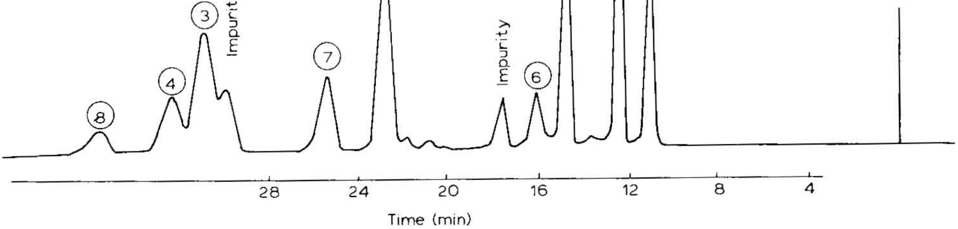

Fig. 4. Separation of chlorocresols by HPLC. For identification of peaks see Table III. Conditions: 300 × 4 mm I.D. column of μPorasil; pressure, 285 p.s.i.; eluent, chloroform–cyclohexane (7:3); flow-rate, 0.4 ml/min.

Separation of chlorocresols by HPLC is shown in Fig. 4. It is evident that all the chlorocresols can be separated by HPLC. *m*-Cresol and *p*-cresol were separated by HPLC (Fig. 4) but not by GLC (Table III, Fig. 2).

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CHROM. 11,398

Note

Separation and quantification of trace isomeric hydroxyphenols in aqueous solution by high-performance liquid chromatography

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(Received August 22nd, 1978)

In order to understand fully the reaction of hydroxyl radical, generated radiolytically, with phenol, it was necessary to develop a rapid and sensitive method for the detection and quantification of the low yields of the products formed. In the presence of radical scavenger, *p*-benzoquinone, under certain radiolytic conditions the products are exclusively *o*-, *p*- and *m*-hydroxyphenols. Separation of the isomeric hydroxyphenols on a column of Merckogel PGM 200 has been reported recently[1]. Wulf and Nagel[2] have used a μBondapak C_{18} column for the analysis of phenolic acids and flavonoids by high-performance liquid chromatography. Due to widespread use of the μBondapak C_{18} columns, we have used this stationary phase for the separation of isomeric hydroxyphenols.

EXPERIMENTAL

The main features of the liquid chromatograph used have been previously described[3,4]. The apparatus contained the following components· Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A pump, a 150-μl six-port injection valve. The chromatographic column used was a μBondapak C_{18} obtained from Waters Assoc. The detector was a Vari-Chrom UV–Vis variable-wavelength detector obtained from Varian Instruments (Palo Alto, Calif., U.S.A.). The photometric range of the detector corresponds to 0.005–2.0 a.u.f.s. In most of the studies, 0.02 a.u.f.s., 100 mV, was used. The flow-rate was 1.4 ml/min at an operating pressure of 1000 p.s.i. The detection was primarily carried out at 280 nm, where phenol has a maximum absorbance and all the dihydroxyphenols also have high absorbance. Detection was also made at 260 nm where *p*-benzoquinone has a higher extinction coefficient than at 280 nm. The absorption spectra were taken using a Cary 219 UV–visible spectrophotometer.

RESULTS AND DISCUSSION

Using water containg 0.01 *M* phosphate buffer at pH 7.0 as the mobile phase, the chromatogram obtained at $\lambda = 280$ nm of a synthetic mixture of isometic hydroxyphenols, phenol and benzoquinone is shown in Fig. 1a. As evident from this figure, the retention times of resorcinol and catechol are the same and the rest of the com-

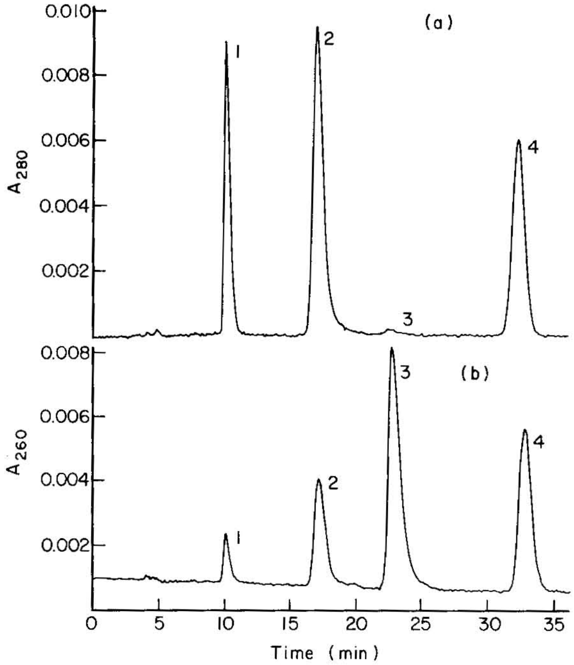

Fig. 1. (a) Separation of isomeric hydroxyphenols from phenol, and benzoquinone on a μBondapak C_{18} column with water containing 0.01 *M* phosphate buffer at pH 7.0 as the mobile phase. Peaks: 1 = hydroquinone (31 μ*M*), 2 = resorcinol (22 μ*M*) + catechol (37 μ*M*), 3 = benzoquinone (38 μ*M*), 4 = phenol (20 μ*M*). The detection was carried out at 280 nm. (b) Experimental conditions are the same as in (a) except the detection was carried out at 260 nm.

ponents are well separated. Benzoquinone has very low absorbance at this wavelength and, hence, elutes as a small spike. The extinction coefficient of benzoquinone is quite high at 260 nm compared to 280 nm and, hence, in the chromatogram obtained at 260 nm the peak due to benzoquinone is much more pronounced (Fig. 1b). In order to separate resorcinol (*meta* isomer) from catechol (*ortho* isomer), ethanol–water mixtures and acetonitrile–water mixtures as mobile phases were tried and the separation was not achieved, even though all the peaks became sharper compared to the peaks obtained using water as the mobile phase. The separation of resorcinol from catechol was achieved using 4% (v/v) of 1,4-dioxane–water mixture as the mobile phase and the chromatogram obtained at 280 nm is shown in Fig. 2a. The chromatogram obtained at 260 nm is shown in Fig. 2b. The retention times of all the components have become shorter using dioxane–water mixtures as the mobile phase. Moreover, the peaks are much sharper. With increasing concentration of dioxane the retention times become shorter and above 15% dioxane–water mixture, both hydroquinone and benzoquinone eluted very close to the solvent front. It was possible to detect 2 μ*M* of each of the components within ± 2% accuracy. The peak heights were linear with concentration from 1 μ*M* tp 1 m*M*. It was possible to detect 10 ppb*

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* Throughout this article the American billion (10^9) is meant.

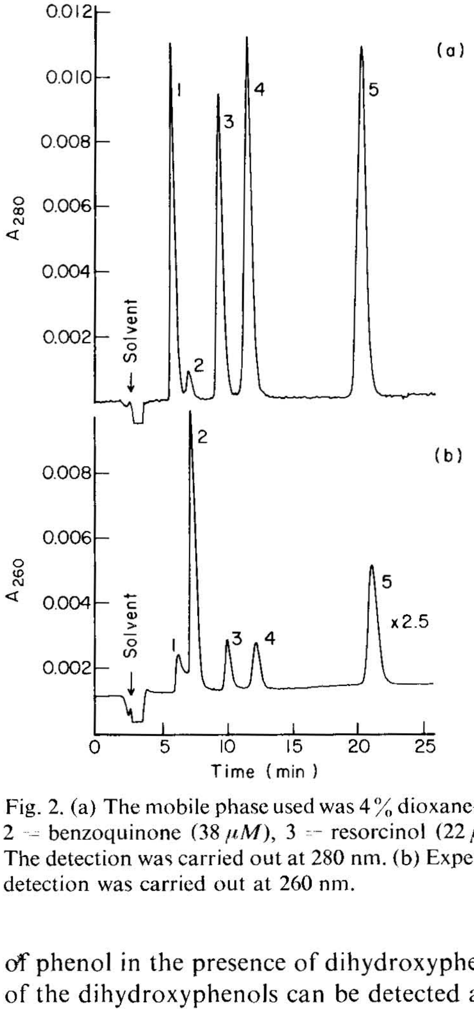

Fig. 2. (a) The mobile phase used was 4% dioxane–water mixture. Peaks: 1 = hydroquinone (31 μM), 2 = benzoquinone (38 μM), 3 = resorcinol (22 μM), 4 = catechol (37 μM), 5 = phenol (20 μM). The detection was carried out at 280 nm. (b) Experimental conditions are the same as in (a) except the detection was carried out at 260 nm.

of phenol in the presence of dihydroxyphenols within ± 4% accuracy. Similarly, any of the dihydroxyphenols can be detected at a level of 10 ppb within ± 4% accuracy.

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Note

Method for fluorescence detection in the high-performance liquid chromatography of Δ^4-3-ketosteroids

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(Received June 20th, 1978)

We have recently developed a fluorometric method for the determination of Δ^4-3-ketosteroids[1]; the steroids react with isonicotinylhydrazine (INH) in a methanolic aluminium(III) salt solution to form hydrazones, which fluoresce owing to complex formation with aluminium ions.

This paper reports the application of the reaction to fluorescence detection in high-performance liquid chromatography of Δ^4-3-ketosteroids, where the UV detection method has previously been exclusively used.

EXPERIMENTAL

Reagents

Isonicotinylhydrazone of testosterone was prepared by the procedure of Umberger[2]. Other chemicals were obtained from commercial sources. A 16 m*M* INH solution was prepared by dissolving INH in methanol, and a 80 m*M* aluminium chloride solution by dissolving $AlCl_3 \cdot 6H_2O$ in methanol. The solutions were stable for several weeks at room temperature in brown bottles.

Liquid chromatography

Fig. 1 shows the complete liquid chromatographic system. Instruments and materials constituting the system were obtained from Kyowa Seimitsu Co. (Tokyo, Japan) unless otherwise stated.

The column for the separation of cortisol (F), corticosterone (B), deoxycorticosterone (DOC), 4-androstene-3,17-dione (AD), and progesterone (P) was 500 × 2 mm I.D. Hitachi Gel 3011 (Hitachi, Tokyo, Japan), a porous styrene–divinylbenzene copolymer of average particle diameter *ca.* 10 μm. The resin was packed in a stainless steel tube by a slurry technique at 2000 p.s.i. The steroids were eluted with *n*-hexane–methanol (5:95) at a flow-rate of 0.26 ml/min. The column inlet pressure was 780 p.s.i.

A Zorbax-Sil column, 250 × 2.1 mm I.D. (DuPont, Wilmington, Del., U.S.A.), and the eluent reported by Trefz *et al.*[3] were employed with a slight modification to

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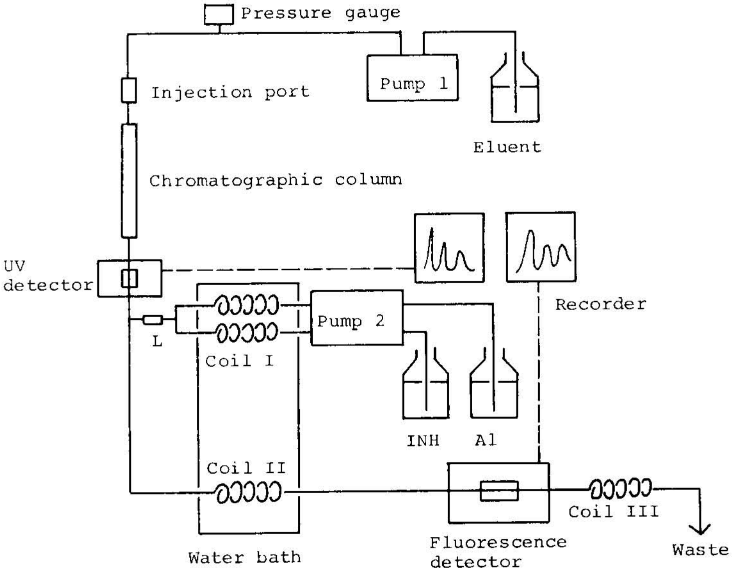

Fig. 1. Schematic diagram of the chromatographic system.

separate B, cortisone (E), F, and prednisolone (PL). The eluent was prepared as follows: 7 ml of distilled water and 12 ml of ethanol in a volumetric flask were made up to 500 ml with dichloromethane. The mixture was stirred vigorously for 1 h at 18° with a magnetic stirrer and allowed to stand overnight; the separated organic phase was used. The flow-rate for elution was 0.42 ml/min at 1200 p.s.i.

Each column was fitted with an on-column injection port, and sample solutions were injected through a PTFE rubber septum using a 5-μl high-pressure Hamilton syringe. The columns were operated at 18°.

Detection system

The column effluent was first monitored by a UV detector (Yanaco UV-212, Yanagimoto Co., Kyoto, Japan) at 254 nm, then added to a mixture of INH and aluminium chloride solution. Each reagent solution, heated for *ca.* 3.7 min in a PTFE tube (Coil I, 0.5 mm I.D.) immersed in a water bath maintained at 70°, was delivered separately by a double plunger pump (Pump 2) at half the flow-rate of the effluent. The solutions were combined just before the mixing with the effluent. The reaction mixture was passed through a reaction coil (Coil II, 20 m × 0.25 mm I.D. PTFE tube) in the same water bath and was monitored by a FLD-1 fluorescence detector (Shimadzu, Kyoto, Japan), which was equipped with a low-pressure mercury lamp of 360 nm and a secondary filter that transmits above 450 nm. The outlet of the detector was attached to Coil III (10 m × 0.25 mm I.D. PTFE tube), which provided enough back pressure to prevent bubble formation in the flow-cell. The recorders were EPR-2TC Electronic Polyrecorders (Toa Electronics, Tokyo, Japan).

