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JOURNAL OF CHROMATOGRAPHY

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See page 3 of cover for Publication Schedule, Information for Authors, and information on the News Section and Advertisements.

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CONTENTS

(Continued overleaf)

Contents (continued)

Notes

Collected Reprints on Electrophoresis from the JOURNAL OF CHROMATOGRAPHY 1979

An experiment in Scientific Publishing

This year the *Journal of Chromatography* extends to 22 volumes, each of some 550 printed pages, and it seems as if the number of papers submitted is still increasing.

Unfortunately this growth of the journal from a total of about 700 printed pages in 1958 to about 12,000 pages this year has long put the journal out of reach of the private subscriber, not only because of its price but also because few subscribers can add 20-odd volumes per year to their private library without having to look for a more spacious house. Returning to the price, printing has become much more expensive in the last twenty years, and not only printing but also language correction, copy editing, posting of manuscripts to referees, etc., etc., and it is rather surprising that the price per volume has but little more than doubled since 1958 (that is, it has not even kept up with general inflation).

These considerations do not however change the fact that nobody can afford to take the journal home and read it after dinner, and the editor thinks that this is one of the functions of a good journal.

What we want to try now is as follows: If we avoid the overhead and production expenses of a large operation we could still produce derived volumes at a price suited to the reader's pocket. The idea is to create Benchmark Paper issues containing chosen papers of wide interest by offset-printing selected papers from the *Journal of Chromatography*. Our first project is to issue selected papers dealing with electrophoretic techniques (that is electrophoresis, isotachophoresis and isoelectric focusing) which were published during 1979.

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7/83

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INFLUENCE OF SORPTION ON THE SHAPE OF CHROMATOGRAPHIC ELUTION CURVES

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(First received September 24th, 1979; revised manuscript received June 19th, 1980)

SUMMARY

An expression for the chromatographic elution curve in the presence of the sorption effect and axial diffusion is obtained, and corresponding expressions for the first three statistical moments are derived. The shape of the chromatographic elution curve is considered for different values of the initial parameters, and the influence of non-linearity of the adsorption isotherm is also discussed.

INTRODUCTION

Recently, attention was paid again[1] to the importance of the sorption effect in chromatographic peak broadening and distortion when the concentration of the solute in the mobile phase becomes large. The band shape problem was considered for the case when the sorption effect and non-linearity of the adsorption isotherm were taken into account, but axial diffusion and kinetic effects were excluded.

An attempt to develop a general approach to such a problem, including the sorption effect, non-linearily of the isotherm and band broadening due to non-ideality (in terms of the concept of theoretical plates) was made by Haarhoff and Van der Linde[2] for the case when the column length tends to infinity.

The influence of the sorption effect on retention time in chromatography with a non-linear isotherm was evaluated by moments analysis by De Clerk and Buys[3].

In this paper we present a solution of the basic chromatography equation which includes the sorption effect and effective axial diffusion, first for the case of a linear isotherm. Although it seems that the combination of sorption effect with linearity of the isotherm is difficult to realize in practice, it is useful to start with this case for a clear and rigorous analysis of the influence of the sorption effect on the shape of chromatographic elution peaks. Moreover, the above solution describes an important case of chromatography with a vapour-phase eluent when sorption of the latter should be taken into account. Further, we have studied the shape of chromatographic peaks when both the sorption effect and non-linearity of the isotherm are present.

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THE BASIC DIFFERENTIAL EQUATION

In gas chromatography, the flow velocity is known to depend on the solute concentration[4–7], and it therefore varies throughout the column length. When both solute and eluent adsorption with linear isotherms is allowed, the mobile phase flow velocity in the solute zone is given by the following expression[8,9*]:

$$u = \frac{u_0}{1 - (1 - \Gamma_2/\Gamma_1) N_1} \tag{1}$$

where u_0 is the mobile phase flow velocity in the absence of solute ($N_1 = 0$) and Γ_1, Γ_2 are Henry coefficients for the solute and eluent, respectively ($\Gamma = \varepsilon + \mu k$, where ε and μ are the column volume fractions for the mobile and stationary phases, respectively, and k is the distribution coefficient for the latter).

If $\alpha = 1 - \Gamma_2/\Gamma_1$, then for the usual case, when adsorption of the eluent is absent, $\Gamma_2 = \varepsilon < \Gamma_1$, so $\alpha > 0$. However, for chromatography with a vapour-phase eluent, α may be of arbitrary sign.

The equation describing the mass transport of the mobile phase in a chromatographic column when non-equilibrium effects are taken into account by an effective diffusion coefficient, D, has the following form for the linear isotherm:

$$\Gamma_1 \cdot \frac{\partial}{\partial t} \cdot N_1 + \frac{\partial}{\partial x}(uN_1) = D \cdot \frac{\partial^2}{\partial x^2} \cdot N_1 \tag{2}$$

where t is time and x is an axial distance coordinate. It should be noted that eqn. 2 is equivalent to the following for eluent N_2:

$$\Gamma_2 \cdot \frac{\partial}{\partial t} \cdot N_2 + \frac{\partial}{\partial x}(uN_2) = D \cdot \frac{\partial^2}{\partial x^2} \cdot N_2 \tag{3}$$

if one takes into account eqn. 1 and the relationship

$$N_1 + N_2 = 1 \tag{4}$$

for the molar fractions of the solute and eluent[8]. Let us suppose the value of $|\alpha| N_1$ to be small: $|\alpha| N_1 \ll 1$; then, from eqns. 1 and 2 one obtains the basic differential equation

$$\frac{\partial}{\partial t} \cdot N_1 + \frac{u_0}{\Gamma_1}(1 + 2\alpha N_1) \frac{\partial}{\partial x} \cdot N_1 = \frac{D}{\Gamma_1} \cdot \frac{\partial^2}{\partial x^2} \cdot N_1 \tag{5}$$

We use it in this paper for studying chromatographic band development started by releasing a solute pulse into a stream of eluent at $t = 0$ (initial condition):

$$N_1(t = 0, x) = f(x) \tag{6}$$

---

* A brief derivation of eqn. 1 is given in the Appendix.

with the boundary conditions

$$N_1(t, x = \pm\infty) = 0 \tag{7}$$

Therefore, we consider here for convenience an infinite column but assume that $f(x) \geqslant 0$ only for $0 \leqslant x$.

After redefining the variables (moving reference frame)

$$\tau = t,\ \xi = \frac{\Gamma_1}{u_0} \cdot x - t$$

eqn. 5 for the function

$$C(\tau,\xi) = 2\alpha N_1[t(\tau,\xi), x(\tau,\xi)] \tag{8}$$

reduces to the well known non-linear Burgers equation[10]:

$$\frac{\partial}{\partial\tau} \cdot C + C \cdot \frac{\partial}{\partial\xi} \cdot C = \frac{\Gamma_1 D}{u_0^2} \cdot \frac{\partial^2}{\partial\xi^2} \cdot C \tag{9}$$

with the initial condition

$$C(\tau = 0,\xi) = 2\alpha f(\xi) \equiv F(\xi) \tag{10}$$

and the zero boundary conditions at infinity. Let $\nu = \Gamma_1 D/u_0^2$, then the Cole–Hopf transformation[10]

$$C(\tau, \xi) = -2\nu \cdot \frac{1}{\varphi} \cdot \frac{\partial}{\partial\xi} \cdot \varphi \tag{11}$$

reduces eqn. 9 to the parabolic diffusion-type equation:

$$\frac{\partial}{\partial\tau} \cdot \varphi = \nu \cdot \frac{\partial^2}{\partial\xi^2} \cdot \varphi$$

with the initial condition (see eqns. 10 and 11)

$$\varphi(\tau = 0, \xi) = \varphi_0 \exp\left[-\frac{1}{2\nu}\int_0^{\xi} d\eta F(\eta)\right] \tag{12}$$

The Cole–Hopf procedure[11] allows one to obtain a general solution of the non-linear problem (eqns. 5–7):

$$N_1(t, x) = \frac{1}{2\alpha t} \cdot \frac{\int_{-\infty}^{+\infty} d\omega\varphi(0, \omega)\left(\frac{\Gamma_1}{u_0} \cdot x - t - \omega\right) \exp\left[-\left(\frac{\Gamma_1}{u_0} \cdot x - t - \omega\right)^2/4\nu t\right]}{\int_{-\infty}^{+\infty} d\omega\varphi(0, \omega) \exp\left[-\left(\frac{\Gamma_1}{u_0} \cdot x - t - \omega\right)^2/4\nu t\right]} \tag{13}$$

For $\alpha \to 0$ the solution in eqn. 13 correctly reduces to the one corresponding to eqn. 5 with $\alpha = 0$ and initial condition 6.

SOLUTIONS AND MOMENTS ANALYSIS

In this section we study the shape of outlet chromatographic peaks for the particular initial condition

$$f(x) = \begin{cases} N_0, & 0 \leqslant x \leqslant x_0 \\ 0, & x < 0, x_0 < x \end{cases} \tag{14}$$

This inlet solute band approximates the rapid sample injection technique used in gas chromatography. From the general solution 13 for initial condition 14 one obtains

$$N_1(t, x) = \frac{N_0}{2\alpha} \cdot \frac{[\operatorname{erf}(a + d) - \operatorname{erf}(b + d)] \exp c}{1 - \operatorname{erf} a + [\operatorname{erf}(a + d) - \operatorname{erf}(b + d)] \exp c + [1 + \operatorname{erf} b] \exp h} \tag{15}$$

where

$$\operatorname{erf} y = \frac{2}{\sqrt{\pi}} \int_{-\infty}^{y} d\zeta e^{-\zeta^2}$$

and

$$\left.\begin{aligned} a &= \xi/\sqrt{4vt} \\ b &= \left(\xi - \frac{\Gamma_1}{u_0} \cdot x_0\right)/\sqrt{4vt} \\ c &= -2\alpha N_0 \left(2t\xi - 2\alpha N_0 t^2 + \frac{\Gamma_1}{u_0} \cdot x_0 t\right)/4vt \\ d &= -2\alpha N_0 t/\sqrt{4vt} \\ h &= -2\alpha N_0 \cdot \frac{\Gamma_1}{u_0} \cdot x_0/2v \end{aligned}\right\} \tag{16}$$

Solution 15 and 16 takes into account a finite inlet volume effect: $x_0 > 0$. This is very important for a rapid development because in this instance the shape of a chromatographic peak depends crucially on the width of the inlet solute band (eqn. 14).

If $x_0 \to 0$ with the condition $x_0 N_0 =$ constant (infinitely narrow inlet solute band), then the finite inlet volume effect disappears and solution 15 and 16 takes the following form[9]:

$$N_1(t, x) = \frac{1}{2\alpha} \sqrt{\frac{\Gamma_1 D}{t u_0^2}} \cdot \frac{(e^R - 1) \exp\left[-\frac{(x - u_0 t/\Gamma_1)^2}{4Dt/\Gamma_1}\right]}{\sqrt{\pi} + (e^R - 1)\sqrt{\pi}\left[1 - \frac{1}{2} \cdot \operatorname{erf}\left(\frac{x - u_0 t/\Gamma_1}{\sqrt{4Dt/\Gamma_1}}\right)\right]} \tag{17}$$

where

$$R = \frac{Q}{D} \equiv \frac{\alpha N_0 x_0 u_0}{D} \tag{18}$$

Let the diffusion be more essential than the sorption effect, *i.e.*, $|R| \ll 1$. Then solution 17 gives a Gaussian-type shape for the outlet chromatographic peak:

$$N_1(t, x) = \frac{N_0 x_0}{\sqrt{4\pi D t/\Gamma_1}} \cdot \exp\left[-\frac{(x - u_0 t/\Gamma_1)^2}{4Dt/\Gamma_1}\right] \tag{19}$$

In contrast, for $|R| \gg 1$ solution 17 gives an essentially asymmetric outlet peak (for details, see ref. 9). It should be noted that the above asymmetry depends crucially on the sign of α. For $\alpha > 0$ (sorption of the solute is easier than that of the eluent) tailing of the outlet peak is observed:

$$N_1(t, x) = \left\{ \begin{array}{cc} 0(R^{-1/2}) & \text{for} \quad Z = \dfrac{x - u_0 t/\Gamma_1}{\sqrt{4Qt/\Gamma_1}} < 0 \\ \dfrac{1}{2\alpha u_0}\sqrt{\dfrac{4Q\Gamma_1}{t}} \cdot \dfrac{Z}{1 + 2Z\sqrt{\pi R}\exp\left[R(Z^2 - 1)\right]} & \text{for} \quad 0 \leqslant Z \end{array} \right\} \tag{20}$$

If $R \to \infty$ the outlet peak (eqn. 20) has a shock-wave shape:

$$N_1(t, x) = \left\{ \begin{array}{cc} \dfrac{\Gamma_1}{2\alpha u_0} \cdot \dfrac{(x - u_0 t/\Gamma_1)}{t} & \text{for} \quad 0 < \dfrac{x - u_0 t/\Gamma_1}{\sqrt{4Qt/\Gamma_1}} < 1 \\ 0 & \text{for other } t, x \end{array} \right\} \tag{21}$$

For $\alpha < 0$ (sorption of the eluent is easier than that of the solute) asymmetry of the outlet peak is of the apposite type:

$$N_1(t, x) = \left\{ \begin{array}{cc} 0(|R|^{-1/2}) & \text{for} \quad Z = \dfrac{x - u_0 t/\Gamma_1}{\sqrt{4|Q|t/\Gamma_1}} > 0 \\ \dfrac{1}{2\alpha u_0}\sqrt{\dfrac{4|Q|\Gamma_1}{t}} \cdot \dfrac{Z}{1 - 2Z\sqrt{\pi|R|}\exp\left[|R|(Z^2 - 1)\right]} & \text{for} \quad Z \leqslant 0 \end{array} \right\} \tag{22}$$

and for $R \to \infty$ one obtains

$$N_1(t, x) = \left\{ \begin{array}{cc} \dfrac{\Gamma_1}{2|\alpha|u_0} \cdot \dfrac{|x - u_0 t/\Gamma_1|}{t} & \text{for} \quad -1 < \dfrac{x - u_0 t/\Gamma_1}{\sqrt{4|Q|t/\Gamma_1}} < 0 \\ 0 & \text{for other } t, x \end{array} \right\} \tag{23}$$

The outlet peaks for different values of parameters and fixed coordinates x (the column length) are shown in Fig. 1.

The explicit solution 17 allows us to develop moments analysis for the outlet peak. This gives information about the shape of the chromatographic elution curves. We start with dispersion (the second moment), $\sigma^2 = < (x - \langle x \rangle)^2 >$, which has the following expression:

$$\sigma^2 = \frac{4D(e^R - 1)\, t}{R\Gamma_1}\left\{I_2 - \frac{e^R - 1}{R} \cdot I_1^2\right\} \tag{24}$$

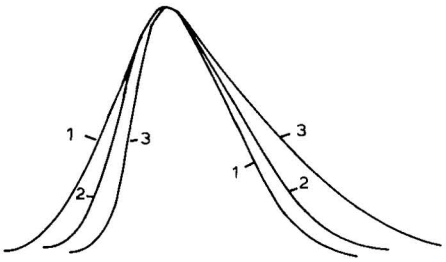

Fig. 1. Peaks at $\mu k_1 = 5.0$; $u_0 = 2$ cm/sec; $x = 50$ cm; $\varepsilon = 0.2$; $D = 0.1$ cm^2/sec. 1, $N_0 = 0.01$ ($R = 0{,}384$); 2, $N = 0.1$ ($R = 3.84$); 3, $N = 0.3$ ($R = 11{,}52$).

