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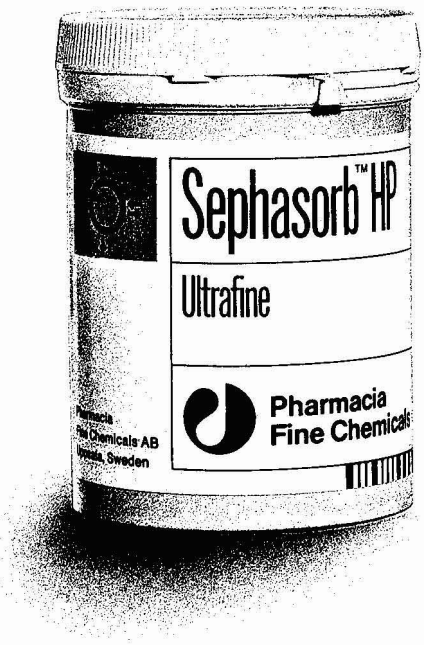

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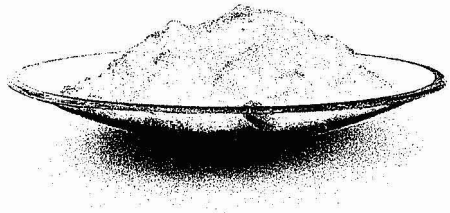

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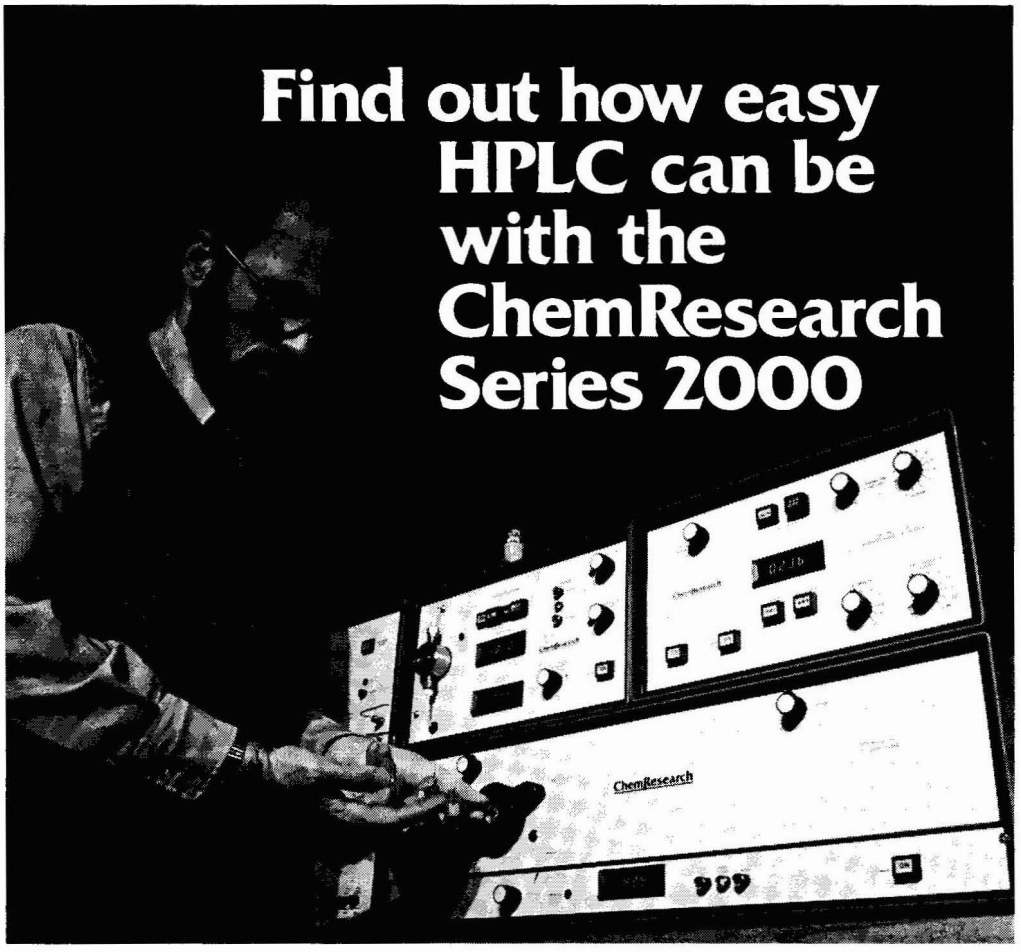

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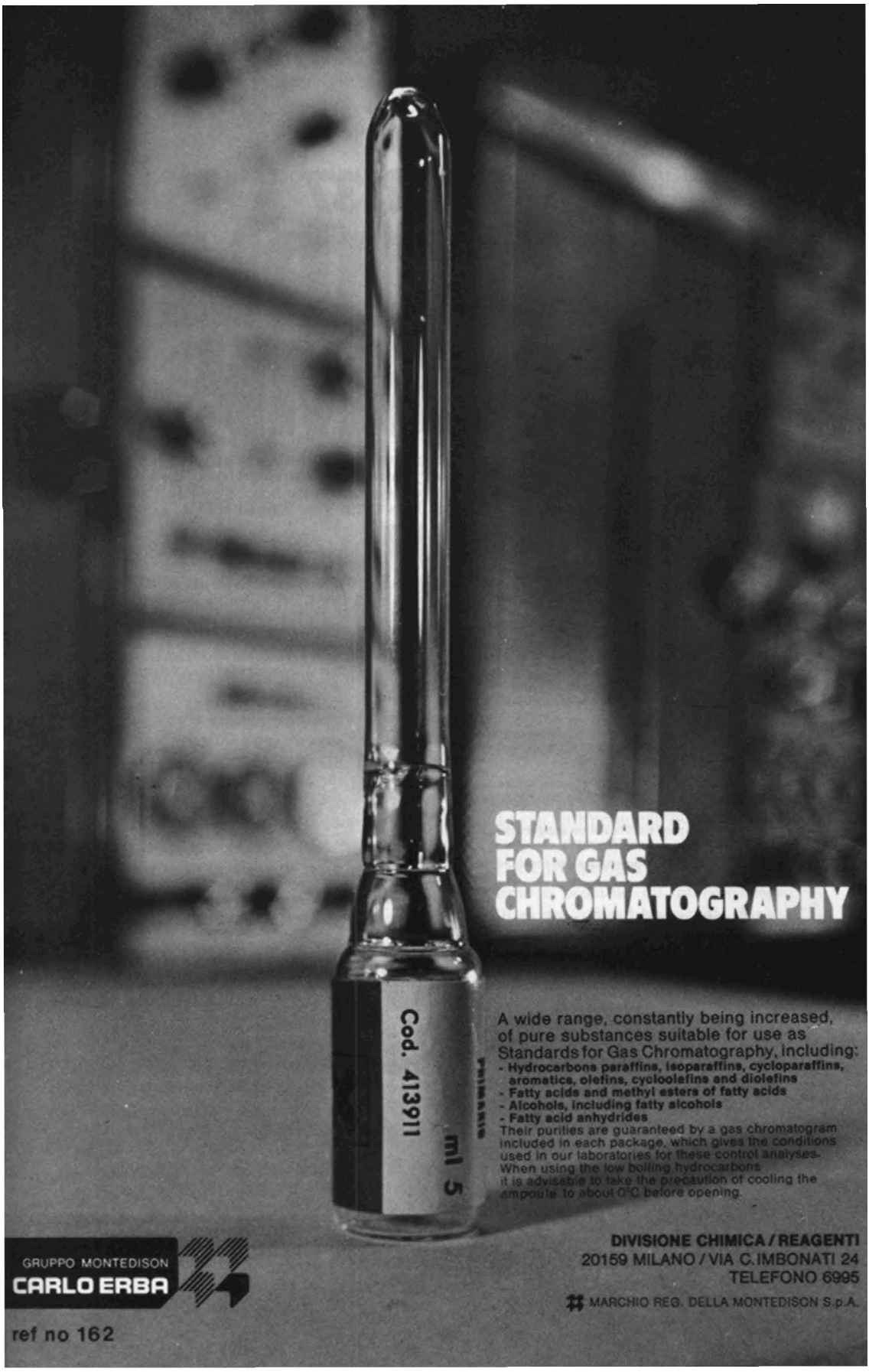

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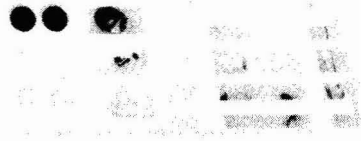

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GRADIENT OPTIMIZATION IN ELUTION LIQUID CHROMATOGRAPHY

I. THEORETICAL CONSIDERATIONS CONNECTED WITH EVALUATION OF THE CONCENTRATION–TIME FUNCTION FOR STEPWISE ELUTION

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(First received June 13th, 1977; revised manuscript received November 18th, 1977)

SUMMARY

Using the theoretical treatment of Jandera and Churáček, problems connected with evaluation of the concentration–time function are discussed. It is shown that the linear relationships of the type capacity factor *versus* concentration of the more efficient eluting component in the binary-solvent mobile phase, measured for different chromatographed compounds, can be used to predict the concentration–time function for stepwise elution with a mobile phase of constant composition in each step.

INTRODUCTION

Liteanu and Gocan wrote[1], "Gradient chromatography has developed in the general context of evolution of chromatography and is in full progress owing to the possibility of automation in the programming of certain parameters". The use of gradients is a basic means of optimizing the process of chromatographic separation, *i.e.*, achievement of the best resolution in as short a time as possible. Of many known types of gradients, the greatest experimental possibilities are offered by mobile phase gradients.

The theory of isocratic and gradient elution chromatography has been discussed by many workers[1–5]. The most advanced studies, both theoretical and experimental, have been carried out by Snyder[2] on adsorption liquid chromatography. Jandera and Churáček[6,7], using the fundamental relationships of Snyder defining distribution coefficients in adsorption chromatography for single- and binary-solvent mobile phases, derived theoretically the relationship between the capacity factor and the concentration of the more efficient eluting component in a binary-solvent mobile phase. A simplified version of this relationship is

$$\log k'_{(AB)i} = \log a_i - n_i \log x_B \qquad (1)$$

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where $k'_{(AB)i}$ is the capacity ratio for the ith component of the mixture in the binary-solvent mobile phase A–B, x_B is the molar fraction (the term "concentration" will subsequently be used) of the more efficient eluting component B in the binary-solvent mobile phase A–B and a_i and n_i are constants. It has also been shown[6] that eqn. 1 is valid for ion-exchange chromatography. However, for partition mechanisms (liquid–liquid chromatography, salting-out chromatography and solubilization chromatography on ion exchangers in mixed aqueous–organic media), a slightly different relationship has been derived[6]:

$$\log k'_{(AB)i} = \log b_i - m_i x_B \tag{2}$$

where b_i and m_i are constants. The constants a_i, n_i and b_i, m_i can either be calculated theoretically by means of the parameters that characterize the sample being chromatographed, the components of the mobile phase and the stationary phase, or can be determined directly from experimental data.

Eqns. 1 and 2, derived theoretically by Jandera and Churáček[6], have been obtained experimentally by several workers[8–11]. The systematic experimental studies of Bieganowska and Soczewiński[12] showed that the classification of chromatographic systems, as suggested by Jandera and Churáček[6], is not sufficiently accurate. The experimental dependences of $\log k'_{(AB)i}$ *versus* $\log x_B$ and $\log k'_{(AB)i}$ *versus* x_B, published in the present literature, have been summarized[12] and on this basis the types of chromatographic systems to which eqns. 1 and 2 apply have been distinguished.

Eqns. 1 and 2 describe satisfactorily a large number of experimental relationships between the capacity ratio and the concentration of component B. Hence the functions $\log k'_{(AB)i}$ *versus* $\log x_B$ and $\log k'_{(AB)i}$ *versus* x_B, measured for different compounds, may be very useful in evaluating the optimal concentration–time function for the separation of a mixture. In this paper, we discuss the problems connected with the numerical evaluation of the concentration–time functions in chromatography using elution with a mobile phase (binary-solvent mixture) with a constant composition in each step (stepwise elution chromatography). Such a type of gradient (stepwise function) is more effective than a continuous function in many chromatographic separations[13,14].

EQUATIONS CHARACTERIZING OPTIMAL SEPARATION OF THE CHROMATOGRAPHED SAMPLE

The theoretical determination of resolution for a multi-component mixture is difficult[3,15]. In order to describe the efficiency of separation fully, the resolution for each successive pair of compounds, i and $i+1$, must be calculated. If the resolution of each such pair were at least greater than unity, then the separation of the given mixture would be good. Another problem is the time of analysis. It may happen that the resolution is very high for a pair of compounds i and $i+1$ which indicates a large distance between the maxima of their chromatographic peaks, and consequently a considerable increase in the time of analysis. The optimal time for the separation of a multi-component mixture can be obtained if the resolutions of consecutive pairs of components[3] are the numbers from the interval (1, 1.5). Taking into consideration

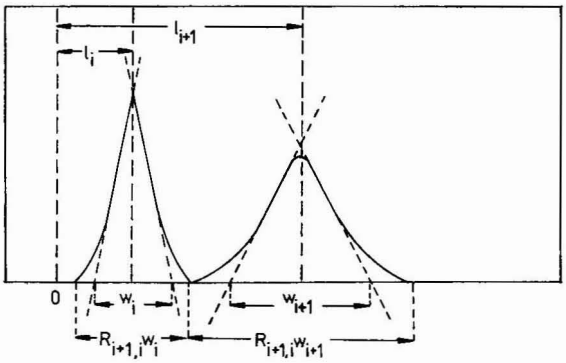

Fig. 1. Schematic diagram for eqn. 3; for $R_{i+1,i}$ the value 1.5 is assumed.

these two aspects (good resolution and a short time of analysis), a condition can be written that should be satisfied for two successive symmetrical peaks:

$$\Delta l_{i+1,i} = l_{i+1} - l_i = 0.5\, R_{i+1,i}\,(w_i + w_{i+1}) \tag{3}$$

where l_i is the distance of the peak maximum from the start for the ith component, w_i is the peak width for the ith component and $R_{i+1,i}$ is the optimal resolution for two successive peaks i and $i+1$. The optimal resolution, $R_{i+1,i}$, should be chosen so that the distances $R_{i+1,i} \cdot w_i$ and $R_{i+1,i} \cdot w_{i+1}$ are the maximal widths of the peaks of i and $i+1$, respectively (Fig. 1). Eqn. 3 can be re-written in a slightly different form:

$$\Delta V_{R_{i+1,i}} = V_{R_{i+1}} - V_{R_i} = D\, R_{i+1,i}\,(V_{R_i} + V_{R_{i+1}}) \tag{4}$$

where $D = 2/\sqrt{N}$, V_{R_i} is the retention volume of the ith component in the mixture and N is the total number of plates in the column. N is assumed to be independent of the type of compound and the composition of the mobile phase. Expressing the retention volume by means of the free volume of the column, V_m, and the capacity factor, k'_i:

$$V_{R_i} = V_m\,(1 + k'_i) \tag{5}$$

from eqn. 4 we obtain:

$$\Delta k'_{i+1,i} = k'_{i+1} - k'_i = D\, R_{i+1,i}\,(k'_i + k'_{i+1} + 2) \tag{6}$$

where k'_i and k'_{i+1} denote the capacity factors of the ith and $(i+1)$th component, respectively, and refer to the binary-solvent mobile phase A–B, *i.e.*, $k'_i = k'_{(AB)i}$ and $k'_{i+1} = k'_{(AB)i+1}$.

Now, we shall show the usefulness of eqn. 6 in the determination of the concentration–time function for two-step elution chromatography. Eqns. 1 and 2 will be used.

Firstly, we shall discuss eqn. 1. If we wish to use the concentration–time function to perform the separation of a given mixture, it is essential to know the concentration of the solvent B at which the elution of the first two components guarantees a resolution $R_{2,1}$ within the range 1–1.5. This concentration can be defined numerically from the equation that is obtained on substituting eqn. 1 in eqn. 6:

$$a_2 x_1^{-n_2}(1 - D R_{2,1}) - a_1 x_1^{-n_1}(1 + D R_{2,1}) = 2 D R_{2,1} \tag{7}$$

where x_1 is the molar fraction at which components 1 and 2 are eluted. When $n_1 = n_2$, eqn. 7 has an analytical solution[6]:

$$x_1 = \left(\frac{a_2}{2}\right)^{\frac{1}{n_1}} \cdot \left(\frac{\alpha_{2,1} - 1}{D R_{2,1} \alpha_{2,1}} - \frac{\alpha_{2,1} + 1}{\alpha_{2,1}}\right)^{\frac{1}{n_1}} \tag{8}$$

where

$$\alpha_{2,1} = a_2/a_1 \tag{9}$$

The next step is often necessary in order to shorten the retention time of a component, *i.e.*, to approximate the peak of component $i+1$ to that of component i. This effect can be achieved by increasing the concentration of the solvent B directly after elution of component i. The concentration at which component $i+1$ should be eluted can be calculated from a modified eqn. 6 and eqn. 1. For this purpose, eqn. 6 should be re-written in the form

$$\bar{k}'_{i+1} - k'_i = D R_{i+1,i}(k'_i + \bar{k}'_{i+1} + 2) \tag{10}$$

where $\bar{k}'_{i+1}$ is an average capacity factor for the $(i+1)$th component which was initially eluting at the concentration x_1 and subsequently at the concentration x_2.

Let us consider migration of component $i+1$ through a chromatographic column of length L (see Fig. 2b). The elution time of the component $i+1$ is given by

$$\bar{t}_{R_{i+1}} = \gamma_{i+1,i}\, t_{R_{(1)i+1}} + (1 - \gamma_{i+1,i})\, t_{R_{(2)i+1}} \tag{11}$$

where

$$\gamma_{i+1,i} = \frac{L_{(1)i+1}}{L} \tag{12}$$

$t_{R_{(1)i+1}}$ and $t_{R_{(2)i+1}}$ are the retention times of component $i+1$ at concentrations x_1 and x_2, respectively and $\bar{t}_{R_{i+1}}$ is the time of elution of component $i+1$ corresponding to the capacity factor $\bar{k}'_{i+1}$. Substituting into eqn. 11 the fundamental relationship

$$k'_i = \frac{t_{R_i} - t_{R_0}}{t_{R_0}} \tag{13}$$

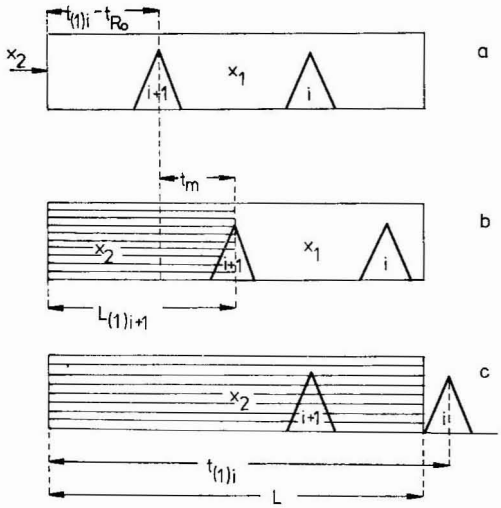

Fig. 2. Illustration of migration of components i and $i + 1$ in solvent programming.

we obtain

$$t_{R_{i+1}} = t_{R_0}(1 + \bar{k}'_{i+1}) \tag{14}$$

where

$$\bar{k}'_{i+1} = \gamma_{i+1,i}\, k'_{(1)i+1} + (1 - \gamma_{i+1,i})\, k'_{(2)i+1} \tag{15}$$

In eqns. 13–15, $k'_{(1)i+1}$ and $k'_{(2)i+1}$ denote the capacity factors for the component $i+1$ at the concentrations x_1 and x_2, respectively; $t_{R_0} = L/v$, where v is the linear velocity of the mobile phase. Combination of eqns. 10 and 15 leads to

$$k'_{(2)i+1} = \frac{k'_{(1)i}(1 + D\,R_{i+1,i}) - \gamma_{i+1,i}\, k'_{(1)i+1}(1 - D\,R_{i+1,i}) + 2\,D\,R_{i+1,i}}{(1 - \gamma_{i+1,i})(1 - D\,R_{i+1,i})} \tag{16}$$

Substituting eqn. 1 into eqn. 16 and taking into account the definition of $\gamma_{i+1,i}$ (see eqn. 21), we obtain the expression for the concentration x_2 at which component $i+1$ should be eluted:

$$x_2 = \left[\frac{a_{i+1}(1 - \gamma^a_{i+1,i})(1 - D\,R_{i+1,i})}{2\,D\,R_{i+1,i}(1 + k'_{(1)i})}\right]^{\frac{1}{n_{i+1}}}$$

$$= \left[\frac{a_{i+1}(1 - \gamma^a_{i+1,i})(1 - D\,R_{i+1,i})}{2\,D\,R_{i+1,i}(1 + a_i\, x_1^{-n_i})}\right]^{\frac{1}{n_{i+1}}} \tag{17}$$

Now we derive an expression for the parameter $\gamma^a_{i+1,i}$, for which purpose the distance $L_{(1)i+1}$ should be calculated. Let us consider the migration of components i

and $i+1$ through the chromatographic column (see Fig. 2). Solvent of concentration x_2 should be introduced into the column at such a time that its front will reach the end of column just as the peak of component i has been eluted completely, *i.e.*, at the time $t_{(1)i} - t_{R_0}$. Hence the time of migration of component $i+1$ at concentration x_1, $t^*_{(1)i+1}$, is given by

$$t^*_{(1)i+1} = t_{(1)i} - t_{R0} + t_m$$

$$= \frac{1}{\vartheta}\left(1 + \frac{1}{k'_{(1)i+1}}\right)[(L + 0.5\, w_{(1)i})\, k'_{(1)i} + 0.5\, w_{(1)i}] \tag{18}$$

The distance $L_{(1)i+1}$ can be calculated from the expression

$$L_{(1)i+1} = t^*_{(1)i+1}\left[\frac{\vartheta}{1 + k'_{(1)i+1}}\right] \tag{19}$$

From eqns. 19, 18 and 12, we have

$$\gamma_{i+1,i} = \frac{1}{k'_{(1)i+1}} \cdot \left[\frac{0.5\, w_{(1)i}}{L} + k'_{(1)i}\left(1 + \frac{0.5\, w_{(1)i}}{L}\right)\right] \tag{20}$$

If $L \gg w_{(1)i}$, this expression can be reduced to

$$\gamma_{i+1,i} = \frac{k'_{(1)i}}{k'_{(1)i+1}} \tag{21}$$

Substituting eqn. 1 into eqn. 20, we obtain an exact numerical value of the parameter $\gamma_{i+1,i}$, which is necessary for calculating the concentration x_2 (see eqn. 17). A very simple expression for $\gamma_{i+1,i}$ can be obtained from eqns. 21 and 1:

$$\gamma^a_{i+1,i} = \frac{a_{(1)i}}{a_{(1)i+1}} \cdot x_1^{n_{i+1} - n_i} = \frac{1}{\alpha_{i+1,i}} \cdot x_1^{n_{i+1} - n_i} \tag{22}$$

For chromatographic systems that satisfy eqn. 2, the following equations are analogous to eqns. 7, 8, 17 and 22:

$$b_2\, 10^{-m_2 x_1}(1 - D\, R_{2,1}) - b_1\, 10^{-m_1 x_1}(1 + D\, R_{2,1}) = 2\, D\, R_{2,1} \tag{23}$$

$$x_1 = \frac{1}{m_1} \cdot \log\left[\frac{b_2}{2}\left(\frac{\beta_{2,1} - 1}{D\, R_{2,1}\, \beta_{2,1}} - \frac{\beta_{2,1} + 1}{\beta_{2,1}}\right)\right] \tag{24}$$

for $m_1 = m_2$

$$x_2 = \frac{1}{m_{i+1}} \cdot \log\left[\frac{b_{i+1}(1 - \gamma^b_{i+1,i})(1 - D\, R_{i+1,i})}{2\, D\, R_{i+1,i}(1 + b_i\, 10^{-m_i x_1})}\right] \tag{25}$$

where

$$\beta_{i+1,i} = b_{i+1}/b_i \tag{26}$$

and

$$\gamma^b_{i+1,i} = \frac{b_1}{b_{i+1}} \cdot 10^{(m_{i+1} - m_i)x_1} = \frac{1}{\beta_{i+1,i}} \cdot 10^{(m_{i+1} - m_i)x_1} \tag{27}$$

The parameter $\gamma^b_{i+1,i}$ is evaluated from eqns. 2 and 21. Eqn. 24 has been also proposed by Jandera and Churáček[6].

EVALUATION OF THE CONCENTRATION–TIME FUNCTION

Let us now discuss the chromatographic separation of an n-component mixture. Assume that we know the relationships between the capacity factors and the concentration of solvent B in the mixture A–B for all components contained in the chromatographed sample. If the chromatographic system satisfies eqn. 1, then eqns. 7, 8, 17 and 22 are useful for programming the gradient. Eqns. 23–27 are used for programming the gradient in chromatographic systems that satisfy eqn. 2. For the sake of illustration, we shall discuss eqns. 7, 8, 17 and 22. Let the sequence of elution of the components be

$$l_1 < l_2 < \ldots < l_i < l_{i+1} < \ldots < l_n \tag{28}$$

Series 28 is equivalent to the following series of capacity factors:

$$k'_1 < k'_2 < \ldots < k'_i < k'_{i+1} < \ldots < k'_n \tag{29}$$

However, for the determined concentration of solvent B, if $n_1 = n_2 = \ldots = n_n$, the following inequality is satisfied:

$$a_1 < a_2 < \ldots < a_i < a_{i+1} < \ldots a_n \tag{30}$$

For the sake of simplification, let us consider a mixture of compounds for which $n_i = 1$ (from numerous experimental studies it appears that for many compounds n_i is close or equal to unity[16]). In Fig. 3, the capacity factor (k^1_i) *versus* molar fraction of solvent B, *i.e.*, $k^1_i = a_i/x$, are presented for different values of a_i. The initial concentration, x_1, at which compounds 1 and 2 should be eluted is calculated from eqn. 8, assuming $R_{2,1} = 1$. In order to obtain the resolution for the interval (1, 1.5) during separation of compounds i and $i+1$ (where $i \geqslant 2$), the distance between the curves i and $i+1$ at the point $x = x_1$ should satisfy the inequality

$$D[(a_i + a_{i+1})/x_1 + 2] \leqslant a_{i+1,i} \leqslant 1.5\, D[(a_i + a_{i+1})/x_1 + 2] \tag{31}$$

for $i = 1, 2, 3, \ldots$ This inequality is equivalent to

$$\frac{a_i(1 + D) + 2\,Dx_1}{1 - D} \leqslant a_{i+1} \leqslant \frac{a_i(1 + 1.5\,D) + 3\,Dx_1}{1 - 1.5\,D} \tag{32}$$

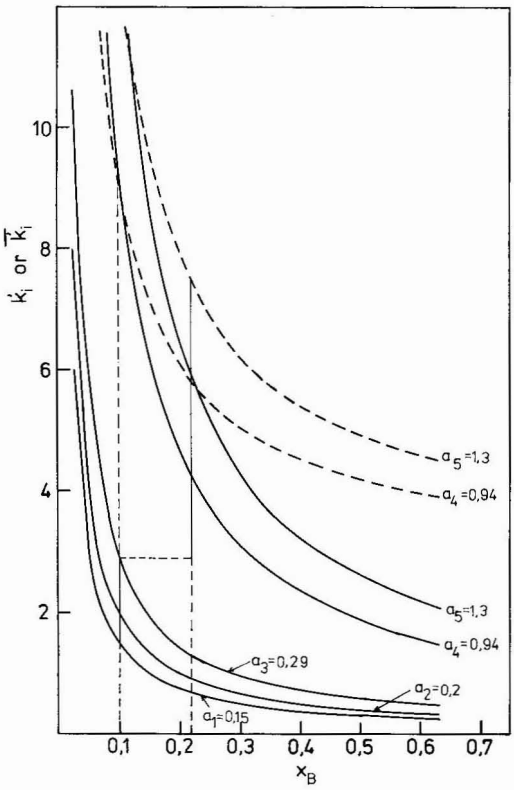

Fig. 3. Dependences between the capacity factor and the molar fraction of the more efficient eluting component calculated for different values of a_i. The solid lines denote the functions $k' = a_i/x$ (i = 1,2,3,4,5) and the broken lines denote the functions $\bar{k}'_j = k'_{(1)3} + (1 - \gamma_{j,3})\, a_j/x$ (i = 4,5). The subscripts i and j denote a given component of the chromatographed sample.

for $i = 1, 2, 3, \ldots$ If the distances between the successive curves at the point $x = x_1$, beginning from $i \geqslant 2$, satisfy inequality 31, then these compounds will be separated at concentration x_1. From Fig. 3, it appears that inequality 31 is satisfied by compounds from 1 to 3 inclusive. The distance between the curves 3 and 4 at the point x_1 already exceeds the upper limit of the distance calculated for a resolution of 1.5. Hence the concentration x_2 at which component 4 will be eluted should be determined. This concentration is determined by means of eqn. 17, assuming the optimal value of the resolution $R_{4,3}$, which should be greater than unity but approximate to unity if the concentration x_2 increases. The capacity ratio of each successive peak j, for $j \geqslant 4$, is given by the equation

$$\bar{k}'_j = \gamma^a_{j,3}\, k'_{(1)j} + (1 - \gamma^a_{j,3})\, k'_{(2)j}$$

$$= k'_{(1)3} + \left[1 - \frac{k'_{(1)3}}{k'_{(1)j}}\right] a_j\, x_2^{-nj} \qquad (33)$$

for $j \geqslant 4$. In Fig. 3, the broken lines present the functions $\bar{k}'_j$ *versus* x_2 for $j \geqslant 4$ and $n_j = 1$.

In order to obtain the resolution for the interval (1, 1.5) for components with $j \geqslant 4$, the distances $d_{j+1,j}$ between the curves j and $j+1$ at the point $x = x_2$ should be in the following range:

$$D\,(\bar{k}'_j + \bar{k}'_{j+1} + 2) \leqslant d_{j+1,j} \leqslant 1.5\,D\,(\bar{k}'_j + \bar{k}'_{j+1} + 2) \tag{34}$$

$j = 4,5, \ldots$ From eqn. 34, an interesting inequality can be deduced:

$$\frac{\bar{k}'_j\,(1 + D) + 2\,D}{1 - D} \leqslant \bar{k}'_{j+1} \leqslant \frac{\bar{k}'_j\,(1 + 1.5\,D) + 3\,D}{1 - 1.5\,D} \tag{35}$$

or

$$\left[\frac{\bar{k}'_j\,(1 + D) + 2\,D}{1 - D} - k'_{(1)3}\right] \frac{x_2}{1 - \gamma^a_{j+1,3}} \leqslant a_{j+1} \leqslant$$

$$\leqslant \left[\frac{\bar{k}'_j\,(1 + 1.5\,D) + 3\,D}{1 - 1.5\,D} - k'_{(1)3}\right] \frac{x_2}{1 - \gamma^a_{j+1,3}} \tag{36}$$

The latter inequality is especially important in determining the resolutions for further pairs of components. From eqn. 36, the boundary parameters a_{j+1} ($j \geqslant 4$) are obtained, which guarantee the resolution of further compounds for the interval (1, 1.5). From Fig. 3, it follows that at concentration x_2 component 5 can be eluted. Proceeding in this way, elution concentrations can be found for other sample components. Knowing these concentrations as well as parameters a and n, the elution time (from the start to the end of the peak) for individual components can be calculated. They are

$$t_{(1)i} = t_{R(1)i} + D\,R_{i+1,i}\,t_{R(1)i} = t_{R_0}\,(1 + k'_{(1)i})\,(1 + D\,R_{i+1,i}) = t_{R_0}\,G_{(1)i} \tag{37}$$

for $i = 1, 2, 3$ and (see Appendix)

$$t_{(1,2)j} = t_{R(1,2)j} + D\,R_{j+1,j}\,t_{R(2)j}$$

$$\approx t_{R_0}\,(1 + \bar{k}'_j)\,(1 + D\,R_{j+1,j}) = t_{R_0}\,G_{(1,2)j} \tag{38}$$

Thus, the concentration–time function presented in Fig. 4 corresponds to Fig. 3. In the above discussion, we assumed the resolutions for successive pairs of the components at a given concentration for the interval (1, 1.5). The upper limit of this interval may be higher than 1.5, depending on the particular mixture being chromatographed.

The evaluation of the optimal concentration–time function may be difficult for many real chromatographic systems. Then, the position of successive peaks can

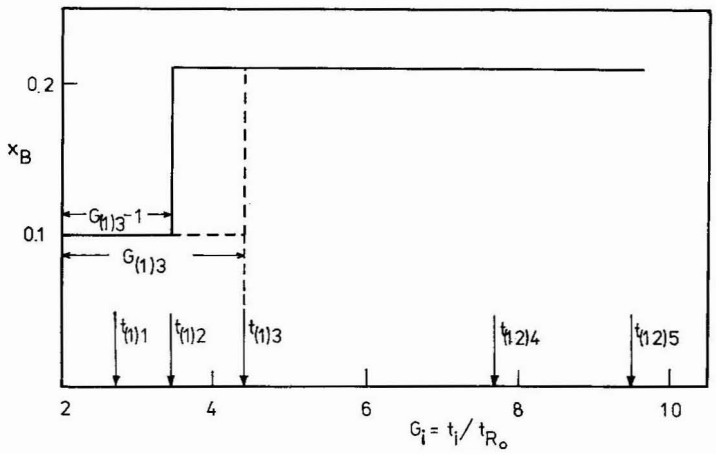

Fig. 4. Two-step gradient corresponding to Fig. 3.

be regulated by changing the concentration x or by changing the solvents in the mobile phase.

APPENDIX

Eqn. 38 defines approximately the elution time $t_{(1,2)j}$, *i.e.*, the time from the start to the end of the jth peak, for two-step elution. The first term of this equation denotes the retention time of the jth component, *i.e.*, the time from the start to the peak maximum, and it is defined by means of the average capacity factor, $\bar{k}'_j$. The other term in eqn. 38 defines approximately half of the peak width for the jth component and it is usually small in comparison with the first term. Although the jth peak is formed during all steps of elution, it is formed mainly in the last step. Therefore, the peak width for stepwise elution may be equal to or greater than that for isocratic elution at concentration x_2. Thus, the second term in eqn. 38 can be determined by means of $t_{R(2)j}$ or $t_{R(1,2)j}$.

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GRADIENT OPTIMIZATION IN ELUTION LIQUID CHROMATOGRAPHY

II. THEORY OF MULTI-STEP ELUTION WITH A MOBILE PHASE OF CONSTANT COMPOSITION IN EACH STEP

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SUMMARY

Equations describing multi-step elution with a mobile phase of constant composition in each step have been derived. These equations are very useful for calculating the concentration–time function if the experimental relationships between the capacity factors and the concentration of the more efficient eluting component in the binary-solvent mobile phase are known for different sample compounds.

INTRODUCTION

In Part I[1], theoretical problems connected with evaluation of the concentration–time function for two-step elution with a mobile phase were considered and a method for calculating two-step concentration–time functions was proposed. In this method, the experimental relationships of the type capacity factor *versus* concentration of the more efficient eluting component in the binary-solvent mobile phase, measured for different pure components of the chromatographed sample, were applied. These experimental dependences may be presented in a linear form. Knowing the parameters that characterize these linear functions, the capacity factor can be calculated for an arbitrary composition of the mobile phase.

In this paper, the procedure for calculating the concentration–time function for multi-step elution in liquid chromatography is discussed. The theoretical discussion relates to elution with a mobile phase of constant composition in each step.

GENERAL CONSIDERATIONS

Theoretical problems connected with the determination of the resolution of multi-component mixture were discussed in Part I[1]. It was shown that the efficiency of separation of a given multi-component mixture can be characterized by means of the resolutions of successive pairs of components. Assuming optimal resolutions for each

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pair of components of the mixture (for the interval 1–1.5 or 1–2.0), the composition of the mobile phase can be calculated.

Here, chromatography of an s-component mixture is discussed. Let A–B denotes the mobile phase, where B is the more efficient eluting solvent. Let us assume an r-step elution, *i.e.*, in the ith step the molar fraction (concentration) of B is x_i and at this concentration the components from $j_{i-1}+1$ to j_i will be eluted; thús, $j_r = s$ (see Fig. 1). The fundamental assumption, which makes the change of the concentration x_B at the optimal moment possible, is as follows: mobile phase with a concentration x_i of solvent solvent B should be introduced into the column at such a time that its front reaches the end of the column just as the peak of the component j_{i-1} has been eluted, *i.e.*, at the time $t_{R(1,\ldots,i-1)\,j_{i-1}} - t_{R_0}$, where $t_{R(1,\ldots,i-1)\,j_{i-1}}$ is the retention time for the last component, which is eluted in the $(i-1)$th step and $t_{R_0} = L/v$ (L is the length of the column and v is the linear velocity of the mobile phase). Hence this component migrated through the column at $i-1$ different concentrations of x_B. In such a model, we assume that the time of elution of a given peak can be neglected in comparison with the retention time, *i.e.*, the width of the peak is very small. Furthermore, small concentrations of the chromatographed compounds and symmetrical peaks are considered.

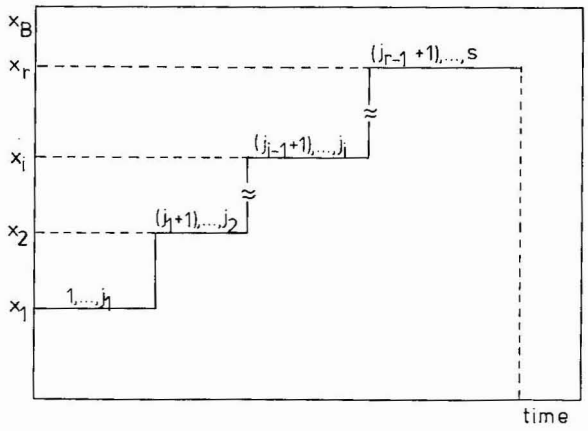

Fig. 1. Schematic diagram for r-step elution when an s-component mixture is separated.

EQUATIONS FOR RETENTION VOLUME AND RETENTION TIME IN STEPWISE ELUTION

In gradient elution chromatography, the reduced retention volume, $V'_{R_j} = V_{R_j} - V_m$ (V_{R_j} is the retention volume of the jth sample compound and V_m is the total volume of the mobile phase in the column) can be calculated by means of the following relationships[2–4]:

$$\int_0^{V_m} \mathrm{d}V_m = \int_0^{V'_{R_j}} \frac{\mathrm{d}V}{k'_j} \tag{1}$$

where k'_j is the capacity factor equal to the ratio of the total amount of the jth compound in the stationary phase to that in the mobile phase A–B under equilibrium conditions; k' is a function of the concentration of solvent B. For r-step elution with a mobile phase of constant composition in each step, eqn. 1 can be re-written as

$$V_m = \sum_{i=1}^{l-1} V'_{(i)}/k'_{(i)j} + V'_{(l)j}/k'_{(l)j} \tag{2}$$

where

$$V'_{R(1,\ldots,l)j} = \sum_{i=1}^{l-1} V'_{(i)} + V'_{(l)j} \tag{3}$$

for $l \leqslant r$; $V'_{(i)}$ is the volume of mobile phase of concentration x_i of $i > 1$; however, for $i = 1$ $V_{(1)} = V'_{(1)} + V_m$ and $k'_{(i)j}$ is the capacity factor of the jth component for mobile phase A–B with a concentration x_i of solvent B. The volume $V'_{(i)}$ is proportional to the capacity factor $k_{(i)j}$:

$$V'_{(i)} = V^i_{m\,j,j_i} \cdot k'_{(i)j} \tag{4}$$

for $j \geqslant j_i$ where $V^i_{m\,j,j_i}$ is the proportionality factor and j_i denotes the last component which is eluted in the ith step (see Fig. 1). $V_{(l)j}$ denotes the volume of mobile phase A–B with a concentration x_l in which the sample compound j migrated. Substituting eqn. 4 into eqn. 3, we obtain

$$V_{R(1,\ldots,l)j} = V_m\Big[1 + \sum_{i=1}^{l-1} \gamma^i_{j,j_i} \cdot k'_{(i)j} + \Big(1 - \sum_{i=1}^{l-1} \gamma^i_{j,j_i}\Big)k'_{(l)j}\Big] \tag{5}$$

where

$$\gamma^i_{j,j_i} = \frac{V'_{mj,j_i}}{V_m} \tag{6}$$

for $i < l$ and $j > j_i$ and l is the last step of elution for the jth compound. Eqn. 5 can be re-written in the following form:

$$V_{R(1,\ldots,l)j} = V_m(1 + k'_{(1,\ldots,l)j}) \tag{7}$$

or

$$t_{R(1,\ldots,l)j} = t_{R0}\Big(1 + k'_{(1,\ldots,l)j}\Big) \tag{8}$$

where

$$k'_{(1,\ldots,l)j} = \sum_{i=1}^{l-1} \gamma^i_{j,j_i} k'_{(i)j} + \Big(1 - \sum_{i=1}^{l-1} \gamma^i_{j,j_i}\Big)k'_{(l)j} \tag{9}$$

The symbol $k'_{(1,\ldots,l)j}$ denotes the capacity factor of the jth sample compound, which migrated through the column with the mobile phase of different concentrations x_B from x_1 to x_l inclusive. The capacity factor $k'_{(1,\ldots,l)j}$ is an average of the capacity factors $k_{(i)j}$ for $i = 1,2,\ldots,l$.

DETERMINATION OF THE PARAMETERS γ

For the purpose of calculating the parameter γ^i_{j,j_i} the volume $V^i_{m\,j,j_i}$ should be evaluated. Considering the definition of $V'_{(i)}$ (see eqn. 3) and eqn. 4, we obtain

$$V^i_{m\,j,j_i} = \frac{V'_{(i)}}{k'_{(i)j}} = \frac{V_{R(1,\ldots,i)j_i} - V_{R(1,\ldots,i-1)j_{i-1}}}{k'_{(i)j}} \tag{10}$$

Substitution of eqns. 7 and 10 in eqn. 6 leads to

$$\gamma^i_{j,j_i} = \frac{k'_{(1,\ldots,i)j_i} - k'_{(1,\ldots,i-1)j_{i-1}}}{k'_{(i)j}} \tag{11}$$

$$= \frac{\sum_{p=1}^{i} \gamma^p_{j_i,j_p} k'_{(p)j_i} - \sum_{p=1}^{i-1} \gamma^p_{j_{i-1},j_p} k'_{(p)j_{i-1}}}{k'_{(i)j}}$$

$$= C + \frac{\gamma^i_{j_i,j_i} k'_{(i)j_i}}{k'_{(i)j}}$$

where

$$C = \frac{1}{k'_{(i)j}} \sum_{p=1}^{i-1} \left[\gamma^p_{j_i,j_p} k'_{(p)j_i} - \gamma^p_{j_{i-1},j_p} k'_{(p)j_{i-1}}\right] \tag{12}$$

It follows from eqn. 4 that

$$V'_{(p)}/V_m = \gamma^p_{j_i,j_p} k'_{(p)j_i} = \gamma^p_{j_{i-1},j_p} k'_{(p)j_{i-1}} \tag{13}$$

for $p \leqslant i-1$. According to eqn. 13, C = 0. Then, from eqn. 11 we obtain

$$\gamma^i_{j,j_i} = \frac{\gamma^i_{j_i,j_i} k'_{(i)j_i}}{k'_{(i)j}} = \frac{k'_{(i)j_i}}{k'_{(i)j}} \left(1 - \sum_{p=1}^{i-1} \gamma^p_{j_i,j_p}\right) \tag{14}$$

Knowing the parameters γ^1_{j,j_1}, γ^2_{j,j_2},..., $\gamma^{l-1}_{j,j_{l-1}}$, the capacity factor $k'_{(1,\ldots,\ j)j}$ can be calculated by means of eqn. 9. These parameters are as follows:

$$\gamma^1_{j,j_1} = \frac{k'_{(1)j_1}}{k'_{(1)j}} \tag{15}$$

$$\gamma^2_{j,j_2} = \frac{k'_{(2)j_2}}{k'_{(2)j}}\left(1 - \frac{k'_{(1)j_1}}{k'_{(1)j_2}}\right)$$

$$\gamma^3_{j,j_3} = \frac{k'_{(3)j_3}}{k'_{(3)j}}\left(1 - \frac{k'_{(1)j_1}}{k'_{(1)j_3}} - \frac{k'_{(2)j_2}}{k'_{(2)j_3}}\left[1 - \frac{k'_{(1)j_1}}{k'_{(1)j_2}}\right]\right)$$

. . . etc.

EVALUATION OF THE CONCENTRATION–TIME FUNCTION

As Part I[1], we applied the following equations for the capacity factors k'_j:

$$k'_j = a_j x^{-n_j} \tag{16a}$$

or

$$k'_j = b_j \cdot 10^{-m_j x} \tag{16b}$$

where a_j, n_j, b_j and m_j are constants, which can be calculated from the experimental values of k'_j presented in a linear form[5–7].

The concentration of solvent B at which the elution of two arbitrary components guarantees the optimal resolution $R_{j+1,j}$ (for example, from 1.0 to 1.5) can be calculated from eqn. 16a or 16b and the following expression:

$$k'_{(1,2,\ldots)j+1} - k'_{(1,2,\ldots)j} = DR_{j+1,j}\left(k'_{(1,2,\ldots)j+1} + k'_{(1,2,\ldots)j} + 2\right) \tag{17}$$

where $D = 2/\sqrt{N}$, N is the total number of plates in the column, which is assumed to be independent of the type of compound and the composition of the mobile phase, and j and $j+1$ denote two arbitrary successive sample compounds.

The first step in the evaluation of the concentration–time function is the calculation of the first concentration, x_1, at which sample compounds 1 and 2 can be eluted with optimal resolution, $R_{2,1}$. This concentration can be evaluated numerically from the following equations:

$$a_2 x_1^{-n_2}(1 - DR_{2,1}) - a_1 x_1^{-n_1}(1 + DR_{2,1}) = 2DR_{2,1} \tag{18a}$$

or

$$b_2 10^{-m_2 x_1}(1 - DR_{2,1}) - b_1 \cdot 10^{-m_1 x_1}(1 + DR_{2,1}) = 2DR_{2,1} \tag{18b}$$

It follows from Fig. 1 that the sample compounds from 1 to j_1 will be eluted at the concentration x_1. The number of compounds that can be eluted at the concentration x_1 is calculated so as to give optimal resolutions for two successive peaks.

The next step is the calculation of a new concentration, x_i, at which the sample compounds from $j_{i-1}+1$ to j_i will be eluted. The concentration x_i can be evaluated from the following equation:

$$k'_{(i)j_{i-1}+1} = \left[\frac{k'_{(1,\ldots,i-1)j_{i-1}}(1 + DR_{j_{i-1}+1,j_{i-1}}) + 2DR_{j_{i-1}+1,j_{i-1}}}{1 - DR_{j_{i-1}+1,j_i}} - \sum_{p=1}^{i-1} \gamma^p_{j_{i-1}+1,j_p} k'_{(p)j_{i-1}+1}\right] \Big/ \left(1 - \sum_{p=1}^{i-1} \gamma^p_{j_{i-1}+1,j_p}\right) \tag{19}$$

Substituting in eqn. 9 for $k_{(i)j_{i-1}+1}$, from eqns. 16a and 16b we obtain

$$x_i = \left\{a_{j_{i-1}+1}\left(1 - \sum_{p=1}^{i-1} \gamma^p_{j_{i-1}+1,j_p}\right) \Big/ \left[\frac{k'_{(1,\ldots,i-1)j_{i-1}}(1 + DR_{j_{i-1}+1,j_{i-1}}) + 2DR_{j_{i-1}+1,j_{i-1}}}{1 - DR_{j_{i-1}+1,j_{i-1}}} - \right.\right.$$

$$\left.\left. - \sum_{p=1}^{i-1} \gamma^p_{j_{i-1}+1,j_p} k'_{(p)j_{i-1}+1}\right]\right\}^{\frac{1}{nj_{i-1}+1}} \tag{20a}$$

or

$$x_i = \frac{1}{m_{j_{i-1}+1}} \log\left\{b_{j_{i-1}+1}\left(1 - \sum_{p=1}^{i-1} \gamma^p_{j_{i-1}+1,j_p}\right) \Big/ \left[\frac{k'_{(1,\ldots,i-1)j_{i-1}}(1 + DR_{j_{i-1}+1,j_{i-1}}) + 2DR_{j_{i-1}+1,j_{i-1}}}{1 - DR_{j_{i-1}+1,j_{i-1}}} - \sum_{p=1}^{i-1} \gamma^p_{j_{i-1}+1,j_p} k'_{(p)j_{i-1}+1}\right]\right\} \tag{20b}$$

At a given concentration, x_i, of solvent B, the sample compounds from $j_{i-1}+1$ to j_i should be eluted, if the following inequality is satisfied:

$$\frac{k'_{(1,\ldots,i)p}(1 + DR^{\min}_{p+1,p}) + 2DR^{\min}_{p+1,p}}{1 - DR^{\min}_{p+1,p}} \leqslant k'_{(1,\ldots,i)p+1} \leqslant$$

$$\leqslant \frac{k'_{(1,\ldots,i)p}(1 + DR^{\max}_{p+1,}) + 2DR^{\max}_{p+1,p}}{1 - DR^{\max}_{p+1,p}} \tag{21}$$

for $j_{i-1} + 1 \leqslant p \leqslant j_i - 1$, where R^{min} and R^{max}, the values for the interval (1.0, 2.0), can be assumed. This inequality can be presented in slightly different forms, which can be obtained from eqns. 21 and 16a or 16b (see Part I[1]).

Knowing the concentrations x_i ($i = 1,2,...,r$) in the successive steps of the elution and the time of these steps, the concentration–time function for an r-step elution can be presented in graphical form (in Part I[1], such a function for a two-step elution was calculated).

The elution time of the p-th compound, measured from the start of the analysis to the end of the pth peak, can be calculated as follows:

$$\begin{aligned} t_{(1,\ldots,i)p} &= t_{R(1,\ldots,i)p} + DR_{p+1,p}t_{R(i)p} \\ &\approx t_{R_{(1,\ldots,i)p}}(1 + DR_{p+1,p}) = t_{R0}(1 + k'_{(1,\ldots,i)p})\,(1 + DR_{p+1,p}) \\ &= t_{R0}G_{(1,\ldots,i)p} \end{aligned} \tag{22}$$

for $j_{i-1} + 1 \leqslant p \leqslant j_i - 1$. It follows from eqn. 22 that the sample component p is eluted at the concentration x_i. The time t_{i+1} when mobile phase with a concentration x_{i+1} of solvent B enters the column is

$$t^{in}_{(i+1)} = t_{(1,2,\ldots,i)j_i} - t_{R0} = t_{R0}[G_{(1,2,\ldots,i)j_i} - 1] \tag{23}$$

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SYMBOLS

A	less efficient eluting component in the binary-solvent mobile phase;
B	more efficient eluting component in the binary-solvent mobile phase;
D	parameter equal to $2\sqrt{N}$;
L	length of the column;
N	number of theoretical plates in the column;
$R_{j+1,j}$	resolution of two compounds, j and $j+1$;
V_m	total volume of the mobile phase in the column;
$V_{R(1,\ldots,l)j}$	retention volume of the jth sample compound, which migrated through the column with mobile phase having different concentrations, x_B, from x_1 to x_l;
V'_{R_j}	reduced retention volume for the jth sample compound;
$V'_{(i)}$	total volume of the mobile phase of concentration x_i of $i > 1$;
$V'_{(l)j}$	volume of mobile phase A–B of concentration x_l for the jth sample compound, where $j_{l-1} < j < j_l$;
$V^i_{mj.j_i}$	proportionality factor in eqn. 4;
a, b	constants in eqns. 16a and 16b;

$k'_{(i)j}$ capacity factor of the jth component for mobile phase A–B with a concentration x_i of solvent B;

$k'_{(1,\ldots,l)j}$ average capacity factor of the jth sample compound, which migrated through the column with mobile phase having different concentrations, x_B, from x_1 to x_l (see eqn. 9);

m constant in eqn. 16b;

n exponent in eqn. 16a;

r total number of elution steps;

s number of sample compounds;

t_{R_o} equal to L/v;

t_R retention time of the jth compound;

$t_{(1,\ldots,l)j}$ elution time of the jth compound, measured from the start of analysis to the end of the jth peak;

γ^i_{j,j_l} parameter defined in eqn. 14;

v linear velocity of the mobile phase.

Subscripts

$(i)j$ refers to jth compound migrating through the column with mobile phase of composition x_i;

$(1,\ldots,i)j$ refers to jth compound migrating through the column with mobile phase having different compositions from x_1 to x_i;

j_i last sample compound eluted at concentration x_i;

p summation index in eqns. 11–13, 19 and 20 or a component in the range $\langle j_{i-1} + 1, j_i - 1 \rangle$.

Superscript

i refers to mobile phase of concentration x_i.

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A MATHEMATICAL MODEL OF FREE-FLOW ELECTROPHORESIS

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SUMMARY

A computerized mathematical model has been developed of a free-flow electrophoresis cell operating under conditions of no convection and no sedimentation of sample. The complex interactions of the various system parameters have been identified and included in this model. Data inputs representing existing equipment have been processed with the theoretical results comparing well with experimental results. Data were also processed for an experimental electrophoresis cell designed to allow optimum resolution and/or sample throughput while operating in a zero *g* environment. Theoretical results are presented along with some experimental ground-based data.

INTRODUCTION

The fact that particles dispersed in a solution could be influenced by an electric field was first described by Lodge[1] in 1886. Six years later, Picton and Linder[2] related their systematic studies of the phenomenon. However, as with most new techniques, there was a dormant period, and it was not until the work of Tiselius[3] in 1937 that electrophoresis began to receive increased attention. The Tiselius method was originally of interest only to biochemists and medical researchers. However, with the introduction of lower cost equipment and advances such as supporting media, biologists, chemists and engineers use the technique for analysis, separation, identification and purification.

During the nineteen fifties and sixties, men such as Barrollier *et al.*[4] and Hannig[5] proposed preparative electrophoretic techniques based on a flowing system in which both the buffer and the sample were continuously admitted to the electrophoresis chamber, with the separated fractions being collected in individual containers. Such electrophoresis systems are now categorized as "free-flow". Because these systems are made thin to minimize convection problems and to maintain stable temperature gradients, the sample fractions are generally distorted due to both hydro-

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dynamic and electro-osmotic flow profiles. These types of distortion were recognized by Kolin[6] in his magnetically driven electrophoretic separator, and by Strickler and Sacks[7] and Hannig *et al.*[8] in the usual free-flow electrophoresis systems.

Distortion of the sample bands can be decreased by decreasing the thickness of the sample stream or by increasing the thickness of the electrophoresis cell. In principle the sample stream thickness could approach zero; in practice the thickness is a significant fraction of the cell thickness. An alternative method of obtaining less distorted sample bands is to increase the electrophoresis cell thickness. This would result in "flatter" profiles for both buffer curtain flow and electro-osmotic flow, but would aggravate the problem of convection since the temperature difference would be greater in a thicker cell. A thin cell can be expected to have severe distortion of the sample due to steep flow profiles, while a thick cell will suffer from convection problems.

A solution is possible. Since convection and sedimentation are attributable to a gravity field, these problems may be alleviated, at least theoretically, if the cell system were to be operated in a zero g environment. The advantages would be: a thicker cell to flatten the flow profiles and the absence of convective mixing and sedimentation of samples at high concentration.

This work describes a mathematical model of an electrophoresis cell which operates under the conditions of no convection and no sedimentation (absence of gravitational effects).

THEORETICAL FOUNDATION

General

In electrophoresis the item of interest is the rate of migration. Provided the migration path is of sufficient length a mixture of components may be separated. The rate of migration (electrophoresis) is a function of net charge, size and shape of the particles, and retarding factors such as viscosity. A particle which has no net charge or is uncharged should not migrate. However, a liquid flow occurs, induced by the applied field, which causes all species present to migrate. This is known as electro-osmosis.

The following sections will discuss the ζ-potential, electrophoretic velocity and mobility, and electro-osmosis.

ζ-Potential

The charge and potential near a phase boundary have been considered in detail by Debye and Hückel[9], Audubert[10], Gouy[11], Chapman[12], Stern[13] and others. The application of these equilibrium properties to electrokinetics has led to the concept of a "slipping plane" displaced somewhat from the actual phase boundary. Electrokinetic phenomena are controlled by the potential at this slipping plane called the ζ-potential as indicated in Fig. 1. The concept of the slipping plane and its attendant potential is useful in measurements and calculations relating to electrophoresis, but the relationship to more fundamental properties of the phase boundary is somewhat tenuous. More detailed descriptions of ζ-potentials and their application in electrophoresis can be found in refs. 14–17, and in the many references cited therein.

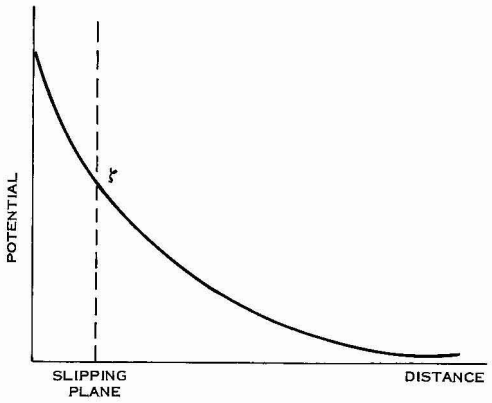


Fig. 1. Variation of potential with distance from a charged surface.

Electrophoresis and mobility

Elementary analysis indicates that if an electric field, $\bar{E}$, is applied to a particle of net charge C, the force producing electrophoretic migration is $\bar{E}$C. The resisting force is given by Stokes' law, *i.e.*, $F = 4\pi a\eta V$ for a spherical particle where a is the particle radius, η is the bulk viscosity of the medium and V is the particle velocity. If the particle has mass m, and neglecting electrostatic interactions, the motion is described by

$$\bar{E}C = \frac{md^2x}{dt^2} + 4\pi\eta a \frac{dx}{dt} \tag{1}$$

The transient response is rather small (10^{-14} sec) and the particle accelerates to its limiting velocity almost instantly. The limiting velocity or the electrophoretic velocity (V_{ep}) is given by:

$$V_{ep} = \frac{\bar{E}C}{4\pi\eta a} \tag{2}$$

The mobility μ (velocity in unit field) is given by:

$$\mu = \frac{V_{ep}}{\bar{E}} = \frac{C}{4\pi\eta a} \tag{3}$$

and it can be shown that the mobility is related to the ζ-potential in the following manner:

$$\mu = \frac{\zeta D}{4\pi\eta} \tag{4}$$

where D is the dielectric constant of the solution.

It has, of course, been shown that the constant, 4π, in eqns. 1–4 is valid only when the radius of the phase boundary is large compared to the thickness of the

electrical double layer. Under other circumstances the constant can range up to 6π[18,19], depending on particle size and the composition and ionic strength of the surrounding medium. For our present purpose the use of the constant, 4π, will suffice. The extension to other circumstances is obvious.

Electro-osmosis

The phenomenon known as electro-osmosis is due to the potential difference existing between the wall of a chamber and the layer of liquid lying next to it; that is to the double layer at the boundary between solid and liquid. The application of an electric field must cause a displacement of the charged layers, and since the wall cannot move the liquid must, and a flow results. The direction of flow depends on whether the ions in this double layer are positive or negative.

Suppose that the wall of the chamber is negatively charged and the layer of liquid adjacent is positively charged. If a field $\bar{E}$ is applied and the surface density of charge is given by σ the force acting on unit surface is $\bar{E}\sigma$. The viscous forces opposing flow are given by $\eta(V_{eo}/\delta)$ where δ is the double layer thickness and V_{eo} is the electro-osmotic velocity. For a steady flow the two forces must be equal.

$$\bar{E}\sigma = \eta \frac{V_{eo}}{\delta} \tag{5}$$

As in electrophoresis, the electro-osmotic velocity at the wall, V_w, can be related to ζ-potential (of the wall) and is given as

$$V_w = \frac{\zeta_w D\bar{E}}{4\pi\eta} \tag{6}$$

where D is the dielectric constant of the solution, η is its viscosity and ζ_w is the ζ-potential of the wall surface with respect to the bulk solution. The fundamentals of electro-osmosis in a closed system are well known[19], and while a free-flow electrophoresis system is, by definition, not a closed system it is closed in the direction of electro-osmosis and the recirculation characteristic of a closed system is observed. Nee has recently re-examined in detail the fundamental equations describing electro-osmosis[20].

ASSEMBLY OF THE COMPUTERIZED MODEL

Introduction

A useful electrophoresis system designed to operate in a zero-g environment should be flexible enough to handle some of the very different biological materials which remain unseparated by present terrestrial electrophoretic methods. The resolution necessary to obtain useful material will vary for each species. This implies an electrophoresis unit with considerable operational latitude in sample flow-rate, sample residence time, field potential, wall ζ-potentials and separation resolution.

The entire mathematical model is based on a criterion called the separation resolution and defined as $\Delta\mu$, the minimum difference in sample component mobility which will result in the complete separation of two adjacent sample components by

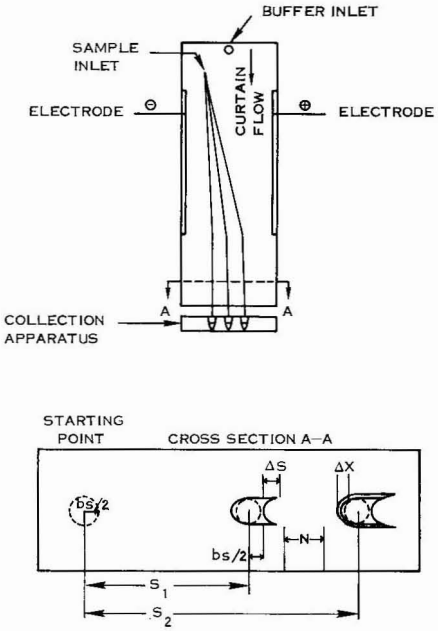


Fig. 2. Schematic, free-flow electrophoresis and definition of separation resolution.

an amount equal to the spacing of the product fraction collection tubes. This is illustrated in Fig. 2.

The term ΔS is the crux of the matter. This term is calculated by taking a sample particle at two locations on the outer edge of the sample stream and calculating net displacements at those points. Fig. 3 illustrates the concept.

The displacement at either point is given by

$$S_x = V_{net(A)} \cdot t_{r(A)} = (V_{ep(A)} + V_{eo(A)}) \cdot t_{r(A)} \tag{7}$$

$$S_0 = V_{net(B)} \cdot t_{r(B)} = (V_{ep(B)} + V_{eo(B)}) \cdot t_{r(B)} \tag{8}$$

$$t_r = L/V_b \tag{9}$$

$$\Delta S = S_x - S_0 = [(V_{ep(A)} + V_{eo(A)})\, t_{r(A)} - (V_{ep(B)} + V_{eo(B)})\, t_{r(B)}] \tag{10}$$

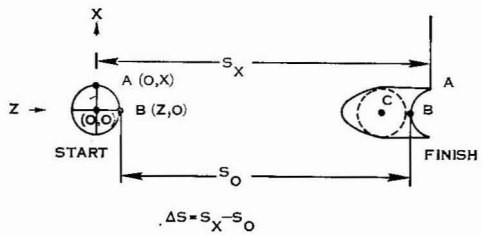


Fig. 3. Definition of sample distortion, ΔS.

where

V_{ep} = electrophoretic velocity,
V_{eo} = electro-osmotic velocity,
V_{net} = algebraic sum of electrophoretic and electro-osmotic velocities,
t_r = residence time in the field,
V_b = the buffer velocity at x and
L = length of the field.

Eqn. 10 is simplified in that it does not take into account diffusion effects. If diffusion were to occur with displacements on the order of the electrophoretic displacements then the A term would be constantly changing during the time particle A is in the field. This effect is built into this model.

In eqn. 10, all of the velocity terms have a dependence upon viscosity, and viscosity is in turn dependent upon temperature. Thus, temperature becomes the most important parameter in the model for separation resolution. Temperature is also important to sensitive (biological) materials, and it is probably necessary to keep the maximum temperature at or below physiological temperature (37°). Therefore, before an attempt can be made to calculate any of the velocities (V_{ep}, V_{eo}, or V_{el}) it is necessary to determine the temperature profile through the cell thickness and the maximum temperature at the cell centerline.

It is now possible to take an overview of the system and identify the interdependence of the cell variables. Fig. 4 shows this interdependence.

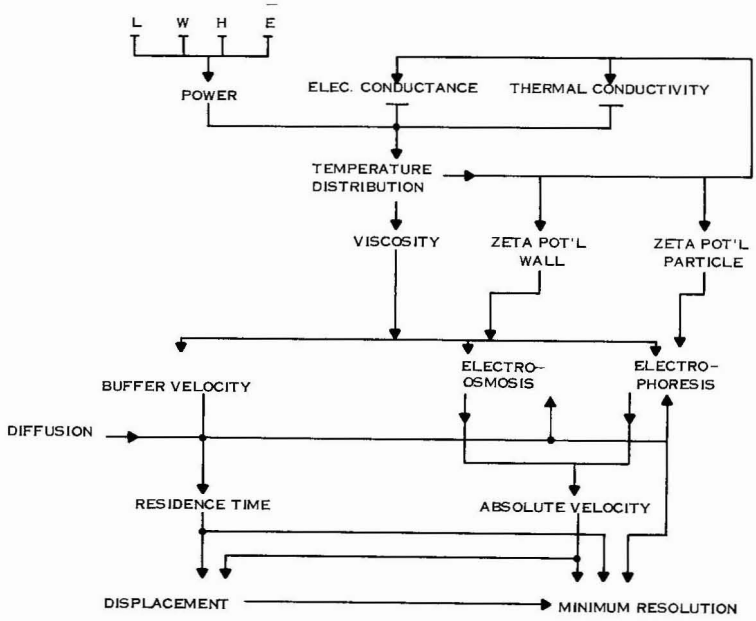


Fig. 4. Interdependence of cell variables.

Temperature distribution and cell thickness: step 1

As mentioned before, it is the cell thickness that is the crucial factor in a successful free-flow system. This is due to several factors: (1) heat transfer occurs through this dimension; (2) the hydrodynamic flow profile is determined by the cell thickness; and (3) the extent of electro-osmotic distortion is determined by the thickness also. The most important parameters here are the temperature distribution and the maximum temperature at the cell centerline. Since these increase rapidly with cell thickness, a trade-off must be made between large temperature gradients and flatter flow profiles.

The first step in the development of the model was to describe accurately the temperature gradient through the cell thickness and from the maximum temperature at centerline to choose an appropriate cell thickness. A similar analysis was performed by Brown and Hinckley[21] subsequent to completion of this work. Their conclusions were generally similar to ours except that we did not consider the wall thickness. In designing equipment we strive for the highest practical thermal conductivity in the walls. Some standard textbook equations were solved first to acquire a "feel" for the solutions (see Figs. 5A, 5B and 6A). Fig. 6B shows the data resulting from the finalized mathematical description of the temperature distribution.

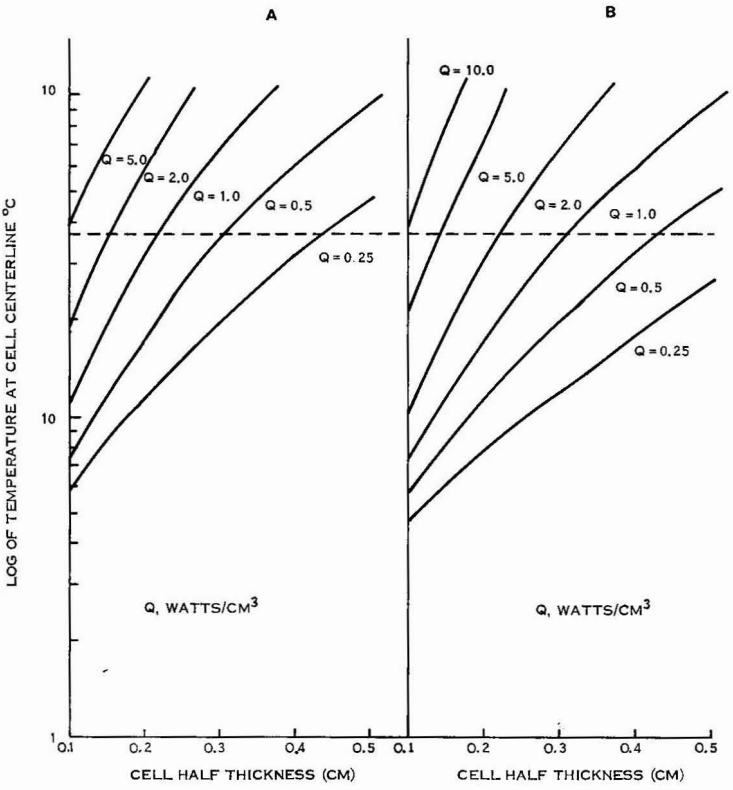


Fig. 5. A, planar heat source; B, distributed heat source, both cases having fixed thermal and electrical conductivities.

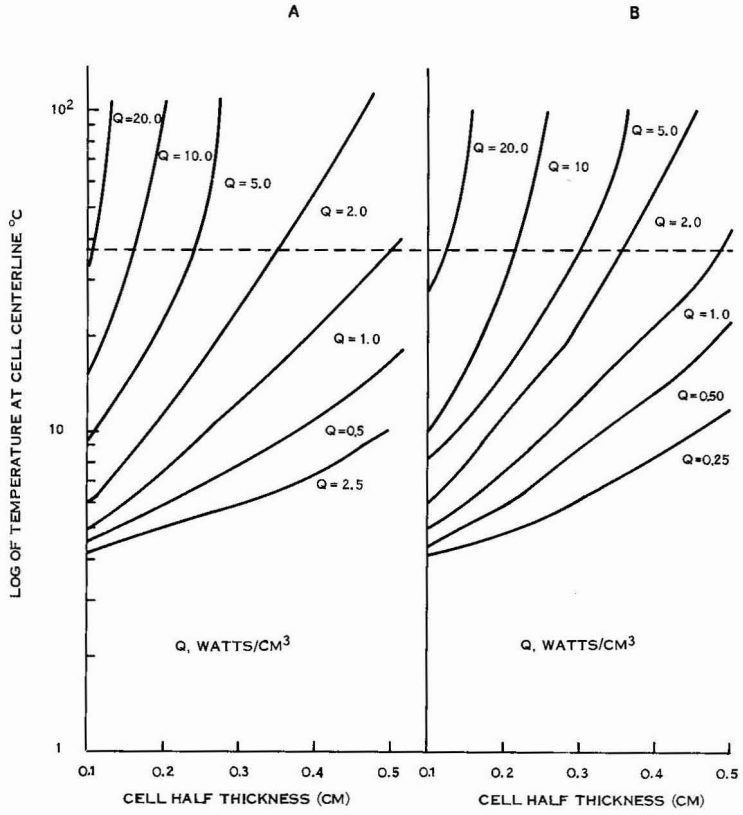


Fig. 6. A, distributed heat source with fixed thermal conductivity and variable electrical conductivity. B, Distributed heat source having variable thermal and electrical conductivity.

The equation governing this distribution may be written as

$$(\alpha + \beta y)\frac{d^2y}{dx^2} + \beta\left(\frac{dy}{dx}\right)^2 + Q(1 + \varepsilon y) = 0 \tag{11}$$

where y = temperature difference from wall at x, x = distance from cell centerline, α = thermal conductivity of buffer at 4°, β = temperature coefficient of the buffer thermal conductivity, ε = temperature coefficient of the buffer electrical conductivity and Q = power density in W/cm^3 containing both the field, $\bar{E}$, and the buffer electrical conductivity, ke.

Eqn. 11 is a boundary value problem (of the second kind) rather than an initial value problem. The boundary conditions for eqn. 11 are: the derivative of the temperature at the cell centerline be equal to zero, $y'(0) = 0$ and the temperature difference at the wall (x_f) be equal to zero, $y(x_f) = 0$. The distribution is assumed to be a symmetrical function with respect to the cell centerline. The sought-for value is the temperature at the cell centerline, $y(0)$. Some sort of iterative technique must be used to solve this equation, with the additional condition that the solution converge

reasonably rapidly. The method used here is a variant of the so called "shooting-method", in which a value for $y(0)$ is assumed, and with $y'(0) = 0$, the equation is then integrated over $[0,x_f]$, and a $y(x_f)$ is calculated. This result, $y(x_f)$, is compared with the condition $y(x_f) = 0$, and the comparison is used to derive a better estimate of $y(0)$. The process is repeated until successive iterations converge. It is clear that some mechanism must be provided to establish how much $y(0)$ is to be incremented on a given iteration and the Newton-Raphson technique was chosen for this purpose.

Referring to Figs. 5 and 6, note how each refinement of the model has affected the maximum temperatures at the cell centerline. From these data a cell thickness of 0.5 cm (0.25 cm half thickness) was chosen. The horizontal dashed line on each graph represents physiological temperature, 37°. An additional output from this step is the temperature profile through a cell of a given thickness. Figs. 7, 8 and 9 are examples of this output for cells of thickness 0.07 cm, 0.16 cm and 0.5 cm, respectively. These data are read onto a file and used in subsequent calculations.

The data inputs to step one are: buffer conductivity and its temperature coefficient, thermal conductivity and its temperature coefficient, voltage gradient and one-half the cell thickness.

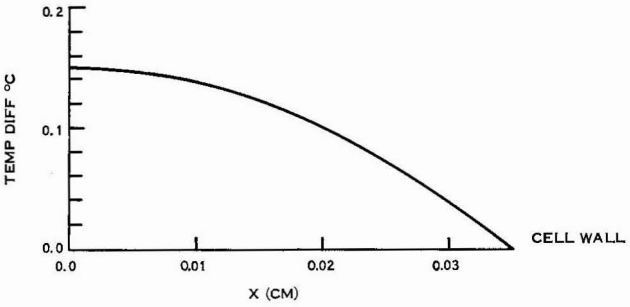


Fig. 7. Temperature gradient *vs.* distance from cell centerline for a field of 40 V/cm and a conductance of $8.7 \times 10^{-4}\,\Omega^{-1}\,\mathrm{cm}^{-1}$. Cell thickness, 0.07 cm.

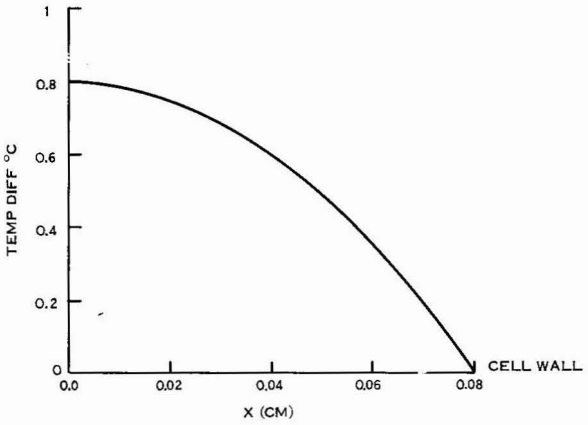


Fig. 8. Temperature gradient *vs.* distance from cell centerline for a field of 40 V/cm and a conductance of $8.7 \times 10^{-4}\,\Omega^{-1}\,\mathrm{cm}^{-1}$. Cell thickness, 0.16 cm.