The reaction times for the systems of Hitachi Gel 3011 column (System I) and Zorbax-Sil column (System II) were about 2.0 and 1.2 min, respectively.

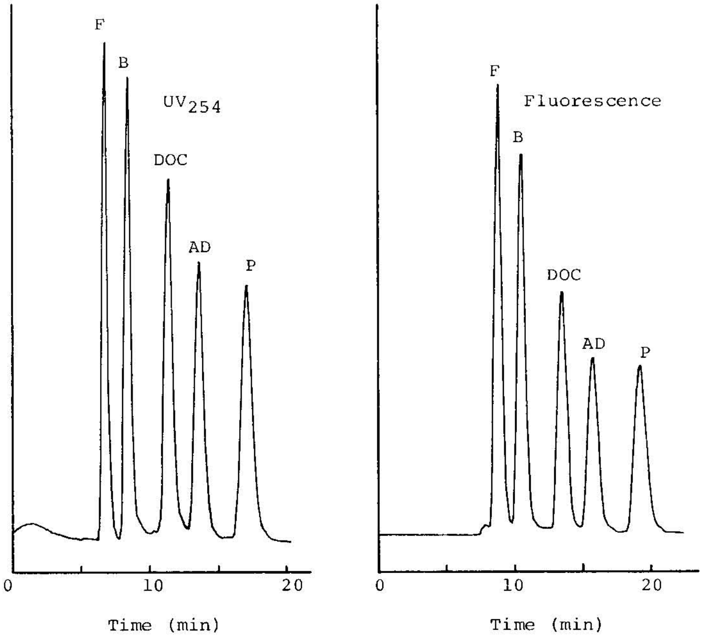

Fig. 2. Chromatograms of Δ^4-3-ketosteroids by UV and fluorescence detection. Column: Hitachi Gel 3011 (500 × 2 mm I.D.). 1 nmol of each steroids was injected.

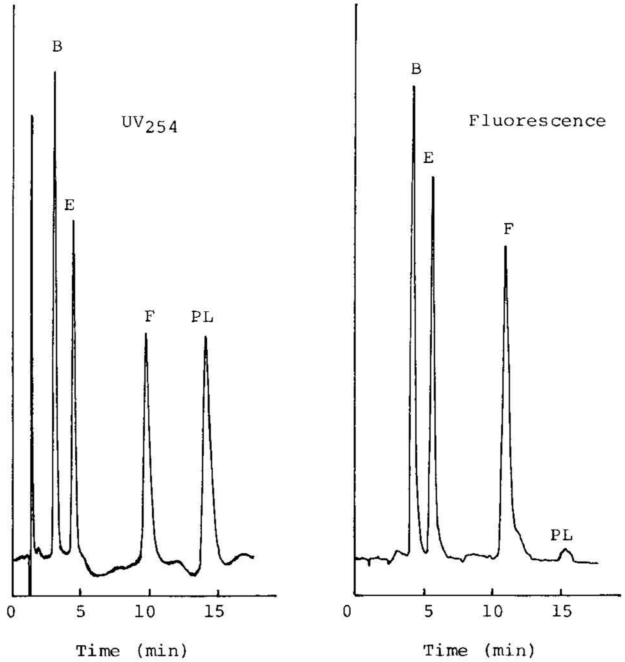

Fig. 3. Chromatograms of Δ^4-3-ketosteroids and prednisolone by UV and the fluorescence detection. Column: Zorbax-Sil (250 × 2.1 mm I.D.). The amount of each steroid injected was 0.5 nmol.

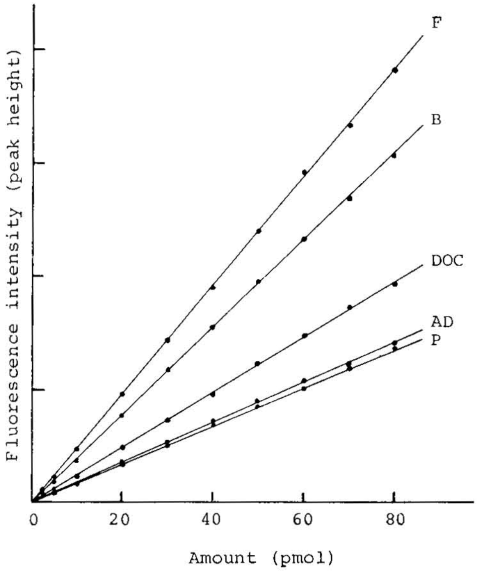

Fig. 4. Calibration curves of Δ^4-3-ketosteroids by fluorescence detection. Column: Hitachi Gel 3011 (500 × 2 mm I.D.).

RESULTS AND DISCUSSION

The steroids were derivatized to the fluorophors without serious distortion of the peak shapes (Figs. 2 and 3). The hydrazone of Δ^4-3-ketosteroid seemed to be formed quantitatively in both Systems I and II under the reaction conditions, as 50 pmol methanolic solutions of testosterone and isonicotinylhydrazone of testosterone injected into the reaction device by a line sample injector (L in Fig. 1) both gave equal fluorescence intensities in each system. The very weak response of the fluorescence detector to prednisolone (PL in Fig. 3) compared with those to Δ^4-3-ketosteroids was mainly due to the slower hydrazone formation of $\Delta^{1,4}$-3-ketosteroid with INH.

In System I, the calibration curves of peak heights against amounts of Δ^4-3-ketosteroids by the fluorescence detection were linear up to several nmol, and 2.5 pmol of cortisol could be detected (Fig. 4). The peak heights were reproducible and the coefficient of variation was 0.9% with 50 pmol of cortisol for five determinations. In System II, the fluorescence intensity of Δ^4-3-ketosteroid decreased to less than one-sixth of that in System I owing to the solvent effect of dichloromethane on the fluorescence[1] and to the greater flow-rate of eluent.

Though the sensitivity of the fluorescence detection is no better than that reported for UV detection[3,4], the selectivity of the fluorescence reaction will be useful for the analysis of the steroids in complex biological samples.

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CHROM. 11,372

Note

Determination of caprolactam and its oligomers by means of liquid chromatography

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(Received July 3rd, 1978)

The separation of a mixture of caprolactam oligomers and their identification by paper chromatography was investigated by Katorzhnov[1]. The determination of the components of such a mixture by various analytical methods has been reviewed[2]. Caprolactam has been analyzed by gas chromatography[3–7], and its oligomers separated by gel permeation chromatography[7–10] using various types of Sephadex and Bio-Gel. For this study, we have used liquid chromatography in columns packed with silica gel.

EXPERIMENTAL

Chemical

Technical caprolactam (purity 99.9%, Spolana, Neratovice, Czechoslovakia) was purified by crystallization three times from benzene and dried at 260 Pa; m.p. 67–69°. The cyclic dimer of caprolactam was obtained by repeated crystallization of a technical mixture of caprolactam oligomers from water; m.p. 342°. These substances were employed as the standards.

A mixture of caprolactam oligomers was prepared from the water extract obtained in the production of polycaprolactam chips (Považské Chemické Závody, Žilina, Czechoslovakia), which comprises a suspension of 5.8% of undissolved compounds. The solid part, containing[11] 49% of caprolactam and 51% of prevalently cyclic oligomers, was filtered, dried and repeatedly extracted with benzene. The content of monomer decreased after each extraction. For the testing of the analytical method, the monomer and cyclic dimer were added to the final mixture. Paper chromatography[12] was used to detect the presence of individual oligomers.

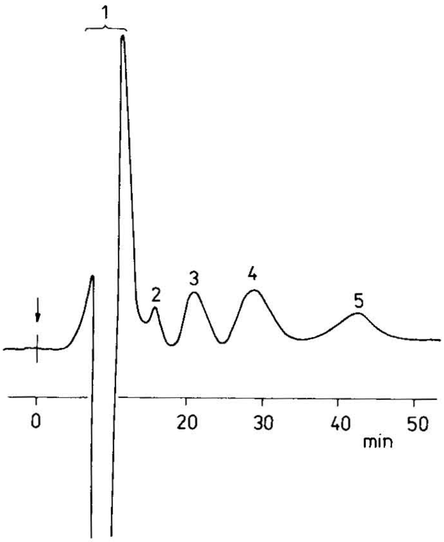
Liquid chromatography

A simple apparatus constructed in the Institute of Macromolecular Chemistry was equipped with a Model 2025-50 through-flow differential refractometer (Knauer, Oberursel, G.F.R.) and with two types of glass column. The column (120 cm × 2 mm) used for the eluent tetrahydrofuran–heptane–water (93:7:5) was packed with Silpearl (Kavalier, Votice, Czechoslovakia), particle size 30–40 μm; the operating pressure was 2.5 MPa, the flow-rate 0.5 ml/min and the temperature 25°. The column

(30 cm × 5 mm) used with the system 1-butanol–glacial acetic acid–water (7:3:1) was packed with the globular porous silica gel SG50I1-7.5KP, which was prepared in the Institute of Macromolecular Chemistry; the operating pressure was 1.5 MPa and the flow-rate 0.7 ml/min at 26°. The amount of injected material ranged from 5 to 10 μg.

RESULTS AND DISCUSSION

The analysis of caprolactam and its oligomers was investigated by means of liquid chromatography in columns packed with silica gel. The results for the oligomers were obtained with the eluent tetrahydrofuran–heptane–water (93:7:5). Owing to the limited solubility of the oligomers, it was necessary to add several drops of formic acid to complete the dissolution of the analyzed substrate. The presence of formic acid does not interfere with the determination of the individual oligomers (Fig. 1). However, if the monomer is also present in the mixture, its identification is disturbed by the peak of formic acid. An unambiguous assignment of the presence of the cyclic dimer of caprolactam in a mixture with the monomer is also difficult due to the small difference between their elution times. The dimer peak overlapped that of the caprolactam present in large excess in the mixture. The tetrahydrofuran–heptane–water eluent may be used for determining the composition of a mixture of caprolactam oligomers free from monomer.



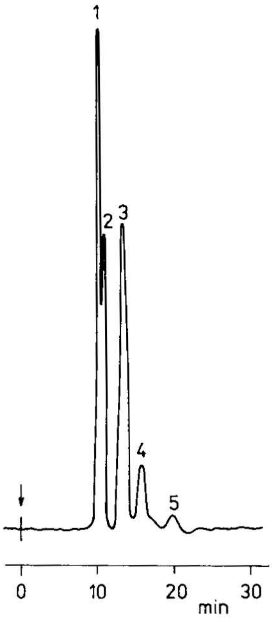


Fig. 1. Chromatogram of the separation of a mixture of caprolactam oligomers on silica gel using tetrahydrofuran–heptane–water (93:7:5) as eluent in the presence of formic acid. Peaks: 1 = formic acid; 2 = dimer; 3 = trimer; 4 = tetramer; 5 = pentamer.

Fig. 2. Typical chromatographic separation of a mixture of the monomer and oligomers of caprolactam on silica gel using 1-butanol–glacial acetic acid–water (7:3:1) as eluent. Peaks: 1 = monomer; 2 = dimer; 3 = trimer; 4 = tetramer; 5 = pentamer.

A complete dissolution and separation was attained with the eluent 1-butanol–100% formic acid–water (75:20:10). However, formic acid is a potential source of corrosion of some parts of the chromatographic apparatus, and acetic acid is more suitable. In 1-butanol–glacial acetic acid–water (7:3:1), complete dissolution of caprolactam and its oligomers occurred and a good separation was achieved (Fig. 2). The applicability of the method was verified with mixtures containing the monomer and oligomers up to the pentamer and having a known ratio of monomer to dimer.

ACKNOWLEDGEMENT

The authors are indebted to Považské Chemical Works (Žilina) for providing the technical mixture of caprolactam oligomers, to Dr. J. Stehlíček for an authentic sample of cyclic dimer and to Drs. M. Kubín and S. Vozka for the silica gel SG50 used as column packing.

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CHROM. 11,435

Note

High-performance liquid chromatographic determination of metrizamide in plasma

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Metrizamide, 2-{[3-(acetylamino)-5-(acetylmethylamino)-2,4,6-triiodobenzoyl] amino}-2-deoxy-D-glucose, Amipaque®, is a non-ionic, water-soluble radiographic contrast medium intended for lumbar, thoracic and cervical myelography, ventriculography and cisternography. The absorption of metrizamide from cerebrospinal fluid to blood has been reported[1] using a technique based on the analysis of the specimen for its iodine content by a non-specific ashing technique[2].

This paper describes the development of a specific high-performance liquid chromatographic (HPLC) method for metrizamide in plasma. This assay technique has been used to measure quantitatively metrizamide in the plasma of human subjects undergoing radiographic evaluation in subarachnoid diagnostic applications.