Here, by definition

$$\langle x^k \rangle = \frac{\int_{-\infty}^{+\infty} \mathrm{d}x x^k N_1(t, x)}{\int_{-\infty}^{+\infty} \mathrm{d}x N_1(t, x)}$$

and therefore

$$I_k = \int_{-\infty}^{+\infty} \mathrm{d}x \cdot \frac{x^k \exp(-x^2)}{\sqrt{\pi} + (\mathrm{e}^R - 1)\sqrt{\pi}\left(1 - \frac{1}{2}\operatorname{erf} x\right)} \tag{25}$$

As a consequence of eqn. 24, dispersion of the outlet elution curves in the presence of a sorption effect increases linearly with time. A detailed analysis shows that for $|R| \gg 1$ (with good accuracy for $R > 13$) one has

$$\sigma^2 = \frac{2|Q|t}{9\Gamma_1} \tag{26}$$

When diffusion dominates, *i.e.*, $|R| \ll 1$, one has correspondingly (see eqn. 19)

$$\sigma^2 = \frac{4Dt}{\Gamma_1} \tag{27}$$

The second moment, σ^2, gives the law of outlet band broadening, and the third moment, $\chi = \langle (x - \langle x \rangle)^3 \rangle$, its degree of asymmetry:

$$\chi = \left(\frac{4Dt}{\Gamma_1}\right)^{3/2} \frac{\mathrm{e}^R - 1}{R}\left\{I_3 + 2 \cdot \frac{(\mathrm{e}^R - 1)^2 I_1^3}{R^2} - 3 \cdot \frac{(\mathrm{e}^R - 1) I_1 I_2}{R}\right\} \tag{28}$$

For $|R| \ll 1$ the third moment, χ, is naturally zero, (see eqn. 19), but for $|R| \gg 1$ one obtains from eqn. 28 the following expression:

$$\chi = -(\operatorname{sign} R)\frac{1}{5}\left(\frac{4|Q|t}{9\Gamma_1}\right)^{3/2} \tag{29}$$

where sign R corresponds to two different situations (eqn. 21 and 23).

NON-LINEAR CHROMATOGRAPHY

A well known standard approach for explaining the real shape of gas chromatographic elution curves is based on the isotherm non-linearity assumption (non-linear chromatography)[11–13]. In spite of the significant attention paid in many papers to non-linearity of the isotherm an exhaustive description of experimental data cannot be given without taking into account the above-mentioned sorption effect. An explicit example has been described[14,15] for a solute which, with high accuracy, has a linear isotherm but an asymmetric outlet chromatographic peak. Therefore, it is important to study the sorption effect in non-linear chromatography.

Let a non-linear solute isotherm to be of the polynomial form

$$a_1 = g_1(N_1) = \mu k_1 N_1 + \delta_1 N_1^2 \tag{30}$$

Then eqn. 2 changes to

$$(\Gamma_1 + 2\delta_1 N_1)\frac{\partial}{\partial t} \cdot N_1 + \frac{\partial}{\partial x}(uN_1) = D \cdot \frac{\partial^2}{\partial x^2} \cdot N_1 \tag{31}$$

Here we again assume adsorption of the eluent with $N_2 = 1 - N_1$, so that equations similar to eqns. 30 and 31 can be written. As in the section The Basic Differential Equation, these equations together with eqns. 31 and 4 immediately give us the expression for the mobile phase flow velocity, u:

$$u = u_0 \exp\left(-\int_0^{N_1} \mathrm{d}y \cdot \frac{n + my}{k + ly + my^2}\right) \tag{32}$$

where

$$k = \Gamma_1;\ l = \Gamma_2 - \Gamma_1 + 2(\delta_1 + \delta_2);\ m = -2(\delta_1 + \delta_2);\ n = \Gamma_2 - \Gamma_1 + 2\delta_2 \tag{33}$$

Let $\Delta = 4km - l^2$ and $4\Gamma_1\Gamma_2 < (\Gamma_1 + \Gamma_2 - m)^2$, then $\Delta < 0$, and therefore

$$u = u_0 \exp\left\{-\frac{n}{\sqrt{-\Delta}} \cdot \ln\left|\frac{(l + 2mN_1 - \sqrt{-\Delta})(l + \sqrt{-\Delta})}{(l + 2mN_1 + \sqrt{-\Delta})(l - \sqrt{-\Delta})}\right|\right.$$
$$-\frac{1}{2}\ln\left|\frac{k + lN_1 + mN_1^2}{k}\right| + \frac{1}{2} \cdot \frac{l}{\sqrt{-\Delta}}$$
$$\left. \cdot \ln\left|\frac{(l + 2mN_1 - \sqrt{-\Delta})(l + \sqrt{-\Delta})}{(l + 2mN_1 + \sqrt{-\Delta})(l - \sqrt{-\Delta})}\right|\right\} \tag{34}$$

The general expression 34 seems to be difficult to handle, but it shows that with the approximations

$$|\delta_1| + |\delta_2| \ll |\Gamma_1 - \Gamma_2|,\ |\delta_1 N_1| \ll \Gamma_1 \tag{35}$$

eqn. 1 remains unchanged. Hence, the basic differential equation takes the following form (compare eqn. 5):

$$\frac{\partial}{\partial t} N_1 + \frac{u_0}{\Gamma_1}\left[1 + 2\left(\alpha - \frac{\delta_1}{\Gamma_1}\right) N_1\right] \frac{\partial}{\partial x} N_1 = \frac{D}{\Gamma_1} \cdot \frac{\partial^2}{\partial x^2} N_1 \tag{36}$$

A similar equation was considered by De Clerk and Buys[3], where only adsorption of a solute was allowed and the investigation was based on moments analysis.

From eqn. 36 it follows that the theory developed above can be adapted to include isotherm non-linearity with the approximation (eqn. 35) by introducing

$$\tilde{\alpha} = \alpha - \delta_1/\Gamma_1 = 1 - (\Gamma_2 + \delta_1)/\Gamma_1 \tag{37}$$

Therefore, there are no drastic changes in the shape of the outlet band in comparison with the case $\delta_1 = 0$, because $\tilde{\alpha}$ has the same sign as α. Hence for the approximations 35 the sorption effect has a prevailing effect on the non-linearity of the isotherm, which gives only small corrections to the solution 15 or 17.

In the opposite case:

$$|\delta_1| + |\delta_2| \gg |\Gamma_1 - \Gamma_2|, \; |\delta_1 N_1| \ll \Gamma_1 \tag{38}$$

one obtains from eqn. 34 for the mobile phase flow velocity:

$$u = \frac{u_0}{1 + N_1(\delta_1 + \delta_2)/\Gamma_1} \tag{39}$$

Then the basic differential equation 31 takes the form

$$\frac{\partial}{\partial t} \cdot N_1 + \frac{u_0}{\Gamma_1}\left[1 + 2(\beta_1 + \beta_2) N_1\right] \frac{\partial}{\partial x} \cdot N_1 = \frac{D}{\Gamma_1} \cdot \frac{\partial^2}{\partial x^2} N_1 \tag{40}$$

As above, this case can be easily adapted by introducing the parameter

$$\hat{\alpha} = \beta_1 + \beta_2$$
$$\beta_1 = -(\delta_1 + \delta_2)/\Gamma_1, \; \beta_2 = -\delta_1/\Gamma_1 \tag{41}$$

An important consequence of eqns. 38 and 40 is a non-trivial interference of the sorption effect, when both solute and eluent adsorption are allowed, with non-linearity of the isotherm. When the sorption effect is excluded, $\beta_1 = 0$, and

$$\hat{\alpha} = -\delta_1/\Gamma_1 \tag{42}$$

The sorption effect (see eqns. 38 and 41 can change the value and sign of $\hat{\alpha}$ (eqn. 42) and, as a consequence, the shape of the outlet peak (see Solutions and Moments Analysis). This means that the investigation of the sorption effect in non-linear chromatography for the case in eqn. 38 is very relevant.

CONCLUSION

In contrast to other papers (*e.g.*, ref. 13) this work takes into account a new aspect of the sorption effect by allowing both solute and eluent sorption within the framework of non-ideal and non-linear chromatography. This case can be realized experimentally in chromatography with a vapour-phase eluent[14,15].

The main results of our research are the following.

(i) The above-mentioned sorption effect allows the elution curve to have both types of asymmetry (even in the case of linear isotherms) which depends on the relationship between the Henry coefficients Γ_1 and Γ_2 corresponding to the solute and eluent.

(ii) There is a non-trivial interference between the sorption effect and the isotherm non-linearity, which for appropriate conditions can change the initial asymmetry due to isotherm non-linearity into the opposite one for different signs of α. These results are obtained on the basis of the following main assumptions:

(a) the chromatographic process is supposed to be non-ideal, which is taken into account by means of a standard concept of effective diffusion with a longitudinal coefficient D incorporated into the well known sorption dynamic equations (eqns. 2, 3 and 31) (in general, the coefficients should be different for the solute and the eluent);

(b) the chromatographic process corresponds to a constant mass flow-rate of carrier gas at the column inlet;

(c) non-linear distribution isotherms (non-linear chromatography) with a small deviation from linearity are allowed for solute and eluent (see eqn. 30).

In this paper, our main purpose has been to obtain an explicit solution of the problem instead of, *e.g.*, pure moments analysis. This is possible, of course, only with some limitations as defined by the model. Our study of the sorption effect in non-linear chromatography is performed, for instance, only in the two extremal cases. The approximations implied can be illustrated in detail (see also Appendix) by the second case. The sorption dynamic equation 31 for the solute contains two types of non-linearity:

$$(\Gamma_1 + 2\delta_1 N_1)\frac{\partial N_1}{\partial t} + \frac{\partial}{\partial x}\left[\frac{u_0}{1 + N_1(\delta_1 + \delta_2)/\Gamma_1} \cdot N_1\right] = D \cdot \frac{\partial^2}{\partial x^2} \cdot N_1 \qquad (43)$$

The first corresponds to isotherm non-linearity (eqn. 30) and the second to the sorption effect (eqn. 39), when linear terms of the isotherms are close (eqn. 38). The assumption (c) [here $|\delta_1 N_1| \ll \Gamma_1$ and $|(\delta_1 + \delta_2)N_1| \ll \Gamma_1$] allows one to rewrite eqn. 43 in the form of eqn. 40. It is for this case that the result (ii) follows for appropriate values of the parameters. The assumptions above are known to be natural and easily realized for gas–liquid chromatography[7,14]. Therefore, results (i) and (ii) are readily applicable to an explanation of experiments described elsewhere[14,15], where "shock-wave"-type elution curves (for $|R| \gg 1$) and asymmetry inconsistent with non-linear chromatography without a sorption effect were observed.

Now, it should be noted that the considerations in the last two sections essentially reduce to analysis of the Burgers-type equations. Therefore, the validity of our approximations has been adequately tested by means of computer simulation[3,12,13]. For intermediate cases, which are not discussed in the previous section,

the vindications of our approach will have to await both computer and experimental verification.

APPENDIX

We turn now to the sorption effect when both solute and eluent adsorption are allowed. For the convenience of readers we derive here the expression for the mobile phase flow velocity, $u = u(N_1)$, in the sorption zone (see eqns. 1 and 32).

For the last stage of the chromatographic process, owing to the broadening of the elution zone, diffusion is expected to be of secondary importance in the equations of sorption dynamics[8]. Therefore, they can be rewritten in the form

$$\left.\begin{aligned} \frac{\partial}{\partial t} a_1(N_1) + \frac{\partial}{\partial x}(uN_1) = 0 \\ \frac{\partial}{\partial t} a_2(N_2) + \frac{\partial}{\partial x}(uN_2) = 0 \end{aligned}\right\} \quad \text{(A1)}$$

where $a_1 = g_1(N_1)$ and $a_2 = g_2(N_2)$ are the distribution isotherms for the solute and eluent, respectively. The molar fractions of the latter satisfy eqn. 4, so from eqn. A1 on obtains

$$\frac{\mathrm{d}a_2}{\mathrm{d}N_2} \cdot \frac{\partial}{\partial x}(uN_1) + \frac{\mathrm{d}a_1}{\mathrm{d}N_1} \cdot \frac{\partial}{\partial x}(uN_2) = 0 \quad \text{(A2)}$$

Eqn. A2 is equivalent to the equation

$$(B - A)\, u \cdot \frac{\partial N_1}{\partial x} + (BN_1 + AN_2) \frac{\partial u}{\partial x} = 0$$

where

$$A = \frac{\mathrm{d}a_1}{\mathrm{d}N_1} \quad \text{and} \quad B = \frac{\mathrm{d}a_2}{\mathrm{d}N_2},$$

which can be easily integrated for $u = u(N_1)$ with initial condition $u_0 = u(N_1 = 0)$:

$$u = u_0 \exp\left(-\int_0^{N_1} \mathrm{d}N_1 \cdot \frac{B - A}{BN_1 + AN_2}\right) \quad \text{(A3)}$$

As a consequence, one obtains eqn. 1 for linear isotherms and eqn. 32 for non-linear isotherms (eqn. 30).

LIST OF SYMBOLS

a = convenient parameter in eqn. 16;
a_1 = solute concentration in stationary phase (eqn. 30);
b = convenient parameter in eqn. 16;

c = convenient parameter in eqn. 16;
C = convenient function in eqn. 8;
d = convenient parameter in eqn. 16;
D = effective longitudinal diffusion coefficient;
g_1 = solute concentration in stationary phase (eqn. 30);
f = value of solute concentration at time $t = 0$;
F = value of C at time $\tau = 0$;
k = Γ_1, Henry coefficient for solute (eqn. 33);
k_i $(i = 1,2)$ = mass distribution coefficients for solute and eluent;
I_k $(k = 1,2,3)$ = value of integrals (eqn. 25);
l = convenient parameter in eqn. 33;
m = convenient parameter in eqn. 33;
n = convenient parameter in eqn. 33;
N_0 = amplitude of solute concentration for inlet band;
N_i $(i = 1,2)$ = concentration of solute and eluent in mobile phase;
Q = convenient parameter in eqn. 18;
R = convenient parameter in eqn. 18;
t = time;
u_0 = mobile phase flow velocity in the absence of solute;
u = mobile phase flow velocity;
x = axial distance coordinate;
x_0 = width of inlet solute band (eqn. 14);
$\langle x \rangle$ = mean (first moment) of concentration–distance distribution;
Z = convenient parameter in eqns. 20 and 22;
α = $(1 - \Gamma_2/\Gamma_1)$, convenient parameter;
$\tilde{\alpha}$ = convenient parameter in eqn. 37;
$\hat{\alpha}$ = convenient parameter in eqn. 41;
β_i $(i = 1,2)$ = parameters in expression for $\hat{\alpha}$;
Γ_i = $\varepsilon + \mu k_i$ $(i = 1,2)$ = Henry coefficients for solute and eluent;
Δ = determinant (eqn. 34);
δ_i $(i = 1,2)$ = isotherm non-linearity parameters;
ε = column volume fraction for mobile phase;
μ = column volume fraction for stationary phase;
ν = $\Gamma_1 D/u_0^2$, convenient parameter in eqn. 11;
ξ = moving reference frame;
σ^2 = second moment;
τ = t, moving reference frame;
φ = convenient function in Cole–Hopf transformation (eqn. 11);
φ_0 = $\varphi(0,0)$, initial value of φ;
χ = third moment.

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PEAK IDENTIFICATION IN CAPILLARY GAS CHROMATOGRAPHY BY SIMULTANEOUS FLAME IONIZATION DETECTION AND ^{14}C-DETECTION

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SUMMARY

The simultaneous peak identification by radiocarbon and flame ionization detection in the effluents from capillary gas chromatography is reported. A constant portion of the effluent is split off, the organic matter converted into CO_2 and the $^{14}CO_2$ monitored using an anthracene flow cell. Adsorption and condensation in the detection system ("memory" effects) are thereby avoided and identical conditions can be used for a variety of compounds. The system proved to be suitable for the analysis of pesticide metabolites with a specific activity down to 37 kBq/mg, the limit of detection being 7 Bq.