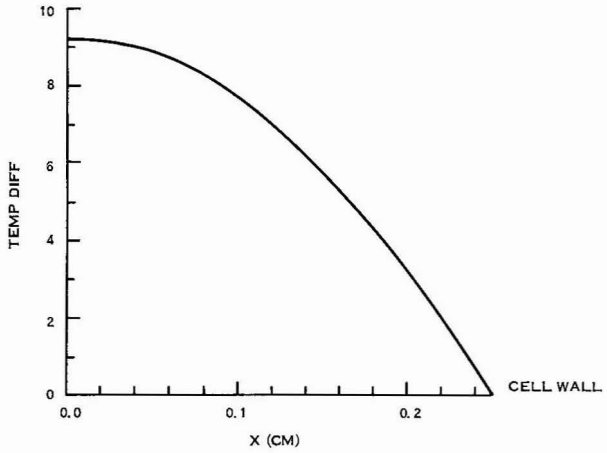


Fig. 9. Temperature gradient *vs.* distance from cell centerline for a field of 40 V/cm and a conductance of $8.7 \times 10^{-4}\,\Omega^{-1}\,\mathrm{cm}^{-1}$. Cell thickness, 0.50 cm.

Curtain velocity profile: step 2

The linear velocity of the buffer curtain determines the residence time, t_r, of a particle in the electric field, and therefore determines, in part, the lateral displacement of the particle. In a constant temperature system, the velocity profile would be parabolic due only to viscous friction. However, viscosity decreases with increasing temperature (for a liquid) and since there is a distribution of temperature in the cell, a distortion of the parabolic flow profile results. It is important to know the buffer velocity at all points through the cell thickness, since a sample stream has a finite diameter and therefore particles at the outer edge of the stream move with a lower velocity than particles at the center. The slower parts of the stream have longer residence times and therefore experience different lateral electrophoretic displacements. This ultimately affects resolution.

The equation used to model the flow profile in the cell can be written as:

$$\frac{\partial}{\partial x}\left(\eta \frac{\partial V_b}{\partial x}\right) + \frac{\mathrm{d}P}{\mathrm{d}z} = 0 \tag{12}$$

where η is the viscosity of the buffer, $\mathrm{d}P/\mathrm{d}z$ is the pressure gradient causing flow, V_b is the linear buffer velocity at x, a distance from the cell centerline.

Since it is not practical to measure $\mathrm{d}P/\mathrm{d}z$ in a real system, this quantity must somehow be related to the volumetric flow-rate of the system, a quantity easily measured and controlled. This quantity $\mathrm{d}P/\mathrm{d}z$ can be written as:

$$-\frac{\mathrm{d}P}{\mathrm{d}z} = \frac{\bar{\eta} 4F}{(4/3)\,ab^3 - (\delta/b)\sum_{n=0}^{\infty} N_n^{-5} \tanh N_n a} \tag{13}$$

$$N_n = (2n + 1)\pi/2b \tag{14}$$

where $\bar{\eta}$ is the average viscosity, a and b are one-half of the cell width and thickness, respectively, and F is the volumetric flow-rate[22].

Figs. 10, 11 and 12 show the outputs for cells 0.07, 0.16 and 0.5 cm thick, respectively. The flow-rates in all three cases were adjusted to give comparable residence times in each cell.

The inputs to step 2 are: flow-rate, cell width, cell thickness and temperature points from the data file created in step 1 to calculate variations in viscosity.

Profile of electro-osmotic velocity: step 3

Electro-osmosis occurs normal to the direction of hydrodynamic flow. Since the cell is a closed system in the direction of electro-osmosis, this flow must be recirculating. Depending upon the sign and magnitude of the applied field and the ζ-potential at the wall, this electro-osmotic flow affects the lateral displacement of a particle undergoing electrophoresis. It is necessary to know the profile of this flow, so that a net horizontal displacement can be calculated for particles at various positions in the cell.

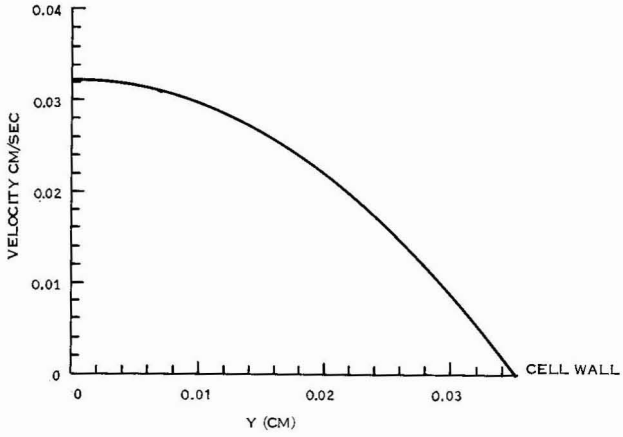


Fig. 10. Buffer curtain velocity *vs.* distance from cell centerline. Cell thickness, 0.07 cm.

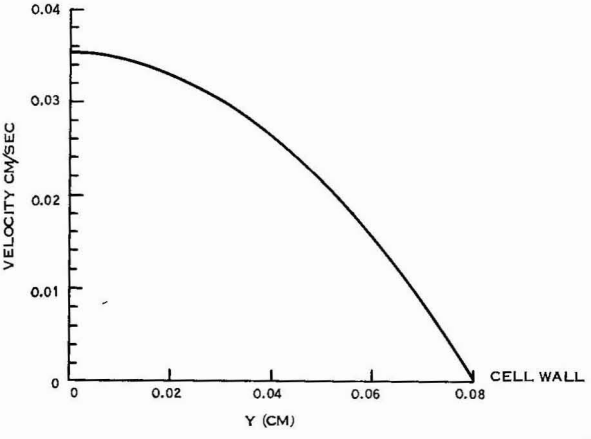


Fig. 11. Buffer curtain velocity *vs.* distance from cell centerline. Cell thickness, 0.16 cm

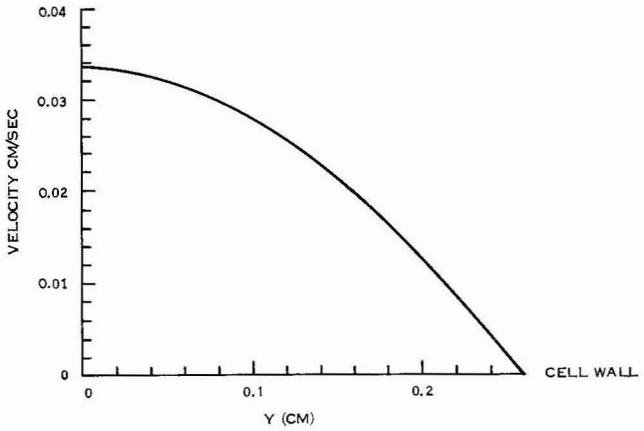


Fig. 12. Buffer curtain velocity *vs.* distance from cell centerline. Cell thickness, 0.50 cm.

The equation used to derive this profile is almost identical to that in step 2 and is written as:

$$\frac{\partial}{\partial x}\left(\eta \frac{\partial V_{eo}}{\partial x}\right) + F = 0 \tag{15}$$

where η is the viscosity, V_{eo} is the electro-osmotic velocity at distance x from the cell centerline and F is the driving force for electro-osmotic flow. An expression is needed to relate the ζ-potential of the wall to the force driving the fluid. If an average viscosity is assumed, then eqn. 15 becomes

$$-F = \bar{\eta} \frac{\mathrm{d}^2 V_{eo}}{\mathrm{d}x'^2} \tag{16}$$

and reduces to

$$V_{eo} = -\frac{Fx'^2}{2\bar{\eta}} + C_1 x' + C_2 \tag{17}$$

If the cell is described through its thickness as shown in Fig. 13, the boundary conditions are at $x' = 0$; $V_{eo} = V_w$. At $x' = s$, $V_{eo} = V_w$, so that eqn. 17 becomes:

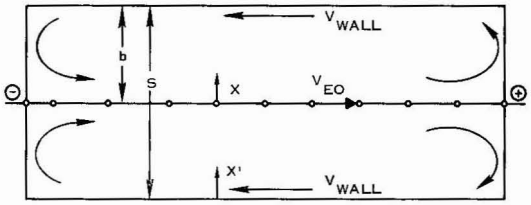
$$V_w = -\frac{F(0)^2}{2\bar{\eta}} + C_1(0) + C_2 \tag{18}$$

so that $C_2 = V_w$ and

$$V_w = -\frac{F(s)^2}{2\bar{\eta}} + C_1(s) + V_w \tag{19}$$

so that

$$C_1(s) - \frac{F(s)^2}{2\bar{\eta}} = 0 \tag{20}$$



b = HALF THICKNESS = 1/2 S
X = O AT CENTERLINE
Y = O AT BOTTOM WALL; Y = S AT TOPWALL

Fig. 13. Closed-flow electro-osmosis.

and

$$C_1 = \frac{Fs}{2\tilde{\eta}} \tag{21}$$

It follows that eqn. 15 is now transformed to

$$V_{eo(x')} = -\frac{Fx'^2}{2\tilde{\eta}} + \frac{Fsx'}{2\tilde{\eta}} + V_w \tag{22}$$

From Smoluchowski's equation for a rectangular cross section cell:

$$V_{eo(x')} = V_w - 6\,V_w\left(\frac{x's - x'^2}{s^2}\right) \tag{23}$$

Setting equations 22 and 23 equal:

$$-6\,V_w\left(\frac{x's - x'^2}{s^2}\right) = \frac{F}{2\tilde{\eta}}(x's - x'^2) \tag{24}$$

$$-\frac{6\,V_w}{s^2} = \frac{F}{2\tilde{\eta}} \tag{25}$$

$$F = -\frac{12\,V_w\,\tilde{\eta}}{s^2} \tag{26}$$

Since $s = 2b$

$$F = -\frac{3\,V_w\,\tilde{\eta}}{b^2} \tag{27}$$

From eqn. 6

$$V_w = \frac{\zeta_w\,D\bar{E}}{4\pi\eta} \tag{28}$$

Substituting into eqn. 27, the result is:

$$F = -\frac{3}{4}\frac{\zeta_w D\bar{E}}{\pi b^2} \tag{29}$$

where D is dielectric constant, $\bar{E}$ is field, b is 1/2 cell thickness and ζ_w is the ζ-potential. Now eqn. 15 can be written as:

$$\frac{3}{4}\frac{\zeta_w}{\pi b^2} = \frac{\partial}{\partial x}\left(\eta \frac{\partial V_{eo}}{\partial x}\right) \tag{30}$$

This last equation is the one used to calculate the final electro-osmotic velocity profile in the cell. Figs. 14, 15 and 16 are the results of these calculations for cells of 0.07, 0.16 and 0.5 cm thickness, respectively. Note that in each case there is a point at which V_{eo} is zero, and beyond that the flow direction reverses. This correlates well with the "real world" situation.

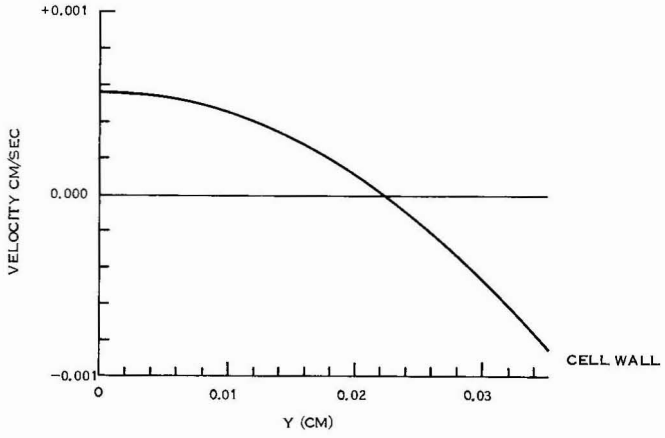


Fig. 14. Electro-osmotic velocity (V_{eo}) *vs.* distance from cell centerline for a field of 40 V/cm and a wall ζ-potential of 5 mV. Cell thickness, 0.07 cm.

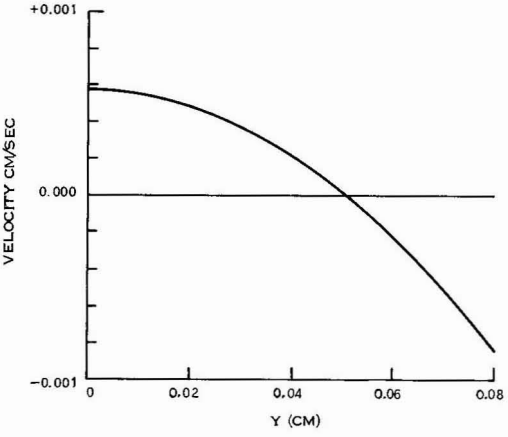


Fig. 15. Electro-osmotic velocity (V_{eo}) *vs.* distance from cell centerline for a field of 40 V/cm and a wall ζ-potential of 5 mV. Cell thickness, 0.16 cm.

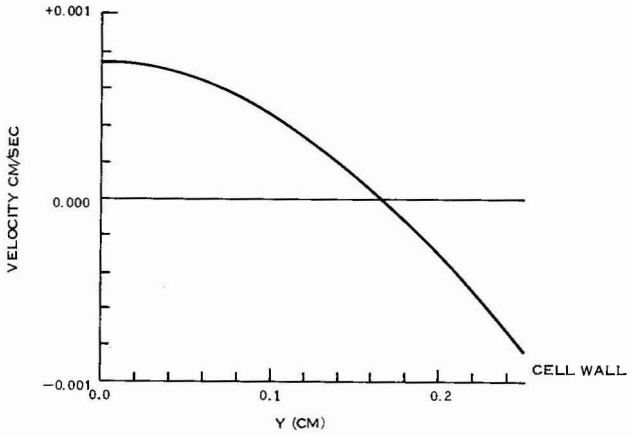


Fig. 16. Electro-osmotic velocity (V_{eo}) *vs.* distance from cell centerline for a field of 40 V/cm and a wall ζ-potential of 5 mV. Cell thickness, 0.50 cm.

Inputs to step 3 are: ζ-potential of the wall surface, dielectric constant of the buffer, the field gradient, 1/2 cell thickness and the temperature from the data file created in step 1 to calculate the variation in viscosity.

Diffusion effects and residence time (t_r): step 4

Depending on the kinds of particles in the sample stream and on the residence time within the field, diffusion effects may play an important role in the separation and resolution achieved. Diffusion will cause the sample stream to increase in diameter as it traverses the length of the cell. If the diffusion time is short compared to the residence time, sample particles will move into slower curtains and the residence times will increase. The effect of diffusing into a slower stream can be compared to a decelerating force and the increase in residence time can be calculated by using the following equation:

$$L = V_0 t_r - 1/2\, A\, t_r^2 \tag{31}$$

where L is the length of the electrophoresis cell, V_0 is the initial velocity of a particle at the outer edge of the sample stream, A is the change in velocity with respect to time (due to diffusion) and t_r is the residence time.

The effect of diffusion can be related to eqn. 31 in the following manner: if the mean increase in sample diameter is expressed as[23]

$$\Delta r = (6\, D_1\, t)^{1/2} \tag{32}$$

where D_1 is the diffusion coefficient and t is time, then the change in ΔV with respect to time is given as

$$\frac{d\Delta r}{dt} = \left(\frac{3}{2}\frac{D_1}{t}\right)^{\frac{1}{2}} \tag{33}$$

Since Δr in this case corresponds to a change in x, the position through the thickness, it is possible to substitute dx/dt for $d\Delta r/dt$. Now, acceleration or deceleration in this case is defined as

$$a = \frac{dV}{dt} \tag{34}$$

However, with a change of variables

$$a = \frac{dV}{dx}\frac{dx}{dt} \tag{35}$$

or

$$\frac{1}{2} a t^2 = \frac{1}{2}\frac{dV}{dx}\left(\frac{3D_1}{2t}\right)^{\frac{1}{2}} t^2 \tag{36}$$

from eqn. 35. Rearrangement brings

$$\frac{1}{2} at^2 = \frac{1}{2} At^{3/2} \tag{37}$$

where

$$A = \frac{dV}{dx}\left(\frac{3D_1}{2}\right)^{1/2} \tag{38}$$

Starting with the initial estimate of $t_r = L/V_0$ a distance is calculated from eqn. 31 and compared to L, the actual length of travel. Using the Newton-Raphson routine an increment, DEL, is generated and added to the old residence time and the cycle begins again until the difference between the calculated distance and the actual distance meets the convergence criterion.

Table I contains the data obtained for three cells of thickness 0.07, 0.16 and 0.5 cm.

TABLE I

RESIDENCE TIME t_r AT EDGE OF SAMPLE STREAM

In all cases $L = 10.16$ cm and $D_1 = 5 \times 10^{-9}$ cm²/sec.

Parameter	*Value*		
Thickness (cm)	0.07	0.16	0.50
V_0 (cm/sec)	0.008	0.030	0.033
A (cm/sec²)	−1.576	−0.331	−0.033
t_r 1st guess (sec)	1163	335	307
1st iteration	1679	338	308
2nd iteration	1720	converges	converges
3rd iteration	1721	converges	converges
ΔX (cm)	0.007	0.003	0.003

For particle remaining at the cell centerline, the residence time is simply the length, L, divided by the curtain velocity at $x = 0$.

The inputs to step 4 are: active cell length, buffer velocity at the edge of the sample stream ($bs/2$) (from step 2), the deceleration factor, A (from step 2), and the diffusion constants of the particles.

Total lateral displacement due to electrophoresis and electro-osmosis: step 5

The total lateral displacement of a particle in the field is the result of electrophoresis, electro-osmosis and residence time. In step 5, the net lateral velocities for the particles at points A and B in Fig. 3 are calculated. For the particle at point B, the net velocity is simply the sum of the electrophoretic and electro-osmotic velocities at $x = 0$. This sum times the residence time at $x = 0$ will yield S_0, the lateral displacement at $x = 0$. Calculation of the similar term, S_x, for the particle at point A, involves integrating the electrophoretic velocity, V_{ep}, and the electro-osmotic velocity, V_{eo}, over the increase in sample diameter. S_x can be written as

$$S_x = \int_0^{tr} \left\{ V_{ep}(x_{(t)}) + V_{eo}(x_{(t)}) \right\} dt \tag{39}$$

where $x_{(t)} = bs/2 + (6D_1\, t)^{1/2} = bs/2 + \Delta x$ ($bs/2$ is the sample stream radius). The boundary conditions on $x_{(t)}$ are: when $t = 0$, $x_{(t)} = bs/2$ and when $t = t_r$, $x_{(t)} = bs/2 + \Delta x$, and from eqn. 35 $dx/dt = ([3/2]\,[D_1/t])^{1/2}$. The following substitution can be made:

$$dt = \frac{2\,(x - bs/2)}{6D_1}\, dx \tag{40}$$

From eqn. 39 and with a change in the limits of the integration, S_x can be written as:

$$S_x = \frac{1}{3D_1} \int_{bs/2}^{bs/2+\Delta x} \left\{ V_{ep(x)} + V_{eo(x)} \right\} \left\{ x - \frac{bs}{2} \right\} dx \tag{41}$$

The inputs to step 5 are the diffusion constant of the particle, the sample radius increase and the residence time. The electro-osmotic velocity is taken as necessary from the data file created in step 3. The electrophoretic velocity is calculated from viscosity variations due to temperature gradient, and particle zeta potential.

Minimum resolution: step 6

Going back to Fig. 2, the separation resolution $\Delta\mu$ can now be calculated from the data available:

$$\Delta\mu = \Delta S + N + \Delta x' + bs/2 \tag{42}$$

where $\Delta\mu$ is the minimum difference in sample mobility which will result in the complete separation of two sample components, N is the collection tube spacing,

$\Delta x'$ is the adjacent sample increase in radius due to diffusion and $bs/2$ is the original sample stream radius. ΔS, which is a measure of the sample distortion, is calculated from step 5 data by taking $\Delta S = S_x - S_0$. The absolute minimum is determined by two factors alone, the initial sample stream radius and the collection tube spacing, since it is conceivable to have a case where ΔS and $\Delta x'$ are both zero.

APPLICATION AND RESULTS

Several realistic, yet hypothetical, cases were examined with the completed model. A sample containing four components was theorized. These components had ζ-potentials of 25, 29, 30 and 34 mV, corresponding to the mobilities measured for the fixed red blood cells of chicken, human A, human B and dog, respectively. In each case, the active cell width and length are 5.08 $\times$ 10.16 cm. The thickness was varied. The flow-rate through each cell was adjusted so that a particle at the centerline would have a residence time comparable to the other cases. The sample stream diameter, 0.06 cm, the wall ζ potential, 5 mV, and the field, 40 V/cm, were the same in all cases.

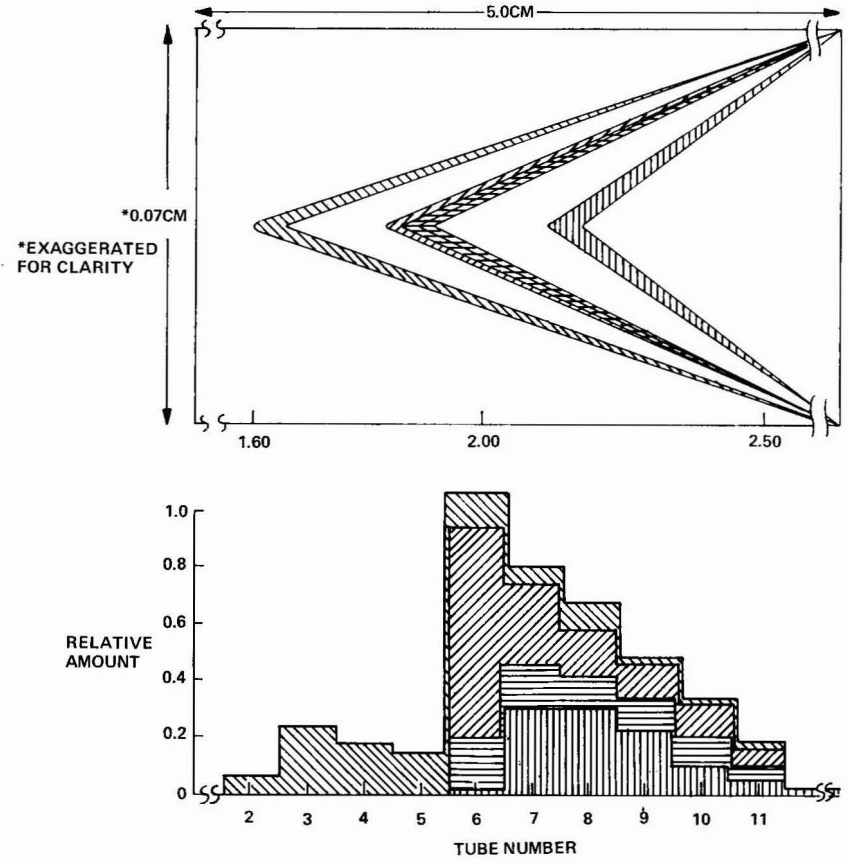


Fig. 17. Graphic illustration of separation and resolution for a 4-component mixture. Cell, 10 $\times$ 5 cm; thickness, 0.07 cm; sample diameter, 0.06 cm; field, 40 V/cm; wall potential, 5 mV; particle potential, 25, 29, 30 and 34 mV; centerline velocity, 0.032 cm/sec.

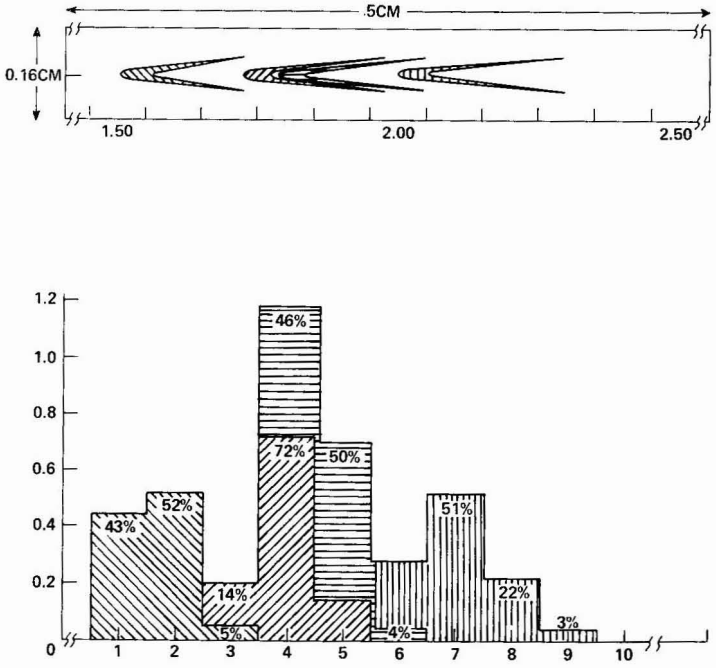


Fig. 18. Graphic illustration of separation and resolution for a 4-component mixture. Cell, 10 × 5 cm; thickness, 0.16 cm; sample diameter, 0.06 cm; field, 40 V/cm; wall potential, 5 mV; particle potential, 25, 29, 30 and 34 mV; centerline velocity, 0.035 cm/sec.

Figs. 17, 18 and 19 are the results of these calculations for cells of 0.07, 0.16 and 0.5 cm thickness, respectively.

The first case, a 0.07-cm thick cell (Fig. 17), is very close to the thickness of the electrophoresis cells described by Barrollier *et al.*[4] and Hannig[5]. The effects of electro-osmosis and the buffer profile are profound. In addition, referring to Table I, the very long residence time for particles at the outer edge of the sample stream has caused the sample stream to diffuse to the cell walls even though a very small diffusion constant was used, *ca.* 10^{-9}. The result of moving to the wall is that the particles are now caught in the reverse flow caused by electro-osmosis and further remixing of the sample occurs. As can be seen from the collection graph, no separated material can be collected in any large amount.

The second case, a 0.16-cm cell (Fig. 18), is similar to equipment used in this laboratory. The crescent effect is still quite pronounced and it is still not possible to obtain a complete separation between any of the components.

The last case, 0.5 cm (Fig. 19), is a proposed "thick" zero *g* experiment cell. A cell of this dimension cannot sustain the resultant temperature gradient in a 1*g* environment without convection setting in rapidly. Although the crescent effect is still present, it is greatly reduced and it now becomes possible to obtain two components of 100% purity. Of the other two components, 95% of one component can be collected free of other material, while the fourth will contain some 4% (of the total amount) contamination.

In all three cases the ζ-potential of the wall was assumed to be 5 mV. This

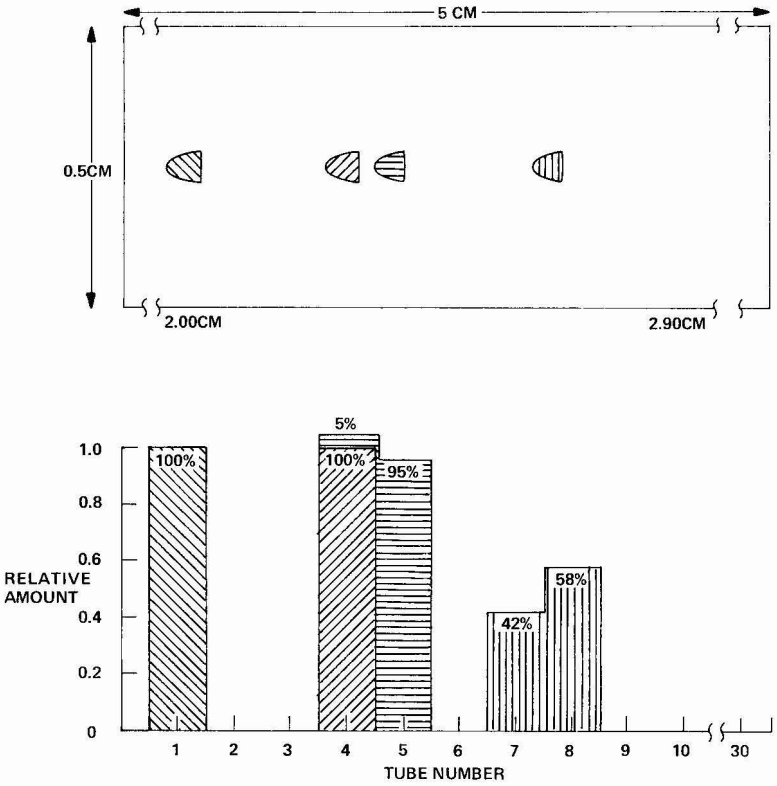


Fig. 19. Graphic illustration of separation and resolution for a 4-component mixture. Cell, 10 × 5 cm; thickness, 0.5 cm; sample diameter, 0.06 cm; field, 40 V/cm; wall potential, 5 mV, particle potential, 25, 29, 30 and 34 mV; centerline velocity, 0.033 cm/sec.

would result in very little electro-osmotic flow and the bulk of the crescent effect is due to the buffer flow profile.

The crescents, in these cases, point from right to left, indicating that particles at the outer edge of the sample stream had more lateral electrophoretic displacement due to longer residence times.

If the wall ζ-potential is changed, for example from 5 to 50 mV, by some treatment of the wall surface, the results would look like Fig. 20. The other parameters are unchanged from case 3. The crescent is now pointing from left to right due to the increased influence of electro-osmosis. Overall, the electro-osmotic velocity at any point is greater than the electrophoretic velocity. As a result, the entire sample band moves farther than previously and particles near the centerline move farther than others in the stream.

Table II lists some data pertaining to these examples. The distortion, ΔS, is given and the separation resolution, $\Delta\mu$, is given in both $\mu m \cdot cm \cdot V^{-1} sec^{-1}$ and in mV.

The first two examples were modeled after existing equipment to check the reliability of the predictions made with the mathematical model. The last two cases are, at present, unable to be verified experimentally, since they require a "zero g" environment. An electrophoresis cell of the dimensions stated for cases 3 and 4 has

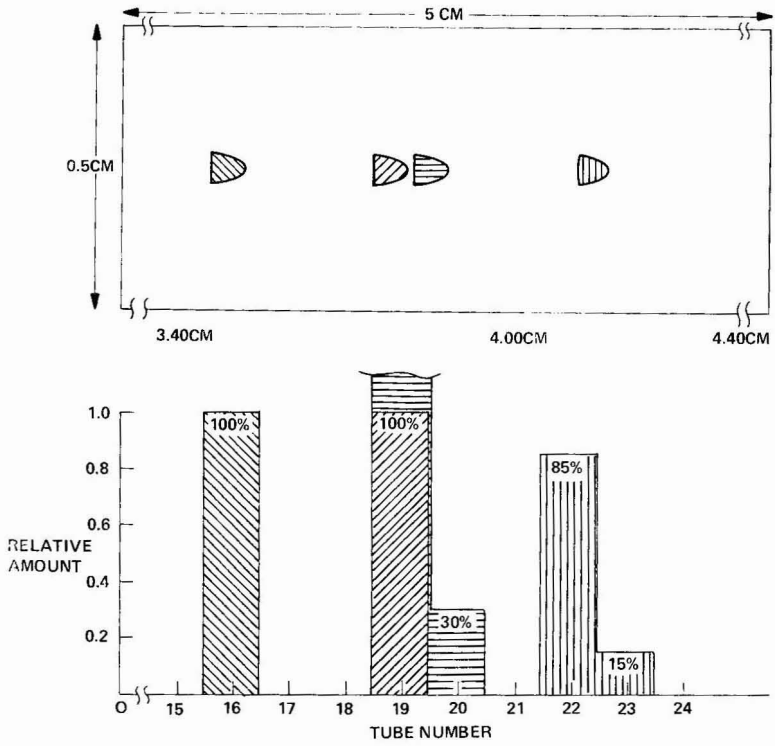


Fig. 20. Effect of a change in the wall ζ-potential. Conditions are the same as in Fig. 19 except the wall ζ-potential has been increased by a factor of 10.

TABLE II

HYPOTHETICAL ELECTROPHORETIC SEPARATIONS

ΔS = cm

$\Delta\mu$ = μm·cm·V^{-1}·sec^{-1}

Separation parameters	*ζ-potential*				*Other parameters*
	25 mV	*29 mV*	*30 mV*	*34 mV*	
ΔS	4.96	6.00	6.27	7.31	0.07 cm thick; ζ_w = 5 mV
$\Delta\mu$	4.08	4.89	5.11	5.94	
$\Delta\zeta$ (mV)	78.77	94.42	98.66	114.69	
ΔS	0.19	0.23	0.24	0.27	0.16 cm thick; ζ_w = 5 mV
$\Delta\mu$	0.28	0.31	0.32	0.35	
$\Delta\zeta$ (mV)	5.41	5.98	6.17	6.75	
ΔS	0.024	0.026	0.027	0.032	0.50 cm thick; ζ_w = 5 mV
$\Delta\mu$	0.137	0.137	0.137	0.137	
$\Delta\zeta$ (mV)	2.645	2.645	2.645	2.645	
ΔS	0.034	0.029	0.028	0.024	0.50 cm thick; ζ_w = 50 mV
$\Delta\mu$	0.138	0.137	0.137	0.137	
$\Delta\zeta$ (mV)	2.664	2.645	2.645	2.645	

been constructed and some ground-based data were obtained. These data were then compared to data for thinner cells and then extrapolated to thicker cells. Fig. 21 relates these data, power density, residence time and cell thickness to an arbitrary stability standard. This standard was defined as: an undisturbed flow of neutral density polymer latex for a minimum of 3 min with the field applied. The result is the curved surface shown in Fig. 21. In a 1*g* environment stable operation of the system will occur only for points lying below the surface. Operation at the surface or above it suggests a reduction in the gravity field to decrease convection.

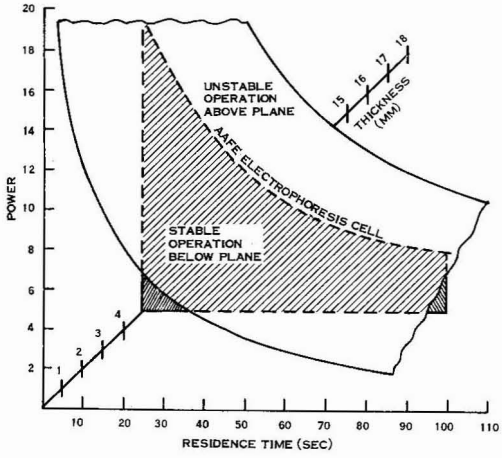


Fig. 21. Experimentally determined regions of stable and unstable operation of an electrophoresis cell as a function of the power (in W) and the residence time for a 0.50-cm thick cell with other conditions as indicated.

Sedimentation of sample at high concentrations is another problem experienced by early researchers. In a preparative system sample throughput would be of prime concern. One solution to the sedimentation problem is to operate the system vertically. However, this orientation tends to maximize convection. Again, a reduced gravity field would serve to overcome these problems. Data were obtained for existing equipment and extrapolated to thicker cells. Fig. 22 shows throughput in g/h *versus* cell thickness for two kinds of sample streams. The first kind of stream is very thin in width, less than the inside diameter of a collection tube, and this width is kept constant. The height of the stream varies with the cell thickness. This kind of stream would be used when resolution of components is the main concern, recalling the criterion for separation resolution. The second kind of sample stream is round in cross section and its diameter is *ca.* 80% of the cell thickness. This kind of stream would be used when high throughput of sample is the objective. Comparing the two kinds of streams, at 10% sample concentration in a 10-mm thick hypothetical cell, the increase in throughput from the rectangular cross section to the round cross section would be greater than a factor of 20.

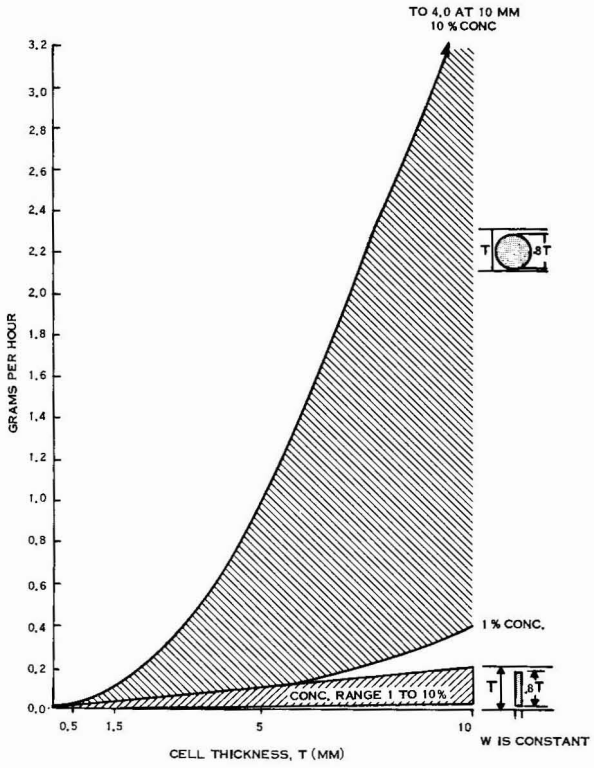


Fig. 22. Throughput *vs.* cell thickness. Lower curves represent sample concentrations for high resolution. Upper curves represent sample concentrations for maximum throughput.

CONCLUSIONS

A mathematical model of an electrophoresis cell operating under conditions of no convection and no sedimentation has been assembled. Through the use of computers, the complex interactions of the various parameters are able to be modeled realistically. The temperature gradient is probably the most important factor affecting a given separation, since this directly affects the buffer curtain profile and the electro-osmotic profile through the temperature dependence of the fluid viscosity. For a cell of given length and width, the thickness has the most profound effect on the temperature gradient since the heat transfer occurs through this dimension. For temperature stability a thinner cell is advisable. However, the buffer profile and electro-osmotic profiles are adversely affected in thinner cells except under special circumstances[7,8]. Therefore, a trade-off must be made between temperature and the two flows for systems operating in 1*g* environments. If the problem of convection in thick cell systems is eliminated by operation in a zero *g* environment the upper limit for cell thickness is governed by the maximum temperature the sample can withstand. In this way, the buffer curtain profile and electro-osmotic profile are kept as flat as possible.

Sedimentation of sample at high concentrations is another problem which plagues terrestrial free flow electrophoresis systems. By operating the system in zero *g*, the sedimentation of sample is negligible, allowing higher throughput.

When the inputs to the model correspond to existing equipment, the theoretical results of the model compare favorably with actual data. These results provide the basis for predicting the separation, separation resolution and throughput for thick cell systems to be operated in a zero g environment.

ACKNOWLEDGEMENT

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DETERMINATION OF COMPLEX ASSOCIATION CONSTANTS FROM GAS CHROMATOGRAPHIC DATA

IV. HYDROGEN BONDING OF AMINES, PROPYL MERCAPTAN AND BUTYNE WITH *n*-HEXADECYL CYANIDE AND *n*-HEXADECYL BROMIDE

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(Received October 27th, 1977)

SUMMARY

Weak hydrogen bridge complexes have been investigated by gas chromatography. A correction for the influence of dipolar interactions between solvent and solute on the retention of the latter has been evaluated, and applied in the calculation of the association constants. Indications have been found of the existence of very weak complexes between mercaptans or alkynes and *n*-hexadecyl bromide, and the occurrence of complex formation between amines and *n*-hexadecyl bromide has been definitely established. Association constants of the stronger complexes between amines and *n*-hexadecyl cyanide have been evaluated with a precision of about 5%.

INTRODUCTION

In previous work[1], hydrogen bonding between alcohols and a number of hexadecyl derivatives and dioctyl compounds was investigated. In this work, weaker hydrogen bridges have been investigated, *viz.*, between amines, propyl mercaptan and butyne on the one hand, and *n*-hexadecyl cyanide and *n*-hexadecyl bromide on the other.

When weak complexes are investigated by means of gas chromatography (GC), great care must be exercised in correcting for dipolar interactions between the solvent and solute, as the retention of the solute is increased not only by hydrogen bonding, but also by dipolar interactions with the solvent. Therefore, an analysis was made of the influence of dipolar interactions between solvent and solute on the retention of the latter, prior to the investigation of hydrogen bonding.

THEORETICAL

By means of GC, association constants, K, for 1:1 complex formation between a volatile solute A and an involatile component B of the stationary phase in the

column can be determined. To do so, the net retention volumes, V, of A, and of a volatile solute, A*, that is closely related to A but does not form a complex, must be measured for a column containing an inert stationary phase, S, and a series of columns containing mixtures of S and B. K is calculated[2,3] from the equation

$$\left[\left(\frac{V_A}{V_{A^*}}\right)_{S+B}\left(\frac{V_{A^*}}{V_A}\right)_S F^{-1} - 1\right] W_B^{-1} = K\left(\frac{f_A f_B}{f_{AB}}\right)_{S+B} \quad (1)$$

where F is a correction factor for dipolar interactions between A and B or A* and B, W_B is the weight fraction of component B and f is the activity coefficient. By extrapolation and interpolation of the values of the left-hand side, the right-hand side can be determined as a function of W_B over the whole concentration range, including the solvents S ($W_B = 0$) and B ($W_B = 1$). The activity coefficients, f, can be defined as unity either in the solvent S or in the solvent B, depending on the convention chosen. If only the value of the association constant in the solvent B is required, retention data for the solvents S and B suffice.

Influence of dipolar interactions between solvent and solute on retention

The correction factor, F, is given by

$$F = \left(\frac{f_{A^*}}{f_A}\right)_{S+B}\left(\frac{f_A}{f_{A^*}}\right)_S \quad (2)$$

In the general case, where A, A* and B have a dipole moment, the calculation of F is beyond the reach of theory. However, F can be estimated empirically as described below. It follows from Littlewood's[4] work that the following equation can be expected to hold for alkanes (alk) and polar solutes (pol) dissolved in *n*-hexadecane (S) or a mixture of *n*-hexadecane and a monofunctional *n*-hexadecyl derivative (S+B):

$$\frac{V_{alk(S)}}{V_{alk(B)}}\cdot\frac{V_{pol(B)}}{V_{pol(S)}} = \frac{f_{alk(B)}}{f_{alk(S)}}\cdot\frac{f_{pol(S)}}{f_{pol(B)}} \equiv \frac{1}{r_a}\cdot\frac{f_{pol(S)}}{f_{pol(B)}} \equiv r_b = \exp\left[g(w_B)\,\mu_B^c\,\mu_{pol}^d\right] \quad (3)$$

where r_a and r_b are symbols used by Littlewood, g denotes a functional relationship, w_B is the concentration of the functional group of B in the solvent, c and d are constants and μ is the dipole moment. When eqn. 3 is applied to the polar solutes A and A*, then

$$\frac{f_{A(S)}}{f_{A(B)}}\cdot\frac{f_{A^*(B)}}{f_{A^*(S)}} = F = \exp\left[g(w_B)\,\mu_B^c\,(\mu_A^d - \mu_{A^*}^d)\right] \quad (4)$$

To avoid large errors in the calculation of F from eqn. 4, a reference solute A* having about the same dipole moment as A should be chosen. In this study, alkyl chlorides ($\mu_{A^*} \approx 2.05$ D) were used as reference solutes for the investigated amines ($\mu_A \approx 1.39$ D). For propylamine, diethyl ether ($\mu_{A^*} = 1.15$ D) was also used as a reference solute, to examine the effect of varying the reference substance. For propyl mercaptan ($\mu_A = 1.58$ D) and butyne ($\mu_A = 0.80$ D), propyl bromide ($\mu_{A^*} = 2.05$ D) and diethyl ether, respectively, were used as reference solutes.

Previously[1], $g(w_B)$ was approximated by $g(w_B) = aw_B$, where a is a constant, and c was set equal to unity, following Littlewood[4]. However, when weak complexes are investigated, as in this work, the correction for dipolar interactions is important and the utmost attention should be paid to the evaluation of $g(w_B)$ and of the constants c and d in eqn. 3.

In the following, w_B is defined as the ratio of the weight fraction of the functional group in the stationary phase to that in the corresponding pure C_{16} compound. We used Littlewood's[4] retention data at 60° for the stationary phases *n*-hexadecane, *n*-hexadecene, *n*-hexadecyl chloride, *n*-hexadecyl bromide and palmitonitrile, and Littlewood and Willmott's[5] data (interpolated to 60°) for squalane–lauronitrile mixtures as stationary phases. It appeared that r_a depends slightly on the identity of the alkane*. Therefore, in the calculation of r_b from Littlewood's data, the value of r_a for the alkane homomorph of the polar solute was used, rather than an average r_a value for all investigated alkanes (Littlewood and Willmott[5] tabulated only average r_a values). We calculated separately for ethyl and propyl derivatives (Tables I and II).

In eqn. 3, $g(w_B)\mu_B^c$ depends only on the identity of the stationary phase (i), and μ_{pol}^d depends only on the identity of the solute (j). Hence eqn. 3 can be written as follows:

$$x_{i,j} = p_i q_j \tag{5}$$

where x represents log r_b, p is proportional to $g(w_B)\mu_B^c$ and q is proportional to μ_{pol}^d. The appropriate mathematical technique for determining the values of p and q that

TABLE I

LOG r_b VALUES OF ETHYL DERIVATIVES, CALCULATED FROM RETENTION DATA OF REF. 4 AT 60° AND REF. 5 INTERPOLATED TO 60°

Stationary phase	*Solute*						
	Diethyl ether	*Ethyl acetate*	*Methyl ethyl ketone*	*Ethyl bromide*	*Ethyl iodide*	*Nitroethane*	*Ethyl cyanide*
$C_{16}H_{32}$	−0.011	0.080	0.035	0.049	0.048	0.077	0.084
$C_{16}H_{33}Cl$	0.060	0.220	0.259	0.164	0.170	0.384	0.395
$C_{16}H_{33}Br$	0.052	0.225	0.268	0.177	0.196	0.390	0.419
$C_{16}H_{33}N$	0.157	0.458	0.557	0.304	0.280	0.879	0.846
$C_{12}H_{25}N$:							
100%	0.262	0.509	0.666			0.992	1.000
48.4%	0.152	0.316	0.444			0.698	0.666
28.6%	0.121	0.222	0.306			0.529	0.483
14.0%	0.097	0.130	0.189			0.361	0.334

* The mean values of r_a of 10 alkanes investigated by Littlewood[4], and their standard deviations, are as follows: *n*-hexadecene, 1.004 ± 0.002; *n*-hexadecyl chloride, 1.264 ± 0.009; *n*-hexadecyl bromide, 1.578 ± 0.019; and palmitonitrile, 1.517 ± 0.019. The standard deviation for *n*-hexadecene is probably caused only by experimental error, but the larger standard deviations for the other stationary phases reflect genuine differences in the individual r_a values. It appears that r_a increases slightly with increasing chain length of the alkane.

TABLE II

LOG r_b VALUES OF PROPYL DERIVATIVES CALCULATED FROM RETENTION DATA OF REF. 4 AT 60° AND REF. 5 INTERPOLATED TO 60°

Stationary phase	*Solute*						
	Dipropyl ether	*Propyl acetate*	*Propionaldehyde*	*Propyl chloride*	*Propyl bromide*	*Nitropropane*	*Propyl cyanide*
$C_{16}H_{32}$	0.012		0.053	0.039	0.026		0.100
$C_{16}H_{33}Cl$	0.060		0.235	0.142	0.153		0.408
$C_{16}H_{33}Br$	0.057		0.253	0.155	0.170		0.416
$C_{16}H_{33}N$	0.145		0.515	0.289	0.283		0.872
$C_{12}H_{25}N$:							
100%	0.192	0.522		0.339	0.329	0.936	0.957
48.4%	0.129	0.329		0.217	0.198	0.632	0.651
28.6%	0.101	0.244		0.149	0.132	0.478	0.476
14.0%	0.083	0.160		0.087	0.080	0.240	0.362

gives the best description of the experimental value of x is factor analysis[6]. Classical factor analysis can be applied only to complete matrices of $x_{i,j}$ data, but De Ligny *et al.*[7] have developed procedures that are suitable for incomplete data matrices such as those in Tables I and II. The ensuing values of p and q are given in Tables III and IV**.

Regression analysis of the values of log q on those of μ_{pol} yielded $d = 1.2 \pm 0.2$ for both ethyl and propyl derivatives.

TABLE III

VALUES OF p_i AND q_j DERIVED FROM THE DATA IN TABLE I

Stationary phase	p_i	*Solute*	q_j
$C_{15}H_{32}$	0.051	Diethyl ether	0.374
$C_{16}H_{33}Cl$	0.235	Ethyl acetate	0.857
$C_{16}H_{33}Br$	0.245	Methyl ethyl ketone	1.104
$C_{16}H_{33}N$	0.505	Ethyl bromide	0.624
$C_{12}H_{25}N$:		Ethyl iodide	0.596
100%	0.593	Nitroethane	1.719
48.4%	0.396	Ethyl cyanide	1.685
28.6%	0.286		
14.0%	0.190		

** Factor analysis also yields the values of r_i, s_j, ... in the more general equation

$$x_{i,j} = p_i q_j + r_i s_j + \ldots \quad (6)$$

However, eqn. 5 gives a good description of the experimental data. Further terms, added to its right-hand side, account only for experimental error, but do not describe any genuine physical effect, as there is no correlation between the values of r_i (or s_j) derived from the data in Tables I and II.

TABLE IV

VALUES OF p_i AND q_j DERIVED FROM THE DATA IN TABLE II

Stationary phase	p_i	*Solute*	q_j
$C_{15}H_{32}$	0.064	Dipropyl ether	0.309
$C_{15}H_{33}Cl$	0.260	Propyl acetate	0.893
$C_{15}H_{33}Br$	0.275	Propionaldehyde	0.992
$C_{16}H_{33}N$	0.533	Propyl chloride	0.566
$C_{12}H_{25}N$:		Propyl bromide	0.550
100%	0.572	Nitropropane	1.669
48.4%	0.382	Propyl cyanide	1.674
28.6%	0.282		
14.0%	0.182		

In Fig. 1, the ratio of p for squalane–lauronitrile mixtures to p for lauronitrile is plotted as a function of w_B for ethyl and propyl derivatives. This ratio is equal to the ratio of the corresponding $g(w_B)$ values. It follows that the ratio of $g(w_B)$ values is identical for ethyl and propyl derivatives, *i.e.*, it is independent of the solute.

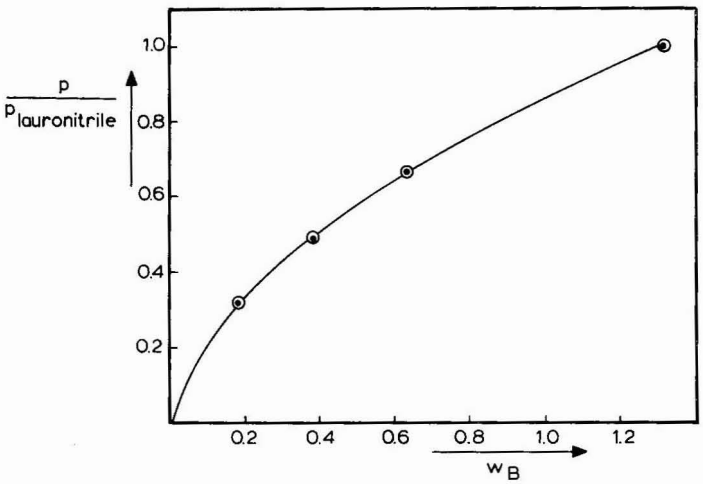

Fig. 1. Influence of solvent composition on dipolar interactions in squalane–lauronitrile mixtures. ●, Ethyl derivatives; ○, propyl derivatives.

To investigate whether the ratio of $g(w_B)$ values depends on the properties of the polar and the apolar component of the solvents, values of $V_{\text{methyl ethyl ketone}} / V_{\text{isopentane}}$ and of $V_{\text{propionaldehyde}} / V_{\text{butane}}$ were determined for mixtures of *n*-hexadecane with *n*-hexadecyl cyanide and with *n*-hexadecyl bromide at 62.6°. From these data, values of r_b for methyl ethyl ketone and propionaldehyde, and values of $g(w_B)/g$ ($w_B = 1$) can be calculated by means of eqn. 3. The results are given in Tables V and VI and in Fig. 2.

Comparison of the data in the fourth columns of Tables V and VI shows once again that $g(w_B)/g$ ($w_B = 1$) does not depend on the investigated solute.

TABLE V

RETENTION DATA ON THE SOLUTES METHYL ETHYL KETONE AND ISOPENTANE FOR STATIONARY PHASES CONSISTING OF MIXTURES OF *n*-HEXADECANE WITH *n*-HEXADECYL BROMIDE AND *n*-HEXADECYL CYANIDE AT 62.6°

Stationary phase	$V_{methyl\ ethyl\ ketone}/V_{isopentane}$	r_b	$g(w_b)/g(w_b = 1)$
$C_{16}H_{34}$	1.64		
$C_{16}H_{33}Br$:			
10%	1.71	1.04	0.05
25%	1.93	1.18	0.25
50%	2.38	1.45	0.56
75%	2.76	1.68	0.78
100%	3.20	1.95	1
$C_{17}H_{33}N$:			
10%	2.07	1.26	0.17
25%	2.65	1.62	0.36
50%	3.75	2.28	0.62
75%	4.81	2.93	0.81
100%	5.91	3.60	0.96
Extrapolated, $w_B = 1$		3.80	1

TABLE VI

RETENTION DATA ON THE SOLUTES PROPIONALDEHYDE AND BUTANE FOR STATIONARY PHASES CONSISTING OF MIXTURES OF *n*-HEXADECANE WITH *n*-HEXADECYL BROMIDE AND *n*-HEXADECYL CYANIDE AT 62.6°

Stationary phase	$V_{propionaldehyde}/V_{butane}$	r_b	$g(w_B)/g(w_B = 1)$
$C_{16}H_{34}$	1.34		
$C_{16}H_{33}Br$:			
10%	1.38	1.03	0.05
25%	1.53	1.14	0.21
50%	1.90	1.42	0.57
75%	2.15	1.61	0.77
100%	2.49	1.86	1
$C_{17}H_{33}N$:			
10%	1.66	1.24	0.17
25%	2.09	1.56	0.35
50%	2.90	2.16	0.62
75%	3.67	2.74	0.81
100%	4.47	3.33	0.97
Extrapolated, $w_B = 1$		3.47	1

Comparison of the data for $g(w_B)/g(w_B = 1)$ for *n*-hexadecane–*n*-hexadecyl cyanide mixtures and for squalane–lauronitrile* mixtures, plotted in Fig. 2, shows that it is immaterial whether the apolar component of the solvent mixture is a straight-

* These data were calculated from the log r_b values for methyl ethyl ketone in the fourth column of Table I and the r_b value at $w_B = 1$ in the third column of Table V.

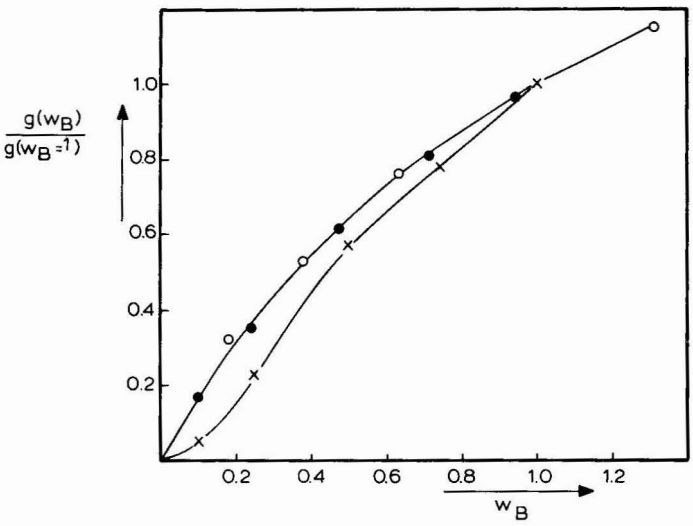

Fig. 2. Influence of solvent composition on dipolar interactions. The effect of changing the type of polar or apolar solvent component is shown. ○, Squalane–lauronitrile mixtures; ●, *n*-hexadecane–*n*-hexadecyl cyanide mixtures; ×, *n*-hexadecane–*n*-hexadecyl bromide mixtures.

or a branched-chain alkane. However, comparison of the data for $g(w_B)/g$ ($w_B = 1$) for *n*-hexadecane–*n*-hexadecyl cyanide mixtures and for *n*-hexadecane–*n*-hexadecyl bromide mixtures, either in the fourth columns of Table V or VI, or in Fig. 2, shows that the functional group of the polar component of the solvent mixture has a large influence.

It follows that the influence of the solvent composition on log r_b (and on F) must be assessed separately for each polar solvent component. It is best estimated graphically, from graphs such as those in Fig. 2.

The value of c was estimated by the following procedure. p, proportional to $g(w_B)\mu_B^c$, was approximated by

$$p = (t + uw_B)\,\mu_B^c \tag{7}$$

where t and u are constants. Starting with $c = 1$, t and u were estimated by regression analysis of the data for p/μ_B^c for squalane–lauronitrile mixtures from Tables III and IV, on the values of w_B. Using these estimates of t and u, c was estimated by regression analysis of the data for log $[p/(t+u)]$ for *n*-hexadecene, *n*-hexadecyl chloride, *n*-hexadecyl bromide and palmitonitrile from Tables III and IV on the values of log μ_B. This procedure was repeated until the values of c, t and u converged. The results were $c = 1.09 \pm 0.05$ for ethyl derivatives and $c = 0.97 \pm 0.05$ for propyl derivatives.

In pure C_{16} compounds as solvents, it holds that

$$r_b = \exp\,(v\mu_B^c\,\mu_{pol}^d) \tag{8}$$

where $v = t+u$. For ethyl derivatives $v = 0.040 \pm 0.004$ and for propyl derivatives $v = 0.04 \pm 0.01$. There is little, if any, difference between the results for ethyl and propyl derivatives. In further calculations we used the values $v = 0.04$, $c = 1.03$ and $d = 1.2$ for all substances.

TABLE VII

RELATIVE RETENTION VOLUMES, V_A/V_{A*}, WHERE A IS A 2-SUBSTITUTED DERIVATIVE OF ETHYLAMINE AND A* IS THE CORRESPONDING SUBSTITUTED DERIVATIVE OF ETHYL CHLORIDE, AT 62.6°, AND RELATIVE RETENTION VOLUMES OF PROPYLAMINE, PROPYL MERCAPTAN AND BUTYNE AT 40°, 60° AND 80°

Stationary phase	*Substituted amines*					$V_{propylamine}$			$V_{propyl\ mercaptan}$			V_{butyne}		
	–H	$-CH_3$	$-C_2H_5$	$=CH_2$	$-OCH_3$	$V_{diethyl\ ether}$			$V_{propyl\ bromide}$			$V_{diethyl\ ether}$		
						40°	*60°*	*80°*	*40°*	*60°*	*80°*	*40°*	*60°*	*80°*
$C_{16}H_{34}$	0.82	0.79	0.81	0.85	0.85	1.08	1.12	1.20	0.86	0.87	0.88	0.34	0.39	0.44
$C_{16}H_{33}Br$:														
10%	0.77	0.78	0.78	0.82	0.81									
25%	0.80	0.78	0.80	0.82	0.83									
50%	0.83	0.80	0.83	0.85	0.83									
75%	0.90	0.86	0.86	0.93	0.88									
100%	0.97	0.90	0.96	0.96	0.98	1.74	1.64	1.61	0.87	0.87	0.88	0.35	0.40	0.45
$C_{17}H_{33}N$:														
10%	0.80	0.78	0.81	0.85	0.83									
25%	0.88	0.84	0.86	0.87	0.87									
50%	0.95	0.90	0.93	0.94	0.93									
75%	1.03	0.93	1.01	1.02	0.98									
100%	1.14	0.97	1.09	1.07	1.05									

TABLE VIII

VALUES OF $Kf_A f_B/f_{AB}$ FOR 2-SUBSTITUTED DERIVATIVES OF ETHYLAMINE AT 62.6° AND OF PROPYLAMINE, PROPYL MERCAPTAN AND BUTYNE AT 40°, 60° AND 80° (CALCULATED FROM THE DATA IN TABLE VII BY EQN. I)

Stationary phase	*Substituted amines*					*Propylamine*			*Propyl mercaptan*			*Butyne*		
	-H	*$-CH_3$*	*$-C_2H_5$*	*$=CH_2$*	*$-OCH_3$*	*40°*	*60°*	*80°*	*40°*	*60°*	*80°*	*40°*	*60°*	*80°*
$C_{16}H_{33}Br$:														
10%	—*	—*	—*	—*	—*									
25%	—**	—**	—**	—*	—*									
50%	0.13	0.13	0.16	0.10	0.06									
75%	0.22	0.21	0.17	0.22	0.14									
100%	0.29	0.24	0.29	0.23	0.25	0.61	0.46	0.34	0.06	0.05	0.05	0.06	0.06	0.05
$C_{17}H_{33}N$:														
10%	0.1	0.2	0.3	0.3	0.1									
25%	0.62	0.57	0.57	0.40	0.40									
50%	0.60	0.56	0.58	0.49	0.46									
75%	0.61	0.49	0.60	0.53	0.45									
100%	0.67	0.48	0.62	0.52	0.49									

* Negative value.

** Zero value.

EXPERIMENTAL

All chemicals were obtained from Fluka (Buchs, Switzerland). A Becker gas chromatograph equipped with a katharometer detector and stainless-steel columns (2 m × 4 mm I.D.) were used.

The same procedure as described earlier[1] was followed.

RESULTS AND DISCUSSION

The results are given in Tables V–VIII.

The positive entries in the last six columns of Table VIII are an indication that propyl mercaptan and butyne may form very weak complexes with *n*-hexadecyl bromide.

The association of propylamine with *n*-hexadecyl bromide decreases with increasing temperature, as expected. The precision of these data was estimated earlier[1] to be about 5%. The accuracy is not as good, however: when dimethyl ether is used as the reference solute, $Kf_Af_B/f_{AB} = 0.46$ at 60°; when propyl chloride is used, $Kf_Af_B/f_{AB} = 0.24$ at 62.6°.

It is remarkable that the values of Kf_Af_B/f_{AB} for the association of the substituted amines with *n*-hexadecyl bromide are strongly dependent on the solvent composition. On extrapolation to *n*-hexadecane as the solvent, the association constants are found to be about zero. Probably, the association of amines with *n*-hexadecyl bromide is too weak to be assessed quantitatively by gas chromatography.

Association constants for hydrogen bonding of substituted amines with *n*-hexadecyl cyanide were determined by linear regression analysis using the values of W_B^2 as statistical weights. The results are given in Table IX. The standard deviation of the association constants is about 6% in the solvent *n*-hexadecane, and 4% in the solvent *n*-hexadecyl cyanide.

TABLE IX

ASSOCIATION CONSTANTS OF 2-SUBSTITUTED DERIVATIVES OF ETHYLAMINE FOR HYDROGEN BONDING WITH *n*-HEXADECYL CYANIDE AT 62.6° (MOLAR FRACTION SCALE)

Substituent	*Solvent*	
	$C_{16}H_{34}$	$C_{17}H_{33}N$
–H	0.50	0.67
$-CH_3$	0.60	0.48
$-C_2H_5$	0.54	0.62
$=CH_2$	0.49	0.52
$-OCH_3$	0.40	0.49

Previously it was found[1], for hydrogen bonding of substituted alcohols with *n*-hexadecyl derivatives, that the 2-methoxy substituent causes a large decrease in the association constant. This was attributed to competition between intermolecular hydrogen bonding of the alcoholic hydroxyl group with the *n*-hexadecyl derivative and intramolecular hydrogen bonding with the 2-methoxy substituent. Inspection of

Table IX shows that the association constant of 2-methoxyethylamine is not particularly small. Apparently, little or no intramolecular hydrogen bonding occurs with this molecule, in contrast to the situation with its alcoholic counterpart.

CONCLUSIONS

Indications have been found that mercaptans and alkynes form very weak hydrogen bonds with *n*-hexadecyl bromide. The association constants found are of the order of 0.05 at 60° (molar fraction scale).

Amines form hydrogen bonds with *n*-hexadecyl bromide. The calculated association constants depend on the reference solute that is used to correct for dipolar interactions between the amine and *n*-hexadecyl bromide, and they also depend strongly on solvent composition. The association is too weak to be assessed quantitatively by GC.

Association constants for hydrogen bonding of amines with *n*-hexadecyl cyanide can be assessed by GC with a precision of about 5%.

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CHROM. 10,758

OPTIMIZED ISOCRATIC CONDITIONS FOR ANALYSIS OF CATECHOLAMINES BY HIGH-PERFORMANCE REVERSED-PHASE PAIRED-ION CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

We studied high-performance liquid chromatographic separation of the catecholamines epinephrine, norepinephrine, 3,4-dihydroxybenzylamine and dopamine utilizing an isocratic aqueous mobile phase flowing over a bonded octadecylsilane (μBondapak C_{18}) solid phase, with amperometric detection of the eluate. Separation was not dependent upon ionic strength of the mobile phase, while a definite dependence upon solvent pH was observed. Detector response showed a marked dependence upon both ionic strength and pH of the liquid phase, with optimum conditions for signal response occurring at pH 5.8 in an aqueous medium of 0.07-mole/l salt concentration. To achieve a baseline separation of the four catecholamines, inclusion of an aliphatic sulfonate as a paired ion was necessary. Heptanesulfonate was found to be the paired ion of choice. In the presence of the paired ion, a sensitivity was achieved which gave a peak more than ten times baseline noise when 100 fmoles of norepinephrine were injected on the column.

INTRODUCTION

The analysis of biological extracts for identification and quantitation of the catecholamines has been carried out by a variety of chemical techniques. In 1949 von Euler and Hamberg[1] introduced a colorimetric procedure which led to much research on the physiology of the neuron. At about the same time, the observation was made[2] that the adrenochrome identified by von Euler could be chemically modified to an adrenolutin with characteristic fluorescent properties. The fluorescence assay yielded the sensitivity necessary for study of the small amounts of adrenergic amines in tissue samples. This procedure has been widely used in the analysis of catecholamines[3].

In 1970, Engelman and Portnoy[4] introduced the concept of enzyme-catalyzed transfer of radiolabelled methyl groups to the catechol ring as a means for analyzing

very small quantities of individual catecholamines. This technique, while very useful in the research laboratory, has a major drawback in that it is very tedious, and requires considerable technical skill. For this reason the assay has not been popular in routine laboratories involved in day-to-day analysis of many biological specimens.

The advent of high-performance liquid chromatography (HPLC) has afforded the laboratory yet another approach to the analysis of catecholamines. Kissinger *et al.*[5–8] reported the use of an electrochemical detector with a cation-exchange liquid chromatograph to analyze catecholamines in the picogram range in the column effluent. Other workers[9,10] have used reverse-phase chromatography with an ultraviolet detector to analyze catecholamines. Preliminary studies in this laboratory with the cation-exchange system proposed by Kissinger *et al.*[5] as well as the reverse-phase system described by Molnár and Horváth[9] indicated that 3,4-dihydroxybenzylamine could not be included as an internal standard in the chromatogram as a separate, unfused peak. In each case, 3,4-dihydroxybenzylamine was found to co-chromatograph with epinephrine. In this paper we report the chromatographic behavior of catecholamines on a reversed-phase chromatographic medium coupled with a detector of the electrochemical type. The goal of this study was to determine the optimal conditions for performance of the electrochemical detector and separation of 3,4-dihydroxybenzylamine as a discrete entity in the chromatogram.

MATERIALS AND METHODS

Chemicals

The catechol standards norepinephrine (NE), epinephrine (E), and dopamine (DA) were purchased from Sigma (St. Louis, Mo., U.S.A.). The internal standard 3,4-dihydroxybenzylamine (DHBA) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Aliphatic sulfonates, butanesulfonate, pentanesulfonate, hexanesulfonate, heptanesulfonate and octanesulfonate were from Eastern Organic Chemicals (Rochester, N.Y., U.S.A.). All reagents, including ethylenediaminetetraacetic acid (EDTA), were reagent grade from Fischer Chemicals (Chicago, Ill., U.S.A.).

Apparatus

The chromatographic system consisted of a 2-l glass solvent reservoir with a PTFE stir bar, a Model 6000A solvent delivery system, a Model U6K injector and a 4 mm × 30 cm μBondapak C_{18} column from Waters Assoc. (Milford, Mass., U.S.A.). The detection device was a Model LC-10 electrochemical detector from Bioanalytical Systems (West Lafayette, Ind., U.S.A.). The detector electrode consisted of a wax-impregnated carbon paste with flow cell defined by the 5 *M* (0.005 in) gasket. The electrode potential was maintained at 0.5 V *versus* a silver–silver chloride cell. The signal generated by the detector was converted by a Houston Omniscribe recorder to a chromatographic trace. All quantitation was performed by determination of peak heights. Response of peak height to concentration has been previously shown to be linear over a wide range of concentrations[5,8]. This was confirmed in our laboratory.

Solvents

Aqueous chromatographic solvents were prepared with double glass-distilled water. After salts were added and pH adjusted, solvents were filtered through a 0.3-

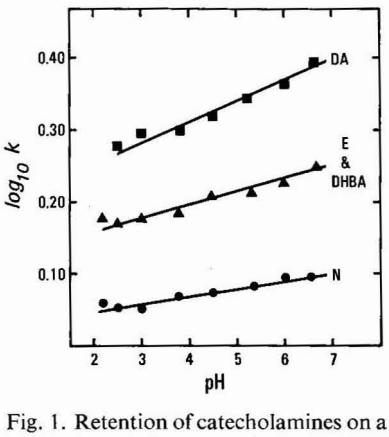
μm Millipore filter. Degassing of solvents was achieved by vacuum filtration and constant slow stirring of the solvent in the reservoir.

Standards

All stock catecholamine standards were prepared in 0.05 *M* $HClO_4$, 0.005 *M* $Na_2S_2O_5$ at a concentration of 100 μg/ml and stored at 4° C in the dark. Appropriate concentrations of the stock standards listed later in the text were diluted with 0.05 *M* H_3CCOOH, 0.005 *M* $Na_2S_2O_5$, and stored for no more than 12 h at room temperature. An injection volume of 25 μl, made with a Precision microsyringe with stainless-steel needle, was used throughout this text, except where noted.

RESULTS AND DISCUSSION

Separation of the catechol group (NE, E, DHBA, and DA) was directly affected by changes in the solvent pH (Fig. 1). These results are comparable to those previously demonstrated by Molnár and Horváth[9]. The theory for this phenomenon has been previously discussed[11]. The detector response, as measured by peak height, was also affected by change of pH (Fig. 2). Since the response is dependent upon oxidation of the catechol ring, which has been shown to be enhanced by increased pH[12], the trend indicated in Fig. 2 would be expected. As the pH rises from 2 to 6, the ring undergoes increased oxidation at the electrode surface. At a pH above 6, the catechol ring, in the presence of metal surfaces present in this chromatographic system, may undergo auto-oxidation prior to entering the detector. This is reflected by the disappearance of the signal observed in Fig. 2.



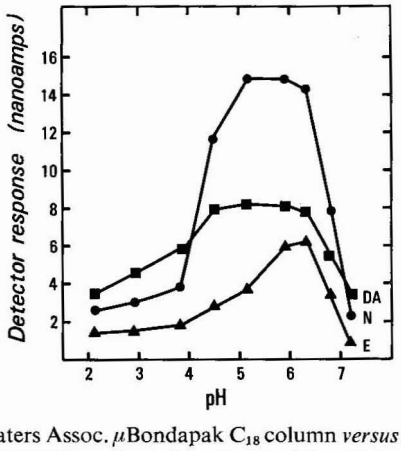


Fig. 1. Retention of catecholamines on a 4 mm × 30 cm Waters Assoc. μBondapak C_{18} column *versus* solvent pH. Solvent consisted of 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, adjusted to appropriate pH by titration with NaOH. Solvent flow-rate, 1.5 ml/min. The catecholamine mixture consisted of 1 μg/ml each of NE, E, DHBA and DA.

Fig. 2. Signal response generated by the electrochemical detector *versus* solvent pH. The solvent consisted of 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, adjusted to the appropriate pH by titration with NaOH. Solvent flow-rate, 1.5 ml/min. 25 μl of the catecholamine mixture, consisting of 1.0 μg/ml each of NE, E, DHBA and DA were injected at each different pH.

Molnár and Horváth[9] reported the effect of ionic strength on retention of catechol compounds on a μBondapak C_{18} column. They concluded that ionic interactions beteeen the aliphatic solid phase and the charged species of the solute are minimal. Our observations (Fig. 3) support those of Molnár and Horváth[9] and are shown here as confirmation of that work. However, the signal output of the electrochemical detector in our studies showed a marked dependence on ionic strength (Fig. 4). The electrochemical reaction occurring in the detector involves the interaction of the solute molecule with the surface of the carbon paste electrode. Thus, at finite current, the electrochemical measurement within the flow cell is limited by mass transport, defined by Fick's first law of diffusion:

$$J = -D\left(\frac{\partial c}{\partial x}\right)$$

where J is the flux, D the diffusion coefficient, and $\partial c/\partial x$ is the partial derivative of the distance (x) the solute molecule (c) must travel to contact the electrode. Thus the signal generated by the detector was anticipated to be dependent on the flow-rate which is regulated by the dimensions of the flow cell, as well as the diffusion rate. Under the conditions of this experiment, the dimensions of the flow cell were constant. Alteration of the solvent flow-rate produced the anticipated effect, *i.e.* at increased flow-rates, the relative signal response was observed to decrease. Under conditions indicated for Fig. 4, the flow-rate was constant. Thus, the only variable was the diffusion coefficient, D, defined by the Nernst equation:

$$D = \frac{RT}{F^2}\frac{\lambda}{|Z_i|}$$

where R is the Rydberg gas constant, T the temperature in °K, F the Faraday constant, λ the equivalent ion conductivity, and Z_i the number of ions in solution. The Nernst

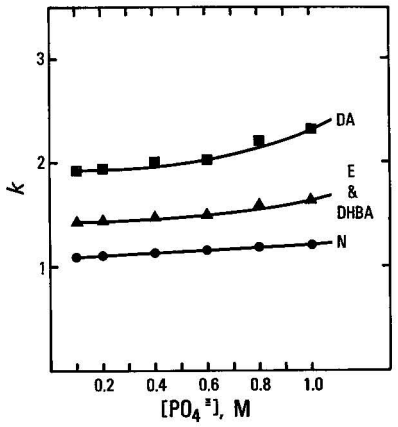


Fig. 3. Retention of catecholamines on a 4 mm × 30 cm Waters Assoc. μBondapak C_{18} column *versus* ionic strength of the solvent. The solvent consisted of varying concentrations of NaH_2PO_4 (pH 4.0) each with 0.1 m*M* EDTA. The catecholamine mixture consisted of 1 μg/ml each of NE, E, DHBA and DA.

equation was initially intended for study of a single ion species, and therefore application to the problem presented in Fig. 4 would be a gross oversimplification. It is introduced here only to promote the interpretation that the diffusion of an ion through an aqueous solution should be inversely proportional, in some manner, to the number of charged species in solution (Z_i). This is generally suggested by the trend in Fig. 4 at a phosphate concentration greater than 0.07 *M*.