EXPERIMENTAL

Materials

Metrizamide is a product of Winthrop Laboratories (New York, N.Y., U.S.A.). The internal standard for the assay, 3-dimethylaminomethyleneimino-2,4,6-triiodobenzoic acid, was synthesized at the Sterling-Winthrop Research Institute. The column was purchased from Whatman (Clifton, N.J., U.S.A.). Other chemicals were obtained commercially (reagent grade) and used without further purification.

Preparation of samples

Plasma standards were prepared by supplementing 1.0 ml of control human plasma with aliquots of an aqueous stock solution of metrizamide (1 mg/ml). Duplicate standards at concentrations of 0, 2, 4, 8, 12, 16 and 24 μg/ml of metrizamide were prepared.

Two sets of randomized and coded plasma samples, to be analyzed under single blind conditions, were prepared as described above. Each set contained quadruplicate plasma samples at final concentrations of 0, 2.5, 5.5, 9, 15 and 19 μg/ml in control human plasma. One set of samples was analyzed immediately and the other set was analyzed following a 5-day storage period in a −4° freezer. Freshly prepared plasma standards were extracted and analyzed concommittantly with each set of unknown samples.

Protein precipitation and analysis

To 1.0 ml of human plasma, containing the disodium salt of N,N′-1,2-ethanediylbis-[N-(carboxymethyl)glycine] (EDTA), as the anticoagulant, was added 50 μl internal standard solution (1.5 mg/ml) and 0.2 ml of zinc sulfate solution (20%, w/v in water). The sample was mixed and 0.2 ml of a saturated aqueous solution of barium hydroxide was added. The tube was again mixed and a marble was placed over the opening of the test tube which was heated on a steam bath for 2 min. The tube was cooled and centrifuged.

A 25-μl aliquot of the clear supernatant solution was analyzed under the following HPLC conditions. Column: Partisil 10 ODS (250 mm × 4.6 mm I.D.) with a Corasil C_{18} precolumn (60 mm × 4 mm I.D.). Detector: Schoeffel Model SF 770 variable wavelength UV detector set at 244 nm. Mobile phase: distilled water–methyl alcohol–glacial acetic acid (94.5:5:0.5). Flow-rate: 2.0 ml/min. Retention time: metrizamide, 3.9 min; internal standard, 11.2 min. Pump: Milton Roy Model 709, 1000 p.s.i. Temperature: 20°, ambient.

Clinical study

Eight patients received a single injection of metrizamide in the subarachnoid space as part of a routine neuroradiological procedure. Blood specimens were obtained at 0, 2, 4, 6, 8, 24 and 48 h after administration. These were collected in vacuum tubes containing EDTA as the anticoagulant. The samples were centrifuged and the plasmas were separated and frozen until the analysis for metrizamide was performed.

RESULTS AND DISCUSSION

Recorder tracings from the HPLC analysis of both a control plasma sample and a sample supplemented with 16 μg/ml of metrizamide are shown in Fig. 1.

A linear regression model was used to describe the relationship between the peak-height ratio of metrizamide to the internal standard and the concentration of

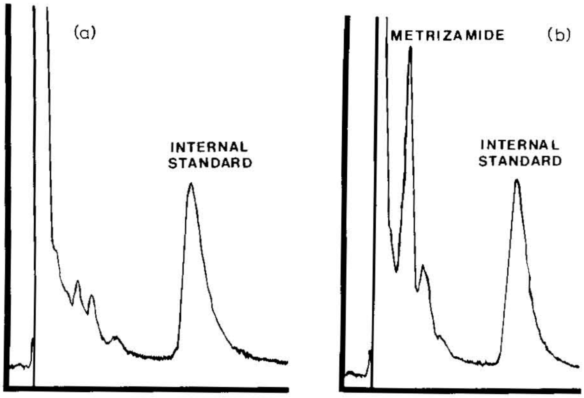

Fig. 1. Chromatogram of control human plasma containing the internal standard (a) and the same sample containing 16 μg/ml of metrizamide (b).

metrizamide in the plasma standards. To estimate assay sensitivity, accuracy, precision, and to determine whether metrizamide concentration changes upon storage of frozen plasma samples, identical sets of plasma samples were assayed immediately and after storage; the plasma parameters were in good agreement. The minimum quantifiable level was determined by inverse prediction[3] as that concentration whose lower 80% confidence limit just encompasses zero[4]. The overall mean sensitivity for metrizamide in plasma was 0.72 μg/ml. A representative plot of the relationship between relative peak height and the concentration of metrizamide added to plasma, is presented in Fig. 2.

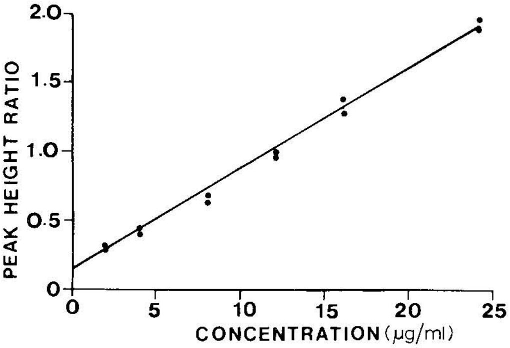

Fig. 2. Extracted standard curve of control human plasma augmented with metrizamide; each concentration was prepared and chromatographed in duplicate.

For the range of concentrations used in the single blind study, results were precise, accurate and reproducible. Table I summarizes the results for the analysis of the "unknown" plasma concentrations. There was no statistically significant difference among concentration groups with respect to mean percent differences. Over the range of these samples the method has an estimated precision of 5.1% (standard deviation). The estimated accuracy ranged from 3% low to 10% high over the concentration range. A logarithmic transformation was applied to the plasma data prior to statistical analysis to minimize the observed correlation between mean response and variance of the quadruplicate sets[5].

The mean concentrations in the plasma of subjects at intervals after subarachnoid administration of metrizamide is shown in Fig. 3. Although the dose varied from 2.0 to 3.2 g of total iodine (4.1 to 6.6 g metrizamide), when the mean plasma concentrations are plotted on a semi-logarithmic scale against time, a linear relationship appears. This suggests first-order kinetics with an apparent half-life of about 11 h. Golman[1], using mean values of his measurements, has reported that "the biological half-life of metrizamide in the subarachnoid space was found to be 11 h". The mean rate constant for excretion of metrizamide was reported as 0.09 ± 0.01 (ref. 1) which corresponds to a half-life of 7.7 h; the range of reported values was from 0.23 (half-life of 4 h) to 0.04 (half-life of 17.3 h). The agreement with our estimated half-life in plasma suggests that the rate-limiting step in the excretion of metrizamide is the transfer from the subarachnoid space into the plasma.

TABLE I

CONCENTRATIONS FOUND IN PLASMA SAMPLES SUPPLEMENTED WITH METRIZAMIDE

Mean % difference = Mean of [antilog ($\log_{10}$ assayed − $\log_{10}$ seeded) − 1] × 100. MQL = minimum quantifiable level.

Seeded levels ($\mu g/ml$)	*Assayed level ($\mu g/ml$)*	
	Day 1	*Day 2*
0	<MQL	<MQL
0	<MQL	<MQL
0	<MQL	<MQL
0	<MQL	<MQL
2.50	2.55	2.61
2.50	2.37	2.71
2.50	2.46	2.89
2.50	2.34	2.81
Geometric mean	2.43	2.75
% S.E.	1.96	2.24
Mean % difference	−2.9	+10.1
5.50	5.39	5.41
5.50	5.31	5.51
5.50	5.83	5.45
5.50	6.12	4.98
Geometric mean	5.65	5.33
% S.E.	3.40	2.34
Mean % difference	+2.8	−3.0
9.0	9.04	9.38
9.00	9.65	9.37
9.00	9.40	9.38
9.00	10.3	11.1
Geometric mean	9.59	9.78
% S.E.	2.80	4.33
Mean % difference	+6.5	+8.7
15.0	17.2	16.6
15.0	15.5	15.7
15.0	15.9	16.4
15.0	15.7	16.5
Geometric mean	16.1	16.3
% S.E.	2.37	1.27
Mean % difference	+7.1	+8.6
19.0	20.3	20.9
19.0	20.9	21.2
19.0	20.3	19.7
19.0	21.3	19.8
Geometric mean	20.7	20.4
% S.E.	1.19	1.88
Mean % difference	+8.9	+7.3

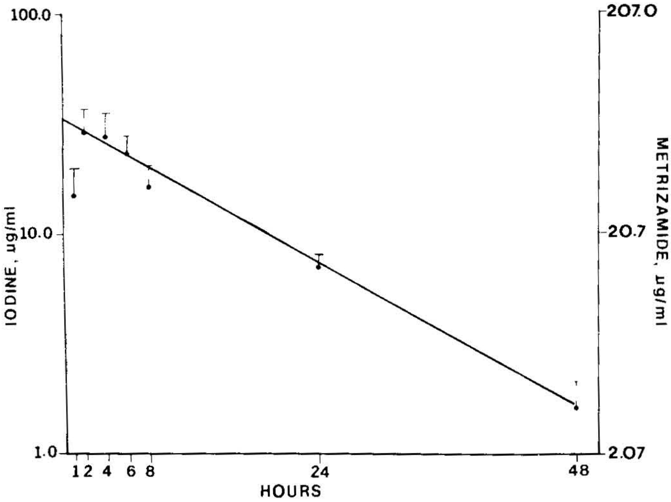

Fig. 3. Mean concentration of metrizamide in plasma of human subjects. Vertical lines denote 1 S.E. ($n = 8$).

In summary, an accurate, specific, reproducible and precise HPLC assay has been developed for the measurement of metrizamide concentration in human plasma. This method permitted estimation of the apparent first-order terminal elimination half-life in the plasma of human subjects that received metrizamide as a diagnostic agent.

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CHROM. 11,462

Note

Ester artefacts formed during isolation of ω-hydroxy fatty acids by DEAE-Sephadex ion-exchange chromatography

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(Received September 13th, 1978)

Ion-exchange chromatography of complex lipids derived from natural sources provides a simple and quantitative method for separating acidic and neutral compounds[1]. The isolation of mixtures of diterpene acids from wood extracts has been achieved by use of DEAE-Sephadex ion-exchange beads, without isomerisation of labile diene acids[2]. After elution of the neutral compounds using diethyl ether–methanol–water (89:10:1), the acids were eluted quantitatively with the same solvent mixture either saturated with carbon dioxide, or containing 4% formic acid[2].

During an investigation of *Pinus radiata* needle epicuticular wax composition, it was required to separate minor amounts of ω-hydroxy fatty acids and diterpene acids from wax esters and estolides, and also to quantitatively separate C_{12}, C_{14}, and C_{16} ω-hydroxy fatty acids from *n*-alkanols and α,ω-diols obtained after basic hydrolysis of estolides[3]. DEAE-Sephadex was the ion-exchange material employed for these separations, but using chloroform–methanol–water as solvent, as the lipids were insoluble in the diethyl ether–methanol–water solvent. This paper describes the anomalous behaviour of the ω-hydroxy fatty acids during ion-exchange chromatography.

EXPERIMENTAL

AnalaR solvents (BDH, Poole, Great Britain) were used. DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) was treated with 2 *N* sodium hydroxide solution, immediately washed with distilled water to remove excess of base, then with methanol, finally with chloroform, and then suspended in chloroform–methanol–water (89:10:1). The slurry was poured into chromatography columns, and the DEAE-Sephadex held in place with one or two discs of Whatman GFB (Whatman, Maidstone, Great Britain). This was necessary, since the ion-exchange beads floated in this solvent mixture.

The epicuticular wax, or hydrolysate, was dissolved in the minimum volume of chloroform–methanol–water, and the solution was transferred to the column (*ca.* 100 mg lipid in 1.5 ml solvent added to 2 ml bed volume of ion-exchange resin). Neutral compounds were eluted with chloroform–methanol–water, and the acids with chloroform–methanol–water–carbon dioxide or chloroform–methanol–water–formic acid.

Thin-layer chromatography (TLC) of the methyl esters of the acid fraction was carried out on layers (0.25 mm) of silica gel G using chloroform–petrol (1:1).

For gas–liquid chromatographic (GLC) analysis, the acids were esterified (diazomethane in diethyl ether–ethanol) and residual hydroxyl groups converted [with bis(trimethylsilyl)acetamide] to trimethylsilyl (TMSi) ethers. The methyl-TMSi derivatives were chromatographed on 1.2 m × 2 mm I.D. glass columns packed with 3% OV-17 on Gas-Chrom Q, temperature programmed from 150° to 250° at 8°/min, injector temperature 180°, flame-ionization detector temperature 280°.

Infrared (IR) spectra were recorded in chloroform solutions, and proton magnetic resonance (PMR) spectra in [^{2}H]chloroform, with TMS internal standard.

RESULTS AND DISCUSSION

Neutral compounds were eluted from the DEAE-Sephadex within ten column volumes of solvent. Use of carbon dioxide-saturated solvent mixture for recovery of the acidic fraction (which was visible as a pale yellow band at the top of the column) only partially eluted the acids, even after 50 column volumes of solvent. When the formic acid-containing solvent mixture was used, the acids were readily eluted, usually within five column volumes.