INTRODUCTION

Gas chromatography–mass spectrometry (GC–MS) is a reliable and effective method for evaluation of the structure of metabolites[1–6]. Biological samples containing metabolites are complex mixtures of components. Open-tubular capillary columns are capable of separating the individual fractions by GC, and the pure components are then introduced into the mass spectrometer for structure analysis. However, the GC fraction consisting of the unknown metabolites is usually not precisely defined, and therefore a set of fractions must be analyzed by MS. The amount of data produced accumulates and the selection of reliable structural information becomes increasingly difficult.

In metabolism studies using radiolabelled compounds it is therefore desirable to detect the ^{14}C-labelled metabolite fractions during GC separation, as only these fractions need to be analyzed in MS. Simultaneous detection of radioactivity and mass in GC has been reported by several authors[7–10], but not in capillary gas chromatography. Therefore, we have developed capillary GC with simultaneous and continuous detection of mass and radioactivity.

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EXPERIMENTAL

Principle of the method

A schematic view of the apparatus is presented in Fig. 1. The effluent of the glass capillary column was split at a ratio of 9:1. The minor portion was introduced into the flame ionization detector (FID), the major one combusted to CO_2 and measured continuously in a radioactivity flow monitor using an anthracene cell.

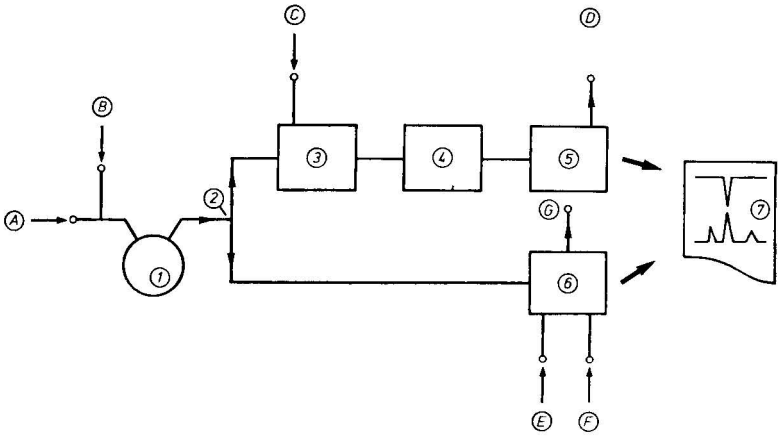

Fig. 1. Schematic description of gas flow and apparatus used for ^{14}C-detection in capillary GC. A = Carrier gas; B = injection; C = oxygen; D = outlet pump; E = air; F = hydrogen; G = exhaust. 1 = Capillary column; 2 = outlet splitter; 3 = combustion furnace; 4 = ^{14}C-flow cell; 5 = pump; 6 = FID; 7 = recorder.

Column oven and splitter

A Model 2151 Fractovap (Carlo Erba, Milan, Italy) was used, equipped with an injection system according to Grob and Grob[11]. Capillary glass columns (10–50 m × 0.3 mm I.D.) were purchased from Jaeggi (Trogen, Switzerland). Helium served as carrier gas (0.5–1.0 bar, 1–5 ml/min) and as purge gas for the injector port (17 ml/min). The columns were usually operated between 50°C and 250°C with various temperature programs. The volumes injected were 0.5–3 μl. The outlet splitter was a modification of the Neuner-Jehle splitter[12] as illustrated in Fig. 2. The splitting ratio was predetermined by the inner diameter of the outlet platinum–iridium capillaries. A ratio of 9:1 was found suitable for our purposes. One tube of the splitter was connected directly with the FID, the other one with the combustion furnace. The splitter was regularly cleaned according to the methods described by Christiansen[6].

Combustion furnace

The combustion furnace is shown in Fig. 3. The splitter effluents (1–3.5 ml/min) were transferred through a heated transfer line (300°C) into a quartz tube (inner volume 200 μl) which contained the combustion catalyst (cobaltous oxide). Oxygen gas was fed at 1.5 ml/min into the tube which was maintained at 650°C by a heating coil (platinum) powered by a variable transformer. The combustion tube is open to the

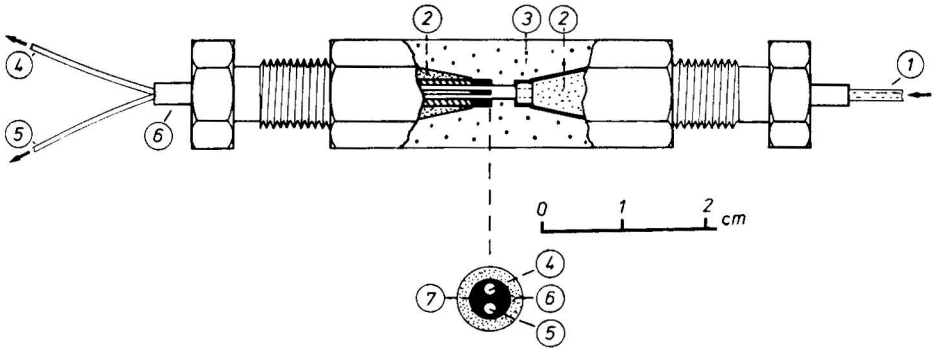

Fig. 2. Design of the outlet splitter. 1 = Glass capillary; 2 = 1/16-in. Swagelock GC ferrules; 3 = Swagelock GC union; 4, 5 = Pt–Ir capillary tube; 6 = 1/16-in. steel capillary tube; 7 = seal of silver solder. Below: exploded view of vertical cross-section of te splitter.

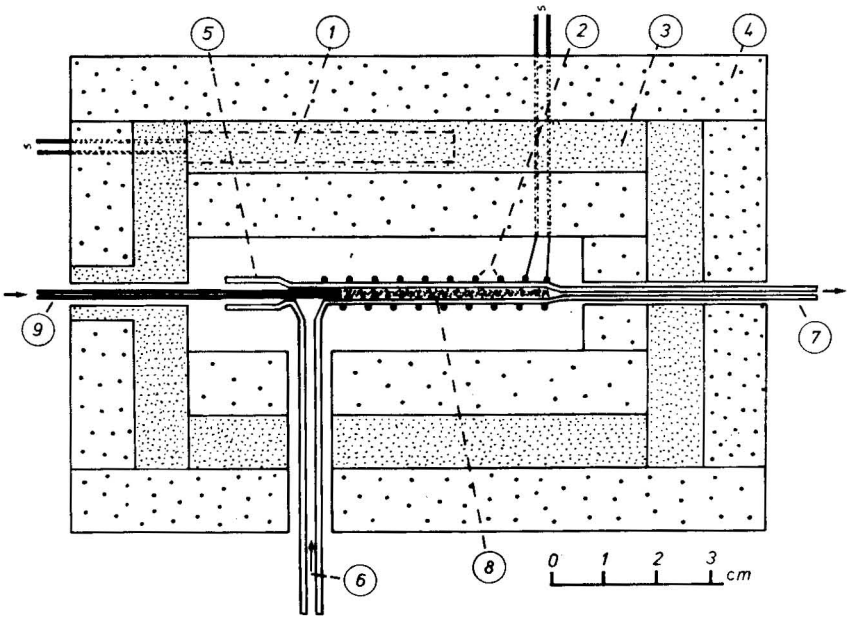

Fig. 3. Design of combustion furnace. 1 = Heating cartridge; 2 = heating coil; 3 = aluminium block; 4 = marinite housing; 5 = quartz combustion tube; 6 = oxygen inlet; 7 = transfer tube to flow cell; 8 = oxidation catalyst; 9 = inlet capillary.

atmosphere along the inlet capillary, which itself impinges directly on the combustion catalyst, back pressure onto the splitter thereby being avoided.

The aluminium block (3) heated by the cartridge (1) is maintained at a minimum temperature of 200°C. Condensation in the transfer capillary and in the combustion tube is thereby avoided.

Radioactivity flow monitor

^{14}C-Monitoring was carried out by a BF 5026 flow monitor (Dr. Berthold, Wildbad, G.F.R.) equipped with an anthracene cell (total volume 7 ml). The effluents from the combustion furnace (2.5–5 ml/min) were transferred to the cell by PTFE tubing (1/16 in. O.D.). A suction pump, Type AL No. 17 KL (W. Wirth, Basel, Switzerland), connected to the flow cell furnished a negative pressure (suction rate, 12–14 ml/min). The BF 5026 was usually operated with a sensitivity of 1000 cpm and a time constant of 2 sec. The lower and the upper thresholds of the analyzer channel A were 250 and 850 respectively. Using the time constant of 2 sec, the background of the operating system was characterized by a standard deviation of 0.37 Bq.

The anthracene (Fluka, Buchs, Switzerland) was recrystallized from toluene–ethyl acetate (8:2) and washed with ethyl acetate, then with 5% aqueous formic acid.

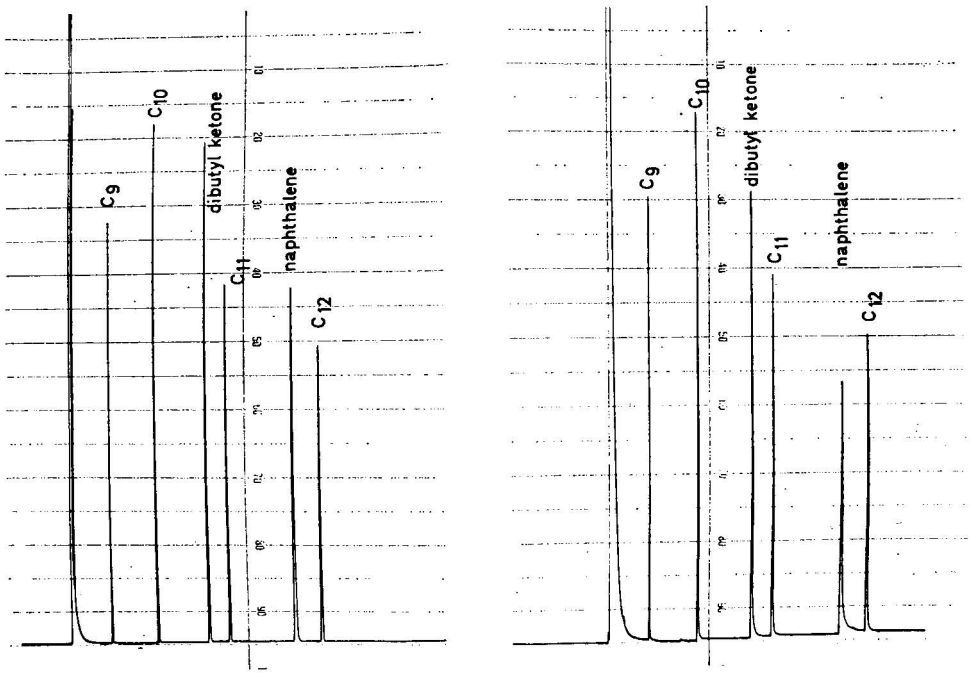

Fig. 4. Separation of a test mixture without (left) and with (right) effluent splitter. Order of elution: nonane, decane, dibutyl ketone, undecane, naphthalene and dodecane.

RESULTS

Influence of the effluent splitter on the GC resolution

As demonstrated in Fig. 4, the separation of the test mixture is slightly affected by the effluent splitter. The strong tailing of the naphthalene peak is probably caused by Carbowax remaining in the effluent splitter after its deactivation.

Reproducibility and sensitivity of the ^{14}C-detection

[^{14}C]2,6-Dimethylaniline (32 Bq) was injected repeatedly onto the capillary

column and detected with a peak height eight times the "noise" of the detector (defined as 4 × SD background, *i.e.* 1.5 Bq). Under these experimental conditions the ^{14}C-peak height of four consecutive injections was calculated to be 12 ± 0.4 Bq (mean $\pm$ SD, $n = 4$).

Application

GC analyses of typical samples are presented in Figs. 5–7.

DISCUSSION

Simultaneous detection of ^{14}C-radioactivity and mass (FID) in capillary GC was successfully performed largely due to the following features:

(1) An outlet splitter with a dead volume of approximately 10 μl.

(2) A combustion tube with a dead volume less than 100 μl in a total volume of 200 μl.

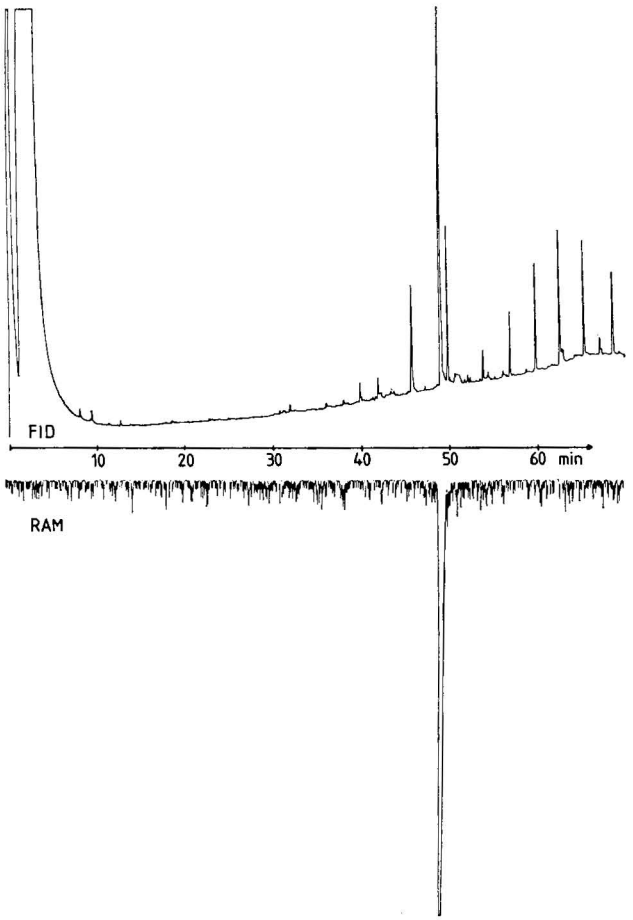

Fig. 5. Analysis of a non-polar metabolite of an agrochemical isolated from potato leaf tissue and partially purified. 167 Bq (1 μl) of radioactive material (specific activity, 800 kBq/mg) were analyzed Column, 25 m × 0.25 mm SE 54; temperature programmed from 60°C to 250°C at 3°/min.

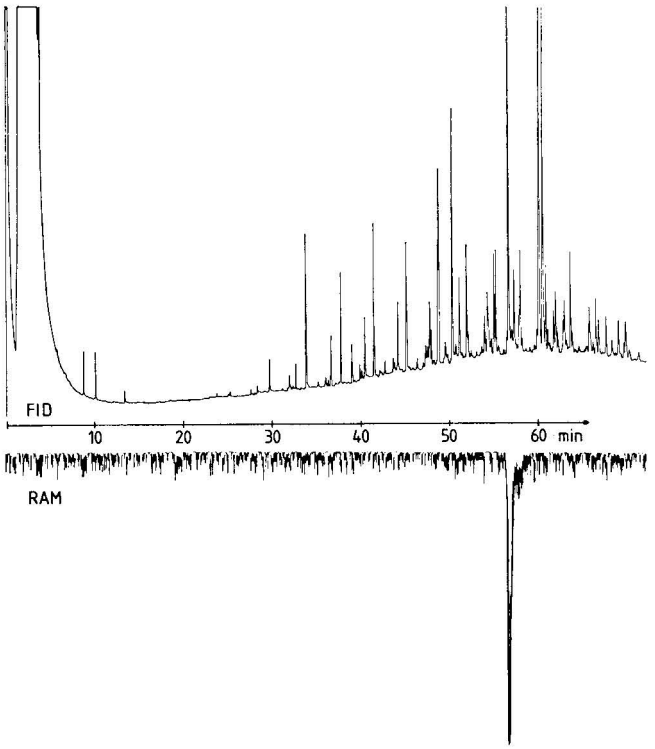

Fig. 6. Analysis of a polar metabolite of an agrochemical isolated as in Fig. 5. After methylation using diazomethane, 67 Bq (1 μl) of the radioactive material (specific activity, 318 k Bq/mg) were analyzed. Column as in Fig. 5.