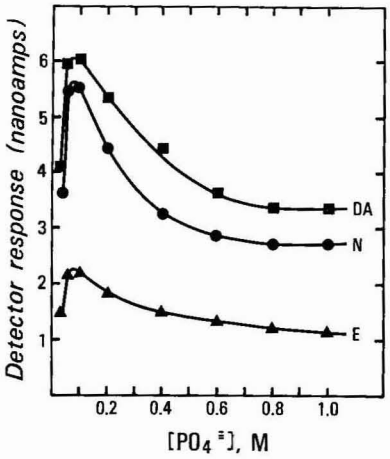
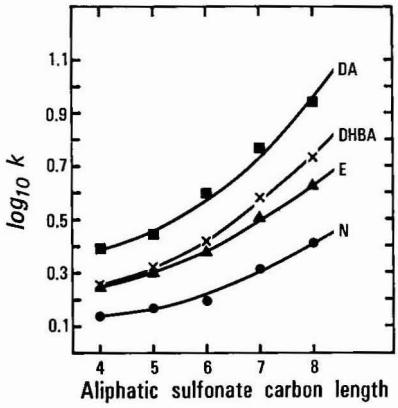


Fig. 4. Signal response generated by the electrochemical detector *versus* solvent ionic strength. The solvent consisted of varying concentrations of NaH_2PO_4 (pH 4.0) with 0.1 m*M* EDTA. 25 μl of the catecholamine mixture, 1.0 μg/ml each of NE, E, DHBA and DA, were injected at each different ionic strength indicated in the figure.

The diminished detector response at low ionic strength indicated in Fig. 4 must be explained by an entirely different mechanism. The detector was composed of a two-loop circuit with amplification. One loop served as a reference; the second loop (working electrode) provided an electron sump at which oxidation occurred, with concurrent generation of a signal voltage proportional to current at the working electrode. To complete the circuit, the reference and working loops must make electrical contact via an ionic medium. Under the constraints of diminished ionic strength, resistance to very small current flow (nA) was increased, thereby decreasing the relative response of the detector.

To enhance the resolution of catecholamines, and particularly to effect a baseline separation between E and DHBA, a useful internal standard, the technique of ion pairing was studied. Coupled with reverse-phase HPLC, ion pairing with aliphatic counter ions provided a very powerful tool for positioning peaks in a chromatogram where desired. This technique promoted the best features of reversed-phase columns (high resolution and column stability) with the strong separation characteristics of ion-exchange chromatography. In view of the success that Knox and Jurand[13] have had with soap chromatography in the separation of biogenic amines, a variety of aliphatic sulfonates were studied as possible ion-pairing agents to achieve resolution between E and DHBA. The results of adding paired ions of varying straight carbon chain length are shown in Fig. 5. In this study, ionic strength of both the buffer and

aliphatic sulfonate were constant. The only physical factor that was changed that could affect resolution was the number of carbons in the aliphatic portion of the pairing species. No change in relative detector response was observed. From Fig. 5, it was deemed that the most practical paired ion would be either heptanesulfonate or octanesulfonate. The effect of heptanesulfonate concentration on retention (k) is shown in Fig. 6. The trend in Fig. 6 closely resembles that seen by Knox and Jurand[13].



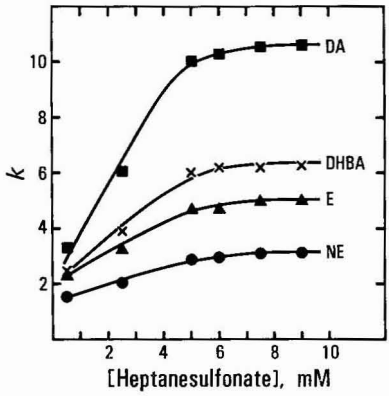


Fig. 5. Retention of catecholamines on a 4 mm × 30 cm Waters Assoc. μBondapak C_{18} column as affected by the carbon chain length of aliphatic sodium sulfonate paired ions. The solvent (1.5 ml/min) consisted of 0.1 M NaH_2PO_4 (pH 5.0), 0.1 mM EDTA with 210 mM aliphatic sulfonate as implied in the figure. The sulfonates were butanesulfonate, pentanesulfonate, hexanesulfonate, heptanesulfonate, or octanesulfonate. The catecholamine mixture consisted of 100 ng/ml each of NE, E, DHBA and DA.

Fig. 6. Retention of catecholamines on a 4 mm × 30 cm Waters Assoc. μBondapak C_{18} column as affected by the concentration of the paired ion, heptanesulfonate. The solvent (1.5 ml/min) was 0.1 M NaH_2PO_4 (pH 5.0), 0.1 mM EDTA containing varying amounts of heptanesulfonate. The catecholamine mixture consisted of 100 ng/ml each of NE, E, DHBA and DA.

CONCLUSION

Determination of solute concentration after separation by a chromatographic method requires application of an optical or chemical method to the eluate. Two factors influence the sensitivity of a chromatographic technique. They are (1) the sensitivity of the detection mechanism for concentration of the solute of interest in the column eluate, and (2) the amount of solute spreading that occurs during the separation procedure. By diminishing the solute dilution that occurs during chromatography, the concentration per unit volume of the solute in the eluate may be increased, which effectively increases the sensitivity of the entire system.

HPLC, using a solid support of small, uniform particle size offers sharp solute resolution. When this type of separation is coupled with a sensitive, specific method of detection, such as amperometry, the system becomes a powerful tool for analysis of a very low concentration of the solute of interest. This is evidenced by the chromatographic trace shown in Fig. 7. Trace A shows the results when 25 μl of a catecholamine standard mixture containing 100 ng/ml each of NE, E, DHBA and DA were introduced

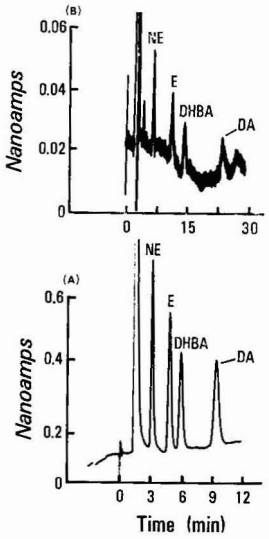


Fig. 7. Chromatographic trace generated by the electrochemical detector. A, 25 μl of a mixture containing 100 ng/ml each of NE, E, DHBA and DA; the solvent (2.0 ml/min) was 0.1 *M* NaH_2PO_4 (pH 5.5), 0.1 m*M* EDTA, 5.0 m*M* heptanesulfonate. B, 5 μl of a mixture containing 5 ng/ml each of NE, E, DHBA and DA; the same solvent as in A flowed at 0.8 ml/min.

into the system. Trace B shows the sensitivity of the method. The peaks in trace B represent 20 pg (*ca.* 100 fmoles) of each of the catecholamine standards.

To accomplish the degree of sensitivity indicated in Fig. 7, the pH and ionic strength must be controlled. Maximum detector response was observed at an ionic strength of 0.07 *M*, pH 5.5–5.8. Under these constraints, it is necessary to add an aliphatic counter ion to the separation solvent to achieve an isocratic separation that gives baseline resolution between the catecholamines of interest. The obvious advantage of the system described here is that a paired ion may be added or removed to alter resolution between peaks of interest and interfering peaks that may appear during the analysis of biological specimens.

Classical approaches to handling of biological specimens for analysis of catecholamines have incorporated a preliminary clean-up step in which the catecholamines are adsorbed onto aluminum oxide under mildly alkaline conditions, followed by elution with acid. We have found this to be necessary prior to analysis using the system described here. Our experience with the aluminum oxide clean-up step has convinced us of the need to include an internal standard with elution characteristics similar to the endogenous catecholamines. Recovery of the catecholamines from biological specimens by adsorption on aluminum oxide under strictly controlled conditions was observed to vary by up to 20%. For this reason, we feel it is essential that an internal standard be incorporated into the procedure. Inclusion of DHBA, a synthetic catecholamine, throughout the clean-up and HPLC steps provides a correction factor to account for recovery of sample during the preliminary clean-up step, a feature not available previously[1–4]. This is a significant contribution toward the faster, more sensitive method for accurate analysis of catecholamines presented here.

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REACTIVE POLYMERS

XVI*. SURFACE-MODIFIED POLYMERIC SORBENTS BASED ON GLYCIDYL ESTERS

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SUMMARY

Copolymers based on glycidyl methacrylate–co-ethylenedimethacrylate were modified by reaction with a number of amines, and by acid hydrolysis followed by cyanoethylation. The physical and chromatographic properties of the modified copolymers suggest that a single copolymer with reactive functional groups can yield a number of sorbents possessing various polarities and various sorption properties.

INTRODUCTION

Surface treatment of polymeric sorbents influences the retention characteristics of individual sorbates. One procedure used in such treatment is the introduction of a stationary phase[1–4], so that absorption becomes the dominant separation process. Use of a variety of polymer sorbents and stationary phases showed that in most cases, the highest separation efficiencies could be obtained with a 2–7% coating[5,6].

The other modification procedure alters the chemical character of the sorbent by a chemical reaction on its surface. The most frequently used treatment of this type is silanization. Silanized copolymers[7] exhibit a higher separation efficiency and considerably smaller tailing of peaks. Polymeric sorbents with reactive functional groups can be modified by polymer-analogous reactions to yield sorbents with completely different separation properties. This procedure was used in the modification of Spheron[8], the free hydroxy groups of which were used in polymer-analogous transformations with stearoyl chloride and acrylonitrile.

This paper reports the investigation of the surface properties of macroporous polymeric sorbents based on glycidyl methacrylate–co-ethylenedimethacrylate (GMA–co-EDMA), modified by polymer-analogous reactions of the epoxy groups of glycidyl methacrylate units.

* For Part XV, see ref. 9.

EXPERIMENTAL

Modification of copolymers by polymer-analogous reactions

Reactions with amines. The starting copolymer in reactions with amines was sample 1, containing 55% (w/w) of GMA. Ammonia, methylamine, dimethylamine, ethanolamine and ethylenediamine were used as reagents. The reaction with ammonia was carried out in a stainless steel autoclave, whereas reactions with volatile amines (methylamine, dimethylamine) were performed in ampoules placed in a thermostat. Reactions with less volatile amines (ethanolamine, ethylenediamine) were carried out in a flask under reflux, heated on a water bath. The reaction conditions, the conversion of GMA and the basic physical characteristics of copolymers modified with the amines are given in Table I.

Hydrolysis of the copolymer GMA–co-EDMA. The epoxide ring was opened by means of the acid hydrolysis of sample 7 with a 0.1 *N* sulphuric acid solution. The reaction was carried out at 90° for 3 h. The product was decanted with water to neutral reaction and dried. Vicinal hydroxy groups were formed, and no epoxy groups were detected in the copolymer after such hydrolysis.

Cyanoethylation of the hydrolysed copolymer. The hydrolysed sample was cyanoethylated after being swollen in a 9 *M* NaOH solution. The reaction with acrylonitrile (AN) was carried out at 25°, with the molar ratio of reagent to the reactive hydroxy unit being 3.2:1. As the copolymer was in the swollen state, the reaction proceeded not only on the surface but also in the bulk.

Chromatographic and other measurements

The chromatographic measurements required for calculation of the specific retention volumes, the Kováts retention indices and the modified Rohrschneider constants were carried out in the same way and under the same conditions as described in the previous paper[9]. The 150–200 μm fraction was used in all cases.

The measurement of specific surface areas and thermal stabilities and the determination of the epoxy groups were also carried out in the same way as in the previous paper[9]. The nitrogen content of the copolymers was determined by the method of Kjeldahl[10].

RESULTS AND DISCUSSION

The starting copolymer used in the polymer-analogous reactions with ammonia and with a number of amines was sample 1, obtained by the copolymerization of 60% (w/w) of GMA and 40% (w/w) of EDMA. This proportion of the cross-linking EDMA ensures the required mechanical strength, small volume contractions due to the temperature and medium, and a sufficiently large surface area of the resulting copolymer. GMA introduces into the copolymer the required concentration of reactive epoxide groups.

The conditions for the polymer-analogous reactions and the basic characteristics of modified copolymers with chemically bonded primary, secondary, and tertiary amines are given in Table I. Although the reactions were carried out in a multiple excess of amines, the conversion of GMA with ammonia and amines containing one nitrogen atom varied from 64 to 79 mole%. In the case of sample 6, when the

TABLE 1

REACTION CONDITIONS AND BASIC CHARACTERISTICS OF SAMPLES MODIFIED WITH AMINES

Sample number	*Modified*	*Mol. ratio (amine/GMA)*	*Reaction time (h)*	*Temp. (°C)*	*N content in sorbent (%, w/w)*	*Conversion of GMA (mole%)*	*Thermal stability (°C)*	*Specific surface area (m^2/g)*	*Mean pore diameter (nm)*
1*	—	—	—	—	—	—	213	69	9.9
2	NH_3	16.7	5.0	80	3.36	64.7	210	79**	10.8**
3	CH_3NH_2	28	4.0	80	3.08	61.0	200	75***	10.4***
4	$(CH_3)_2NH$	37	1.5	80	3.78	79.4	undetermined	68***	12.1***
5	$HOCH_2CH_2NH_2$	6	6	95	3.50	76.2	206	59**	11.0**
								61***	11.9***
6	$H_2NCH_2CH_2NH_2$	31	4	80	5.18	53.7§	205	70***	9.8***

* GMA content = 55% (w/w).

** Measured before conditioning.

*** Measured after conditioning.

§ Assuming that only one amino group of ethylenediamine reacts.

reagent contained two nitrogen atoms, the conversion (assuming that only one amino group of ethylenediamine reacts) was only 53.7 mole%. This result may be distorted by the fact that under appropriate steric conditions ethylenediamine could react with both groups. In addition to amino groups and hydroxy groups introduced by ethanolamine in sample 5, the surface of copolymers thus modified also contains epoxy groups of unreacted GMA. Because the specific surface areas of samples of the modified copolymers vary within a small range about the value of the starting sample 1, it is impossible to follow exactly the effect of polymer-analogous reactions on the change in the surface area. The experimental error in the specific surface area can be considerable (±5%). It may be asserted, however, that polymer-analogous reactions lead to an increase in the mean pore diameter; sample 6 is the only exception to this finding.

Compared with the starting sample 1, the thermal stability of this series of modified copolymers tends to decrease somewhat. The lowest thermal stability was observed with sample 4, the thermogravimetric record of which continued to decrease in the linear region, so that at the characteristic breaking point (onset of decomposition) the weight loss amounted to 7.4%.

Subsequent modifications (Table II) were carried out using sample 7 as the starting copolymer. Its acid hydrolysis yielded a macroporous copolymer with free hydroxy groups on the surface (sample 8). Reaction of AN with the hydroxy groups of the hydrolysed copolymer yielded sample 9, which contained strongly polar nitrile groups. The last in the series is sample 10: a terpolymer, GMA–co-AN–co-EDMA. The latter two samples were compared in order to examine how the properties of the copolymer, with the substrate (AN) attached to the surface, differed from those of the terpolymer, into which the substrate had been incorporated as the monomer.

TABLE II

BASIC CHARACTERISTICS OF THE STARTING COPOLYMER GMA–CO-EDMA AND OF SAMPLES MODIFIED BY HYDROLYSIS FOLLOWED BY CYANOETHYLATION

Sample number	*Modified*	*Content in copolymer*		*Thermal stability (°C)*	*Specific surface area (m^2/g)*	*Mean pore size (nm)*
		GMA (%, w/w)	*AN (%, w/w)*			
7	—	53.5*	—	216	60	11.4
8	by hydrolysis with H_2SO_4	—	—	207	62	12.0
9	by hydrolysis and cyanoethylation	—	18.9	216	51	20.5
10	—	36.9**	14.8	272	59	17.1

* GMA–EDMA (60:40, w/w) in the polymerization mixture.
** GMA–AN–EDMA (40:20:40, w/w) in the polymerization mixture.

As with copolymers modified with amines, in this case too the changes in the specific surface areas are comparatively small, and one can also observe an increase in the mean pore diameter (with sample 9, this change is conspicuously large) in relation to the starting copolymer. Comparison of the thermal stabilities of modified

copolymers and the starting sample 7 shows that the stability of the hydrolysed sample has decreased by *ca.* 10°. The reason is the lower stability of the vicinal hydroxy groups. Subsequent cyanoethylation of the hydrolysed copolymer caused an increase in thermal stability to that of the starting sample 7. A considerably higher stability was observed with the terpolymer. A possible cause of the difference in the thermal stabilities of samples 9 and 10 is the ether bond formed in the reaction of AN with the hydroxy groups of the hydrolysed copolymer.

It follows from the specific retention volumes in Table III that modified sorbents have quite different sorption properties. Although the specific surface areas of modified samples do not differ much from those of the original copolymers, the differences in the retention values of compounds on the individual samples are considerable. With the majority of samples (2–6 and 8) the retention values of not only polar, but also non-polar compounds are increased compared with the starting copolymers (1 and 7). Thus important changes in the pore distribution probably occur in the polymer-analogous reactions. Of the modified copolymers, only sample 9 containing AN is an exception: as with the terpolymer 10, the retention values on sample 9 are shorter than those on sample 7.

TABLE III

SPECIFIC RETENTION VOLUMES OF SORBATES ON COPOLYMER SAMPLES

Column temperature 150°, flow-rate of argon 25 ml/min; injected amount 0.1 μl.

Sorbate	*Sample number*									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Pentane	2.8	5.4	4.4	3.2	1.6	1.6	3.0	6.1	0.9	1.7
Hexane	5.8	9.7	8.9	6.4	2.5	2.9	4.3	11.3	1.6	3.2
Heptane	11.4	18.0	16.5	11.6	4.0	5.0	8.1	19.4	3.3	5.8
Octane	22.1	—	32.8	21.2	7.4	9.2	15.0	—	5.2	9.3
Nonane	44.2	—	65.8	38.6	13.0	17.0	26.7	—	7.8	13.4
Methanol	5.3	18.8	13.7	10.9	10.2	16.4	6.2	18.8	4.8	5.7
Ethanol	8.8	32.3	25.6	15.8	13.7	21.5	7.6	37.3	5.5	7.6
Propanol	17.1	68.2	42.6	29.4	22.9	37.3	14.4	86.3	8.4	13.3
Butanol	34.3	147.2	93.4	56.7	41.2	65.9	27.5	—	13.7	23.4
Benzene	17.3	40.0	27.4	20.3	11.9	12.3	15.5	44.9	6.0	12.6
Methyl ethyl ketone	18.4	—	40.1	24.1	17.2	—	14.7	71.5	8.1	14.4
Nitromethane	27.8	61.6	44.2	36.3	28.8	43.8	26.2	60.9	18.9	27.9
Pyridine	57.1	266.9	159.7	92.9	70.2	107.0	45.4	—	25.4	41.8

The dynamic conditions in columns packed with modified copolymers based on glycidyl esters are analogous to the starting copolymers[9]; the separation efficiency is somewhat reduced only in the series modified with amines. This finding is confirmed by the fact that the structure of porous particles is essentially unchanged during the modification.

The polar character of the sorbent, which is the cause of major or minor specific interactions with the sorbate molecules, is reflected in the basic chromatographic data, retention times, or volumes. This is also why the Kováts retention indices, which are interpolated logarithms of the retention of compounds related to the homologous series of alkanes, express the chromatographic properties of the separation materials.

TABLE IV

MODIFIED ROHRSCHNEIDER CONSTANTS[9] OF THE COPOLYMER SAMPLES

Related to Kováts indices determined at 150° on Carbopack B: $I_{benzene} = 561$, $I_{ethanol} = 296$; $I_{methyl\ ethyl\ ketone} = 476$, $I_{nitromethane} = 358$, $I_{pyridine} = 547$.

Sample number	x'	y'	z'	u'	s'
1	2.02	3.65	2.96	4.75	3.93
2	2.69	5.02	—	5.43	5.92
3	2.13	4.67	3.52	4.84	4.80
4	2.32	4.58	3.47	5.33	5.04
5	3.23	6.12	4.74	6.83	6.53
6	2.89	6.40	—	6.98	6.54
7	2.45	3.88	3.22	5.39	4.37
8	2.93	5.24	4.64	5.52	—
9	2.71	5.14	4.29	7.13	5.81
10	3.26	4.64	4.43	7.00	5.97

The polarity of the samples of sorbents under investigation was expressed using the modified Rohrschneider constants[9], the calculations of which are based on the Kováts retention indices of the Rohrschneider standards (benzene, ethanol, methyl ethyl ketone, nitromethane, pyridine) on the given sorbent and on non-polar Carbopack B. Table IV shows that all polymer-analogous reactions lead to an increase in the polarity of modified copolymers. The highest polarity was obtained with samples prepared by reaction with ethanolamine (5), ethylenediamine (6), and by hydrolysis (8) followed by cyanoethylation (10). Compared with sample 9, the specific interactions of terpolymer 10 are stronger in the separation of aromatic (x'), weaker in the separation of alcohols (y'), and approximately the same in the separation of the other types of sorbate (z', u', s').

Because the retention values are smallest on samples 9 and 10, these samples are the most suitable for application. Moreover, the separation efficiency, thermal

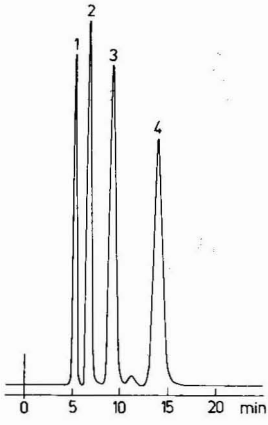

Fig. 1. Separation of carboxylic acids C_2–C_5 on sample 9. Glass column (100 × 0.3 cm I.D.); injection, 0.15 μl; column temperature, 165°; flow-rate of nitrogen, 30 ml/min. Peaks: 1 = acetic acid; 2 = propionic acid; 3 = butyric acid; 4 = valeric acid.

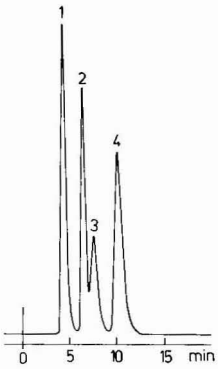

Fig. 2. Separation of mixture of polar compounds with close boiling points on sorbent 9. Glass column (100 × 0.3 cm I.D.); injection, 0.1 μl; column temperature, 90°; flow-rate of nitrogen, 30 ml/min. Peaks: 1 = 1-chlorobutane; 2 = ethanol; 3 = butyraldehyde; 4 = methyl ethyl ketone.

stability and lifetime of these packings in the chromatographic column are the highest. The reactivity of the epoxy groups in the copolymer GMA–co-EDMA rules out the separation of carboxylic acids and amines. On the other hand, on copolymers subjected to surface modification with AN the separation of carboxylic acids proceeds without problems (Fig. 1), as is the case with the terpolymer GMA–co-AN–co-EDMA. The separation of mixtures of polar compounds with close boiling points on sample 9 is shown in Fig. 2. This modified copolymer exhibits inversion in the separation of 1-chlorobutane and ethanol compared with the starting copolymer[9]. The separation of chlorinated hydrocarbons on this sorbent is highly selective, and the peaks obtained are highly symmetrical. This is illustrated by the separation of isomers of trichlorobenzene (Fig. 3).

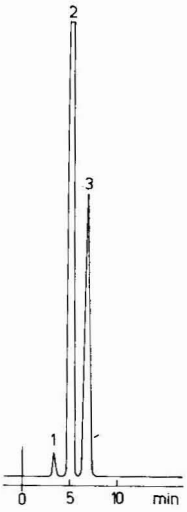

Fig. 3. Separation of trichlorobenzene isomers on sorbent 9. Glass column (100 × 0.3 cm I.D.); injection, 0.1 μl; column temperature, 190°; flow-rate of nitrogen, 30 ml/min. Peaks: 1 = 1,3,5-trichlorobenzene; 2 = 1,2,4-trichlorobenzene; 3 = 1,2,3-trichlorobenzene.

The results described in this paper show that by modifying polar polymeric sorbents based on glycidyl esters by means of polymer-analogous reactions, one can obtain a great number of sorbents possessing various polarities and various sorption properties.

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CHROM. 10,815

SCREEN FOR THE EVALUATION OF CHEMICALLY BONDED SUPPORTS USED IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A screen is proposed for the evaluation of chemically bonded supports; for use in reversed-phase high-performance liquid chromatography. Application of the screen to a range of test mixtures has revealed that, in general, octadecyl-bonded silica is the most useful.

INTRODUCTION

A chemically bonded support is the normally accepted terminology for describing the chemical linking of organic moieties on to chromatographic adsorbents such as alumina and silica gel. The advantages of chemically bonded supports in routine analysis stem from their long-term stability as well as compatibility with gradient elution techniques. Although widely used, the logic behind the choice of a bonded support for a particular separation has become very vague.

Conventionally, a stationary phase was chosen after partition experiments had indicated the desired selectivity. Such experiments are difficult to perform with bonded phases and in any case, the effect of the monolayer so formed is significantly less than the multilayer film associated with the adsorbed stationary phase. Consequently, comparisons between adsorbed and bonded phases are probably meaningless. Consistent with this viewpoint, difficulties have been encountered when reversed-phase chromatography has been used in an attempt to determine dynamic partition coefficients[1–3].

Applications of chemically bonded supports for reversed-phase chromatography are legion[4–11]. Many use octadecyl-bonded silica. Recently, a vast range of silanes has been made generally available thus prompting an escalation of the number of bonded silicas evaluated and made commercially available. It is interesting to note

that the majority of applications advocating the use of such bonded materials employ adsorption chromatography with non-aqueous solvent systems[4–7,12–15] and no real effort, apart from the investigation of chain length of the bonded alkyl groups[16,17], appears to have been made to see if a better separation could have been achieved on more readily available supports. It was to this end that a column screen was instigated.

EXPERIMENTAL

Apparatus

Modular equipment was used comprising: constametric I pump (LDC); variable-wavelength UV detector (Cecil Instruments, Model CE 212); syringe loading injection valve (Rheodyne, Model 7120) and a Servoscribe I.S. recorder (Smiths Industries).

Reagents

Silanes were purchased from Petrarch Systems (Magnus Scientific, Sandbach, Cheshire, Great Britain) and Aldrich (Milwaukee, Wisc., U.S.A.).

Preparation of bonded supports

In all cases, the following procedure was used. Silica (10 g) was slurried in toluene (100 ml) to which the appropriate silane was subsequently added (1 g). With occasional stirring, the reaction was allowed to proceed at ambient temperature for approximately 24 h. The supernatant solvent was subsequently decanted off. Toluene (100 ml) was added and the bonded silica was again dispersed and allowed to settle. The supernatant was decanted-off. This procedure was repeated several times using methanol as solvent (usually 3–5 washes) until no residual silane was observed in the supernatant. Finally, the methanol dispersion was filtered, washed with methanol and dried at 40°.

Column packing

The procedure used has been described previously[18]. All columns were packed by the same procedure and under the same conditions (*i.e.* 7500 p.s.i. for 30 min). Extreme care was taken to ensure that all the end fittings, column tubing (Apollo, from Accles and Pollock, Warley, Worcestershire, Great Britain), dimensions and support material (Partisil 5) were the same for each column, thus reducing the possibility of ascribing peculiar chromatography to factors other than chemical modifications of the support.

Chromatography

All chromatography was performed under isocratic conditions using a solvent composition found to be suitable for the application but not necessarily optimal. Test mixtures used and the corresponding eluent composition are given in Table I.

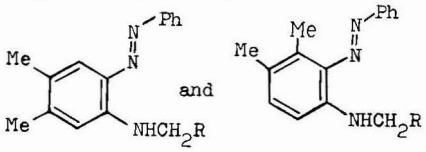
The fundamental chromatographic parameter chosen to characterise the chemically modified supports is that of resolution R, determined from the expression

$$R = \frac{2\Delta t}{W_1 + W_2}$$

TABLE I

COMPOSITION OF TEST MIXTURES AND THEIR CORRESPONDING ELUENTS

Me = methyl, Ph = phenyl.

Test mixture		*Methanol*	*Water*
PAH	benzene, naphthalene, diphenyl and anthracene	60	40
Phenol-1	phenol, *p*-cresol and 2,4-xylenol	20	80
Phenol-2	phenol, *p*-cresol, *p*-bromophenol, *p*-iodophenol	20	80
Mefruside	see Fig. 2	20	80
2-NI	see Fig. 5	10	90
Ketone	see Fig. 1	40	60
Sugar	arabinose, xylose, fructose and sucrose	90*	10
Azo compounds	*n*-(I) and *iso*-(II) azo dyes	90	10
			
	where R = (HOCH$_2$–CHOH–CHOH–CHOH)–		

* Acetonitrile.

where Δt is the difference in retention time of the solutes and W_1 and W_2 are the peak widths at the base of the peak respectively. Unlike the commonly used capacitance factor (k^1) we feel that R is of more practical use as well as being easier to comprehend.

The column screen

The column screen involves the evaluation of a wide range of bonded supports for each separation under investigation.

Test mixtures were chosen to represent relatively simple molecules of similar functional group together with more complex mixtures of drugs and their metabolites. The latter are included in order to justify the relevance of the screen to real applications.

An evaluation of the screen must take into account the following parameters: alkyl chain length; steric effects due to bulky groups; basicity; acidity; polarity of the bonded phase.

The use of the screen should be aimed at providing the following information: the best column for a given separation; trends which can be used to probe the underlying mechanism of reversed-phase liquid chromatography; and a limited number of bonded supports suitable for use in a more restricted screen.

Furthermore, application of the screen would be expected to restrict the proliferation of publications describing novel bonded phases.

Application of the screen

The proposed screen has been applied to at least 20 bonded supports (the number is being increased continuously) based upon various alkyl, aryl, acidic, polar and unsaturated functional groups. Identification of the bonded supports mentioned in Table III is given in Table II.

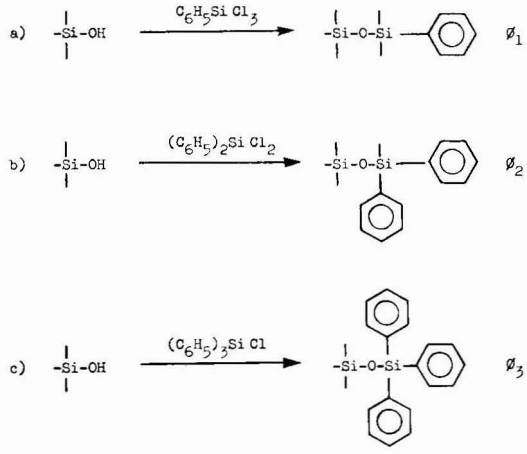
TABLE II

IDENTIFICATION OF SOME BONDED SUPPORTS LISTED IN TABLE III

Column packing		*Identification*
Support	*Modification*	
Partisil-5	Hexyl	C_6
	Nonyl	C_9
	Tetradecyl	C_{14}
	Octadecyl	C_{18}
	Monophenyl	φ_1
	Diphenyl	φ_2
	Triphenyl	φ_3
	Diphenyl, sulphonic acid	φ_2/SCX
	Diphenyl, sulphonamide	φ_2/S-NH_2
	Amino propyl	NH_2
	Propyl ethylenediamine	en
	Propionitrile	CN
	Methacrylate	MA
	Allyl	All
	Glycidoxypropyl	GLY
	Octadecyl, sulphonic acid	C_{18}/SCX
	n-Butyl germanium	BuGe
Alox-T, 5μ	Octadecyl	Al-T/C_{18}

Alkyl bonded supports. Three different separations (illustrated by Figs. 1, 2 and 3) have been used to demonstrate the relationship between resolution R and alkyl chain length C_n. In each case, R was found to increase linearly with C_n (Fig. 4). These examples show zero resolution on silica alone ($n = 0$). Of the examples studied so far only the nitroimidazole separation gives a positive resolution on silica alone. It is conceivable that the degree to which a group of compounds is affected by changes in C_n could be utilised to improve some complex separations.

Steric effects due to bulky groups. Three phenyl-bonded supports were made according to the scheme



Reaction scheme for chemical bonding with mono-, di- and tri-phenyl silanes.

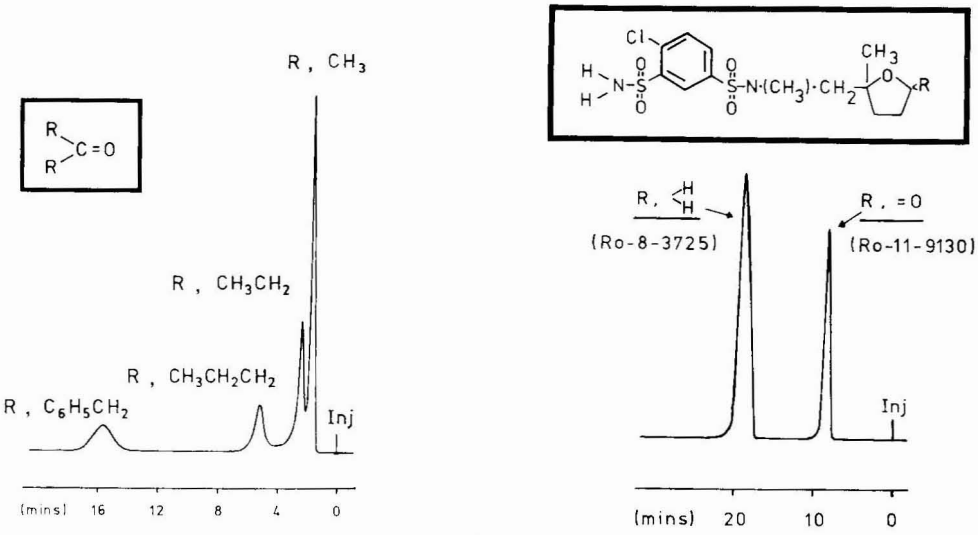
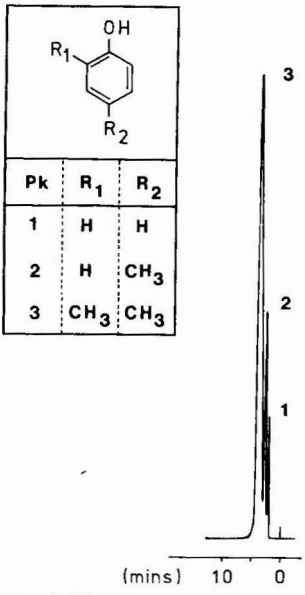


Fig. 1. Ketone separation on octadecyl Partisil (5 μm). Eluent, methanol–water (4:6); flow-rate, 1 ml/min; detection, UV (254 nm).

Fig. 2. Sulphonamide separation on octadecyl Partisil (5 μm). Eluent, methanol–water (2:8); flow-rate, 1.5 ml/min; detection, UV (248 nm).

As the bulkiness of the bonded phenyl groups increases from $\varphi_0 \rightarrow \varphi_1 \rightarrow \varphi_2 \rightarrow \varphi_3$ (where $\varphi_0 = C_0 =$ unbonded silica) steric exclusion of the solute from the support surface can be expected to increase far more dramatically than that due to increasing chain length of similar carbon number. This provides a tool for investigating the elution mechanism of reversed-phase chromatography.



Pk	R_1	R_2
1	H	H
2	H	CH_3
3	CH_3	CH_3

Fig. 3. Phenol-1 separation on octadecyl Partisil (5 μm). Eluent, methanol–water (2:8); flow-rate, 0.8 ml/min; detection, UV (275 nm).

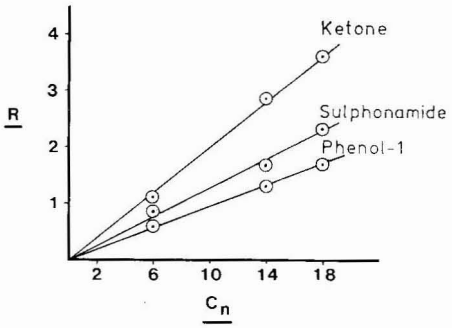


Fig. 4. Relationship between resolution R and alkyl chain length C_n.

If liquid partition was the dominating mechanism an increase in retention and resolution would be expected for increasing alkyl and aryl coverage of the silica surface. On the other hand, if adsorption was the dominating mechanism an increase in support coverage would be expected to decrease retention and resolution. At first sight, the separation of the 2-nitroimidazole radiosensitiser [Ro-07-0582] from its metabolite [Ro-11-9130] (Figs. 5 and 6) would fall into the latter category. If, however, the resolution data is superimposed on the corresponding resolution data for the alkyl coverage (Fig. 7) in such a manner that phenyl corresponds to hexyl, diphenyl corresponds to dodecyl and triphenyl corresponds to octadecyl, a more complex situation is observed. Going from phenyl to triphenyl the general decrease in retention and resolution upholds the adsorption as the dominating mechanism but the increase in retention and resolution in going from silica to phenyl can be attri-

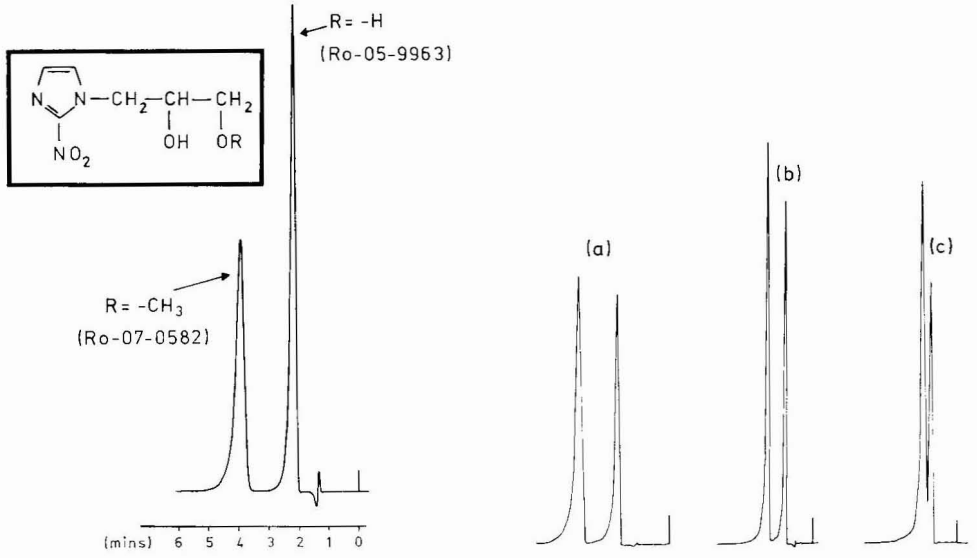
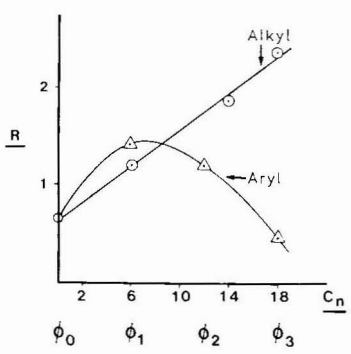


Fig. 5. Nitroimidazole separation on octadecyl Partisil (5 μm). Eluent, methanol–water (1:9); flow-rate, 1 ml/min; detection, UV (324 nm).

Fig. 6. Nitroimidazole separation on mono- (a), di- (b) and tri- (c) phenyl-bonded silicas.



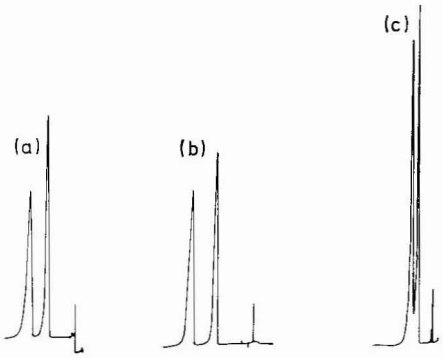


Fig. 7. Comparison between the effects of carbon number C_n and phenyl carbon number φ_n on resolution for the nitroimidazole separation.

Fig. 8. Sulphonamide separation on mono- (a), di- (b) and tri- (c) phenyl-bonded silicas.

buted either to partition effects or to adsorption modified by hindrance to the desorption process. The problem is magnified by the continued increase in retention and resolution by increasing alkyl coverage. Perhaps the increase in carbon number merely increases the hindrance to the desorption process. A similar result[18] is obtained with the separation of the sulphonamide, Mefruside [Ro-8-3725] from its metabolite [Ro-11-9130] (Fig. 8) except that the maximum retention and resolution occurs nearer to diphenyl (Fig. 9).

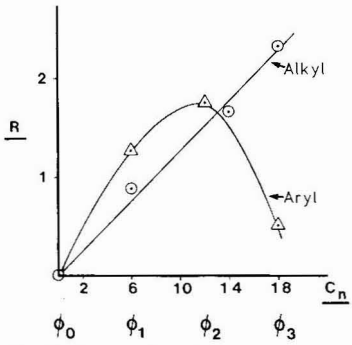


Fig. 9. Comparison between the effects of carbon number C_n and phenyl carbon number φ_n on resolution for the sulphonamide separation.

Acidic and basic bonded supports. At this stage it is worth referring to a summary of the screen to date (Table III) which attempts to classify the performance of the bonded supports investigated for each application. Not every bonded support is listed, but those that are have been chosen as being representative or illustrative of a point of particular interest. Most of the bonded supports investigated fall into the zero–moderate category.

In general, the acidic and basic bonded supports gave poor resolution. The only application giving significant separation with these supports was that of the nitroimidazoles. However, this separation gave a similar separation on unbonded

TABLE III

SUMMARY OF COLUMN SCREEN

Performance	*PAH*	*Phenol-1*	*Phenol-2*	*Mefruside*	*2-NI*	*Ketone*	*Sugar*	*Azo compounds*
Zero	CN	NH_2	$\varphi_{(1\text{-}3)}$	CN	NH_2	CN	C_{14}	C_{18}
Poor	C_{18} | SCX	C_6	CN	C_6	BuGe	$\varphi_{(1\text{-}3}$	CN	—
Mod.	(Al-T) | C_{18}	C_{14}	All	C_{14}	CN	MA	BuGe	—
Good	C_{14}	MA	C_{14}	φ_2	φ_1	C_{14}	NH_2	C_{18} | SCX
Best	C_{18}	(Al-T) | C_{18}	C_{18}	C_{18}	C_{18}	C_{18}	en	SCX

silica. Consequently, one can conclude that the bonded groups have made very little difference to the separation.

Exceptions relating to these bonded supports occur in the applications where ion-exchange or ion-pair formation can be invoked (*i.e.* sugars and azo compounds). An interesting trend to emerge from the sugar screen is the improvement in resolution as the number of bonded amino groups is increased. This work will be reported in greater detail in a future publication[19].

Polar bonded supports. The general remarks referring to acidic and basic bonded supports also apply to polar supports such as nitrile and methacrylate. The major exception to these comments refers to the resolution of the phenol-1 mixture where methacrylate produces a good resolution. However, the resolution offers no advantage over the octadecyl-bonded supports of either silica or alumina. To date no application has been found for this group of supports which cannot be better performed by other supports.

Miscellaneous. Apart from the octadecyl Alox T support, which is comparable to octadecyl Partisil for the phenol-1 separation, the only noteworthy result is that of *n*-butyl germanium in the sugar separation. This application serves to emphasize the importance of screening several supports prior to submitting a new application for publication. This is the only potential application we have found for *n*-butyl germanium so far. If we had published on the basis that it was superior to octadecyl Partisil a misleading situation would have arisen through not taking into account the even better resolution of aminopropyl Partisil and ethylenediamine-bonded Partisil.

CONCLUSIONS

Apart from applications involving ion-exchange or ion-pair mechanisms, octadecyl-bonded supports appear to be the most useful for reversed-phase chromatography.

Although inconclusive at this stage, there is some evidence that the predominant role of the bonded phase in reversed-phase chromatography is to modify the adsorption effects of the support. Almost certainly there is more than one mechanism acting and it is hoped that more conclusive evidence will be forthcoming.

Already there is sufficient evidence to propose a restricted column screen. It would appear that silica, octadecyl silica, aminopropyl silica, ethylenediamine-bonded silica and a strong cation-exchange-bonded silica should suffice to give the maximum of information for the minimum of effort.

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CHROM. 10,716

HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF PEPTIDES AND PROTEINS

II. THE USE OF PHOSPHORIC ACID IN THE ANALYSIS OF UNDERIVATISED PEPTIDES BY REVERSED-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

The chromatographic properties of a range of peptides varying in size from di- to decapeptide have been investigated by reversed-phase high-pressure liquid chromatography. A new set of conditions, namely, the addition of phosphoric acid to the mobile phase, has been found to have very real advantages in the analysis of underivatised peptides. These conditions allowed marked alterations in retention times, improvement in reproducibility and excellent resolution of peptides differing by as little as a single amino acid. A major advantage of phosphoric acid is that it can be used successfully in the range 195–220 nm which makes it compatible with the use of variable wavelength UV monitors as sensitive detectors in high-pressure liquid chromatography. In addition, the use of phosphoric acid permits the significant lowering of concentrations of organic solvents in the mobile phase, thus reducing the possibility of denaturation or precipitation.

INTRODUCTION

In the past, reversed-phase liquid–liquid partition chromatography has found limited application, but recent developments in the preparation of chemically bonded stationary phases have shown that highly efficient columns can be prepared for a variety of applications which include the analysis of pharmaceuticals[1], metabolites[2], pesticides[3], derivatised amino acids[4–7] and derivatised peptides[8]. Recently the use of

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ammonium acetate[9] and other buffers[10] has been reported for the analysis of peptides by reversed-phase high-pressure liquid chromatography (HPLC).

We recently reported a reversed-phase method for the analysis and resolution of a series of underivatised peptides on 37–50 μm pellicular packings[11]. Improved retention times with consequently improved peak separations should result by using reversed-phase packings of less than 10 μm particle diameter. These fully-porous silica based packings are noted for their significantly higher plate counts. It was found, however, that retention times of known peptides were extremely long and reproducibility was difficult to obtain with these micro-reversed-phase packings. In order to overcome these problems a series of ion-pairing and ionic suppression reagents were examined as additives to the mobile phase. As a result of these studies we found that several reagents, and in particular phosphoric acid, when added to the eluant, gave a marked improvement in reproducibility and retention times of peptides on commonly used reversed-phase systems. In addition, the use of phosphoric acid allows detection of eluted peptides at wavelengths in the range of 200–220 nm.

It is the purpose of this paper to describe the effectiveness of phosphoric acid in the analysis of a variety of peptides.

MATERIALS AND METHODS

High-pressure liquid chromatography

A Waters high-pressure liquid chromatography system was used which included two M-6000 solvent delivery units, an M-660 solvent programmer and a U6K universal liquid chromatograph injector, coupled either to a Cecil 212 variable wavelength UV monitor with an 8 μl flow-through cell and a Linear Instruments Corp. double channel chart recorder, or to a Series 440 Waters UV detector and Rikadenki double channel chart recorder.

The Bondapak C_{18}-Corasil and Bondapak Phenyl-Corasil (37–50 μm) were purchased prepacked in stainless steel columns (61 cm $\times$ 2 mm I.D.) from Waters Assoc. (Milford, Mass., U.S.A.). The μBondapak-Fatty acid analysis and μBondapak-C_{18} columns (10 μm, 30 cm $\times$ 4 mm I.D.) were also 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 by the method of Walter and Ramaley[12]. Orthophosphoric acid was from May & Baker (Dagenham, Great Britain), potassium dihydrogen phosphate and benzoic acid from BDH (Poole, Great Britain). Water was glass-distilled and de-ionised. Samples in Fig. 3 (A, B, D) and the protected hexapeptide (Fig. 2) were produced in this laboratory[13] by the solid phase method using standard procedure[14]. The peptides described in Table I were purchased from Research Specialities (Richmond, Calif., U.S.A.). All amino acids were of the L-configuration.

Methods

A flow-rate of 1.5 ml/min was used for each column which was maintained by a pressure of 1100 p.s.i. for the C_{18}-Corasil column, 2000 p.s.i. for the phenyl-Corasil column, 2100 p.s.i. for the μBondapak-C_{18} column and 2300 p.s.i. for the μBondapak-Fatty acid analysis column. All tests were at room temperature (*ca.* 22°). Sample sizes varied between 0.1 and 10 ng of peptide material injected in volumes 1–100 μl. Detection was in the range 205–225 nm, 254 nm or 280 nm depending on the nature of the sample and the mobile phase. All peptides were taken up in de-ionised, distilled water and, prior to injection, made up in the eluting solvent. All bulk solvents were degassed separately for the following times; water for at least 30 min, organic solvents for 1.5 min. The solvents were mixed in the required volumes, degassed for 1.0 min and then equilibrated to room temperature. This preciseness was necessary for reproducibility. All solvents were stirred magnetically during equilibration and elution. All columns were equilibrated to new solvents for at least 30 min. The mobile phase solutions were routinely filtered using 0.5 μm Millipore filters as were all peptide samples.

RESULTS

In Fig. 1 the marked decrease in retention time of a tetrapeptide (Leu-Trp-Met-Arg) on C_{18}-Corasil after the addition of 0.1% phosphoric acid to the mobile phase is shown. The pH of the acetonitrile–water mobile phase decreased from 6.5 to 2.2. These are apparent pH values obtained using a glass electrode. Thus, the addition of phosphoric acid will have a dual effect of a change in pH and of the equilibria of the ionisable groups in the peptide. The very basic guanidino side chain of the arginine residue (pK_a 12.48) will remain protonated over the pH ranges used in reversed-phase chromatography. The α-amino group and C-terminal carboxyl group will, however, both be affected by this change in pH.

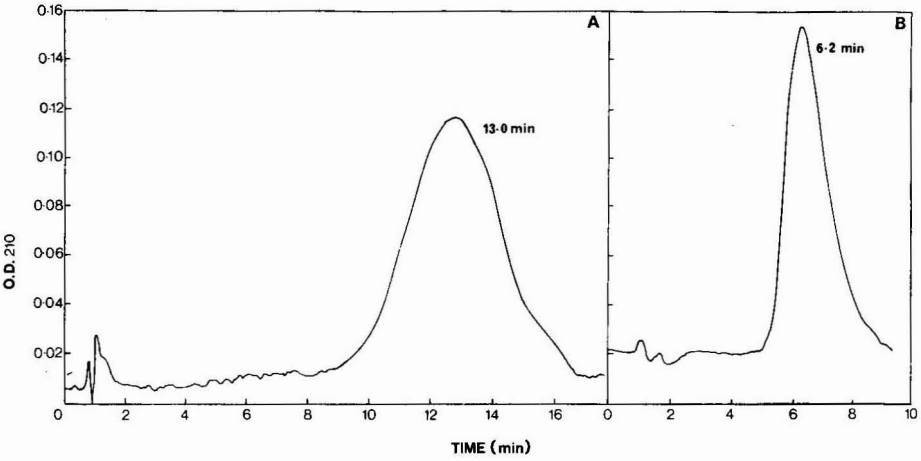

Fig. 1. Elution profile of the tetrapeptide Leu-Trp-Met-Arg on a C_{18}-Corasil column. (A) with acetonitrile–water (1:3) as mobile phase (B) with acetonitrile–water (2.5:97.5) and 0.1% phosphoric acid as mobile phase.

In an attempt to distinguish between these effects a model peptide (a derivative of the amino terminal hexapeptide fragment of Acyl Carrier Protein) Ac-Ser-Thr-Ile-Glu(OBzl pNO_2)-Asp(OBzl pNO_2)-Arg(NO_2)OH, was examined under the same conditions. Since the amino terminus has been blocked by acetylation and all side chain functional groups are protected, the only ionisable group is the C-terminal carboxyl group. In this case decreasing the pH of the mobile phase increases the retention time of the peptide (Fig. 2). Apparently, suppression of the ionisation of the carboxyl group has reduced the polarity of the peptide. A variety of concentrations of added phosphoric acid were tested, and 0.1 % (v/v) was found to be optimal and was used in all subsequent studies. A similar result was observed for acetic acid in that at the same pH of the mobile phase, both acetic and phosphoric acid resulted in increased retention times for the peptide. Similar results were obtained in the analysis of the amphoteric molecule, *p*-aminobenzoic acid[15].

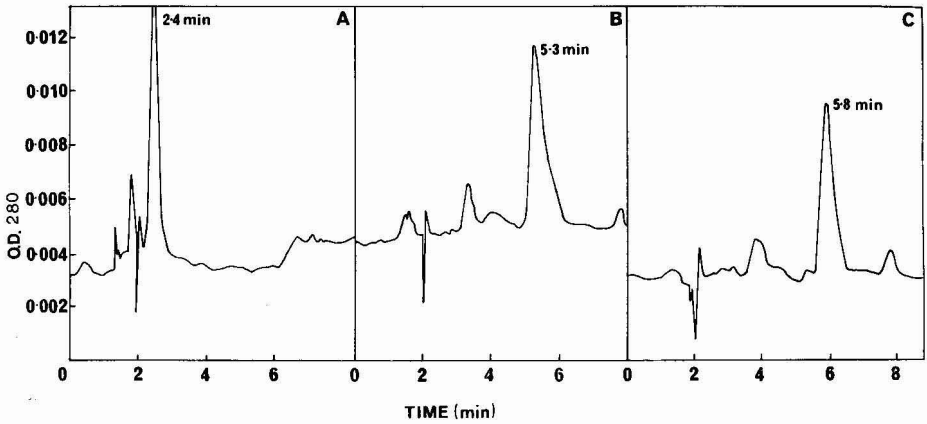

Fig. 2. Elution profile of the protected hexapeptide Ac-Ser-Thr-Ile-Glu (OBzl pNO_2)-Asp(OBzl pNO_2)-Arg(NO_2)OH on a μBondapak-Fatty acid analysis column. In each case the mobile phase was methanol–water (48:52) with the following additions: (B) 0.1 % acetic acid; (C) 0.1 % phosphoric acid.

The dramatic reduction in retention time for peptides which contain free amino groups was confirmed with a variety of other examples (Column 1, Table I). The retention time of the pentapeptide Leu-Trp-Met-Arg-Phe with a mobile phase consisting of 40 % methanol–water decreased from 104 to 12.4 min on the addition of phosphoric acid. Many peptides which contain aromatic amino acids are strongly retained on reversed-phase columns. Such an example is shown in Table II with the peptide linear antamanid where the use of phosphoric acid greatly facilitates the chromatography of such materials. Fig. 3 shows the elution profiles of four peptides on a μBondapak-Fatty acid analysis column. Again in each case the addition of phosphoric acid allowed rapid analysis of the peptide by reversed-phase HPLC.

It is of interest to note that phosphate buffers also mimic the effect of phosphoric acid on retention times. As is shown in Table II with linear antamanid as an example, the effect of the $H_2PO_4^-/HPO_4^{2-}$ system on retention time is greatest at the lower pH values. However, 0.1 *M* potassium dihydrogen–monohydrogen phosphate at pH 7 still causes a significant decrease in retention time for this peptide from 100 to 4.7 min.

TABLE I

THE EFFECT OF PHOSPHORIC AND ACETIC ACID ON THE RETENTION TIME OF VARIOUS PEPTIDES

The analysis was carried out on a μBondapak alkyl-phenyl column with 50% methanol as the eluent.

*Peptide**	*Retention time*	
	*0.1% H_3PO_4, pH 2.5***	*0.1% CH_3COOH, pH 4***,****
L-W-M-R	2.3	4.1
L-G-M-R-F	5.1	8.3
G-F	2.4	3.5
G-G-Y	1.9	3.0
M-R-F	2.5	3.8
F-S-K-L-G-D-G	2.4	3.8
G-L-Y	2.4	3.6
R-F-A	2.05	2.2

* The code for amino acids is as used by Dayhoff[23]. A = alanine, D = aspartic acid, F = phenylalanine, G = glycine, K = lysine, L = leucine, M = methionine, R = arginine, S = serine, W = tryptophan, Y = tyrosine.

** Apparent pH values measured with a glass electrode.

*** Broad peaks unsuitable for analytical separations were observed with this reagent.

TABLE II

THE RETENTION TIMES OF THE DECAPEPTIDE LINEAR ANTAMANID[1] ON A μBONDAPAK-C_{18} COLUMN

Amino acid sequence Val-Pro-Pro-Ala-Phe-Phe-Pro-Pro-Phe-Phe.

Mobile phase Methanol–water	*Retention time (min)*
50:50	∞
55:45	∞
60:40	100
Methanol–water + 0.1% phosphoric acid	
50:50	6.01
55:45	2.74
60:40	2.37
Methanol–water (55:45) + 0.1% phosphoric acid + 0.1 M potassium dihydrogen dipotassium hydrogen phosphate	
pH 2.5	2.45
pH 3.0	2.70
pH 4.0	3.30
pH 5.1	4.95
pH 7.0	4.07

DISCUSSION

In a recent publication[11] the separation of underivatised peptides on reversed-phase C_{18}- or alkyl-phenyl columns (37–50 μm) was described. Although useful results could be obtained with this system, it was noted that peptides characteristically give broad peak shapes indicating a lower theoretical plate count, N, than those observed

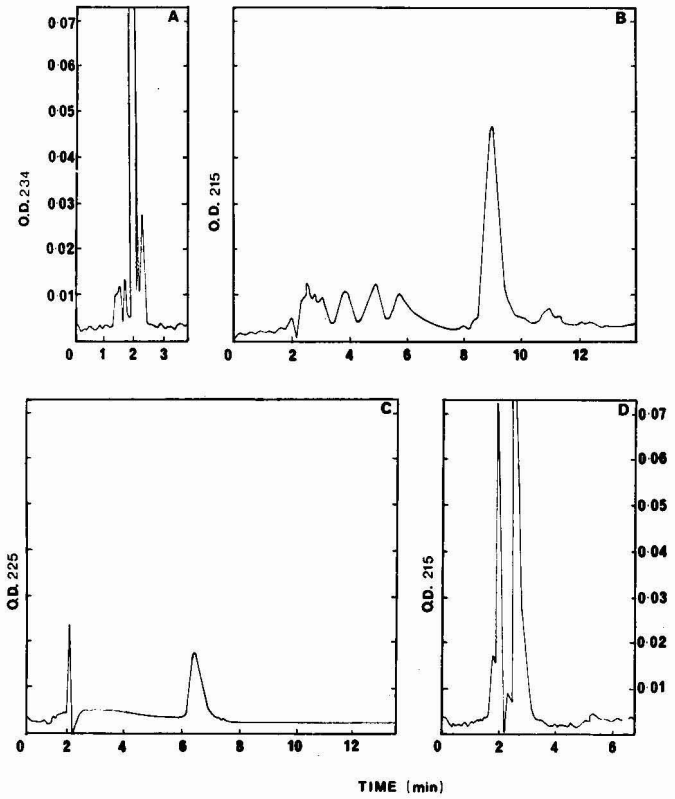

Fig. 3. Elution profiles of four different peptides on a μBondapak-Fatty acid analysis column. (A) The synthetic peptide linear antamanid with a mobile phase consisting of methanol–water (4:1) and 0.1% phosphoric acid. (B) The synthetic peptide Val-Ile-His-Pro-Phe, an angiotensin fragment, with a mobile phase consisting of methanol–water (1:1) and 0.1% phosphoric acid. (C) The tetrapeptide Met-Arg-Phe-Ala with mobile phase acetonitrile–water (1:3) and 0.1% phosphoric acid. (D) The synthetic peptide Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl) with mobile phase methanol–water (3:7) and 0.1% phosphoric acid.

for most other organic molecules. For example, benzoic acid has a value of N 2.5 times that of the tetrapeptide Leu-Trp-Met-Arg when compared on the same chromatogram. This broader peak shape can possibly be attributed to the complex contribution of ionic groups to the polarity of the peptide. This phenomenon has been recently observed with other highly polar molecules[16,17].

The use of the micro columns, μBondapak-Fatty acid analysis and μBondapak-C_{18}, has now been investigated in an attempt to solve this problem. These packings have the stationary phase chemically bonded to silica particles of 10 μm. Unfortunately, in preliminary experiments with these micro columns, peptides often gave irreproducible elution times when chromatographed under standard conditions with methanol–water mobile phases. In some cases, very long retention times were obtained, as was the case with linear antamanid which was eluted at 100 min even with a high concentration of an organic solvent in the mobile phase.

The addition of phosphoric acid to the eluant resulted in dramatic decreases

in the retention time of a range of peptides of widely different chemical structures (Figs. 1(b) and 3; Tables I and II) as well as giving good reproducibility. This general phenomenon of decrease in retention time is, presumably, due to an increase in polarity of peptides when analysed by reversed-phase chromatography in the presence of phosphoric acid.

A possible explanation for the effect of phosphoric acid on the polarity of a peptide can be obtained by a consideration of the ionic equilibria involved. In the case of the unblocked peptide Leu-Trp-Met-Arg, addition of acid will not only have the effect of protonating the carboxyl group, but also will simultaneously affect other equilibria, such as:

$$R\text{–}NH_2 \rightleftharpoons R\text{–}NH_3^+ \quad (1)$$

It appears that protonation of the amino group, by driving the equilibrium (1) to the right, overwhelms the contribution of the neutral carboxyl group, the formation of which should make the peptide less polar.

The effect of phosphoric acid cannot, however, be solely explained on the basis of a simple acid effect. As can be seen for the case of linear antamanid the addition of $H_2PO_4^-$ at constant pH causes a large decrease in the retention time (Table II). With methanol–water (60:40) and 0.1 % phosphoric acid as the eluant this decapeptide gave a retention time of 2.37 min on the μBondapak-C_{18} column. The addition of 0.1 *M* potassium dihydrogen phosphate to this eluant decreased this retention time further to 1.9 min. Other polar anions such as perchlorates[18,21], picrates[22], and methyl sulphonates[20] cause a similar decrease in retention time of peptides[21] and polar pharmaceuticals[22], which was attributed to ion-pairing between the polar anion and the solute molecules.

By analogy it is possible that the formation of an ion-pair between the peptide $R\text{–}NH_3^+$ and the hydrophilic anion $H_2PO_4^-$ is responsible for the large increase in polarity observed for solute molecules in the present study. Other possible explanations seem less likely, particularly as less polar acids such as acetic acid (column 2, Table I) do not show this dramatic effect.

CONCLUSION

The purpose of this paper has been to report the advantages of phosphoric acid in the analysis of underivatised peptides by reversed-phase HPLC: namely, marked alterations in retention times, improvement in reproducibility and excellent resolution of peptides differing by as little as a single amino acid (Fig. 3 B, C).

Since phosphoric acid can be used successfully in the range 195–220 nm, it is compatible with the use of variable wavelength UV monitors as sensitive detectors in HPLC[11]. In addition phosphoric acid is readily available, inexpensive, and allows the use of significantly lower concentrations of organic solvents in the mobile phase (with less chance of precipitation or denaturation of peptides). The development of a system for the routine analysis of underivatised peptides and amino acids using such reagents is being vigorously pursued.

ACKNOWLEDGEMENTS

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FLÜSSIGCHROMATOGRAPHISCHE PARAMETER HERBIZIDER WIRKSTOFFGRUPPEN

II*. CHLORPHENOXYALKANSÄUREN

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(Eingegangen am 24. November 1977)

SUMMARY

Liquid chromatographic parameters of groups of herbicidal active substances. II. Chlorophenoxyalkanoic acids

The liquid chromatographic properties of the most important active ingredients of the chlorophenoxyalkanoic acid herbicides were analysed on unmodified silica gel and on some reversed phases. An effective retention of the substances as well as good separation on the reversed phase "RP-2" could be obtained by adding 60 mmoles of tetramethylammonium halogenide per litre of mobile phase. The UV spectra of seven active ingredients and three chlorophenols were recorded. In many cases they show an absorption minimum near the most-applied UV wavelength of 254 nm.

EINLEITUNG

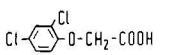
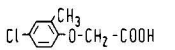
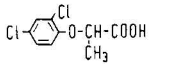
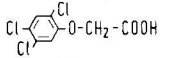
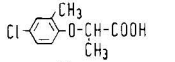
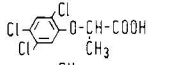
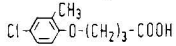
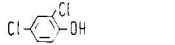
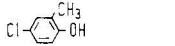
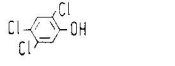
Die Rückstandsanalytik der Chlorphenoxyalkansäureherbizide wird heute meist gaschromatographisch (GC) durchgeführt. Da die Verbindungen als Carbonsäuren niedrige Dampfdrucke besitzen, müssen sie vor der Aufgabe auf die GC-Säule derivatisiert werden. Derartige GC-Verfahren zeichnen sich auf diese Weise zwar durch hohe Nachweisempfindlichkeiten aus, jedoch sind Umsetzungsvorgänge im Nano- oder Picomolbereich nicht immer ohne Probleme zu bewältigen und zudem bieten sie infolge der Verlängerung und Komplizierung des Analysenganges die zusätzliche Gefahr einer Fehler- und Fremdstoffeinschleppung. Wir haben deshalb die Möglichkeit untersucht, diese Säuren direkt zu bestimmen, und zwar auf dem Wege der Hochdruck-Flüssigchromatographie mit Detektion durch UV-Absorption.

Neben den Chlorphenoxyalkansäuren selbst wurden auch einige Chlorphenole untersucht, da diese einerseits als Rohstoffe oder Zwischenprodukte bei der Herstellung von Phenoxysäuren dienen, andererseits als Metaboliten beim Abbau der Phenoxysäureherbizide in der Natur auftreten.

* Erster Teil: *J. Chromatogr.*, 125 (1976) 487–494.

TABELLE I

UNTERSUCHTE CHLORPHENOXYALKANCARBONSÄUREN UND CHLORPHENOLE

Chemische Verbindung (Wirkstoff-Namen)	*Strukturformel*	*F.P. (°C)**	*Wasserlöslichkeit (ppm)*	*Extinktionskoeffizient (cm^2/mg)*			
				Max. 1	*254 nm*	*Max. 2*	*Max. 3*
2,4-Dichlorphenoxyessigsäure (2,4-D)		141	620* (25°)	10 (283 nm)	3	44 (228 nm)	91 (204 nm)
4-Chlor-2-methylphenoxyessigsäure (MCPA)		119	825* (20°)	10 (278 nm)	3	55 (227 nm)	88 (202 nm)
2-(2,4-Dichlorphenoxy)-propionsäure (2,4-DP,Dichlorprop)		118	350* (20°)	11 (284 nm)	3	52 (229 nm)	94 (206 nm)
2,4,5-Trichlorphenoxyessigsäure (2,4,5-T)		158	278* (25°)	12 (288 nm)	4	kein Maximum	101 (209 nm)
2-(4-Chlor-2-methylphenoxy)-propionsäure (MCPP,Mecoprop)		94–95	620* (20°)	10 (278 nm)	3	51 (228 nm)	88 (202 nm)
2-(2,4,5-Trichlorphenoxy)-propionsäure (2,4,5-TP,Fenoprop)		179–181	140* (25°)	10 (290 nm)	2	kein Maximum	150 (210 nm)
4-(4-Chlor-2-methylphenoxy)-buttersäure (MCPB)		99–100	44* (20°)	10 (278 nm)	3	50 (227 nm)	87 (202 nm)
2,4-Dichlorphenol		45	8320 (25°)	10 (286 nm)	0	kein Maximum	53 (205 nm)
4-Chlor-2-methylphenol		49	7390 (25°)	12 (282 nm)	0	47 (226 nm)	53 (202 nm)
2,4,5-Trichlorphenol		66–67	485 (25°)	10 (290 nm)	3	kein Maximum	64 (212 nm)

* Lit. 7.

Die Literatur über die flüssigchromatographische Bestimmung der Phenoxysäuren ist —im Gegensatz zu den GC-Methoden— nicht sehr umfangreich. Es handelt sich vielfach um Firmenschriften.

Auf Kieselgel werden mit Hexan–Essigsäure-Gemischen auf zum Teil sehr langen Säulen zufriedenstellende Trennungen von Kombinationen der hier behandelten Phenoxysäuren erzielt[1,2]. Von merklichem Einfluss ist dabei die Grösse der Oberfläche des Trägers. Eine Bestimmung neben verschiedenen Organochlorinsektiziden zeigte hingegen trotz komplizierter Gradienten-Elution nur mässigen Erfolg[3].

Von den sogenannten Umkehrphasen (Reversed-Phasen, RP) wurden Sorbentien beschrieben, die mittels Polyamidgruppierungen[4] oder Alkylgruppen verschiedener Kettenlänge[5,6] substituiert waren. Hier wurde mit Methanol–Wasser- oder Methanol–Wasser–Essigsäure-Gemischen eluiert. Auch dabei war die Trennung trotz hoher Säulenlänge nicht immer zufriedenstellend. Die eingespritzten Substanzmengen lagen, soweit Angaben darüber gemacht werden, recht hoch: 5, 25 und 50 μg pro Verbindung. Ausserdem wurde mitunter für die Messung UV-Strahlung gewählt, deren Wellenlänge —wie wir anhand der aufgenommenen UV-Spektren feststellten— keine volle Ausschöpfung der Nachweis-Empfindlichkeit zuliess.

PARAMETER

Die wichtigsten physikalischen Eigenschaften der von uns untersuchten Verbindungen sind in Tabelle I aufgeführt. Hier sind neben der chemischen Bezeichnung der Verbindungen jeweils ihre im Pflanzenschutz gebräuchlichen Wirkstoff-Bezeichnungen angeführt. Betrachtet man die chemische Zusammensetzung, so ist die offenbar obligate Substitution der 2- und 4-Stellung im Benzolkern durch Chlor bzw. Methylgruppen bemerkenswert. Die Wasserlöslichkeit bezieht sich stets auf die freie Säure und nicht auf deren Salze, die in der Praxis meist eingesetzt werden und vielfach eine unvergleichlich höhere Löslichkeit im Wasser zeigen. Die bei den Extinktionskoeffizienten in Klammern angegebenen Werte stellen die Wellenlänge dar, bei denen gemessen wurde. Wie aus den Spektren (Fig. 4) ersichtlich, sind meist drei Maxima vorhanden.

Die Möglichkeiten der Flüssigchromatographie sind infolge der Variabilität der mobilen Phase vielfältiger als die der Gaschromatographie, zumal auch hinsichtlich der Anzahl stationärer Phasen eine zunehmende Auswahl zur Verfügung steht. Wir haben jedoch die Erfahrung gemacht, dass sich für das vorliegende Problem mit Kieselgelsorbentien und einfachen Reversed-Phasen brauchbare Resultate erzielen lassen. Die Beschickung der Säulen geschah zum Teil durch Einrütteln des trockenen Materials mit Hilfe eines Vibrators und der Wasserstrahlpumpe, zum grösseren Teil jedoch nach verschiedenen Slurry-Techniken. Eine Weiterentwicklung dieser Techniken mit dem Ziele der Optimierung der Säulenleistung wurde in diesem Zusammenhang nicht angestrebt.

Es wurden prinzipiell von jedem Säulentyp zwei Exemplare hergestellt, und für die Ermittlung der chromatographischen Daten dann die leistungsfähigste und damit für die Charakterisierung ihrer Füllung typischste Säule ausgewählt. Diese Säulen wurden auf Trennleistung sowie auf allgemeine und selektive Retentionswirkung gegenüber Phenoxysäuren und Chlorphenolen getestet.

Stationäre Phasen

Kieselgel. Kieselgel als das am häufigsten verwendete Adsorbens hält durch Wasserstoffbrückenbildung die relativ polaren Moleküle der Phenoxysäuren stärker zurück. Um sie zu eluieren, müssen sehr polare, meist wasserhaltige Elutionsmittel verwendet werden. Die Phenoxysäuren werden dann nach zunehmender Polarität eluiert.

Für den Dauerbetrieb ist das wässrig–alkoholische Elutionsmittel sicherlich nicht problemlos (Reproduzierbarkeit des Wassergehaltes der Mischung, Haltbarkeit der Säule auf Grund der Löslichkeit des Kieselgels). Das trifft jedoch für das verschiedentlich empfohlene und hinsichtlich seiner Polarität ausserordentlich gegensätzliche und zudem aggressive Gemisch Hexan–Essigsäure mindestens ebenso zu.

Umkehrphasen. Umkehrphasen mit fest verankerten Kohlenstoffketten (Bürsten) halten Stoffe geringerer Polarität stärker zurück. Die hier untersuchten Verbindungen werden teilweise auf Grund ihrer hohen Polarität kaum festgehalten. So wandern 2,4-DP, MCPA, 2,4,5-T und MCPP bereits mit Wasser oder wässrigen Kochsalzlösungen ohne Alkoholzusatz durch die RP-Säule. Andererseits werden die etwas weniger polaren Vertreter wie MCPB, 2,4,5-TP, 2,4,5-Trichlorphenol, 4-Chlorphenol und 2,4-Dichlorphenol so stark an die RP-Packung sorbiert, dass Wasser nicht ausreicht, die Stoffe zu eluieren.

Eine Lösung für dieses Problem fanden wir in der Zugabe bestimmter quartärer Amine zum Elutionsmittel. Durch eine sogenannte Ionenpaarbildung werden hierdurch die chromatographischen Eigenschaften der Phenoxysäuren entscheidend beeinflusst. Auf diese Weise können Unterschiede in den Eigenschaften der besonders stark polaren Phenoxysäuren infolge einer "Maskierung" der vermutlich für das einheitliche chromatographische Verhalten verantwortlichen stark dominierenden Carboxylgruppen besser zur Geltung kommen. Die Auftrennung der einzelnen Phenoxysäuren voneinander konnte nach diesen Verfahren wesentlich verbessert werden. Die Polarität der Phenoxysäuren wurde dabei zum Teil so stark abgeschwächt, dass sie sich nicht mehr allein mit Wasser (plus Tetraalkylammoniumhalogenid) eluieren liessen und die Elutionsstärke durch Alkoholzusatz erhöht werden musste.

Molekularsiebe. Molekularsiebe bewirken bekanntlich auf Grund ihres spezifischen Porendurchmessers eine Aufteilung nach Molekülgrösse. Wird die mögliche Restadsorption des Siebes ausgeschaltet, so kommt die Trennung durch Molekulargrössenunterschiede zustande. Da die Unterschiede zwischen kritischen Molekulardurchmessern relativ gering sind, lassen sich, wie auch unsere Messergebnisse gezeigt haben, die Molekularsiebe für die Trennung der hier untersuchten Pflanzenschutzmittel voneinander nicht gut verwenden. Für eine vorherige Abtrennung ("cleanup") der Phenoxysäuren von verschiedenen höhermolekularen Stoffen, z.B. aus Bodenextrakten, sind die Molekularsiebe sicherlich geeignet, jedoch wurde die Untersuchung dieser Frage nicht vertieft.

Mobile Phasen

Die Auswahl einer geeigneten mobilen Phase hat einen entscheidenden Einfluss auf das Gelingen einer Trennung. In Fig. 1 wird dies verdeutlicht. Die in Tabelle II aufgeführten Lösungsmittel erfüllen die gestellten Ansprüche; sie sind im UV-Bereich meist schon ab 220 nm von ausreichender Transparenz. Die verwendete Konzentration (30–60 m*M*) des "Komplexierungsstoffes" entspricht einem erheblichen

Überschuss schon an der Eintrittsstelle in das chromatographische System (Einspritzblock).

ERGEBNISSE

Die besten Ergebnisse wurden mit Säulenfüllungen aus Perisorb RP-2 in einer Stahlsäule von 50 cm × 3.5 mm I.D. sowie mit einem Elutionsmittel aus 5 bis 15% wässrigem Methanol bei Zusatz von 30 bis 60 m*M* Tetraäthylammoniumbromid in Wasser erzielt.