GLC of the acid fraction eluted from five ion-exchange columns with chloroform–methanol–water–formic acid showed three extra peaks in addition to those of the C_{12}, C_{14}, and C_{16} ω-hydroxy fatty acid derivatives. The ratio of the total area of the three ω-hydroxy fatty acid methyl ester GLC peaks to that of the three new peaks varied from *ca.* 4:1 to 1:1. It was apparent that the ω-hydroxy fatty acids were being modified during the ion-exchange chromatography. Two classes of compounds from the acid fraction (as methyl esters) were separated by TLC on silica gel G. GLC confirmed the separation of the two classes, each containing three compounds. The IR spectrum of each class showed carbonyl absorption at 1735 cm^{-1} due to carboxylic acid ester, but only the more polar one had absorption at 3600 cm^{-1} due to hydroxyl. The PMR spectrum of the more polar compounds showed these to be ω-hydroxy fatty acid methyl esters (δ 3.62, s, OCH_3; 3.60, t, J = 7 Hz, CH_2–O; 2.32, t, J = 7 Hz, CH_2–C=O). The PMR spectrum of the less polar class showed these to be formate esters of the ω-hydroxy fatty acid methyl esters (δ 8.02, s, H–C=O; 4.18, t, J = 7 Hz, CH_2–O–C=O; 3.62, s, OCH_3; 2.22, t, J = 7 Hz, CH_2–C=O). The formate methyl esters were readily hydrolysed with base to the original ω-hydroxy fatty acids.

Formate esters were also formed (but to only *ca.* 5% of the total acid fraction) when the ω-hydroxy fatty acids were dissolved in chloroform–methanol–water–formic acid alone and kept for 12 h at 20°. To compare the behaviour of ω-hydroxy fatty acids with other hydroxy fatty acids, 2-hydroxypalmitic acid, and 12-hydroxystearic acid were committed to DEAE-Sephadex ion-exchange chromatography. Both these hydroxy fatty acids were only very slightly ($< 1\%$) esterified compared with the ω-hydroxy fatty acids when treated in an identical manner, as shown by GLC of their methyl esters. Neither 2-hydroxypalmitic acid nor 12-hydroxystearic acid was esterified when dissolved in chloroform–methanol–water–formic acid alone.

The unusual reactivity of the primary alcohol of ω-hydroxy fatty acids towards acylation with carboxylic acid can possibly be compared with its facile alkylation with diazomethane. Treatment of di- and trihydroxy fatty acids with ethereal diazomethane resulted in partial alkylation of the primary hydroxyl groups, but not of the secondary hydroxyl groups[4]. Similar esterification of primary hydroxyl groups of hydroxy fatty acid methyl esters, but not of secondary hydroxyl groups, has been shown to occur during their GLC on polyester phases[5].

In the present case, ready formylation of the primary hydroxyl group of ω-hydroxy fatty acids when eluted from DEAE-Sephadex columns with chloroform–methanol–water–formic acid appears due to its unusual reactivity, and possibly also due to acid catalysis by the protonated DEAE-Sephadex.

ACKNOWLEDGEMENT

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CHROM. 11,429

Note

Two-dimensional thin-layer chromatography of *Digitalis* cardenolides using a continuous development technique

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Since Stahl and Kaltenbach[1] first used thin-layer chromatography (TLC) on silica gel to separate cardioactive glycosides, numerous TLC systems have been published for this pharmacologically important group of compounds. Sjöholm[2] recognised that separation of complex mixtures of cardioactive glycosides could not be achieved by conventional TLC, and obtained improved resolution by using two-dimensional TLC. Bennett and Heftmann[3] improved TLC resolution by use of a continuous development technique whereby a trough filled with adsorbent material was fixed to the top edge of the TLC plate to take up the mobile phase. Lisboa and co-workers[4,5] used a similar technique for TLC of steroids, including cardenolides, and found that continuous development gave better resolution of some closely related steroids than multiple developments in the same mobile phase.

The application of TLC to the analysis of cardiac glycosides and their genins has been reviewed by Nover[6]. This article includes a summary of Nover's papers in which the relationship between chemical structure and chromatographic behaviour has been explored.

This paper describes the use of continuous development TLC in two directions to obtain resolution of highly complex mixtures of *Digitalis* cardenolides, including an extract of *Digitalis lanata* leaf. The spots obtained are compact, and the cardenolides can be detected with great sensitivity and specificity. In addition to its use in identifying known components of a mixture, the method described can also be used to furnish structural information on unidentified constituents of natural product extracts.

EXPERIMENTAL

Reagents and reference materials

All solvents used were of analytical grade.

The chromogenic reagent employed was 20% (v/v) aqueous phosphoric acid.

Samples of the mono- and bis-digitoxosides of diginatigenin, digoxigenin, gitoxigenin and digitoxigenin were prepared according to the methods of Kaiser *et al.*[7]. Other compounds were obtained from commercial sources as indicated in Table I.

The particular sample of dried *D. lanata* leaf reported here was obtained from Tabandru (Uden, The Netherlands).

Extraction of D. lanata leaf

The cardenolides were extracted by maceration and percolation of the leaf with 20% aqueous ethanol, and the extract enriched by partition with chloroform as described in an earlier paper[8].

TLC technique

The TLC plates used were Kieselgel 60 DC-Fertigplatten, 20 × 20 cm with a layer thickness of 0.25 mm (Merck, Darmstadt, G.F.R.).

Reference samples were applied as 0.05% solutions in chloroform–methanol (1:1). The extract from 10 g of dried *D. lanata* leaf was dissolved in 0.5 ml of chloroform–methanol (1:1).

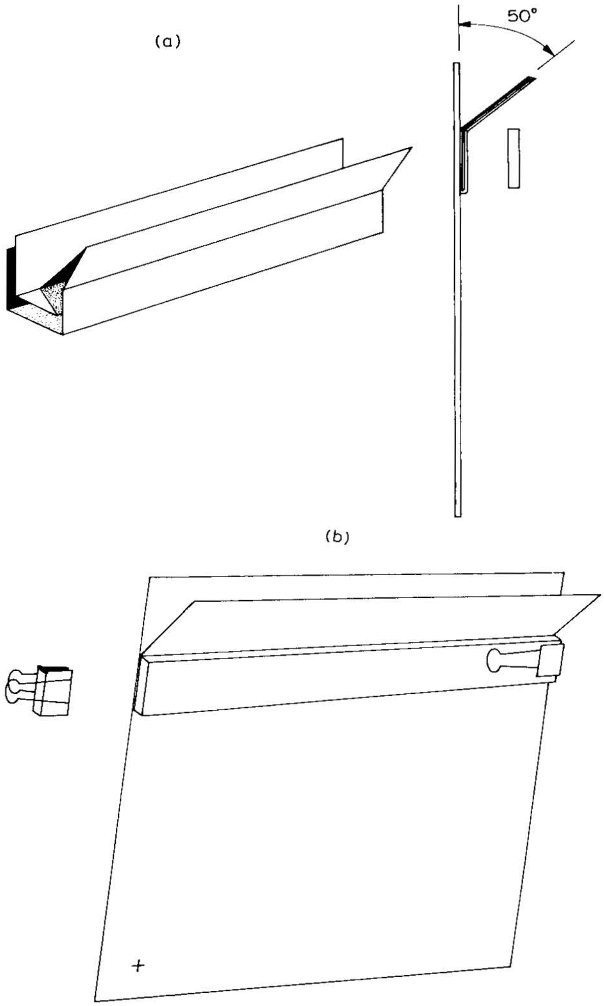


Fig. 1. Preparation of the trough for continuous development TLC. (a) The folded paper is placed against the adsorbent surface; (b) the folded paper is secured to the plate with a glass strip and spring clips.

The sample (0.5 μl) was applied by means of a 7101-SN Point style 3, microliter syringe (Hamilton, The Hague, The Netherlands) to the bottom left-hand side of the plate about 2 cm from the corner on the diagonal.

A trough of adsorbent was fitted to the top edge of the plate in the following manner. A sheet of chromatography paper (20 × 20 cm) was folded three times until its dimensions were (20 × 2.5 cm). The last fold was opened out, and the paper was placed against the adsorbent surface at the top of the plate (Fig. 1a). The bottom half of the folded paper was secured to the plate using a glass strip (20 × 2.5 cm) held by spring clips, and the top half was hinged outwards (Fig. 1b). The open ends of the trough were sealed with adhesive tape and the completed trough was filled with TLC-grade silica gel (20 g).

The plate was developed for 3 h in a conventional TLC chamber using ethyl acetate–dichloromethane–methanol–water (120:72:7:4) as mobile phase. The plate was then removed from the chamber and the trough detached. After drying in air at room temperature the plate was turned through 90° and a new trough attached to the top edge. The plate was then developed for 2 h in the second direction using dichloromethane–methanol (9:1) as mobile phase. After the second development the plate was removed from the chamber and the trough detached. The plate was then dried in air before immersing it in the phosphoric acid reagent for 5 sec (*care:* use gloves). The cardenolides were visualised by heating the plate at 100° for 15 min then viewing under UV light at 366 nm.

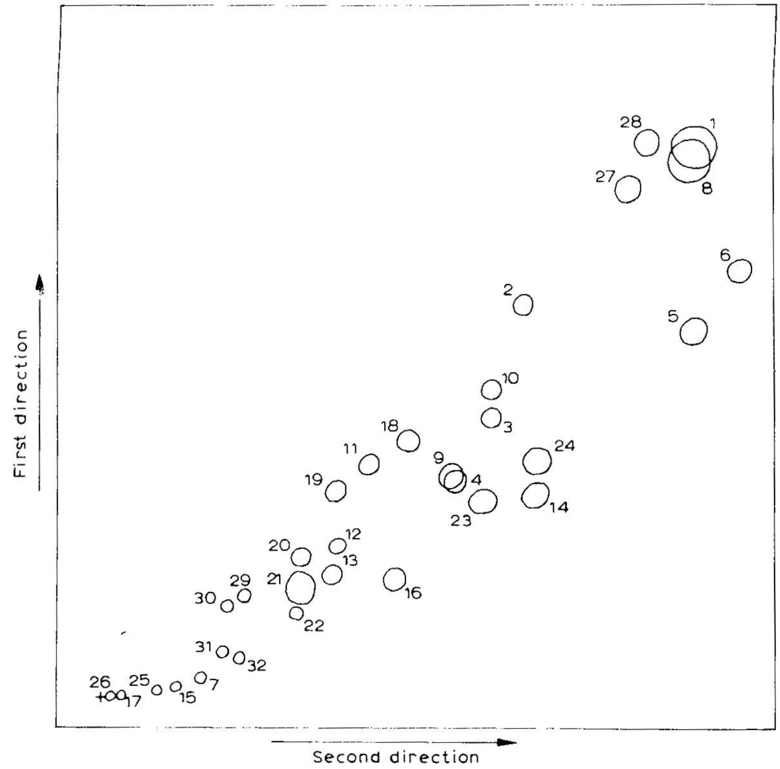
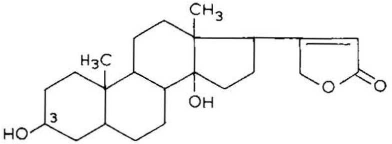
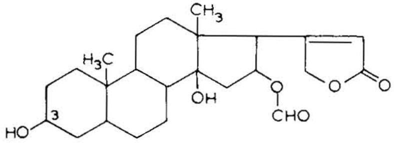
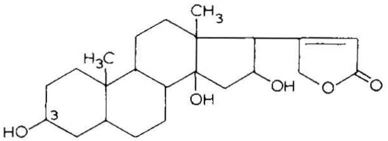
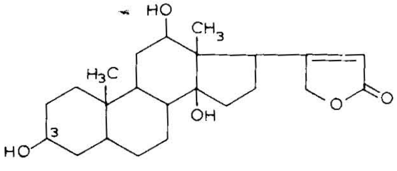
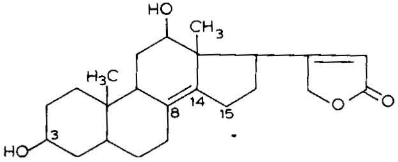
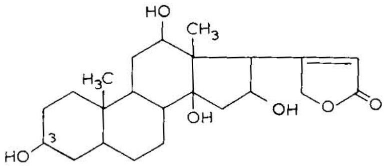


Fig. 2. Chromatogram of a mixture of cardenolide standards. The identity of the spots is given in Table I.