Since organic materials in the GC effluents are not oxidized completely in this small volume, addition of oxygen is necessary. The catalyst would be contaminated and its activity soon exhausted without the supply of oxygen.

(3) Pressure waves generated by combustion in the tube are prevented from being tranferred back into the GC system (splitter and column) by the fact that the combustion tube is open to the atmosphere and by the presence of the suction pump.

(4) Oxidation of total organic material to CO_2 proved to have the following advantages: no contamination of the ^{14}C-detection system by organic matter, and hence no "memory" effects; no ^{14}C-recovery problems caused by the individual organic compounds; and the ^{14}C-detection must be optimized for only one compound namely CO_2. Furthermore, combustion to $^{14}CO_2$ allowed the use of an anthracene cell, which represents an improvement over glass and liquid scintillators with regard to counting efficiency and handling.

Under practical conditions the peak resolution R of the ^{14}C-detection system

$$R = \Delta t/w$$

where t = retention times, w = peak width at half peak height, was about one-half that of the FID detection. 6.7 Bq of injected radioactivity resulted in a peak height

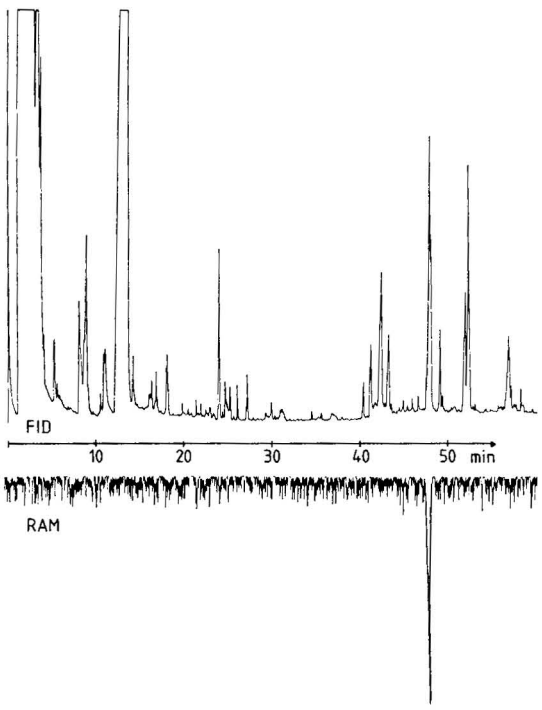

Fig. 7. Analysis of a polar metabolite of an agrochemical isolated from grape leaf tissue and partially purified. After ethylation using diazoethane, 34 Bq (1 μl) of the radioactive material (specific activity, 185 kBq/mg) were analyzed. Column as in Fig. 5, but programmed from 50°C to 250°C at 3°/min.

above background equivalent to six times the standard deviation of the background. Thus, 0.2 μg of a metabolite with a specific activity of k Bq/mg were detected during capillary gas chromatography. This amount was considered to represent the "limit of detection" of our system.

In conclusion, it was possible to measure continuously the ^{14}C-radioactivity in capillary GC effluents simultaneously with mass detection. Thus the presence of a ^{14}C-labelled metabolite in a given GC fraction could be verified immediately, and the MS data directly related to it.

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HYDROPHOBIC CHROMATOGRAPHY WITH DYNAMICALLY COATED STATIONARY PHASES

III. NON-IONIC SURFACTANT EFFECTS

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SUMMARY

The effects of addition to aqueous–organic eluents of four members of a group of chemically similar non-ionic surfactants (Tweens) on the chromatographic properties of silicon(IV) oxide gels has been investigated. It was found that small amounts of Tween added to eluent altered the silica surface so that hydrocarbon, phenolic and ketonic eluites were retained to give separations very similar to those obtained with an alkyl (C_{18}) covalently bonded silica.

INTRODUCTION

Knox and co-workers[1–3] demonstrated that retention of charged analytes on columns of alkyl-bonded silica gels could be increased by inclusion in the mobile phase of suitably hydrophobic "counter-ions". This technique is often referred to as "soap" or "reversed-phase ion-pair" chromatography. These adducts to the mobile phase in this technique are often charged surfactants such as alkyl sulphonates and sulphates or tetraalkylammonium salts. However, little information is available on the possible effects of addition of non-ionic detergents to these separation systems.

Gilbert and Wall[4] found that porous ceria column packings gave eluite retention from aqueous methanolic eluents containing hexadecyl trimethylammonium bromide similar to that reported by Knox and Laird[1] on SAS-silica, an alkyl-bonded high-performance liquid chromatography (HPLC) column packing. Ghaemi and Wall[5] reported separations of some aromatic hydrocarbons, ketones and sulphonic acids on columns of silica and zirconia modified by dynamic interaction with quaternary ammonium cationic surfactants dissolved in aqueous–organic eluents, and also separations[6] of peptides on columns of silica modified by reaction with solutions of mixtures of non-ionic and anionic detergents.

In the present study the effects of addition of some non-ionic surfactants to aqueous methanolic eluents of silica columns are described. This class of surface-active compound is also shown to be deposited onto the silica gel surface. Furthermore, data presented here show that as the hydrophobicity of the surfactant increases

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(*i.e.*, as the hydrophobic carbon chain length increases), retention of aromatic hydrocarbons and ketones also increases.

EXPERIMENTAL

Instrumentation

Chromatographic systems were assembled as required from M6000A (Waters Assoc., Milford, MA, U.S.A.) or 110A (Beckman, Irvine, CA, U.S.A.) pumps; CE212 (Cecil Instruments, Cambridge, Great Britain) or SF650 (Applied Chromatography Systems, Luton, Great Britain) ultraviolet absorption detectors; septum injectors and columns (125 × 4.6 mm I.D.) made in the laboratory; and guided plunger microsyringes (Scientific Glass Engineering, Melbourne, Australia).

Column packings and reagents

The silica gels used in these experiments were Hypersil (Shandon Southern Products, Runcorn, Great Britain; $d_p = 5\ \mu m$, $S_{BET} \approx 200\ m^2\ g^{-1}$) and GA 43, an experimental spherical material ($d_p = 9\ \mu m$, $S_{BET} \approx 170\ m^2\ g^{-1}$).

Columns were packed by the "upward slurry" technique described by Bristow *et al.*[7] at 300 bar. The silica packing materials were suspended and packed in methanol. Solvent methanol was either AnalaR grade (BDH, Poole, Great Britain) or HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). Tweens 20, 40, 60, and 80 were purchased from Sigma (London, Great Britain) and were all described as of industrial quality. No manufacturers' batch numbers were quoted by the vendors.

Measurement of "adsorbed" surfactant

Samples (*ca.* 1 g) of silica gel (GA 43) were weighed into tared, stoppered flasks and dried *in vacuo* for 30 min at 120°C. Flask and contents were cooled in a desiccator and reweighed to determine the dry weight of the silica. Methanol–water (1:1, 200 cm^3) containing a known concentration of surfactant was mixed with the dried silica for 5 min in an ultrasonic cleaning bath to ensure complete dispersion of the particles. The mixture was filtered by suction through a sintered glass funnel and the excess solvent carefully sucked from the retained silica. The "filter-dry" silica was weighed and then dried *in vacuo* at 120°C, cooled in a desiccator and weighed again to obtain both the dry weight and the "wet" weight. In no case was the amount of detergent adhering to "filter-dry" silica as excess *solution* greater than a few per cent of the amount bound via surface interaction.

The above procedure was repeated for each determination with a fresh 200 cm^3 sample of surfactant solution to ensure complete equilibration.

RESULTS AND DISCUSSION

Adsorption of Tweens

Table I shows that the amount of non-ionic surfactant sorbed on the silica surface is a function of detergent concentration in the contacting solution, as has been shown by Rupprecht[8]. A general tendency to *decreasing* adsorption with increasing hydrophobicity was also observed, which will be discussed later in greater detail. The highest degree of sorption shown in Table I (197 mg of Tween 20 per gram of

TABLE I

SORPTION OF TWEEN ON GA 43 SILICA

	10^3[Tween]/kg dm^{-3}	Tween adsorbed (mg per g SiO_2)	Hydrophobic alkyl chain
Tween 20	2.0	123	$(CH_2)_{11}CH_3$
	3.0	197	
Tween 40	2.1	65	$(CH_2)_{15}CH_3$
	4.3	150	
Tween 60	2.2	52	$(CH_2)_{17}CH_3$
Tween 80	5.0	85	$(CH_2)_8CH=CH(CH_2)_7CH_3$

SiO_2) corresponds to a surface coverage of *ca.* 1.5 μmol m^{-2}, which is about half the value reported by Roumelotis and Unger[9] for C_8 and C_{18} alkyl-bonded silicas.

Separations on silica gel

Methanol–water (1:1) was used as the eluent base in all experiments reported here, with addition of the appropriate Tween to give concentrations between 0.5 and

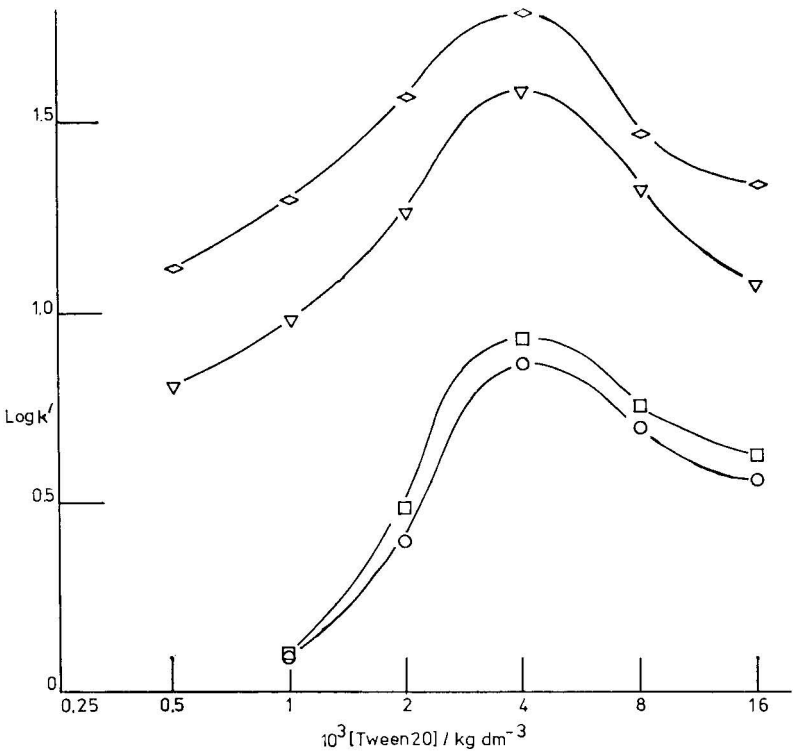


Fig. 1. Relationship of log k' to concentration of Tween 20 in methanol–water (1:1) eluent. Column packed with Hypersil ($d_p \approx 5\,\mu$m). Flow-rate 1 cm^3 min^{-1} at ambient temperature. Eluites: ○ = 9-fluorenone; □ = naphthalene; ▽ = anthracene; ◇ = pyrene.

16×10^{-3} kg dm^{-3}. All separations were carried out at ambient temperature (15–23°C) with an eluent flow-rate of 1 cm^3 min^{-1}.

Passage of between 100 and 150 cm^3 of solvent containing Tween 20 was necessary to completely equilibrate a column (125 × 4.6 mm I.D.) packed with Hypersil. Fig. 1 shows the dependence of the capacity factor, k', of some aromatic hydrocarbons and ketones on the Tween 20 modified silica gel columns as a function of detergent concentration. Fig. 2 shows the same retention/[surfactant] relationship for Tween 80, which has an unsaturated carbon chain.

The composite graph, Fig. 3, shows that the Tween concentration at which maximum retention is observed for two eluites is a function of surfactant hydrophobicity. As would be expected by analogy with alkyl-bonded silicas[10], the largest values of k' are observed with the most hydrophobic Tween, and, moreover, the surfactant concentration needed for maximum retention is progressively *reduced* as the carbon chain length is increased. Hemetsberger *et al.*[10] showed that k' was a linear

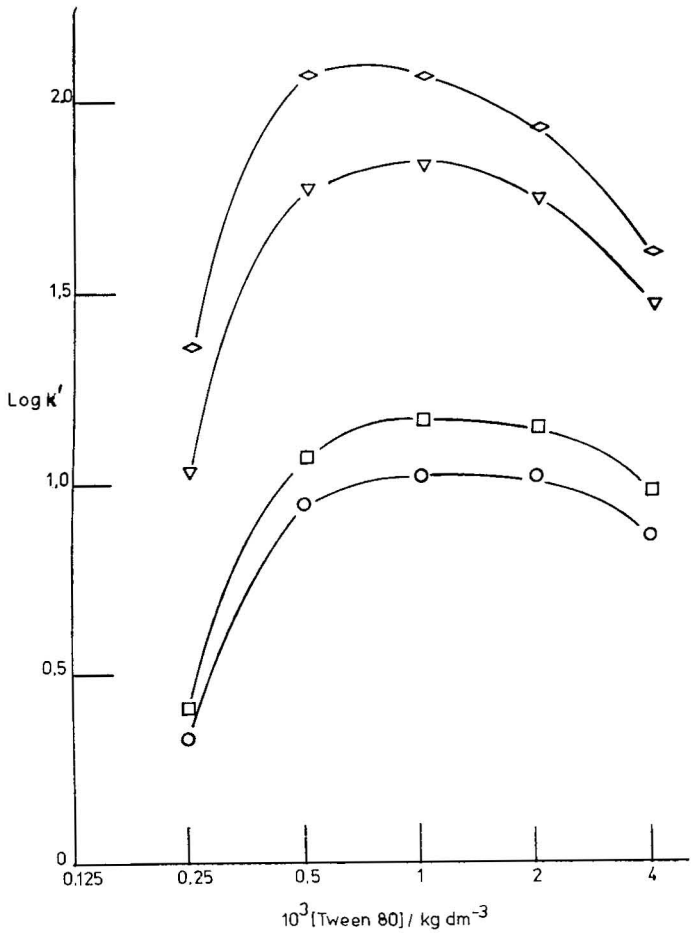


Fig. 2. Relationship of log k' to concentration of Tween 80 in eluent. Chromatographic conditions and eluites as in Fig. 1.

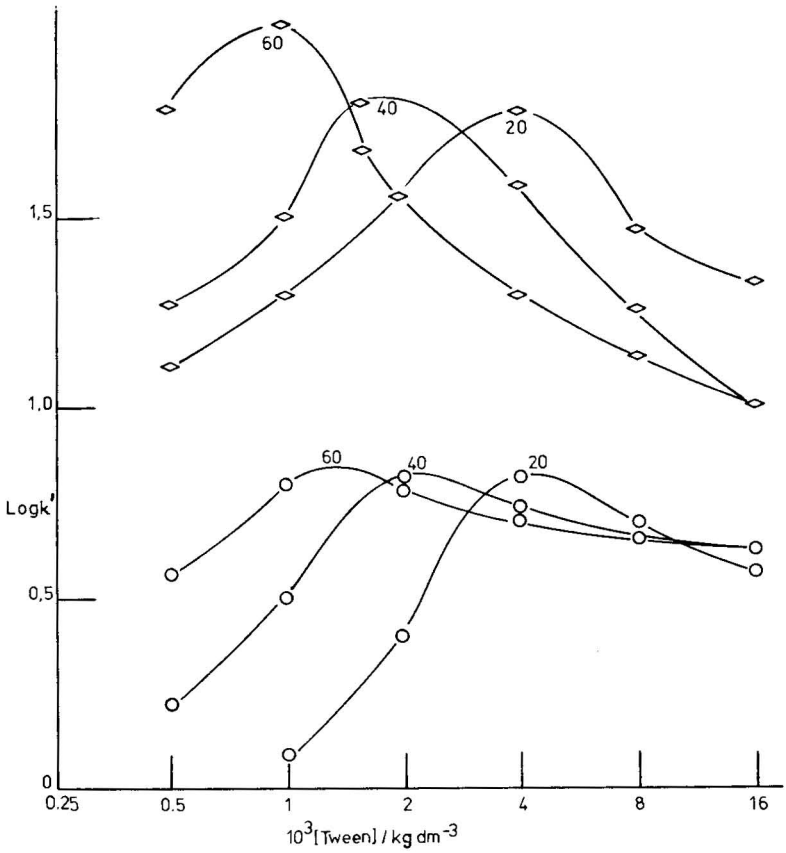


Fig. 3. Relationship of log k' to concentration of Tweens 20, 40, and 60 in the eluent. Samples and conditions as in Fig. 1.

function of percentage carbon content on a series of alkyl methyl silyl "bonded-phase" packings, and a similar trend is observed with these dynamically coated silica surfaces.