Fig. 1 zeigt die Chromatogramme eines Gemisches von fünf Substanzen. Die Proben wurden in Wasser oder 30%igem wässrigem Methanol gelöst und eingespritzt;

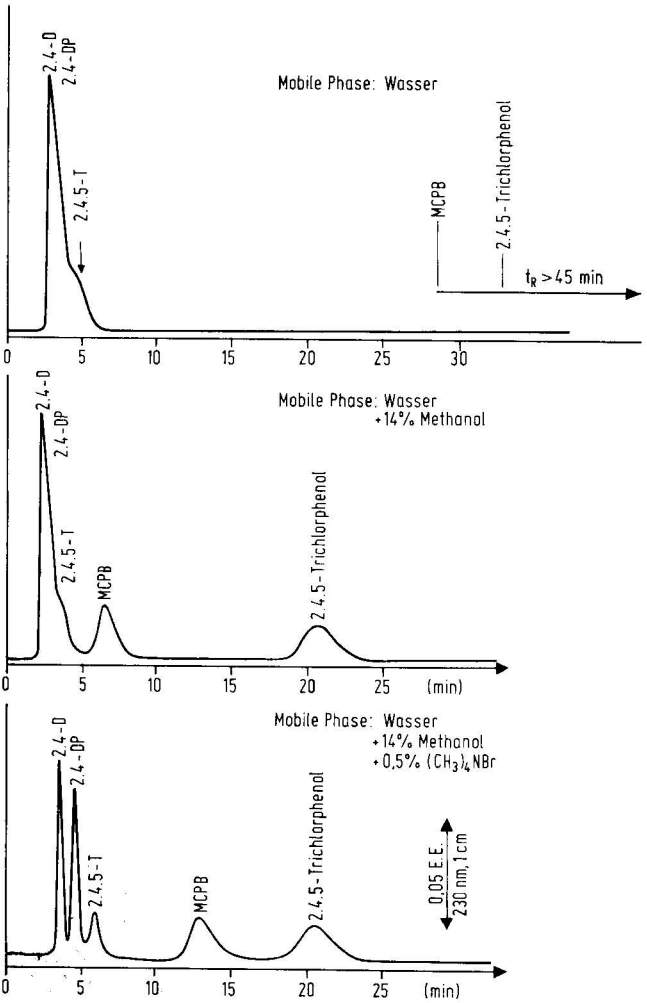


Fig. 1. Einfluss des Gehaltes an Methanol und tertiärem Ammoniumsalz auf die Trenneigenschaften der Säule (500 × 3.5 mm); stationäre Phase: Perisorb RP-2, 30–40 μm; Fliessgeschwindigkeit 2 ml/min; isocratisch.

das Einspritzvolumen betrug überwiegend 10 μl mit je 1–2 μg Substanz. Mit reinem Wasser als Elutionsmittel (oberes Beispiel in Fig. 1) laufen drei der Substanzen ungetrennt mit der Front; die restlichen beiden verlassen die Säule erst nach *ca.* 45 min. Wird der mobilen Phase 14% Methanol zugesetzt (Mitte), so verlassen die beiden sonst schwer eluierbaren Stoffe die Säule in vertretbaren Zeiten; am Frontpeak verbessert sich jedoch nichts. Der Zusatz von 0.5% Tetramethylammoniumsalz (unteres Beispiel) ändert das Bild grundlegend: Die Reihenfolge der Elution ändert sich nicht, aber die vier Phenoxysäuren werden jetzt gut voneinander getrennt; das Phenol ändert seine Laufzeit nicht, da es naturgemäss von den organischen Kationen weniger beeinflusst wird.

So war es auch gleichgültig, welches Tetramethylammoniumsalz eingesetzt wurde; wir verwandten das Bromid, weil es bei uns von Anfang an in ausreichender Reinheit verfügbar war. Fig. 2 verdeutlicht nochmals die Abhängigkeit der Retention vom Methanol-Gehalt der mobilen Phase bei unveränderter Konzentration an Tetramethylammoniumsalz.

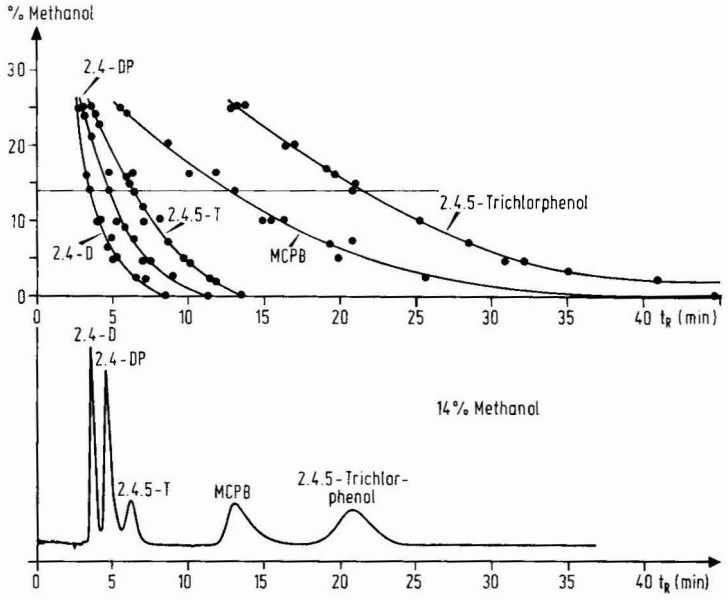


Fig. 2. Abhängigkeit der Retentionszeiten von der Zusammensetzung der mobilen Phase (wässriges Methanol mit 30 mMol Tetramethylammoniumbromid); übrige Bedingungen siehe Fig. 1.

Für das Retentionsverhalten nicht gleichgültig war die Länge des an der Reversed-Phase angelagerten Alkylrestes. Es zeigt sich, dass die RP-2-Phase geeigneter was als das analoge RP-8-Produkt; das letztere wiederum erwies sich als geeigneter als RP-18. In der gleichen Reihenfolge ist auch eine Zunahme der Retention zu verzeichnen (siehe Fig. 3). Die Reduzierung der Säulenlänge von 50 auf 25 cm ergab erwartungsgemäss schlechtere Resultate; das gleiche gilt für die Verwendung geringerer Säulenquerschnitte (2 mm anstatt 3.5 mm). Schliesslich erhielten wir mit Methanol günstigere Ergebnisse als mit Äthanol.

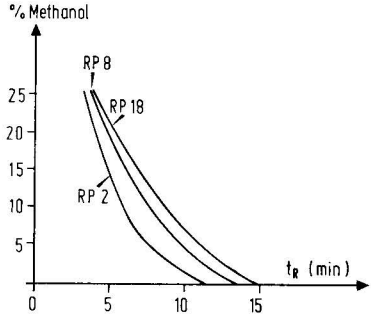


Fig. 3. Retentionsverhalten von 2,4-DP bei Variation des Methanolgehaltes bei Einsatz verschiedener Perisorb-Umkehr-Phasen; die mobile Phase enthält 30 mMol Tetramethylammoniumbromid; übrige Bedingungen siehe Fig. 1.

Die Bedeutung des Trennsystems für das Retentionsverhalten verdeutlicht Tabelle II. Hier sind die von uns untersuchten Herbizide und ihre Aniline unter den verschiedenen geprüften Bedingungen in der Reihenfolge ihrer Elution von der Säule zusammengestellt. Wie aus den beiden linken Spalten ersichtlich, verlassen die stark polaren Verbindungen eine Kieselgel-Säule zuletzt, während die Reihenfolge auf

TABELLE II

ELUTIONSREIHENFOLGE DER GEPRÜFTEN VERBINDUNGEN AUF DEN STATIONÄREN PHASEN (KIESELGEL UND RP) BEI VERSCHIEDEN ZUSAMMENGESETZTER MOBILER PHASE

	Kieselgele		*RP-2, RP-8, RP-18*			
	Essigsäure–Hexan	*Wasser–Propanol–CH_2Cl_2*	*Wasser–ROH*	*Wasser–ROH, R_4NX*	*Wasser–ROH, NH_4Br, NaCl, Na_3PO_4, Triäthanolamin*	*Wasser–ROH, R-NH-NH-R · 2HCl*
Elutionsreihenfolge	2,4,5-Trichlorphenol	2,4,5-Trichlorphenol	2,4,5-Trichlorphenol	2,4,5-Trichlorphenol	2,4,5-Trichlorphenol	MCPB
	2,4-Dichlorphenol	2,4-Dichlorphenol	2,4-Dichlorphenol	2,4-Dichlorphenol	2,4-Dichlorphenol	2,4,5-TP
	4-Chlor-2-methylphenol	4-Chlor-2-methylphenol	4-Chlor-2-methylphenol	4-Chlor-2-methylphenol	4-Chlor-2-methylphenol	2,4,5-Trichlorphenol
	2,4-DP	MCPB	MCPB	MCPB	MCPB	MCPP
	2,4,5-TP	2,4,5-TP	2,4,5-TP	2,4,5-TP	2,4,5-TP	2,4,5-T
	MCPB	MCPP	MCPP	MCPP	MCPP	2,4-DP
	MCPP	2,4-DP	2,4,5-T	2,4,5-T	2,4,5-T	MCPA
	MCPA	2,4,5-T	2,4-DP	2,4-DP	2,4-DP	2,4-DP
	2,4,5-T	MCPA	MCPA	MCPA	MCPA	2,4-Dichlorphenol
	2,4-D	2,4-D	2,4-D	2,4-D	2,4-D	4-Chlor-2-methylphenol

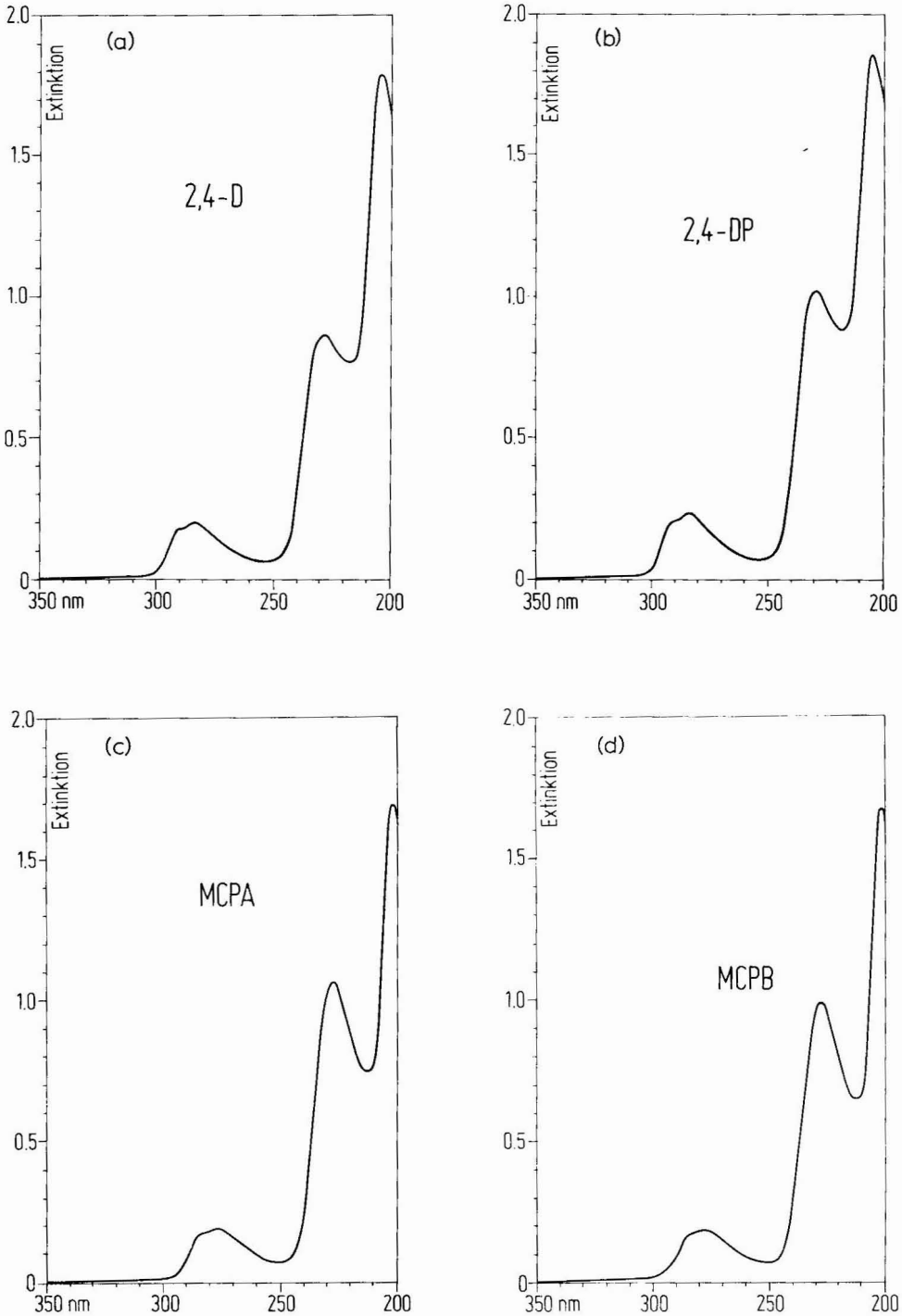

(a)
Extinktion
2,4-D
2.0
1.5
1.0
0.5
0
350 nm
300
250
200
(b)
Extinktion
2,4-DP
2.0
1.5
1.0
0.5
0
350 nm
300
250
200
(c)
Extinktion
MCPA
2.0
1.5
1.0
0.5
0
350 nm
300
250
200
(d)
Extinktion
MCPB
2.0
1.5
1.0
0.5
0
350 nm
300
250
200

Fig. 4

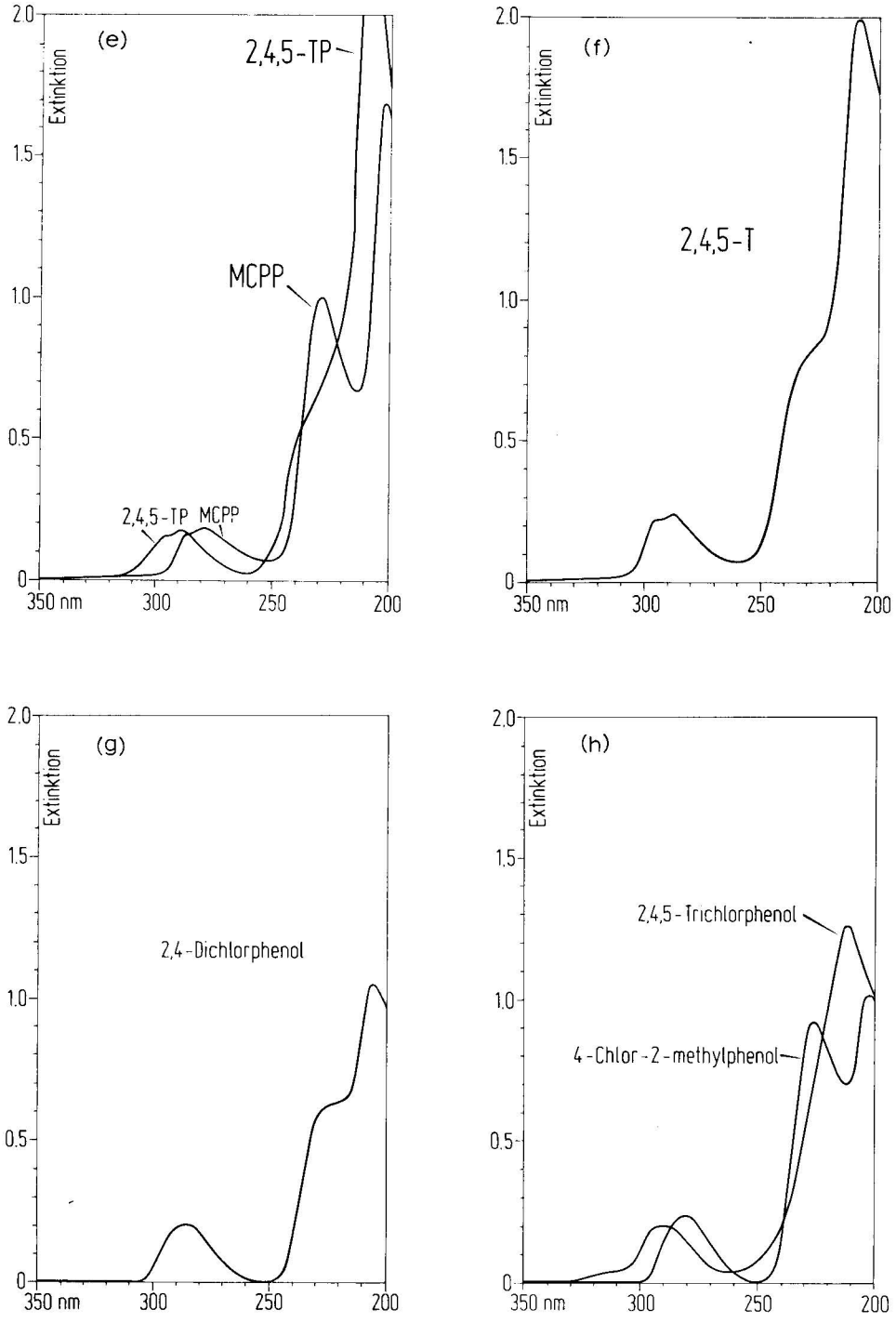


Fig. 4. UV-Spektren der untersuchten Verbindungen; 1-cm-Küvette; Konzentration: 20 μg/ml Wasser.

RP-Säulen sich umkehrt. Verschiebungen innerhalb der Reihenfolge wurden durch Schräglinien gekennzeichnet. So tauschen auf Kieselgelsäulen eine Reihe von Wirkstoffen ihre Plätze, wenn als mobile Phase Hexan–Essigsäure anstatt eines Gemisches von Wasser, Propanol und Methylenchlorid verwendet wird. Bei den RP-Säulen, die allgemein mit wässrigem Alkohol als Elutionsmittel verwendet werden, ändert sich an der (umgekehrten) Reihenfolge nichts, wenn der pH-Wert der wässrig–methanolischen Phase verändert wurde oder verschiedene Salze für eine etwaige Ionenpaarbildung, wie z.B. Natriumchlorid, Natriumphosphat, Ammoniumbromid, Tetraalkylammoniumhalogenide oder Triäthanolammoniumsalz, zugesetzt wurden. Eine Ausnahme machte hier überraschenderweise Dimethylhydraziniumdichlorid, das sogar die drei Phenole zu unterschiedlichem Retentionsverhalten veranlasste.

Die Messungen wurden an einem Varian-Gerät des Typs LC 8500 mit Spektralphotometer 635 durchgeführt. Um die Nachweisempfindlichkeit zu optimieren, wurden die UV-Spektren der untersuchten Verbindungen aufgenommen (siehe Fig. 4). Hierfür wurden die Phenoxysäuren als Natriumsalze im Wasser aufgelöst (20 μg/ml).

Die Eichkurve verlief im untersuchten Bereich von 10–3000 ng linear. Bei Versuchen mit Dosiervolumina zwischen 3 und 60 μl wurden keine unterschiedlichen Ergebnisse beobachtet. Aus der Streuung der Messwerte errechnete sich eine relative Standardabweichung von 6%. Die Nachweisgrenze liegt für 2,4-D bei etwa $4 \cdot 10^{-11}$ Mol (10 ng), naturgemäss etwas abhängig vom Trennsystem.

ZUSAMMENFASSUNG

Die flüssigchromatographischen Eigenschaften der wichtigsten Wirkstoffe der Chlorphenoxyalkancarbonsäure-Herbizide auf unmodifiziertem Kieselgel und auf einigen Umkehr-Phasen wurde untersucht. Eine wirksame Rückhaltung der Wirkstoffe sowie eine gute Trennung auf der Reversed-Phase "RP-2" konnte durch Zusatz von 60 Millimol Tetramethylammoniumhalogenid pro Liter mobiler Phase erzielt werden. Die UV-Spektren von sieben Wirkstoffen und drei Chlorphenolen wurden aufgenommen. Sie zeigen in der Nähe des gebräuchlichsten UV-Bereiches von 254 nm vielfach ein Extinktions-Minimum.

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LABORATORY PREPARATION AND APPLICATIONS OF MODIFIED CARBOWAX 20M BONDED SUPPORTS TO THE GAS CHROMATOGRAPHY OF PESTICIDES

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SUMMARY

Five gas chromatographic (GC) solid supports, modified by treatment to give surface bonded Carbowax 20M, were evaluated for their suitability by GC stationary phases for several pesticides both before and after coating with OV-210. Even though all the modified supports were far superior to non-treated supports, there were appreciable differences between the supports for some of the more difficult to chromatograph pesticides. Carbowax 20M bonded to Gas-Chrom P generally gave the best performance for the pesticides tested. Preparation of the support for optimum response required extensive refluxing in 9 *N* hydrochloric acid to remove all traces of acid, coating with 5% Carbowax 20M, conditioning overnight at 280° followed by Soxhlet extraction with methyl alcohol and dichloromethane to remove all unbound Carbowax 20M. It was demonstrated that certain modified packings could play a useful role in the analysis of such compounds.

INTRODUCTION

The novel development of polymer-deactivated adsorbents for use in gas–liquid chromatography has been described previously by Hastings and Aue[1]. These authors applied an ultrathin film of Carbowax 20M on gas chromatographic (GC) supports equivalent to a monomolecular layer[2] as they nominally described it. The result of this treatment was a "deactivated" support which greatly improved the GC behavior of polar and/or relatively unstable organic compounds. These authors[1] went one step further by coating the "deactivated" support with different liquid phases. The purpose of these different liquid phases was to improve separations beyond that attainable with Carbowax treatment alone, thereby permitting an analyst to choose selectively that phase(s) which would give desired separations and at the same time be less concerned with the poor chromatographic behavior attributed to non-deactivated supports.

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In the analysis of pesticides, one is faced with many compounds that are very difficult to separate and/or are often unstable on commerically prepared supports. The objective of this study was to determine what factors are most critical in the preparation of Carbowax 20M surface-modified solid supports and the practical application of these supports to pesticide separations.

EXPERIMENTAL

Preparation of supports for gas chromatography

The principle used for preparing the supports was the same as that described by Aue *et al.*[2]. The supports evaluated in this study (Table I) included Chromosorb P, 80–100 mesh (Analabs, North Haven, Conn., U.S.A.); Chromosorb G, 80–100 mesh (Analabs.); acid-washed Chromosorb W, 80–100 mesh (Analabs); Gas-Chrom Q, 80–100 mesh (Applied Science Labs., State College, Pa., U.S.A.); and Gas-Chrom P, 80–100 mesh (Applied Science Labs.). Each commercial diatomaceous earth support was acid washed in a Soxhlet apparatus with 9 *N* hydrochloric acid until all traces of a yellow hue were removed. This generally required a minimum of 1–3 weeks, depending on the support. The supports were then washed to neutrality with distilled water and dried. Each support was gently screened through a 120-mesh Tyler stainless-steel screen to remove fines, and coated with Carbowax 20M (usually 5%) using rotary evaporation until a good uniform coating was achieved.

TABLE I

SUPPORT MATERIALS AS RECEIVED FROM THE MANUFACTURER PRIOR TO MODIFICATION BY CARBOWAX 20M TREATMENT

Source, Diatomite; mesh size, 80–100.

Support	*Color*	*Surface area (m^2/g)*	*Treatment by manufacturer*	*Free fall density (g/ml)*
Chromosorb P	Pink	4.0	Calcined	0.38
Chromosorb G	Oyster white	0 5	–	0.47
Chromosorb W	White	1.0	Flux calcined, acid washed	0.18
Gas-Chrom P	White	1.0	Flux-calcined, acid-washed, base-washed	0.22
Gas-Chrom Q	White	1.0	Flux calcined, acid washed, base washed, DMCS treated	0.22

The coating of the support with the Carbowax was accomplished by mixing the support with Carbowax 20M dissolved in dichloromethane and allowing to stand overnight before evaporating the solvent on a rotary evaporator. Solvent was then added to the coated support, the mixture stirred gently and allowed to set one hour before solvent removal by rotary evaporation. This procedure was carried out two more times before the coating was considered uniform. The dried supports were transferred to a 50-ml volumetric pipette containing a glass wool plug at the restricted end and capped with an additional glass wool plug at the top of the packing. The pipette containing the support was placed in a 4-in. long circular heating block which had an I.D. sufficient to hold the pipette at a flow-rate approximating 200 ml/min

for at least 30 min. The flow of nitrogen was then reduced to 5 ml/min. The temperature was then raised to 270°–280° (unless specified otherwise) over a period of about 2 h and maintained at that temperature for 20 h before cooling to room temperature. The supports were then transferred to a Soxhlet apparatus and successively extracted with methanol and dichloromethane (unless otherwise stated) for at least seven days. All supports were then dried and divided into two portions, one of which was coated with OV-210 and the other used without further treatment for general comparisons. OV-210 coated supports were prepared in the same manner as the Carbowax 20M treated supports.

Pesticides evaluated

The pesticides selected for evaluation (Table II) vary greatly in polarity and suitability as solutes for GC on conventional silicone coated columns[3]. Many of these compounds are generally considered thermally unstable, yield unfavorable separations, and/or give less than optimum chromatographic behavior as often characterized by peak tailing or broadening.

TABLE II

PESTICIDES USED IN THE EVALUATION OF MODIFIED SUPPORTS

Common name	*Chemical name*
Aldrin	1,2,3,4,10,10-Hexachloro-1,4,4*a*,5,8,8*a*-hexahydro-endo-1,4-exo-5,8-dimethanonaphthalene
Atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine
Azinphos-methyl	O,O-Dimethyl S-[4-oxo-1,2,3-benzotriazin-3(4H)-ylmethyl] phosphorodithioate
Benefin	N-Butyl-N-ethyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine
Chlorpyrifos	O,O-Diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate
Chlorpyrifos oxygen analogue	O,O-Diethyl O-(3,5,6-trichloro-2-pyridyl) phosphate
Diazinon	O,O-Diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate
Dioxathion	2,3-*p*-Dioxanedithiol-S,S-bis(O,O-diethyl phosphorodithioate)
Disulfoton	O,O-Diethyl-S-2-(ethylthio)-ethyl phosphorodithioate
Lindane	γ-Isomer of 1,2,3,4,5,6-hexachlorocyclohexane
Methamidophos	O,S-Dimethyl phosphoramidothioate
Methyl Parathion	O,O-Dimethyl O-*p*-nitrophenyl phosphorothioate
Mevinphos	O,O-Dimethyl 1-carbomethoxy-1-propen-2-yl phosphate (60% *trans*, 30% *cis* isomer)
Monocrotophos	*cis*-3-(dimethoxyphosphinyloxy)-N-methylcrotonamide
Parathion	O,O-Diethyl-O-*p*-nitrophenyl phosphorothioate
Paraoxon	O,O-Diethyl O-*p*-nitrophenyl phosphate
Phosphamidon	2-Chloro-N,N-diethyl-3-hydroxycrotonamide dimethyl phosphate
p,p'-DDT	1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl) ethane
Simazine	6-Chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine
Tepp	Tetraethyl pyrophosphate
Trifluralin	α,α,α-Trifluoro-2,6-dinitro-N,N-dipropyl-*p*-toluidine

Gas chromatography

All supports were packed in 1.8 m × 2 mm I.D. U-shaped glass columns and evaluated in a Varian Aerograph Model 2100 gas chromatograph equipped with ^{3}H electron-capture detectors (ECDs) and a Microtek Model 220 gas chromatograph

equipped with a ^{63}N ECD. The Varian Aerograph gas chromatograph was equipped for on-column injection while the Microtek Model 220 was equipped for off-column injection utilizing a demisting trap as received from the manufacturer. Only two column temperatures were used in this study, 175° and 200°, to keep operating conditions as static as possible. Nitrogen was used as the carrier gas and flow-rates were maintained at 25 ml/min. As a precautionary procedure it is very important that the carrier gas be free of oxygen in order to prevent removal of the film through oxidation[4].

RESULTS AND DISCUSSION

The unique behavior of a thin film of Carbowax 20M on a support such as Chromosorb W or G, very adequately described by previous investigators[1,2,5,6], has shown potential for the analysis of a limited number of pesticides[4,5,7]. The primary purpose of this study was to ascertain if a micro-thin layer of Carbowax 20M was useful as a stationary phase for the GC analysis of multi-pesticide residues, and to ascertain more carefully what precautions might be necessary in preparing coated supports for studies involving pesticide residues. Five commonly employed GC supports were selected (Table I) for deactivation using the Carbowax 20M treatment. These treated supports were then compared with non-deactivated supports by observing differences in their ability to efficiently chromatograph unstable and/or difficult to separate pesticides. Gas chromatographs equipped with ^{3}H and ^{63}Ni ECDs were employed in this study because of their wide use and sensitivity to many types of pesticides and their inherent ability to detect degradation products, chromatographic peak tailing, and artifacts not always observed with other GC detection systems. Therefore, it is not unreasonable to believe that if the various columns performed well with the ECD system, they should perform even better with element-selective detectors.

Many of the steps in the original procedure[1,2] for preparing the supports were subjected to systematic variation in this study; examples include the percent load of Carbowax 20M, the choice of solvent used to remove the excess Carbowax, the temperature selected for overnight conditioning of the coated Carbowax on the solid support, and the necessity for the acid treatment. Only one variable was evaluated at a time while the others were kept constant according to the original procedure, unless stated otherwise.

When 2.5 and 10% (w/w) coatings of Carbowax 20M on Chromosorb G were compared, no significant GC differences could be observed. Therefore, this variable was not considered critical and a 5% Carbowax treatment was used for subsequent evaluations.

The next variable tested was the extracting solvent used to remove excess Carbowax 20M. The original extracting solvent was methanol[2]. Since Carbowax 20M is not readily soluble in methanol, dichloromethane was also used. Acid-washed Chromosorb G was coated with 10% Carbowax and heat treated as usual. The material was then divided into two portions. One portion was extracted with methanol and the other with dichloromethane. No differences in chromatographic behavior were observed for the compounds tested except for azinphos-methyl and diazinon. The latter showed a much greater relative retention time (*RRT*) with the methanol-

extracted support but peak shape was improved slightly with the dichloromethane extracted support. Azinphos-methyl appeared to decompose on the dichloromethane treated column. Therefore, a third treatment was carried out by extracting first with methanol for 3 or 4 days followed by dichloromethane for the remainder of a total period of seven days. The dual-solvent treatment gave identical retention times as for the single methanol extraction but at the same time provided the better peak shapes characteristic of the single dichloromethane extraction. Consequently, the dual-solvent extraction was utilized for the remainder of this study.

The temperature used for conditioning the Carbowax coated solid support appeared to be somewhat critical based on previous studies. Aue *et al.*[4] reported that temperatures in excess of 280° resulted in polymer deterioration but gave improved chromatographic behavior for the alcohols and hydrocarbons tested. Two different batches of Carbowax 20M coated on acid-washed Chromosorb G were subjected to 250° and 270° heat treatments. No real differences were observed for the pesticides tested, with the exception that the support from the lower temperature treatment produced an extra peak equivalent to *p,p'*-DDE following injection of DDT. This was not evident with the 270° treated support. Whether this was due to the unique difference in the Carbowax polymer resulting from the heat treatment or a difference in actual distribution of the Carbowax on the diatomaceous earth support is difficult to conclude since no load determinations were attempted.

A fourth aspect of this study challenged the necessity of the initial acid treatment of the solid support prior to Carbowax coating. Since acid-washed supports are commercially available it would be advantageous to omit this process. To evaluate adequately the need for the acid treatment, a commercially prepared batch of Gas-Chrom P (which is acid-washed by the manufacturer) coated with 15% Carbowax 20M was heat treated and solvent extracted. Another batch of Gas-Chrom P was treated similarly except that it was acid washed in this laboratory prior to treatment with Carbowax 20M. For many of the compounds tested the commercially coated packing compared favorably with our acid-washed and coated support (Table III). However, phosphimadon, paraoxon, and azinphos-methyl were not eluted, and mevinphos and *p,p'*-DDT gave broader peaks at different retention times on the commercial phase than with the other packings (Table IV). Several other compounds also gave different *RRT* values on these two supports.

It was important to compare the differences between a commonly employed commercial-prepared packing with the same packing which had been reconditioned prior to coating with the stationary phase. Therefore, a commercial packing of 10% OV-210 on Gas-Chrom Q was compared to a batch of Gas-Chrom Q reconditioned in our laboratory. The Gas-Chrom Q was extracted for four days with methanol–hydrochloric acid (1:1) followed by our usual extraction with hydrochloric acid and Carbowax 20M treatment. The reconditioned Gas-Chrom Q was coated with 5% OV-210 and compared with the commercially prepared OV-210 on Gas-Chrom Q. The commercial packing produced much broader peaks, severe tailing, and different *RRT* values (Table III) for many of the pesticides tested. In general, the laboratory-prepared Carbowax 20M treated packing was far superior. It was quite evident from this portion of the study that the acid treatment of supports greatly improved the chromatographic performance, particularly for many of the difficult to chromatograph pesticides.

TABLE III

THE EFFECT OF VARIOUS TREATMENTS ON THE CHROMATOGRAPHIC BEHAVIOR OF MODIFIED CARBOWAX 20M SUPPORTS

Peak shape is defined by numbers: 1 = sharp peak with little or no tailing; 2 = sharp but tailing; 3 = broad but symmetrical with little or no tailing; 4 = moderate tailing; 5 = severe tailing; N.P. = no peak. *RRT* = relative retention time (parathion = 1.00). Gas-Chrom P columns were operated at 175°; Gas-Chrom Q columns were operated at 200°.

Pesticide	*Commercial Gas-Chrom P coated with 15% Carbowax 20M, heat treated and solvent extracted*		*Acid-washed Gas-Chrom P coated with Carbowax 20M, heat treated and solvent extracted*		*Commercial Gas-Chrom Q coated with 10% OV-210*		*Modified Gas-Chrom Q acid washed, heat treated, solvent extracted, and coated with 5% OV-210*	
	Peak shape	*RRT*	*Peak shape*	*RRT*	*Peak shape*	*RRT*	*Peak shape*	*RRT*
Phosphamidon	N.P.	—	1	0.52, 0.78	2	0.10	4	0.12, 0.15
Mevinphos	3	0.13	1	0.14	5	0.20	1	0.17
Methamidophos	3	0.23	3	0.28	—	—	—	—
Diazinon	1	0.23	1	0.29	1	0.17	1	0.15
Lindane	1	0.31	1	0.31	1	0.17	1	0.26
Disulfoton	1	0.23	1	0.29	1	0.21, 0.72	1	0.27
Atrazine	1	0.63	1	0.65	2	0.20	1	0.30
Simazine	1	0.98	1	0.82	2	0.20	1	0.32
Benefin	1	0.10	1	0.17	—	—	—	—
Trifluralin	1	0.10	1	0.17	—	—	—	—
Aldrin	1	0.24	1	0.26	1	0.21	1	0.29
Dioxathion	3	0.52	3	0.37	2	0.23, 0.44	1	0.32
Chlorpyrifos	1	0.55	1	0.55	1	0.38	1	0.46
Monocrotophos	4	0.34, 0.43	4	1.49	—	—	—	—
Methyl Parathion	1	1.01	1	0.90	4	0.74	1	0.80
Parathion	1	1.00	1	1.00	1	1.00	1	1.00
Chlorpyrifos oxygen analogue	1	1.90	1	0.78	—	—	—	—
p,p'-DDT	3	1 01	1	2.39	—	—	—	—
Paraoxon	N.P.	—	1	1.04	5	1.69	1	1.33
TEPP	1	2.56	1	2.51	—	—	—	—
Azinphos-methyl	N.P.	—	1	13.00	4	4.55	3	7.10

TABLE IV

PEAK SHAPE AND RELATIVE RETENTION ON UNCOATED CARBOWAX 20M-MODIFIED SUPPORTS

Peak shape is defined by numbers: 1 = sharp peak with little or no tailing; 2 = sharp but tailing; 3 = broad but symmetrical with little or no tailing; 4 = moderate tailing; 5 = severe tailing; 6 = peak poorly distinguished; N.P. = no peak. *RRT* = relative retention time (Parathion = 1.00). All column temperatures were held constant at 175° with the exception of Chromosorb P which had a column temperature of 220°.

Pesticide	*Chromosorb P*		*Chromosorb G*		*Chromosorb W*		*Gas-Chrom P*		*Gas-Chrom Q*	
	Peak shape	*RRT*	*Peak shape*	*RRT*	*Peak shape*	*RRT*	*Peak shape*	*RRT*	*Peak shape*	*RRT*
Phosphamidon	4	0.13	*	0.05, 0.07	1	0.08	1	0.52, 0.78	1	0.11, 0.85
Mevinphos	1	0.21	1	0.12	1	0.16	1	0.14	1	0.14
Methamidophos	4	0.17	4	0.32	4	0.62	3	0.28	4	0.18
Diazinon	3	0.24	3	0.31	1	0.37	1	0.29	1	0.35
Lindane	1	0.27	3	0.26	1	0.24	1	0.31	1	0.31
Disulfoton	1	0.28	3	0.28	1	0.31	1	0.29	1	0.30
Atrazine	4	0.32	3	0.61	4	0.62	1	0.65	1	0.65
Simazine	4	0.33	3	0.72	4	0.70	1	0.82	1	0.86
Benefin	1	0.29	1	0.15	1	0.18	1	0.17	1	0.18
Trifluralin	1	0.29	1	0.14	1	0.18	1	0.17	1	0.18
Aldrin	1	0.32	3	0.25	1	0.26	1	0.26	1	0.28
Dioxathion	1	0.32	3	0.35	4	0.36	3	0.37	1	0.38
Chlorpyrifos	1	0.46	3	0.59	1	0.60	1	0.55	1	0.56
Monocrotophos	4	0.78	4	0.16, 1.48	5	2.22	2	1.49	2	1.46
Methyl Parathion	1	0.82	3	0.84	1	0.72	1	0.90	1	0.90
Parathion	1	1.00	3	1.00	1	1.00	1	1.00	1	1.00
Chlorpyrifos oxygen analogue	6	—	6	—	6	—	1	0.78	1	0.72, 1.84
p,p'-DDT	3	1.11	3	2.32	3	0.82, 1.99	1	2.39		2.34
Paraoxon	N.P.	—	3	1.06	4	1.35	1	1.04	1	1.06
TEPP	1	2.36	3	2.90	1	3.17	1	2.51	1	2.58
Azinphos-methyl	N.P.	—	3	12.06	3	10.17	1	13.00	1	11.75

* Peaks on a solvent front; could not distinguish peak shape.

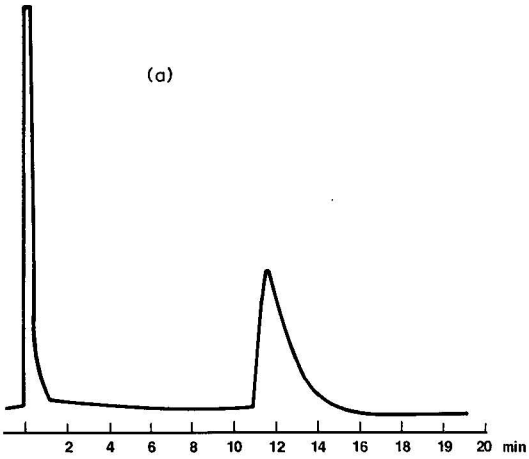
TABLE V

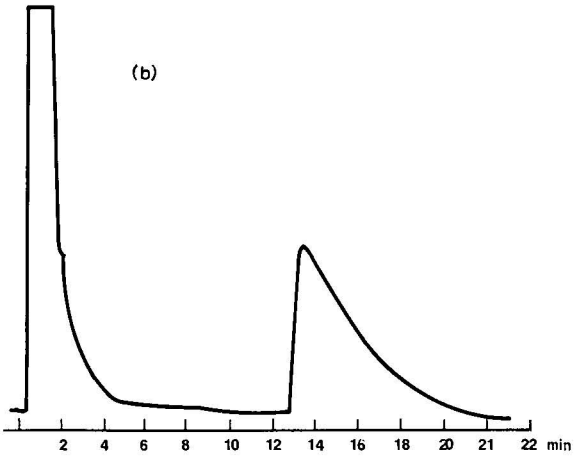
PEAK SHAPE AND RELATIVE RETENTION ON CARBOWAX 20M MODIFIED SUPPORTS COATED WITH OV-210

Peak shape is defined by numbers: 1 = sharp peak with little or no tailing; 3 = broad but symmetrical with little or no tailing; 4 = moderate tailing; 5 = severe tailing; N.P. = no peak. *RRT* = relative retention time (Parathion = 1.00). All column temperatures were held constant at 200°.

Pesticide	*10% OV-210 on Chromosorb P*		*10% OV-210 on Chromosorb G*		*5% OV-210 on Chromosorb W*		*5% OV-210 on Gas-Chrom P*		*5% OV-210 on Gas-Chrom Q*	
	Peak shape	*RRT*	*Peak shape*	*RRT*	*Peak shape*	*RRT*	*Peak shape*	*RRT*	*Peak shape*	*RRT*
Phosphamidon	3	0.10	1	0.10, 0.12	1	0.08, 0.10	1	0.12, 0.14	4	0.12, 0.15
Mevinphos	3	0.15	1	0.15	1	0.17	1	0.17	1	0.17
Methamidophos	—	—	3	0.15	5	0.17	3	0.15	4	0.15, 0.26
Diazinon	3	0.20	1	0.23	1	0.20	1	0.21	1	0.15
Lindane	3	0.23	1	0.24	1	0.23	1	0.25	1	0.26
Disulfoton	3	0.24	1	0.25	1	0.25	1	0.26	1	0.27
Atrazine	3	0.29	1	0.30	1	0.26	1	0.28	1	0.30
Simazine	3	0.33	1	0.34	1	0.28	1	0.29	1	0.32
Benefin	—	—	1	0.25	1	0.28	1	0.29	1	0.28
Trifluralin	—	—	1	0.25	1	0.29	1	0.29	1	0.28
Aldrin	3	0.27	1	0.28	1	0.28	1	0.29	1	0.29
Dioxathion	3	0.28	3	0.30	1	0.29	1	0.31	1	0.32
Chlorpyrifos	3	0.43	1	0.45	1	0.44	1	0.46	1	0.46
Monocrotophos	—	—	3	0.25, 0.84	4	0.76	3	0.71	4	0.26, 0.74
Methyl Parathion	3	0.79	1	0.80	1	0.78	1	0.80	1	0.80
Parathion	3	1.00	1	1.00	1	1.00	1	1.00	1	1.00
Chlorpyrifos oxygen analogue	—	—	3	0.86	*		1	0.23, 1.00	*	
p,p'-DDT	3	1.18	3	1.26	1	1.06	1	1.15	1	0.62, 1.17
Paraoxon	N.P.	N.P.	1	1.30	1	1.35	1	1.34	1	1.33
TEPP	—	—	3	1.47	1	1.40	1	1.41	1	1.39
Azinphos-methyl	—	—	1	7.36	1	5.74	1	6.06	3	7.10

* Very poor response with many small peaks and a large hump.





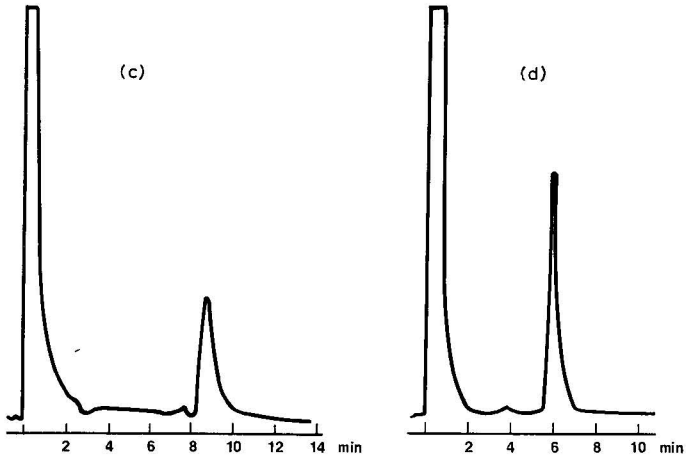


Fig. 1. Chromatogram of monocrotophos on Carbowax 20M-modified (a) Chromosorb G, (b) Chromosorb W, (c) Gas-Chrom Q, and (d) Gas-Chrom P. Column temperature, 175°.

Tables IV and V compare *RRT* values and chromatographic efficiencies for 21 pesticides on each of the five Carbowax 20M modified supports evaluated both with and without OV-210 coating. Fig. 1a–d illustrate typical chromatograms obtained for monocrotophos on four of the five non-coated Carbowax 20M modified supports.

When comparing the five reconditioned supports it was found that the most desirable chromatographic behavior was obtained with the Carbowax 20M modified Gas-Chrom P and Q. Modified Chromosorb G was also good with the exception that peaks were generally broader, indicating lower column efficiency. Chromosorb W modified with Carbowax 20M was not as good as was originally anticipated. More tailing was observed for many of the compounds tested and peaks were broad, indicating poor column efficiency. Some of the pesticides also gave additional large peaks indicating thermal breakdown of the parent compound. Coating the modified supports with OV-210 generally improved the chromatographic behavior of the pesticides. Figs. 1b and 2 depict a typical improvement using the OV-210 coated support on Chromosorb W. Monocrotophos still tailed some but a significant improvement over the non-coated Chromosorb W was noted.

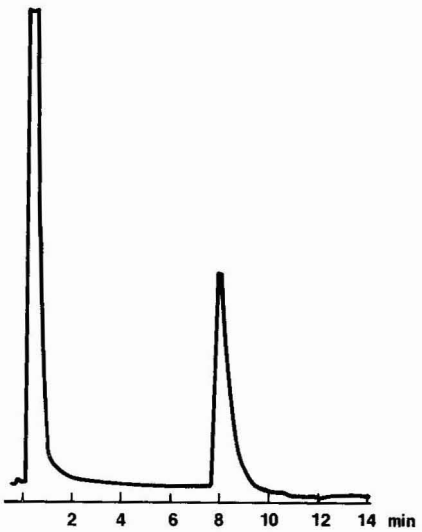


Fig. 2. Chromatogram of monocrotophos on Carbowax 20M-modified Chromosorb W coated with 5% OV-210. Column temperature, 200°.

Separation of the pesticides, as expressed by retention times relative to parathion, was generally comparable for the deactivated supports, with the exception of Chromosorb P (Table IV). However, resolution of the pesticide was not always as good as might be desired even though there was an improvement over most published reports, particularly with lindane, diazinon, atrazine and simazine. Coating the supports with OV-210 changed the *RRT* for many of the compounds as well as improved the separations not possible with the deactivated support alone (Table V).

When comparing *RRT* values and chromatographic behavior expressed by peak shape with coated OV-210 non-treated and coated OV-210 deactivated supports,

there was considerable improvement in favor of the coated deactivated supports (Fig. 3a–h). Most of the *RRT* values of the pesticides were similar between the coated deactivated supports while this was not always true for the non-treated supports. Commercially prepared packings including a 3% OV-210 on Chromosorb W (Pierce, Rockford, Ill., U.S.A.) and a 10% OV-210 on Gas-Chrom Q (Applied Science Labs.) were also compared in this portion of the study (Fig. 3c and Table III). These materials were used as received from the supplier after conditioning for 24 h at 225°. With few exceptions, the deactivated supports coated with OV-210 were superior to the non-treated supports for those chemicals tested (Fig. 3a–h).

The results of this study indicate the importance of a well deactivated solid support when attempting GC separation of certain pesticides. The use of silylating reagents has traditionally provided a relatively inert support surface, but this work indicates that for some specific applications, Carbowax 20M modified supports offer significant advantages.

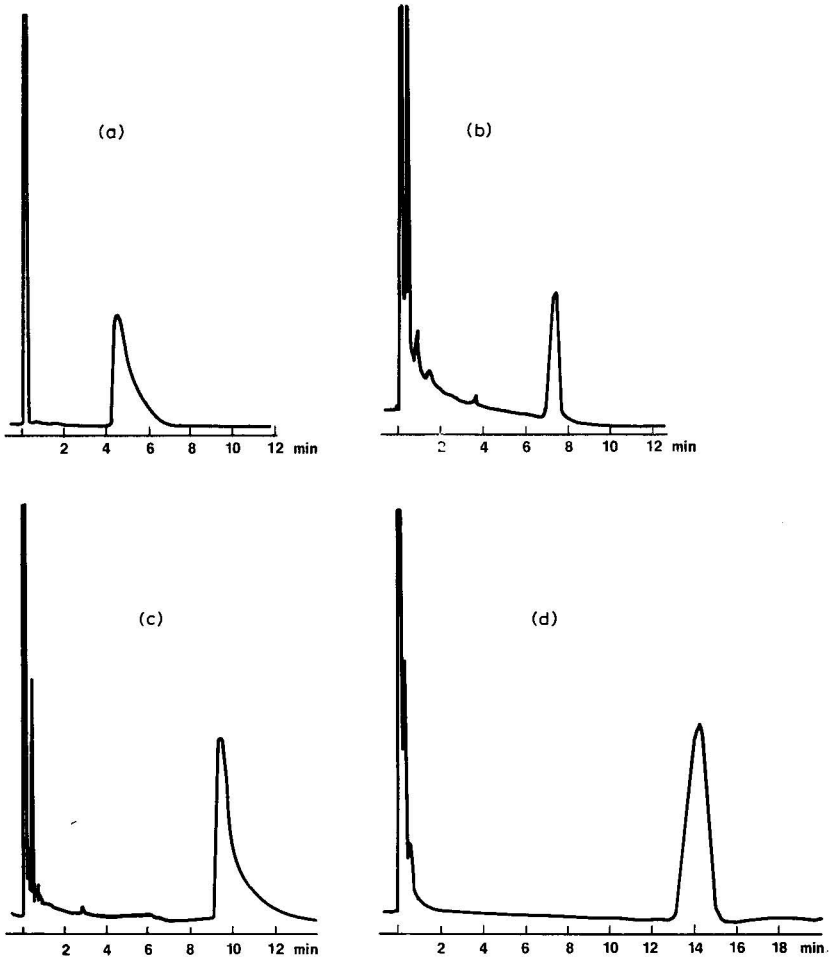


Fig. 3.

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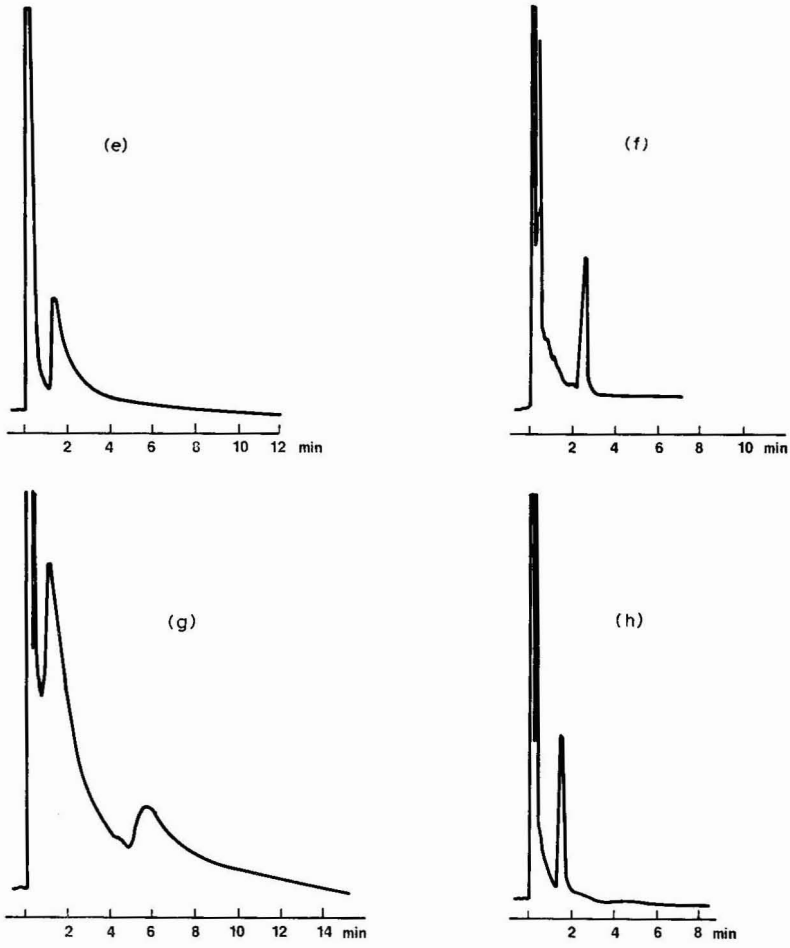

Fig. 3. Chromatogram of disulfoton (a) on non-treated Chromosorb P coated with 10% OV-210 (column temperature, 200°); (b) on Carbowax 20M-modified Chromosorb coated with 10% OV-210 (column temperature, 200°). (c) Chromatogram of mevinphos on non-treated Chromosorb G coated with 10% OV-210 (column temperature, 200°). (d) Mevinphos on Carbowax 20M-modified Chromosorb G coated with 10% OV-210 (column temperature, 200°). (e) Mevinphos on non-treated Chromosorb W HP coated with 3% OV-210 (column temperature, 175°). (f) Mevinphos on Carbowax 20M-modified Chromosorb W coated with 5% OV-210 (column temperature, 175°). (g) Mevinphos on non-treated Gas-Chrom Q coated with 5% OV-210 (column temperature, 175°). (h) Mevinphos on Carbowax 20M-modified Gas-Chrom Q, coated with 5% OV-210 (column temperature, 175°).

Where optimum separations of various compounds have been attempted in the past there have been two basic chromatographic considerations, one dealing with the stationary phase and the other with the solid support. By deactivating a support one can reduce the first factor to a minimum. The characteristics attributed to the stationary phase can then serve as the primary consideration for separating various compounds.

It should be added that since this work was completed there are now many suppliers that can provide such deactivated supports commercially. However, these

materials are very expensive and it might be advantageous for some laboratories to prepare their own modified supports following the procedure and precautions described.

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IN VITRO DEGRADATION OF DIFLUBENZURON (DIMILIN) DURING DERIVATIZATION WITH PERFLUOROANHYDRIDES*

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SUMMARY

Diflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea) and one of its metabolites, 4-chlorophenylurea were found to undergo cleavage during reaction with trifluoroacetic and heptafluorobutyric anhydrides. The products, N-monoperfluoroacyl-2,6-difluorobenzamide (from diflubenzuron) and N-monoperfluoroacyl-4-chloroaniline (from diflubenzuron and 4-chlorophenylurea) were identified as occurring during derivatization and not from thermal degradation of the 1- or 3-N-monoperfluoroacyl derivatives of the intact diflubenzuron in the gas–liquid chromatography (GLC) injection port or on the GLC column. Elucidation of an *in vitro* cleavage reaction and a proposed degradation scheme is presented based on electron capture–GLC, thin-layer chromatography, GLC–mass spectrometry and direct-inlet probe mass spectrometric analysis.

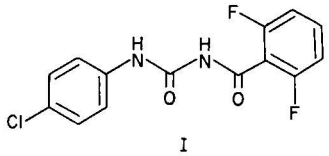
INTRODUCTION

Use of derivatization techniques in the gas–liquid chromatography (GLC) of pesticides is now widely accepted. The functional group transformations are usually straightforward reactions carried out prior to injection into the chromatograph and often produce more thermally stable and detector-sensitive derivatives of the intact parent compounds. Perfluoroacylation of insecticidal carbamates[1,2] and herbicidal ureas[2–5] have been previously reported and reviewed.

With certain carbamates and ureas, however, perfluoroanhydrides may form unforeseen derivatives of the parent compound. Khalifa and Mumma[6] reported that reaction of N-hydroxymethylcarbaryl with trifluoroacetic or heptafluorobutyric anhydrides resulted in the formation of only the corresponding perfluoroacyl derivative of 1-naphthol. VandenHeuvel *et al.*[7] investigated the reaction of heptafluorobutyric anhydride with a mono-substituted urea drug and a substituted carbamate drug. In both cases they showed that the urea and carbamate moieties were cleaved

* Contribution No. 11, Pesticide Research Laboratory.

during the reaction with concomitant conversion to the corresponding N- and O-heptafluorobutyramide derivatives.



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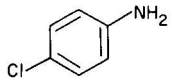

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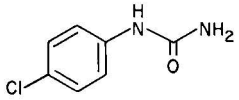


II

IV

Diflubenzuron, I (Dimilin, 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea), a new growth regulating insecticide, is thermally unstable to temperatures normally used in GLC as previously reported[8]. The data in this report was obtained during an attempt to develop a GLC method for the simultaneous determination of diflubenzuron and three of its metabolites, 4-chlorophenylurea (II), 4-chloroaniline (III) and 2,6-difluorobenzamide (IV), at residue levels. The use of trifluoroacetic or heptafluorobutyric anhydrides as derivatization reagents to produce more volatile, thermally stable and/or electron-capture sensitive derivatives resulted in the formation of unexpected reaction products for diflubenzuron and 4-chlorophenylurea. The formation of these unexpected products were confirmed to be the results of an *in vitro* cleavage reaction and not of GLC thermal degradation of N-monoperfluoroacyl derivatives of the parent ureas.

EXPERIMENTAL

Materials and reagents

All solvents were of glass-distilled residue-free grade. Trifluoro and heptafluorobutyric anhydrides were obtained from Aldrich, Milwaukee, Wisc., U.S.A. Diflubenzuron, 4-chlorophenyl urea, 4-chloroaniline and 2,6-difluorobenzamide were supplied by Thompson Hayward (Kansas City, Kan., U.S.A.). Each was recrystallized from benzene and its identify was confirmed by mass spectrometry. Standard solutions of each compound containing 100 ng/μl were prepared in ethyl acetate.

Gas–liquid chromatography

All columns [electron capture–GLC (GLC–ECD) and GLC–mass spectrometry (MS)] were of Pyrex and packed with 5% OV-101 Chromosorb W AW DMCS, 100–120 mesh. Dimensions used are reported with results.

Electron-capture detection

A Varian 2440 gas chromatograph equipped with a tritium foil detector was

used; operating conditions: injector 180°, column 150°, detector 200°; nitrogen carrier gas flow-rate was 40 ml/min.

Mass-spectrometry

Mass spectra were obtained with either a DuPont Dimaspec or Finnigan 1015 mass spectrometer using either GLC–MS or direct-inlet probe (DIP) techniques. GLC–MS and DIP–MS conditions are given in the legends to tables and figures.

Thin-layer chromatography (TLC)

TLC was carried out on 0.75 mm thick silica gel G plates (20 × 20 cm, pH 7) and developed in chloroform–ethylacetate–acetic acid (85:10:15). Visualization was obtained using a very light spray of 0.01 % fluorescene in ethanol, followed by ultraviolet detection of spots. Elution of spots was carried out using ethyl acetate.

Preparation of derivatives

Diflubenzuron, 4-chlorophenylurea, 4-chloroaniline, or 2,6-difluorobenzamide (10–100 μg/0.2 ml ethyl acetate in a 5-ml graduated glass-stoppered centrifuge tube) was treated with trifluoroacetic or heptafluorobutyric anhydride (0.2 ml) at 50° for 30 min in a water bath. The reaction mixture was then evaporated just to dryness under nitrogen and the residue taken up in an appropriate volume of ethyl acetate for analysis.

RESULTS AND DISCUSSION

In an attempt to extend the trifluoroacetic anhydride reaction previously reported for I[9] to three of its metabolites, II, III and IV, it was found that all three metabolites reacted producing single highly sensitive peaks, products IIC and IIIA having identical retention times by GLC–ECD (Fig. 1). Reaction of diflubenzuron (I) with trifluoroacetic anhydride yielded two peaks, IB having the same retention time as reaction product IVA, and IC corresponding in retention time to reaction products IIC and IIIA (Fig. 1). The use of several GLC columns of various polarities under a variety of column temperatures and flow-rates failed to resolve peaks IC, IIC, and IIIA.

As mentioned earlier the *in vitro* cleavage of certain ureas and carbamates in the presence of perfluoroanhydride derivatization reagents (trifluoroacetic and heptafluorobutyric anhydrides) has been demonstrated[6,7]. Thus it was necessary to elucidate in this instance whether the reaction products of I and II were the result of on-column degradation or *in vitro* cleavage.

The first approach was to identify all reaction products of I to IV via the reaction with trifluoroacetic anhydride using GLC–MS. The total ion monitor chromatograms (Fig. 2) revealed four peaks from the reaction of I with trifluoroacetic anhydride having parent ions at *m/e* 153 (IA), 253 (IB), 223 (IC), and 157 (ID). II yielded three products with parent ions at *m/e* 153 (IIA), 127 (IIB), and 223 (IIC); III, one product with *m/e* 223 (IIIA); and IV, two products, with *m/e* 253 (IVA) and 157 (IVB). Table I lists fragmentation ions and their relative abundances for each of their products. The total ion monitor chromatograms indicated that ID and IVB

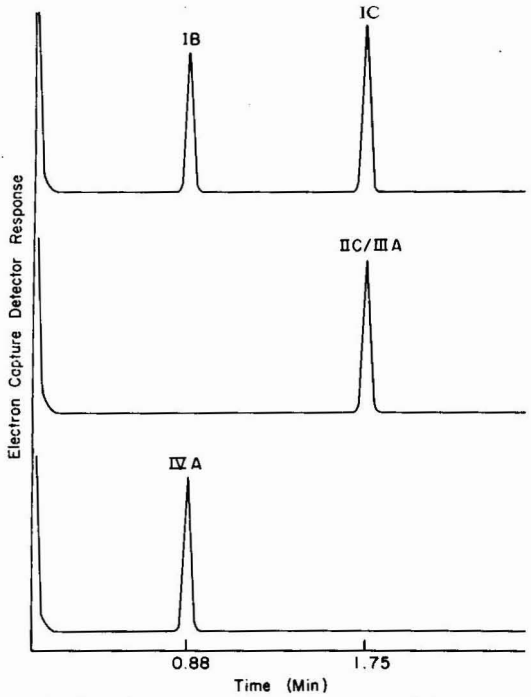


Fig. 1. Gas chromatograms of products of the reaction of I, II, III and IV with trifluoroacetic anhydride using electron capture detection. (Reaction of II or III yields the same product and therefore the same chromatogram). Column conditions: 1.7 m × 2 mm I.D. glass column; 5% OV-101 on Chromosorb W AW DMCS, 100–120 mesh; column temperature = 150°.

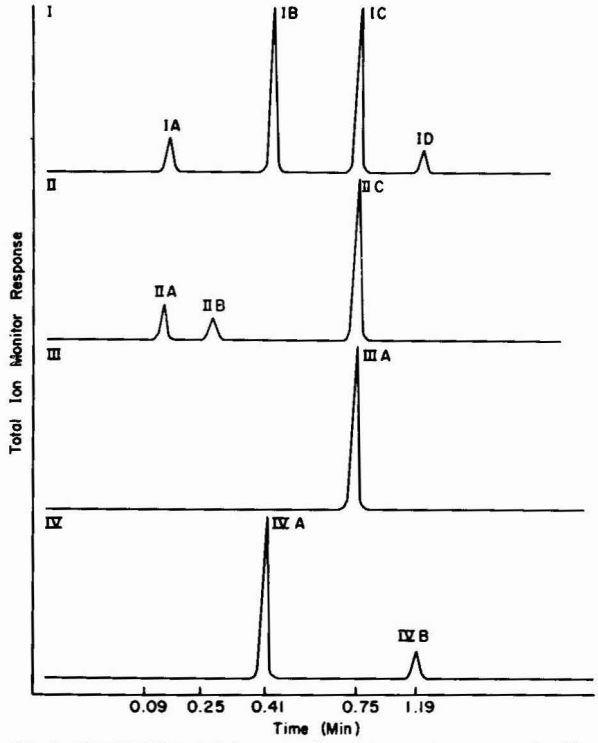


Fig. 2. GLC–MS total ion monitor chromatograms of trifluoroacetic anhydride reaction products from I, II, III and IV. GLC–MS conditions: 5% OV-101, 1.6 m × 2 mm I.D. glass column; ionization potential = 70 eV; temp. prog. = 50–150° at 8°/min.

TABLE I

THE MASS SPECTRA OF TRIFLUOROACETYL DERIVATIVES OF DIFLUBENZURON AND METABOLITES FROM FIG. 2

Ions of less than 3% intensity or less than *m/e* 100 are not included. P = parent ion.

TIM peak (m/e)	*Compounds*									
	I				*II*			*III*	*IV*	
	A	*B*	*C*	*D*	*A*	*B*	*C*	*A*	*A*	*B*
253		23.1P							34.2P	
225			33.0				33.3	33.6		
223			100.0P				100.0P	100.0P		
157				40.0P						60.5P
156			13.1				9.6	10.7		
155	33.3				40.5					
154			39.8				30.6	32.8		
153	100.0P				100.0P					
141		100.0		100.0					100.0	100.0
139		3.1							4.14	
129						45.8				
128			33.5				24.7	27.1		
127	17.8				18.9	100.0P				
126			47.7				34.6	37.3		
125	53.3				51.4					
113		22.3	5.7	50.0			3.4	3.5	32.9	31.6
111			14.2				10.2	10.7		

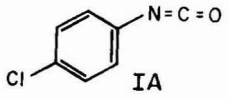
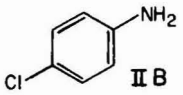
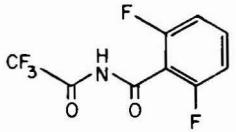
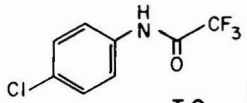
(*m/e* 157), IA and IIA (*m/e* 153), and IIB (*m/e* 127) are minor components. Structural assignments for all parent ions are shown in Table II.

The fact that degradation of I and II had occurred during trifluoroacetylation was shown by TLC of the reaction products of compounds I–IV. Products from I and II yielded spots IC and IIC with the same R_F (0.90) as product IIIA (Fig. 3). Elution and GLC–MS of these spots showed single chromatographic peaks for each with parent ions in each case at *m/e* 223. DIP–MS analysis at ambient temperature of the same material showed only *m/e* 223. It was evident that product IVA, the imide derivative, detected by GLC–MS with parent ion at *m/e* 253, had decomposed on the TLC plate since no IV A could be detected from the TLC separation of derivatized IV; the only compound detected had a parent ion at *m/e* 157 (IVB). In addition, the area from I (ID) with the same R_F (0.43) as spot IVB showed a parent ion at *m/e* 157. The fact that no IVA or IB could be isolated by TLC is to be expected since asymmetrical imides are very sensitive to hydrolysis in much the same way as asymmetrical anhydrides, and the TLC plate was not activated. The GLC–MS data for all TLC spots are listed in Table III.

Finally, as shown in Table IV, DIP–MS of the entire trifluoroacetic anhydride reaction product from I showed very minor peaks at *m/e* 406 (N-1- or N-3-trifluoroacetyl derivative of I) and *m/e* 502 N,N-1,3-ditrifluoroacetyl derivative of I). Intense parent ions at *m/e* 253, 223, and a strong peak at *m/e* 183 corresponding to 2,6-difluorobenzoylisocyanate were also evident. The product with parent *m/e* 183 was not detected by GLC–MS of the reaction products of I and therefore may arise in an ion-

TABLE II

PRODUCTS FROM THE TRIFLUOROACETYLATION REACTION OF COMPOUNDS I, II, III AND IV DETECTED BY GLC–MS, AND/OR DIP–MS

Parent m/e	*I*	*II*	*III*	*IV*
153,155	 IA	IIA	–	–
127,129	—	 IIB	–	–
253	 IB	–	–	IVA
223,225	 IC	IIC	IIIA	–
157	 ID	–	–	IVB
183*	 IE	–	–	–

* Product IE seen only on DIP–MS of reaction product of I.

molecule reaction in the mass spectrometer, or as a product of the *in vitro* degradation reaction which does not elute from the GLC or elutes with a very long retention time. Ambient DIP–MS (7 eV) analysis showed very strong *m/e* 253 and 223 confirming these ions as parent ions of the *in vitro* reaction products. The fact that the intensity of the *m/e* 406 peak was much less than *m/e* 253 or 223 was taken to indicate that it was not the parent ion for peaks at *m/e* 253 and 223. Previous mass spectra of substituted phenylureas have shown strong parent ions[11,12]. In addition, the fact that the *m/e* 406 peak was not present at 5 eV indicates that it was unlikely to be a parent ion. Fragmentation of either the mono- or the di-trifluoroacetyl derivatives (*m/e* 502) could be expected to yield daughter ions with even mass numbers, *i.e.*, 252 and 222 respectively, unless a hydrogen atom rearrangement occurred. (There are none available to be rearranged in the di-derivative and it is unlikely that an N–H would be

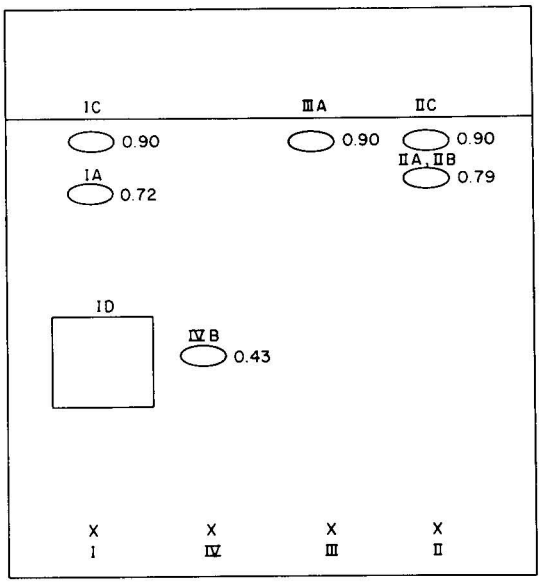


Fig. 3. TLC of diflubenzuron (I) and its metabolites II, III, IV after reaction with trifluoroacetic anhydride.

TABLE III

THE MASS SPECTRA OF TLC ELUATES FROM FIG. 3

Ions of less than 3% intensity or less than *m/e* 100 are not reported. Ionization potential = 70 eV, GLC–MS, 5% OV-101, column temperature, 150°.

TIM peak (m/e)	*Eluate*							
	IVB	*IA*	*ID*	*IC*	*IIIA*	*IIC*	*IIA*	*IIB*
253								
225				33.3	33.3	33.3		
223				100.0P	100.0P	100.0P		
157	60.5P		41.8P					
156				4.2	4.2	3.7		
155		27.3					35.0	
154				12.5	12.5	11.0		
153		100.0P					100.0P	
141	100.0		100.0					
129								42.9
128				11.7	11.7	7.4		
127		9.1					15	100.0P
126				15.0	15.0	11.0		
125		36.4					35	
113	31.6		20.9					
111								

rearranged easily.) Thus it was concluded that *m/e* 253 and 223 were parent ions corresponding to products of an *in vitro* cleavage during the trifluoroacetylation step.

With the trifluoroacetic anhydride reagent, the imide peak (*m/e* 253), IB, increased in intensity (GLC–ECD) with time and temperature of the reaction. How-

TABLE IV

THE DIRECT-INLET PROBE MASS SPECTRA OF THE TRIFLUOROACETYL DERIVATIVES OF DIFLUBENZURON (I)

Ion intensity reported as millimeters peak height. Mass spectrometer source temperature, 150°. IP = ionization potential.

m/e	*DuPont, IP = 70 eV*	*Finnigan, IP = 70 eV*	*Finnigan, IP = 7 eV*	*Finnigan, IP = 5 eV*
504	—	2	4	2
502	—	5	12	5
408	—	4.5	—	—
406	0.5	13	3	—
253	200	2350	950	150
225	440	1900	3100	400
223	1290	5800	9000	1200
183	240	3100	5100	500

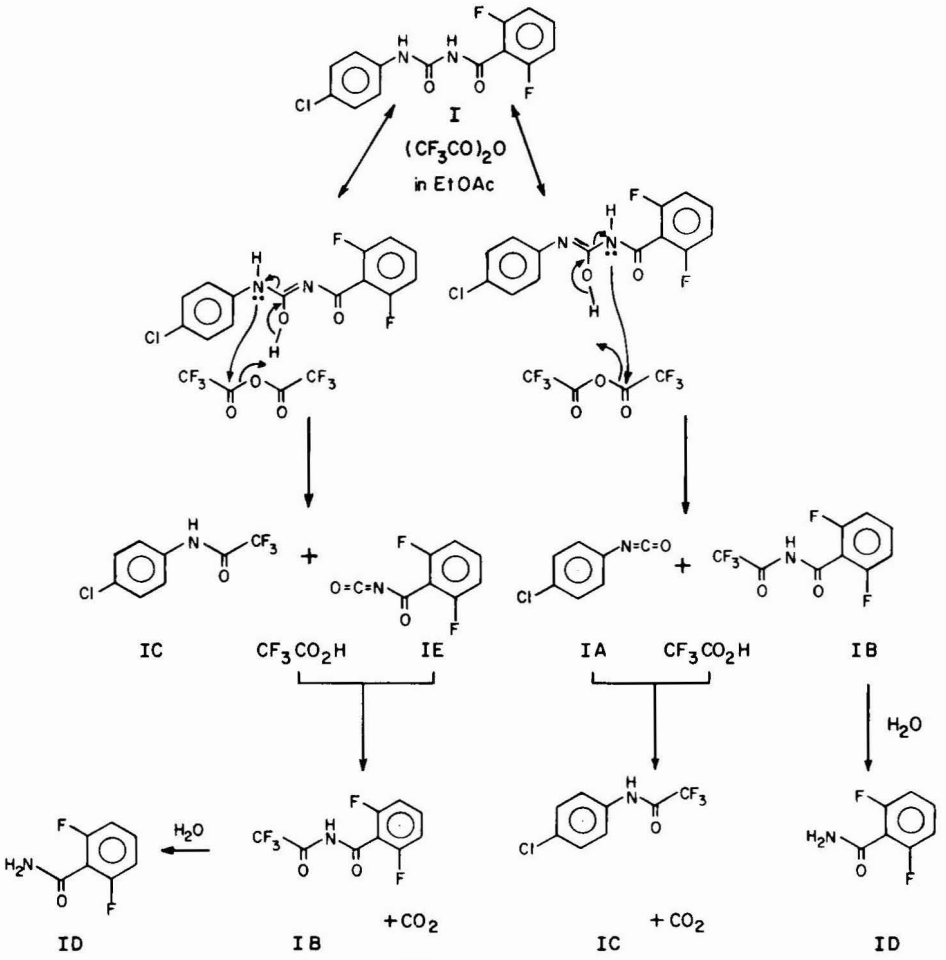


Fig. 4. Suggested reaction pathways yielding products observed from trifluoroacetylation of diflubenzeron (I).

ever, it decreased with prolonged evaporation to dryness, and on standing in ethyl acetate. No effect was observed whether 20 or 200 μl of the derivatization reagents were used.