TABLE I

MOBILITIES OF A RANGE OF CARDENOLIDES RELATIVE TO DIGOXIN

Compound	Symbol	Source*	Aglycone	Glycosidically linked residue at C_3**	UV appearance	Mobility relative to digoxin	
						1st direction	2nd direction
Series A							
Digitoxigenin	1	a		—	Yellow/brown	5.09 S.D. = 0.15	2.82 S.D. = 0.23
Digitoxigenin mono-digitoxoside	2	a		-D	Yellow/brown	3.59	2.14
Digitoxigenin bis-digitoxoside	3	a		-D-D	Yellow/brown	2.57	1.94
Digitoxin	4	a		-D-D-D	Brown	2.00 S.D. = 0.03	1.64 S.D. = 0.07
α-Acetyldigitoxin	5	d		-D-D-D	Brown	3.31	3.11
β-Acetyldigitoxin	6	d		Ac	Brown	4.00	3.29
Lanatoside A	7	b		-D-D-D-G			
				Ac	Brown	0.16	0.46
Series E							
Gitaloxigenin	8	c		—	Brown	4.90	3.06
Gitaloxin	9	c		-D-D-D	Brown	1.97	1.88
Series B							
Gitoxigenin	10	f		—	Blue/white	2.78	1.99
Gitoxigenin mono-digitoxoside	11	a		-D	Blue/white	2.14 S.D. = 0.06	1.25 S.D. = 0.05
Gitoxigenin bis-digitoxoside	12	a		-D-D	Blue/white	1.36	1.16
Gitoxin	13	a		-D-D-D	Blue/white	1.12 S.D. = 0.04	1.14 S.D. = 0.01
β-Acetylgitoxin	14	g		-D-D-D			
				Ac	Yellow	1.98	2.25
Lanatoside B	15	b		-D-D-D-G			
				Ac	Light brown	0.08	0.29
Strospeside	16	c		-L	Yellow/brown	1.11	1.45
Digitalinum verum	17	b		-L-G	Brown	0.05	0.13

Series C							
Digoxigenin	18	a		—	Green	2.36	1.55
Digoxigenin mono-digitoxoside	19	a		-D	Yellow/green	1.93	1.15
Digoxigenin bis-digitoxoside	20	a		-D-D	Yellow/green	1.31	1.02
Digoxin	21	a		-D-D-D	Yellow/brown	1.00	1.00
Digoxoside	22	c		-D-D-D-D	Yellow	0.68	0.95
α-Acetyldigoxin	23	c		-D-D-D	Yellow	1.81	1.94
β-Acetyldigoxin	24	c		Ac	Yellow	2.22	2.24
Lanatoside C	25	b		-D-D-D-G			
				Ac	Brown	0.06	0.23
Deacetyl lanatoside C	26	b		-D-D-D-G	Brown	0.05	0.08
α-Anhydro digoxigenin	27	a		—	Blue/mauve	4.79	2.65
β-Anhydro digoxigenin***	28	a		—	Yellow	5.10	2.77
Series D							
Diginatigenin	29	b		—	Blue/white	0.98	0.68
Diginatigenin mono-digitoxoside	30	a		-D	Blue/white	0.90	0.61
Diginatigenin bis-digitoxoside	31	a		-D-D	Blue/white	0.41	0.57
Diginatin	32	b		-D-D-D	Blue/white	0.33 S.D. = 0.03	0.68 S.D. = 0.02

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* a = Burroughs Wellcome (Dartford Great Britain); b = Sandoz (Basle, Switzerland); c = Boehringer (Mannheim, G.F.R.); d = K & K Labs. (Plainview, N.Y., U.S.A.); e = Aldrich (Milwaukee, Wisc., U.S.A.); f = Fluka (Buchs, Switzerland); g = USPC (Rockville, Md., U.S.A.).

** D = digitoxose; G = glucose; L = digitalose; Ac = acetyl.

*** β-Anhydro digoxigenin has a double bond in the 14–15 position.

RESULTS AND DISCUSSION

The cardenolides examined are listed together with their mobilities relative to digoxin in Table I.

A typical chromatogram obtained for a mixture of cardenolides is represented in Fig. 2, and Fig. 3 shows the chromatogram obtained from an extract of *D. lanata* leaf. The spots produced are compact and symmetrical and it is possible to detect 0.015 μg of most of the cardenolides. The reproducibility of the positions of the spots with respect to digoxin was determined using 8 chromatograms developed on separate occasions. Five cardenolides were chosen for this experiment and the standard deviations obtained are shown in Table I. These results demonstrate good reproducibility.

Continuous development is necessary to give the high degree of resolution required in the analysis of complex mixtures of cardenolides. This means that some non-cardenolide components of *Digitalis* extracts run into the trough and are not observed. This represents no disadvantage, however, since conventional two-dimensional TLC can be used if these more mobile compounds are to be studied (Fig. 4).

Some correlation between the chromatographic mobility of the cardenolides and their chemical structure has been observed. The spots due to corresponding cardenolides from each series, *e.g.* diginatin, digoxin, gitoxin and digitoxin lie on straight lines (Fig. 5a). Regular curves can be drawn through the spots from

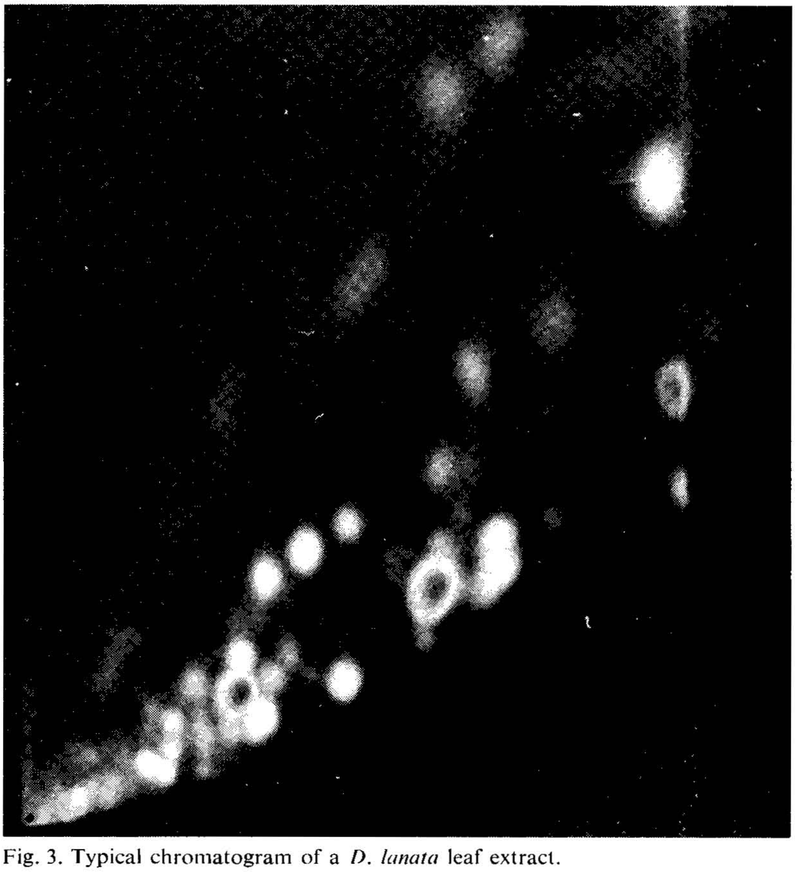

Fig. 3. Typical chromatogram of a *D. lanata* leaf extract.

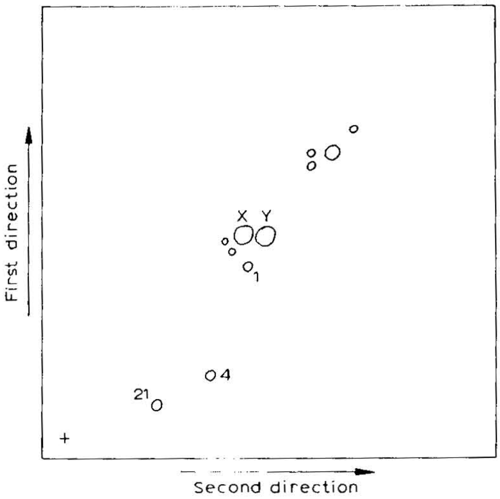

Fig. 4. Two-dimensional chromatogram of a *D. lanata* leaf extract developed without using adsorbent troughs. X = 5,7,4′-trihydroxy-3′-methoxyflavone; Y = 5,7,4′-trihydroxy-3′,6-dimethoxyflavone.

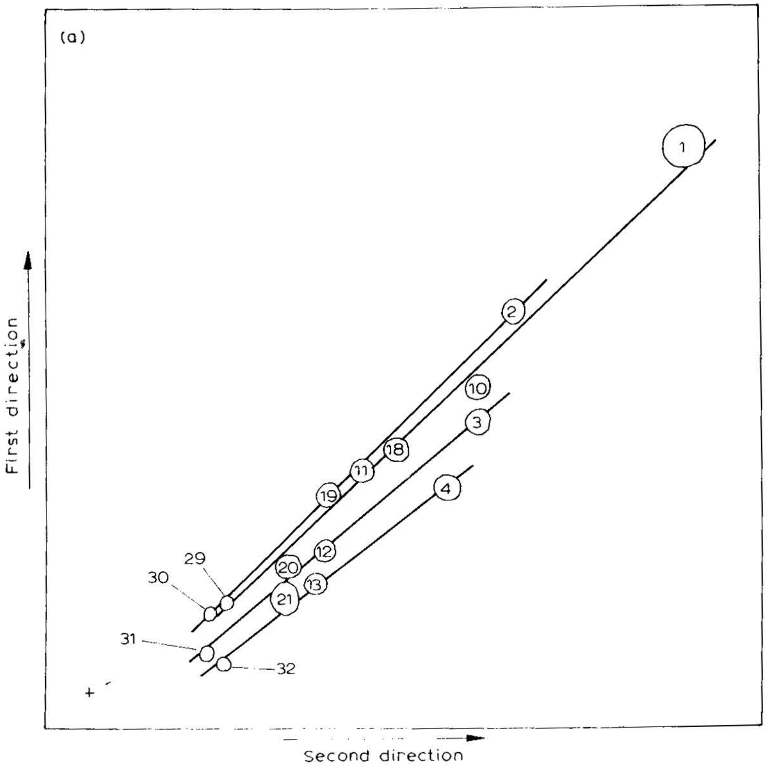

Fig. 5.

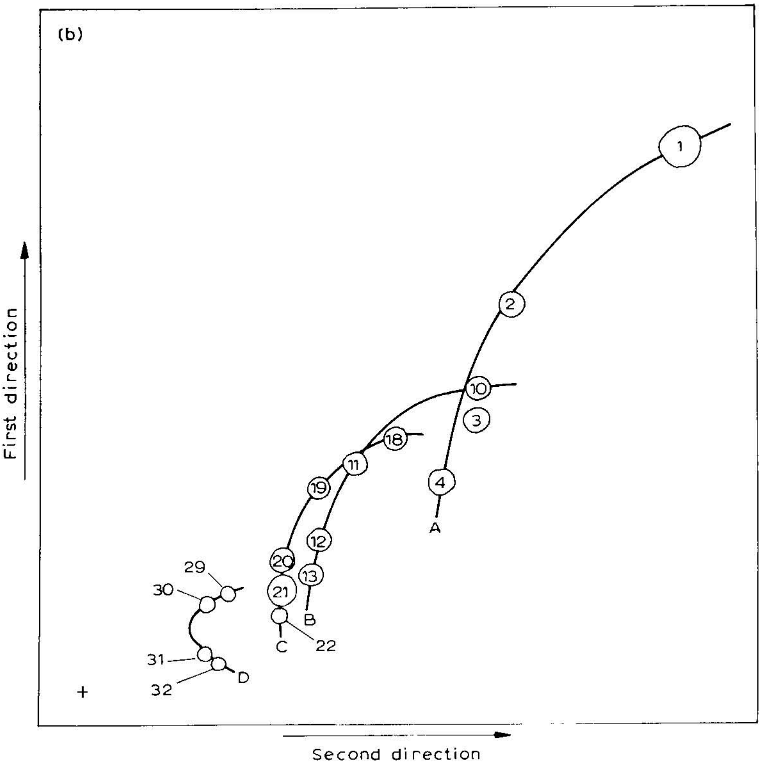

Fig. 5. Relationship between chromatographic mobility and chemical structure. (a) Relationship between corresponding cardenolides from different series; (b) relationship between homologues. The identity of the spots is given in Table I.

homologous compounds, *e.g.* digoxigenin, digoxigenin mono-digitoxoside, digoxigenin bis-digitoxoside and digoxin (Fig. 5b). These empirical relationships were used to identify the positions of digoxoside, diginatigenin mono-digitoxoside and diginatigenin bis-digitoxoside before reference samples became available.

This chromatographic technique has proven extremely useful in the qualitative monitoring of the commercial extraction of digoxin from *D. lanata* leaf. It has also made possible the isolation of previously unknown glycosides from extracts of *D. lanata* and these will be reported in a later paper.

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Note

Determination of lysinoalanine by densitometry

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Lysinoalanine (LAL), the trivial name of N$^\varepsilon$-(DL-2-amino-2-carboxyethyl)-L-lysine, is an amino acid which can arise in food proteins from lysyl and seryl residues[1,2]. This compound has recently attracted considerable attention because of its ability to induce renal lesions in rats[3–5]. In most studies on LAL, this compound was determined by means of an amino acid analyzer[1,3,5,6]. Recently, however, Sternberg *et al.*[7] have described a method consisting of thin-layer chromatography (TLC) and densitometry.

This note describes our experience with this method, in particular the protein hydrolysis, the chromatographic procedure and the colouring with ninhydrin.