In view of the observations by Hemetsberger[10] on the relationship of eluite retention to chain length of *bonded* alkyl residue, one would expect k' values measured in those surfactant solutions giving maximum retention induced by that surfactant to increase by a relatively large factor for an increase of two or four methylene groups in the alkyl chain length of the detergent molecule. However, it can be seen from Table I that surface sorption of Tweens varies *inversely* as chain length. So the greater hydrophobicity of the longer chains is counterbalanced by lower surface coverage of the hydrophilic silica. Fig. 3 demonstrates that either no increase in k' (fluorenone) or a relatively minor increase in k' (pyrene) is observed with increasing hydrophobicity of the surfactant. It is noteworthy that introduction of a single double bond into the 18-carbon chain of Tween 60 to produce Tween 80 (compare Figs. 2 and 3) significantly increased retention of all eluites shown, but did not apparently change the detergent concentration at which maximum k' was observed.

Figs. 4 (Fig. 4a is without surfactant and Fig. 4b with surfactant) and 5 are representative chromatograms of aromatic hydrocarbons, phenols and ketones on silica. Separations of one of the standard test mixtures suggested by Knox[11] for determination of the efficiency of columns of alkyl-bonded silica columns are shown in Figs. 6 and 7. The order of elution and relative retention of the analytes on the ODS-Hypersil column (Fig. 6) is very similar to that observed in aqueous methanolic Tween 40 elution from a silica column (Fig. 7).

The similarity in elution order and relative retention of the various analytes shown in Figs. 6 and 7 suggests a similarity in separation mechanism. As suggested in earlier studies in this series, the result of interaction between silica and the polyol terminus of the Tween molecule is probably a modified surface bearing "brushes" of surfactant molecules. As the concentration of surfactant in the eluent is increased, two competing processes occur. Firstly, coverage of the silica by "brushes" increases and, secondly, association of "bound" and free surfactant molecules (probably by hydrophobic bonding of alkyl chains) also increases.

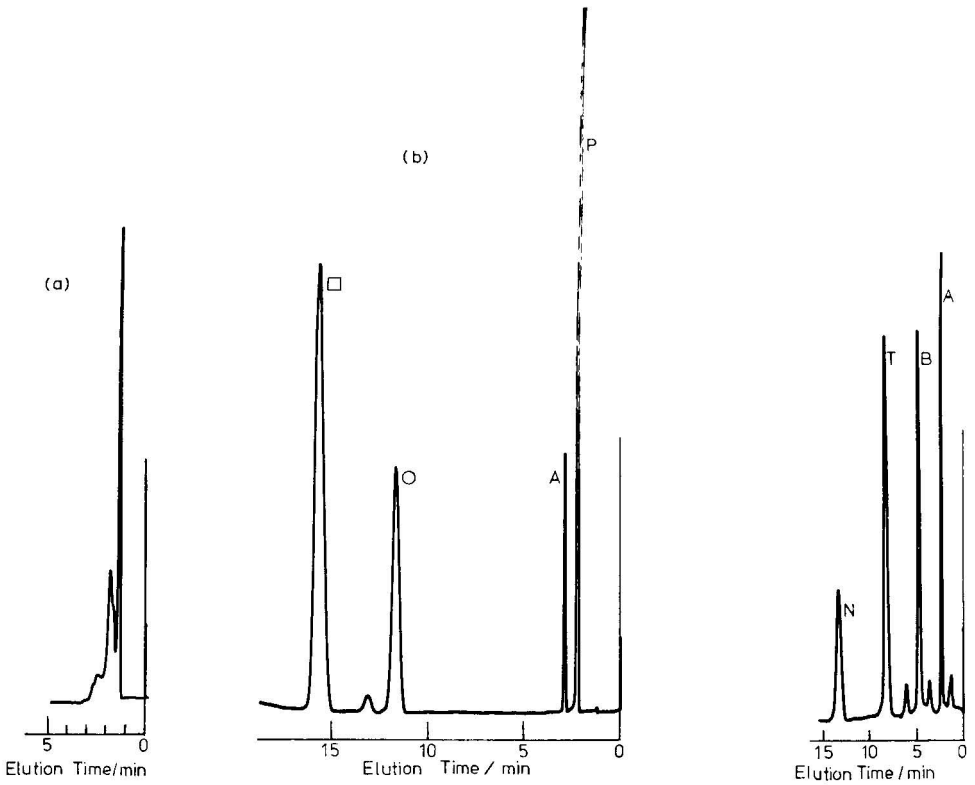
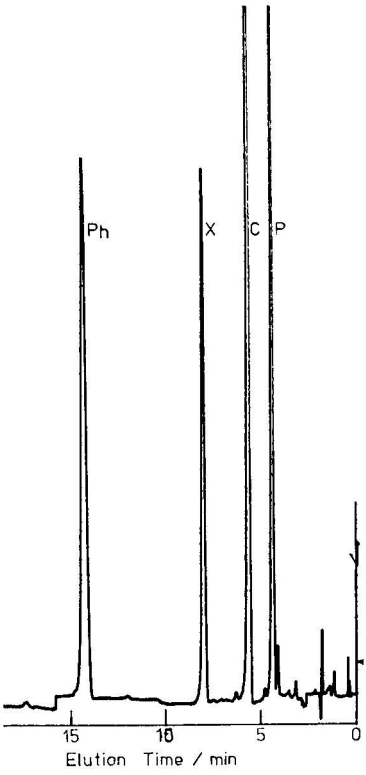


Fig. 4. (a) Demonstration of minimal retention of sample mixture of Fig. 1 on Hypersil from methanol–water (1:1) solvent system in absence of surfactant. (b) Separation of fluorenone (○) and naphthalene (□) on column of Fig. 4a in equilibrium with a solution of Tween 40 ($2 \cdot 10^{-3}$ kg dm^{-3}) in methanol–water (1:1). Less retained eluites are acetophenone (A) and phenol (P), respectively.

Fig. 5. Separation of acetophenone (A), benzene (B), toluene (T), and nitrobenzene (N). Column, eluent, and conditions as in Fig. 4b.



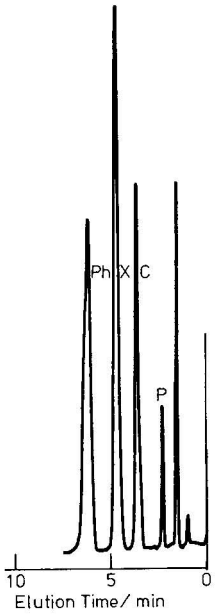


Fig. 6. Separation of phenol (P), *para*-cresol (C), 3,5-xylenol (X), and phenetole (Ph) on a column (125 mm × 4.6 mm I.D.) of ODS-Hypersil ($d_p \approx 5\ \mu m$). Methanol–water (6:4) was passed through the column at ambient temperature at 1 $cm^3\ min^{-1}$.

Fig. 7. Separation of sample mixture as shown in Fig. 7 with column and solvent system as in Fig. 4b.

The consequence of the first process would be an increase in retention of hydrophobic solutes with increasing brush density, but the second process would lead to an increasingly *polar* surface, studded with brushes with polyol termini instead of aliphatic hydrocarbon chains. Horváth *et al.*[12] have shown that the elution order of solutes from columns of alkyl-bonded silica packing materials is largely a function of the balance of surface and solute hydrophobicities. Accordingly, since essentially the same elution order is observed from the Tween–silica system as from octadecyl silica packings, retention in the former system should be largely due to the same properties of solute and surface.

If this hypothesis does accurately reflect the retention mechanism, then a steady increase in retentive power for chemically similar analytes should be observed with increasing brush density. This increase in retentive character will be counter-balanced by a decline in brush hydrophobicity which would ultimately lead to a decline in k' as surfactant concentration is increased. This behaviour has been observed with all single surfactant systems examined so far (*cf.* Figs. 1, 2 and 3 and refs. 1, 4 and 5), but may not be the case when ionic and non-ionic surfactants are used in combination[6].

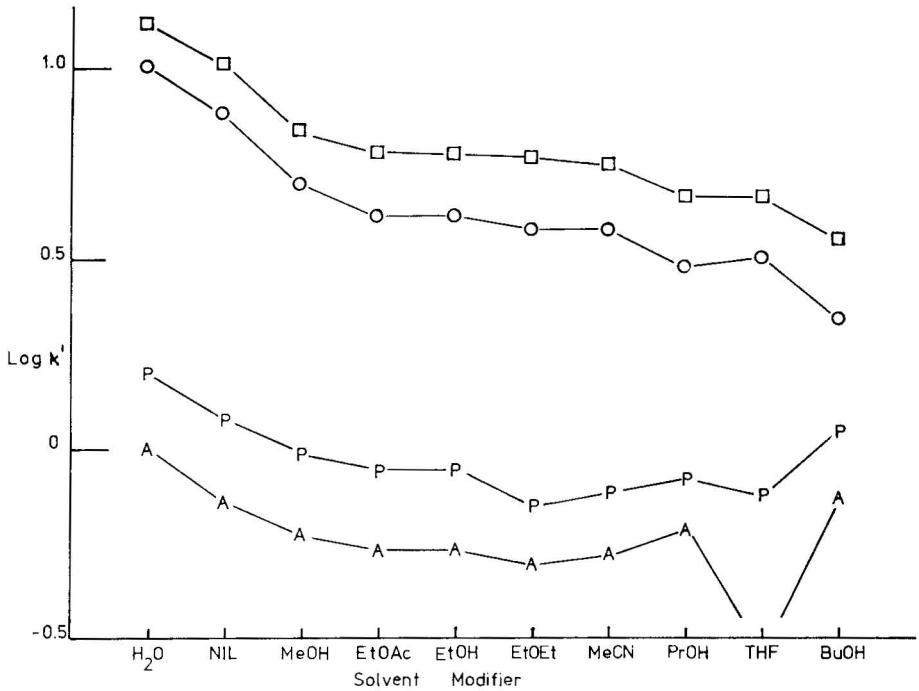


Fig. 8. Relationship of log k' to 10% (v/v) addition of a range of solvent modifiers. Conditions as in Fig. 4b, except solvent now 10% additive plus 90% [methanol–water (1:1) containing Tween 40 ($2 \cdot 10^{-3}$ kg dm^{-3})]. NIL = No addition; MeOH = methanol; EtOAc = ethyl acetate; EtOH = ethanol; EtOEt = diethyl ether; MeCN = acetonitrile; PrOH = propan-1-ol; THF = tetrahydrofuran; BuOH = butan-1-ol.

Control of both absolute and relative retention of several different eluites is usually maintained by choice of organic modifier and its concentration in "reversed-phase" chromatography. Tanaka *et al.*[13] and Bakalyar *et al.*[14] have clearly shown that selectivity in this LC mode is critically dependent on the nature and proportion of organic modifier in the eluent system. Fig. 8 is a composite diagram showing the effects on retention of four eluites of small changes in eluent composition at constant surfactant concentration. It shows that, as an added third solvent component decreases in polarity, there is a general trend to more ready elution. However, as would be expected by analogy with alkyl-bonded silica separations, the effects of solvent change are by no means equal on all four solutes. Clearly, control of organic modifier nature and proportion allows considerable control of selectivity in any separation attempted in this new HPLC mode.

CONCLUSIONS

Previous investigation[1,4,5] of the interaction between cationic surfactant and/or mixed non-ionic–anionic surfactants[6] dissolved in the eluent phase and LC column packings prepared from porous silicon(IV) and zirconium(IV) oxides and the present data on similar interactions of non-ionic surfactants with silicon(IV) oxide have

shown that this new mode of HPLC may be used to separate a wide range of analytes varying from sulphonic acids through neutral aromatic hydrocarbons and ketones to basic amines, amino acid esters, and peptides.

Selectivity in these separations may be enhanced by control of pH, solvent composition, ionic strength, chemical nature of the oxide surface, and selection of the appropriate surfactant. In the present work, Tweens (alkyl sorbitan polyethyl-enoxy polyols) were shown to be strongly bonded to silica gel surfaces; indeed, they proved to be impossible to wash off the column packing with several litres of water or aqueous alcoholic solvents. This technique of separation is rapidly and easily set up, and many of the GPLC separations presently done by "reversed-phase" chromatography on alkyl-bonded silicas could be carried out with equal efficiency using this new oxide–surfactant approach.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY OF ANTHRACYCLINE ANTIBIOTICS

SEPARATION AND IDENTIFICATION OF COMPONENTS OF THE DAUNORUBICIN COMPLEX FROM FERMENTATION BROTH*

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SUMMARY

A new solvent system composed of methanol–acidic water (pH 2.0 with phosphoric acid) has been developed for high-performance liquid chromatography of the daunorubicin complex from fermentation broth on a μBondapak C_{18} column. Application of this solvent system in conjunction with thin-layer chromatography in the identification of anthracycline antibiotics from the fermentation broth is discussed.

INTRODUCTION

High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have become two of the most important techniques for the separation, identification and isolation of synthetic and natural products. Both methods have certain limitations but, in conjunction, they complement each other.

This paper discusses HPLC and its application, in conjunction with TLC, for the identification of anthracyclines formed during the microbial production of daunorubicin (1) (NSC-82151).

Anthracycline antibiotics[1–4], in particular daunorubicin (also known as rubidomycin, daunomycin and rubomycin)[4–10], adriamycin (2) (NSC-123127; also known as doxorubicin)[11–13] and carminomycin I (3)[14,15] have aroused considerable interest in recent years because of their impact on cancer chemotherapy. Of particular significance is their potency against acute lymphocytic and myelogenous leukemias[16–18]; they are also active against a broad spectrum of solid tumors[13,19,20]. However, restricting their usefulness, is their dose-limiting cardiotoxicity[21,22].

In an attempt to eliminate, or at least to minimize, toxic side effects (especially cardiotoxicity) and to increase the clinical usefulness of these antibiotics, various structural modifications have been carried out[4]. Because of the demand for and the

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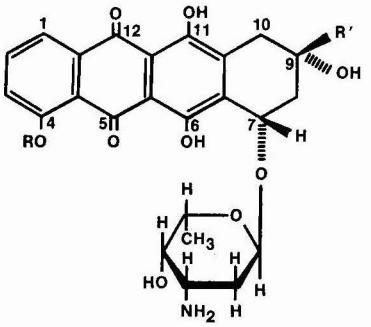


1: Daunorubicin: $R = CH_3$, $R' = -\overset{O}{\overset{\|}{C}}-CH_3$

2: Adriamycin: $R = CH_3$, $R' = -\overset{O}{\overset{\|}{C}}-CH_2OH$

3: Carminomycin I: $R = H$, $R' = -\overset{O}{\overset{\|}{C}}-CH_3$

cost of producing these products, efforts have also been directed towards strain development for higher yields. During this program at the Frederick Cancer Research Center[23,24], it was necessary to identify daunorubicin precursors and co-metabolites. Based on this knowledge, fermentation conditions could then be modified to enhance daunorubicin production.