The importance of a careful investigation into the chemistry occurring during a derivatization step and subsequent GLC is evident. Indeed, a better understanding of these phenomena may lead to a more simplified and/or rational approach to analysis. For example, knowledge that the urea moiety of I and II is cleaved during perfluoroacylation with concomitant conversion of the cleavage products to perfluoroamides eliminates the necessity for a separate hydrolysis step. By this method I, and its metabolites, II and III can be simultaneously analyzed for as the mono-N-perfluoramide derivative of III. The method published by us for analysis of diflubenzuron in pond water as its N-trifluoroacetyl derivative[9], in fact proceeds via this pathway. Hydrolysis of herbicidal phenyl ureas to a substituted aniline product or carbamates to the corresponding phenol followed by derivatization is commonly used as an approach to residue analysis by GLC[3].

Thus, when dealing with derivatization techniques, one must be alert to the possibility of the unexpected, during both the GLC and the derivatization steps.

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PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THYROTROPIN-RELEASING HORMONE ANALOGUES

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SUMMARY

A high-performance liquid chromatographic system for the preparative separation of peptide mixtures using strongly acidic ion-exchange resins and volatile elution media is described. About 5% of the effluent is subjected to continuous basic partial hydrolysis in order to increase the sensitivity and then detected with ninhydrin. Several examples have demonstrated that this method is suitable for the isolation of peptides with high purity from multi-component mixtures.

INTRODUCTION

Pyroglutamic acid (Pyr) peptides have received increasing attention recently because of their natural occurrence as biologically active compounds. Eledoisin (Pyr–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2)[1], physalaemin (Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2)[2], thyrotropin-releasing hormone (Pyr–His–Pro–NH_2)[3] and follicle-stimulating hormone-releasing hormone (Pyr–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2)[4,5] are some examples of naturally occurring pyroglutamyl peptides.

Several syntheses of the tripeptide hormone Pyr–His–Pro–NH_2 (TRH) have been reported using glutamyl (Glu) or glutaminyl (Gln) peptides as intermediates. The first synthesis of TRH was achieved by the treatment of Glu(OCH_3)–His–Pro–OCH_3 with ammonia[3]. In the TRH syntheses of Rivaille and Milhaud[6], Folkers *et al.*[7] and Inouye *et al.*[8], Glu–His–Pro–NH_2 was treated with acid to give the desired tripeptide TRH. Several TRH syntheses were based on the discovery of König and Geiger[9] that N-4,4′-dimethoxybenzhydryl(Mbh)-L-glutamine derivatives form pyroglutamic acid in boiling trifluoroacetic acid. For example, Z–Gln(Mbh)–His–Pro–NH_2 (Z = benzyloxycarbonyl) can be converted into TRH in good yields in boiling trifluoroacetic acid[9].

In the natural occurring pyroglutamyl peptides, the Pyr residue is always attached to the N-terminal side of the chain. For investigations of the structure–activity relationship, Pyr^2–TRH or Pyr^3–TRH analogues are of general interest[10,11]. Therefore several tripeptides with Glu, Gln and isoglutaminyl (Isogln) residues at the C-terminal end or at position 2 were synthesized and treated with trifluoroacetic acid

(TFA) or hydrogen fluoride in order to achieve the formation of the pyroglutamic acid residue. In most instances, however, the crude products could not be purified by conventional methods used in peptide chemistry such as recrystallization, ion-exchange chromatography on CM-cellulose or partition chromatography on Sephadex. To solve this problem, we have developed a preparative high-performance liquid chromatographic separation system.

EXPERIMENTAL

Reagents for peptide synthesis

For the synthesis of the peptides, all solvents were purified according to ref. 12. Dimethylformamide (DMF) and tetrahydrofuran (THF) were products of Merck (Darmstadt, G.F.R.). All amino acids except pyroglutamic acid, which was a product of Aldrich-Europe (Beerse, Belgium), were purchased from Merck. For the syntheses of amino acid derivatives and peptides, the following reagents were used: dicyclohexylamine (Merck), dicyclohexylcarbodiimide (DCC, Merck), 2,4,5-trichlorophenol (Merck), isobutyl chloroformate (Merck), 1-hydroxybenzotriazole (Aldrich-Europe) and N-hydroxysuccinimide (Fluka, Buchs, Switzerland). *tert.*-Butyloxycarbonyl(Boc) azide and benzyloxycarbonyl chloride were synthesized according to refs. 13 and 14. For thin-layer chromatography, Kieselgel 60 F_{254} plates (Merck) and the solvent systems *n*-butanol–acetic acid–water (3:1:1) and chloroform–methanol–benzene–water (60:40:40:5) were used.

Syntheses of the peptide materials

TRH (Pyr–His–Pro–NH_2). TRH was synthesized as described in ref. 15.

D-*His^2*–*TRH (*L-*Pyr*–D-*His*–L-*Pro–NH_2).* The synthesis of D-*His^2*–TRH was carried out according to ref. 16.

Phe^2–TRH (Pyr–Phe–Pro–NH_2). The synthetic tripeptide[11] had the following characteristics: m.p., 182–185°; $[\alpha]_D^{23}$, −43.06° (c = 1.46/MeOH).

Reaction products after treatment of Boc–Phe–Glu(OBzl)–Pro–NH_2 with trifluoroacetic acid and hydrogen fluoride[11]. The starting material Boc–Phe–Glu(OBzl)–Pro–NH_2 had the following characteristics: m.p., 57–61°; $[\alpha]_D^{23}$, −15.3° (c = 1.04/DMF). Elemental analysis: calculated, C 64.12, H 6.94, N 9.65%; found, C 63.00, H 7.12, N 8.66%.

A 400-mg amount of Boc–Phe–Glu(OBzl)–Pro–NH_2 was dissolved in 45 ml of TFA, the mixture was refluxed for 2.5 h, evaporated to dryness and the residue subjected to chromatography. In a second study, 580 mg of Boc–Phe–Glu(OBzl)–Pro–NH_2 were dissolved in 45 ml of liquid hydrogen fluoride and the mixture was stirred for 2 h at room temperature. After evaporation, the product was dried at 10^{-2} torr over solid potassium hydroxide and separated by chromatography. To obtain the reference peptide H–Phe–Glu(OH)–Pro–NH_2, Boc–Phe–Glu(OBzl)–Pro–NH_2 was treated with TFA at room temperature for 90 min. After evaporation to dryness, the resulting product, H–Phe–Glu(OBzl)–Pro–NH_2, was saponified with a 2 *M* excess of potassium hydroxide for 90 min. The subsequent neutralization was effected with 1 *N* hydrochloric acid. After evaporation to dryness, the product was separated by chromatography.

Reaction products after treatment of Boc–Pro–Phe–Isogln(OBzl) with trifluoroacetic acid[11]. The starting material Boc–Pro–Phe–Isogln(OBzl) had the following characteristics: m.p., 158–162°; $[\alpha]_D^{23}$, −57.17° (c = 1.2/DMF). Elemental analysis: calculated, C 64.12, H 6.94, N 9.65%; found, C 63.27, H 7.12, N 9.90%.

A 250-mg amount of Boc–Pro–Phe–Isogln(OBzl) was refluxed in 35 ml of TFA for 2.5 h. After evaporation to dryness, the product was subjected to chromatographic separation.

Mass spectrometry

All mass spectra were obtained with a Varian-MAT F11 instrument operating at 70 eV.

Materials for chromatographic separation

Pyridine was distilled over solid potassium hydroxide and then at least three times over ninhydrin in order to remove all impurities that react with the reagent. For the separations, the cation-exchange resin DC-1A (Durrum, Palo Alto, Calif., U.S.A.), which is an 8% crosslinked polystyrene of particle size 18 ± 3 μm, was used. Pyridine–acetic acid buffers were prepared from pyridine and deionized, doubly distilled water. The pH was adjusted with distilled acetic acid. For partial hydrolysis after chromatographic separation, 5 *N* sodium hydroxide solution was used. The ninhydrin solution was prepared from a mixture of the reagent prepared by Spackman *et al.*[17] and distilled acetic acid (1:1). The peptide mixture (up to 250 mg) was dissolved in 2–5 ml of the starting buffer.

Amino acid analysis

The fractions from the fraction collector were evaporated to dryness, hydrolysed with 6 *N* hydrochloric acid at 110° for 12 h and used for amino acid analysis.

RESULTS AND DISCUSSION

Fig. 1 shows a schematic diagram of the preparative peptide analyser, developed in collaboration with Biotronik, Frankfurt, G.F.R.

A Milton Roy Dosapro micro-pump, which is connected with the buffer reservoirs, magnetic valves and an air-bubble trap, pumps the pyridine–acetic acid buffers (1.5 ml/min) via a 2 × 3-way valve on a Biotronik glass-jacketed high-performance glass column (550 × 9 mm) filled with DC-1A cation-exchange resin. To the effluent from the column, distilled water is added (1:2.5) in order to reduce the loss of separated peptides. From this diluted effluent a small amount (0.2 ml/min) is used for detection. After dilution with 5 *N* sodium hydroxide solution (1:2), partial hydrolysis in a PTFE reaction coil (20 m × 0.7 mm) at 100° is carried out and the mixture is subjected to reaction with ninhydrin reagent. The colour is finally developed in a second PTFE reaction coil (30 m × 0.7 mm) at 100° and detected and recorded with a Biotronik photometer at 570 nm. About 95% of the effluent is brought to a fraction collector.

Figs. 2 and 3 show chromatograms of synthetic TRH and TRH derivatives with an N-terminal pyroglutamic acid residue. The synthetic steps used for the samples subjected to chromatographic separation are also given in Figs. 2 and 3. The

chromatograms demonstrate the high purity of TRH and the derivatives and thus the suitability of the strategy chosen for their synthesis. The chromatogram of D-His^2–TRH shows clearly that the compound is contaminated with about 8% of the natural hormone (L-Pyr–L-His–L-Pro–NH_2).

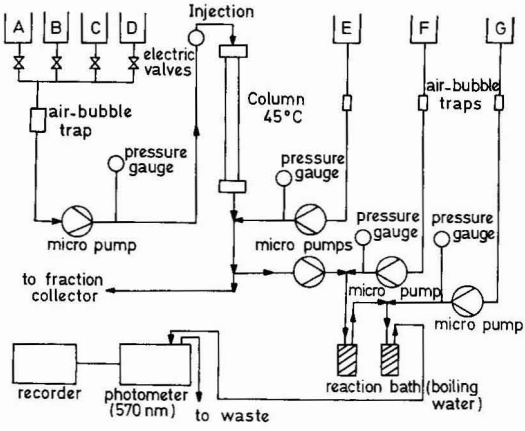

Fig. 1. Schematic diagram of the peptide analyser. A, B, D, D = Buffer reservoirs; E = water reservoir; C = 5 *N* NaOH reservoir; D = ninhydrin reagent–acetic acid (1:1) reservoir.

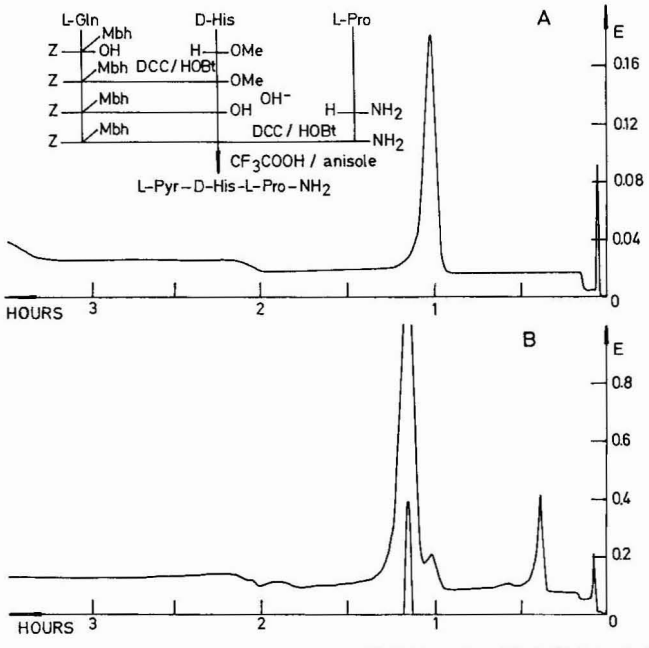

Fig. 2. Preparative chromatograms of TRH and D-His^2–TRH. Column: 550 × 9 mm, DC-1A resin (18 ± 3 μm). Elution buffers: A, 0.1 *M* pyridine acetate, pH 3.2 (15 min); B, 0.2 *M* pyridine acetate, pH 3.5 (45 min); C, 0.3 *M* pyridine acetate, pH 4.0 (60 min); D, 0.6 *M* pyridine acetate, pH 4.2 (60 min); E, 1.0 *M* pyridine acetate, pH 4.6 (80 min). Flow-rate, 1.5 ml/min; back-pressure, 70–90 bar; temperature, 43°; detection with ninhydrin (570 nm) after partial hydrolysis (5 *N* NaOH). Samples: A, 8.7 mg; B, 250 mg. Sensitivity: A, 0.2 a.u.; B, 1.0 a.u.

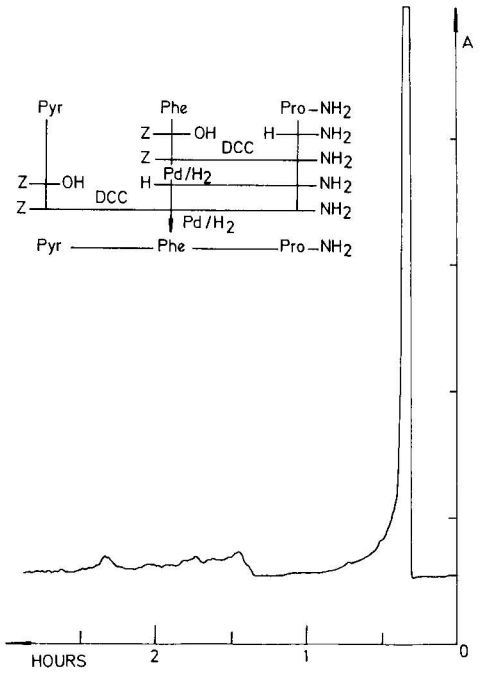

Fig. 3. Chromatogram of synthetic Phe²–TRH. Column: 550 × 9 mm, DC-1A resin. Pyridine–acetic acid buffers: A, 0.2 *M* pyridine, pH 3.4 (60 min); B, 0.5 *M* pyridine, pH 4.25 (120 min); C, 1.0 *M* pyridine, pH 5.0 (120 min); D, 4.0 *M* pyridine, pH 5.6 (180 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 = 4.4% loss of sample for detection; recorder range, 0–0.5 A; amount injected, 150 mg.

According to several workers[10], D-His²–TRH should have considerable biological activity. However, we were able to demonstrate that the chromatographically pure D-His²–TRH is completely inactive. Hence this example clearly shows the suitability of our chromatographic system for separating peptide diastereomers.

Contrary to the synthesis of Pyr¹-tripeptides, that of Pyr²-tripeptides seems to be much more difficult, as is demonstrated in Figs. 4–6.

Figs. 4 and 5 demonstrate the wide variety of compounds that are formed when Glu²-tripeptides react with boiling TFA (Fig. 4) or hydrogen fluoride (Fig. 5). Therefore, it is not surprising that the reaction of TFA or hydrogen fluoride with Boc–Phe–Glu(OBzl)–Pro–NH_2 yields reaction products that could not be purified by crystallization[11]. Under similar conditions, Glu¹-tripeptides form Pyr¹-tripeptides in large amounts. The formation of the pyroglutamic acid ring seems to be sterically hindered in Glu²-tripeptides and fission and rearrangement products seem to be formed instead (Fig. 7).

According to amino acid analysis, many peaks correspond to fractions with the expected amino acid content Phe–Glu–Pro = 1:1:1 (Figs. 4 and 5, solid line). However, most of the fractions contain peptides with protected amino functions and therefore give rise to no peaks in the chromatogram performed without partial hydrolysis (Fig. 4).

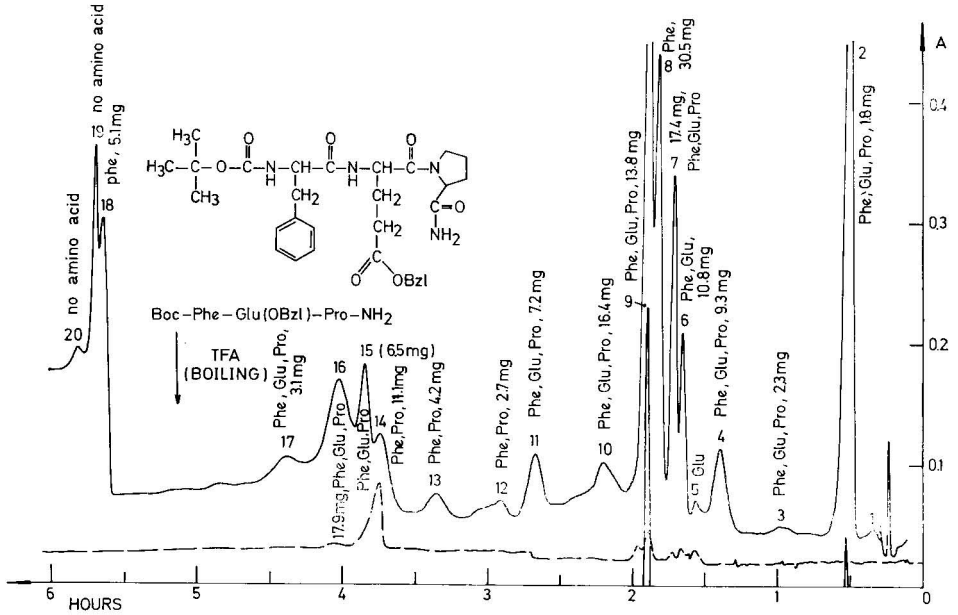

Fig. 4. Chromatogram of the reaction mixture of Boc–Phe–Glu(OBzl)–Pro–NH_2 after treatment with boiling TFA (2.5 h). Column: 550 × 9 mm, DC-1A resin. Pyridine–acetic acid buffers: A, 0.2 *M* pyridine, pH 3.4 (60 min); B, 0.5 *M* pyridine, pH 4.25 (120 min); C, 1.0 *M* pyridine, pH 5.0 (120 min); D, 4.0 *M* pyridine, pH 5.6 (180 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 = 4.4% loss of sample for detection; recorder range, 0–0.5 A. Solid line: amount injected, 166 mg; separated material collected, 155 mg; detection after partial hydrolysis. Broken line: amount injected, 109 mg; detection without partial hydrolysis.

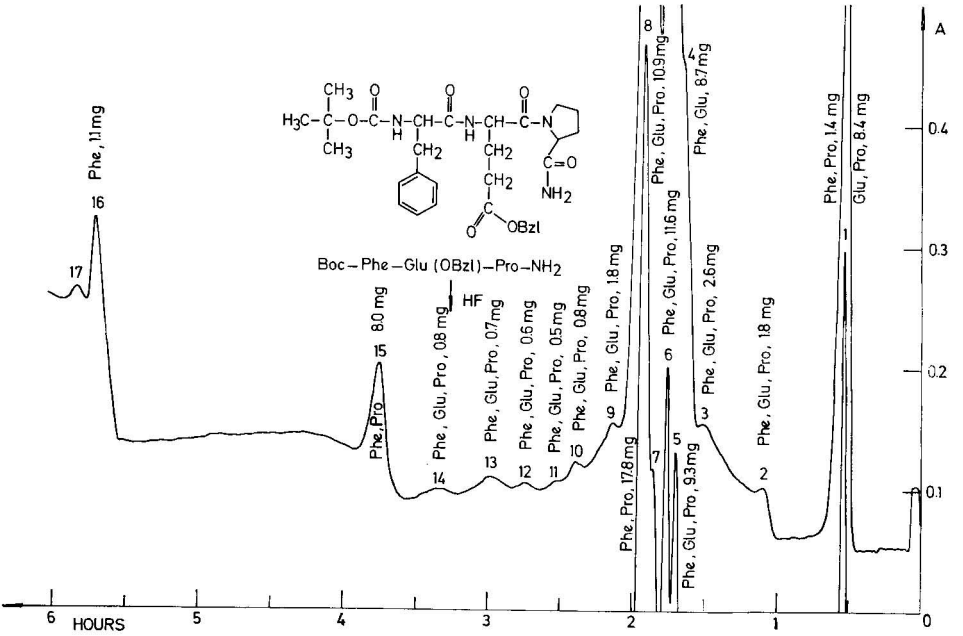

Fig. 5. Chromatogram of the reaction mixture of Boc–Phe–Glu(OBzl)–Pro–NH_2 after treatment with hydrogen fluoride. Column: 550 × 9 mm, DC-1A resin. Pyridine–acetic acid buffers: A, 0.2 *M* pyridine, pH 3.4 (60 min); B, 0.5 *M* pyridine, pH 4.25 (120 min); C, 1.0 *M* pyridine, pH 5.0 (120 min); D, 4.0 *M* pyridine, pH 5.6 (180 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 = 4.4% loss of sample for detection; recorder range, 0–0.5 A; amount injected, 139.5 mg; separated material collected, 86.1 mg.

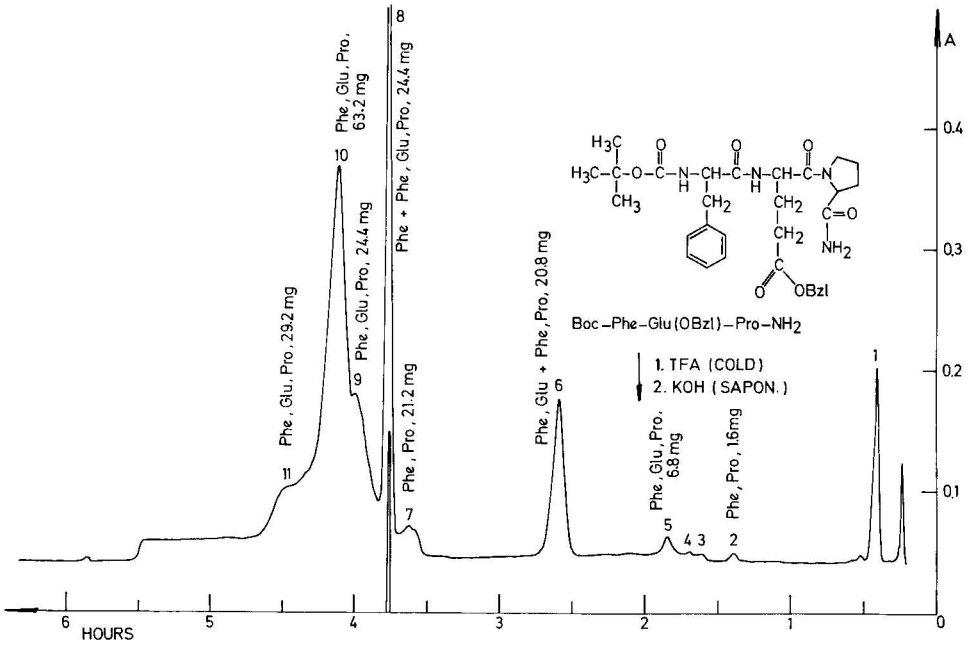

Fig. 6. Chromatogram of the reaction mixture of Boc–Phe–Glu(OBzl)–Pro–NH_2 after treatment (1) with TFA in the cold and (2) with 1 *N* KOH solution. Column: 550 × 9 mm, DC-1A resin. Pyridine–acetic acid buffers: A, 0.2 *M* pyridine, pH 3.4 (60 min); B, 0.5 *M* pyridine, pH 4.25 (120 min); C, 1.0 *M* pyridine, pH 5.0 (120 min); D, 4.0 *M* pyridine, pH 5.6 (180 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 – 4.4% loss of sample for detection; recorder range, 0–0.5 A; amount injected, 207 mg; separated material collected, 192 mg.

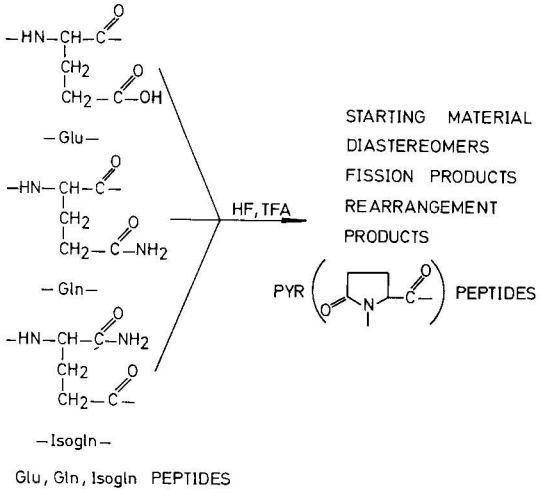

Fig. 7. Possible reaction products of Glu, Gln and Isogln peptides.

Common reactions in peptide chemistry are cleavage of the *tert.*-butyloxycarbonyl group by dichloromethane–TFA or saponification of esters with 1 *N* potassium hydroxide solution. Boc–Phe–Glu(OBzl)–Pro–NH_2 was subjected to both reactions and the resulting product separated by ion-exchange chromatography (Fig. 6). Although the chromatogram in Fig. 6 shows a much smaller number of peaks than those in Figs. 4 and 5, it demonstrates clearly that commonly used peptide reagents may also lead to cleavage of the peptide chain.

The products of several fractions corresponding to peaks in the chromatograms in Figs. 4 and 5 were investigated by mass spectrometry. These studies proved unequivocally that peak 7 in Fig. 4 is caused by the pyroglutamylpeptide Phe–Pyr–Pro–NH_2 (Fig. 8).

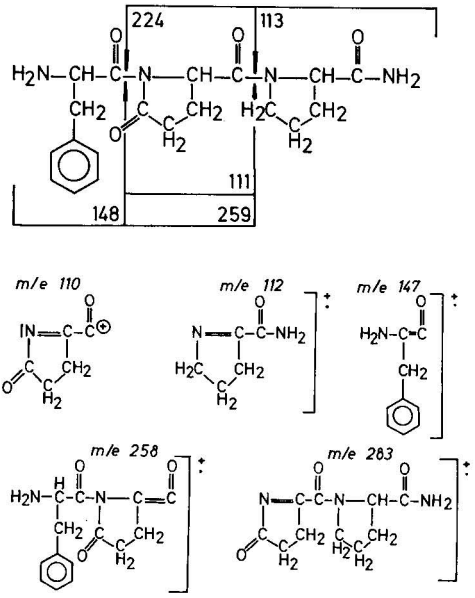

Fig. 8. Principal ions formed upon electron impact of the peptide fraction corresponding to peak 7 in Fig. 4.

The mass spectrum of the peptide fraction corresponding to peak 7 in Fig. 4 shows ions at *m/e* 110, 111, 112, 113, 147, 148, 224, 258, 259 and 283. The peaks at *m/e* 111, 224 and 259 are characteristic of Pyr, Pyr–Pro–NH_2 and Phe–Pyr moieties.

These investigations clearly demonstrate that Glu[2]-tripeptides form partly Pyr[2]-tripeptides in boiling TFA, which can be separated by the chromatographic technique described here.

Contrary to glutamyl[2]-tripeptides, C-terminal glutamyl[3]-tripeptides form more readily the pyroglutamyl residue in boiling TFA, as is demonstrated in Fig. 9.

In boiling TFA, Boc–Pro–Phe–Isogln(OBzl) forms a product that shows one main peak (peak 6) in the chromatogram in Fig. 9. Mass spectrometric studies led to the unequivocal conclusion that this peak is caused by the pyroglutamyl peptide Pro–Phe–Pyr–NH_2 (see Fig. 10, the principal ions upon electron impact of the peptide fraction corresponding to peak 6 in Fig. 9).

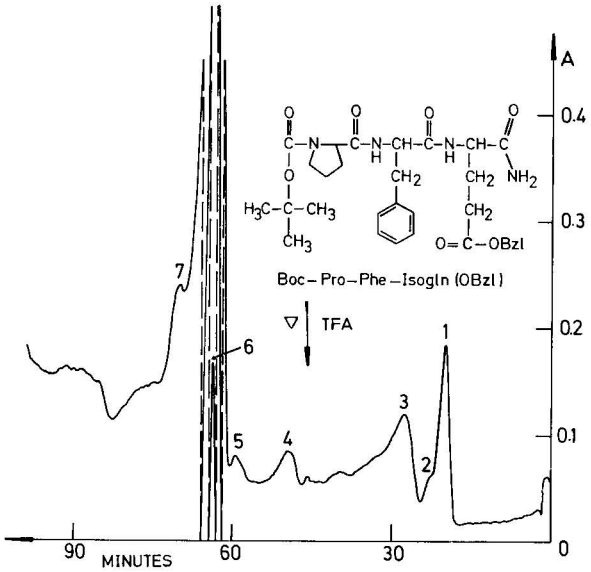

Fig. 9. Chromatogram of the reaction mixture of Boc–Pro–Phe–Isogln(OBzl) after treatment with boiling TFA (3 h). Column: 550 × 9 mm, DC-1A resin. Pyridine acetic acid buffers: A, 0.2 *M* pyridine, pH 3.2 (30 min); B, 0.5 *M* pyridine, pH 4.6 (30 min); C, 1.0 *M* pyridine, pH 5.9 (60 min); D, 4.0 *M* pyridine, pH 5.2 (60 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 ≈ 4.4% loss of sample for detection; recorder range, 0–0.5 A; amount injected, 200 mg.

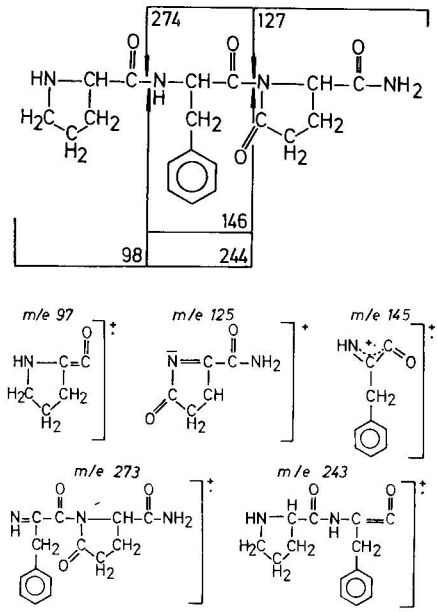

Fig. 10. Principal ions formed upon electron impact of the peptide fraction corresponding to peak 6 in Fig. 9.

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DERIVATIZATION PROCEDURE FOR THE DETERMINATION OF CHLOROACETYL CHLORIDE IN AIR BY ELECTRON CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic method is described for the determination of chloroacetyl chloride (CAC) in air in the presence of monochloroacetic acid (MCA). Air containing CAC was bubbled through a reactant solution of 2,4,6-trichlorophenol (TCP) and pyridine in toluene to produce the derivative 2,4,6-trichlorophenyl chloroacetate (TCPC). Excess TCP was removed by extractions with 1 *N* sodium hydroxide, and TCPC was determined via electron capture gas chromatography. An average recovery of 76 ± 4% (mean ± S.D.) was obtained when CAC was added directly to the reactant solution. When air samples containing CAC were scrubbed and analyzed, recoveries averaged 70 ± 5% (mean ± S.D.). The quantitative detection limit (2.5 × noise) was 1 ng CAC/ml in the extracted organic layer [1 ppb** in air for a 5-l sample]. The general applicability of this method for volatile acid halides was also explored.

INTRODUCTION

Chloroacetyl chloride (CAC) is a member of a very reactive class of compounds used in industry as chemical intermediates. Because of their highly reactive nature, acid halides may present a significant personnel hazard[1], although no threshold limit value (TLV) for CAC has been proposed by the American Conference of Governmental Industrial Hygienists. In order to determine CAC in industrial environments and to monitor test chamber concentrations during inhalation toxicology studies, it was necessary to develop an analytical method more sensitive and specific than any of those presently available.

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** Throughout this article the American billion (10^9) is meant.

Indirect methods involving reactions and subsequent colorimetric determination[2] or dechlorination of acid chlorides and determination as chloride ion in solution[3,4] have been described. Typically these methods lack specificity and the desired sensitivity. However, Rusch *et al.*[5] claim a detection limit for dimethylcarbamoyl chloride in air in the low ppb range by reaction with 4-(*p*-nitrobenzyl)pyridine to form the highly colored dihydropyridine derivative and Crummett and McLean[6] obtained similar sensitivity for phosgene by ultraviolet spectrometry after derivatization with aniline.

Methods for measuring acid halides directly by gas chromatography have been described[7,8]; however, these typically involve difficult and/or exotic chromatographic techniques. Espisito *et al.*[9] have recently circumvented most of these problems in determining phosgene to sub-ppb levels in air.

Greatly enhanced chromatographic properties and increased response can be imparted to acid halides by derivatization prior to analysis. Acid chlorides have been analyzed as their methyl esters[10] and their N,N-diethyl amides[11]. Dahlberg and Kihlman[12] converted acid chlorides to their isopropyl esters before analysis via electron capture gas chromatography. They claim detection limits of 10^{-11} mole/μl (1 μg/ml) for CAC and 10^{-12} mole/μl for dichloroacetyl chloride and trichloroacetyl chloride.

Although the highly specific and sensitive method described herein has been tailored specifically for the determination of CAC, several other acid chlorides were derivatized under identical reaction conditions and the derivatives identified to emphasize the general utility of this procedure.

EXPERIMENTAL

Materials

Calcium hydride and sodium hydroxide and the organic solvents acetone, hexane, methanol, pyridine and toluene were all of analytical reagent grade. The toluene was stored over calcium hydride and pyridine over sodium hydroxide pellets to reduce and maintain the water content at a consistent and acceptable level. Dimethyldichlorosilane (DMCS), used for deactivating glassware, was obtained from Pierce (Rockford, Ill., U.S.A.).

Chloroacetyl chloride (97% pure) and 2,2,3-trichloropropionyl chloride were supplied by Dow Chem. (Midland, Mich., U.S.A.). Acetyl chloride, dichloroacetyl chloride and 2,4,6-trichlorophenol (TCP) (98% pure) were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Pentafluorophenol was obtained from Peninsular Chem. Research (Gainesville, Fl., U.S.A.). 1,4-Dibromonaphthalene (DBN), the internal standard, was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) and was used without further purification.

The 2,4,6-trichlorophenyl chloroacetate (TCPC) was prepared by scaling up the derivatization procedure described below. The TCPC was purified by recrystallization twice from hexane. The other reaction products, 2,4,6-trichlorophenyl 2,2,3-trichloropropionate (TCPT), 2,4,6-trichlorophenyl acetate (TCPA), 2,4,6-trichlorophenyl dichloroacetate (TCPD) and pentafluorophenyl chloroacetate (PFPC), were prepared as was TCPC above, but were not recrystallized.

Apparatus

A Varian Series 2400 gas chromatograph equipped with a ^{63}Ni electron capture detector was used. A 1.7 m × 2 mm I.D. glass column was packed with a bonded polyester liquid phase on Chromosorb W-AW (100–120 mesh) and conditioned prior to use for 24 h at 200° with an argon–methane (95:5) gas flow-rate of 30 ml/min. A Hewlett-Packard 5750 gas chromatograph equipped with a flame ionization detector (FID) was used for purity confirmation of synthesized compounds. A 1 m × 2 mm I.D. glass column was packed with 3% SP-2100 on Supelcoport (80–100 mesh) and conditioned prior to use for 24 h at 230° with a helium gas flow-rate of 30 ml/min.

A Finnigan Model 3200 mass spectrometer equipped with a chemical ionization source and a Model 6110 data package was used for structure confirmation. A Perkin-Elmer R-32 nuclear magnetic resonance (NMR) spectrometer was also employed for structure elucidations and purity estimations.

A Model G portable pump (Mine Safety Appliance Co.) was used in the air sampling system. The air flow-rates were determined with a Brooks rotameter, Model 1355-00ClFAA.

Air sampling

Air was passed through two 30-ml impingers in series at a rate of 0.5 l/min for 10.0 min. Each impinger contained 20.0 ml of a dry toluene reactant solution (0.14 g TCP, 0.1 ml pyridine and 4.0 μg DBN per 20 ml solution). No volume corrections were necessary after sampling. All glassware used in the air sampling apparatus was deactivated with 30% DMCS in toluene followed by washes with methanol and acetone.

Gas chromatography (GC)

Samples were extracted with 2 × 20 ml of aqueous 1 *N* sodium hydroxide solution. Aqueous extracts were discarded. 2 μl of the extracted toluene solution were injected onto the analytical chromatographic column under the following conditions: argon–methane (95:5) carrier gas at 30 ml/min; column temperature, 150°; injection port temperature, 175°; ^{63}Ni electron capture detector at 220°; electrometer at 8×10^{-10}. The retention times for TCP, DBN, TCPC and TCPD were 1.3 min, 2.1 min, 2.6 min and 2.8 min, respectively (Fig. 1).

The purity of the synthesized ester was estimated by area percent calculations of the flame ionization responses for solutions of *ca.* 10 mg/ml ester in acetone under the following conditions: 1 μl injection onto the SP-2100 column; helium carrier gas at 30 ml/min; column temperature, 200°; injection port temperature, 200°; flame ionization detector at 250°; electrometer, $8 \cdot 10^3$.

Recoveries

Recoveries were determined in two different experiments. First, synthetic samples were prepared by spiking 20.0 ml of reactant solution with 0.2–2.0 μl of solutions containing 5.64 mg or 11.28 mg (4.0 or 8.0 μl) CAC per 20.0 ml dry toluene (Table I). After 10 min the samples were extracted as described above. Secondly, the air sampling apparatus was used to simulate actual sampling conditions by injecting 0.2–2.0 μl of the above dilute CAC solutions into the inlet arm of the leading impinger.

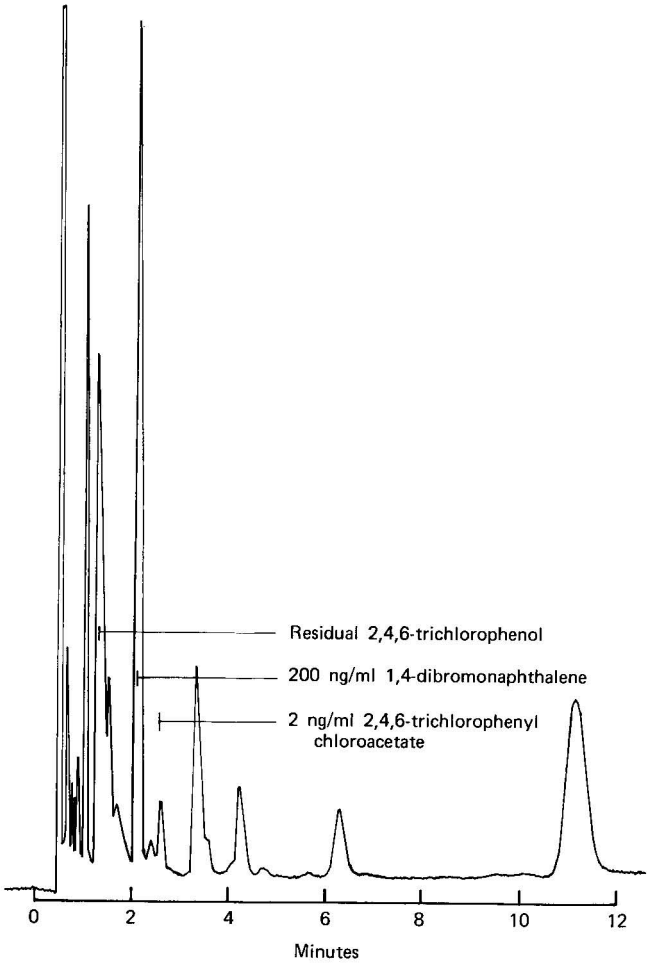

Fig. 1. Typical chromatogram. Instrument: Varian 2400 ECD (^{63}Ni); column: 1.7 m × 2 mm I.D. glass packed with a bonded polyester packing on Chromosorb W-AW; carrier gas: argon–methane (95:5) at 30 ml/min; temperatures: column 150°, injector 175°, detector 220°; range: 10^{-10}; attenuation: 8; injection volume: 2.0 μl.

Air was passed through the system at 0.5 l/min for 10 min after the injection. The samples were then extracted as described above (Table II).

Spectrometry

Identification of the reaction products was performed via the direct probe on the Finnigan Model 3200 chemical ionization mass spectrometer (CI–MS). The probe temperature was increased gradually from 70° to 400°. The methane reactant gas was adjusted to yield an ion source pressure of 450 μm.

Structures and approximate purities of the reaction products were determined by nuclear magnetic resonance (NMR) using the following conditions: 90 MHz proton NMR; reference standard, tetramethylsilane; temperature, 37°; sweep time,

TABLE I

RECOVERY DATA OBTAINED BY SPIKING CAC DIRECTLY INTO REACTANT SOLUTION

Sample no.	*Equivalent concentration of TCPC added as CAC (ng/ml)*	*TCPC found (ng/ml)*	*Recovery (%)* *
1	68.5	51.1	75
2	68.5	52.9	77
3	34.3	26.6	78
4	102.8	73.4	71
5	68.5	52.4	76
6	48.0	33.2	69
7	89.1	61.8	69
8	68.5	55 0	80
9	68.5	50.9	74
10	68.5	52.4	76
11	68.5	51.4	75
12	20.6	17.3	84
13	116.5	80.6	69
14	27.4	22.3	81
15	54.8	43.8	80
16	68.5	56.0	82
17	68.5	53.6	78
18	82.2	62.9	76

* Average recovery = 76; $\delta = 4$; $2\delta = 9$.

TABLE II

RECOVERY DATA OBTAINED BY SPIKING CAC INTO AIR SAMPLING STREAM

Sample no.	*TCPC added as CAC (μg)*	*TCPC found in impinger 1 (μg)*	*TCPC found in impinger 2 (μg)*	*Carry-over (%)* **	*Total TCPC found (μg)*	*Recovery (%)* ***
1	2.06	1.40	0.05	3	1.45	70
2	1.37	0.90	0.08	8	0.98	72
3	1.37	0.79	0.08	10	0.87	64
4	0.96	0.70	0.04	5	0.74	77
5	0.41	0.34	ND*	0	0.34	82
6	1.10	0.65	0.06	9	0.71	65
7	0.55	0.35	0.04	10	0.39	70
8	1.37	0.88	0.07	7	0.95	69
9	1.78	1.15	0.02	2	1.17	66
10	1.78	1.17	0.10	8	1.27	71
11	2.06	1.39	0.04	3	1.43	70
12	1.37	0.85	0.04	5	0.89	65

* ND = Not detected with a detection limit of 0.02 μg TCPC in 20 ml solution.
** Average carry-over = 6%.
*** Average recovery = 70; $\delta = 5$; $2\delta = 10$.

300 sec; filter 2; H_1 level 9; sensitivity 4; solvents, deuterated acetone for TCPC and deuterated chloroform for the others; concentration, 10–50% by weight; 4-mm sample tubes.

RESULTS

The response for TCPC was shown to be linear from 2 ng/ml to at least 20(ng/ml in toluene. The response for the internal standard, DBN, was shown to b(linear from 20 ng/ml to at least 460 ng/ml. The recovery results obtained by spikin(CAC directly into the reactant solution (Table I) averaged 76 ± 4% (mean ±S.D.) When the CAC was added directly to the air stream ahead of the first impinger (Tabl(II) the average recovery dropped to 70 ± 5% (mean ±S.D.). This decrease in re- covery may be due to the reaction of CAC with atmospheric moisture.

To test the effect of moisture build-up in the reactant solution during sampling six recoveries were obtained using toluene saturated with water as the solvent fo(the reactant solution. The average yield for these six samples dropped by 5% relative to yields obtained under the same conditions but with dry toluene.

The average carry-over of reaction product into the second impinger was 6% (Table II). This carry-over could possibly be diminished by replacing the impinger(with the generally more efficient fritted-glass sparger-tubes.

The TCPC prepared in bulk and used as the authentic standard for this work was characterized by NMR, CI–MS and FID–GC. It is a white solid melting at 69.0–69.5° and yields a single peak by FID–GC. The proton NMR spectrum was consistent with the proposed structure showing a two-proton singlet at 4.85 δ corresponding to the chloroacetyl protons and another two-proton singlet at 7.78 δ corresponding to the aromatic protons. No evidence of the aromatic protons of TCP was observed at 7.38 δ. The M + 1 ion (*m/e* 273) was the base peak in the chemical ionization mass spectrum of TCPC. The isotope ratios for the M + 1 ion were indicative of four chlorine atoms. No M + 29 or M + 41 reactant ions were observed. The major fragmentation ions were *m/e* 196 and *m/e* 197, each with three chlorine atoms.

The structures of TCPT, TCPA, TCPD and PFPC were likewise confirmed by NMR and CI–MS, but no recovery data were generated for these compounds.

DISCUSSION

Acid halides react with halogenated phenols in the presence of pyridine to give stable ester derivatives which exhibit excellent gas chromatographic properties. A halogenated phenol was chosen as the derivatization reagent because: (1) the resulting ester yields a very strong response for electron capture detection, and (2) the excess reagent can be quantitatively removed by extraction. The retention times for the species of interest may be varied by choosing a different halogenated phenol (*e.g.*, using a fluorinated phenol for the less volatile aromatic acid halides, etc.).

The TCPC recovery was not increased by allowing the reaction to continue after the 10-min sampling period; however, the recovery was proportional to the excess of TCP in the reactant solution with 53% recovery for a 0.10 g/20 ml concentration and 76% recovery for a concentration of 0.14 g/20 ml. The limiting factor for increasing the recovery this way is the amount of TCP which may be removed by extraction prior to analysis by electron capture GC.

Changing the concentration of pyridine in the reactant solution had no effect on recoveries. However, if a catalytic amount of pyridine was not added, recoveries decreased drastically and were inconsistent. Pyridine is thought to catalyze this re-

action by forming an acylpyridinium chloride salt with CAC which then reacts with the phenol. Pyridine also acts as an acid scavenger in the system. TCPC was shown to be stable for at least 2 days when the reactant solution was left in contact with the 1 *N* sodium hydroxide solution; however, the presence of an excess of HCl will cause rapid hydrolysis of the ester.

It is suspected that monochloroacetic acid (MCA) will be present in finite amounts in air samples containing CAC due to water hydrolysis of the acid chloride. MCA was added to the reactant solution at concentrations as high as 50 μg/ml with no TCPC being detected. The detection limit for TCPC was 2 ng/ml or 0.001 mole % of the MCA added. However, the corresponding chloroacetic anhydride did react with a high yield apparently forming one mole of ester per mole of anhydride added. In fact, anhydrides have been applied previously as derivatizing agents for the determination of phenols[13].

The detection limit (2.5× noise) for CAC is 1 ng/ml reaction solution (2.4 ng TCPC/ml). This corresponds to 1 ppb CAC in air when using 20 ml of reactant solution and 5 l of air. The absolute detection limit can conceivably be lowered to the sub-ppb range, if needed, by increasing the air volume collected.

The bonded polyester gas chromatographic column packing used in this procedure was prepared at The Dow Chemical Co. Publication of the preparation and applications of this and other bonded chromatographic packings is planned for the near future[14]. This packing permits significantly more efficient separations as compared to conventional coated packings and exhibits shorter retention times for most compounds which allows the use of much lower column temperatures. Conventional packed columns can be substituted in this procedure but will result in longer analysis times and less sensitivity.

CONCLUSIONS

The proposed method is a sensitive and specific procedure for the determination of chloroacetyl chloride in air. Recoveries in two different experiments were 76 ± 4% (mean ±S.D.) and 70 ± 5% (mean ±S.D.). A quantitative detection limit (2.5× noise) of 1 ppb can be achieved with a 5-l air sample. This technology may be feasible for the determination of other acid halides in air.

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GAS–LIQUID CHROMATOGRAPHIC PROPERTIES OF CATECHOLAMINES, PHENYLETHYLAMINES AND INDOLALKYLAMINES AS THEIR PROPIONYL DERIVATIVES

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SUMMARY

The gas chromatographic properties of the biogenic amines, catecholamines, phenylethylamines and indolalkylamines as their propionyl derivatives were studied. These derivatives are readily formed in an aqueous medium. Propionylated amines are more stable than their parent compounds and increasingly lipophilic, so that they can be extracted quantitatively into an organic solvent.

The propionyl derivatives of the biogenic amines show good gas chromatographic properties. They can be well separated on OV-101 and OV-17 silicones. Care must be taken of certain interactions of the compounds during the chromatographic procedures. Pre-treatment of the column with thionyl chloride inhibits decomposition of β-O-propionylated catecholamines and prevents their interference with other amines.

Propionylation is a useful means for the isolation and determination of a wide range of biogenic amines from biological materials by gas chromatography.

INTRODUCTION

In biological fluids, the concentration of biogenic amines is normally very low and very sensitive methods must therefore be used for their qualitative and quantitative analysis. In this respect, gas–liquid chromatography has been widely used for the separation and determination of catecholamines, phenylethylamines and indolalkylamines of various origin. For the gas chromatographic analysis of polar compounds such as these biogenic amines, their derivatization is essential. Most investigators have used trimethylsilyl[1–3], trifluoracetic[4,5] or pentafluorpropionyl[5–7] derivatives of the amines. These derivatives are volatile and have good chromato-

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graphic properties. However, they are stable only in the absence of water[5]. For their analysis in biological fluids, the amines have to be transferred into an organic solvent before derivatization. During this procedure, especially at an alkaline pH, polar amines such as noradrenaline and dopamine may decompose, sometimes resulting in a loss of up to 50%[8–10].

In a preceding paper[11], we considered acylated amine derivatives (propionyl-, *n*-butyryl-, isobutyryl- and pivaloylamines), which can be prepared in aqueous solution and which are stable. They also are increasingly lipophilic so that they can be extracted quantitatively into ethyl acetate or other organic solvents. Of these acyl derivatives, propionylated amines appear to be the most advantageous. In this investigation we have studied the gas chromatographic properties of 23 propionylated catecholamines, phenylethylamines and indolalkylamines. Special emphasis was directed towards the stability of the derivatives in gas chromatography and towards any possible interactions or interferences between the various compounds during analytical procedures.

MATERIALS AND METHODS

Substances used

The biogenic amines used were as follows: N,N-dimethyltyramine (hordenin), N,N-dimethyl-5-methoxytryptamine and N-methyl-5-hydroxytryptamine, obtained from Aldrich, Beerse, Belgium; D,L-metanephrine hydrochloride and D,L-normetanephrine hydrochloride, obtained from Calbiochem, Frankfurt, G.F.R.; N-methylphenylethylamine, L-noradrenaline hydrochloride, phenylpropylamine, bufotenin hydrogenoxalate, N-methyltryptamine and 5-hydroxytryptamine hydrogenoxalate, obtained from Fluka, Neu-Ulm, G.F.R.; L-adrenaline, 3,4-dimethoxyphenylethylamine and tryptamine hydrochloride, obtained from Koch-Light, Colnbrook, Great Britain; 3,4-dihydroxyphenylethylamine hydrochloride (dopamine), tyramine hydrochloride, phenylethylamine, 3-methoxy-4-hydroxyphenylethylamine and 5-methyltryptamine obtained from Serva, Heidelberg, G.F.R.; *p*-methoxyphenylethylamine and 5-methoxytryptamine, obtained from Sigma, Munich, G.F.R.; and N,N-dimethyltryptamine and N-methyltyramine, kindly donated by Dr. Rimek, Institute of Pharmacy, University of Bonn, Bonn, G.F.R.

All reference compounds were obtained in the highest purity available from commercial sources and used without further purification.

Abbreviations

The abbreviations used for the amines in the figures are listed in Table I.

Reagents

Propionic anhydride (puriss.) and thionyl chloride (puriss.) were obtained from Fluka.

Preparation of propionylated amines

A 5-μmole amount of the amines is dissolved in 1.0 ml of 0.1 *N* hydrochloric acid and the solution is saturated with solid sodium carbonate. Between intervals of constant shaking for 5 min, 0.05 ml of propionic anhydride are added three times. The propionylated amines are then extracted three times with 1.0 ml of ethyl acetate.

TABLE I

ABBREVIATIONS FOR AMINES

Amine	*Abbreviation*	*Amine*	*Abbreviation*
Phenylethylamine	PE	Tryptamine	TR
N-Methylphenylethylamine	MPE	N-Methyltryptamine	MTR
N,N-Dimethyltyramine	DMTY	Dopamine	DA
Phenylpropylamine	PP	5-Methoxytryptamine	MOTR
p-Methoxyphenylethylamine	MOPE	Normetanephrine	NMN
N,N-Dimethyltryptamine	DMT	Metanephrine	MN
5-Methoxydimethyltryptamine	MODMT	Noradrenaline	NA
3,4-Dimethoxyphenylethylamine	DMPEA	Adrenaline	ADR
Tyramine	TY	5-Methyltryptamine	5MTR
N-Methyltyramine	MTY	Serotonin (5-hydroxytryptamine)	HT
3-Methoxy-4-hydroxyphenylethylamine	MOHPE	N-Methylserotonin	MHT
Bufotenin	BUFO		

The combined organic extracts are evaporated to dryness at 25–30° under a stream of dry air, the residue is dissolved in 0.2 ml of pyridine–propionic anhydride (3:1, v/v) and the solution is heated for 15 min at 100° in PTFE-capped vials. After cooling to room temperature, excess of pyridine and propionic anhydride is evaporated. The propionylated amine derivatives are then dissolved in 1.0 ml of acetonitrile to give solutions ready for gas chromatographic analysis.

RESULTS AND DISCUSSION

Comparing different acyl derivatives of biogenic amines, Kauert *et al.*[11] found that propionic anhydride readily reacts with primary and secondary amino groups and with hydroxyl groups. In preliminary experiments, we found that quantitative derivatization takes place even in an aqueous medium if the amines have no or only one phenolic hydroxyl group (in addition to the amino groups). For quantitative propionylation of all functional groups of biogenic amines such as catecholamines, phenylethylamines and indolalkylamines, it is neccessary to carry out the derivatization reaction under anhydrous conditions in the presence of pyridine and propionic anhydride. The reaction of propionylation of tyramine and bufotenin is shown in Fig. 1.

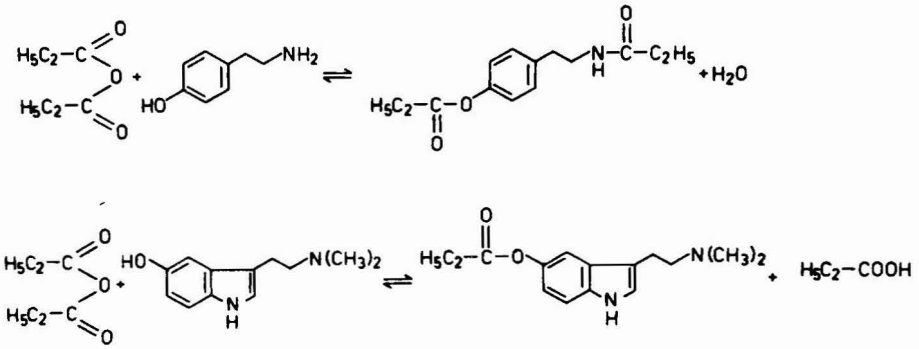

Fig. 1. Acylation of tyramine and bufotenin with propionic anhydride.

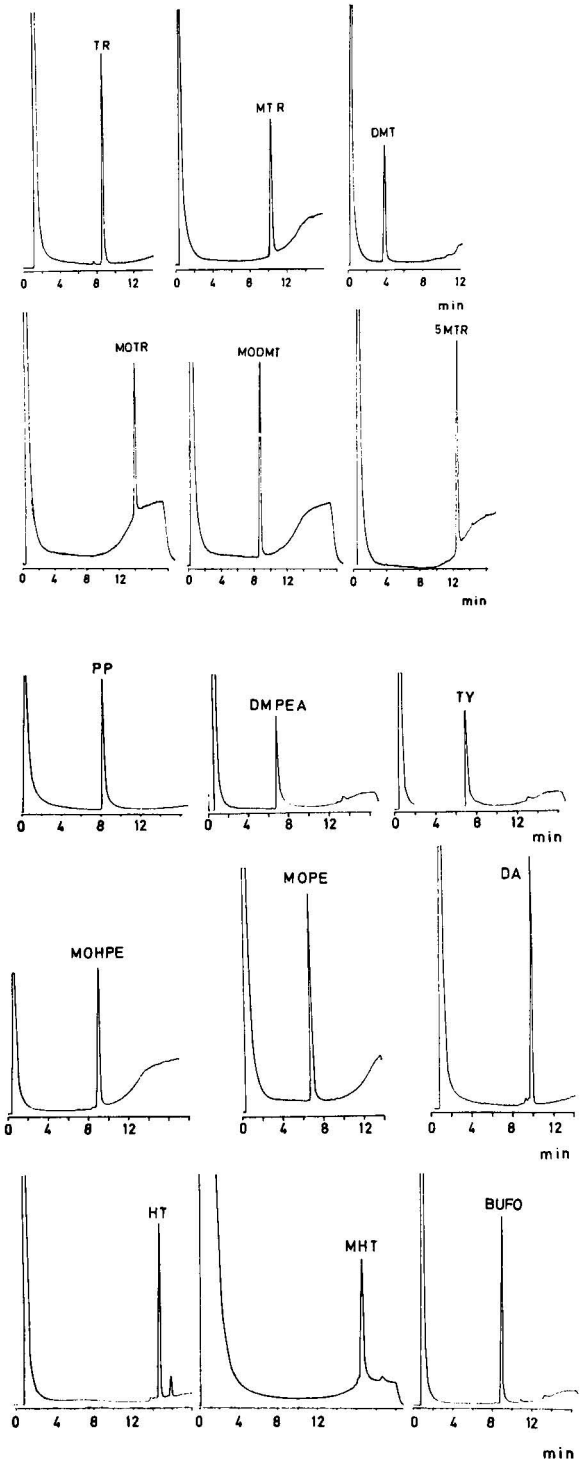

Fig. 2. Gas chromatographic assay of phenylethylamines, catecholamines and indolalkylamines as propionyl derivatives.

If quantitative propionylation of the various biogenic amines occurs, only a single peak for each should appear in the gas chromatogram. In Fig. 2, gas chromatograms of 15 propionylated amines are shown, clearly indicating that each substance is eluted in a single peak. The only exception is 5-hydroxytryptamine (which will be discussed later).

Gas chromatographic analysis of amines containing β-hydroxyl groups

We found that biogenic amines with a β-hydroxyl group (adrenaline, noradrenaline, metanephrine, normetanephrine) were not eluted in a single peak when chromatographed as propionyl derivatives. As shown in Fig. 3 (left), noradrenaline appeared as three peaks. In other gas chromatographic separations we found that the number of peaks may vary under different conditions. This indicates that the derivatives are destroyed during the gas chromatographic procedure. In order to prevent destruction, we tried to transform the β-hydroxycatecholamines into more stable derivatives. Kauert[12] was successful in forming oxazoline derivatives by the use of thionyl chloride. Accidentally we found that treating the gas chromatographic column with small amounts of thionyl chloride resulted in deactivation of the column. When the propionylated catecholamines are then injected and chromatographed, they appear as single peaks and show no signs of destruction (see Fig. 3, right).

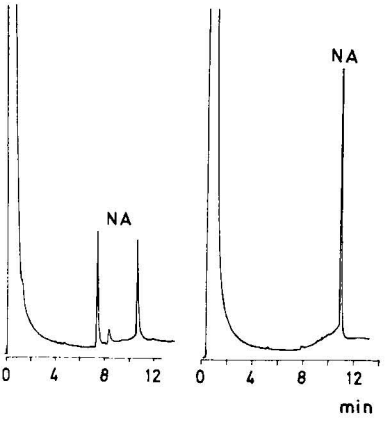

Fig. 3. Gas chromatographic assay of noradrenaline on OV-101 without (left) and with (right) pretreatment of the column with thionyl chloride.

Deactivation of the gas chromatographic column was also obtained with other chemicals such as silylating reagents, propionic acid anhydride and N-methyl-bistrifluoracetamide, but deactivation was not as complete as with thionyl chloride.

We were interested in establishing the cause of reactions that occur during the gas chromatographic separation of the amines within the column. The following can be stated: (1) only those biogenic amines which have a β-O-propionyl group are not eluted as a single peak; (2) all reagents that were able to prevent destruction of these amines react with hydroxyl groups; and (3) glass, the support material and silicones (used as the stationary phase) contain hydroxyl (silanol) groups. We concluded that a propionyl group is transfered from the β-hydroxyl position of the catecholamine to a silanol group (Fig. 4).

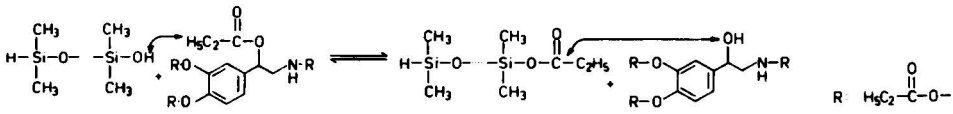

Fig. 4. Proposed interaction of a silanol group with a β-O-propionylated catecholamine (NA).

Pre-treatment of the column with thionyl chloride chlorinates the silanol groups[13] according to the following equation:

$$\text{–Si–OH} + SOCl_2 \rightarrow \text{–Si–Cl} + HCl + SO_2$$

By this reaction, the column is deactivated for an exchange with β-O-propionyl groups, so that all catecholamines can be chromatographed without any problems (Fig. 5).

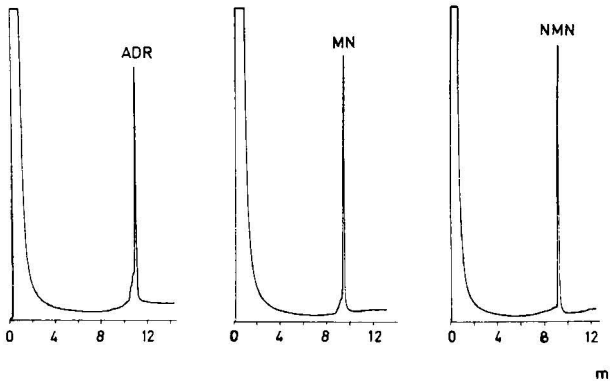

Fig. 5. Gas chromatographic assay of β-O-propionylated catecholamines after deactivation of the column with thionyl chloride.

Gas chromatographic analysis of 5-hydroxytryptamines

Further investigations of the gas chromatographic properties of propionylated biogenic amines have shown that 5-hydroxylated tryptamines such as serotonin and N-methyl-5-hydroxytryptamine behave differently when chromatographed alone (Fig. 6, I) or with propionic anhydride (Fig. 6, II) or together with a catecholamine such as adrenaline (Fig. 6, III). As can be seen in Fig. 6, II and III, an additional peak (B) can be detected.

Mass spectrometric analysis showed that the additional peak (B) represents a tripropionylated hydroxytryptamine with the third propionyl group at the indole-N position. The donor of the third propionyl group may be propionic anhydride as well as β-O-propionyladrenaline, whereas the indolalkylamine is the acceptor. Catalyst of the reaction is again the material of the column, because when it is deactivated with thionyl chloride as described above, the propionyl transfer is inhibited (Fig. 6, IV).

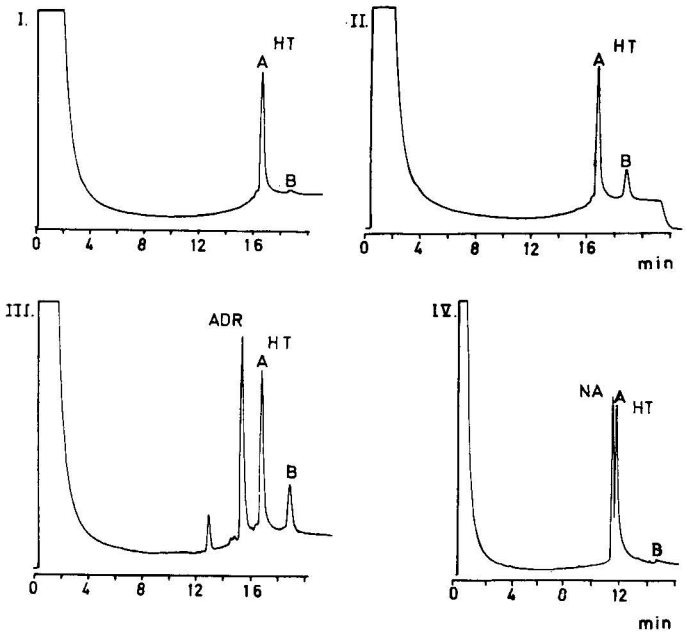

Fig. 6. Gas chromatographic assay of 5-hydroxytryptamine. I, HT dissolved in acetonitrile; II, HT dissolved in propionic anhydride; III, HT + ADR dissolved in acetonitrile; IV, HT + NA dissolved in acetonitrile, column deactivated with thionyl chloride.

It was further found that the effect of deactivation by thionyl chloride is not permanent. Injection of small amounts of water into the column will restore the silanol groups according to the equation

$$\text{-Si-Cl} + H_2O \rightarrow \text{-Si-OH} + HCl$$

and the column becomes active again. Trace amounts of water are present in any sample. In order to prevent reactivation, we injected 1–2 μl of ethyl acetate containing 0.5% of thionyl chloride before each gas chromatographic run, which ensured that the propionylated amines are eluted in single peaks.

Gas chromatographic analysis of mixtures of biogenic amines

In studying the gas chromatographic properties of biogenic amines as their propionyl derivatives, further experiments were performed to find the optimal conditions for the separation of amines in a complete mixture. For these experiments, an equimolar mixture containing 21 propionylated amines (5 μm/ml) was prepared. A 5-nmole (1-μl) amount was injected into the column. Because the propionylated amines have high boiling points, stationary phases with high thermal stability (maximum temperature *ca.* 300°) must be used. Various silicone phases with different polarity were tested, and the silicones OV-17 and OV-101 proved to be the most suitable. Gas chromatograms obtained on these two phases are shown in Figs. 7 and 8.

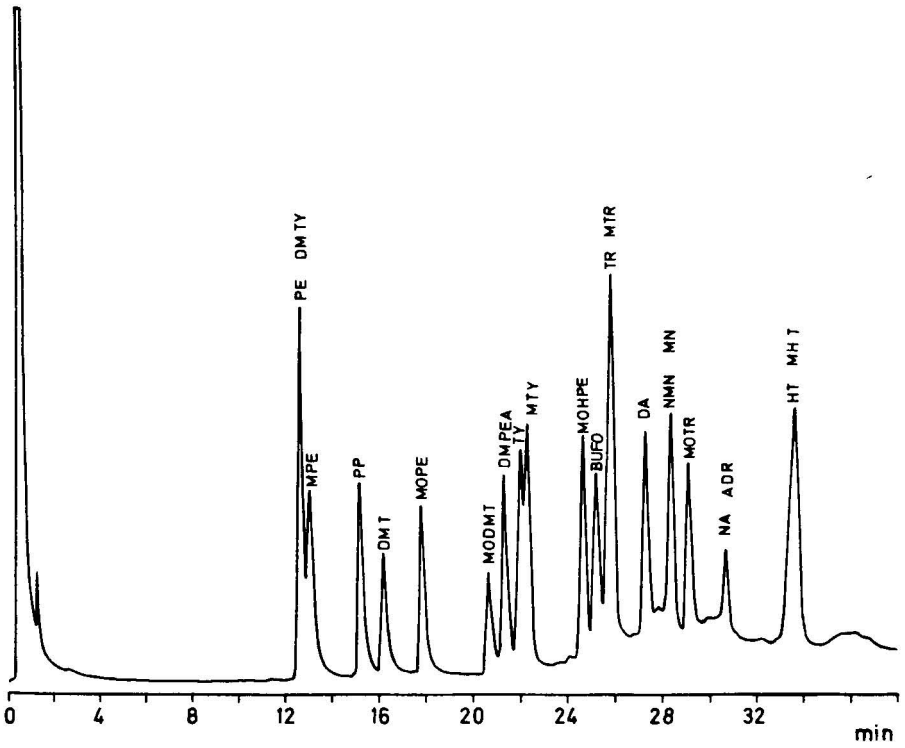

Fig. 7. Gas chromatographic separation of 21 biogenic amines as their propionyl derivatives. Stationary phase, 3% OV-101 on Chromosorb Q (80–100 mesh).

The retention times of the derivatives increase with the number of propionyl groups that are bound to the amines. A complete separation of all amines could not be obtained on packed columns, but this will be possible if capillary columns are used.

CONCLUSION

The properties of propionylated amines, in comparison with those of other derivatives, especially silyl, trifluoracetyl and pentafluorpropionyl derivatives, have considerable advantages. Propionylation is performed in aqueous solution; the resulting derivatives are more stable than the basic amines and increasingly lipophilic, so that they can be extracted quantitatively from the aqueous medium; the propionylation step leads to uniform derivatives that are eluted in a single gas chromatographic peak and that can be well separated; and storage of the propionylated amines for several weeks at room temperature does not lead to measurable decomposition.

Hence propionylation provides a useful means not only for the gas chromatographic separation and determination but also for the isolation of a wide range of catecholamines, phenylethylamines and indolalkylamines. Preliminary analyses of human urines have given very useful results[14].

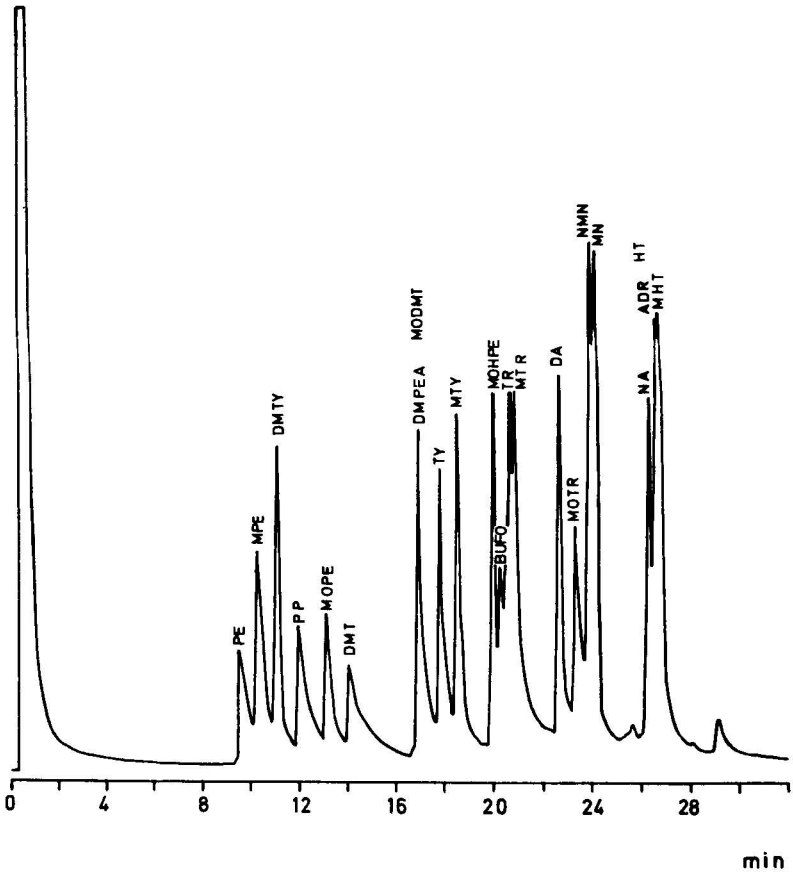

Fig. 8. Gas chromatographic separation of 21 biogenic amines as their propionyl derivatives. Stationary phase, 3% OV-17 on Chromosorb Q (80–100 mesh); glass column, 1.9 m × 2 mm I.D; oven temperature, 100–280°, increased at 6°/min; injection port temperature, 270°; detector temperature, 300°; flow-rate of carrier gas (helium), 30 ml/min.

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SIMPLE GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF DISOPYRAMIDE IN BLOOD-PLASMA OR SERUM AND IN URINE

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SUMMARY

A simple method has been developed for the measurement of disopyramide in blood-plasma or serum at the concentrations attained during therapy. A relatively small (200 μl) sample volume is made basic and extracted with 50 μl of chloroform containing an internal standard, and the extract is analysed directly by gas–liquid chromatography with flame-ionization detection. The instrument calibration is linear and passes through the origin of the graph. Neither solvent transfer nor evaporation steps are used in the extraction procedure, which takes less than 3 min to complete, and urine specimens may be analysed by an analogous technique. No interference from either endogenous sample constituents or other drugs has been observed, although a simple back-extraction procedure is described which eliminates potential interference from a small number of basic and neutral drugs.

INTRODUCTION

Disopyramide is reported to suppress ventricular arrhythmias in patients who have suffered acute myocardial infarction[1], and it may prove of value in the prophylactic treatment of such patients. Plasma drug concentrations between 2.8 and 7.5 mg/l are thought to be required for optimal clinical effect, but adverse reactions may occur at concentrations greater than 3.6 mg/l[2]. The apparent ineffectiveness of disopyramide in trials[3,4] in which the plasma drug concentrations attained were not monitored may have been due to inadequate dosage.

The spectrophotofluorimetric assay[5] for plasma disopyramide does not differentiate between the drug and its mono-*N*-dealkylated metabolite (MND), and variable "blank" values are produced by some specimens[6]. Of the published gas–liquid chromatographic (GLC) methods[6–8], all incorporate solvent evaporation steps, and that of Hutsell and Stachelski[6] required prolonged extraction times and an extensive extract purification procedure. Moreover, the calibration graphs obtained had non-zero intercepts, indicating that either adsorption or decomposition of the drug had occurred on-column. This latter problem has been avoided by use of liquid chromatography (LC)[9], although the extraction procedure used in this method was still

relatively long. Disopyramide and an internal standard were extracted into diethyl ether at an alkaline pH and subsequently back-extracted into dilute acid. In the LC method this extract was analysed directly using ion-paired chromatography on an octadecylsilane reversed-phase column. However, we have found that a simple re-extraction into a small volume of chloroform followed by the direct analysis of this latter extract using GLC with flame ionization detection can be easily performed. On-column decomposition of disopyramide has been prevented by prior treatment of the column with γ-glycidoxypropyltrimethoxysilane, and thus the instrument calibration obtained was linear with zero intercept.

Subsequently, it was found that the extraction procedure could be simplified considerably with no loss of sensitivity. Thus, a 200 μl volume of plasma or serum was made basic, extracted with 50 μl of chloroform containing the internal standard and analysed directly. Urinary disopyramide concentrations, which are approximately 10-fold higher than those found in plasma[9], may be measured by a similar technique, but only a 1:1 ratio of sample to solvent is required. No interference has been observed with either type of extraction procedure, but the capacity to perform the back-extraction procedure was retained in view of potential interference from a small number of basic or neutral drugs.

EXPERIMENTAL

Materials and reagents

Disopyramide free base (4-diisopropylamino-2-phenyl-2-(2-pyridyl)butyramide), an aqueous solution of disopyramide phosphate (equivalent to 200 mg/l disopyramide base), its MND; 4-isopropylamino-2-phenyl-2-(2-pyridyl) butyramide) and the internal standard, *p*-chlorodisopyramide (CDP; 4-diisopropylamino-2-*p*-chlorophenyl-2-(2-pyridyl)butyramide) (200 g/l in methanol) were all supplied by Roussel Laboratories, Wembley, Great Britain. The aqueous disopyramide solution was used to prepare a "quality control" sample in heparinized human plasma at a concentration of 4.0 mg/l. γ-Glycidoxypropyltrimethoxysilane (A-187) was supplied by Union Carbide U.K., Southampton, Great Britain. Diethyl ether, chloroform, sodium hydroxide, sulphuric acid and tris(hydroxymethyl)aminomethane (tris) were all analytical-reagent grade; the last three compounds were used as 2.0, 0.05 and 2.0 mole/l aqueous solutions, respectively.