MATERIALS AND METHODS

Amino acids

Lysinoalanine, ornithinoalanine [N$^\delta$-(DL-2-amino-2-carboxyethyl)-L-ornithine, OAL] and lysinomethylalanine [N$^\varepsilon$-(DL-1-methyl-2-amino-2-carboxyethyl)-L-lysine, LAL (Me)] were synthesized by the Central Institute for Nutrition and Food Research TNO, according to the method of Okudo and Zahn[8]. S-β-(4-Pyridylethyl)-L-cysteine (PEC) was also synthesized by the same Institute according to the method of Cavins and Friedman[9]. The following amino acids were purchased: AA$_5$ standard amino acid solution (Calbiochem, Los Angeles, Calif., U.S.A.), containing lysine, histidine, arginine, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine; lanthionine, DL- and *meso*-forms (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.); mixed DL- and DL-*allo*-δ-hydroxylysine hydrochloride (Sigma, St. Louis, Mo., U.S.A.); L-ornithine monohydrochloride (Fluka, Buchs, Switzerland); DL-methioninsulphon (Fluka); DL-norleucine (Fluka); L-1-methylhistidine monohydrate (Calbiochem); L-3-methylhistidine (Calbiochem); DL-2,3-diaminopropionic acid hydrochloride (Fluka); DL-tryptophan (E. Merck, Darmstadt, G.F.R.); DL-cysteic acid (Koch-Light, Colnbrook, Great Britain); DL-γ-aminobutyric acid (Hoffmann-La Roche, Basle, Switzerland); D-galactosamine hydrochloride (J. T. Baker, Phillipsburgh, N.J., U.S.A.), D-glucosamine hydrochloride (Biochem. Res. Corp., Los Angeles, Calif., U.S.A.) and N-ε-methyl-L-lysine hydrochloride (Sigma).

Reagents

Precoated cellulose layers (0.1 mm thickness) were used (Macherey, Nagel & Co., Düren, G.F.R.; Cel 300-10). The plates were densitometrically tested; plates of irregular thickness were not used. The ninhydrin solution was prepared by dissolving 1 g of ninhydrin in a mixture of 97 ml of *n*-butanol and 3 ml of glacial acetic acid. A copper sulphate solution was obtained by dissolving 2 mg of $CuSO_4 \cdot 5H_2O$ in 10 ml of distilled water and then adding 30 ml of 95% ethanol. Ninhydrin, acetic acid, copper sulphate, ethanol and 37% hydrochloric acid were purchased from E. Merck, *n*-butanol and formic acid from J. T. Baker. All the reagents used were analytical-reagent grade.

The samples investigated, *i.e.*, alkali-treated soy proteins, sodium caseinate and gelatine, were commercial products.

Sample preparation

The sample (200–250 mg dry weight) was hydrolyzed by boiling in 200 ml of 6 *N* hydrochloric acid for 22 h. The hydrolysate was evaporated to dryness in a rotary evaporator at 50°. The residue was redissolved in 20 ml of water and the evaporation was repeated twice. In some cases it was necessary to filter the solution before the last evaporation. The final residue was dissolved in 10 ml of water. This solution is suitable for chromatography. Solutions obtained from samples with a content higher than 800 ppm of LAL (on a dry weight basis) have to be diluted with water.

Chromatography

The samples were applied by repeated spotting using a 5-μl SGE syringe fitted with a PTFE nozzle, with the aid of a stream of warm air. The maximum allowable amount of hydrolysate per spot corresponds to 125 μg dry weight of the sample. The spots should be as small as possible (diameter *ca.* 3 mm). LAL was applied to each plate, in amounts up to 0.10 μg, in separate spots for calibration.

Ascending chromatography was performed[7] in the solvent system 90% formic acid–*n*-butanol–water (4:23:5) in an unsaturated tank (Desaga, Heidelberg, G.F.R.). The distance travelled by the solvent was 17 cm from the start. The development was performed three times, the elution time for each run being 4 h.

Measurements of spots

After drying the developed plate for 0.5 h at room temperature, it was sprayed with the ninhydrin solution and dried for 10 min at 110°. The layer was sprayed subsequently with the copper sulphate solution and dried for 30 min at room temperature. The spots were scanned with a Vitatron TLD 100 densitometer (Vitatron, Dieren, The Netherlands) connected to a Vitratron UR 402 integrating recorder. The operating conditions were: transmittance mode log(—); filter, 525 nm; level d; diaphragm, 0.25 mm; stroke distance, 5 mm; scan speed, 3 cm/min; recorder chart speed, 10 cm/min; integrator 7; span variation from 9/25 to 9/90, dependent on the spot intensity and background signal of the sample. Peak areas were calculated by means of the integrator readings and corrected for fluctuations in the background absorption due to variations in layer thickness as indicated by the manufacturer[10]. The amount of LAL in each hydrolysate was calculated from the mean of three determinations made on one plate.

RESULTS AND DISCUSSION

Hydrolysis

Protein hydrolysis can be carried out in sealed tubes or under total reflux. We think the sealed-tube technique is less convenient for routine determinations than the open-flask hydrolysis under total reflux. Moreover, disadvantage of the sealed-tube technique is the limited sample size, which may result, in some cases, in samples which are not representative of the material investigated. Both the sealed-tube method, followed by removal of the bulk of HCl in a desiccator over NaOH as used by Sternberg, and the open-flask hydrolysis were applied to three samples, *i.e.*, alkali-treated casein, a commercial product containing a whipping agent and alkali-treated soy protein. Differences in chromatographic separation were not observed. Slump[6] has already demonstrated that, for most proteins, hydrolysis in 6 N hydrochloric acid for 22 h under total reflux is sufficient for complete liberation of LAL.

Chromatography

A prerequisite for densitometry is the complete separation of the given compound from interfering substances. In this respect, both Eastman cellulose sheets (catalogue number 13255) used by Sternberg *et al.* and the plates of Macherey, Nagel & Co. (MN), which we used, were suitable. However, in the case of MN plates, the R_F value of LAL is somewhat larger, and LAL is further removed from other ninhydrin-positive substances, resulting in a better chromatogram. Moreover, we found that MN cellulose plates were in general more homogeneous than Eastman sheets, which results in a lower background noise. An example of a densitogram, showing the results of LAL determinations on MN plates and on Eastman sheets, is provided in Fig. 1.

Not all types of cellulose plates are suitable for separation: it was found, that under the same chromatographic conditions, LAL interferes with other ninhydrin-positive substances, *i.e.*, lanthionine and cysteic acid, when Eastman cellulose sheets containing a fluorescence indicator (catalogue number 13254) were used. Precoated cellulose layers of Merck (catalogue number 5716) are also unsuitable. The Gelman chamber applied by Sternberg is not essential for LAL separation.

The method was also tested on ornithinoalanine (OAL), a compound which, like LAL, may arise during alkali treatment of proteins[11]. It was observed that, on the Eastman sheet as well as on the MN plate, the spots of both compounds coincide. However, in alkali-treated products (*i.e.*, soy proteins, caseinates and foaming agents), investigated on an amino acid analyzer, OAL was found to be absent or present only in negligible amounts[12]. If the presence of both OAL and LAL has still to be demonstrated, the ratio of the solvent components should be changed to 4:23:4. This system was not investigated further.

LAL (Me), an amino acid which theoretically can be formed from threonyl and lysyl residues[6], shows a slightly higher R_F value than LAL. Furosine and pyridosine, compounds arising in acid hydrolysates of heated, proteinaceous products rich in reducing sugars, were also tested for their possible interference with the LAL determination. A hydrolysate of a lactose-hydrolyzed skimmed milk powder, in which the presence of both furosine and pyridosine was demonstrated by an amino acid

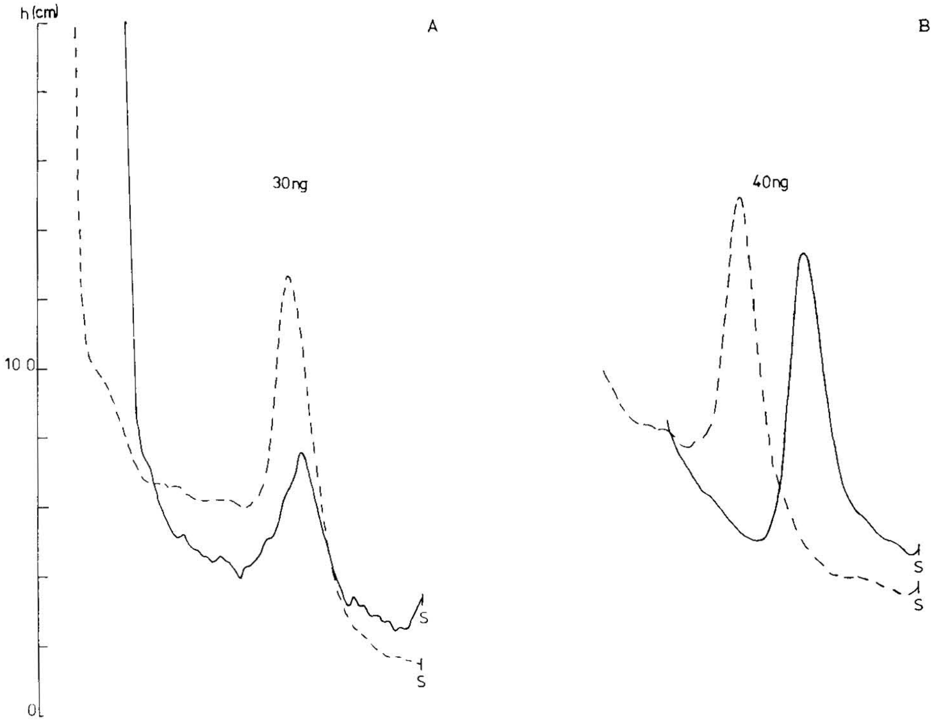

Fig. 1. Densitograms, showing LAL determinations on MN cellulose plates (– – –) and on Eastman cellulose sheets (———). A — Calcium caseinate (270 ppm LAL); B — sodium caseinate (340 ppm LAL); S = start.

analyzer, was submitted to chromatography. No ninhydrin-positive compound was found with a R_F value equal to that of the LAL spot.

A complete chromatogram showing the tested amino acids and some samples is shown in Fig. 2. The R_F value of LAL in a sample was slightly lower than that of reference LAL.

Precoated silica gel plates (E. Merck, silica gel 60 F_{254}; catalogue number 5715; thickness 0.25 mm) were also investigated because of their better homogeneity as compared to cellulose plates. The eluent was *n*-butanol–isopropanol–acetic acid–water (4:4:4:5). After prewashing the plates with the eluent, ascending chromatography was performed for 16 h. Ninhydrin colouring was carried out as described above (absorbance maximum, 497 nm). LAL was separated from most other amino acids usually present in protein hydrolysates, with the exception of OAL, LAL (Me), 3-methylhistidine and N-ε-methyllysine. However, colour development is much poorer compared to that on the cellulose layer, resulting in a detection limit four times that observed on cellulose. The colour is also less stable. On other silica gel plates (Woelm silica gel $F_{254/366}$; Macherey, Nagel & Co. silica gel N-HR/UV_{254}; Bakerflex IB-F and Merck silica gel 60 without fluorescence indicator) LAL was not separated from one of the amino acids of the standard amino acid mixture, under the same chromatographic conditions.

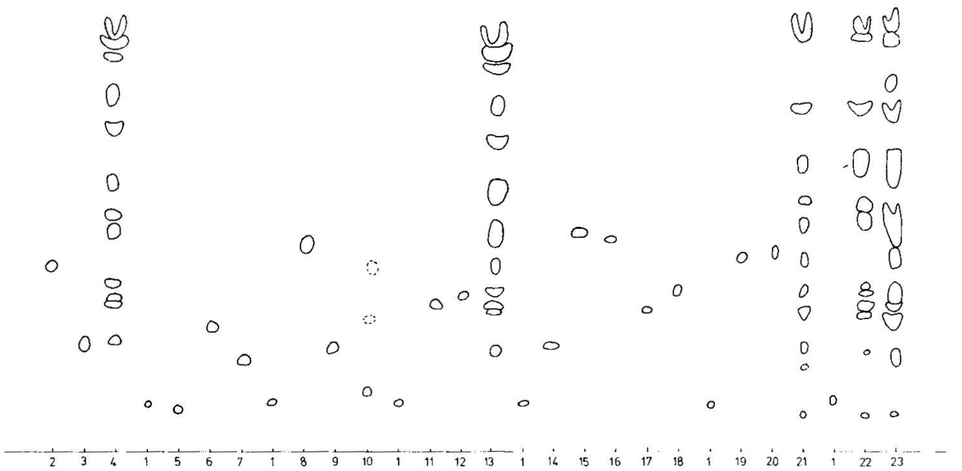

Fig. 2. Thin-layer chromatogram of amino acids on MN cellulose plates. Spots: 1 = LAL; 2 = PEC; 3 = hydroxylysine; 4 = standard amino acid mixture; 5 = OAL; 6 = ornithine; 7 = lanthionine; 8 = γ-aminobutyric acid; 9 = 2,3-diaminopropionic acid; 10 = LAL(Me); 11 = galactosamine; 12 = glucosamine; 13 = lactose-hydrolyzed skimmed milk powder; 14 = cysteic acid; 15 = methioninsulphon; 16 = tryptophan; 17 = 1-methylhistidine; 18 = 3-methylhistidine; 19 = N-ε-methyl-L-lysine; 20 = norleucine; 21 = alkali-treated soy protein (2800 ppm LAL); 22 = sodium caseinate (550 ppm LAL); 23 = alkali-treated soy protein (40 ppm LAL).