TLC has been used to monitor daunorubicin and other anthracyclines in fermentation broths, but there are certain limiting factors, and often more than one mobile phase must be used for the TLC[25–27]. In order to eliminate the use of multiple mobile phases in TLC, and also because of the high sensitivity of HPLC, it was decided to develop an HPLC system that could be used to monitor daunorubicin fermentation, determine the number of components in a particular fermentation, and, finally, quantitate daunorubicin and/or its precursors and co-metabolites.

EXPERIMENTAL

General

Daunorubicin, daunorubicinone, 7-deoxydihydrodaunorubicinone, ε-rhodomycinone, glycoside I (baumycin A_2), 30-8-1M (an unknown) and other similar compounds used in the present investigation were purified by column chromatography and preparative TLC on silica gel. Their identities were confirmed by ultraviolet–visible (UV–VIS), infrared (IR), proton nuclear magnetic resonance (^1H NMR), carbon magnetic resonance (^{13}C NMR), electron-impact mass spectrometry (EI-MS) and field-desorption mass spectrometry (FD-MS).

All chemicals were of analytical grade and were used without further purification.

The mobile phases were prepared from methanol, acetonitrile [both from Burdick and Jackson (Muskegon, MI, U.S.A.), distilled in glass], PIC B-7 (Waters .

Assoc., Milford, MA, U.S.A.), phosphoric acid (Mallinckrodt, St. Louis, MO, U.S.A.; analytical grade) and glass-distilled water.

All HPLC work was carried out on a μBondapak C_{18} column using a UV detector (254 nm; 0.04 a.u.f.s.).

Apparatus

A Waters Assoc. Model 6000A solvent-delivery system, with Model 660 solvent programmer, a Waters Assoc. U6K universal injector with a sample loop of 2 ml and a Schoeffel SF 770 Spectroflow variable-wavelength detector were used for all HPLC work. The detector was set at 254 nm and 0.04 a.u.f.s. (cell volume 8 μl; path length 10 mm). The μBondapak C_{18} columns (30 cm × 3.9 mm I.D.; particle size 10 μm) used were manufactured by Waters Assoc.

A London Co. pH meter (The London Co., Cleveland, OH, U.S.A.), Model 64, equipped with a Sensorex (Sensorex, Westminster, CA, U.S.A.) electrode was used for pH measurements. TLC plates used were of pre-coated silica gel 60 F-254 (Merck, Darmstadt, G.F.R.). Millipore filters (type HA, pore size 0.45 μm and FH, pore size 0.5 μm) (Millipore, Bedford, MA, U.S.A.) were used for filtering solvents and samples.

Preparation of mobile phase

The glass-distilled water was adjusted to pH 2.0 with phosphoric acid and was filtered through a Millipore filter (type HA); methanol or acetonitrile was also filtered through a Millipore filter (type FH). Measured amounts of the organic and aqueous phases were mixed by stirring. The PIC B-7 solvent system was also prepared in a similar manner. Filtration was avoided, after mixing the solvents, to eliminate the possibility of a change in ratio of low- and high-boiling solvents (see Results and discussion).

Preparation of samples for HPLC injection

Authentic samples of daunorubicin, daunorubicinone, ε-rhodomycinone, baumycin A_2 and other structurally related compounds were carefully weighed and dissolved in methanol (*ca.* 15 mg/l). The solutions were filtered through Millipore filters (type FH), and 10-μl aliquots were injected onto the HPLC column.

Preparation of samples for TLC

Standard samples of daunorubicin, daunorubicinone, ε-rhodomycinone, baumycin A_2, 7-deoxydihydrodaunorubicinone and others were taken in *ca.* 1–2 ml of methanol or chloroform to give pale orange-colored solutions. These solutions were applied in a range of 2 μl to 20 μl to give well-defined spots on development. Butanol extracts from whole-broth samples were spotted without further preparation; generally, 20–30 μl of butanol extract were necessary in order to see all the compounds present.

Extraction of fermentation broth

General procedure. In a typical experiment, the whole broth is mixed with an equal volume of 1-butanol; the pH of the mixture is adjusted to 8.5 with 50% aqueous sodium hydroxide solution, then the mixture is stirred vigorously for 30 min and

filtered through Celite. Aqueous and butanol layers are separated, and the aqueous layer and cake are mixed and re-extracted with fresh 1-butanol. The combined butanol extracts are washed with water, then the pH is adjusted to 5.5 with aqueous 6 *N* hydrochloric acid, and the butanol is concentrated under vacuum at *ca.* 40°C. The concentrate is then precipitated with heptane, filtered, and dried under high vacuum to yield the crude daunorubicin complex, which is analysed by TLC and HPLC. This procedure is summarized in Fig. 1.

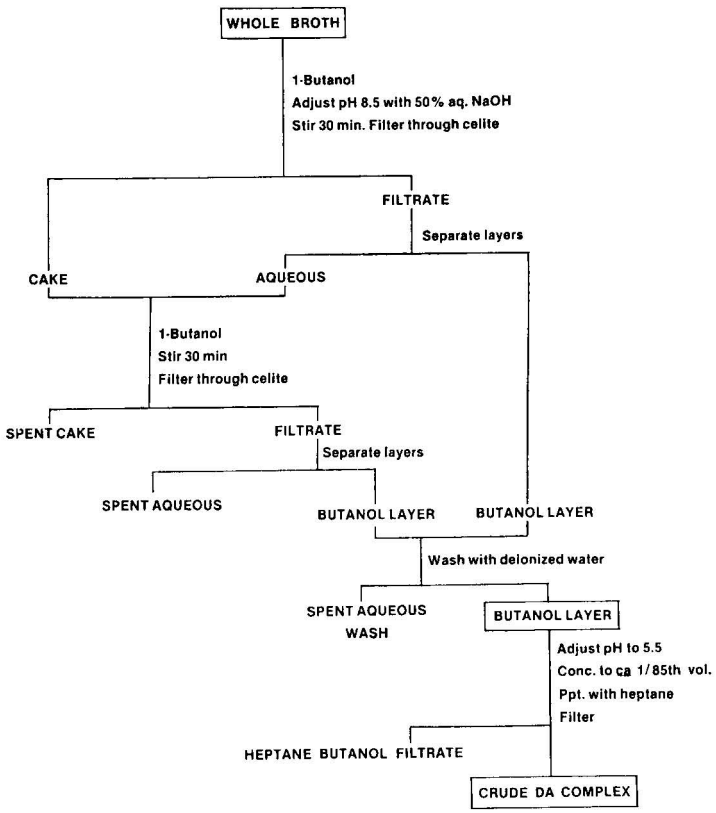


Fig. 1. General procedure for isolation of total crude daunorubicin complex from fermentation broth.

The above general procedure has been modified, and, for most of the work, the following three procedures (summarized in Fig. 2) were developed for the partial or total extraction of the fermentation broth for HPLC work.

(A). Whole broth is adjusted to pH 8.5 and stirred with 1-butanol for 15 min. Spent cake is removed by filtration, and the butanol layer is separated and filtered through a Millipore filter (type FH); the filtrate is injected onto the HPLC column to determine the composition of the unhydrolysed broth (Fig. 2A).

(B). Whole broth is mixed with 1-butanol and adjusted to pH 1.5 with hydrochloric, sulphuric or oxalic acid, with stirring. The mixture is heated at 40°C for 30 min, then cooled to room temperature, and its pH is adjusted to 8.5 with aqueous

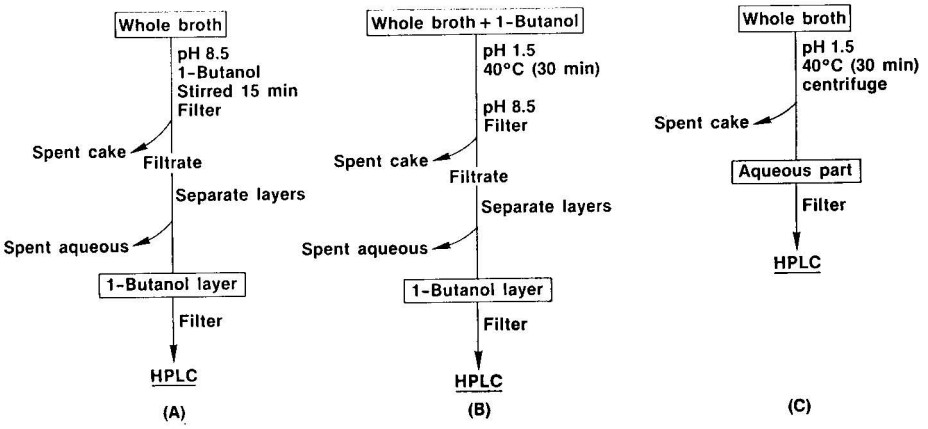


Fig. 2. Modified procedures for extraction of daunorubicin fermentation broth under different conditions.

sodium hydroxide. The spent cake is removed by filtration, and the butanol layer is separated, filtered through a Millipore filter (type FH), and injected onto the HPLC column to determine the composition of the hydrolysed broth (Fig. 2B).

(C). Whole broth is adjusted to pH 1.5 with aqueous hydrochloric, sulphuric or oxalic acid and heated at 40°C for 30 min. The broth is then centrifuged, and the aqueous part is filtered through a Millipore filter (type HA) and injected onto the HPLC column to determine the daunorubicin content (Fig. 2C).

RESULTS AND DISCUSSION

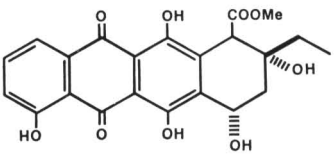
In the course of these studies, it was necessary to identify and determine the relative percentages of the different components in a particular fermentation.

TLC has been used for the separation of anthracycline antibiotics, but quantitation has been difficult because of the complexity of the fermentation mixture[25–27]. HPLC systems have been reported in the literature[28–33] for such clinically useful anthracyclines as daunorubicin and adriamycin, and their metabolites, but they have not proved useful for complex mixtures.

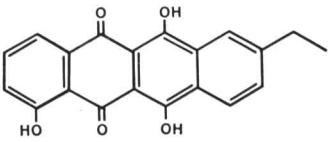
The present investigation was directed toward development of one or more simple HPLC and TLC systems for the separation, quantitation and identification of various components in a daunorubicin fermentation broth. The daunorubicin is produced as its higher glycosides, along with other anthracyclines, during the course of a fermentation. Subsequently, the higher glycosides are partially hydrolysed, and the resulting daunorubicin is then purified by solvent extraction at different pH values and crystallized[24].

In order to determine the peak production of the glycosides that give rise to daunorubicin, samples are taken at different time intervals, worked up and hydrolysed (or *vice-versa*), and assayed for daunorubicin in the broth by HPLC[33] or TLC[27].

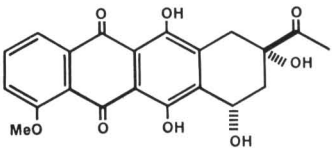
Presently known methods (HPLC and TLC) are suitable for such quantitation if the assay samples contain two or three components. In any crude mixture, it is difficult to quantitate exactly when known HPLC mobile phases are used, because of



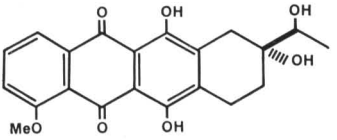
4: ε-Rhodomycinone



5: Descarbomethoxybisanhydro-ε-rhodomycinone



6: Daunorubicinone



7: 7-Deoxydihydrodaunorubicinone

the overlap of certain peaks. Further, in order to determine the composition of a fermentation broth at various time intervals or after certain genetic manipulations, it became necessary to separate the components. TLC in various solvents, particularly in chloroform–methanol–formic acid (80:20:2) and hexanes–chloroform–methanol (5:5:1), always indicated (Fig. 3) that the whole broth was a complex mixture of at least eleven to fifteen components. Some of these, ε-rhodomycinone (4), descarbomethoxybisanhydro-ε-rhodomycinone (5), daunorubicinone (6) and 7-deoxydihydrodaunorubicinone (7) were isolated (Table I and Fig. 4) by repeated silica gel column chromatography and preparative TLC. Because of the close proximity of several spots

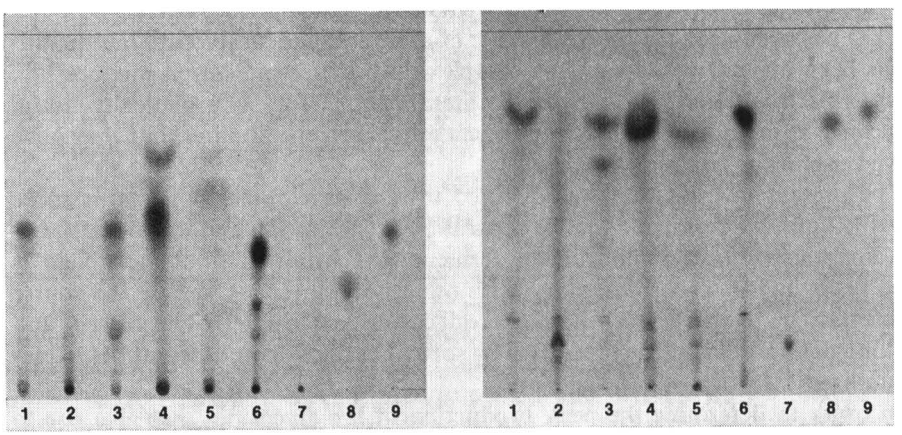


Fig. 3. Thin-layer chromatograms of various butanol extracts. Plate: silica gel G (20 × 20 cm, 0.25 mm thick). Solvent systems: left, chloroform–methanol–formic acid (80:20:2); right, chloroform–hexanes–methanol (5:5:1). 1 = Total butanol extract of whole daunorubicin broth; 2 = butanol extract after acid hydrolysis; 3 = total butanol extract of 2-week-old broth; 4 = total butanol extract of 30-8 broth; 5 = total butanol extract of DAMCT-1 broth; 6 = total ethanol extract of A-21 cake; 7 = daunorubicin; 8 = daunorubicinone; 9 = ε-rhodomycinone.

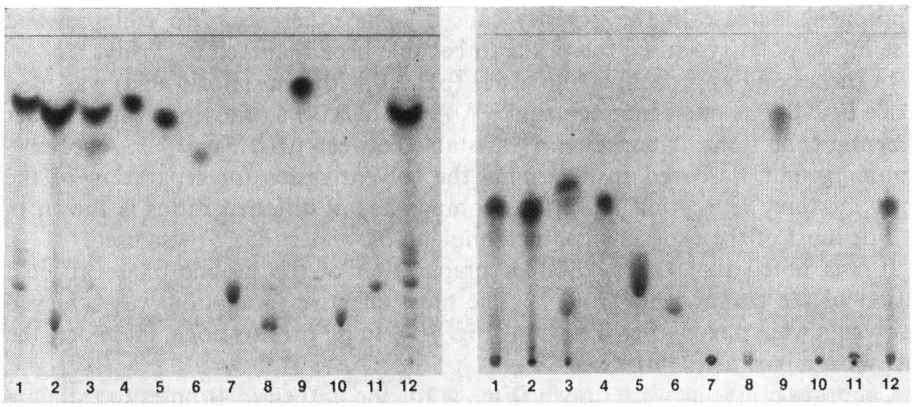


Fig. 4. Thin-layer chromatograms of various isolated anthracyclines. Plates as in Fig. 3. Solvent systems: left, chloroform–methanol–formic acid (80:20:2); right, chloroform–heptane–methanol (5:5:1). 1 and 12 = DAF-33; 2 = DAF-33-1; 3 = DAF-33-2, 4 = ε-rhodomycinone; 5 = daunorubicinone; 6 = 7-deoxydihydrodaunorubicinone; 7 = glycoside I (baumycin A_2); 8 = 30-8-1M (unknown); 9 = descarbomethoxybisanhydro-ε-rhodomycinone; 10 = daunorubicin; 11 = DAMCT-1-1 (unknown).

in TLC (Fig. 4), quantitation was difficult, although TLC provided an excellent qualitative analytical method. In order to develop an efficient mobile phase for separation of the daunorubicin complex by HPLC, preliminary investigations were started on a synthetic mixture (prepared from pure components isolated from daunorubicin fermentation broth) with the known solvents used for assay of daunorubicin. The separation of such a mixture on a μBondapak C_{18} column with methanol–water–PIC B-7 (65:35:1.8) is shown in Fig. 5. This mobile phase did not separate daunorubicin and 7-deoxydihydrodaunorubicinone, both of which are daunorubicin fermentation co-metabolites, under different conditions[34]. Thus, we decided to change or modify the solvent system.