Gas–liquid chromatography

A Pye Series 204 gas chromatograph equipped with a flame ionization detector and linked to a 10 mV recorder was used. Integration of peak areas was performed using a Hewlett-Packard 3352 data system. The column and detector oven temperatures were 240° and 300°, respectively; injection block heaters were not employed. The nitrogen (carrier gas) flow-rate was 40 ml/min, and the flame was supplied by air and hydrogen at inlet pressures of 16 and 21 p.s.i., respectively, giving flow-rates of approximately 440 and 40 ml/min.

A coiled glass column (1.5 m × 4 mm I.D.) was silanized by immersion in 5% dichlorodimethylsilane in toluene for 1 h, rinsed in methanol and dried at 100°. The column was packed with 3% OV-1 on 80–100 mesh Supelcoport, purchased ready-prepared from Chromatography Services, Merseyside, Great Britain. The

packed column was conditioned at 300° with a nitrogen flow of 40 ml/min for 15 h, and was treated subsequently by injection of from 10 to 20 μl of A-187. Thereafter, occasional injections of 5–10 μl of this compound were performed to maintain the column in the deactivated form.

The retention times of disopyramide and of some other compounds on this system measured relative to CDP are given in Table I. The chromatography of a chloroform solution containing both disopyramide and CDP is illustrated in Fig. 1.

TABLE I

RETENTION DATA OF DISOPYRAMIDE AND SOME OTHER COMPOUNDS ON THE OV-1 COLUMN SYSTEM

Compound	*Retention time (relative to CDP)*
Procainamide*	0.27–0.31
MND**	0.48
Diazepam	0.48
Dipipanone	0.56
Chlorprothixene	0.58
Chlorpromazine	0.58
Methixine	0.60
Disopyramide	0.62
Methotrimeprazine	0.64
Pecazine	0.64
Trimethoprim*	0.67–0.80
N-Acetylprocainamide*	0.76–0.80
Chloroquine	0.80
Metoclopramide	0.89
Trifluoperazine	0.95
Acepromazine	0.98
CDP	1.00
Phenazocine	1.04
Propiomazine	1.04
Quinine	1.24
Quinidine	1.26

* Positively skewed peak —retention times measured on analysis of 5 and 0.05 μg, respectively.
** Principal peak; other compounds eluted at relative retention times of 0.26 and 0.30 (*cf.* Fig. 5).

Extraction procedures

Direct extraction of plasma or serum. Sample (200 μl), tris solution (20 μl) and internal standard solution (50 μl of 20 mg/l CDP in chloroform) were added to a clean Dreyer tube (Poulten, Selfe and Lee, Wickford, Great Britain). The last two additions were performed using Hamilton repeating mechanisms fitted with 1.0 ml and 2.5 ml Hamilton gas-tight luer fitting glass syringes, respectively (Field Instruments, Richmond, Great Britain). Everett stainless-steel needles (No. II serum) were affixed to these syringes.

The contents of the tube were mixed thoroughly on a vortex mixer for 30 sec and the tube was centrifuged for 2 min at 9950 *g* in an Eppendorf centrifuge 5412 (obtained from Anderman and Co., East Molesey, Great Britain, and modified to accept Dreyer tubes by slight drilling-out of the 0.4 ml test tube centrifuge adaptors).

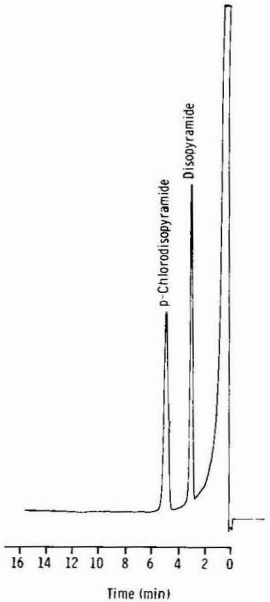


Fig. 1. Chromatogram obtained on analysis of a solution of disopyramide (16 mg/l) and CDP (20 mg/l) in chloroform; 3-μl injection.

Subsequently, a 1–5 μl-portion of the chloroform phase was obtained and injected on to the column of the gas chromatograph. This latter portion was obtained by taking 5 μl of air into a gas-chromatographic syringe and passing the syringe needle through the basic layer into the chloroform. The air was expelled, and 1–5 μl of the extract were taken up for injection.

The extraction was performed in duplicate and the mean result was taken. If the difference between the duplicates was greater than 10%, both the extractions and the analysis were repeated.

Direct extraction of urine. The procedure was identical to that described above except that (i) 50 μl of sample were taken, and (ii) 50 mg/l CDP in chloroform was used as the extraction solvent.

Back-extraction of plasma or serum. Sample (500 μl), sodium hydroxide solution (100 μl) and diethyl ether (5 ml) were added to a 10 ml tapered glass tube. Subsequently, 50 μl of the internal standard solution (50 mg/l aqueous CDP) were added using a Hamilton repeating mechanism. The tube was sealed using a ground-glass stopper, the contents were vortex-mixed for 20 sec and the tube was then centrifuged in a windshielded instrument at 1800 *g* for 4 min. The ether layer was transferred by aspiration to a second tapered tube containing sulphuric acid (500 μl), and the contents of this tube were similarly vortex-mixed and centrifuged. Subsequently, the organic layer was removed by aspiration and any residual solvent eliminated under a stream of air. The acidic solution was made basic with sodium hydroxide solution (300 μl), and chloroform (50 μl) was added. After vortex mixing and centrifugation at 1250 *g* for 4 min, a portion of the chloroform extract was injected on to the column of the gas chromatograph. This portion was obtained by an analogous method to

that described for direct-extraction analyses. Plasma samples were assayed in duplicate and the mean results taken.

Instrument calibration and calculation of results

In the case of the direct-extraction analyses, standard solutions containing both disopyramide and CDP were prepared in chloroform and were used to obtain calibration graphs of peak area ratio (disopyramide/internal standard) against disopyramide concentration. The sample disopyramide concentration was calculated from the peak area ratio obtained on analysis of the extract and by the use of a previously calculated recovery factor. In contrast, solutions prepared in heparinized human plasma and containing disopyramide at a range of concentrations were analysed by the back-extraction procedure, together with each batch of specimens, and the sample drug concentration was obtained directly from the calibration graph.

The "quality control" specimen containing disopyramide (4.0 mg/l), which was obtained from an independent source of the drug, was analysed along with each batch of plasma specimens. If a mean result was obtained which differed by more than $\pm 5\%$ from the true value, the batch of analyses was repeated.

Direct extraction: plasma or serum analyses. Standard solutions containing disopyramide at concentrations of 2.0, 4.0, 6.0, 8.0, 12.0, 16.0, 24.0 and 32.0 mg/l were prepared by dilution of a 1 g/l solution of the drug in chloroform. (*N.B.* These concentrations were four times higher than the equivalent sample concentrations since a sample–solvent ratio of 4:1 was used in the extraction). Each standard solution also contained CDP (20 mg/l) which was obtained from a similar stock source. A linear calibration graph with zero intercept was obtained on analysis of these solutions (Fig. 2); the calibration gradient (peak area ratio/plasma drug concentration) normally obtained was 0.24 l/mg. (*N.B.* Analogous graphs were obtained from the other sets of standard solutions). The results of sample analyses were multiplied by a factor of 1.13 to compensate for the incomplete extraction of the drug.

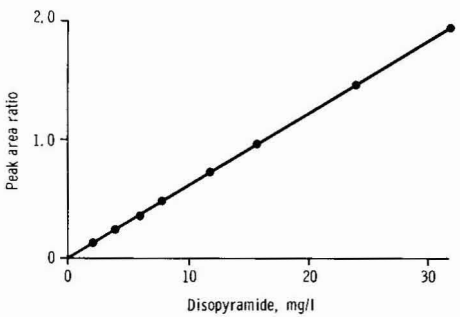


Fig. 2. Calibration graph obtained on analysis of the standard disopyramide solutions in chloroform used in conjunction with the direct extraction of plasma specimens.

Direct extraction: urinary analyses. The instrument calibration procedure was identical to that given above except that the range of disopyramide solutions ran from 20 to 100 mg/l, in increments of 20 mg/l, and each contained CDP at a concentration of 50 mg/l. The calibration gradient normally obtained was 0.023 l/mg, and the results of sample analyses were multiplied by a factor of 1.12.

Back-extraction procedure: plasma or serum analyses. Standard solutions of disopyramide at concentrations of 0.5, 1.0, 3.0, 5.0 and 8.0 mg/l were prepared in heparinized human plasma by dilution of an aqueous solution (1 g/l) of disopyramide free base. The calibration gradient normally obtained on analysis of these solutions was 0.28 l/mg.

RESULTS AND DISCUSSION

Column deactivation

The relationship between the disopyramide concentration in the chloroform standards and the peak area ratio of drug to internal standard was non-linear, especially at low drug concentrations, if the column was not treated with A-187. Moreover, the chromatogram obtained using an untreated column on analysis of a 1 g/l solution of disopyramide in chloroform showed clear evidence of on-column decomposition of the drug; the baseline rose before the elution of the disopyramide, *i.e.* a negatively skewed peak was obtained. Column treatment with A-187 to prevent the degradation of compounds such as disopyramide during GLC analysis has been discussed by Averill[10]. This silane is used to promote the adhesion of organic materials to inorganic substrates, and it may act by promoting a relatively complete stationary phase coating of the support material, thus masking catalytically active sites.

Column treatment with a silylating agent (Rejuv-8, obtained from Chromatography Services) did not noticeably reduce the on-column decomposition of disopyramide, although the injection of 5–10 μl portions of a 5 g/l solution of DL-α-phosphatidylcholine dipalmitoyl (Sigma London, Kingston-upon-Thames, Great Britain) in chloroform was partially effective. However, daily injections of 5–10 μl of this solution were required to maintain the column in the deactivated form, and since relatively large peaks were obtained for up to 1 h after treatment, this was considered unsatisfactory. Although peaks were normally obtained following column treatment with A-187, such treatment was only required infrequently (at most, weekly with a relatively new column, and monthly thereafter). The column was maintained at 140° with nitrogen flow of 40 ml/min when not in use, in order to minimize the need for further treatment.

Recovery studies

Detailed recovery studies with the back-extraction procedure were not necessary since standard disopyramide solutions prepared in heparinized human plasma were analysed together with each batch of samples, and were used to provide the calibration graph. However, in the case of the direct-extraction analyses, recovery factors were calculated in order to facilitate calibration using standard solutions prepared in chloroform, with a consequent reduction in the total analysis time.

Plasma analyses. The calibration standards used here contained disopyramide at a concentration four-fold higher than in the corresponding plasma solutions since a sample: solvent ratio of 4:1 was used in the extraction procedure. Thus, solutions containing disopyramide at concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 mg/l were prepared in heparinized bovine plasma by dilution of a 1 g/l aqueous solution of the drug. The quintuplicate analysis of each of these solutions revealed a mean recovery of 88.5 $\pm$ 5.3 (SD) % which was uniform over the range of concentra-

tions studied. The results of sample analyses were therefore multiplied by a factor of 1.13.

In order to assess the validity of this factor when applied to human plasma assays, 50 specimens obtained from patients treated with disopyramide were analysed in duplicate by the direct- and back-extraction procedures. There was a good correlation ($r = 0.95$) between the results given by each method, and the mean of all of the analyses obtained using the back-extraction (3.87 $\pm$ 1.41 (S.D) mg/l) was the same as that obtained from the direct extraction (3.87 $\pm$ 1.38 (S.D) mg/l).

Urinary analyses. In this case, the calibration standards contained disopyramide at concentrations equivalent to the corresponding urinary solutions since a sample–solvent ratio of only 1:1 was required in the extraction. Thus, solutions containing disopyramide at concentrations of 20–100 mg/l, in increments of 20 mg/l, were prepared in drug-free human urine. The triplicate analysis of these solutions revealed a mean recovery of 89 $\pm$ 4 (S.D)%, which was uniform over the range studied; the results of sample analyses were therefore multiplied by a factor of 1.12.

Assay reproducibility

The coefficient of variation (C_v) of the plasma direct-extraction procedure assessed from the difference between duplicates of 50 sample analyses was 3.8% in the range 1.4–7.0 mg/l. The intra-assay C_v at 4.0 mg/l was 2.2% ($n = 20$).

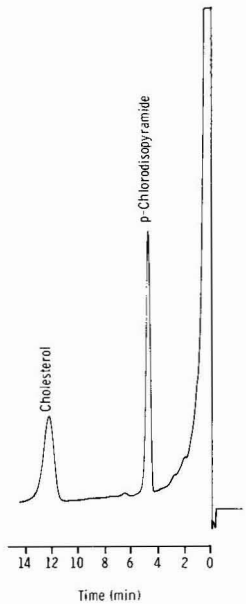
The C_v of the back-extraction procedure assessed from the difference between duplicates of 50 analyses was 4.0% in the range 1.2–7.5 mg/l. The intra-assay C_v of this procedure at 3.0 mg/l was 4.1% ($n = 10$). The inter-assay C_v at 2.6 mg/l was 3.2% ($n = 15$) and at 4.0 mg/l was 3.0% ($n = 22$). The similarity of these results to those obtained with the LC disopyramide assay[9] was not unexpected in view of the similarities between the extraction procedures used in each method.

Specificity

No interference has been observed in either direct- or back-extracts of drug-free heparinized human plasma, or in direct extracts of drug-free human urine, and an example of such an analysis is given in Fig. 3. Analogous extractions performed without the addition of CDP have not revealed the presence of compounds that could elute with this standard. In addition, specimens of either plasma or urine obtained from patients treated with disopyramide have shown a similar absence of interference in both direct- and back-extraction analyses (Figs. 4–6). Again, no compounds have been observed which could elute with CDP.

The major metabolite of disopyramide (MND)[11] is reported[6] to be unstable under similar GLC conditions to those used here. Indeed, three compounds eluting before disopyramide were represented on the chromatogram obtained on analysis of a 1 g/l solution of MND in chloroform, and an analogous pattern of peaks has been observed on analysis of urine specimens obtained from patients treated with disopyramide (*cf.* Fig. 5). Since disopyramide is excreted largely unchanged in the urine of healthy subjects[11], and MND is reported to be not only inactive against ventricular arrhythmias but also less active than disopyramide against atrial arrhythmias[12], the plasma or urinary assay of MND appears unlikely to be of clinical value.

A number of basic and neutral drugs were investigated as possible sources of interference. Mexiletine and lignocaine both eluted with the solvent under the GLC



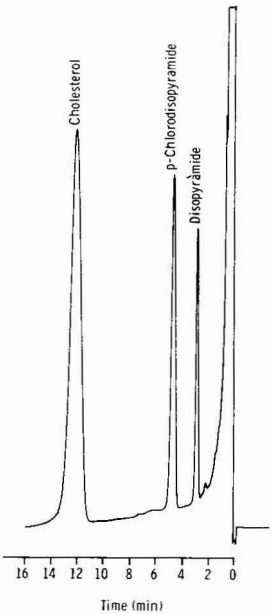
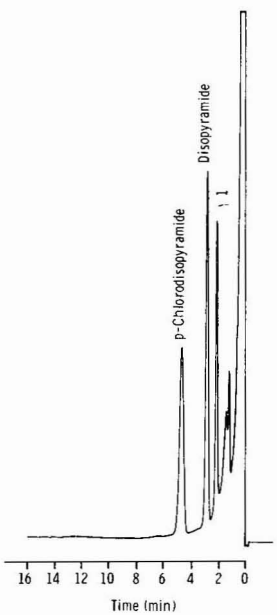


Fig. 3. Chromatogram obtained on analysis of a direct extraction of drug-free human plasma; 4-μl injection. The CDP concentration was 20 mg/l.

Fig. 4. Chromatogram obtained on analysis of a direct extract of plasma obtained immediately prior to dosage from a patient treated with disopyramide (200 mg, 8 hourly); 4-μl injection. The CDP concentration was 20 mg/l, and the plasma disopyramide concentration was found to be 2.3 mg/l.



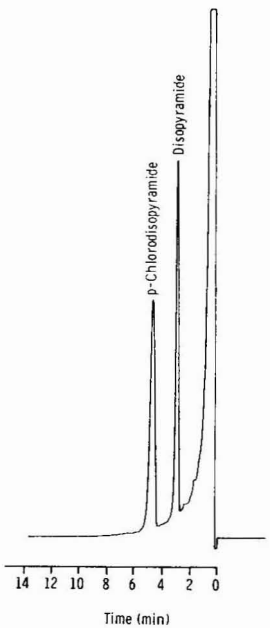


Fig. 5. Chromatogram obtained on analysis of a direct extract of urine obtained from a patient treated with disopyramide (100 mg, 6 hourly); 2-μl injection. The CDP concentration was 50 mg/l and the urinary disopyramide concentration was found to be 52 mg/l. (1 = Degradation products of MND.)

Fig. 6. Chromatogram obtained on analysis of a back-extract of plasma obtained 3 h after dosage from a patient treated with disopyramide (100 mg, 6 hourly); 3-μl injection. The plasma disopyramide concentration was found to be 3.3 mg/l.

conditions used, and the retention times of the remaining compounds measured relative to the retention time of CDP are given in Table I. None of the compounds likely to be present in the plasma of patients receiving disopyramide (*i.e.* procainamide, *N*-acetylprocainamide, diazepam, trimethoprim, metoclopramide and quinidine) interfered in the assay. Most of the remaining drugs are administered at low dosage (less than 100 mg/day) or are extensively metabolized and are thus unlikely to interfere in either the direct- or back-extracts of plasma. Nevertheless, the capacity to perform the back-extraction was retained in order to counter possible interference from compounds not surveyed in the present work.

Limits of sensitivity

The limit of sensitivity of the direct-extraction plasma assay was taken to be 0.2 mg/l if 200 μl of sample were used. Although the use of a larger sample volume could produce a corresponding decrease in the limit of sensitivity of the assay, this was not thought to be necessary in view of the plasma disopyramide concentrations associated with effective therapy[2]. The use of relatively small volumes of plasma is especially useful where further analyses are to be performed using the same specimen or where sample size may be limited. The back-extraction plasma assay has a similar limit, but 500 μl of sample are required. Since the urinary disopyramide concentrations attained during therapy are approximately 10-fold higher than in plasma, the calibration range and limit of sensitivity of the direct-extract urinary assay were correspondingly higher, and this minimized the need for dilution of the specimen prior to analysis. However, increased sensitivity could easily be obtained if required by adopting the assay procedure used for plasma.

Choice of direct-extraction conditions

Initially, the conditions used in the direct-extraction procedure were similar to those used prior to back-extraction in that 2 mole/l sodium hydroxide was employed. However, emulsions were obtained on analysis of some plasma specimens under these conditions. No emulsions were obtained following the use of 2 mole/l tris in place of the sodium hydroxide solution, whilst the recovery of disopyramide was unchanged. The pH of a mixture of 1 volume of tris solution and 10 volumes of plasma was found to be 9.4.

An extraction time of 30 sec was chosen on the basis of results obtained with other direct-extraction drug assay procedures performed using Dreyer tubes[13–15].

Advantages of the direct-extraction procedures

Disopyramide has an elimination half-life of *ca.* 7 h in patients who have suffered a myocardial infarct[16]. A plasma disopyramide concentration may be accurately measured using the direct-extraction procedure within *ca.* 20 min of receipt of the specimen, provided that the instrument calibration has been accomplished previously, and thus the result may be made available with sufficient speed to have relevance to therapy. In contrast, an analysis using the back-extraction procedure takes *ca.* 40 min to complete. The direct-extract analysis of urine also minimizes the time required for the assay, especially since previous methods[6,9] advocated the dilution of the urine specimen prior to disopyramide measurement by the same procedure as for plasma.

In addition to the rapidity and small sample requirement of direct-extract drug assays, there are other advantages to the use of such procedures. These have been discussed in detail elsewhere[13–15], and include the requirement of minimal apparatus and reagents, the absence of interference derived from solvent transfer and evaporation stages or from inadequately cleaned glassware and the good accuracy and reproducibility of the techniques.

A disadvantage to the direct-extraction procedure was that cholesterol was represented on all of the chromatograms of plasma or serum extracts (*cf.* Figs. 3 and 4). Although this compound did not interfere in the analysis, its presence did reduce the rate at which these analyses could be performed. Nevertheless, the advantages of the direct-extraction procedure outweighed this latter consideration. The use of nitrogen-selective detection, or indeed LC analysis, in place of flame ionization could serve to prevent the detection of cholesterol should this be required.

CONCLUSIONS

The direct-extraction procedures described represent improvements over previously published methods for the measurement of either plasma or urinary disopyramide at the concentrations achieved during therapy. A complete quantitative analysis can be performed, in duplicate, within 20 min and with the use of a relatively small sample volume. The extraction may be completed in less than 3 min and is performed in a single tube, and thus solvent transfer and evaporation stages are not required. No interference from endogenous sample constituents, other drugs or drug metabolites has been observed.

It has proved possible to use an analogous direct-extraction procedure to that described here in the measurement by GLC of two other antiarrhythmic drugs, mexiletine and lignocaine, at the plasma concentrations attained during therapy (D. W. Holt, A. M. Hayler, M. Loizou and R. J. Flanagan, unpublished results). In addition, preliminary results suggest that the method described here may prove to be applicable to the measurement of the plasma concentrations of not only disopyramide but also procainamide, *N*-acetylprocainamide and quinidine which are achieved during therapy.

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ANALYSIS OF CHLORMETHIAZOLE, ETHCHLORVYNOL AND TRICHLOROETHANOL IN BIOLOGICAL FLUIDS BY GAS–LIQUID CHROMATOGRAPHY AS AN AID TO THE DIAGNOSIS OF ACUTE POISONING

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SUMMARY

A simple method has been developed whereby chlormethiazole, ethchlorvynol and trichloroethanol can be simultaneously detected and measured in biological fluids. The procedure is based upon the rapid extraction of a small (50-μl) sample volume with an equal volume of chloroform containing an internal standard, followed by the gas–liquid chromatographic analysis of this extract. Specimens of blood plasma or serum, urine and gastric contents can be used, and no interference from either endogenous or exogenous sources has been observed. The method is suitable for the measurement of the plasma concentrations of these compounds attained after overdosage.

INTRODUCTION

Chlormethiazole, ethchlorvynol and trichloroethanol (the pharmacologically active metabolite of chloral hydrate[1,2]) can all cause coma if ingested in sufficient quantity, and thus the detection and identification of these compounds may be of clinical relevance. Ethchlorvynol and trichloroethanol can be detected by simple chromogenic reactions which are applicable essentially to urine and only provide qualitative information[3]. Moreover, in the case of this latter compound, the test[4] is not specific and will detect other trichloro-compounds such as chloroform. Chlormethiazole and its metabolites may be detected by the thin-layer chromatographic analysis of a chloroform extract of alkaline urine, but identification may prove difficult, especially if some other drugs have been ingested.

A gas–liquid chromatographic (GLC) technique for the analysis of ethchlorvynol in specimens of either plasma or urine obtained from poisoned patients has been described[5]. This method is rapid, specific, sensitive and requires only 100 μl of sample to enable a duplicate analysis to be performed. The investigation of possible sources of interference in this assay showed that not only were chlormethiazole and

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trichloroethanol resolved from both ethchlorvynol and the internal standard on the chromatographic system used, but also that they were extracted into chloroform under the same conditions as this latter drug. Subsequently, a modification of this technique has been found to be applicable to the analysis of all three compounds in specimens obtained from poisoned patients.

EXPERIMENTAL

Chemicals and reagents

Chlormethiazole free base (Astra Chemicals, Watford, Great Britain) and 2,2,2-trichloroethanol (Aldrich, Gillingham, Great Britain) were stored at −20° prior to use. The source and purity of the ethchlorvynol used have been discussed previously[5]. The internal standard, 2-methylnaphthalene (Hopkin and Williams, Chadwell Heath, Great Britain) was used as a 20 mg/l solution in chloroform (analytical-reagent grade).

Gas–liquid chromatography

A Pye 104 model 24 dual-column gas chromatograph fitted with flame-ionisation detectors was used throughout. The column and detector oven temperatures were 140° and 200°, respectively, and the injection port-setting was 2. The carrier gas (nitrogen) flow-rate was 60 ml/min and the hydrogen and oxygen inlet pressures were 15 and 10 p.s.i., respectively, giving flow-rates of approximately 45 and 200 ml/min. The column, a 1.5 m × 4 mm I.D. coiled glass tube, was packed with 2% (w/w) Carbowax 20 M (Field Instruments, Richmond, Great Britain) and 5% (w/w) KOH on HP Chromosorb W, 80–100 mesh[5]. On this system, trichloroethanol, ethchlorvynol and chlormethiazole had retention times of 0.52, 0.69 and 1.28, respectively, relative to 2-methylnaphthalene.

Extraction procedure

The sample (50 μl) was introduced into a Dreyer tube (Poulten, Selfe and Lee, Wickford, Great Britain) by means of a semi-automatic pipette; specimens of gastric contents containing large amounts of solid material were centrifuged prior to analysis in order to obtain a clear fluid. Subsequently, 50 μl of the internal standard solution were added via a 2.5-ml Hamilton gas-tight luer-fitting glass syringe fitted with a Hamilton repeating mechanism (both available from Field Instruments). An Everett stainless-steel needle (No. 11 serum) was affixed to this syringe. The contents of the tube were mixed thoroughly on a vortex mixer for 30 sec and the tube was centrifuged for 30 sec. at 9950·g in an Eppendorf centrifuge 5412 (Anderman and Co., East Molesey, Great Britain and modified to accept Dreyer tubes by slight drilling-out of the 0.4 ml test tube centrifuge adaptors). Subsequently, a 3- to 5-μl portion of the chloroform phase was obtained as described[5] and injected onto the column of the gas chromatograph.

The extraction was performed in duplicate and a mean result obtained. If the difference between the duplicates was greater than 10% both the extractions and analysis were repeated.

TABLE I

DRUG STANDARD SOLUTIONS AND CALIBRATION GRADIENTS

Each solution also contained 2-methylnaphthalene at a concentration of 20 mg/l.

Compounds	*Calibration gradient (l/mg)*	*Standard drug solutions available (mg/l)*												
		5	*10*	*15*	*20*	*25*	*30*	*40*	*50*	*75*	*100*	*150*	*200*	*250*
Chlormethiazole	0.016	×	×	×	×	×	×	×	×					
Ethchlorvynol	0.035		×		×		×	×	×	×	×	×		
Trichloroethanol	0.013								×		×	×	×	×

Instrument calibration and calculation of results

Standard solutions containing each drug were prepared in chloroform by dilution of a 1 g/l stock solution in this same solvent (Table I). Each standard also contained 2-methylnaphthalene at a concentration of 20 mg/l, obtained from a separate stock source. The ratio of the peak height of each drug to the peak height of 2-methylnaphthalene bore a linear relationship to the drug concentration over the ranges studied. The normal calibration gradients obtained (*i.e.* peak height ratio–drug concentration) are shown in Table I. The results of sample analyses were multiplied by a "recovery factor" to compensate for the incomplete extraction of each drug. The factors used for either plasma or urine analyses are given in Table II.

TABLE II

RECOVERIES OF ADDED DRUG FROM EITHER HEPARINISED BOVINE PLASMA OR DRUG-FREE HUMAN URINE

Compounds	*Standard solutions*		*Plasma*		*Urine*	
	Range (mg/l)	*Increment (mg/l)*	*Mean ± SD (%)*	*Recovery factor*	*Mean ± SD (%)*	*Recovery factor*
Chlormethiazole	10– 50	10	96 ± 4	1.04	100 ± 2	1.00
Ethchlorvynol	20–100	20	95 ± 4	1.05	95 ± 2	1.05
Trichloroethanol	50–250	50	75 ± 4	1.33	78 ± 3	1.28

RESULTS AND DISCUSSION

Recovery studies

Standard solutions were prepared in 10.0 ml of either heparinised bovine plasma or drug-free human urine by dilution of a 2-g/l solution of each drug in ethanol, and the range of concentrations thus obtained is shown in Table II. The quintuplicate and triplicate analyses of the plasma and urine solutions, respectively, revealed the mean drug recoveries given in Table II. Each recovery was uniform over the range studied.

Sources of interference

The method has been applied primarily to the analysis of plasma or serum specimens obtained from poisoned patients and no interference from either endogenous sample constituents or other drugs has been encountered. Examples of the

chromatograms obtained on analysis of drug-free human plasma and of plasma obtained from an ethchlorvynol overdose patient have been given previously[5]. The analysis of a plasma specimen from a patient who had ingested a large amount of dichloralphenazone is illustrated in Fig. 1.

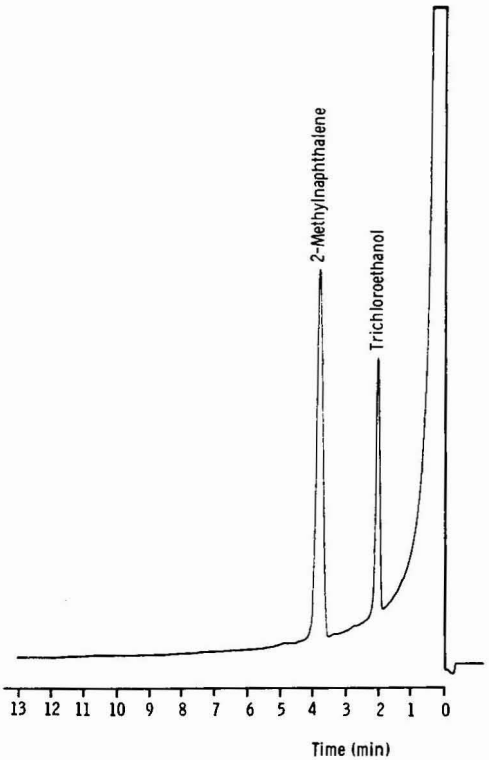

Fig. 1. The analysis of an extract of plasma obtained from a dichloralphenazone overdose patient on the Carbowax 20M–KOH column system; 3-μl injection. The plasma trichloroethanol concentration was found to be 71 mg/l.

A feature of the analyses of urine (and in some cases of plasma) from chlormethiazole overdose patients is the presence of several compounds which elute after chlormethiazole on the Carbowax 20 M–KOH column system (Fig. 2). These compounds are probably chlormethiazole metabolites, but specific identifications have not been attempted. Although up to five metabolites of this drug have been identified in human urine[6,7], all thought to result from oxidation of the 2-chloroethyl moiety of chlormethiazole[7], the fate of only approximately 20% of the dose has been defined. The possibility that metabolites of chlormethiazole might interfere in the assay has been investigated. Analyses on a second GLC column system (2.1 m $\times$ 4 mm I.D. glass column packed with 10% Apiezon L–2% KOH on 80–100 mesh Chromosorb W AW (obtained ready-prepared from Chromatography Services Ltd., Hoylake, Great Britain)) at 160° have given identical quantitative results to those obtained from the same extract on the Carbowax 20 M–KOH column system. The retention times relative to 2-methylnaphthalene of the peak corresponding in area to "metab-

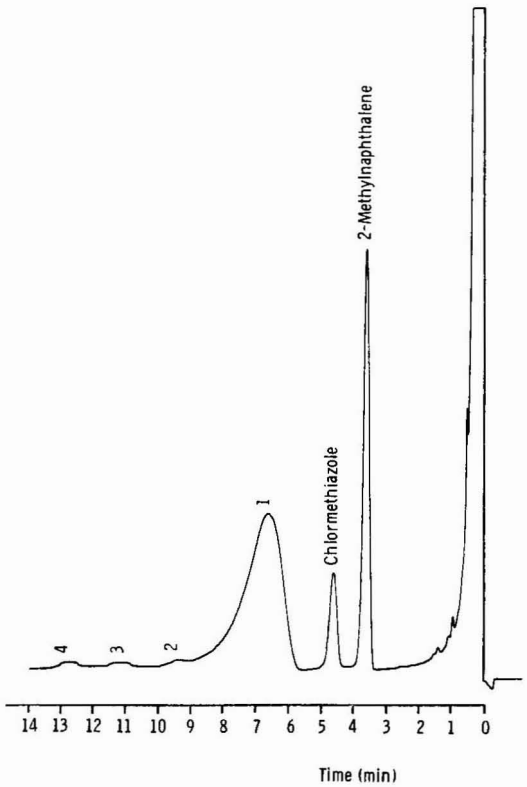

Fig. 2. The analysis of an extract of a urine specimen obtained from a chlormethiazole overdose patient on the Carbowax 20M–KOH column system; 3-μl injection. Compound 1 (and possibly compounds 2–4) are metabolites of chlormethiazole (see text).

olite 1" (*cf.* Fig. 2) and of chlormethiazole were 0.40 and 0.51, respectively, on the Apiezon L–KOH column system. All of these compounds gave rise to sharp, symmetrical peaks, but no other metabolites were observed on this latter system.

It has been found that both chlormethiazole and ethchlorvynol may be readily detected and identified in gastric content specimens by use of this procedure, and an example of such an analysis is given in Fig. 3. However, chloral hydrate was neither extracted[8] nor chromatographed under the conditions of this assay.

Limits of sensitivity

When using a sample–solvent ratio of 1:1, the minimum sensitivities of the technique to chlormethiazole, ethchlorvynol and trichloroethanol were 2, 2 and 10 mg/l, respectively, at the amplifier attenuation normally used ($5 \cdot 10^{-10}$ A). The plasma concentrations of both ethchlorvynol and trichloroethanol attained in overdose are above these limits[5,9], and the available data suggested that this also applied for chlormethiazole; the intravenous infusion to six volunteer subjects of from 1.20 to 2.25 g of chlormethiazole ethanedisulphonate at rates varying from 11.9 to 25.0 mg/min gave rise to plasma drug concentrations between 3 and 40 mg/l at the cessation of infusion[10]. The results from nine patients who had ingested an overdose of chlor-

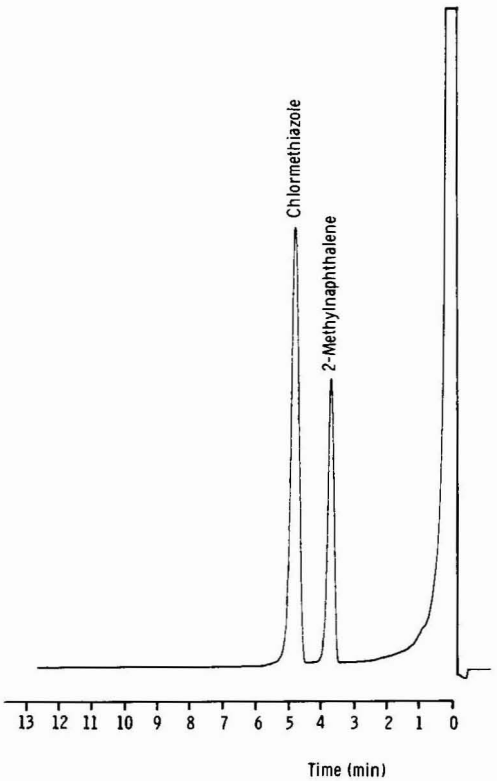

Fig. 3. The analysis of an extract of a 1:20 aqueous dilution of a specimen of gastric contents obtained from a chlormethiazole overdose patient on the Carbowax 20M–KOH column system; 2-μl injection.

TABLE III

PLASMA CONCENTRATIONS AND ADDITIONAL DATA FROM NINE PATIENTS WHO HAD INGESTED AN OVERDOSE OF CHLORMETHIAZOLE

Patient No.	*Age (yr)*	*Sex*	*Plasma chlormethiazole (mg/l)*	*Grade of coma at time of sampling*	*Other drugs detected*
1	63	F	16	IV	Diazepam
2	38	F	9	II	Nil
3	26	F	66	IV	Dextropropoxyphene, Nitrazepam
4	40	M	11	III	Nil
5	61	F	8	III	Nil
6	34	M	8	II	Nil
7	47	M	37	IV	Chlorimipramine, Diazepam
8	48	F	14	IV	Nitrazepam
9	77	F	12	III	Nil

methiazole either alone or together with other drugs supported this view (Table III). All of these patients recovered uneventfully.

The use of electron-capture detection has been advocated recently[9] for the measurement of the plasma trichloroethanol concentrations attained in overdose. However, the results presented here clearly show that flame-ionisation detection is satisfactory even though only 50 μl of specimen are required. Indeed, smaller volumes of both sample and solvent can be used with no decrease in sensitivity. On the other hand, greater sensitivity to all of the drugs studied with the present technique was attainable if required. The use of a higher instrument sensitivity together with a less concentrated internal standard solution served to increase the minimum sensitivities of the method 10-fold without a concomitant increase in the interference observed. In addition, a higher sample–solvent ratio in the cases of both ethchlorvynol[5] and chlormethiazole also increased the minimum sensitivities attainable.

CONCLUSIONS

Analytical techniques which are specific, sensitive, rapid and provide not only qualitative but also quantitative information are advantageous in clinical toxicology. The method described here has been used in the assay of several hundred specimens obtained from poisoned patients during the course of our 24-h drug analysis service over a period of approximately one year. Even quantitative analyses were completed within 20 min and with the use of a very small sample volume. The GLC column system has proved to be extremely stable under the conditions used, and the method represents a considerable improvement over methods used previously in our laboratory for the analysis of chlormethiazole, ethchlorvynol and trichloroethanol in biological fluids.

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CHROM. 10,749

HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF ARYLHYDROXYLAMINES AFTER DERIVATIZATION WITH METHYL ISOCYANATE

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SUMMARY

The quantitative analysis of micromolar levels of arylhydroxylamines in liver homogenates is described. The analyte is extracted from the biological medium with dichloromethane and then converted to the corresponding N-hydroxyurea by reaction with methyl isocyanate. The reaction product is stable both in the dry state and in solution. The derivatized mixture is separated by reversed-phase partition chromatography and the hydroxylamine quantitated as the methylhydroxyurea by spectrophotometric monitoring of the column eluent at 254 nm. The method is specific for arylhydroxylamines in that the product is different from products formed by reaction of methyl isocyanate with alternate amine metabolites and metabonates.

INTRODUCTION

Some primary aromatic amines and amides are thought to induce cancer through interaction of their N-hydroxylated metabolites with nucleic acids and protein[1,2]. The low concentrations at which these compounds are present in biological samples and their ease of oxidation have hindered development of reliable methods for their analysis, particularly in the presence of other metabolites. Most reported methods lack sufficient specificity to differentiate between the arylhydroxylamines and C-hydroxylated metabolites of aryl amines. Furthermore, reported procedures often measure arylhydroxylamines by conversion to nitroso[3] or azo[4] derivatives which are indistinguishable from alternate metabolic products, formed via different metabolic routes. Ultimate detection of these analytes has involved spectrophotometry[5–7], fluorimetry[8], amperometry[9] and isotopic measurements[10].

We have recently described a high-pressure liquid chromatographic (HPLC) separation and quantitation of C- and N-hydroxylated metabolites and metabonates of the aromatic amine, aniline, using reversed-phase partition chromatography with ultraviolet spectrophotometric[11] or amperometric[12] detection of analytes. These

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methods are specific, sensitive (detection limit: 10^{-8} *M*) and reliable for analysis of C-hydroxylated metabolites of arylamines. However, although these procedures can specifically monitor N-hydroxylamines, the instability of these compounds limits the concentration that can be quantitated to $5 \cdot 10^{-5}$ *M* and demands rapid processing and analysis of samples. It is not possible to practically monitor arylhydroxylamines in biological fluids with these methods, because of their short half lives, without stabilization of the analyte early into the analysis scheme. In this report we describe a derivatization procedure for arylhydroxylamines prior to their HPLC separation, which renders the analyte stable in solution and converts the hydroxylamine to a species which can be distinguished from all other amine metabolites and metabonates.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Model M 6000A solvent delivery system, a Model U6K septumless injector and Model 440 absorbance detector operated at 254 nm (Waters Assoc., Milford, Mass., U.S.A.).

Reagents

Methanol was ChromAR grade (Mallinckrodt, St. Louis, Mo., U.S.A.). Distilled water was used throughout. Analytical-reagent grade dichloromethane (Fisher Scientific, Pittsburgh, Pa., U.S.A.) was used without further purification. Methyl isocyanate was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was used as received.

5-Hydroxyaminoindan (HAI) was synthesized by reduction of 5-nitroindan (Aldrich) with zinc and ammonium chloride[13]. The product was isolated as yellowish-white plates with a melting range of 64–66°. Spectrometric data, including ultraviolet and mass spectra, and elemental analysis confirmed the identity of the product (elemental analysis: calculated, C, 72.44%; H, 7.44%; N, 9.39%; found, C, 73.83%; H, 7.50%; N, 9.50%).

1-Hydroxy-1-(5′-indanyl)-3-methylurea (HIMU) was synthesized by established methods[14], mixing a solution of HAI in diethyl ether with a 25 molar excess of an ether solution of methyl isocyanate. The product, after recrystallization from benzene, was white and crystalline with a melting point of 119.5–120.0°. Spectrometric data and elemental analysis confirmed the identity of the product (elemental analysis: calculated, C, 57.81%; H, 6.08%; N, 16.86%; found, C, 58.01%; H, 5.95%; N, 16.98%). Mass spectra gave a peak for the molecular ion at *m/e* 206, and a fragmentation pattern consistent with the behavior of similar substituted hydroxyureas[15].

Liver homogenates

Male Sprague-Dawley rats (weighing 200–250 g) were decapitated, their livers excised and washed in cold 0.02 *M* Tris-HCl buffer (pH 7.4). The liver was homogenized with a PTFE pestle in four volumes of the buffer. The homogenate was centrifuged at 9000 *g* for 20 min at 4°. The supernatant was removed, diluted 10-fold with buffer and used as such in all experiments.

PROCEDURES

Extraction

Varying known amounts of HAI were added to either 5 ml of Tris-HCl buffer (0.02 *M*; pH 7.4) or 5 ml of liver homogenate to give final solutions containing $1 \cdot 10^{-4}$ to $1 \cdot 10^{-6}$ *M* HAI. The mixtures were extracted for 5 min with 8 ml of dichloromethane, and then centrifuged at 800 *g* for 5 min. The aqueous layer and protein precipitate at the solvent interface were aspirated to waste.

Derivatization

Methyl isocyanate (400 μl of undiluted material) was added to a 3-ml aliquot of the dichloromethane extract and the solution was shaken for 1 min at room temperature. The solvent was then evaporated to dryness in a nitrogen atmosphere and the residue redissolved in 250 μl of methanol–water (50:50) containing $4.73 \cdot 10^{-5}$ *M* N,N-dimethylaniline (present as the internal standard).

Chromatography

Samples were analyzed by HPLC using a μBondapak C_{18} column (30 cm $\times$ 4 mm I.D.; Waters Assoc.) operating at a flow-rate of 2 ml/min (2300 p.s.i.) with methanol–water (50:50) as mobile phase. Analytes were detected spectrophotometrically at 254 nm. For quantitative analysis concentrations of hydroxylamine were determined from a standard curve. A stock solution of HIMU ($1 \cdot 10^{-3}$ *M*) was prepared in methanol–water (50:50) and diluted as required with the same solvent. N,N-Dimethylaniline (final concentration $4.73 \cdot 10^{-5}$ *M*) was added to each solution as internal standard. All quantitative measurements were made relative to this constant amount of dimethylaniline. Standard curves were constructed by plotting peak height ratio (analyte: internal standard) *vs.* analyte concentration for 6 concentrations of HIMU in the range from $1 \cdot 10^{-4}$ to $2 \cdot 10^{-6}$ *M*. All measurements were made in triplicate and the data subjected to linear regression analysis.

RESULTS

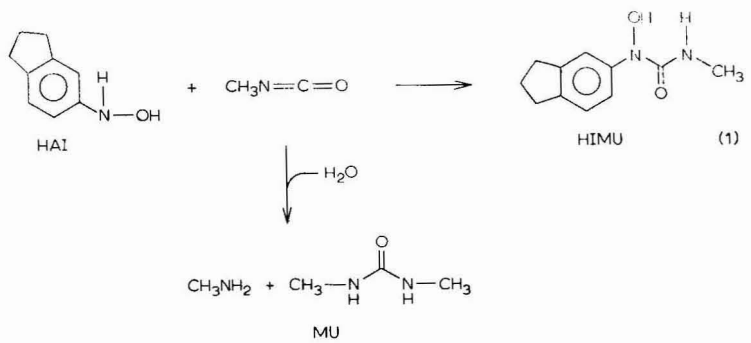
Analysis of the HAI in liver homogenates was performed in three stages: (1) initial extraction of the analyte; (2) subsequent derivatization by reaction with methyl isocyanate; and (3) separation of the derivatized mixture by high-performance reversed-phase partition chromatography.

Extraction

The arylhydroxylamine was removed from aqueous buffer or liver homogenate solutions by extraction with 1.6 volumes of dichloromethane. For biological samples 5 min extractions were required to reach equilibrium. One extraction removed $88 \pm 2\%$ of the analyte from aqueous solutions, and 82% from tissue homogenates, over the concentration range ($1 \cdot 10^{-4}$ to $2 \cdot 10^{-6}$ *M*) studied. Extraction could alternatively be carried out with chloroform, however, this solvent was unsatisfactory for subsequent derivatization. Centrifugation of the biological fluid-dichloromethane system was required after agitation to form two distinct liquid phases, thereby maximizing analyte recovery.

Derivatization

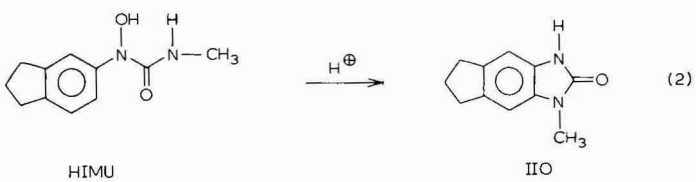
The extracted hydroxylamine was reacted with methyl isocyanate. The reaction product was isolated by HPLC and identified from elemental analysis and mass spectra as the N-substituted monoacylated hydroxylamine (HIMU) (eqn. 1)



For analysis from biological fluids the reaction was carried out in the presence of a 1000-fold excess of isocyanate. This large excess of reagent was required because isocyanates react indiscriminately with nucleophilic species and therefore are consumed by compounds which may co-extract (*e.g.* water, alcohols, amines) with HAI into dichloromethane. Sufficient reagent must be present to totally derivatize the analyte in the presence of these potential interferents. Acylation reactions are, therefore, carried out under anhydrous conditions. In this regard, the derivatizing agent also acts as a dessicant reacting with water to form methyl urea (MU) (eqn. 1). For the convenience of the analyst, reaction was carried out at room temperature in the untreated dichloromethane extract. Under these conditions reaction was complete in less than 1 min and HAI was quantitatively converted to the corresponding hydroxyurea, HIMU.

HIMU was stable at room temperature in the extraction solvent or HPLC mobile phase (methanol–water (50:50)) for more than 1 h, *i.e.* less than 5% loss was observed over this time period. The derivative appears to be indefinitely stable (more than 6 weeks) when stored dry (*i.e.* in the absence of solvent) at room temperature. Reaction must, however, be carried out in the absence of acid to prevent the acid catalyzed conversion of HIMU to the corresponding benzimidazol-2-one (IIO) (eqn. 2)[15].

Chloroform was unsuitable as the reaction medium, because of the relatively high concentration of ethanol which is added to stabilize the commercially available solvent. Ethanol consumes the reagent, converting it to N-methyl-O-ethylcarbamate.



Chromatography

The derivatized hydroxylamine was separated from other species by reversed-phase partition chromatography with isocratic elution of components using methanol–water (50:50) as mobile phase (Fig. 1). To maximize sensitivity, the dichloromethane solvent was removed by evaporation and the residue reconstituted in a minimum volume of mobile phase prior to HPLC analysis. This volume reduction step results in the appearance of unidentified peaks in the chromatographs, apparently attributable to co-extracted materials and decomposition of methyl isocyanate. HAI was quantitated as HIMU (retention volume, $V_R = 7.8$ ml) by measuring peak height relative to a constant amount ($4.73 \cdot 10^{-5}$ *M*) of N,N-dimethylaniline ($V_R = 14.2$ ml), present as internal standard. A linear relationship was found between HAI concentration and peak height ratio over the range from $1 \cdot 10^{-4}$ to $2 \cdot 10^{-6}$ *M*. Linear regression analysis of the data generated the line $y = 0.240$ [HIMU] $- 0.976$ with a correlation coefficient of 0.998. Analysis was carried out with reproducibility of $\pm 5\%$. Accuracy of the determinations, based on the amount of HAI determined by HPLC after extrac-

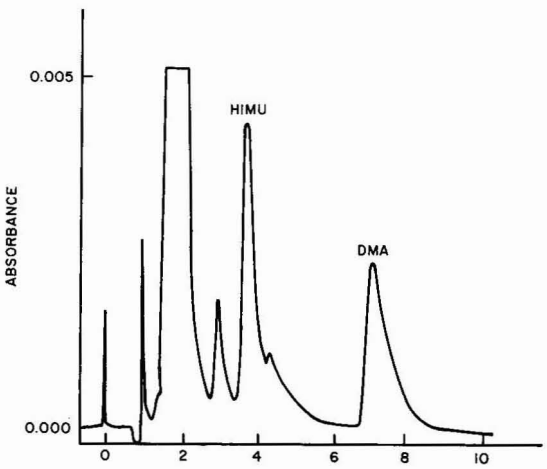


Fig. 1. Chromatography of 1-hydroxy-l-(5′-indanyl)-3-methylurea (HIMU) obtained by extraction of $8 \cdot 10^{-6}$ *M* 5-hydroxyaminoindan (HAI) from liver homogenate and reaction with methyl isocyanate. N,N-Dimethylaniline (DMA; $4.73 \cdot 10^{-5}$ *M*) is present as the internal standard. Components were separated by reversed-phase partition chromatography using methanol–water (50:50) as mobile phase.

tion from liver homogenates and derivatization with methyl isocyanate, was $\pm 4\%$. The minimum concentration of HAI which could be quantitated with this precision and accuracy was $1 \cdot 10^{-7}$ *M* when the analyte was originally dissolved in aqueous buffer solution and $2 \cdot 10^{-6}$ *M* for HAI present in liver homogenates. These limits are all based on 10-μl injection volumes. Larger injection volumes could not be used to enhance sensitivity, because they resulted in an application of greater amounts of interfering substances onto the column, which masked the HIMU peak and decreased column life. Total chromatographic analysis time was less than 9 min.

Methyl isocyanate is a very non-specific reagent and therefore acylatable co-extracted materials will react with it. The products formed will not, however, be

hydroxyureas (unless other arylhydroxylamines are present in the analysis mixture). Analysis of liver homogenate mixtures not containing HAI, but carried through the entire analysis scheme, showed no interfering peaks at the retention volume at which HIMU (V_R = 7.8 ml) and dimethylaniline (V_R = 14.2 ml) elute from the column. Similarly, chromatographic analysis of underivatized (V_R = 12.2 ml) and derivatized (V_R = 10.0 ml) 5-aminoindane, 5-nitrosoindane (V_R = 26.1 ml), 5-nitroindane (V_R = 29.3 ml) and 5,5′-azoxyindane (V_R > 250 ml) (possible metabolic products or metabonates of HAI) did not produce interfering peaks in the region of the peaks of interest. Furthermore, 5-aminoindan (which is also derivatized with methyl isocyanate) and 5-nitroindan, which are the metabolic precursors for HAI, are eluted after the hydroxylamine; therefore, hydroxylamine can be determined in the presence of a large excess of the amine or nitro compound. Under the conditions of methyl isocyanate derivatization, 5-aminoindan was quantitatively converted to the corresponding methylurea, *i.e.* reaction mixtures containing the amine and subjected to isocyanate treatment show no peak at V_R = 12.2 ml; only a peak at 10.0 ml corresponding to the methylurea derivative.

DISCUSSION

A method has been described for the analysis of micromolar levels of the arylhydroxylamine HAI, present in liver homogenates. HAI serves as a model compound having reactivity and solubility characteristics similar to polynuclear aromatic derivatives, but without the proven potent carcinogenicity associated with the latter group. The major difficulty in quantitative analysis of arylhydroxylamines is their instability, *i.e.* at neutral pH they have a half life of *ca.* 20 min; at pH $\geqslant$ 10, the $t_{1/2}$ is less than 2 min[11].

The problem of analysis of such unstable compounds has been handled by either an on-line assay or by chemical conversion of the analyte to a more stable entity. On-line assays have used amperometric transducers[9,16,17] that cannot differentiate between hydroxylamines and aminophenols without prior HPLC separation of components[12]. The procedures are, therefore, either non-specific or time-consuming. Alternatively, arylhydroxylamines have been analyzed by conversion to nitroso[3] or azo[18] derivatives. These products are stable, but are indistinguishable from those formed from alternate amine metabolites or via other routes of biotransformation. The methods are, therefore, non-specific. Furthermore, these procedures are time-consuming and often involve carrying out operations at elevated pH —jeopardizing the stability of the analyte and, therefore, the accuracy of the determination. In this report an analytical procedure is described, in which the arylhydroxylamine is converted to a hydroxyurea by reaction with an isocyanate directly in the extraction solvent. The product is stable, both in the dry state and in solution, so that HPLC analysis of samples can be carried out at a leisurely pace once derivatization is complete. Most importantly, the derivative which is monitored by HPLC is specifically derived from the arylhydroxylamine, *i.e.* the analyte is converted to a product which is different than products formed from other amine metabolites or metabonates. The reagent reacts indiscriminantly with nucleophilic species, but the product that is monitored is only formed by reaction with the arylhydroxylamine. Thus, when

coupled with HPLC separation of components, the method is specific for the arylhydroxylamine.

Sample handling was minimized to insure maximum stability of the underivatized analyte. All operations leading to formation of the hydroxyurea —extraction, aspiration of the aqueous layer and reaction with methyl isocyanate— were carried out in one vessel and as rapidly as possible without compromising efficiency. In this way deterioration of the hydroxylamine was minimized.

The sensitivity limit of the method for the determination of HAI in liver homogenates with methyl isocyanate as the derivatizing agent was $2 \cdot 10^{-6}$ *M*. However, by appropriate selection of isocyanate (*i.e.* R in R–N=C=O), derivatives of hydroxylamines can be formed with radiochemical, strong spectrophotometric, fluorometric or electrochemical properties. Thus, the derivatization reaction can be tailored to the detection system that the investigator wishes to employ and modified to give the sensitivity required for a particular study. This method thus provides advantages for the analysis of arylhydroxylamines over the previously reported chromatographic methods[11,12]: (1) the analyte is converted to a stable species; (2) the hydroxyurea is formed only by reaction with an arylhydroxylamine, so that in this respect, derivatization is highly specific; and (3) the sensitivity of the method is greater than that previously described for analysis with spectrophotometric detection.

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CHROM. 10,796

GENTAMICIN C-COMPONENT RATIO DETERMINATION BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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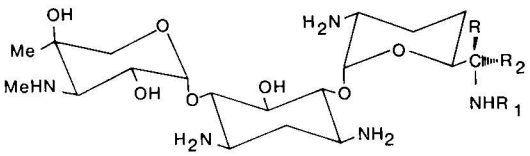
SUMMARY

A rapid and reproducible high-pressure liquid chromatographic determination of gentamicin has been developed. The analysis is performed by a combination of paired-ion chromatography, post-column derivatization and fluorescence detection. The results show gentamicin to be composed of three major components, C_1, C_2 and C_{1a}, and several minor components. The quantitative results are compared to those obtained by a microbiological method and are in excellent agreement. This technique is applicable to other aminoglycoside analyses.

INTRODUCTION

Gentamicin, an aminoglycoside antibiotic produced by the fermentation of *Micromonospora purpurea*, was introduced in 1969 and has a broad spectrum of activities against both gram-positive and gram-negative bacteria. It is composed of three major components, gentamicins C_1, C_2 and C_{1a}, and several minor components including gentamicins A, A_1, B, B_1, C_{2a} and C_{2b} (ref. 1).



Gentamicin C_1: $R = R_1 = Me$; $R_2 = H$

Gentamicin C_2: $R = Me$; $R_1 = R_2 = H$

Gentamicin C_{2a}: $R = R_1 = H$; $R_2 = Me$

Gentamicin C_{2b}: $R = R_2 = H$; $R_1 = Me$

Gentamicin C_{1a}: $R = R_2 = R_1 = H$

Chromatographic methods for analysis of the gentamicin complex have been reported[2–5]. Since these methods do not possess a combination of speed, specificity, sensitivity or precision, the most widely used method for analysis has been a microbiological assay. Microbiological assay, however, is time consuming and lacks specificity.

A rapid and reliable liquid chromatographic assay of each of the three major components of gentamicin has been developed. Most of the minor components are clearly separated and easily identified on each of the chromatograms studied. The procedure utilizes three relatively new techniques of liquid chromatography: (1) fluorescence detection; (2) post-column derivatization; and (3) ion-pairing chromatography. Reagents used in the separation are novel, and the post-column reaction apparatus is composed of commercially available components only slightly modified for the analysis. Detection is based on the reaction of *o*-phthalaldehyde with primary amines to give fluorescent products[6,7]. Recommended reagents for the analysis are all easily obtainable and require no special handling. The use of a similar technique for the assay of gentamicin in serum has recently been reported by one of us[8].

A second liquid chromatographic method of analysis utilizing more conventional chromatography has also been investigated. This method employs normal-phase chromatography and refractive index detection. Since this method incorporates a large volume of aqueous base as the mobile phase, the silica gel column is quickly degraded. This condition and the limited sensitivity make this technique less desirable than the ion-pairing method. However, this method has been acceptably applied to large-scale preparative chromatography[5].

Results from the liquid chromatographic analysis compared favorably with an assay performed according to the USP microbiological method.

EXPERIMENTAL

Reagents

The purified gentamicin components C_1, C_{1a} and C_2 were kindly provided by J. Allan Waitz (Schering Co., Bloomfield, N.J., U.S.A.). Gentamicin sulfate (USP reference standard) was obtained from Schering Co., *o*-phthalaldehyde (Fluoropa, manufactured by Durrum) from Pierce (Rockford, Ill., U.S.A.), 2-mercaptoethanol from Sigma (St. Louis, Mo., U.S.A.), methanol from Burdick & Jackson Labs., (Muskegon, Mich., U.S.A.) and sodium pentanesulfonate from Eastman-Kodak (Rochester, N.Y., U.S.A.). Water was deionized and glass-distilled. All other chemicals were of reagent grade. *o*-Phthalaldehyde reagent (OPA) was prepared by the method of Benson and Hare[7] with the exception that 5% Brij was added to reduce precipitation of polysulfide in the detector. Solutions of antibiotic were freshly prepared in distilled water at a concentration of 500 μg/ml.

Apparatus and chromatography for method I

A Waters M6000A (Waters Assoc., Milford, Mass., U.S.A.) pump was used to deliver the mobile phase. A Ferrand (Valhalla, N.Y., U.S.A.) Model RF-2 fluorometer equipped with 350-nm (excitation) and 450-nm (emission) filters was used to detect the product formed by continuous-flow, post-column derivatization with OPA reagent. The OPA reagent was delivered with a second Waters M6000A pump.

A zero dead volume T union (R. S. Crum, Inc., Springfield, N.J., U.S.A.) was used to introduce the OPA reagent into the chromatographic stream, and a reaction coil comprising a length of PTFE tubing (1.5 m × 0.7 mm I.D.) coiled to a 6-mm diameter was used between the mixing T union and detector. Analysis was performed using a Hibar LiChrosorb RP-8 column (25 cm × 3.0 mm I.D.) (E. Merck, Darmstadt, G.F.R.) with a mean particle size of 7 μm. 2-μl samples containing 500 μg/ml of antibiotic were injected using a Waters U6K injector.

The mobile phase contained 0.015 *M* sodium pentanesulfonate, 0.2 *M* sodium sulfate and 0.1% acetic acid in water. The mobile phase flow-rate was 1.5 ml/min at 3000 p.s.i., and OPA was introduced at 0.5 ml/min at 300 p.s.i. Reagents and mobile phase were filtered and degassed prior to use.

Apparatus and chromatography for method II

This procedure utilized a Waters M6000A pump, a μ-Partisil column (30 cm × 3.9 mm I.D.) (Waters Assoc.), a Waters refractive index detector and the U6K injector system. The mobile phase was composed of water–methanol–diethylamine (60:40:0.5). The mobile-phase flow-rate was 1 ml/min at 3000 p.s.i.

RESULTS AND DISCUSSION

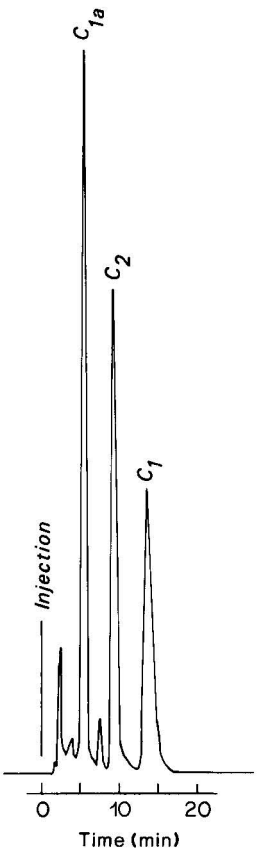
Fig. 1 is representative of the chromatograms obtained by method I. The elution order was C_{1a}, C_2, C_1. Polar impurities appear prior to these three components. Fig. 2 presents the results from method II. Note that the elution order has changed to C_2, C_{1a}, C_1, a reversal of C_2 and C_{1a} compared to the other method. The order and relative position of each component was determined using pure components injected separately. In method I, the retention times can be shortened by the addition of sodium sulfate and/or methanol. Greater retention times may be obtained by addition of greater amounts of sodium pentanesulfonate.

Component ratios of ten samples of gentamicin sulfate were evaluated using method I. These results were compared to those obtained using the official USP microbiological analysis. The results indicate an excellent agreement for the two techniques in almost all cases (Table I). Data from replicate ($N = 7$) assays by method I of USP Reference Standard are presented in Table II. The maximum coefficient of variation (CV) was 2.6%.

The results obtained from method II indicate this technique could be used for qualitative separation of the gentamicin C complex. However, column degradation during prolonged usage negated its usefulness as a reliable quantitative method of analysis. Recently, Sancilio *et al.*[5] investigated the usefulness of this method for preparative chromatographic separation, and small quantities of ultra-pure components of each of the three major gentamicin C components were obtained. The results from these experiments will be presented at a later time.

The geometry of the post-column reaction coil was also studied. Substitution of a stainless-steel coil of 3 mm in diameter gave increased response. All results presented in this report, however, were obtained using the reaction coil described above which was 6 mm in diameter.

In summary, rapid and reliable chromatographic systems for the analysis of gentamicin have been developed. One of these methods is a viable alternative to the



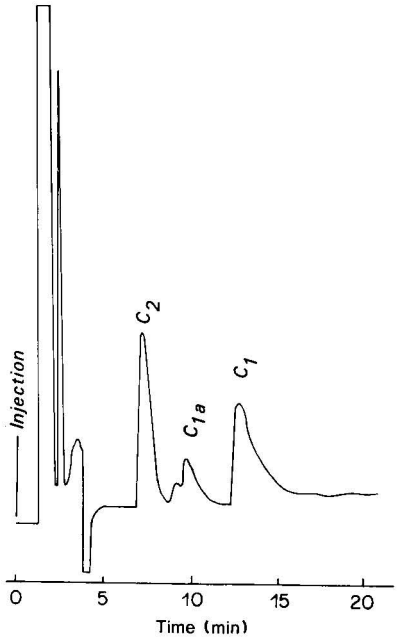


Fig. 1. Representative chromatogram of gentamicin by method I.

Fig. 2. Representative chromatogram of gentamicin by method II.

TABLE I

COMPARISON OF COMPONENT RATIOS FOR TEN BATCHES OF GENTAMICIN DETERMINED BY HPLC AND MICROBIOLOGICAL ASSAY

Batch	*HPLC assay (%)*			*Microbiological assay (%)*		
	C_1	C_2	C_{1a}	C_1	C_2	C_{1a}
1	35.6	35.6	28.8	34.6	33.5	31.9
2	34.5	34.9	30.6	33.5	34.0	32.5
3	36.6	36.0	27.4	33.6	37.0	29.4
4*	37.2	42.1	20.8	33.9	40.5	25.8
5	36.4	34.6	29.0	37.4	33.4	29.2
6	36.7	34.0	29.2	36.3	35.0	28.7
7	35.7	35.4	28.9	35.3	33.8	30.9
8	34.8	35.1	30.1	34.8	34.8	30.4
9	34.3	36.5	29.3	32.9	34.1	33.0
10	35.2	36.2	28.6	35.0	36.4	28.6

* USP Reference Standard.

TABLE II

REPLICATE ANALYSES OF COMPONENT RATIO FOR THE USP REFERENCE STANDARD OF GENTAMICIN SULFATE

Component	*Analysis number*							*Av. ± S.D.*	*CV (%)*
	1	*2*	*3*	*4*	*5*	*6*	*7*		
C_{1a}	20.4	21.2	20.0	21.1	21.0	21.4	20.3	20.8 ± 0.5	2.6
C_2	41.9	41.4	42.5	42.4	42.9	41.3	42.0	42.1 ± 0.6	1.4
C_1	37.7	37.4	37.5	36.5	36.1	37.3	37.7	37.2 ± 0.6	1.7

microbiological method used routinely for the analysis of gentamicin and gives comparable results. It has also been observed that this same methodology can be adapted to the quantitative analysis of other similar antibiotics. Additional work with neomycin, netilmicin, sisomicin and kanamycin has been completed and will be presented in a subsequent publication.

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QUANTITATIVE DÜNNSCHICHTCHROMATOGRAPHISCHE BESTIMMUNG DES MAKROLIDANTIBIOTIKUMS TURIMYCIN H UND EINIGER ABBAUPRODUKTE DURCH DENSITOMETRISCHE *IN-SITU*-MESSUNG MIT DEM BILDANALYSEGERÄT "QUANTIMET 720"

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SUMMARY

Quantitative thin-layer chromatographic determination of the macrolide antibiotic turimycin H and some of its degradation products by densitometric in situ measurement with the image analyser "Quantimet 720"

A method for the quantitative determination of the macrolide antibiotic complex turimycin H, its components turimycin H_2, H_3 and H_5, as well as the degradation products 4″-deacylturimycin H and demycarosylturimycin H, is described. Thin-layer chromatography is used for the separation of the mixtures. The image analyser "Quantimet 720" is employed for densitometric *in situ* quantitation. The reproducibility of evaluation of coloured spots on thin-layer chromatograms by image analysis is studied. Spotting errors, conditions of visualization and the sensitivity of the detector (vidicon tube) were responsible for the variance of repeated determinations. The method is demonstrated by analysis of some synthetic mixtures as well as turimycin complexes obtained from different fermentation batches.

EINLEITUNG

Die densitometrische Auswertung von Dünnschichtchromatogrammen ist eine schnelle und empfindliche Methode zur quantitativen *in-situ*-Bestimmung von Substanzen. Über Faktoren, die Reproduzierbarkeit und Genauigkeit dieser Technik beeinflussen, wurden wiederholt Untersuchungen veröffentlicht[1–4]. In letzter Zeit wurde diese Methode zur quantitativen Analyse der Antibiotika Tetracyclin[5], Erythromycin[6], Gentamycin[7], Everninomicin D[8], Penicillin[9], Antibiotikum SF-837[10] sowie der Polyenmakrolidantibiotika Candidin und Candihexin[11] angewandt.

Das Makrolidantibiotikum Turimycin wird von *Streptomyces hygroscopicus* JA 6599 gebildet und besteht aus einem Gemisch chemisch verwandter Komponenten. Auf Grund der strukturellen Ähnlichkeit der Komponenten sind sowohl spektrometrische als auch chemische und mikrobiologische Bestimmungsmethoden nur zur

Bestimmung des Gesamtkomplexes geeignet. Da die Zusammensetzung des Komplexes in Abhängigkeit von Fermentationsbedingungen und Reinigungsoperationen variieren kann und die einzelnen Komponenten unterschiedliche mikrobiologische Wirksamkeiten besitzen, ist eine Methode zur quantitativen Erfassung der Einzelkomponenten in einem Komplex von allgemeinem Interesse. Für pharmakologische und pharmakokinetische Untersuchungen ist ferner die quantitative Bestimmung von Metabolisierungsprodukten des Antibiotikums von Bedeutung.

In der vorliegenden Arbeit wird über eine dünnschichtchromatographische Methode zur Trennung von Turimycin-H-Komplex (THK) in seine Einzelkomponenten sowie über die Trennung des Turimycin H von 4″-Desacylturimycin H (4″-DATH) und Desmycarosylturimycin H (DMTH) berichtet. Die Struktur der Verbindungen ist in Fig. 1 dargestellt. Zur direkten densitometrischen Quantifizierung dieser Substanzen wurde ein Bildanalyseverfahren erarbeitet, das auf der Bestimmung der Extinktion eines farbigen Fleckes beruht.

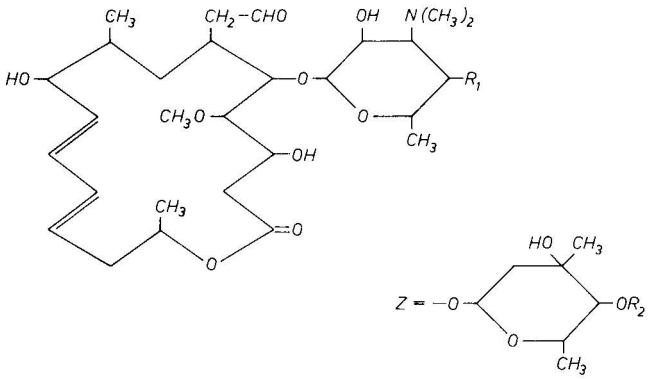

Fig. 1. Struktur von Abbauprodukten und Komponenten des Makrolidantibiotikums Turimycin H.

	R_1	R_2
Desmycarosylturimycin H	OH	-
4″-Desacylturimycin H	Z	H
Turimycin H_2	Z	$-CO-CH_3$
Turimycin H_3	Z	$-CO-CH_2-CH_3$
Turimycin H_5	Z	$-CO-CH_2-CH(CH_3)_2$

THEORIE UND METHODIK

Bildanalyseverfahren zur Substanzmengenbestimmung

In der Dünnschichtchromatographie (DC) ist die Grösse, die Form, die Farbsättigung und das Absorptionsprofil eines Chromatogrammfleckes abhängig von der Verteilung der Masse M einer Substanz am Adsorbens. Nimmt man an, dass die Extinktion bestimmter Bereiche des Fleckes konstant ist, so erhält man

$$M = \frac{1}{\varepsilon_\lambda} \sum_{i=1}^{n} E_{\lambda,i} \Delta F_i$$

wobei ΔF_i die Fläche der zur Extinktion $E_{\lambda,i}$ gehörenden Äquidensite i ist und ε_λ der

Extinktionskoeffizient der Substanz bei der Wellenlänge λ des Messlichtes. Unabhängig von der Objekttextur werden die Äquidensitenflächen ΔF_i mit dem Bildanalysator direct gemessen. Lediglich aus der Annahme der näherungsweise konstanten Extinktion im entsprechenden Extinktionsintervall resultiert ein Messfehler, der von der Empfindlichkeit des Empfängers abhängt. Bei vorgegebenem Kontrastumfang der Chromatogramme erreichten wir mit der Vidiconkamera ein Auflösungsvermögen von etwa 20 Graustufen. Die Eichung der Graustufen, die mit dem 2D-Detector in der Betriebsart "Autodelineation" in 20 äquidistanten Schritten programmierbar eingestellt wurden, ergab einen sehr gut linearen Zusammenhang zwischen Extinktionswert und dem Logarithmus des relativen Grauwertes, d.h. dem Verhältnis des Grauwertes der auf die Durchlässigkeit 100% normierten Umgebung zum aktuellen Grauwert des Objektes. Ein dem Messsystem on-line nachgeschalteter Tischrechner HP 9100 B verarbeitete die Daten nach obiger Gleichung. Die Mess- und Auswertezeit pro Fleck betrug 2 sec.

EXPERIMENTELLER TEIL

Geräte und Materialien

Bildanalysegerät "Quantimet 720" mit Vidiconkamera. Programmierbarer Tischrechner HP 9100 B. Glaschromatographiegefässe (20.5 × 12.5 × 21.0 cm; VEB Glaswerke Ilmenau, Ilmenau, D.D.R.). 5-μl-Einmalkapillarpipetten "capilette" (Labora Mannheim, Mannheim, B.R.D.). Kieselgel-Fertigplatten (20 cm × 20 cm × 0.25 mm; E. Merck, Darmstadt, B.R.D.).

Der THK wurde aus der Fermentationslösung von *Streptomyces hygroscopicus* JA 6599, Mutante R 27, isoliert. Die Einzelkomponenten Turimycin H_2, H_3 und H_5 wurden durch Säulenchromatographie aus dem Komplex abgetrennt und dünnschichtchromatographisch auf Reinheit überprüft. 4″-DATH wurde durch enzymatische Spaltung von Turimycin H_3 mit Hilfe eines Rattenleberhomogenisats hergestellt und säulenchromatographisch gereinigt. DMTH wurde durch saure hydrolytische Spaltung von THK erhalten und säulenchromatographisch gereinigt.

Standards

Je 24 mg THK, 4″-DATH und DMTH wurden gemeinsam in 10 ml Chloroform gelöst (Stammlösung I).

Je 24 mg Turimycinkomponenten H_2, H_3 und H_5 wurden gemeinsam in 10 ml Chloroform gelöst (Stammlösung II). Von beiden Stammlösungen wurden Verdünnungen mit Chloroform hergestellt, die 2, 3, 4 und 6 μg einer jeden Substanz pro 5 μl Lösung enthielten.