Conditions for ninhydrin spraying

A comparison of different ninhydrin reagents demonstrated that the optimal colour intensity and stability was attained by spraying with 1% ninhydrin solution followed by spraying with copper sulphate solution, as described in Materials and methods. Moreover, the standard deviation is lower than that obtained by spraying with ninhydrin alone. The copper sulphate spray shifts the colour maximum from 550 to 525 nm. Spraying with a mixture of ninhydrin and copper sulphate results in a very poor colour formation. For the LAL concentrations investigated, spraying a few days after elution resulted in no colour formation. When this is the case, a good colour can still be obtained by spraying with the eluent, drying in a hood for 0.5 h and treating with ninhydrin and copper sulphate as already described.

Some other spray reagents were investigated with the intention of improving the sensitivity of the determination. Spraying with *o*-phthalaldehyde (Fluka), recently described by Lindberg[13] as a very sensitive reagent for amino acids on thin-layer plates, does not lead to a lower detection limit for LAL as compared to spraying with ninhydrin. This is also true when spraying with fluorescamine (Hoffmann-La Roche). Applying the procedure described by Mendez and Lai[14] to LAL, the minimum detectable amount was 0.02 μg.

Linearity and sensitivity

A linear relationship exists between the amount of LAL spotted up to 0.10 μg and the corresponding peak areas, the limit of detection being 0.005 μg or 40 ppm in the sample (dry weight basis). Amounts of LAL exceeding 0.10 μg per spot were not tested.

Reproducibility and comparison with the amino acid analyzer

The reproducibility of the procedure was tested with four different samples, each being hydrolyzed six times (Table I). For the same hydrolysates, LAL was also measured by means of an amino acid analyzer according to Slump's method A[6]. For both methods the standard deviation is calculated from

TABLE I

COMPARISON OF RESULTS OBTAINED BY TLC DENSITOMETRY AND BY THE AMINO ACID ANALYZER (AAA)

Sample	*Method*	*Amount of LAL (ppm)*	*Range (ppm)*	*Standard deviation*	*Coefficient of variation (%)*
Alkali-treated soy protein	TLC	40	30– 55	10 ($n = 6$)	25
	AAA	140	125– 165	15 ($n = 6$)	11
Alkali-treated soy protein	TLC	2800	2550–3000	220 ($n = 6$)	8
	AAA	3000	2600–3400	320 ($n = 6$)	11
Sodium caseinate	TLC	550	400– 650	87 ($n = 6$)	16
	AAA	680	610– 800	81 ($n = 6$)	12
Gelatine	TLC	250	218– 308	39 ($n = 6$)	16
	AAA	165	148– 170	12 ($n = 6$)	7

$$S = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n - 1}}$$

and the coefficient of variation from

$$v = \frac{S}{\bar{x}} \cdot 100\%.$$

The comparatively large standard deviation of the densitometric method, in particular in the low concentration range, is partly due to the tailing of a ninhydrin-positive compound present in a very high concentration in comparison with LAL, or to incomplete separation of the LAL spot from an unknown ninhydrin-positive compound. This, in some cases, makes it rather difficult to determine the area enclosed by the LAL peak. Except for alkali-treated soy protein (2800 ppm), the results of both methods are, with respect to the products investigated, statistically different (*t*-test; $P = 95\%$). Possibly, in both methods, other unknown ninhydrin-positive compounds, dependent on the sample, are co-determined. A more extensive comparison of both methods in different foods and food products will be published elsewhere[15].

ACKNOWLEDGEMENTS

The authors wish to thank Dr. P. Slump of the Central Institute for Nutrition and Food Research TNO for stimulating discussions, and Dr. A. Ruiter of the University of Utrecht for critical reading of the manuscript. We also thank Mrs. R. Schreuder of the TNO Institute for carrying out the analyses with the amino acid analyzer, and Mrs. R. van Elburg for the experiments with the silica gel plates.

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Journal of Chromatography, 168 (1979) 557–561

CHROM. 11,412

Note

Thin-layer chromatography of conjugated bile acids

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(First received April 7th, 1978; revised manuscript received July 26th, 1978)

Thin-layer chromatography (TLC) has proved to be very useful for the rapid qualitative analysis of free bile acids and solvent systems have been worked out for the separation of most of the bile acids of biochemical interest[1–10]. Little work has, however, been done for the separation of the individual glycine and taurine conjugated bile acids[1–3,8–11], while a group separation of the glycine and the taurine conjugates has recently been reported[12]. In the present paper a solvent system is described which separates the glycine and the taurine conjugates of lithocholic, chenodeoxycholic, deoxycholic and cholic acids from one another. The different glyco- and tauro-conjugates present in human and guinea pig biles have been separated using this system. In addition, sharp differences are demonstrated in the relative mobilities of the various glycine *vs.* taurine conjugated bile acids on changing from an acidic to a basic solvent system.

EXPERIMENTAL

Taurine and glycine conjugated lithocholic, chenodeoxycholic, deoxycholic and cholic acids (Calbiochem, Los Angeles, Calif., U.S.A.) were dissolved in methanol and 2–3 μg were applied to 0.25 mm thick silica gel G plates (Brinkmann, Westbury, N.Y., U.S.A.). The plate was developed in the appropriate solvent system and the solvent was allowed to rise 16–18 cm from the starting line, followed by drying at 110°. When necessary, the plate was developed a second time in the same solvent system. The plate was then sprayed with 20% sulfuric acid and phosphomolybdic acid (3.5% in isopropanol; EM Labs, Westbury, N.Y., U.S.A.) and heated at 110° for 2 min. The spots, thus obtained, generally had a maximum diameter of 1 cm.

RESULTS AND DISCUSSION

Table I illustrates the mobilities of the various conjugated bile acids relative to that of taurocholic acid obtained in a number of acidic and basic solvent systems. Acidic solvent systems gave round spots with better resolution of the components, whereas the basic solvent systems often gave elongated spots and the resolution of the

TABLE I

MOBILITIES OF CONJUGATED BILE ACIDS IN DIFFERENT SOLVENT SYSTEMS RELATIVE TO THAT OF TAUROCHOLIC ACID

The following compositions of the various solvents were used: A_1, chloroform–isopropanol–acetic acid–water (30:30:4:1); A_2, *n*-butanol–acetic acid–water (20:4:3); A_3, ethyl acetate–methanol–acetic acid–water (35:12:2:2); N_1, *n*-butanol–water (20:3); N_2, ethyl acetate–methanol–water (35:12:2); B_1, *n*-butanol–pyridine–water (20:4:3); B_2, ethyl acetate–methanol–pyridine–water (35:12:5:2).

Compound	*Solvent system*						
	A_1	A_2	A_3	N_1	N_2	B_1	B_2
Taurolithocholic acid	4.40	1.47	1.60	1.52	1.22	1.17	1.19
Taurochenodeoxycholic acid	2.50	1.28	1.36	1.36	1.12	1.11	1.12
Taurodeoxycholic acid	2.20	1.28	1.36	1.32	1.12	1.11	1.12
Glycolithocholic acid	13.2	2.10	1.86	1.14	0.84	0.88	0.84
Glycochenodeoxycholic acid	10.5	1.76	1.62	0.94	0.67	0.79	0.73
Glycodeoxycholic acid	9.5	1.76	1.62	0.90	0.67	0.79	0.73
Glycocholic acid	5.20	1.55	1.26	0.64	0.51	0.70	0.56
Mobility of taurocholic acid (cm)	1.0	5.8	8.3	5.0	10.7	8.1	11.2

various components was generally poor. Nevertheless, the basic systems had the advantage of changing dramatically the relative mobilities of the different conjugated bile acids. They showed a clear separation between the glycine and the taurine conjugated bile acids, the latter moving faster.

Separation between the taurine or glycine conjugates of chenodeoxycholic acid and deoxycholic acid was found to be difficult in a single development. System A_1 separated all four, but the two taurine conjugates were separated only partially (Table I and Fig. 1a). In order to achieve complete separation of these two acids, it was necessary to develop the plate twice in the solvent system (A_1) (Fig. 1b). (For a radio-assay, a third development in system A_1 was found to be more desirable.) However, it was easy to differentiate between the conjugated chenodeoxycholic and deoxycholic acids on the TLC plate since, when the plate was sprayed with 20% sulfuric acid after development and heated at 110° for 5 min, the conjugates of chenodeoxycholic acid appeared as brown spots whereas those of deoxycholic acid appeared as yellow spots.

The application of solvent system A_1 in resolving the components of human and guinea pig biles is illustrated in Figs. 2a and b. The major bile acid in guinea pig bile appears to be glycochenodeoxycholic acid, and a smaller amount of taurochenodeoxycholic acid was also observed[13]. Human bile appears to contain glycocholic, glycochenodeoxycholic and taurocholic acids, and small amounts of taurine conjugates of chenodeoxycholic and deoxycholic acids[14].

Recently, Goswami and Frey[11] described a solvent system consisting of chloroform–isopropanol–isobutanol–acetic acid–water (30:20:10:2:1) which could separate the taurine and glycine congates of deoxy- and chenodeoxycholic acids and the R_F values of the various conjugated bile acids were comparable to those obtained in the present study. However, the system A_1 required only two runs of the TLC plate for a complete separation of these four conjugates, whereas Goswami and Frey had to use as many as six runs. System A_1 has also the advantage of exhibiting a group separation of the various glycine conjugated bile acids from their taurine conjugates.

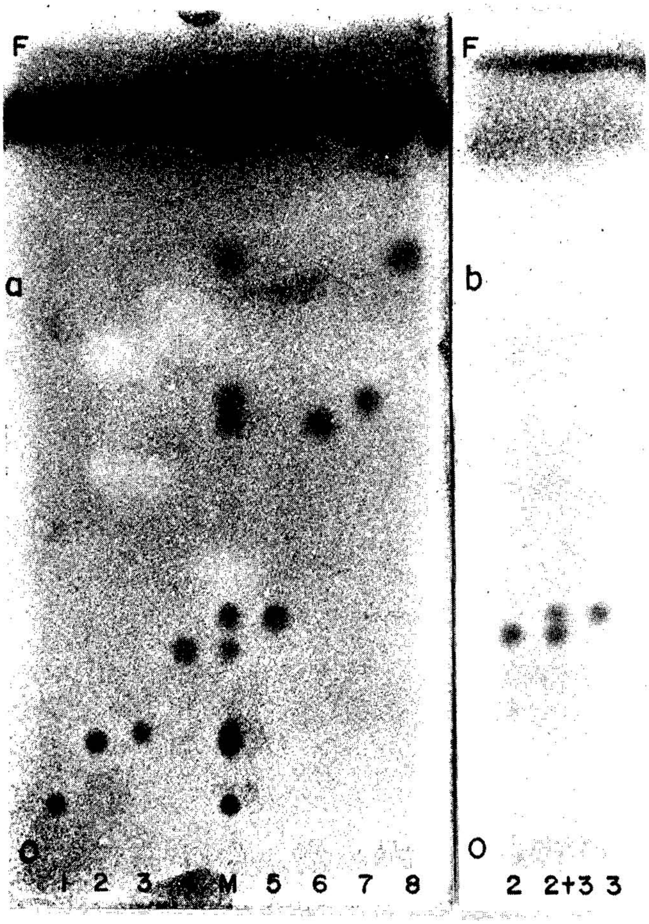

Fig. 1(a) Separation of the conjugated bile acids. Solvent system A_1 (Table I). O = Origin; 1 = taurocholic acid; 2 = taurodeoxycholic acid; 3 = taurochenodeoxycholic acid; 4 = taurolithocholic acid; M = mixture of conjugated bile acids; 5 = glycocholic acid; 6 = glycodeoxycholic acid; 7 = glycochenodeoxycholic acid; 8 = glycolithocholic acid; F = solvent front. (b) Separation of taurodeoxycholic acid and taurochenodeoxycholic acid. Solvent system A_1 (Table I); developed twice. O = Origin; 2 = taurodeoxycholic acid; 3 = taurochenodeoxycholic acid; F = solvent front.

In system A_1 and in systems described earlier[1,2,9–12], the glycine conjugates always moved faster than the corresponding taurine conjugates. When the proportion of acetic acid in the solvent system was reduced, the mobilities of all the conjugated bile acids decreased, but the effect was more pronounced on the glycine conjugates. On eliminating acetic acid completely from the solvent system (system N_1, Table I), the ratio of the mobilities of taurolithocholic acid, the least polar among the taurine conjugates, increased from 1:1 in the acidic solvent system A_2 to 2.4:1 in system N_1 (Figs. 3a and b). On the other hand, the ratio of the mobilities of glycolithocholic acid, the least polar glycine conjugate, and taurocholic acid, the most polar taurine conjugate, was reduced from 2.1:1 in system A_2 to 1.1:1 in system N_1 (Figs. 3a and b).