Three approaches were used: (1) changing the solvent ratio, (2) replacing the PIC B-7 reagent, and (3) replacing both the PIC B-7 and the organic solvent. In the

TABLE I

THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR OF SOME ANTHRACYCLINES ISOLATED FROM DAUNORUBICIN FERMENTATION BROTH

Compound No.	*Anthracycline*	*Structure No.*	*R_F value**	
			Solvent A	*Solvent B*
1	ε-Rhodomycinone	4	0.79	0.48
2	Daunorubicinone	6	0.74	0.25
3	7-Deoxydihydrodaunorubicinone	7	0.63	0.16
4	Glycoside I (baumycin A_2)	8	0.21	0.00
5	30-8-1M	Unknown	0.12	0.00
6	Descarbomethoxybisanhydro-ε-rhodomycinone	5	0.83	0.74
7	Daunorubicin	1	0.15	0.00
8	DAMCT-1-1	Unknown	0.23	0.03

* Silica gel plates (20 × 20 cm; 0.25-mm layer). Solvent A, chloroform–methanol–formic acid (80:20:2); solvent B, chloroform–heptane–methanol (5:5:1).

first approach, increase in the polarity caused some other peaks to collapse, and decrease in the polarity caused the peaks to become broad, and elute slowly.

In the second approach, acidic water (pH 2.0 with phosphoric acid) was used to replace PIC B-7 as one of the solvents[32,33]. A pH of 2.0 was chosen because, under experimental conditions, daunorubicin was stable between pH 1.5 and 2.5. A solvent-programming unit was used to determine the solvent ratios for separation of the authentic mixture. Separation of various components at different ratios is shown in Fig. 6. For most of the experiments, methanol–acidic water (65:35) was used.

It was noted that the method of preparation of the mobile phase affected separation of the components (Fig. 7). The most effective separation was achieved when solvents were mixed after filtration (Fig. 7C). In all preparations, therefore, the solvents were mixed after filtration.

Calibration graphs were drawn (Fig. 8) for the authentic samples to estimate the amount of the components in the fermentation broth.

When the organic solvent was replaced by acetonitrile and used with acidic water (pH 2.0 with phosphoric acid) in different ratios, poorer separations were observed (compare Figs. 7 and 9). The best separation in this solvent was obtained at a acetonitrile–acidic water ratio of 30:70, as shown in Fig. 9.

As a result of these experiments, it was concluded that the mobile phase containing methanol–acidic water (pH 2.0 with phosphoric acid) (65:35) was the best

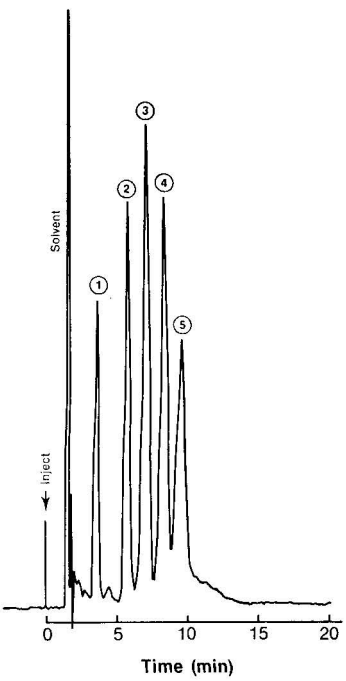


Fig. 5. High-performance liquid chromatogram of a mixture of six anthracyclines. Mobile phase: methanol–water–PIC B-7 (65:35:1.8); flow-rate, 2 ml/min. Peaks: 1 = 30-8-1M; 2 = daunorubicinone; 3 = daunorubicin and 7-deoxydihydrodaunorubicinone; 4 = ε-rhodomycinone; 5 = baumycin A_2.

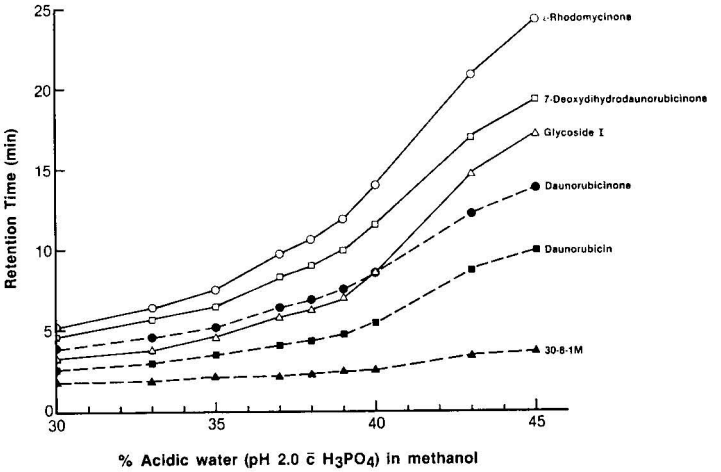


Fig. 6. Plot of HPLC retention times, on a μBondapak C_{18} column (30 cm × 3.9 mm I.D.), of various anthracyclines isolated from daunorubicin fermentation broth *vs.* acidic water (pH 2.0 with phosphoric acid)–methanol at different ratios.

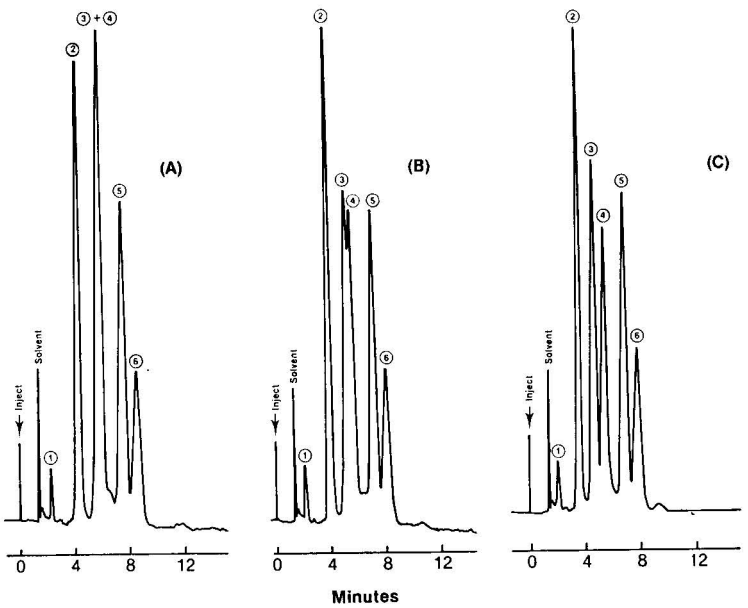
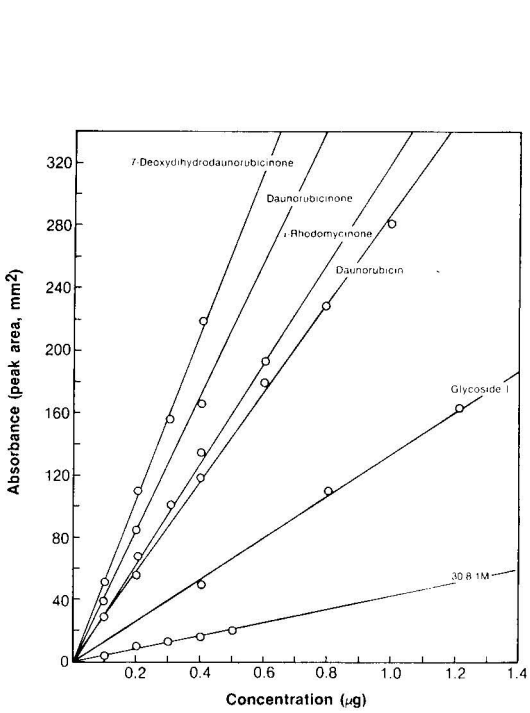


Fig. 7. Effect of solvent preparation on separation of various anthracyclines; mobile phase, methanol–water (pH 2.0 with phosphoric acid); flow-rate, 2 ml/min. Method of solvent preparation: A, mixed and filtered immediately; B, mixed and filtered after some time; C, filtered and then mixed. Peaks: 1 = 30-8-1M (unknown); 2 = daunorubicin; 3 = baumycin A_2; 4 = daunorubicinone; 5 = 7-deoxydihydrodaunorubicinone; 6 = ε-rhodomycinone.

for separation of daunorubicin and its co-metabolites. It was also simple and inexpensive compared with other solvent systems reported in the literature[28–33].

A comparison of HPLC data for butanol extracts of daunorubicin fermentation



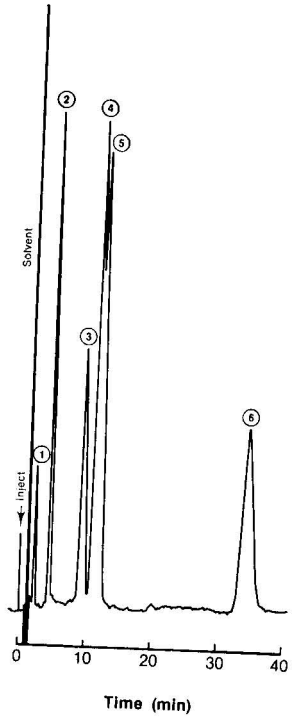


Fig. 8. Calibration graphs for authentic anthracyclines. Mobile phase: methanol–acidic water (pH 2.0 with phosphoric acid) (65:35).

Fig. 9. High-performance liquid chromatogram of six anthracyclines; mobile phase: acetonitrile–acidic water (pH 2.0 with phosphoric acid) (30:70); flow-rate: 3 ml/min. Peaks: 1 = 30-8-1M; 2 = daunorubicin; 3 = baumycin A_2; 4 = daunorubicinone; 5 = 7-deoxydihydrodaunorubicinone; 6 = ε-rhodomycinone.

broth at various time intervals is shown in Table II. From these data, it is evident that little daunorubicin is formed in fermentation; most occurs as its higher glycoside I, identified[35] as baumycin A_2 (8; ref. 36). Further, there is not much difference in the products formed after 140 h. Table II also shows that the ratio of ε-rhodomycinone

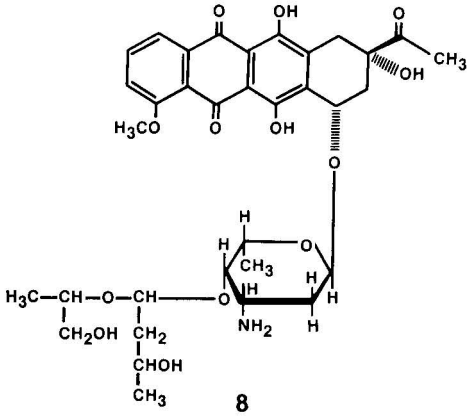


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TABLE II

MONITORING OF PERCENTAGE COMPOSITION OF DAUNORUBICIN FERMENTATION AT VARIOUS TIME INTERVALS

Peak Nos.	*Retention time** (min)*	*Assignment*	*Composition (%) of DAF-37 broth**						
			46 h	*70 h*	*94 h*	*140 h*	*178 h*	*214 h*	*238 h*
1	2.6		4.1	2.9	2.9	1.1	1.6	1.0	1.2
2	3.3	Daunorubicin	8.6	12.1	3.9	2.8	3.7	3.8	3.6
3	4.0								
4	4.8	Glycoside I			10.8	15.5	16.0	15.8	15.2
5	5.8	Daunorubicinone	4.5	8.3	10.2	6.5	5.7	5.7	5.3
6	6.2								
7	7.1	7-Deoxydihydro-daunorubicinone	2.1	1.4		2.9	1.9	1.8	1.6
8	8.0	ε-Rhodomycinone	11.9	25.7	32.8	33.7	30.3	28.5	33.8
9	9.6		6.9	5.8	3.8	3.7	2.9	3.5	2.5
10+11+12 +13	10.4, 11.1, 12.7, 13.8		7.0	5.4	2.6	7.4	6.2	7.2	6.3
14	15.2		8.5	5.3	4.8	3.7	4.2	4.1	3.7
15	16.0		9.8	7.8	5.7	3.9	5.0	5.0	4.7
18	24.8		9.1	6.1	4.8	3.8	5.2	4.7	3.9
19	26.6		11.4	11.2	5.1	4.3	6.1	6.1	5.6
Other peaks	26.6		15.9	6.7	12.4	10.7	11.1	12.7	12.4

* Calculated from the HPLC profile of total butanol extract.

** On a μBondapak C_{18} column (30 cm × 3.9 mm I.D.); mobile phase: methanol–acidic water (pH 2.0 with phosphoric acid) (65:35); flow-rate: 2 ml/min.

to baumycin A_2 at 94 h changes after 140 h; it then remains constant. The change in this ratio could have occurred through some of the ε-rhodomycinone being converted into baumycin A_2 or, after ε-rhodomycinone plateaued, other components were transformed into baumycin A_2. Isolation and characterization of the various components (which are in progress in this laboratory) would confirm either or both possibilities. A comparison of actual HPLC traces at 46, 94 and 238 h and the assignments of various peaks are shown in Fig. 10. From these data, it is very clear that peak 5 (assigned as daunorubicinone) and peak 6 (an unknown) go to peak 4 (assigned as baumycin A_2).

Fig. 11 shows a comparison of a butanol extract of a daunorubicin fermentation broth (batch DAF-33) harvested after 166 h and worked up according to Fig. 2A (top chromatogram) with the acid-treated (pH 1.5) broth (middle chromatogram) and the broth stored at pH 7.5 for 2 weeks (lower chromatogram). These chromatograms clearly indicate that baumycin A_2 (peak 4), as expected, goes to daunorubicin (peak 2) on acid treatment, and, on storage at neutral pH, baumycin A_2 is almost completely converted into 7-deoxydihydrodaunorubicinone.

Fig. 12 shows application of this mobile phase in daunorubicin assay. Two sample broths, Nos. 3508 and 4491, were extracted as described in Fig. 2C and were injected onto the HPLC column using methanol–acidic water ratios of 65:35 (upper chromatograms) and 60:40 (lower chromatograms).