Dünnschichtchromatographie

Die zu untersuchenden Substanzen wurden als Chloroformlösungen in 5-μl-Portionen in 2 cm Entfernung von der unteren Kante und 2.7 cm von den Seitenkanten punktförmig auf die DC-Platten aufgetragen und mit den aus Fig. 2 und 3 ersichtlichen Lösungsmittelsystemen chromatographiert. Die Laufstrecke betrug in allen Fällen 13 cm. Zur Detektion wurden die Platten mit einer 5%igen Lösung von Phosphorwolframsäure in 10%iger Schwefelsäure besprüht und 30 min bei 90° im Trockenschrank erhitzt. Alle Substanzen ergaben violett-graue Flecke. Die fertigen

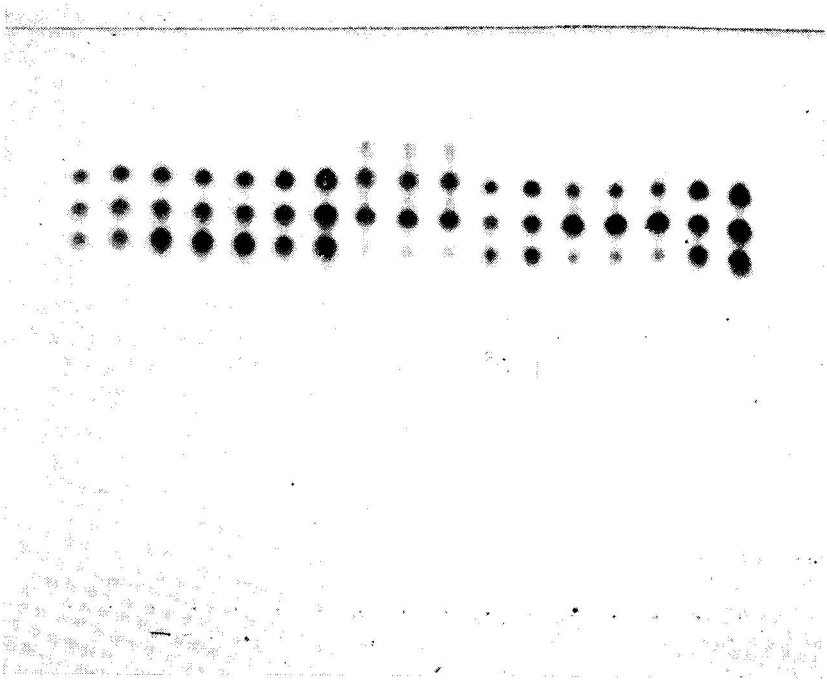

Fig. 2. Trennung von Turimycin-H-Komplexen. Lösungsmittelsysteme: Benzol–Methanol = 3:1 (1×) und Benzol–Methanol (4:1) (2×). Substanzen: Als Standard (St) wurde ein Gemisch der Turimycinkomponenten H_5 (obere Reihe), H_3 (mittlere Reihe) und H_2 (untere Reihe) im Gewichtsverhältnis 1:1:1 verwendet. Von links nach rechts wurden aufgetragen: St_1 (je 2 µg); St_2 (je 3 µg); dreimal synthetisches Gemisch (3 µg H_5, 3 µg H_3, 6 µg H_2); St_3 (je 4 µg); St_4 (je 6 µg); dreimal 9 µg Charge 112; St_1; St_2; dreimal 9 µg Charge 111; St_3; St_4.

Chromatogramme wurden mit Glasplatten abgedeckt und bis zur Messung im Dunkeln aufbewahrt.

ERGEBNISSE UND DISKUSSION

Dünnschichtchromatographische Trennung

Wie Fig. 2 zeigt, führt die DC des Antibiotikum-Komplexes in den angegebenen Systemen zur Auftrennung in die drei Hauptkomponenten Turimycin H_2, H_3 und H_5 mit den R_F-Werten 0.59, 0.65 und 0.71. Als Spurenkomponenten werden Turimycin H_4 (R_F-Wert 0.68) sowie eine unbekannte Verbindung mit dem R_F-Wert 0.77 beobachtet. Um Störungen durch diese Substanzen zu vermeiden, wurden bei Modellversuchen synthetische Gemische der drei Hauptkomponenten untersucht.

Bei der DC-Trennung eines Gemisches von DMTH, 4″-DATH und THK ergeben die beiden ersten Substanzen je einen einheitlichen Fleck beim R_F-Wert 0.10 bzw. 0.38, während eine beginnende Auftrennung des Turimycins zur Bildung eines Doppelflecks (R_F-Wert $\approx$ 0.6) führt (vgl. Fig. 3). Letzterer wird bei der quantitativen Bestimmung wie ein Fleck behandelt.

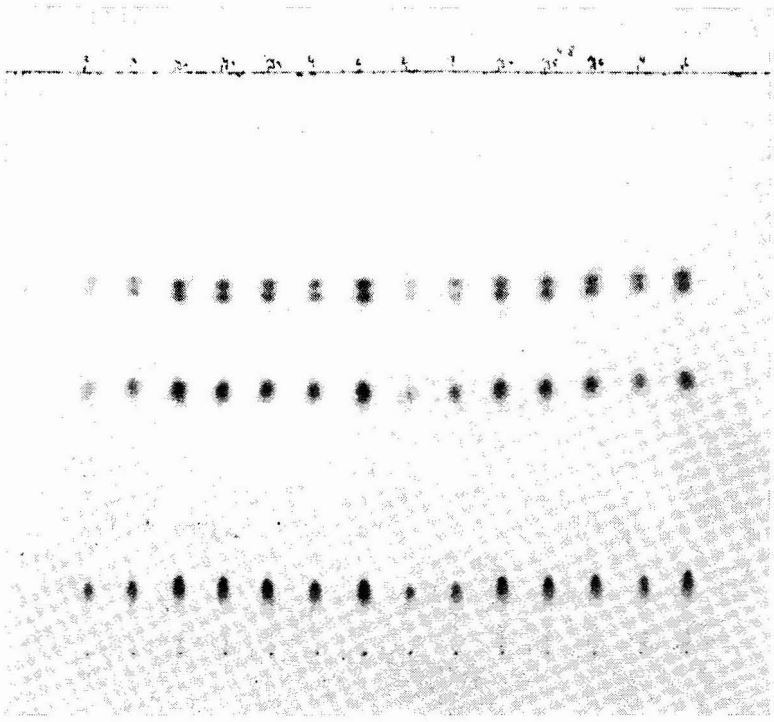

Fig. 3. Trennung von Desmycarosylturimycin H (untere Reihe), 4″-Desacylturimycin H (mittlere Reihe) und Turimycin-H-Komplex (obere Reihe). Lösungsmittelsystem: Benzol–Methanol (2:1). Substanzen: In der Reihenfolge von links nach rechts wurden folgende Mengen als Gemisch der drei Substanzen (Gewichtsverhältnis 1:1:1) aufgetragen: je 2 μg, je 3 μg, dreimal je 4.8 μg, je 4 μg, je 6 μg und Wiederholung in gleicher Reihenfolge.

Reproduzierbarkeit als Funktion der Anzahl der Messungen, der Probenmenge und der Anzahl der aufgetragenen Flecke

Je ein Fleck mit 2, 3, 4 und 6 μg 4″-DATH wurde nach chromatographischer Entwicklung und Detektion mehrmals ($n = 10$) vermessen. Als arithmetische Mittel der Messwerte ergaben sich: 33.37, 51.25, 74.04 und 114.05. Mit relativen Standardabweichungen zwischen 0.49 und 1.76% wird eine sehr gute Reproduzierbarkeit der Auswertung mit dem Quantimet erreicht.

Zur Untersuchung der Abhängigkeit der Messwerte von der Substanzart sowie des Einflusses von Auftragungsfehlern wurden definierte Mengen von DMTH, 4″-DATH und THK in 6facher Wiederholung punktförmig aufgetragen und chromatographiert. Die Flecke wurden zweimal vermessen, wobei die DC-Platten nach der ersten Messung um 180° gedreht wurden, um Unterschiede der Schichtdicke bzw. eine gewisse "Wolkigkeit" der Platten zu eliminieren. Vor jeder Messreihe wurde für jede Platte eine elektronische Ausleuchtungskorrektur vorgenommen.

Den Ergebnissen (Tabelle I) ist zu entnehmen, dass generell grössere Fehler als bei wiederholter Messung am Einzelfleck auftreten. Darüberhinaus werden Unterschiede zwischen den Messreihen a und b beobachtet, wenngleich die Übereinstimmung ausser bei 2.4 μg 4″-DATH im Rahmen der Genauigkeit des Auswertesystems in bezug auf einzelne Flecken befriedigend ist. Die vorhandenen Unterschiede

TABELLE I

REPRODUZIERBARKEIT DER MESSWERTE FÜR VERSCHIEDENE FLECKE GLEICHER SUBSTANZMENGE

Je 2.4 bzw. 4.8 μg THK, 4''-DATH und DMTH wurden 6mal auf derselben Platte aufgetragen; jeder Fleck wurde 1mal vermessen (Messreihe a); nach Drehung der DC-Platten um 180° wurde die Messung wiederholt (Messreihe b). $\bar{y}$ = Arithmetisches Mittel, s = Standardabweichung, $s\%$ = relative Standardabweichung.

	THK				*4''-DATH*				*DMTH*			
	2.4 µg		*4.8 µg*		*2.4 µg*		*4.8 µg*		*2.4 µg*		*4.8 µg*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Messwerte y	48.8	54.8	101.4	106.1	38.7	42.2	91.9	95.6	30.7	30.6	64.3	64.6
	46.2	47.8	97.1	100.7	35.9	39.6	88.3	89.5	31.2	29.6	67.4	66.9
	48.3	49.6	100.0	94.1	41.2	41.1	83.5	83.6	31.1	31.5	69.4	60.4
	51.5	51.5	92.0	94.5	44.6	42.2	77.9	82.0	32.4	27.8	58.1	63.5
	49.6	50.2	89.2	92.5	43.3	40.5	81.7	82.3	34.4	31.2	60.7	57.2
	50.7	46.8	89.7	91.1	45.7	41.8	74.7	72.1	35.1	30.4	64.5	61.0
$\bar{y}$	49.2	50.2	94.9	96.5	41.6	41.2	83.0	86.6	32.5	30.2	64.1	62.3
s	1.9	2.8	5.3	5.7	3.7	1.0	6.4	5.9	1.9	1.3	4.2	3.4
$s\%$	3.86	5.58	5.58	5.91	8.89	2.43	7.71	6.81	5.85	4.30	6.55	5.46
$\bar{s}\%_{oa,b}$	4.72		5.75		5.66		7.26		5.08		6.00	

zwischen beiden Messreihen weisen darauf hin, dass neben den Auftragungsfehlern zusätzlich apparativ bedingte Fehler auftreten. Dabei handelt es sich um ortsabhängige Restfehler der Ausleuchtungskorrektur sowie optische und elektronische Abbildungsfehler der Vidiconkamera. Diese Fehler werden jedoch auf Grund ihrer systematischen Natur bei Drehung der Platten zumindest teilwiese kompensiert. Als Mass für den mittleren Fehler der Substanzmengenbestimmung wird der Mittelwert der relativen Standardabweichung aus beiden Messreihen benutzt. Er liegt für THK bei 4.72 bzw. 5.75%, für 4''-DATH bei 5.66 und 7.26% und für DMTH bei 5.08 bzw. 6.0%.

Messwerte als Funktion der aufgetragenen Substanzmengen

Zur Untersuchung der Abhängigkeit der Messwerte von den aufgetragenen Substanzmengen wurden auf drei verschiedenen DC-Platten je 1–8 μg der drei Substanzen in 3- bzw. 4-facher Wiederholung punktförmig aufgetragen. Die relativen Standardabweichungen lagen für 4, 6 und 8 μg der drei Substanzen unter 5.05%, während für 1 und 2 μg Standardabweichungen bis zu 11% gefunden werden.

Zur Beurteilung der auf verschiedenen DC-Platten erhaltenen Ergebnisse wurden aus den experimentellen Daten einer jeden Platte für die drei Substanzen die Gleichungen der Regressionsgeraden in der allgemeinen Form

$$y = (m \pm s_m)\, x + (b \pm s_b)$$

ermittelt, in der x = aufgetragene Substanzmenge pro Fleck in μg, y = Messwerte, m = Regressionskoeffizient (Steigung der Regressionsgeraden), b = Schnittpunkt der Regressionsgeraden mit der y-Achse, s_m = Standardabweichung von m und s_b = Standardabweichung von b bedeuten (Tabelle II).

TABELLE II

KONSTANTEN DER REGRESSIONSGERADEN FÜR THK, 4''-DATH UND DMTH

	DC-Platte No.	m	s_m	b	s_b	r
THK	I	34.198	0.451	5.114	2.246	0.9986
	II	32.395	0.766	8.677	3.819	0.9956
	III	35.130	0.432	4.857	2.151	0.9988
4''-DATH	I	29.090	0.425	−9.034	2.120	0.9983
	II	33.309	0.531	−6.233	2.644	0.9980
	III	38.620	0.394	−5.026	1.965	0.9992
DMTH	I	15.850	0.149	2.885	0.743	0.9993
	II	19.062	0.283	6.347	1.409	0.9980
	III	17.508	0.231	0.922	1.152	0.9986

Korrelationskoeffizienten zwischen $r = 0.9956$ und 0.9993 lassen für alle drei Substanzen im untersuchten Konzentrationsbereich weitgehende lineare Abhängigkeit der Messwerte von den Substanzmengen erkennen. In Fig. 4 ist der Verlauf der Regressionsgeraden dargestellt. Wie ein Vergleich der Regressionskoeffizienten m und deren Standardabweichungen s_m zeigen, ist die Steigung der Regressionsgeraden

TABELLE III

QUANTITATIVE BESTIMMUNG EINES GEMISCHES VON THK, 4''-DATH UND DMTH

Analysenprobe No. 1–3: Je 4.8 μg der drei Substanzen.

Substanz	*Messreihe*	*Messwerte*							*Regressionsgerade*	*Substanzmenge gefunden (μg)*		
		Eichkurve		*Analysenprobe Nr.*			*Eichkurve*					
		2 μg	*3 μg*	*1*	*2*	*3*	*4 μg*	*6 μg*		*1*	*2*	*3*
THK	a	36.5	60.4	92.0	89.2	89.7	72.9	115.5	$y = 19.3x - 1.1$	4.82	4.68	4.70
	b	37.6	57.9	94.5	92.5	91.9	75.1	114.2	$y = 19.0x + 0.2$	4.98	4.88	4.81
4''-DATH	a	30.9	51.0	77.9	81.7	74.7	57.9	93.8	$y = 15.2x + 1.3$	5.04	5.29	4.83
	b	33.4	49.9	82.0	82.3	72.1	63.2	92.0	$y = 14.5x + 5.2$	5.30	5.32	4.61
DMTH	a	28.2	40.2	58.1	60.7	64.5	54.6	76.6	$y = 12.2x + 4.3$	4.41	4.62	4.93
	b	26.3	38.3	63.5	57.2	61.0	49.1	69.9	$y = 10.8x + 5.3$	5.39	4.81	5.16

für dieselbe Substanz auf verschiedenen DC-Platten unterschiedlich, obwohl die chromatographischen Bedingungen weitgehend standardisiert wurden. Das bedeutet, dass bei der Bestimmung unbekannter Substanzmengen jede DC-Platte durch Eichpunkte normiert werden muss.

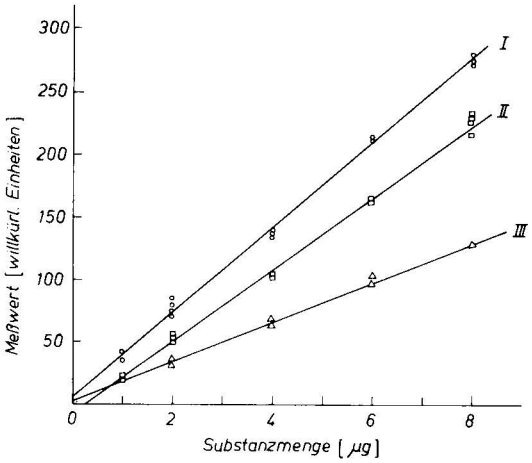

Fig. 4. Regressionsgeraden und Einzelmesswerte. I = Turimycin-H-Komplex, $y = (34.198 \pm 0.451)\, x + (5.114 \pm 2.246)$. II = 4″-Desacylturimycin H, $y = (29.09 \pm 0.425)\, x + (-9.034 \pm 2.12)$. III = Desmycarosylturimycin H, $y = (15.85 \pm 0.149)\, x + (2.885 \pm 0.74)$.

Quantitative Bestimmung von DMTH, 4″-DATH und THK in Gemischen

Aus den vorangehenden Untersuchungsergebnissen lässt sich ableiten, dass die Reproduzierbarkeit und Richtigkeit der Ergebnisse wesentlich von der Anzahl der Eichpunkte, der Anzahl der Analysenpunkte auf einer bzw. mehreren DC-Platten sowie von der aufgetragenen Substanzmenge abhängig ist. Folgender Substanzauftrage- und Auswertemodus hat sich als günstig erwiesen: Die zu analysierenden Proben werden 3fach zwischen je zwei verschiedenen Konzentrationen der Standards aufgetragen. Nach chromatographischer Entwicklung und Detektion werden die Flecke im Bildanalysator zwei mal in der bereits angegebenen Weise vermessen. Aus den Messwerten der Eichpunkte werden die Gleichungen der Regressionsgeraden ermittelt und daraus der Gehalt der Analysenproben errechnet. Zur Demonstration der Methode sind in Tabelle III Messwerte und Ergebnisse eines Modellversuches zusammengestellt.

In zwei weiteren Versuchsserien wurden Gemische von drei Substanzen auf je zwei verschiedenen DC-Platten analysiert (Tabelle IV). Die Korrelationsfaktoren der Eichgeraden variierten zwischen 0.9853 und 1.0. Zum besseren Vergleich wurden aus n Einzelbestimmungen Mittelwerte ($\bar{x}$), Standardabweichungen (s), relative Standardabweichungen ($s\,\%$) und Differenzen zwischen Mittelwert und Sollwert ($\bar{x}-X$) sowohl für jede Platte und Versuchsserie einzeln als auch für je zwei Platten gemeinsam berechnet. Während bei den Platten I/Ia mit 0.27 µg (5.6 %) die grösste Abweichung des Mittelwertes vom Sollwert bei der Bestimmung von 4.8 µg 4″-DATH gefunden wurde, werden bei den Platten II/IIa —insbesondere bei der 2. Versuchsserie— grössere Differenzen beobachtet, die im Falle der Bestimmung von 2.4 µg 4″-DATH 0.32 µg

TABELLE IV

ANALYSE EINES MODELLGEMISCHES VON THK, 4''-DATH UND DMTH BEI ZWEI VERSCHIEDENEN KONZENTRATIONEN

DC-Platte I und II je 2.4 μg; DC-Platte Ia und IIa je 4.8 μg; Abkürzungen siehe Text.

	THK				*4''-DATH*				*DMTH*			
	2.4 µg		*4.8 µg*		*2.4 µg*		*4.8 µg*		*2.4 µg*		*4.8 µg*	
	1	*2*	*1*	*2*	*1*	*2*	*1*	*2*	*1*	*2*	*1*	*2*
Platte I/Ia												
n	6	6	6	6	6	6	6	6	6	6	6	6
$\bar{x}$ (μg)	2.43	2.44	4.93	4.81	2.49	2.48	5.06	5.07	2.38	2.41	4.93	4.89
s (μg)	0.14	0.06	0.24	0.11	0.11	0.08	0.29	0.29	0.07	0.12	0.26	0.36
s%	5.76	2.46	4.87	2.29	4.42	3.23	5.73	5.72	2.94	4.98	5.72	7.36
$\bar{x} - X$ (μg)	0.03	0.04	0.13	0.01	0.09	0.08	0.26	0.27	0.02	0.01	0.13	0.09
Platte II/IIa												
n	6	6	6	6	6	6	6	6	3	2	6	6
$\bar{x}$ (μg)	2.38	2.55	4.84	5.18	2.47	2.08	4.94	4.80	2.33	2.26	5.16	4.81
s (μg)	0.08	0.23	0.13	0.19	0.15	0.21	0.15	0.11	0.12	0.23	0.13	0.09
s%	3.36	9.02	2.69	3.67	6.07	10.1	3.04	2.29	5.15	10.18	2.52	1.87
$\bar{x} - X$ (μg)	−0.02	0.15	0.04	0.38	0.07	−0.32	0.14	0	−0.07	−0.14	0.36	0.01
Platte I + II/Ia + IIa												
n	12	12	12	12	12	12	12	12	9	8	12	12
$\bar{x}$ (μg)	2.41	2.49	4.88	5.00	2.48	2.28	5.00	4.93	2.36	2.37	5.04	4.85
s (μg)	0.11	0.17	0.19	0.24	0.12	0.26	0.23	0.25	0.09	0.15	0.23	0.25
s%	4.55	6.83	3.89	4.80	4.84	11.4	4.60	5.07	3.81	6.33	4.56	5.15
$\bar{x} - X$ (μg)	0.01	0.09	0.08	0.20	0.08	−0.12	0.20	0.13	−0.04	−0.03	0.24	0.05

(13.1%) betragen. Bei der Bestimmung auf zwei verschiedenen DC-Platten werden jedoch für die Abweichungen der Mittelwerte von den Sollwerten in allen Fällen Werte $\leq 5\%$ gefunden. Die relativen Standardabweichungen für 4″-DATH liegen zwischen 4.6 und 11.4%, während die Werte für DMTH und THK zwischen 3.81 und 6.83% variieren.

Quantitative Bestimmung von Turimycin-Komponenten

Bei der quantitativen Bestimmung der Komponenten des Antibiotikums wurde nach den bereits beschriebenen Substanzauftrage- und Auswertemodus gearbeitet. In drei verschiedenen synthetischen Gemischen wurde die Wiederfindungsrate der Turimycin-Komponenten H_2, H_3 und H_5 bestimmt. Der Tabelle V ist zu entnehmen, dass im Mittel 97.8% der aufgetragenen Gesamtmenge wiedergefunden werden. Für die Wiederfindungsraten der Einzelkomponenten in den drei Gemischen ergeben sich im Mittel für Turimycin H_5 101.2%, für Turimycin H_3 99.7% und für Turimycin H_2 98.7%. Die relativen Standardabweichungen lagen bei der Bestimmung der Einzelkomponenten zwischen 1.65 und 9.25%.

TABELLE V

QUANTITATIVE BESTIMMUNG VON TURIMYCINKOMPONENTEN IN GEMISCHEN

Anzahl der Bestimmungen: $n = 6$.

Turimycin	*Substanzmengen der Komponente*						*s*	
	aufgetragen			*gefunden*			*µg*	*%*
	Komponente	*µg*	*%*	*Komponente*	*Mittel*			
					µg	*%*		
Gemisch A	H_5	3	25		3.02	25.17	0.05	1.65
	H_3	3	25		3.17	26.42	0.14	4.45
	H_2	6	50		5.60	46.67	0.11	1.98
Gemisch B	H_5	3	25		3.35	27.92	0.17	5.04
	H_3	6	50		5.45	45.42	0.20	3.58
	H_2	3	25		2.88	24.00	0.27	9.25
Gemisch C	H_5	6	50		5.47	45.58	0.24	4.35
	H_3	3	25		3.08	25.67	0.07	2.37
	H_2	3	25		3.20	26.67	0.07	2.23
Standard		9	100	H_5	2.44	27.11	0.10	4.08
				H_3	4.50	50.00	0.10	2.17
				H_2	1.42	15.78	0.07	4.57
Standard		18	100	H_5	5.67	31.50	0.21	3.69
				H_3	9.16	50.89	0.71	7.76
				H_2	3.28	18.22	0.38	11.65
Charge 111		9	100	H_5	2.45*	27.30	0.17	6.73
				H_3	4.55*	50.67	0.32	7.09
				H_2	1.67*	18.56	0.14	8.10
Charge 112		9	100	H_5	3.55	39.44	0.19	5.33
				H_3	3.35	37.22	0.26	7.67
				H_2	0.59	6.56	0.06	10.10
Charge 130		9	100	H_5	2.17	24.11	0.13	6.12
				H_3	4.98	55.33	0.12	2.30
				H_2	1.27	14.11	0.09	6.89

* $n = 12$.

Als Anwendung der Methode in der Praxis wurde die Zusammensetzung von drei verschiedenen Versuchschargen und einem als Standard verwendeten THK bestimmt. Der Gehalt an den Komponenten H_5, H_3 und H_2 beträgt beim Turimycin-H-Standard im Mittel 29.3, 50.5 bzw. 17.0%. Die Versuchschargen 111 und 130 stimmen in ihrer Zusammensetzung im wesentlichen mit dem Standard überein. Charge 112 unterscheidet sich dagegen eindeutig in der Zusammensetzung von allen anderen untersuchten Proben. Der Gehalt an Turimycin H_5 ist im Vergleich zu den anderen Chargen um 10–15% erhöht, während die Komponente H_3 um 13–18% erniedrigt ist. Diese Erhöhung der biologisch aktiveren Komponente H_5 erklärt zumindest teilweise die grössere antimikrobielle Wirksamkeit dieser Charge (1816 Einheiten/mg) im Vergleich zum Standard (1403 Einheiten/mg). Dagegen vermag die veränderte Zusammensetzung nicht die Unterschiede in der spezifischen Drehung (−72.7° gegenüber −82.0° bis −84.6° bei den übrigen Chargen) sowie das unterschiedliche UV-Verhalten ($E^{1\,cm}_{232\,nm}$ bei $c = 10\ \mu g/ml$ Methanol: 0.245 gegenüber 0.340–0.390) zu erklären. Vielmehr ist dafür eine bisher nicht als Einzelkomponente isolierte Verbindung unbekannter Struktur verantwortlich, die in den Dünnschichtchromatogrammen der Charge 112 nachweisbar ist und einen grösseren R_F-Wert besitzt als Turimycin H_5 (vgl. Fig. 2). Als Differenzbestimmung ergibt sich für diese Komponente ein Gehalt von 16.8%.

ZUSAMMENFASSUNG

Es wird eine Methode zur quantitativen Bestimmung des Makrolidantibiotikums Turimycin-H-Komplex, seiner Einzelkomponenten Turimycin H_2, H_3 und H_5 sowie der Abbauprodukte 4″-Desacylturimycin H und Desmycarosylturimycin H beschrieben. Die Trennung der Substanzgemische erfolgt durch Dünnschichtchromatographie. Zur densitometrischen *in-situ*-Bestimmung wurde das Bildanalysegerät "Quantimet 720" verwendet. Die Reproduzierbarkeit der bildanalytischen Auswertung farbiger Flecke auf Dünnschichtchromatogrammen wird untersucht. Für die Varianz bei Wiederholungsbestimmungen sind Auftragefehler, Detektionsbedingungen und die Empfindlichkeit des Detektors (Vidicon-Röhre) verantwortlich. Am Beispiel der Analyse einiger Modellgemische sowie verschiedener Fermentationschargen des Antibiotikumkomplexes wird die Methode demonstriert.

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CHROM. 10,737

SEPARATION OF METAL IONS USING AN AROMATIC *o*-HYDROXYOXIME RESIN

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SUMMARY

A chelating ion exchanger has been synthesized in which an *o*-hydroxypropiophenoneoxime group is attached chemically to the benzene ring of a divinylbenzene resin. On a short column of such resin, copper(II) is selectively adsorbed from pH 3.5 acetate solution or from pH 5 tartrate solution and can be subsequently eluted sharply by 0.1 *M* hydrochloric acid. Molybdenum(VI) is sorbed selectively from 0.1 *N* sulfuric acid solution and is eluted in a narrow band by aqueous sodium hydroxide. Numerous rapid column-chromatographic separations are reported using the resin, including some used in the analysis of NBS standard samples.

INTRODUCTION

Several hydroxyoximes have found use as analytical chelating reagents. Benzoin α-oxime and, more recently, two aliphatic α-hydroxyoximes[1] were used as selective reagents for solvent extraction of molybdenum(VI) from acid solution and of copper (II) from basic or weakly acid aqueous solution. Copper(II) has been extracted from acidic leach liquors by a commercial product that contains mixed aliphatic α-hydroxyoximes and *o*-hydroxybenzophenoneoximes[2]. Poddar studied *o*-hydroxyacetophenoneoxime as a reagent for copper and nickel[3,4], and Gupta and Malik[5] used this compound as a reagent for molybdenum(VI).

A resin containing an α-hydroxyoxime functional group might be expected to sorb copper(II) and molybdenum(VI) selectively at suitable pH values. Goodkin[6] synthesized such a resin [*Res*-CH(OH)C(=NOH)C_6H_5, where *Res* represents a polymeric divinylbenzene]; her resin was fairly selective for copper(II) at a pH of 5 or greater and for molybdenum(VI) over the pH range 1 to 4.

Here, we describe the synthesis and analytical properties of a chelating resin in which *o*-hydroxypropiophenoneoxime is chemically bonded to the polydivinylbenzene resin XAD-4. The resulting chelating resin is used for the separation and quantitative determination of both copper and molybdenum by high-performance liquid chromatography (HPLC). To check the practical applicability of this method, several NBS standard samples have been analyzed.

EXPERIMENTAL

Liquid chromatograph

A liquid chromatograph has been constructed that is more compact and easier to operate than the apparatus used in previous studies[7]. The entire unit is self-contained in a steel cabinet (14 in. × 14 in. × 14 in.). A schematic diagram of the unit is shown in Fig. 1, and some of the main features of the chromatograph are listed below.

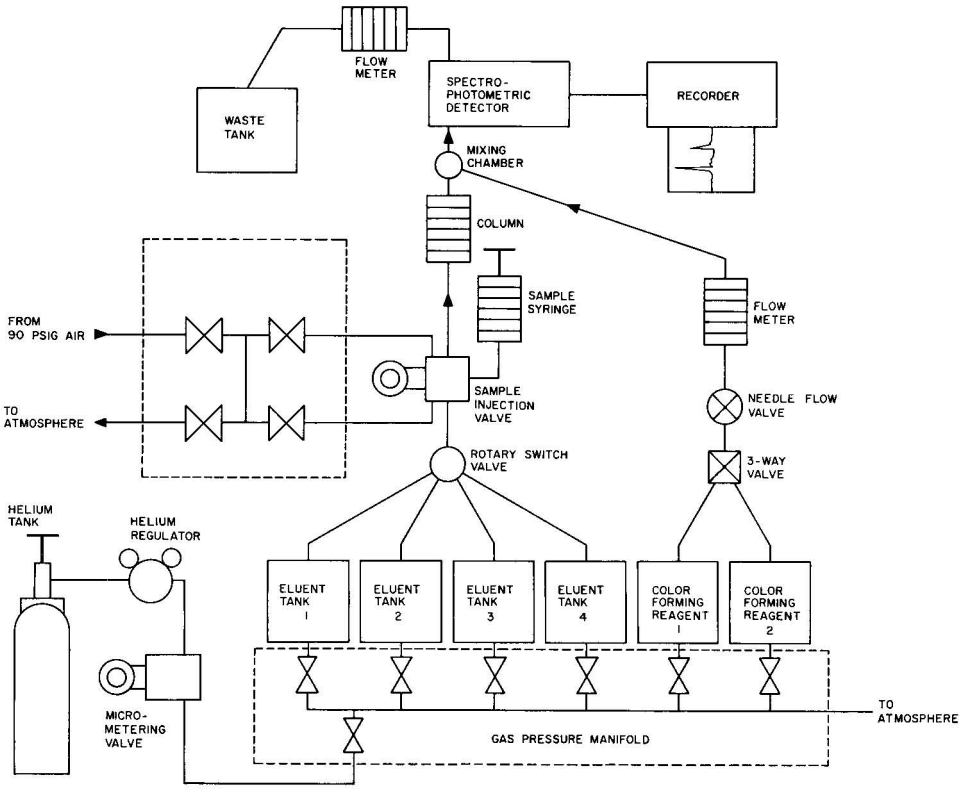


Fig. 1. Schematic diagram of the liquid chromatograph.

(1). The four eluent reservoirs are 500-ml glass reagent bottles with a 24/40 standard taper joint. The stippers for the bottles are machined from Kel-F and are held in place by No. 35 Thomas clamps. The bottles can withstand pressures up to 90 p.s.i. without danger of explosion. With this system, eluent bottles can be changed in less than 1 min.

(2). Eluent selection is effected by a 6-way rotary selector valve (Altex 202-01), which allows quick selection of any of the eluents with a minimum amount of mixing.

(3). There are two reservoirs that contain dye. Either of the two dyes may be used by switching a 3-way valve (Altex 201-51): this makes it possible to use both dyes in the same run without having to stop operation, change the dye bottle, and start again. The dye flow-rate is regulated by a chemically inert needle valve placed after the 3-way valve.

Detector

A Tracor 970 variable-wavelength detector was used for continuous spectrophotometric monitoring of the column effluent. All chromatograms were recorded on a Houston Instruments Omniscribe recorder.

Column

The analytical column was constructed from two Altex 200-28 glass connectors fused together. The column had an I.D. of 2 mm and an over-all length of 70 mm; a helium pressure of 26 p.s.i. was required to attain a flow-rate of 2 ml/min.

Synthesis of the resin

α-Bromo-2-hydroxypropiophenone was prepared by the method of Buu-Hoi and Lavit[8]. *o*-Hydroxypropiophenone (20 g) in 200 ml of glacial acetic acid was cooled in an ice bath, and bromine (22 g) in 30 ml of the same solvent was added dropwise, with stirring. After complete decolorization, the mixture was diluted with water, and the yellow oil formed was dissolved in chloroform. The chloroform layer was dried over magnesium sulfate and vacuum distilled, α-bromo-2-hydroxypropiophenone being collected at 145–155°/15 mm. (Caution, this compound is lachrymatory and should be handled with care.)

XAD-4, a macroporous styrene–divinylbenzene copolymer, was obtained from the Rohm& Haas Co. The beads were extracted overnight with methanol in a Soxhlet apparatus, dried, ground and sifted; the 250–325 mesh fraction was further dried in a vacuum oven at 110° to ensure complete removal of water and methanol. The reaction vessel consisted of a 500-ml 3-neck-flask equipped with a mechanical stirrer, reflux condenser, dropping-funnel and nitrogen inlet. XAD-4 (5 g) was slowly added to 33 g of anhydrous aluminium choride suspended in 100 ml of dichloroethane, and 17 g of α-bromo-2-hydroxypropiophenone in 25 ml of dichloroethane were added (dropwise, with stirring) to the mixture. After complete addition (0.5 h), the mixture was heated gently under reflux for 4 h. The entire reaction was performed under a nitrogen atmosphere to exclude water. The resin was hydrolyzed by being poured into an ice–hydrochloric acid mixture, and was then washed with water, 1 *M* hydrochloric acid and acetone, and dried. The product was oximated by being heated under reflux for 2 g with 15 g of hydroxylamine hydrochloride in 50 ml of absolute ethanol. Upon cooling, the resin was washed several times with water, then with methanol, then with acetone, and dried.

Color-forming reagents

PAR[4-(2-pyridylazo)resorcinol] was used for the detection of manganese(II), iron(II), iron(III), cobalt(II), nickel(II), copper(II), zinc(II), cadmium(II) and uranium(VI); these species were all monitored at 495 nm. The PAR solution was prepared by dissolving 121.1 g of THAM (Tris) in *ca.* 750 ml of distilled water, then adding PAR (0.125 g) and adjusting the pH to 9.0 with concentrated hydrochloric acid. The final solution was diluted to 1 l with distilled water. A red precipitate formed after several days, which necessitated filtering the solution; a corresponding loss in dye strength was also evident after filtering.

Arsenazo I was used for the detection of calcium, magnesium, thorium and

copper(II), all of which were monitoted at 590 nm. The arsenazo I solution was prepared as described above for PAR.

Fritz and Sutton[9] have shown that bis-(2-hydroxyethyl) dithiocarbamate will form a water-soluble yellow complex with copper(II). This reagent was easily prepared by adding 0.82 g of carbon disulfide to 105 g of diethanolamine, diluting to 750 ml with distilled water, adjusting the pH to 9.5 with hydrochloric acid, and diluting the solution to 1 l. Copper(II) was detected at 433 nm, at which wavelength the molar absorptivity is 11,400.

Thiolactic (2-mercaptopropionic) acid was used for detection of molybdenum[10]. The reagent was prepared by adding 57.5 ml of glacial acetic acid to 900 ml of water and adjusting the pH to 4.0 with 20% sodium hydroxide solution. Then 1.90 g of thiolactic acid were added, and the solution was diluted to 1 l. Molybdenum was detected at 365 nm with this reagent.

Separation procedure

Copper. The following procedure was used to separate copper from non-interfering metal ions.

(1) Before application of the sample to the column, allow 0.1 *M* tartrate buffer (pH 5) to flow through the column at 2 ml/min for 5 min.

(2) Inject the sample on to the column.

(3) Allow pH 5 tartrate buffer to flow through the column at 2 ml/min for 5 min to separate the non-interfering ion from copper.

(4) Change the eluent to 0.1 *M* hydrochloric acid at a flow-rate of 2 ml/min for 5 min to elute copper.

When NBS standards were analyzed, steps 1 and 3 were shortened to 2.5 min each; this allowed an analysis to be completed in 10 min.

Molybdenum. The following procedure was used to separate molybdenum from non-interfering metal ions.

(1) Before application of the sample to the column, allow 0.05 *M* sulfuric acid to flow through the column at 2 ml/min for 5 min.

(2) Inject the sample on to the column.

(3) Allow 0.05 M sulfuric acid to flow through the column at 2 ml/min for 5 min to separate the non-interfering ion form molybdenum.

(4) Change the eluent to 0.1 *M* sodium hydroxide at a flow-rate of 2 ml/min for 5 min to elute molybdenum.

When NBS standards were analyzed steps 1 and 3 were shortened to 2.5 min; this allowed the analysis to be completed in 10 min.

Analysis of NBS standards

Copper. Approximately 0.15 g of NBS sample 94A, a nickel–copper alloy, was d,ssolved in 5 ml of aqua regia, and the solution was transferred to a 500-ml volumetric flask, diluted to to the mark with pH 5 tartrate buffer and then analyzed for copper as described in the previous section.

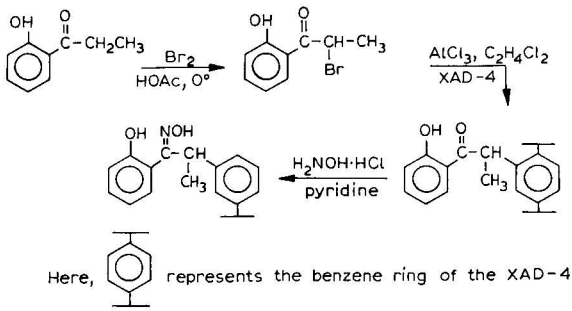
Molybdenum. Approximately 0.95 g of NBS sample 160, a chromium–nickel–molybdenum steel, was dissolved in 50 ml of sulfuric acid (1:6). The solution was cooled, transferred to a 250-ml volumetric flask, diluted to the mark with distilled water, then analyzed for molybdenum as described in the previous section.

Approximately 0.35 g of NBS sample 132 A, a tungsten–chromium–molybdenum–vanadium steel, was dissolved in 5 ml of concentrated hydrofluoric acid and 4 ml of concentrated nitric acid in a platinum dish. After cooling, the solution was transferred to a 250-ml volumetric flask containing 50 ml each of saturated sodium borate solution and 0.5 *M* sodium tartrate. The resulting mixture was diluted to the mark with distilled water; this gave a solution of pH between 2 and 3, in which molybdenum was determined as described in the previous section.

RESULTS AND DISCUSSION

Resin synthesis

To produce a chelating resin with favorable kinetics that can be used in column chromatography, it is necessary to start with a resin matrix that is rigid and highly porous; previous experience had shown Rohm and Haas XAD-4 to be an excellent starting material. Several synthetic routes were tried in an attempt to introduce a hydroxyoxime functional group into this resin in high yield. The Friedel–Crafts reaction was first tried, using α-bromo-2-hydroxyacetophenone. However, Trahanovsky[11] pointed out that this would involve the formation of a primary carbonium ion, which would make the reaction very unfavorable. For this reason, α-bromo-2-hydroxypropiophenone was chosen as the reagent. The addition of a methyl group in a position *alpha* to the bromine allowed a secondary carbonium ion to form, thus making the reaction more favorable.



Elemental analysis gave an average nitrogen content of 1.77%. Assuming that all of the nitrogen is from the oxime, 1 g of resin should contain 1.26 mequiv. of the desired functional group.

The IR spectra of XAD-4 and of the hydroxyoxime resin are shown in Fig. 2. A large hydroxyl stretch from the phenol, and an oxime C=N stretch evident at 1700 cm^{-1}, are evident.

Copper(II) sorption

Previous studies by Fritz *et al.*[1] and Goodkin[6] have shown that *o*-hydroxyoximes can form complexes with copper in basic or slightly acidic (pH 5) medium. In both of these studies, the hydroxyl functional group was an alcohol, which is a much weaker acid than a phenol. The resin used in our work contains a phenolic group and would therefore be expected to sorb copper at a lower pH; this proved to be the case.

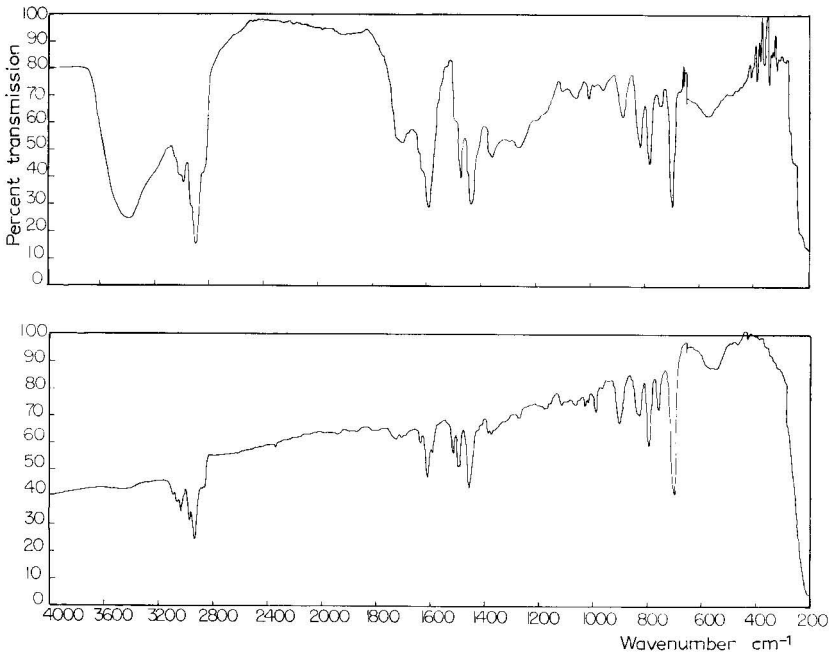


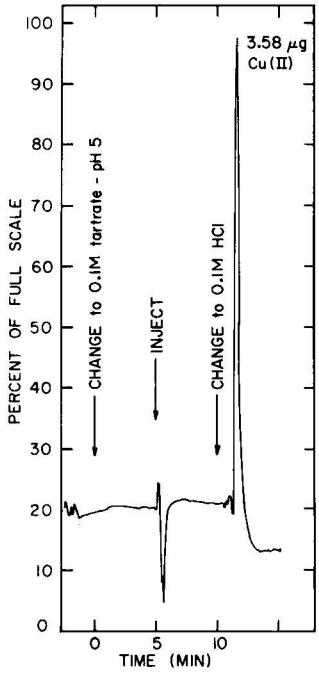
Fig. 2. Infrared spectra. Top: *o*-Hydroxypropiophenone oxime resin. Bottom: XAD-4 resin.

The resin will retain copper completely from a column in a 0.1 *M* acetate buffer down to pH 3.5; below this pH, the copper bleeds off the column. The resin will also completely retain copper from 0.1 *M* tartrate buffer at the same pH. Goodkin found that, when using a tartrate buffer with the α-hydroxyoxime resin, it was necessary to raise the pH from 5 to 10 to retain copper completely. This is further evidence that the *o*-hydroxyxyoximes are stronger complexers for copper. There was no advantage in working at pH 3.5, so all separations were performed using 0.1 *M* tartrate buffer at pH 5. Higher acid concentrations were tried, but the slight increase in peak height was more than offset by the larger base-line shift. A typical elution of copper is shown in Fig. 3.

Attempts to construct a calibration graph for copper(II) using PAR as color-forming reagent in the detector gave a non-linear curve with a very limited copper range (Fig. 4). A reagent with better selectivity for copper, bis-(2-hydroxyethyl) dithiocarbamate, was then tried. This reagent is easy to prepare and works very well for copper(II), as shown in Fig. 4.

Copper(II) can be sorbed selectively on column of resin from aqueous solution of pH 3.5 or higher, or from tartrate solution of pH 5. The latter condition permits separation of copper(II) from a large number of other metal cations. In the tartrate buffer, the resin had essentially no affinity for zinc(II), nickel(II), cobalt(II) or manganese(II), these species being eluted completely from the column in less than 2 min. Fig. 5 shows the separation of copper from equimolar amounts of zinc and nickel. It is of interest to note that copper and nickel, metals whose chemistry is similar, are completely separated into sharp bands.

Quantitative studies in tartrate solution of pH 5 showed that copper(II) could



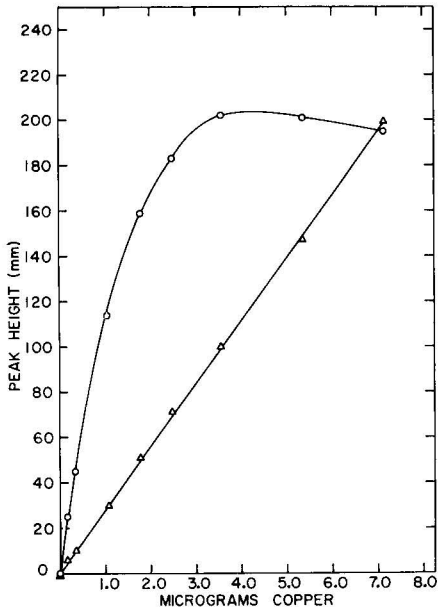


Fig. 3. Sorption and subsequent elution of copper(II).

Fig. 4. Comparison of calibration graphs for determination of copper. ○, with PAR; △, with bis-(2-hydroxyethyl)dithiocarbamate.

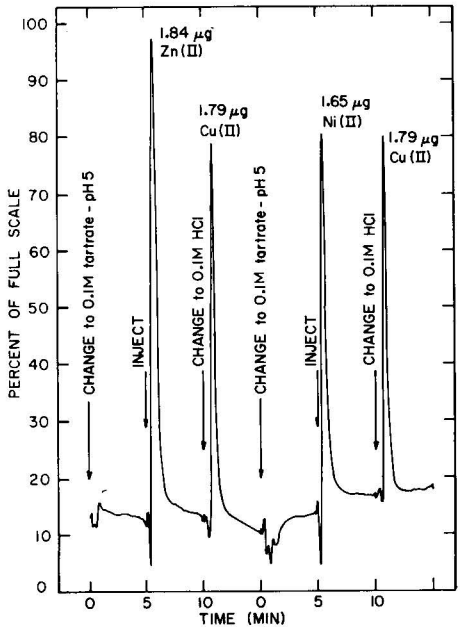


Fig. 5. Separation of copper(II) from equimolar amounts of zinc(II) and nickel(II).

TABLE I
INTERFERENCE STUDY OF FOREIGN IONS ON DETERMINATIONS OF COPPER(II) AND MOLYBDENUM(VI) (EACH 0.0005 *M*)

Ion	*Concn. (M)*	*Copper determination**	*Molybdenum determination**
Acetate	0.10	±	±
Aluminum(III)	0.0005	±	±
Cadmium(II)	0.0005	±	±
Calcium(II)	0.005	±	±
Chloride	0.10	±	±
Chromium(III)	0.0005	±	±
Citrate	0.10	−	
Cobalt	0.001		±
Copper(II)	0.005		±
Fluoride	0.10	−	±
Iron(III)	0.001	+	±
Lead(II)	0.0005	±	±
Magnesium(II)	0.05	±	±
Manganese(II)	0.0005	±	±
Mercury(II)	0.0005	±	±
Molybdenum(VI)	0.0005	±	
Nickel(II)	0.0005	±	±
Nitrate	0.10	±	±
Phosphate	0.10	±	±
Sulfate	0.10	±	
Thorium(IV)	0.0005	±	±
Tungsten(VI)	0.001		±
Uranium(VI)	0.0001	+	±
Vanadium(V)	0.001		±
Zinc(II)	0.0005	±	±

* ± means that the peak height deviated by ±3% or less from that on a calibration graph; + indicates high results and − indicates low results.

be separated from many foreign metal ions and anions (see Table I). Of the metals studied, only iron(III) and uranium(VI) are taken up by the resin under the same conditions as copper(II); these ,like copper, are eluted with 0.1 *M* hydrochloric acid. After sorbing both copper(II) and uranium(VI) from tartrate solution of pH 5, the copper can be eluted with 0.01 *M* EDTA (pH 6.0), leaving the uranium intact; then the uranium can be stripped with 0.1 *M* hydrochloric acid. However, this separation does not lend itself well to automatic chromatic detection.

Iron(III) is by far the most important practical interference. Fluoride was tried unsyccessfully as a masking agent, and attempts to avoid interference by reduction to iron(II) with ascorbic acid also failed. Some iron is eluted from pH 5 tartrate solution, but some is retained and subsequently eluted with the copper.

Molybdenum(VI) sorption

Studies by Gupta and Malik[5], Fritz *et al.*[1] and Goodkin[6] have shown that hydroxyoximes form complexes with molybdenum(VI) in acidic media, the actual complexing species being the molybdyl ion, MoO_2^{+2}.

A series of eluents was prepared in which the pH was varied from 0 to 5, and retention of molybdenum by the resin in each of these eluents was determined.

Molybdenum was completely retained on the column at pH values between 1 and 4, but below and above this range the element slowly bled off the column. The eluent used throughout this work to load the column with molybdenum was 0.1 *M* sulfuric acid.

The molybdenum could be stripped from the column in a narrow band by switching to 0.1 *M* sodium hydroxide. Various concentrations of ammonium hydroxide were also tried as stripping eluents; each of these gave a peak with considerable tailing, indicating that the resin–molybdenum complex was not being broken up as readily as with the sodium hydroxide eluent.

With 0.1 *N* sulfuric acid as eluent, molybdenum(VI) was successfully separated from 17 other metal ions (see Table I); no foreign metal ion investigated interfered. As copper(II) was sorbed by the resin only at a higher pH, molybdenum could also be separated from copper (see Fig. 6). Thiolactic acid is an excellent and delective color-forming reagent for molybdenum(VI). With thiolactic acid, the calibration graph of peak height *vs.* molybdenum content is rectilinear for 1 to 11 μg of molybdenum.

The best proof of any method of analysis is how well actual samples can be analyzed. Results of three independent analyses for each of three NBS standard samples are shown in Table II; these results are in excellent agreement with the certified values.

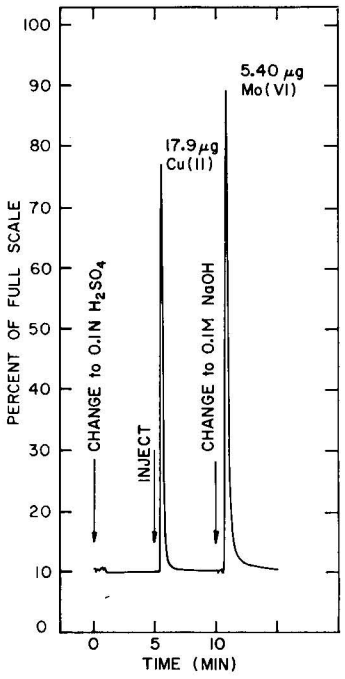


Fig. 6. Separation of molybdenum(VI) from a fivefold molar excess of copper(II).

TABLE II

ANALYSIS OF NBS STANDARDS

Sample number	*Element present*	*Content present (%)*	*Content found (%)*	*Standard deviation*
94A	Ni	66.38		
	Cu	28.93	28.94	0.171
	Mn	2.34		
	Si, Co, Fe, Cr	<1		
	Al, Ti, C, S	<1		
160	Cr	19.12		
	Ni	8.91		
	Mo	2.95	2.96	0.074
	Si	1.13		
	Mn	<1		
	C, P, S, Cu	<0.1		
	V, Co, N	<0.1		
132A	W	6.20		
	Mo	4.51	4.53	0.055
	Cr	4.21		
	V	1.94		
	C, Mn, Si, Cu, Ni	<1		
	P, S	<0.1		

ACKNOWLEDGMENT

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Note

Degradation of pentafluorobenzyl bromide in the extractive alkylation procedure

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(Received December 1st, 1977)

Extractive alkylation with pentafluorobenzyl bromide has been used for the derivatization of organic acids prior to analysis by gas chromatography with electron-capture detection[1–4]. The principle of this one-step extraction and derivatization procedure is the partition of the acid anion as an ion pair with a quaternary ammonium ion to an organic phase containing the alkylating reagent.

In this study, the degradation of pentafluorobenzyl bromide has been studied with methylene chloride as the organic phase and aqueous phases with different pH values, buffer systems and quaternary ammonium ions.

EXPERIMENTAL

Apparatus

A Varian 1400 gas chromatograph with a flame-ionization detector was used. The glass column (150 × 0.2 cm I.D.) was filled with 5% Carbowax terephthalic acid on Chromosorb W (80–100 mesh) or 15% OV-225 on Gas-Chrom Q (100–120 mesh).

Reagents and chemicals

Pentafluorobenzyl bromide was obtained from Pierce (Rockford, Ill., U.S.A.) or P.C.R. (Gainesville, Fla., U.S.A.) and pentafluorobenzyl alcohol from Fluka (Buchs, Switzerland). Tetrabutylammonium hydrogen sulphate was purchased from Labkemi (Gothenburg, Sweden). Tetrapropyl- and tetrapentylammonium iodide were purchased from Eastman (Rochester, N.Y., U.S.A.) and converted into the corresponding hydroxides by shaking with silver oxide[5].

Quaternary ammonium ion solutions (0.1 *M*) were prepared by neutralization of the corresponding hydroxide or hydrogen sulphate solution with sulphuric acid or sodium hydroxide.

Methods

The degradation of pentafluorobenzyl bromide was studied in a system containing equal volumes of a methylene chloride solution of pentafluorobenzyl bromide and an internal standard (mesitylene) and a 0.1 *M* solution of quaternary ammonium ion in sodium hydroxide or buffer. The mixtures were shaken for the appropriate

time at 25°. A suitable volume of the organic phase was withdrawn and shaken with 0.1 *M* sulphuric acid before analysis.

Quantitation was effected with the aid of the internal standard and reference solutions of pentafluorobenzyl bromide and alcohol.

RESULTS AND DISCUSSION

Degradation products of pentafluorobenzyl bromide

Pentafluorobenzyl bromide is degraded to pentafluorobenzyl alcohol and chloride and bispentafluorobenzyl ether. Pentafluorobenzyl chloride is formed from pentafluorobenzyl bromide and chloride ions, liberated by the degradation of methylene chloride under alkaline conditions[6].

The time course of the degradation of pentafluorobenzyl bromide and the formation of degradation products is demonstrated in Fig. 1. The change in the concentration of pentafluorobenzyl alcohol and the ether is small after 8 h, when little pentafluorobenzyl bromide remains.

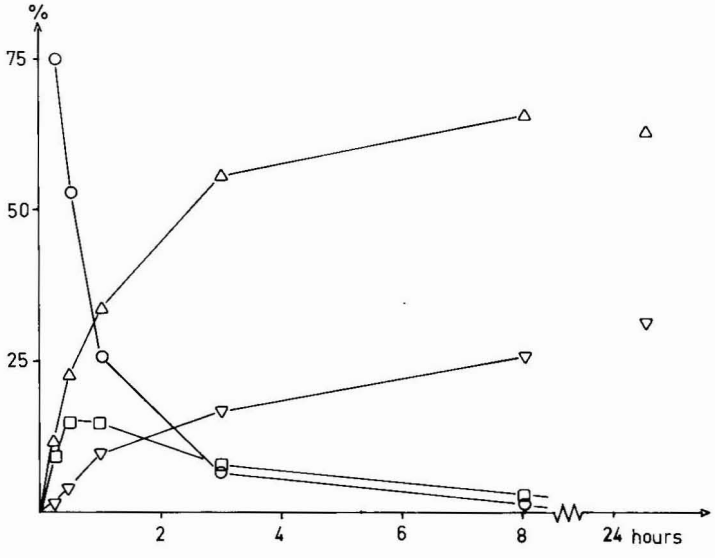

Fig. 1. Degradation of pentafluorobenzyl bromide. Organic phase: methylene chloride with 0.066 *M* pentafluorobenzyl bromide. Aqueous phase: tetrapentylammonium (0.1 *M*) in 0.25 *M* carbonate buffer (pH 10). Equal phase volumes. ○, Pentafluorobenzyl bromide; □, pentafluorobenzyl chloried △, pentafluorobenzyl alcohol; △, bispentafluorobenzyl ether.

Low concentrations (< 1 %) of pentafluorobenzyl alcohol were observed when the aqueous phase contained 0.1 *M* sodium hydroxide and tetrapentylammonium was used as the counter ion. The concentration of bispentafluorobenzyl ether increased as long as pentafluorobenzyl bromide was present. This indicates an almost immediate conversion of the alcohol into the ether at the high pH used.

The recoveries of pentafluorobenzyl bromide and its degradation products were in most instances above 90% of the initial concentration. In the presence of phosphate and tris buffers the recovery was only about 50% (24 h).

Influence of the counter ion and hydroxyl ion concentration

The degradation of pentafluorobenzyl bromide using aqueous phases with different quaternary ammonium ions is demonstrated in Table I. The degradation of pentafluorobenzyl bromide increases with increasing lipophilic character of the counter ion. This is probably due to an enhanced extraction of hydroxyl ions into the organic phase[7], which increase the hydrolysis rate of the reagent. This is supported by the data given in Table II, which shows the degradation of pentafluorobenzyl bromide in the presence of different concentrations of hydroxyl ions with tetrabutylammonium as the counter ion. More than 20% of the initial pentafluorobenzyl bromide concentration has been destroyed after 30 min in 1 *M* sodium hydroxide solution.

TABLE I

INFLUENCE OF THE COUNTER ION ON THE DEGRADATION OF PENTAFLUOROBENZYL BROMIDE

Organic phase: methylene chloride with 0.066 *M* pentafluorobenzyl bromide. Aqueous phase: tetraalkylammonium ion (0.1 *M*) in 0.1 *M* sodium hydroxide solution. Equal phase volumes.

Counter ion	*Pentafluorobenzyl bromide (%) remaining after*			
	0.5 h	*1 h*	*3 h*	*24 h*
Tetrapropylammonium	100	96	104	97
Tetrabutylammonium	99	94	90	56
Tetrapentylammonium	32	21	9	2

TABLE II

INFLUENCE OF THE HYDROXYL ION CONCENTRATION ON THE DEGRADATION OF PENTAFLUOROBENZYL BROMIDE

Organic phase: methylene chloride with 0.066 *M* pentafluorobenzyl bromide. Aqueous phase: tetrabutylammonium (0.1 *M*) in 0.1 or 1 *M* sodium hydroxide solution. Equal phase volumes.

Concentration of sodium hydroxide solution (M)	*Pentafluorobenzyl bromide (%) remaining after*			
	0.5 h	*1 h*	*3 h*	*24 h*
0.1	99	94	90	56
1	78	58	33	6

TABLE III

INFLUENCE OF THE BUFFER SYSTEM ON THE DEGRADATION OF PENTAFLUOROBENZYL BROMIDE

Organic phase: methylene chloride with 0.007 *M* pentafluorobenzyl bromide. Aqueous phase: tetrapentylammonium (0.1 *M*) in 0.25 *M* buffer (pH 10). Equal phase volumes.

Buffer system	*Pentafluorobenzyl bromide (%) remaining after*					*ΔpH*
	0.5 h	*1 h*	*2 h*	*4 h*	*24 h*	
Citrate	64	43	17	0		−0.46
Carbonate	69	43	21	12	0	−0.24
Phosphate	94	91	79	73	24	0.43
Borate	94	82	81	61	20	−0.36
Tris	95	80	75	70	36	−0.28

Influence of the buffer systems

The stability of pentafluorobenzyl bromide was studied using five different buffer systems. The concentration change with time is given in Table III together with the shift in pH observed after 24 h. The initial concentration of pentafluorobenzyl bromide was 10 times lower in these experiments than under ordinary extractive alkylation conditions. Preliminary studies showed pronounced pH deviations (citrate −2.1, phosphate −1.8) in the buffer systems with the normal pentafluorobenzyl bromide concentration.

From the data given in Table III it can be seen that carbonate and citrate buffers are more detrimental to the stability than are borate, phosphate and tris buffers.

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Note

Capillary-column combined gas chromatography–mass spectrometry method for the estimation of nicotine in plasma by selective ion monitoring

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The gas chromatographic (GC) determination of nicotine in plasma has been carried out previously using a nitrogen-selective flame ionization detector (NFID)[1,2]. These methods, although sensitive, are not entirely specific for nicotine. GC peaks, corresponding in retention time to nicotine, were obtained from the plasma of non-smokers[1,2]. Using mass spectrometry (MS), Horning *et al.*[3] were able positively to identify nicotine in the urine of non-smokers, and these authors concluded that non-smokers absorb nicotine (via the lungs) from room air.

GC–MS was used to confirm the identity of nicotine extracted from whole blood, in the development of a GC method for the estimation of nicotine[4]. However, this GC–MS method required 100 ml of whole blood, and quantitation of nicotine was not carried out. A preliminary account of a GC–MS method for the quantitative determination of nicotine in plasma, using selective ion monitoring (SIM), was described briefly by Pilotti *et al.*[5], who used a GC column packed with 8% Carbowax 20M (2% KOH) on Gas-Chrom Q (100–120 mesh), and deuterated nicotine as internal standard.

In the present method, good resolution, sensitivity and specificity were obtained by attaching a glass capillary column directly to a mass spectrometer, which was operated in the SIM mode. Nicotine was extracted from plasma by a modification of the method of Fayerabend *et al.*[2], which allowed the direct addition of the internal standard to plasma prior to extraction. Quinoline used as internal standard in the NFID–GC method[2], was found to be a convenient internal standard in the present method.

Glassware

Glassware was cleaned by immersion overnight in a detergent (Pyroneg) solution, rinsed with water, ethanol and distilled water[4], then dried in an oven in a laboratory where smoking was strictly prohibited.

EXPERIMENTAL

Extraction of nicotine from plasma

An aqueous solution of quinoline (5 μg/ml; 100 μl) was added as internal

standard to plasma (3 ml) in a 12.5-ml glass-stoppered centrifuge tube, followed by sodium hydroxide (5 *N*; 2 ml) and diethyl ether (3 ml). The tube was agitated on a vortex mixer (1 min) and then shaken for 10 min on a mechanical shaker. The organic layer was recovered (after centrifuging) and extraction of the aqueous layer was repeated using a further 3 ml of ether. The combined ether extracts were evaporated in a stream of nitrogen, and concentrated to 200–500 μl. Hydrochloric acid (2 *N*; 200 μl) was added and the mixture was agitated for two min, centrifuged, and the ether removed. The aqueous layer was washed with ether (0.5 ml), the ether removed, and any remaining on top was blown off in a stream of nitrogen. The aqueous solution was transferred to a dryer tube, sodium hydroxide (5 *N*; 400 μl) was added and the tube was agitated by vortex mixer. Benzene (100 μl) was added and the tube was agitated, centrifuged, and 0.8 μl of the benzene layer was injected into the GC–MS.

Gas chromatography–mass spectrometry

A Varian 1400 gas chromatograph equipped with a capillary injector (split ratio 1:10) and a glass capillary column (20 m × 0.3 mm I.D.) coated with SP1000 (Supelco, Bellefonte, Pa., U.S.A.) was used. The column temperature was 160°, and the helium flow-rate 0.5 ml/min.

A V.G. (Altrincham, England) Micromass 12B mass spectrometer was directly coupled via a 0.15 mm I.D. glass capillary restriction to the gas chromatograph. Operating conditions for the mass spectrometer were: interface, 250°; ion source, 200°; ionising potential, 70 eV; accelerating voltage, 4 kV; and source pressure, 10^{-5} torr.

Only the base peak of the nicotine spectrum (*m/e* 84) and the molecular ion of quinoline (*m/e* 129) were monitored (SIM). The accelerating voltage was switched between 2.6 and 4 kV to bring these ions into focus.

A Rikadenki B-36 pen recorder was used to record the *m/e* 84 and *m/e* 129 signals at 20–50 mV and 200 mV respectively. The chart speed was 200 mm/h.

RESULTS AND DISCUSSION

Mass spectra of nicotine and quinoline

The mass spectra of nicotine (a) and quinoline (b) are illustrated in Fig. 1. The base peak of the nicotine spectrum (*m/e* 84), produced by α-cleavage of the α-substituted pyrrolidine with the loss of pyridine[6], was sufficiently abundant to be measured quantitatively by SIM over the required range of nicotine concentrations. The molecular ion of quinoline (*m/e* 129), which is also the base peak, was sufficiently abundant and close enough in mass to the base peak of nicotine for quinoline to be used as the internal standard.

Sensitivity

The mass chromatograms of nicotine standards along with their respective quinoline internal standard, used to construct a calibration curve, are shown in Fig. 2. Nicotine was added to plasma, in duplicate, to give 100, 75, 50 and 25 ng/ml final concentrations, and extracted together with a plasma blank as described above. The lower limit of detection of nicotine was below 5 ng/ml.

To prolong filament life, and lengthen the time between cleanings, the power

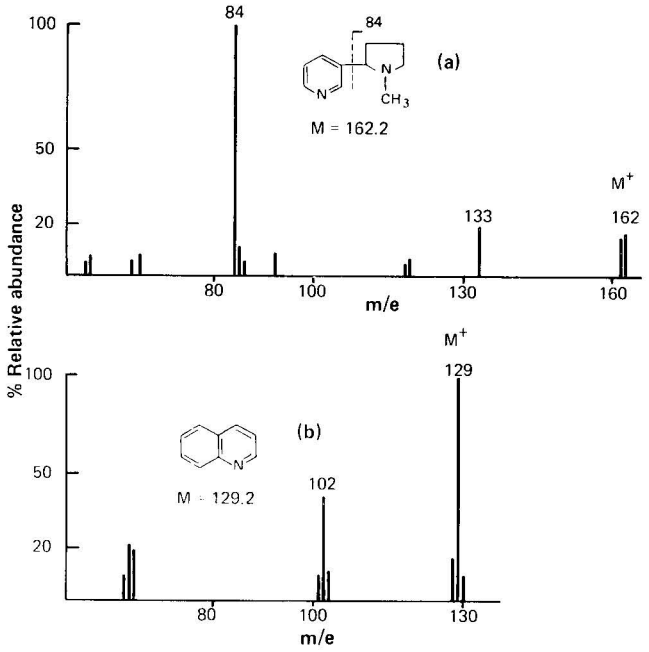

Fig. 1. Electron impact mass spectra of (a) nicotine and (b) quinoline. Only ions with relative abundance > 5% have been plotted.

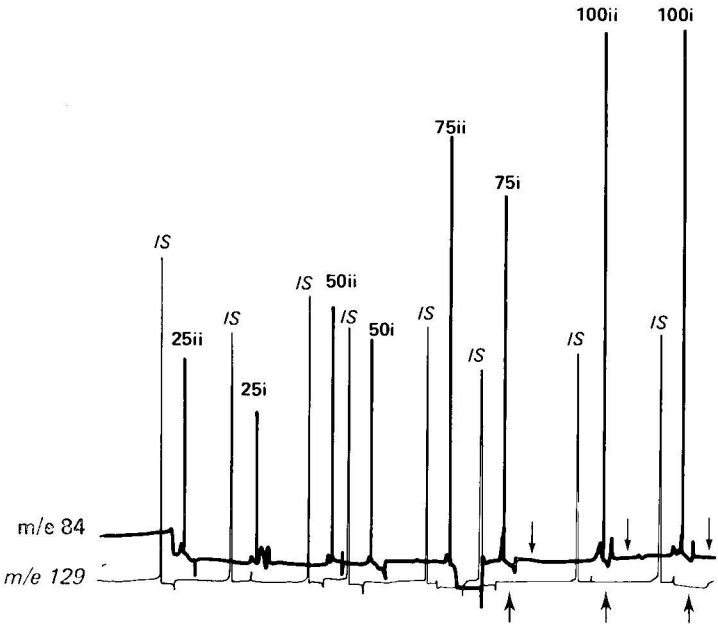

Fig. 2. Mass chromatograms of duplicate (i and ii) nicotine standards and their respective quinoline internal standards (*IS*) extracted from plasma. The arrows indicate the point of injection for the first three samples, and the recorder pens are offset by the distance between the top and bottom arrow. The retention times for nicotine and quinoline were 2.4 and 3 min, respectively.

supply to the source was switched off after each injection, and reset after the solvent peak had been observed on the vacuum gauge. No solvent peaks therefore appear in the chromatograms (Fig. 2).

The calibration curve (Fig. 3) was constructed by plotting the ratio of the peak heights of the *m/e* 84 ion of nicotine, and the *m/e* 129 ion of quinoline (from the chromatograms in Fig. 2) against the concentration of nicotine (ng/ml). This plot is linear over the measured concentration range and was also shown to be linear over the concentration range 5–100 ng nicotine/ml. The line does not pass through the origin as nicotine is detectable in the plasma blank. The concentration of nicotine in plasma was calculated from: (peak height ratio−intercept)/gradient.

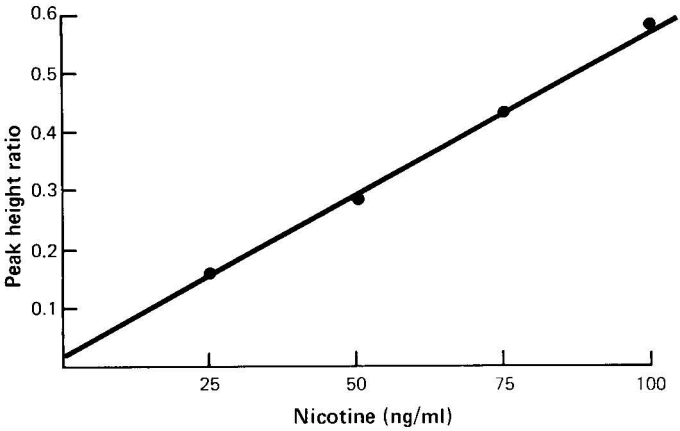

Fig. 3. Calibration curve of duplicate nicotine standards extracted from plasma. The peak height ratio of the *m/e* 84 and *m/e* 129 ions is plotted against concentration of nicotine. This follows the equation for a straight line, with a slope of 0.005, an ordinate intercept of 0.016, and a correlation coefficient of 0.99.

Reproducibility

Nicotine was added to plasma in quadruplet, to give solutions containing 50 and 10 ng/ml. After extraction, the means and standard deviations were (51.9 $\pm$ 2.4) and (10.6 $\pm$ 0.8) respectively. This compares favourably with values of (49.76 $\pm$ 1.04) and (10.23 $\pm$ 0.67) for ten determinations on 50 and 10 ng/ml standards obtained by Fayerabend *et al.*[2] using NFID–GC.

Specificity

Although SIM is not completely specific for a particular molecule, SIM combined with capillary-column GC–MS has a greater specificity due to the high resolution of capillary columns.

The minor tobacco alkaloid anabasine is a potential interfering substance as it also has a base peak at *m/e* 84[6], but since it has double the retention time of nicotine, when analysed on a glass capillary column similar to that used in the present study[5], it is unlikely to interfere.

Measurement of nicotine elimination from plasma

A plot of the decline in plasma nicotine concentration against time is shown in

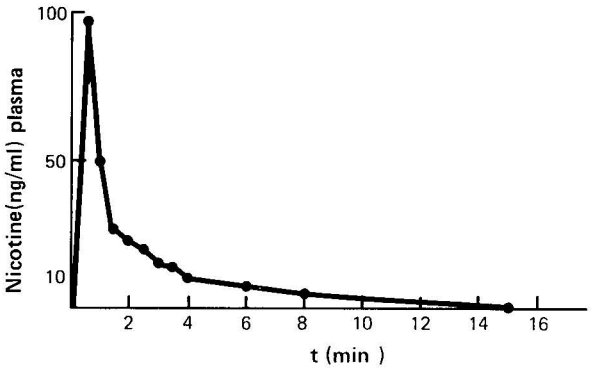

Fig. 4. Decline in plasma nicotine concentration against time in the dog. 10 μg nicotine/kg body weight was injected intravenously, and blood samples taken at the indicated times.

Fig. 4. Nicotine was measured by the present method, which is currently being applied to studies of nicotine elimination in man and experimental animals.

CONCLUSIONS

Nicotine has been estimated in plasma using capillary column GC–MS. This has produced a sensitive and specific method, without the need for deuterated nicotine as internal standard. Although only a small volume (< 0.1 μl) of sample is actually injected into the GC–MS, the sensitivity of the method is enhanced by the sharpness of the mass chromatogram peaks produced by the glass capillary column.

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CHROM. 10,839

Note

Detection of sulphonamides in urine by pyrolysis–gas chromatography–mass spectrometry

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We have previously described the pyrolysis–gas chromatography–mass spectrometry of a series of medicinal sulphonamides[1]. It was shown that each sulphonamide yielded a unique pyrogram and the principal mode of decomposition was fission about the labile sulphonamido group. Fragmentation yielded aniline from all medicinal sulphonamides, and a heterocyclic amine which was characteristic of the sulphonamide under test. Here we wish to describe the detection of these drugs in urine samples and to show that on the basis of fragmentations previously described, the identification of sulphonamides in urine may be accomplished using pyrolysis methods.

EXPERIMENTAL

Preparation of samples

An aliquot of urine (≈25 ml), frozen until required, was freeze dried. The residue was taken up in a small amount of water (0.5 ml) and the resulting paste was coated onto the rotating pyrolysis wire[2]. Wires were then placed in a desiccator, under vacuum, for 30 min to yield a firmly adhering coat.

Apparatus and conditions

A Pye Curie Point pyrolyser was connected to a Pye GCV gas chromatograph (dual columns and flame ionisation detector) or a Pye 104 gas chromatograph interfaced to a Micromass 12B mass spectrometer. Pyrolysis was performed at 770°, maintained for 5 sec and chromatography was carried out using 1.5 m × 4 mm I.D. columns packed with 8% Carbowax 20M and 2% KOH on Chromosorb W AW DMCS (100–120 mesh). The temperature was programmed from 100° (5 min hold) at 5°/min up to 245° (8 min hold). The injection port was held at 275° and the detector oven at 350°. The air pressure was maintained at 0.5 kg/cm², the hydrogen at 1.4 kg/cm² and a flow-rate of 50 ml/min (nitrogen, helium) was used. Mass spectra were collected with an ionisation energy of 22 eV, a trap current of 100 μA and an accelerating voltage of 4 kV.