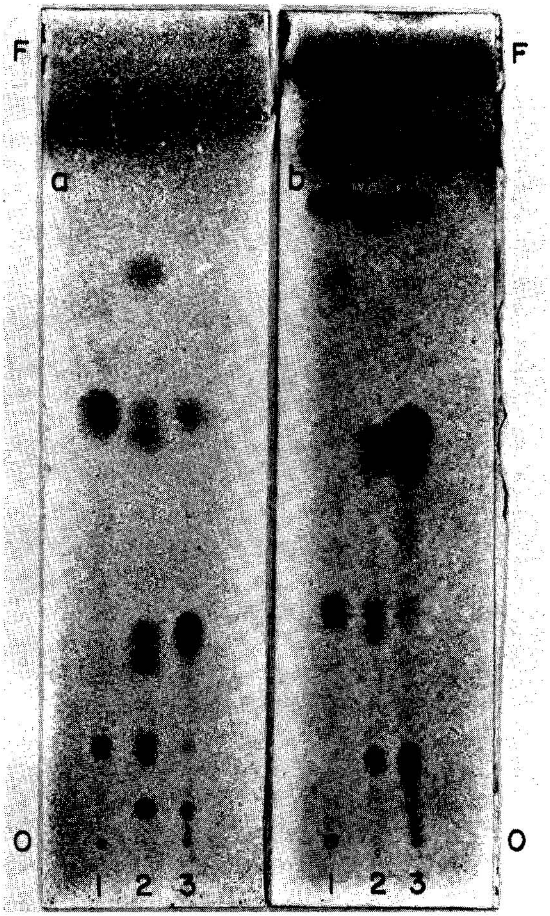

Fig. 2. Separation of biliary conjugated bile acids. Solvent system A_1 (Table I). O = Origin; 1 = guinea pig bile; 2 = mixture of standard bile acid conjugates (M, Fig. 1a); 3 = human bile; F = solvent front. (a) Developed once; (b) developed twice.

The mobilities of the conjugates of the two classes were completely reversed when acetic acid was replaced by pyridine (system B_1, Table I), all the taurine conjugated bile acids moved faster than any of the glycine conjugated ones (Table I). As is seen in Fig. 3c, taurolithocholic acid moved 1.7 times faster than glycocholic acid, whereas taurocholic acid moved 1.1 times faster than glycolithocholic acid. A similar dramatic change in the mobilities of the various conjugated bile acids was observed in solvent systems A_3, N_2 and B_2. Here, the reversal of the mobilities of the taurine and the glycine conjugates was observed even in the neutral system N_2.

It is thus evident that a combination of the acidic and the basic solvent systems can be used to completely separate and analyze a mixture of the taurine and glycine conjugates of lithocholic, deoxycholic, chenodeoxycholic and cholic acids. In addition, the change of pH from acidic to basic in the solvent system can serve as a criterion to establish more positively if a given conjugated bile acid belongs to the taurine or the glycine class.

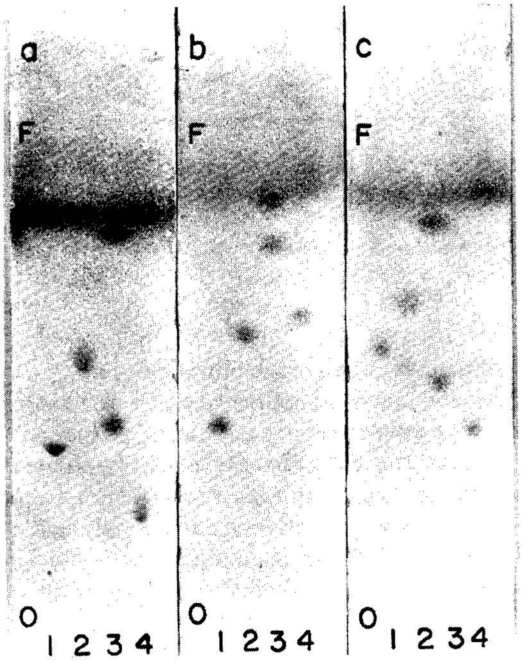

Fig. 3. Thin-layer chromatogram of conjugated bile acids. O = Origin; 1 = taurocholic acid; 2 = taurolithocholic acid; 3 = glycolithocholic acid (upper spot = lithocholic acid); 4 = glycocholic acid; F = solvent front. Solvent systems: (a) N_1 (Table I); (b) A_2; (c) B_1.

ACKNOWLEDGEMENTS

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Journal of Chromatography, 168 (1979) 562

CHROM. 11,403

Note

Thin-layer chromatography and spot test of Paraquat-contaminated marihuana

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(Received August 24th, 1978)

The weed killing herbicide, 1,1′-dimethyl-4,4′-bipyridinium dichloride (Paraquat), has been used extensively to eliminate marihuana illicitly cultivated in the fields. However, this operation has produced some serious side effects, particularly on the cannabis-smoking population, due to the high toxicity of this herbicide. According to the U.S. Federal Government, excessive or even regular smoking of marihuana contaminated with Paraquat can cause permanent lung damage. Moreover, 20% of the marihuana utilized in the U.S.A. is tainted with Paraquat.

A number of thin-layer chromatographic (TLC) methods for the identification of tetrahydrocannabinol (THC) are available[1]. We have developed in our laboratory a TLC method for the isolation and identification of marihuana in the presence of other hallucinogenic compounds[2].

In the present study, the same technique was employed for the separation and identification of Paraquat from herbicide-sprayed marihuana. Small quantities of dry marihuana leaves were washed, in approximately 5 ml of methanol, shaken well for about 20 min and filtered; microquantities of the filtrates were applied on silica gel G-coated plates (250 nm thick, according to Stahl[3]) and developed in chloroform–dioxane–ethyl acetate–conc. ammonium hydroxide (25:60:10:5). The plates were removed, air dried, and the compounds were detected in a tank containing iodine fumes. R_F values for marihuana were 0.17 and 0.95, for THC and cannabidiol, respectively, while Paraquat remained at the baseline ($R_F = 0.02$). When chloroform–acetone (9:1) was used as developing system, Paraquat again gave the same R_F value while THC was detected at $R_F = 0.30$.

The herbicide was easily detected on the second chromatogram by spraying aqueous solution of sodium carbonate (1%), followed by an equal strength solution of sodium hydrosulfite. Paraquat reacted to give a strong blue color. Presumably, the strong basic consistency of the first developing system inhibits the reaction between Paraquat and sodium hydrosulfite.

Further studies indicated that the later reagent could be used successfully for a spot-test method for the herbicide. In this case, the marihuana-washed filtrates were tested by adding sodium carbonate and sodium hydrosulfite solutions. Quantities of up to 0.001% of Paraquat in water were easily detectable by the appearance of a distinctive blue color: sodium carbonate and sodium hydrosulfite could be used also as dry powders instead of solution.

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Journal of Chromatography, 168 (1979) 563

CHROM. 11,559

Letter to the Editor

Comments to the article "Comparison of methods for the deactivation of glass open-tubular columns with PEG 20M"

Sir,

De Nijs *et al.*[1] report a procedure which, provided it is confirmed by future experience, will become an important step forward in column technology.

However, the way the procedure is compared to existing ones, is incorrect. What is still called the "Grob and Grob" procedure has been modified to such an extent that it can no longer carry our name. The essential modifications are the following.

(1) The deactivation, which we have never recommended as a general procedure, but only as an *integral part of the $BaCO_3$ procedure*, is taken out of context and applied to the *bare glass* surface. The basis of our procedure is a very thin polyglycol layer which, as we reported[2], is less thermostable on bare silica. This means that the procedure is blamed for a failure which is due to conditions the authors have erroneously applied.

(2) The PTFE connections the authors use at oven temperatures up to 250° cause a significant *oxygen* uptake into the carrier gas even when the tubings are carefully connected end-to-end. Thin layers of polyglycols are very sensitive to oxygen (hopefully the gas phase deactivation is not). They are fully stable only with H_2 as a carrier gas. Presumably the authors used He or N_2.

(3) A further integral part of our deactivation is heat treatment with Triton or Emulphor which both increase the wettability for apolar phases and reduce the influence of the deactivation on polarity. The authors arbitrarily *omit* this part, while blaming our deactivation for strong influence on polarity.

It is obvious that, under the combined influence of such modifications, our deactivation procedure can no longer make sense. When applied correctly it yields perfect results (see our recent study in this context[3]).

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K. GROB

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(Received October 10th, 1978)

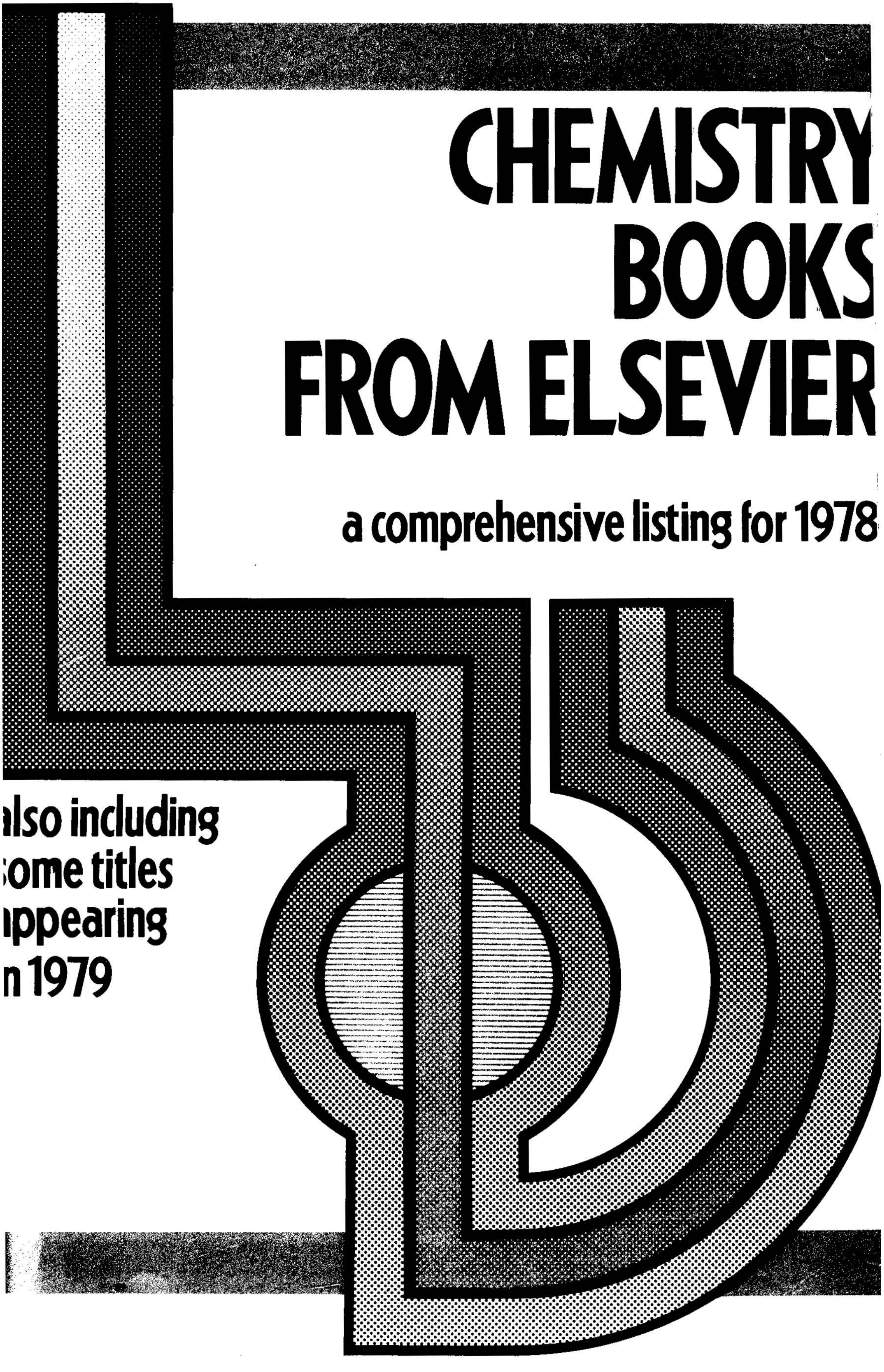
Author Index

Erratum

J. Chromatogr., 155 (1978) 47–68

Page 55, lines 14–15: "50 ml of mercaptoethanol" should read "an equal volume of 0.1 *M* $NaHCO_3$ and 50 g NaSH".

After line 17 the following paragraph should be added: "EP-Sephadex was also converted to MHP-Sephadex according to Carlsson *et al.*, *Eur. J. Biochem.*, 59 (1975) 567–572".



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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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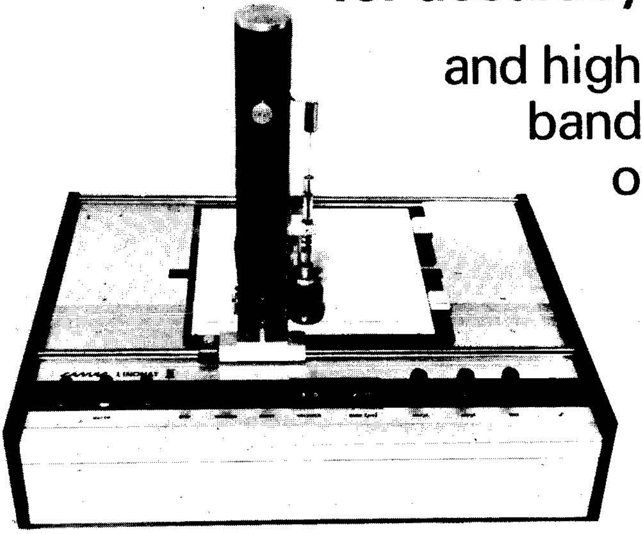
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