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RESULTS AND DISCUSSION

The pyrolysis of an aliquot of the total solids obtained from lyophilised urine yielded a characteristic pyrogram and typical examples may be seen in Figs. 1 and 2. Samples taken from different individuals or samples taken at different intervals during the day showed no great variation in appearance. The overall pyrograms were surprisingly simple at attenuations necessary for the detection of excreted drugs in view of the complex mixture of biochemicals present[3]. A control sample was established using the 24-h total solids obtained from a normal individual. Pyrolysis of the urine solids from a patient undergoing sulphonamide medication yielded a pyrogram in which the peaks due to the urine components could be established by comparison

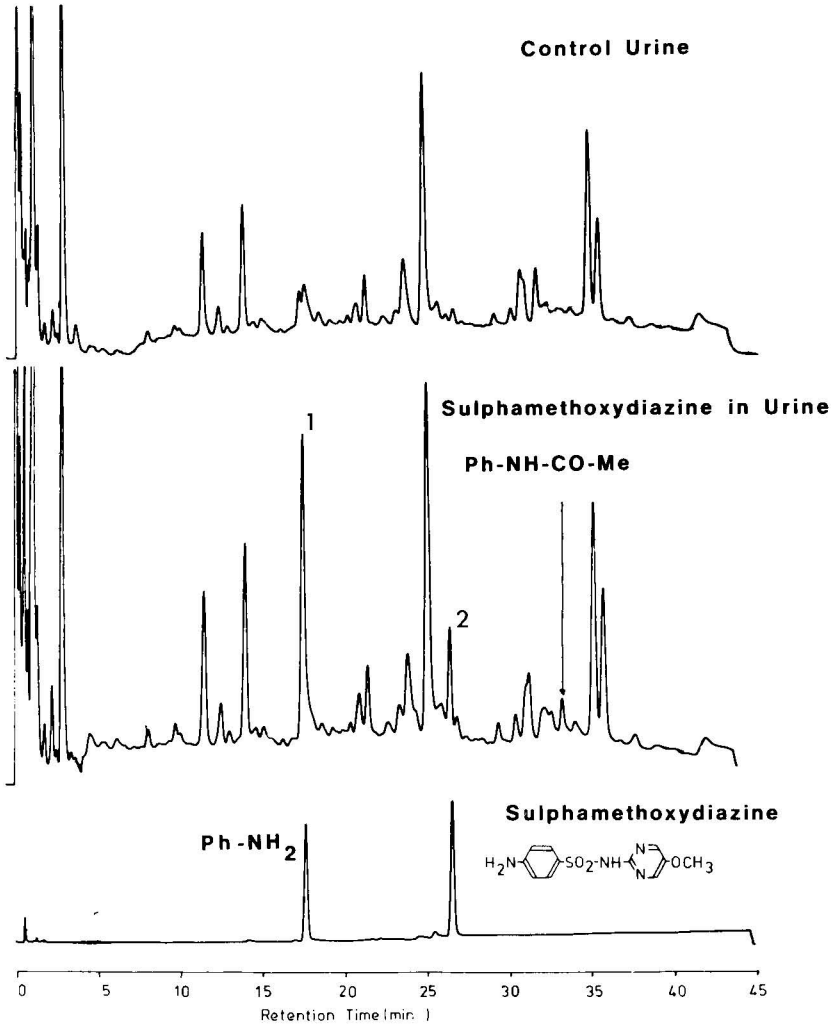

Fig. 1. Detection of sulphamethoxydiazine in urine showing the presence of aniline (1), 2-amino-5-methoxypyrimidine (2) and acetanilide.

with the control sample. In addition, it was seen that the fragmentation of the sulphonamide also present in this sample was essentially identical to that found in the pure drug[1,4]. Thus the sulphonamide could readily be identified in the urine by the presence of the characteristic pyrolysis fragments superimposed upon those of normal urine. Furthermore the presence of the N-acetyl metabolite could also be detected. The pyrolysis mode of this component was similar to that of the parent sulphonamide and yielded the characteristic heterocyclic amine and acetanilide.

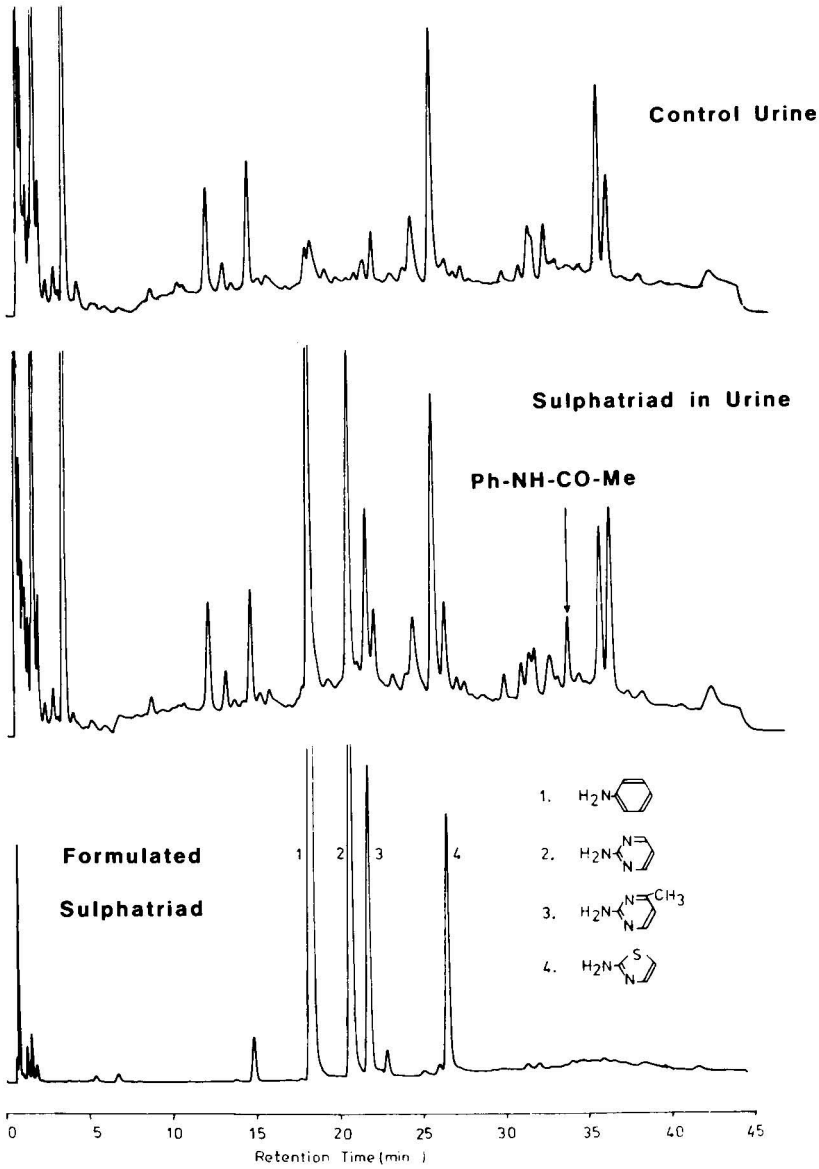

Fig. 2. Detection of sulphatriad in urine showing the presence of aniline (1), 2-aminopyrimidine (2), 2-amino-4-methylpyrimidine (3), 2-aminothiazole (4), and acetanilide.

The factors determining the sensitivity of this method are unusual. The principal consideration is not the detection limit of the sulphonamide (under appropriate conditions 50 ng may be detected and normally 50 μg are pyrolysed) but rather the proportion of drug to total solids which allows the detection of the drug against the urine background. At levels maintained during the clinical use of these drugs, the detection was found to pose no problems. Fig. 1 records the pyrogram obtained from sulphamethoxydiazine. This is relatively low-dosage sulphonamide[5] (maintenance dose 500 mg daily) and a lower than normal attenuation is required in this analysis. The urine background is more significant than in other examples but nevertheless the diagnostic features (*i.e.* aniline, 2-amino-5-methoxy pyrimidine and acetanilide) are clearly visible. The acetanilide is a rather weakly intense peak in this pyrogram as the N-acetyl metabolite is a minor component ($\approx$20%).

This system also enables the presence of mixed sulphonamides to be determined. Fig. 2 shows the pyrogram obtained from the urine of a patient undergoing treatment with sulphatriad. This is a mixed sulphonamide preparation which contains sulphadiazine, sulphamerazine and sulphathiazole to ensure a duration of action. Aniline and acetanilide are again present and act as internal standards. 2-Aminopyrimidine, 2-amino-4-methylpyrimidine and 2-aminothiazole, respectively, are derived from the sulphonamides and enable the identification to be achieved. Quantitative pyrolysis has already been used in the analysis of biologically important molecules and the application of these techniques to the above results may prove of value[6–8].

ACKNOWLEDGEMENTS

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CHROM. 10,836

Note

Gas chromatographic determination of methanal traces in presence of other volatile carbonyl compounds

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(Received November 14th, 1977)

The determination of methanal in products such as flavourants containing large amounts of volatile carbonyl compounds is, in most cases, impossible with the classical optical methods using reagents such as acetylacetone, chromotropic acid, albumin–iron reagent, 4-amino-3-hydrazino-5-mercapto, 1,2,4-triazol, etc. The direct gas-chromatographic detection on molecular sieve is not sufficiently sensitive. For these reasons, we have adapted the gas-chromatographic separation of 2,4-dinitrophenylhydrazone suggested by Soukup *et al.*[1] to obtain a method allowing trace determination at the 10 ppm level.

EXPERIMENTAL

Reagents

The reagents used were as follows: 2,4-dinitrophenylhydrazine (DNP); sulphuric acid; DNP reagent: dissolve 1.0 g DNP in 20 ml sulphuric acid and a few drops of water, and dilute slowly to 100 ml with water; benzene; all were of analytical grade.

Apparatus

The apparatus consisted of: test tubes (3 ml); electric stirrer for test tubes (Vortex-Genie); 100 and 600 μl piston pipettes (Eppendorf) gas chromatograph (Perkin-Elmer 900 or similar); glass column (4 m $\times$ $\frac{1}{8}$ in. I.D.); stationary phase: 4% SE-30 + 4% OV-17 on Chromosorb W HP (100–120 mesh) or 10% OV-101 on Chromosorb W HP (100–120 mesh). The operating temperature was programmed from 150–220° (6°/min) with the injector and manifold at 300°. The carrier gas was nitrogen (30 ml/min) and detection was by flame ionisation.

METHOD

In a test tube, add 600 μl of DNP reagent to 100 μl of sample. Mix well and allow to stand for 15 h (temperature: 1–3°). In the same test tube, just before the injection, extract the derivative in 100 μl benzene. Ensure an excess of reagent has

been used: the water phase remains yellow after the extraction. Inject 2 μl of benzene solution.

For quantitative determination, use internal standards of methanal.

RESULTS

This method was found suitable for determination of methanal at the 10 ppm level in concentrated liquid apple and smoke flavours.

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CHROM. 10,844

Note

Sephadex columns equilibrated with NaCl to purify invertase, acid phosphatase and glycosidases from plants

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During purification of invertase[1], acid phosphatase[2] and β-glucosidase from wheat leaves, forms of these enzymes were encountered that eluted from Sephadex G-200 gels in the void volumes. Other forms of β-glucosidase, β-galactosidase and invertase were found that eluted as if they were quite small proteins. These enzymatically active small proteins could not always be recovered if rechromatographed on the same columns of Sephadex G-200.

The enzymes eluting in the void volume contained soluble polysaccharides (unpublished, *cf.* ref. 3). There are numerous reports that some enzymes bound to cell walls[4–9] and other insoluble cell components[10,11] may be eluted from the debris in homogenates with 1 *M* NaCl solutions. Other enzymes may be disaggregated by high concentrations of monovalent cations[12,13]. Solutions of NaCl can prevent the interactions between glycosaminoglycans and basic polypeptides in some cases[14].

Since the form of invertase that behaves as a small protein on Sephadex G-200, is a basic protein[1], it might adsorb on the few acidic groups of Sephadex G-200 and, consequently, be eluted from the column with 0.01 *M* sodium maleate buffer, pH 6.5, much later than it would if molecular size were the only factor controlling elution volume. This behavior has been observed with several small basic proteins on Sephadex G-50[15–17].

Experiments with Sephadex G-200 columns, equilibrated with NaCl, were done to try to separate those enzymes that were eluted in the void volume of Sephadex G-200 columns from the soluble carbohydrate materials, and to prevent the adsorption of small basic proteins on Sephadex G-200.

EXPERIMENTAL

Juice from the leaves of Kharkov 22 MC winter wheat was clarified, concentrated and chromatographed on a Sephadex G-200 column, eluted with 0.01 *M* sodium maleate buffer pH 6.5[1]. The fractions obtained were assayed for invertase[1], acid phosphatase hydrolyzing adenosine-5-phosphate[18], β-glucosidase, and β-galactosidase to identify those fractions containing activity. The glycosidases were assayed by incubating 0.5 ml of the unknown with 0.5 ml of McIlvaine's buffer[19] containing 2.4 mg/ml of either *p*-nitrophenyl β-D-glucopyranoside or *p*-nitrophenyl β-D-galactopyranoside. The liberated *p*-nitrophenol was assayed colorimetrically (No. 42 filter,

Klett Summerson) after the addition of 4 ml 1 *M* Na_2CO_3. For rechromatography, the appropriate fractions were combined and concentrated by dialysis against 30% aqueous polyethylene glycol (MW 20,000). The concentrate was applied to the same column, equilibrated either with 0.01 *M* sodium maleate buffer, pH 6.5, or maleate buffer containing 1 *M* NaCl, and eluted with the equilibrating solution. Fractions were assayed for invertase, acid phosphatase, and glycosidases.

RESULTS AND DISCUSSION

Disaggregation of large-molecular complexes

The fractions representing the void volume from the first chromatographing on Sephadex G-200, eluted with buffer only, contained "large-molecular" forms of invertase, acid phosphatase, and β-glucosidase. When these fractions were concentrated and rechromatographed under the same conditions, the invertase was eluted again as a single peak in the void volume[1], the bulk of the acid phosphatase was eluted in the void volume[2], and the β-glucosidase was eluted partly in the void volume and partly as two distinct peaks of lower molecular weight (unpublished).

The conditions required to disaggregate the high-molecular-weight form of invertase were explored in experiments using small-bore columns of Sephadex G-50 and G-200 connected in series[20]. Disaggregation was greater in columns equilibrated and eluted with 1 *M* NaCl in buffer than in columns equilibrated and eluted with 0.1 *M* NaCl in buffer. However, even when 1 *M* NaCl was used, aggregated enzyme was sometimes detectable.

When the fraction of wheat-leaf enzymes that was eluted in the void volume from Sephadex G-200 with 0.01 *M* sodium maleate buffer, pH 6.5, was rechromatographed on a similar column previously equilibrated and eluted with 1 *M* NaCl in buffer, the bulk of the invertase, β-glucosidase, and acid phosphatase was eluted as if these enzymes were now smaller molecules than originally (Fig. 1). Invertase consistently gave one peak when eluted with 1 *M* NaCl in buffer, whereas β-glucosidase has sometimes yielded two peaks, one eluting in the same volume as fractions 65–70 and the other in fractions 95–125 as shown in Fig. 1. These results indicate that chromatography on Sephadex G-200 equilibrated and eluted with buffer containing 1 *M* NaCl can disaggregate some of the large molecular complexes present in the crude preparations.

Enzymes present as large aggregates in the crude preparations can be freed of smaller unaggregated proteins by chromatography on Sephadex G-200 in the absence of 1 *M* NaCl. The aggregates may then be broken down during rechromatography in the presence of 1 *M* NaCl. This step also removes any adsorbed polysaccharide that may have been present initially.

Desorption of "small" proteins from Sephadex

During the initial chromatography of processed wheat-leaf juice on Sephadex G-200 columns eluted with 0.01 *M* maleate, pH 6.5, forms of β-glucosidase and β-galactosidase were present in fractions (145–159) that were expected to contain "small" proteins. When these fractions were concentrated and rechromatographed on a Sephadex G-200 column equilibrated and eluted with buffer containing 1 *M* NaCl, the two glycosidases eluted sooner (fractions 95–125) than they did originally (Fig. 2).

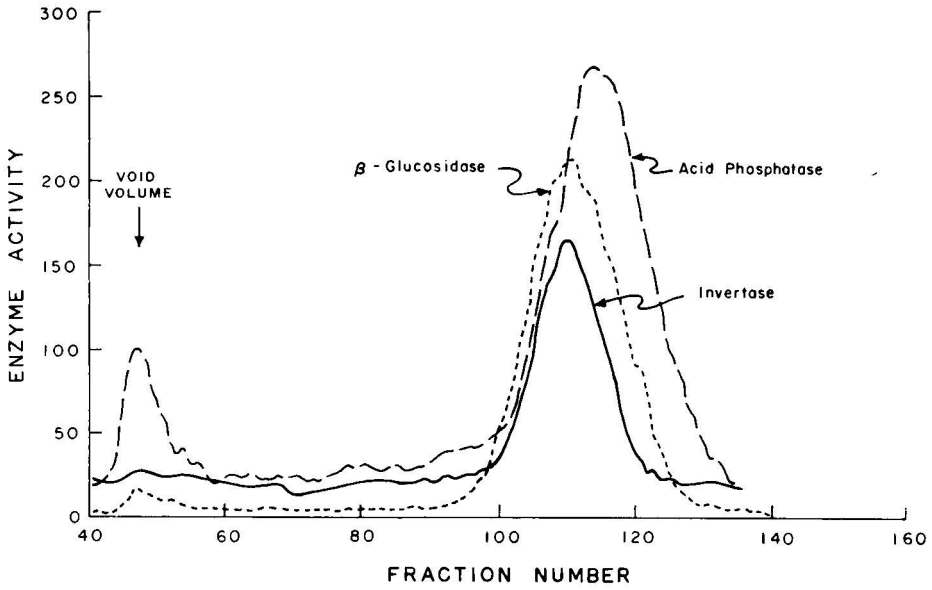

Fig. 1. Rechromatography of high-molecular-weight enzyme fraction from wheat leaves on a column equilibrated and eluted with 0.01 *M* maleate buffer, pH 6.5, containing 1 *M* NaCl. The sample was derived from a wheat-leaf preparation that had been chromatographed on the identical Sephadex G-200 column under identical conditions, except that NaCl had been omitted from equilibrating and eluting buffer. During the initial chromatography, the sample eluted in the void volume.

This behavior probably resulted from the adsorption of these forms of β-glycosidases on Sephadex G-200 during the first chromatography and their failure to adsorb on Sephadex G-200 in the presence of an adequate concentration of NaCl. During initial chromatography, these forms of β-glycosidases are probably eluted by ionic impurities of low molecular weight present in the crude preparations. Enzymes that behave like this give spurious values for their molecular weights when chromatographed on Sephadex G-200 in the absence of sufficiently high salt concentrations.

The differential ability of these enzymes to adsorb on Sephadex under different conditions may be used for their purification. If crude, partially deionized, preparations of wheat-leaf juice are chromatographed on Sephadex G-200, enzymes such as some forms of β-glucosidase and β-galactosidase will be eluted by a large volume of buffer. If such samples are concentrated and rechromatographed in the presence of 1 *M* NaCl, these enzymes will be eluted sooner than during the initial chromatography in the absence of 1 *M* NaCl. This procedure will separate these enzymes from impurities (including small proteins) that do not adsorb on Sephadex G-200. A modification of this procedure has been used successfully on Peak III invertase, a basic protein[1]. In this case, the active eluate from the chromatography of the crude preparation was thoroughly dialyzed to remove ionic impurities during its concentration. This concentrated deionized sample was applied to a Sephadex G-200 column equilibrated and later eluted with 0.01 *M* maleate buffer, pH 6.5. The Peak III invertase was not recovered. Then the column was eluted with 1 *M* NaCl in maleate buffer and the Peak III invertase was recovered in the salt front.

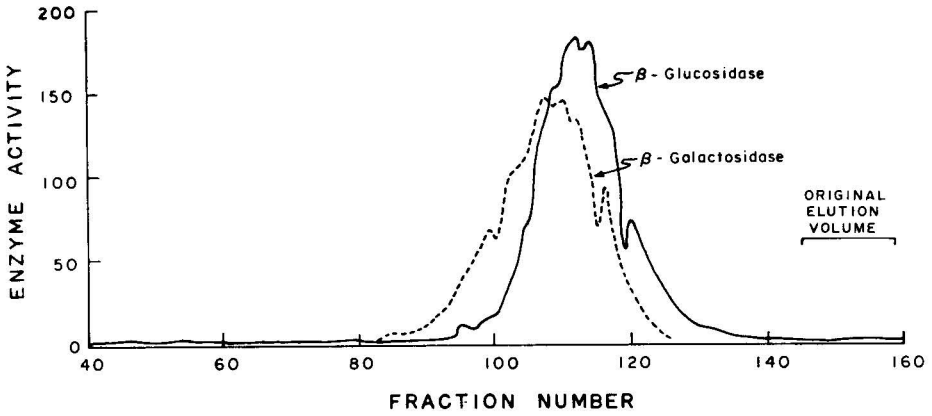

Fig. 2. Rechromatography of low-molecular-weight enzyme fraction from wheat leaves on a column equilibrated and eluted with 0.01 *M* maleate buffer, pH 6.5, containing 1 *M* NaCl. The sample was derived from a wheat-leaf preparation that had been chromatographed on the identical Sephadex G-200 column under identical conditions, except that NaCl had been omitted from equilibrating and eluting buffer. During the initial chromatography, the sample eluted in fractions 145–159.

Regeneration of Sephadex columns

When dealing with enzymes that adsorb on carbohydrates, Sephadex columns should be regenerated before re-use. I have found flushing with buffer containing 1 *M* NaCl useful for this purpose when dealing with wheat-leaf hydrolases. This procedure apparently desorbs the adsorbed enzymes.

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I wish to thank Mr. D. Voth for performing the experiments described above.

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CHROM. 10,846

Note

Analysis of amino acids in plant and fungal extracts by single-column stepwise elution

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Most programmes for amino acid analysis, utilizing modern analysers, are designed for rapid, automated analyses on a routine basis[1,2]. In most instances these simple programmes are useful only if there are a limited number of known amino acids in a sample (*e.g.*, hydrolysates) that have to be identified. If, however, physiological fluids or plant extracts, which contain large proportions of uncommon amino acids, have to be analysed frequently, separations with simple programmes are not sufficient[3].

In our work on extracts from mosses and fungi, it was found necessary to extend an already existing programme in order to identify a further 27 amino acids.

EXPERIMENTAL

The A programme involving five buffers and three temperatures, recently set up with a Biotronic LC 6000[4], with which about 40 common and uncommon amino acids could be analysed, was modified by increasing temperature 1 to 38.5°, decreasing temperature 3 to 68°, extending step 7 to 120 min, shortening step 8 to 20 min and decreasing the buffer flow-rate to 90 ml/h and that of ninhydrin to 40 ml/h. The pH values of the buffers in the extended programme were as follows ways: buffer A, pH 2.72; B, pH 3.04; C, pH 3.14; D, pH 4.33; and E, pH 3.37. In order to maintain the separation capacity of the resin (Durrum DC 1A), after 100 analyses it was removed from the column, suspended in 1.2 *N* lithium hydroxide solution containing 0.5% of EDTA for 20 min at 70°, washed with buffer A, then with 6 *N* hydrochloric acid and finally with buffer A again, then re-packed in the column in buffer D at 68°.

As the column packing settles and the resin shrinks after a fresh preparation, during the first 20 analyses care must be taken to keep the dead volume as small as possible by adjusting of the head of the column. All other conditions remained as specified earlier[4].

RESULTS AND DISCUSSION

To determine the retention times, the pure substances were eluted separately or combined in groups. Then a standard (No. 1) containing 40 substances and an-

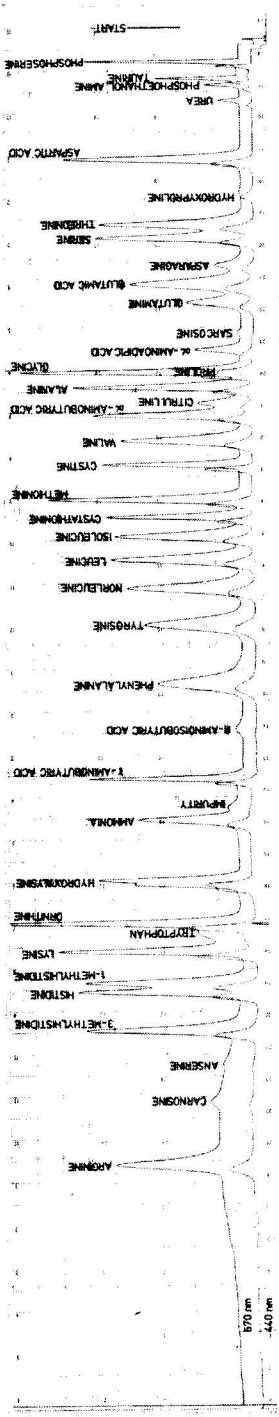

Fig. 1. Chromatogram of standard 1 on a Biotronik LC 6000 using a five-buffer, three-temperature stepwise elution programme. Dual pen recorder with measuring range extension. Distance between two vertical lines = 2 cm ≈ 8 min.

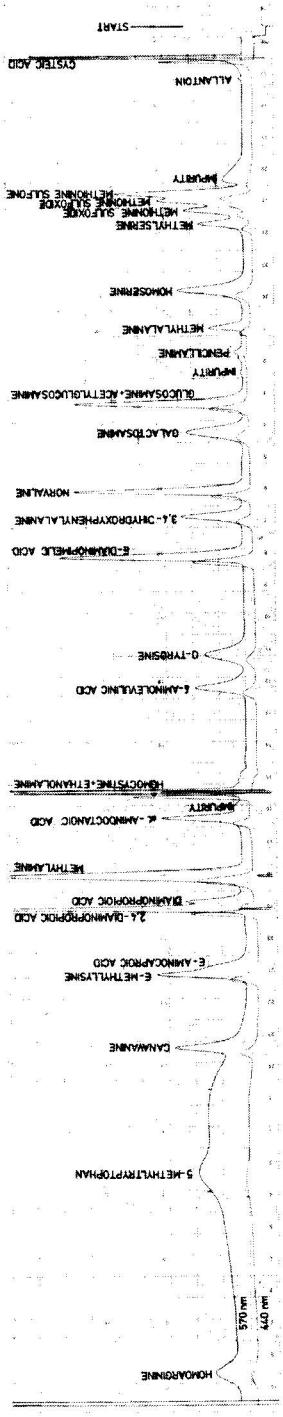

Fig. 2. Chromatogram of standard 2. Conditions as in Fig. 1.

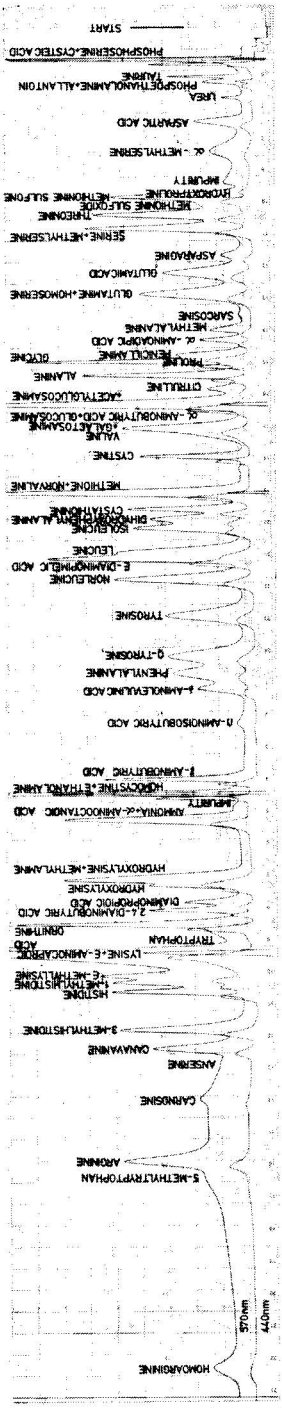

Fig. 3. Chromatogram of standard 3. Conditions as in Fig. 1.

other (No. 2) containing 27 substances were prepared. Standards 1 and 2, when combined, formed standard 3, consisting of 67 ninhydrin-positive substances (amino acids, acid amines and three amino sugars).

Fig. 1 shows a chromatogram of standard 1. Similar compositions are found in commercially available standards (Hamilton, Calbiochem) for so-called physiological fluids. Apart from incomplete separation of tryptophan from ornithine, the separations are good. The programme is already suitable for a further 27 uncommon substances that occur in our extracts.

The chromatogram of standard 2 (Fig. 2) clearly shows that not all ninhydrin-positive substances can be identified by their retention times alone. In addition, substances of great metabolic importance such as glucosamine and acetylglucosamine and, further, homocystine and ethanolamine could not be separated from each other. In all other ranges, however, the separation works well (*e.g.*, the separation of the methionine sulphoxide racemate) or acceptably (*e.g.*, diaminopropionic acid as a shoulder on the diaminobutyric acid peak and ε-aminocaproic acid as a shoulder on the methyllysine peak).

The chromatogram of the combined standards 1 and 2 (standard 3) is shown in Fig. 3. Although 21 substances completely overlap, and thus further procedures must be used for their identification, this is the best that can be achieved with our optimal programme. Thus, it was impossible to lengthen the zones from methylalanine to valine and from aminobutyric acid to histidine, *e.g.*, by using different times of buffer change or by extending the analysis time.

Although not each of the 67 substances can be assigned a retention time, we consider that this programme is useful for survey analyses of complex mixtures of free amino acids and acid amides that may occur in plants, especially in fungi. If higher standards of separation are required, computer analysis[5] or the *o*-phthalaldehyde method[6] may be employed; the latter has the disadvantage that it is inapplicable to some important amino acids, *e.g.*, proline and hydroxyproline. For the analysis of amino sugars one should use a programme in which amino acids are excluded[7].

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CHROM. 10,828

Note

Multi-step time program for the rapid gas–liquid chromatography of carbohydrates

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(Received December 13th, 1977)

Following previously used methods[1,2], the gas–liquid chromatography (GLC) of carbohydrates ranging from C_4 compounds to tetrasaccharides resulted in retention times far longer than 1 h. A reduction in retention time can be achieved by (i) a more rapid temperature program, (ii) high initial temperatures, (iii) higher final temperatures and (iv) the use of more volatile derivatives, but none of these possibilities alone can be applied without causing difficulties. High final temperatures tend to exaggerate column bleeding, higher heating rates can result in a poor resolution of components and high initial temperatures and the use of more volatile derivatives[3,4] give inadequate separations of early emerging substances such as pentoses and tetroses from solvent peaks.

By using a reasonably high initial temperature, a thermally stable stationary phase, not too volatile derivatives and a multi-step time program, excellent resolution of numerous components and short analysis times were achieved.

EXPERIMENTAL

Materials and equipment

N,O-Bis(trimethylsilyl)acetamide +BSA) was obtained from Pierce (Rockford, Ill., U.S.A.). Pyridine was of silylation grade and carbohydrates were obtained from various commercial sources. Phenyl β-D-glucopyranoside (Fluka, Buchs, Switzerland) was used as an internal standard.

Analysis was carried out on a Hewlett-Packard 5835-A terminal-operated gas chromatograph with flame-ionization detectors using nitrogen as the carrier gas (flow-rate 20 ml/min) on 6 ft. × 2.0 mm I.D. glass columns equipped for on-column injection (all-glass system). The stationary phase was 3% Dexsil 300 GC (Applied Science Labs., State College, Pa., U.S.A.) on Chromosorb W AW DMCS (80–100 mesh). The injector and detector temperatures were 275° and 345°, respectively. The initial temperature was 160°. The temperature program was 10°/min for 8 min, 30°/min for 3.66 min, isothermal at 350° for 11.34 min. The run time was 23 min. The injection frequency (run time plus cooling time) was 32.5 min.

Reactions were carried out in 0.2-ml vials (made from ordinary glass-tubing) fitting 1-ml glass flasks (Hewlett-Packard, 62311-S29) and capped with PTFE–rubber

laminated seals (Hewlett-Packard, 5080-8713/1540-0132). Samples of 5 μl were injected using a Hewlett-Packard 7671-A Automatic Sampler (Hamilton 701 syringe).

Derivatization procedure

Approximately 0.5–1.0 mg of sugar mixture in a 200-μl vial was dissolved in 70 μl of pyridine containing 0.21 mg of phenyl β,D-glucopyranoside as internal standard. After 5 min, 130 μl of BSA were added. The vial was capped immediately and kept at room temperature for 30 min. Reactions were complete by that time and the mixture was stable for at least 9 h at room temperature. A 5-μl volume of the reaction product was taken for analysis.

RESULTS

BSA, which is normally used as a silylating agent for organic acids, was introduced into sugar analysis[5] in order to simplify the procedures involved as the GLC separation of both organic acids and carbohydrates is carried out simultaneously on separate instruments in our laboratory. As BSA derivatives of sugars are only moderately volatile, Dexsil, a thermally stable stationary phase which can be heated to 350° without severe column bleeding, was used in detection of tetrasaccharides.

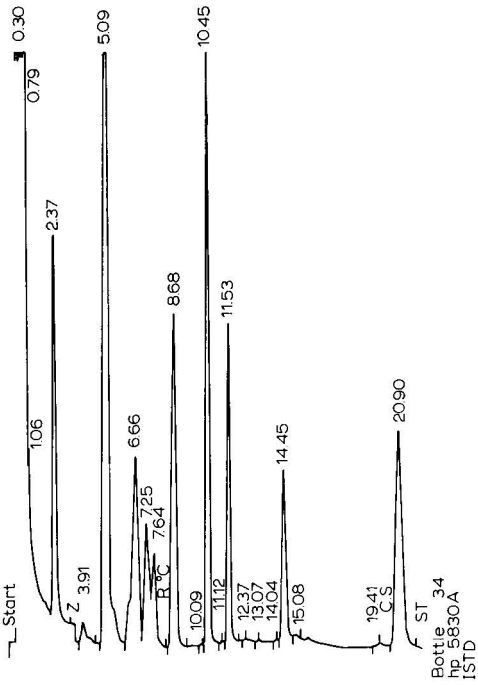

Fig. 1. GLC separation of carbohydrates. Initial temperature, 160°; heating rate, 10°/min for 8 min, 30°/min for 3.66 min; final temperature, 350° (held for 11.34 min); carrier gas (nitrogen) flow-rate, 20 ml/min. Column: 6 ft. × 2.0 mm I.D. all-glass system; 3% Dexsil on Chromosorb W AW DMCS (80–100 mesh). Retention times (min) of peaks: 2.37, erythritol; 5.09, ribose; 6.66, fructose; 7.25, α-glucose; 7.64, β-glucose; 8.68, inositol; 10.45, phenyl β-D-glucopyranoside; 11.53, sucrose; 14.45, raffinose; 20.90, stachyose.

Using our former GLC conditions (initial temperature 130°, heating rate 10°/min, final temperature 350°), the retention times obtained were still too long. Therefore, the initial temperature was raised to 160° and the heating rate was changed to 30°/min. This reduced the retention times of tetrasaccharides to less than 20 min, but had an adverse effect on early emerging components, as could be foreseen. As there was evidence that with a one-phase temperature program satisfactory separation of C_4 and C_5 compounds on the one hand and shorter retention times for tetrasaccharides on the other could not be obtained, a multi-phase time program was adopted.

Starting at 160° with a heating rate of 10°/min minimized the influence of solvent peak tailing on erythritol (Fig. 1). Such a good resolution of a C_4 compound was due to the lower volatility of the BSA derivatives compared with that of the solvent. The low heating rate also improved the separation of all C_6 compounds. After 8 min, elution of all slowly emerging compounds were speeded up by adopting a heating rate of 30°/min. Finally, an isothermal phase at 350° yielded retention times for stachyose close to 21 min without damaging the column. The flat base-line should be noted. After 23 min the heating was shut off and the column oven cooled to the initial temperature. After thermal equilibration, a new run was started automatically. The total run time (heating period, cooling and equilibration) was 32.5 min, allowing 15 runs to be carried out during the time period when the derivatives remained stable (9 h). More than 600 samples have been analysed with the method described without any signs of ageing of the column. Relative retention times of a number of compounds are given in Table I and absolute retention times are shown in Fig. 1.

TABLE I

RELATIVE RETENTION TIMES OF CARBOHYDRATES

RRT = mean relative retention time; n = number of observations; S.D. = standard deviation.

Compound	*RRT*	*n*	*S.D.*
Erythritol	0.228	7	0.0008
α-Xylose	0.450	1	—
β-Xylose	0.510	1	—
Ribose	0.488	7	0.0008
α-Arabinose	0.460	1	—
β-Arabinose	0.530	1	—
Fructose	0.638	12	0.0031
α-Glucose	0.694	12	0.0027
β-Glucose	0.730	12	0.0028
Sedoheptulose	0.710	1	—
Inositol	0.831	12	0.0018
Phenyl β-D-glucopyranoside (internal standard)	1.000	12	0.0131
Sucrose	1.103	12	0.0007
Arbutine	1.131	1	—
Trehalose	1.152	1	—
Raffinose	1.383	12	0.0010
Stachyose	2.000	7	0.0029

As for the wide molecular range the method is well suited for application on complex carbohydrate mixtures like plant extracts and other biological fluids.

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CHROM. 10,816

Note

"C_{22}"-A superior bonded silica for use in reversed-phase high-performance liquid chromatography

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We have recently shown[1] the importance of the screening of several bonded supports prior to the development of a separation involving reversed-phase high-performance liquid chromatography (HPLC). This study revealed that resolution improved with increasing chain length and that the C_{18} (*n*-octadecyl)-bonded silica was the best of the then existing materials. The only exceptions to this generalisation appear to be those applications involving ion-exchange and ion-pair interactions with the support.

As a significant development of this work, we have now extended the length of the carbon chain to C_{22} (dococyl). Details of the superior performance of this new bonded support are now reported.

EXPERIMENTAL

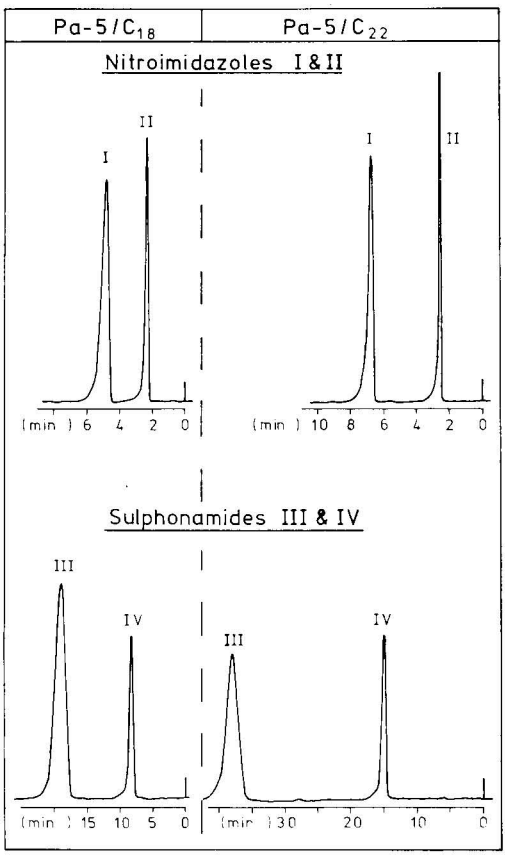
All conditions, reagents, equipment and chemical structures relating to this communication are as previously reported[1].

RESULTS AND DISCUSSION

Our previous study[1] indicated that a linear relationship existed between resolution and alkyl chain length up to C_{18}. We now have established that this linearity extends to at least C_{22} and that substantially better separations have been observed for each of the applications used in our column screen. These comparisons were carried out under identical experimental conditions. Typical results are shown in Figs. 1 and 2.

It is probable that the capacity of preparative, reversed-phase HPLC is greater with C_{22}-bonded silica. This aspect is currently being investigated.

In order to achieve a separation on Pa-5/C_{22} in a time equivalent to that achieved on Pa-5/C_{18}, the level of organic solvent in the eluent must be increased.



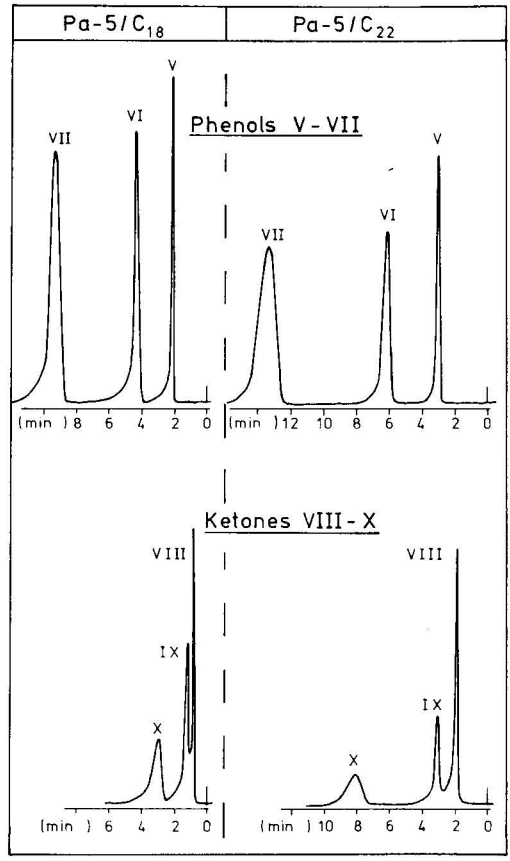


Fig. 1. Chromatograms of the nitroimidazole "misonidazole" I and its metabolite II and of the sulphonamide "mefruside" III and its metabolite IV on Pa-5/C_{18} and Pa-5/C_{22} columns. Eluent compositions for these separations were: methanol–water (1:9) for nitroimidazole separation; methanol–water (2:8) for sulphonamide separation.

Fig. 2. Chromatograms of phenol and ketone mixtures on Pa-5/C_{18} and Pa-5/C_{22} columns. Eluent compositions for these separations were: methanol–water (2:8) for phenols; methanol–water (4:6) for ketones.

TABLE I

IDENTIFICATION OF THE COMPOUNDS SEPARATED IN FIGS. 1 AND 2

Peak No.	*Identification*
I	Misonidazole
II	Metabolite of I
III	Mefruside
IV	Metabolite of III
V	Phenol
VI	4-Methyl phenol
VII	2,4-Dimethyl phenol
VIII	Acetone (dimethyl ketone)
IX	Diethyl ketone
X	Di-*n*-propyl ketone

This is of significance when the compounds to be separated are only sparingly soluble in water. In this category, the sulphonamide "mefruside" III and its metabolite IV are good examples.

The structures of all of the compounds separated can be found in our previous study[1]. An identification of the peaks is given in Table I.

Work designed to extend the investigation to even larger chain lengths and to a much wider range of compounds is now in progress and will be published in due course.

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CHROM. 10,845

Note

High-performance aqueous gel-permeation chromatography of oligomers

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(First received November 23rd, 1977; revised manuscript received December 20th, 1977)

Gel-permeation chromatography (GPC) is a type of liquid chromatography in which the separation is based only on the molecular size of the solute in solution, and hence the setting of the experimental conditions and the interpretation of the results obtained are easy. In general, however, GPC is slow and has a low resolution in comparison with other liquid chromatographic techniques. Therefore, GPC has rarely been applied to the separation of low-molecular-weight compounds and has been mainly employed to separate high-molecular-weight compounds. However, high-resolution columns packed with microparticulate polystyrene gels were developed a few years ago[1], and high-speed GPC with satisfactory resolution was attained[2]. As a result, the possible applications of GPC have been extended to small molecules in organic solvent systems[3,4]. Also in aqueous systems several column packings have been developed for use in high-speed measurements. However, those column packings have adsorptivity, resolution or pore size disadvantages.

Recently, GPC columns packed with microspheres of hydrophilic polymer gels have become commercially available (TSK-GEL, Type-PW; Toyo Soda Manufacturing Co.). These columns can be operated under high pressure in aqueous systems and have a large number of theoretical plates (more than 4000 or more than 6000 plates/ft.). Moreover, several grades of columns with different pore sizes are available. High-speed GPC of ethylene glycol oligomers was performed with two grades of TSK-GEL, Type-PW, with small pore sizes (G2000PW and G3000PW), in order to investigate the resolution and the separation range of these columns. The results are described in this paper.

EXPERIMENTAL AND RESULTS

GPC measurements were carried out at 55° on a commercial gel-permeation chromatograph, HLC-801A (Toyo Soda Manufacturing Co.), with two G2000PW columns or with two G3000PW columns. Each column was 2 ft. long with an I.D. of 0.305 in., all were packed with gel particles of diameter 12–15 μm and had almost the same theoretical plate numbers and pressure drops. In Fig. 1, flow-rate dependences of the theoretical plate number and the pressure drop are shown for G3000PW. Distilled water was used as the solvent, the flow-rate was 1.4 ml/min, the injection volume was 0.18 ml and the sample concentration was varied between 1 and 18 mg/ml depending on the molecular weight distributions of the samples. Narrow molecular

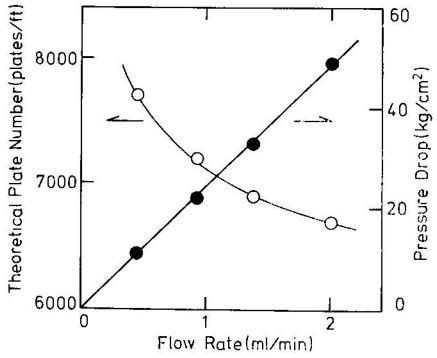

Fig. 1. Flow-rate dependences of the theoretical plate number (○) and the pressure drop (●) for a G3000PW column. The theoretical plate number was measured with ethylene glycol.

weight distribution polyethylene glycols purchased from Wako (Osaka, Japan) shown in Table I, were used as samples.

TABLE I

POLYETHYLENE GLYCOL SAMPLES

Sample No.	*Molecular weight**	*Sample No.*	*Molecular weight**
I	20,000	VI	600
II	7500	VII	400
III	3000	VIII	200
IV	1500	IX	62
V	1000		

* Manufacturer's data.

Figs. 2 and 3 show the elution curves of polyethylene glycols measured with the two-column systems. Samples I, II and III were eluted from the two-G2000PW

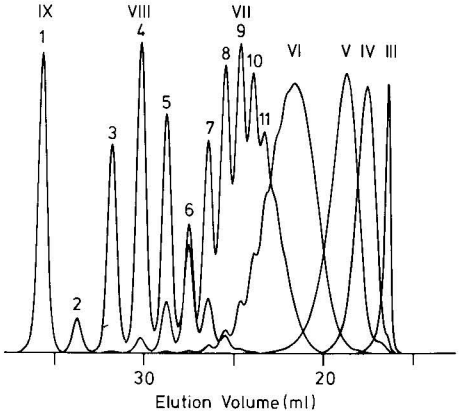

Fig. 2. Elution curves of polyethylene glycols measured with a two-G2000PW column system. Roman numerals (I–IX) are sample numbers and arabic numerals (1–11) are degrees of polymerization of the components.

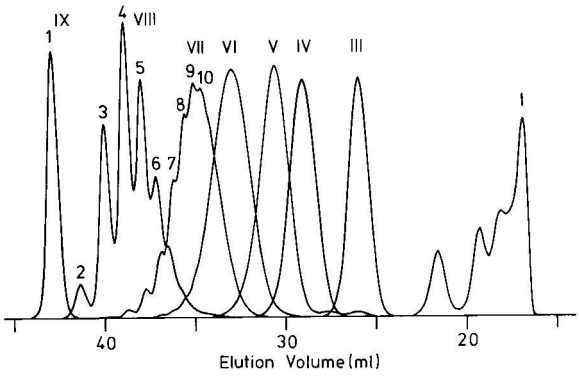

Fig. 3. Elution curves of polyethylene glycols measured with a two-G3000PW column system. Numerals as in Fig. 2.

column system at elution volumes corresponding to the void volume, and the elution curves of samples I and II have been omitted from Fig. 2. Sample II, the elution curve of which is not shown in Fig. 3, was eluted at the same position as the lowest molecular weight component of sample I in the two-G3000PW column system. Although the abscissa is expressed in terms of elution volume, it can be seen that ethylene glycol, which is the sample with the smallest molecular weight, was eluted from the columns within 30 min because the flow-rate was 1.4 ml/min. Peaks of components from the monomer to the decamer or undecamer were observed, which indicates that the resolution in this high-speed GPC is very high. Ethylene glycol oligomers have been measured by aqueous GPC with some other column packings, but resolutions as high as those in Figs. 2 and 3 were not attained even when the analysis time was several hours[5–7]. The specific resolution, R_s, which is useful for comparing the resolutions of different column systems, was calculated from the elution curve of sample VIII in Fig. 2 by using the equation

$$R_s = \frac{2\,(V_2 - V_1)}{(W_1 + W_2)\,(\log M_1 - \log M_2)} \tag{1}$$

where V, W and M represent the elution volumes, peak widths at the base and the molecular weights, respectively, of two components. Values of 12.6 and 15.9 were obtained for the dimer and trimer and for the tetramer and pentamer. These values are comparable to or greater than those obtained by high-speed GPC in organic solvent systems in the same molecular weight region[4,8].

Plots of molecular weight against peak elution volume (calibration graphs) are shown in Fig. 4. In the low-molecular-weight region, plots were made for each component separated. The molecular weight of a component with a degree of polymerization n, $(\mathrm{MW})_n$, was calculated by using the equation

$$(\mathrm{MW})_n = 18 + 44n \tag{2}$$

The void volume of the two-G3000PW column system could be determined because high-molecular-weight components in sample I were totally excluded from

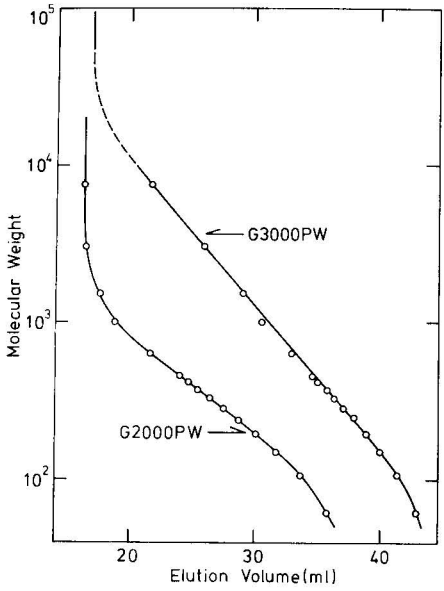

Fig. 4. Semi-logarithmic plots of molecular weight of polyethylene glycol *versus* elution volume for a two-G2000PW and a two-G3000PW column system.

this column system (shown in Fig. 3). Fig. 4 indicates that the molecular weight exclusion limits of G2000PW and G3000PW are 1500 and 20,000, respectively. Although both G2000PW and G3000PW have a resolving power in the region of molecular weights lower than 1000, G2000PW has a higher resolution than G3000PW. This is because the gradient of the calibration graph for G2000PW is smaller than that for G3000PW, and is clearly shown in the elution curves of samples VII and VIII in Figs. 2 and 3.

From the results, it can be concluded that the high-speed aqueous GPC of oligomers and small molecules on TSK-GEL, Type-PW, gives a satisfactory resolution, comparable to that in GPC with organic solvent systems.

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CHROM. 10,879

Note

Separation of glycolipids from neutral lipids and phospholipids

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The most important functions of glycosphingolipids found in biological membranes are antigenicity and cell–cell recognition, which are manifested even at very low concentrations. The concentrations are mostly 5–10 times less than that of the accompanying phospholipids. The chromatographic separation of these substances is difficult because of their similar polarities and solubilities. In order to develop a satisfactory method of separating all glycosphingolipids quantitatively from the phospholipids in one step, use has been made of the ability of boric acid to form complexes with sugars. Using Kieselguhr impregnated with boric acid–disodium borate buffer, all glycosphingolipids except mono-, di- and triglycosylceramides and sulphatides were separated from phospholipids and neutral lipids[1]. In the preparative use of the method, boric acid–borax buffer was always found in the glycosphingolipid fractions and, as this hindered further use of the material, the buffer had to be separated by special purification procedures.

We have studied the possibilities of developing a simple and effective separation procedure. The above disadvantage could be avoided if the boric acid used for complexing were bound on a polymeric matrix. Fréchet and co-workers[2–4] have described the preparation of a resin that contains covalently bound phenylboronic acid held on a cross-linked polystyrene matrix. By the reaction of lithiated polystyrene with triethyl borate they achieved very high capacity of 2–3.5 mmole per gram of resin. The polarity of the non-polar polystyrene matrix was varied by adjusting the proportion of polar, hydrophilic phenylboronic acid. It seemed to us that this resin would be suitable for the separation of glycolipids, which contain both lipophilic and hydrophilic groups. In addition, the mechanical stability of the rigid, porous resin is superior to that of a cellulose[5] or a polyacrylamide support[6,7].

EXPERIMENTAL

The resin was prepared according to Farrall and Fréchet[2] and contained 3.7 mmole per gram of phenylboronic acid. A 7-g amount of resin was swollen in chloroform–methanol (10:2, v/v), the column being 16 cm long with an I.D. of 1.5 cm. A mixture of about 5 mg of phospholipids from egg and bovine brain together with cerebrosides, sulphatides and gangliosides from bovine brain was applied to the column. Elution was performed with chloroform–methanol (10:2, v/v) containing in-

creasing amounts of water (0, 0.3, 0.6, 1.2 and 3.0 ml of water per 100 ml of solvent) and fractions of 25 ml were collected.

For thin-layer chromatography (TLC) (Fig. 1), two corresponding fractions were combined and evaporated *in vacuo* at 40°. Chromatograms were developed as described previously[8] with tetrahydrofuran–water (5:1, v/v), 100 mg of potassium chloride being added per 100 ml of solvent mixture. Using pre-coated silica gel 60 HPTLC plates for nano-TLC (E. Merck, Darmstadt, G.F.R.), very sharp separations were obtained in a particularly short development time of 45 min. The developing solvent was removed completely from the plates in a vacuum oven at 130° before staining the glycosphingolipids with orcinol–sulphuric acid[9]. Phospholipids were detected using a molybdenum spray or by charring the orcinol–sulphuric acid-sprayed plates at 160°.

RESULTS AND DISCUSSION

Fig. 1 shows that all glycosphingolipids (fractions 2–10) were separated from phospholipids and neutral lipids (fraction 1) in a single step. The phospholipids and neutral lipids were stained only slightly by the orcinol–sulphuric acid spray. Less than 1 % of the glycosphingolipids were lost in fraction 1. The separation of the glycosphingolipids from the phospholipids and the neutral lipids was accompanied by a fractionation of the glycosphingolipids according to the number of free hydroxyl groups. Sulphatides (fractions 2–5) were followed by monoglycosylceramides (fractions 6–9) and gangliosides (mainly fractions 9 and 10).

A large number of different column separations, of which Fig. 1 shows only one example, have demonstrated that the addition of water was necessary for the elution of the glycosphingolipids. In fact, even very small amounts of water added to the chloroform–methanol solvent liberated from the resin those glycosphingolipids which contain only a few sugar moities. Using solvent mixtures with minimal differences in water concentration, a further separation of glycosphingolipids might be achieved. However, the separation of brain gangliosides can possibly be obtained more easily by TLC as shown in Fig. 1.

The separation time can be increased or decreased according to the volume of water-free elution solvent used. If an additional fractionation of the glycosphingolipids is not required, the primary separation from the phospholipids can be achieved in 1–2 h. This short separation time is especially recommended for mixtures that contain gangliosides with several sialic acids in order to avoid hydrolysis of sialic acid residues. Some experiments showed that the capacity of the column used was sufficient to separate about ten times as much material as is indicated under Experimental.

An advantage of the method is the separation of all glycosphingolipids from neutral lipids and phospholipids and also from remaining proteins and colouring matter in a single step. Further, it is possible additionally to fractionate and concentrate the glycosphingolipids, which normally occur in only very small amounts, from relatively large amounts of biological material. Of special advantage is the fact that the resin can be regenerated and that no boric acid is eluted.

Hence, on the one hand our procedure might be helpful in obtaining larger amounts of purified glycosphingolipids for chemical studies, and on the other it could be used as an automated process in the routine determination of gangliosidoses.

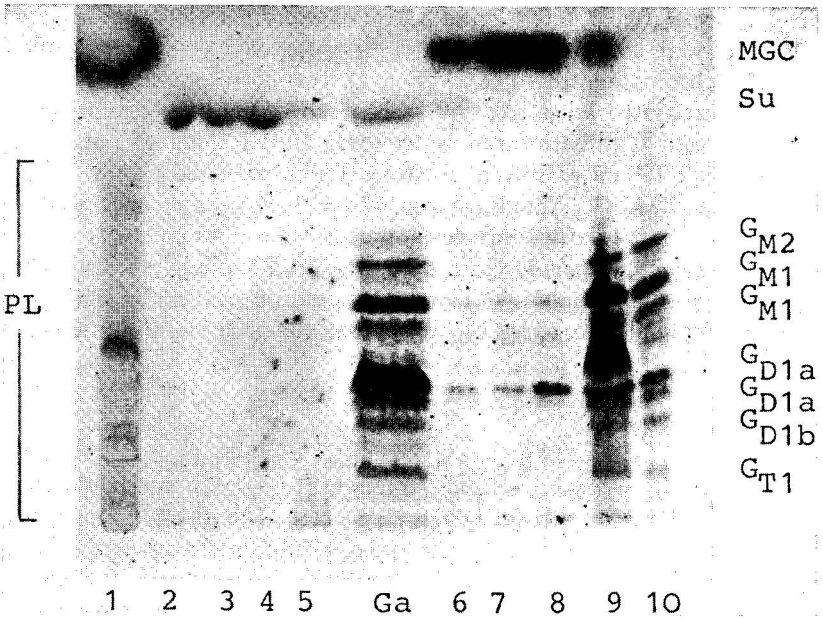

Fig. 1. Thin-layer chromatogram of lipids separated by column chromatography on polyphenylboronic acid. Adsorbent: pre-coated silica gel 60 HPTLC plate for nano-TLC (E. Merck). Solvent: tetrahydrofuran–water (5:1, v/v) containing 0.1% (w/v) of KCl. Spray: 0.2% orcinol in sulphuric acid, heated at 130° for 4 min. Samples: 1–10 = fractions from column chromatography; Ga = gangliosides from bovine brain. Abbreviations: MGC = monoglycosyl ceramide; Su = sulphatide; G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, G_{T1} = gangliosides according to the Svennerholm nomenclature; PL = phospholipids.

ACKNOWLEDGEMENT

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CHROM. 10,919

Book Review

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

This book is one of the more successful examples of a collection of sections from different authors and has achieved a rare balance in depth and comprehensiveness. This is probably because the authors are all members of a gas chromatography (GC) forum group which must have made the coordination of effort much easier than if they had been more dispersed.

The effort is all the more laudable as by nature chromatography involves a much higher element of art, cookery and witchcraft than other instrumental analytical techniques, but the local forum origin has inevitably given rise to some minor deficiencies. In particular the sections on hardware contain some covert commercials and reflect manufacturer's marketing policy. The chapters on applications are somewhat parochial, covering only the foodstuff and medical fields with no mention of the chemicals and fuels industries which probably make up the bulk of the users of GC.

The book is in three sections: Theory and basics, Techniques and instrumentation, and Applications.

Chapter 2 on theory is by the Editor and in my view is admirably presented, blending mathematical, physical and empirical explanations coherently and in just the right depth and is one of the best introductory accounts of GC I have come across.

Chapter 3 on columns is by Supina who is a master of the art of column design and contains a wealth of useful "hints and tips" on basic practice of column design and preparation. I feel this chapter could well have been made longer at the expense of some of the later chapters to give a better coverage of gas–solid columns, capillary columns and particularly more practical data, such as given in the author's Company's commercial cataglgue.

Chapter 4 on qualitative and quantitative analysis is a good working account of the basics albeit with some duplication with Chapter 8 on data handling. In the discussion on peak measurement there is a review of the relative usage of the different techniques in *1966* which is hardly in keeping with the title. The attempt to cover the preparation and use of standard atmospheres for calibration is far too brief and would best be left to specialised books on the subject, such as Nelsons —which is not even referred to.

Chapter 5 on detectors is in two sections, the first giving good balanced accounts of basic aspects and the catharometer and flame ionization detector. The second covers "the rest" in varying detail.

Chapter 6 on instrumentation is no more than adequate and contains a lot of superfluous information and diagrams, the latter being there probably for advertising purposes. There is again some duplication with Chapter 8 on data handling.

Chapter 7 covering trace analysis gives useful information on the problems encountered when working close to the detection limit and with difficult analyses.

The techniques described for sample concentration are not exactly modern and any later editions would benefit by including current developments in environmental measurements of very low levels of vapours in air and water.

Chapter 8, "Selection of analytical data from a GC laboratory", really means Guidelines for choosing a Data Handling System and covers the merits and limitations of hardware from integrators to computers with emphasis on cost effectiveness. This chapter is rather short, and contains very little on the machine logic which is so important when selecting integrators though less so for computers.

Chapters 9, 10 and 12 in the Applications section cover food, clinical and drug analyses. The food chapter is sensibly limited to lipids, proteins and carbohydrates and gives excellent well balanced cover of these topics. The clinical chapter covers steroids and urine and blood analyses in an equally competent manner and Chapter 12 completes the analytical applications with a concise account of drug analysis.

A welcome addition to such a book on chromatography is Mary Kaiser's Chapter 11 on physicochemical measurements by GC which covers a lot of ground, giving the basic principles for a very comprehensive range of applications and useful bibliography of references.

The quality of the book, its indexing, referencing and contents listing are very good. The book must rank as one of the better general handbooks on GC, is good value for its cost and is a must for the GC laboratory bookshelf.

Abingdon (Great Britain) D. T. COKER

Journal of Chromatography, 153 (1978) 555

CHROM. 10,918

Book Review

Liquid chromatography of polymers and related materials, edited by J. Cazes, Marcel Dekker, New York, Basel, 1977, IX + 180 pp., price SFr. 65.00, ISBN 0-8247-6592-3.

This attractively produced eighth volume of Marcel Dekker's Chromatographic Science Series presents the proceedings of the First International Symposium on Liquid Chromatographic Analysis of Polymers and Related Materials. This event took place in October 1976 at Houston, Texas, U.S.A. and was sponsored by Waters Associates (Milford, Mass., U.S.A.). The book contains eleven original papers, a twelfth paper that has already been published elsewhere and by contrast, the introduction and bibliography of the thirteenth paper which had yet to be written.

The most important development in gel permeation chromatography in recent years has been the introduction of microparticulate organic stationary phases which have enabled analysis times to be shortened up to ten-fold without loss in efficiency and resolution; the subject matter of many of the papers presented here reflects the current wide-spread interest in columns packed with such materials. Such topics include calibration, data processing, measurement of intrinsic viscosities and applications to the analysis of polymethacrylates, epoxy resins, polyvinyl chloride and materials associated with the wood fabrication industry.

Fractionation of liquid crystals is also discussed and a water purification kit for reversed-phase chromatography is evaluated. Performances of a new microparticulate stationary phase suitable for exclusion chromatography of hydrophilic polymers and a new mobile phase (hexafluoro-2-propanol) for non-aqueous work are also described. The cost of this solvent is quoted at US $ 550 per gallon —presumably the smaller U.S. gallon— and its use here with μStyragel columns in a commercial chromatograph will further support the cynics' claim that the initials HPLC really stand for "High-Priced Liquid Chromatography"!

Waters Associates have sponsored many successful symposia in the past, but many of the original papers cannot easily be found. Publication of the proceedings of this latest meeting in a single volume is therefore to be welcomed. Nevertheless, these contributions could have been accommodated comfortably in one of the current chromatography journals, thus ensuring a wider audience which, in the event, will now be limited by the high price of the book.

Abingdon (Great Britain) R. AMOS

Author Index

Erratum

J. Chromatogr., 144 (1977) 209–214

Page 214, Fig. 3, third line of legend, should read: 5, galacturonic acid (= methyl galactopyranoside)

Vol. 153, No. 2

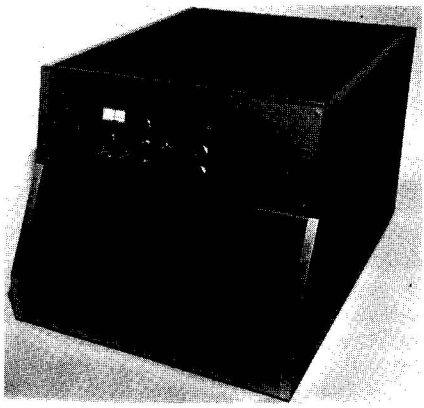
journal of
chromatography news section

APPARATUS

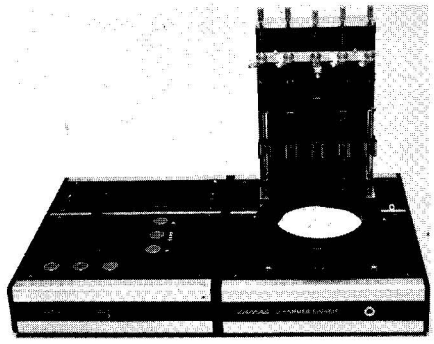
N-1137

TLC/HPTLC SCANNER

CAMAG announce a new quantitative photodensitometer for conventional and high-performance TLC, suitable for linear as well as circular chromatograms. This TLC/HPTLC scanner measures the intensity of diffuse light reflected from the chromatogram allowing unlimited use of the full wavelength range 200–800 nm, and is suitable for absorbance and fluorescence measurements. The detection limit for substances with strong fluorescence is below 50 pg. The chromatogram is scanned with a slit adjustable according to requirements. For the very small zones typical in HPTLC the slit dimensions can be reduced to 0.2 × 1.0 mm.



For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.



N-1132

NEW U-CHAMBER CHROMATOGRAPH

A new variation of the CAMAG U-chamber system for fast HPTLC is announced. The U-chamber chromatograph has four solvent capillaries, each fed by its own syringe in addition to the central solvent capillary of a regular U-chamber. This serves for quick optimization of a developing solvent by running a circular chromatogram with four different solvents simultaneously. The 5-solvent system U-chamber unit is operated by the same control unit that is part of a regular U-chamber chromatograph.

N-1148

SPECTROFLUOROMETER

A 2-page data sheet discusses Varian's new Model SF-330 spectrofluorometer. Design features and the performance advantages including ratio photometer, concave grating monochromators and automatic scanning are described. The range of accessories for special sample handling include those for HPLC requirements.

N-1123

INTELLIGENT SAMPLE PROCESSOR

Waters' intelligent sample processor (WISP™) is a fully automatic sample injection system ideal for use with the Waters Series 200 liquid chromatographs. Flexibility for chromatographic analysis results from the microprocessor-based intelligence and includes continuously variable injection size, automatic needle wash, individual bottle programming, self-diagnosis with responsive logic, compatibility with gradient operation, NoLoss™ sample bottles.

N-1126

COMPUTING INTEGRATORS

Available from Packard-Becker are brochures describing the Model 602 and 603 computing integrators. Model 602 is compatible with any gas or liquid chromatograph and can integrate practically all chromatograms. The Model 603 is a computer-based chromatographic data reduction system designed for gas and liquid chromatographs.

N-1133

GRAPHICS TERMINAL

The 2648A graphics terminal from Hewlett-Packard can help if spectral plots are needed from a computer-controlled gas chromatograph–mass spectrometer system by providing bright display and graphics text composition. During extended computer sessions where spectral plots might be analyzed the bright display would help reduce operator eye fatigue and stress. After analysis the spectral plots can be readily labelled.

N-1144

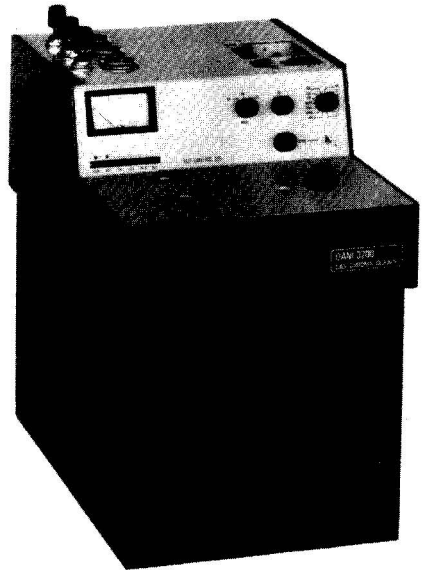
UNIMETRICS CATALOGUE

The 40-page 1977–78 catalogue from Unimetrics gives details of their complete line of microsyringes, valves, fittings, pressure sample injectors, rotary valves, pneumatic actuators, miniature tubing fittings, and other chromatographic accessories. Products include the microsyringe with external scale and the Knauer and Vydac columns for HPLC.

N-1150

DEDICATED GAS CHROMATOGRAPHS

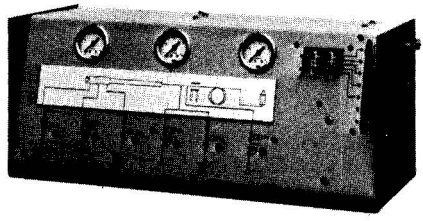
The 3200 Series gas chromatographs from Dani are single-column isothermal instruments dedicated to ECD, HWD or FID applications. The ECD dedicated instrument incorporates the new Dani low-volume ECD detector with frequency-modulated excitation.



N-1151

MULTIPHASE GAS CHROMATOGRAPH

Dani present a new capillary column gas chromatograph which incorporates multi-column dedicated hardware. Model 3920/M features septum flushing, split or splitless automatic injectors, direct calibrating injectors and a valveless flow switching module with matrix selectable programmes for automatic multicolumn operation such as backflushing, peak cutting and trapping.



CHEMICALS

N-1125

MACHEREY-NAGEL PRODUCTS

The complete production and factoring programme of Macherey-Nagel is now available in a single general catalogue. Listed in alphabetical order are amongst others, materials and accessories for all chromatographic methods. Also available are individual product group bulletins on HPLC, GC, paper, column and thin-layer chromatography, precoated plates and sheets for TLC, etc.

N-1129

SILICONE STATIONARY PHASE

A new silicone stationary phase, OV-330, is offered by Supelco, with properties differing from other silicones. It is similar to various polyglycols such as UCON 50-HB-5100 and Triton X-100 but not as polar as Carbowax 20M. With this type of polarity it would be useful for the separation of mixtures of alcohols, ketones, esters, etc. The maximum column temperature for OV-330 is 250° and the material is soluble in acetone or toluene.

MEETING

THE 29TH PITTSBURGH CONFERENCE ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY

The 29th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy was held from February 27 to March 4 this year. Despite its title, this conference has been held for many years in the Convention Centre, Cleveland, Ohio, and is organised annually by the Society for Analytical Chemistry and by the Spectroscopy Society of Pittsburgh.

At this Conference, 667 papers were presented in 58 sessions, compared with 495 papers last year. Not only is the number of papers presented rising every year, but the exhibition area in the spacious Mall Exhibit Area of the Convention Centre is also expanding. The exhibition of modern laboratory equipment which is traditionally organised during the Conference required an increase of 202 standard booth areas this year, and included some 350 instrument manufacturers and trading companies, 12% more than in 1977.

The theme of this 29th Pittsburgh Conference was 'the Service to Humanity through Analytical Chemistry and Applied Spectroscopy', which was underlined by the statement in the conference programme booklet that 'on the shoulders of the analytical chemist and spectroscopist rests a burden of maintaining a clean and healthy workplace and environment as the world strives for a solution to decreasing energy reserves'. More than 13,000 participants attended, from all over the world.

The Perkin-Elmer Corporation from Norwalk, Conn., with their 30 standard booth areas, occupied the largest booth of the exhibition. In the field of chromatography Perkin-Elmer showed only a few new items following last year's introduction of the Sigma series of gas chromatographs and the Series 2 and Series 3 high-performance liquid chromatographs. In the realm of gas chromatography the Corporation showed their new 'head space' version of the Sigma 4, which is obviously meant to replace the German-built Model F-42 head space analyzer. For high-performance liquid chromatography they exhibited a new low cost pumping system, Series 1, which is in fact the stand-alone version of the Series 2/1 pump, and can build up a pressure of 6000 p.s.i. Both Perkin-Elmer and Spectra-Physics, Houston, Texas, showed new automatic sample changers for high-performance liquid chromatography.

E.I. DuPont de Nemours & Company from Wilmington, Del., surprised everyone with the introduction of two new liquid chromatography systems, a new low resolution GC–MS system with a minicomputer-based data system, and completely new instrumentation for thermoanalysis. The Model 860 is a low cost routine type liquid chromatography system. The Model 850 is a research type high-performance liquid chromatograph with microprocessor control and sophisticated data handling and processing. In the pumping system three pumps are used, operating with a phase difference of 120°. The same configuration of pumps is used by the Japanese manufacturer, Jasco, who calls it 'Tri Rotar'. The triple pumping system is claimed to have advantages in smoothing the liquid flow and in gradient work.

Laboratory Data Control, Riviera Beach, Fla., exhibited a new, simple-to-operate, low cost, liquid chromatography system, the Model 301A.

Philips Electronic Instruments, Inc., Mahwah, N.J., showed the new powerful pumping and flow programming system, LC3-XP, with a maximum pressure of 10,000 p.s.i.

The products of ISCO from Lincoln, Nebr., had changed their name; they were exposed under the new label ChemResearch.

The German company E. Merck, Darmstadt, introduced the HIBAR column cassette system for HPLC. The company claims that the system can be used in combination with all commercially available HPLC systems.

Waters Associates, Milford, Mass., introduced the Model ALC 150C, a gel permeation/high-performance liquid chromatograph for analysis at temperatures much higher than ambient. The instrument can be run at 150°C. Waters also showed the second stage after the WISP (the Waters Intelligent Information Processor/System Console), a microprocessor-based system for simultaneous processing of data from as many as four detectors. On the preparative side of HPLC, Waters introduced their PrepPAK-500/C_{18} column cartridges. Finally we mention their new fluorescence detector.

Hewlett-Packard showed a sophisticated spectrophotometric detector for high-performance liquid chromatography. It can be operated at a fixed wavelength, but it can also scan a UV–VIS spectrum by stopped flow.

The Micrometrics Instrument Corporation from Norcross, Ga., also exhibited a spectrophotometric detector, as well as an automatic sample changer and equipment for the packing of columns.

Although almost every manufacturer of HPLC equipment now has a spectrophotometric detector in his programme, there is a trend in the direction of dedicated and sensitive UV detectors operating at a few discrete wavelength values. Laboratory Data Control and Tracor Instruments, Austin, Texas, introduced the type of detector operating at 214 nm, in a region of the UV spectrum where the usual solvents are still relatively transparent and the components of the separated mixture are already strongly absorbing. The light source in this detector is a zinc lamp. Zinc exhibits a strong emission line at 214 nm. Tracor showed also a photo-conductivity detector for HPLC.

The Technicon Instrument Company from Tarrytown, N.Y., introduced in Cleveland two new instruments for liquid chromatography. The first is a highly automated research-type instrument; the second is a dedicated system for vitamin analysis.

In the field of gas chromatography there were far fewer new instruments and accessories than in liquid chromatography. Pye Unicam's Model 204 gas chromatograph was new to the U.S. market, but already well known to the European participants.

HNU Systems, Inc., Newton Upper Falls, Mass., showed an improved version of their photo-ionisation detector; the new detector can be operated at much higher temperatures than the earlier model.

Hewlett-Packard presented the new disc memory unit with their Model 5985 GC–MS system.

Again this year the Pittsburgh Conference showed clearly the progressive trend toward the use of the microprocessor in all kinds of laboratory equipment. In the area of chromatography data processing and presentation there is a growing number of instruments in the market which do the calculation, integration, printing and plotting and which are operated via various types of keyboard. In gas chromatography there is still much interest in capillary work and in splitter systems.

The possibilities for combination of high-performance liquid chromatography with mass spectrometry are improving; work in this field is done by Finnigan Instruments, Sunnyvale, Calif.

Finally, in HPLC there is a lot of interest in auto-samplers and in gradient work. Manufacturers are improving their older systems and are working on new, more sophisticated and programmable devices for gradient work. Every well known manufacturer of HPLC instrumentation has at the moment a complete set of detectors in his programme and is working on sophisticated microprocessor-based systems for data processing and for control of the liquid chromatograph.

KLAAS H.P. BROER

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

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

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

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

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

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

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

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

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

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