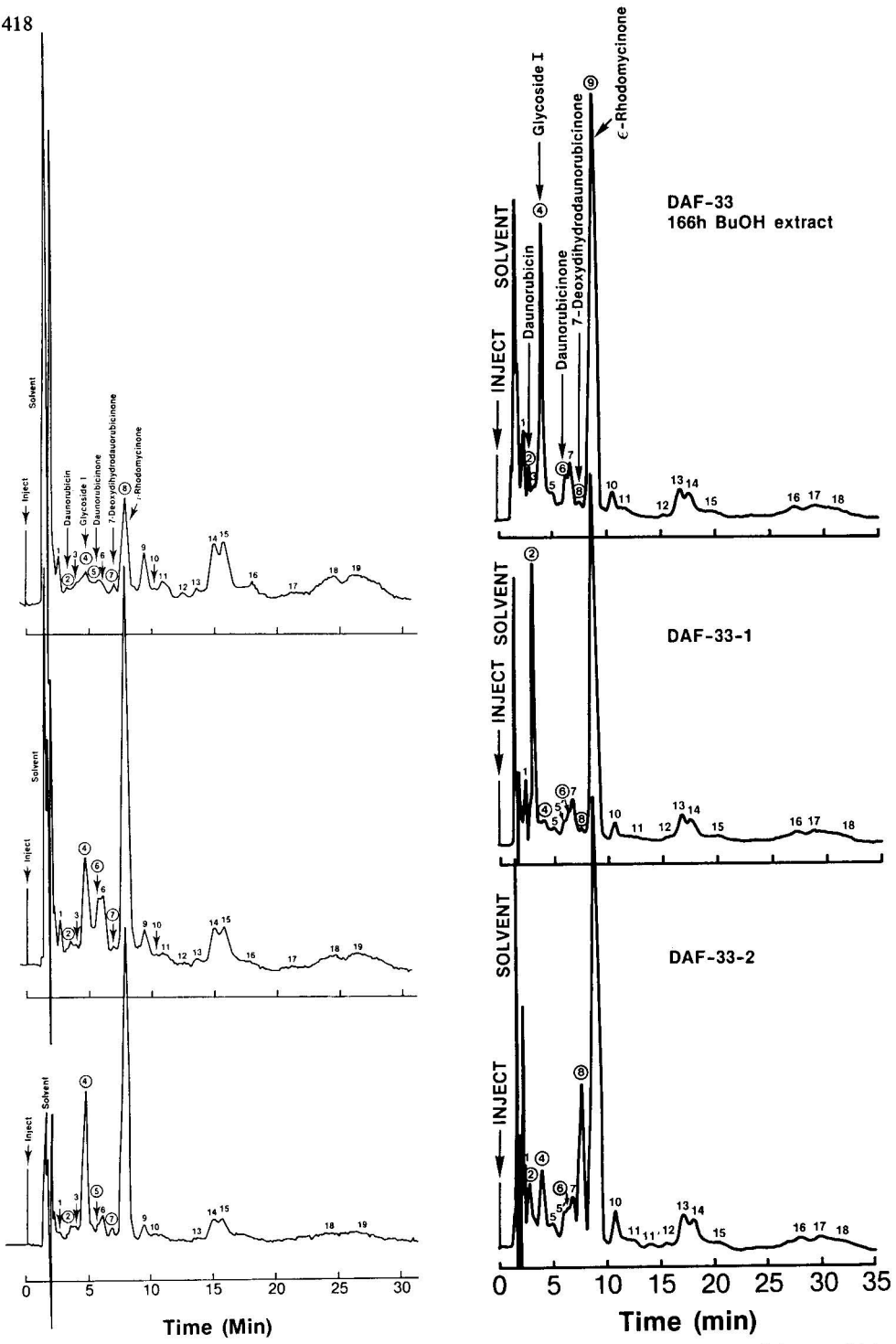

Fig. 10. Comparison of high-performance liquid chromatograms of butanol extracts of daunorubicin fermentation broth (batch DAF-37) at three time intervals: 46 h (top), 94 h (middle) and 238 h (bottom). Mobile phase as in Fig. 8; flow-rate, 2 ml/min.

Fig. 11. High-performance liquid chromatograms of butanol extracts of daunorubicin fermentation broth (batch DAF-33). Top: extracted at pH 8.5 (Fig. 2A); middle: extracted after hydrolysis (Fig. 2B); bottom: extracted as in Fig. 2A after 2 weeks; mobile phase and flow-rate as in Fig. 10.

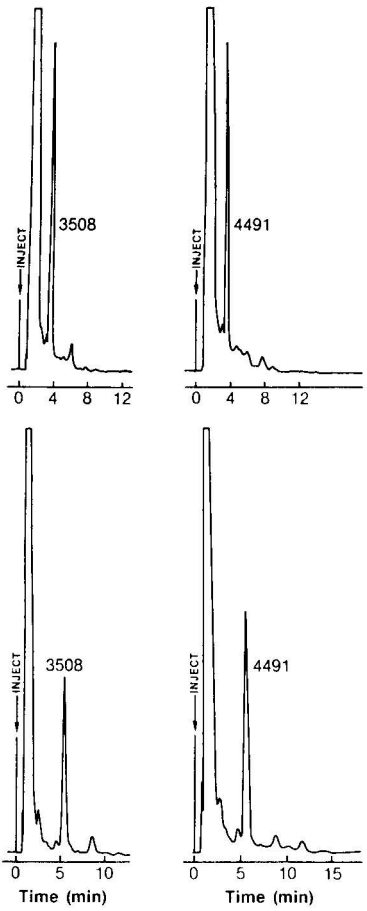

Fig. 12. High-performance liquid chromatograms of extracts of daunorubicin fermentation broths (samples 3508 and 4491) obtained according to Fig. 2C. Mobile phase: top, methanol–pH 2.0 water (65:35); bottom, methanol–pH 2.0 water (60:40); flow-rate, 2 ml/min.

CONCLUSION

We have thus developed a simple, fast and inexpensive HPLC solvent system for the separation of anthracycline antibiotics in daunorubicin fermentation broths; its application to monitoring fermentation broth, identifying various peaks and assaying daunorubicin is discussed.

ACKNOWLEDGEMENTS

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STATIONARY PHASES FOR HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The characteristics of commercially available high-performance thin-layer chromatographic quality thin-layer plates pre-coated with silica gel, cellulose, chemically bonded phases or polyamide are discussed and compared with those of pre-coated layers used in conventional thin-layer chromatography.

INTRODUCTION

In recent years, high-performance thin-layer chromatography (HPTLC) has rapidly gained widespread acceptance. In the literature, the importance of aspects such as improved dosage methods, optimal techniques for layer conditioning and feeding the mobile phase and the use of suitable data acquisition techniques has repeatedly been stressed. Most attention, however, has been devoted to the development of suitable stationary phase materials.

In this paper, information is presented on the characteristics of the pre-coated stationary phases for HPTLC that are commercially available. In addition, experimental results obtained with several of the newer types of material are reported.

EXPERIMENTAL

Commercially available pre-coated thin-layer plates used in this study included HPTLC and conventional TLC silica gel plates from Merck (Darmstadt, G.F.R.; product nos. 5628 and 5715, respectively) and Macherey, Nagel & Co. (Düren, G.F.R.; product nos. Sil-20 UV_{254} and Sil G-25 HR/UV_{254}, respectively), HPTLC silica gel plates from Schleicher & Schüll (Dassel, G.F.R.; product no. G 1570/LS 254), HPTLC and conventional TLC cellulose plates from Merck (product nos. 5787 and 5716, respectively), plates coated with LiChrosorb RP-18 (Merck; product no. 13724), KC_{18} thin-layer plates from Whatman (Springfield Mill, Great Britain; product no. 4803600), plates silanized 50, 75 or 100% with a C_{18} alkylsilane (Macherey, Nagel & Co.; product nos. Sil C 18-50, -75 and -100 UV_{254}, respectively, and designated MN50, MN75 and MN100 in this paper) and plates coated with HPTLC or

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conventional TLC polyamide (Macherey, Nagel & Co.; designation Wang-Polyamid-6 and Polygram Polyamid-6 UV_{254}, respectively). Further details on these and other types of thin-layer plates are given later.

All solvents, model compounds and further chemicals used were of normal analytical-reagent grade.

Details of the chromatographic procedures routinely used in our laboratory have been published recently[1].

RESULTS AND DISCUSSION

Silica gel

Silica gel thin-layer plates were the first pre-coated slides to be offered for sale in HPTLC quality. To-day, Merck sells a complete range of pre-coated plates, in different sizes, with or without an added fluorescent indicator (F_{254}), and with or without a concentration zone. Also, plates are available that have an aluminium instead of a glass backing. Less varied supplies of pre-coated silica gel plates for HPTLC are marketed by Schleicher & Schüll, Macherey, Nagel & Co. and Whatman; again, plates with or without a fluorescent indicator and, occasionally, plates with a concentration zone are offered. HPTLC plates, whether with a silica gel or another type of coating, are frequently termed "Nano Plates" on account of the recommended 10–200-nl dosage volume. It is interesting that, confusingly, in several early papers on HPTLC authors stated they carried out separations on "Nano Plates" without specifying the type of stationary phase material used. However, because at that time only HPTLC plates pre-coated with silica gel were commercially available, it is reasonable to conclude that these were the chromatoplates actually used.

A serious disadvantage of all three types of silica gel HPTLC plates studied (Merck, Schleicher & Schüll, Macherey, Nagel & Co.) is the relatively long development time compared with that observed with conventional TLC plates (Table I; similar results have been obtained with other mobile phases). Using the well-known relationship

$$z_f^2 = kt \tag{1}$$

where z_f is the distance of the run and t is the time of the run, one can calculate that the velocity constant, k, of HPTLC plates is about 40% smaller than on conventional TLC plates. This conclusion is in fair agreement with literature data[2].

TABLE I

DEPENDENCE OF TIME OF RUN ON TYPE OF SILICA GEL USED IN (HP)TLC FOR TWO SOLVENTS

Stationary phase	$t_{5\text{-cm run}}$ *(min)*		$100R_F/R_{F,Me\ HP}$*
	Toluene	*Chloroform*	
Merck TLC	6.5	6	98–102
Macherey, Nagel & Co. TLC	6	6	102–106
Merck HPTLC	10.5	11	100
Macherey, Nagel & Co. HPTLC	10	11	99–101
Schleicher & Schüll HPTLC	10.5	10	96– 99

* $R_{F,Me\ HP}$ denotes R_F values on Merck HPTLC plates; results are based on data for 10–15 compounds.

In an obvious attempt to remedy this disadvantage of silica gel HPTLC plates, Merck has recently announced the production of a new series of plates, which will gradually replace the older type. The new plates display[2] a higher k value than the older plates, *e.g.*, 4.5 *vs.* 3.8 mm^2/sec for toluene as solvent. The velocity constant is, however, still lower than that recorded for conventional TLC silica gel (5.4 mm^2/sec). This agrees with our experimental data: a 5-cm run with toluene took about 8 min on a "new" HPTLC plate compared with 10 min on an "old" HPTLC plate, and 6 min on a conventional TLC plate (*cf.*, Table I).

Another noteworthy aspect is the relative contribution of longitudinal molecular diffusion and mass transfer (plus the quality of the packing) to band broadening in (HP)TLC. On the basis of extensive discussions, Guiochon and co-workers[3,4] have concluded that HPTLC on silica gel, with its relatively low mobile phase velocities, is carried out under conditions such that molecular diffusion largely determines the spot length, *i.e.*, the standard deviation of the spot peak, σ_x, and, therefore, the average plate height, H_{av}, defined as

$$H_{av} = \sigma_x^2/z_x \tag{2}$$

where z_x is the distance migrated by the spot. Conventional TLC, on the other hand, has been shown to work under experimental conditions such that resistance to mass transfer controls the spot length. According to Guiochon and co-workers, as a consequence H_{av} will increase very rapidly with increasing length of run if very fine silica gel particles ($d_p < ca.$ 7 μm, generally stated to be typical for HPTLC; however, see below) are used. With the larger (10–15 μm) conventional TLC type of particles, on the other hand, the increase in H_{av} with increasing length of run will be much slower. If this is true, then the low plate heights said to be commonly achieved in HPTLC can be obtained only if the diffusion coefficient, D, of the solute is very small, *i.e.*, if the solute molecules are relatively large. In other words, to quote Guiochon and Siouffi[4], it may be of some significance "... that most demonstrations of the performance of HPTLC are made using mixtures of dyes which have rather large molecular weight".

Apart from some data recorded in ref. 4, no experimental verification of the above conclusions has been published. We therefore carried out the TLC of two chloroanilines and two, presumably high-molecular-volume, dyestuffs in the system silica gel–toluene. Development of the chromatograms in HPTLC and conventional TLC was done under identical conditions. From Figs. 1 and 2 one can conclude that the above hypotheses are confirmed. With the two relatively low-molecular-volume and, therefore, rapidly diffusing chloroanilines, the plate height in HPTLC is seen to reach its minimum value at a small z_f value and to increase very rapidly with increasing length of run; a similar steep increase is absent in conventional TLC. With the dyestuffs, which probably will have small D values, the curves in TLC and HPTLC are very similar. These experiments were carried out using "old" HPTLC plates; no data have as yet been collected for the "new" plates.

Two more remarks should be made. (1) The H *vs.* z_f curves in Figs. 1 and 2 display minimum plate heights, H_{min}, which are distinctly larger than are those sometimes cited in the literature, *e.g.*, 30 and less than 12 μm for conventional TLC and HPTLC, respectively, in a much quoted table reported in ref. 5 (although inspection

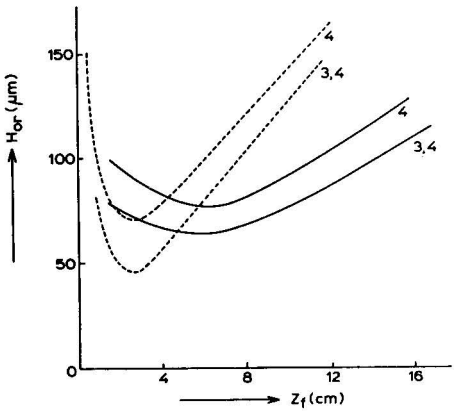

Fig. 1. H *vs.* z_f curves for 4-chloroaniline (R_F 0.20) and 3,4-dichloroaniline (R_F 0.35) in the system silica gel–toluene using HPTLC (broken lines) and TLC (solid lines) silica gel. Sample volume, 200 nl. The experimental H_{av} values have been corrected for the plate-height contribution due to original spot length, using the relationship $H_{or} = H_{av} - \sigma_{or}^2/z_x$.

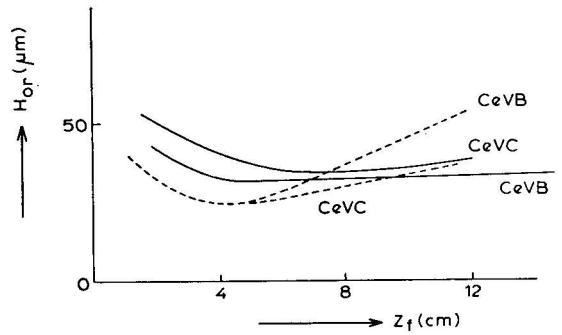

Fig. 2. H *vs.* z_f curves for two Ceres Violet constituents (R_F 0.60 and 0.85) in the system silica gel–toluene using HPTLC (broken lines) and TLC (solid lines) silica gel. Sample volume, 200 nl. Other details as in Fig. 1.

of experimental curves in refs. 2 and 6 suggests values of about 30 and 20 μm, respectively, for comparable conditions). Presumably, this divergence can be partly explained* by the fact that some authors (see, *e.g.*, refs. 7 and 8) present, instead of H_{av} (or H_{or}; *cf.*, legend to Fig. 1), so-called H_{50} data calculated by means of the equation

$$H_{50} = (R_F/0.50)H_{av} \tag{3}$$

where R_F is the R_F value of the model compound. Self-evidently, this mode of calculation considerably affects the H values for compounds that display relatively

* According to information recently received from Dr. H. E. Hauck (Merck), the use of an even slightly imperfect spotting procedure can easily adversely influence spot sizes and hence plate heights.

high retentions, such as the chloroanilines in Fig. 1. (2) The differences observed by us between H_{min} in HPTLC and conventional TLC are rather small, which suggests that the particle size of the HPTLC- and TLC-type silica gels do not differ substantially. In the literature, values such as 7 *vs.* 9 (ref. 9), 7–8 *vs.* 10–11 (ref. 10) and 5 *vs.* 11 (ref. 4) μm have been reported; from data quoted by Kaiser[11], a value of about 7–10 μm for HPTLC silica gel can be calculated. The technical bulletin issued by Merck[12] on the new developments incorporated in their HPTLC layers, next to the use of a narrower particle size classification of the silica gel and optimized layer thickness and quality of layer packing, merely mentions that the particle size classification is below the range of that of the usual TLC pre-coated plates. Macherey, Nagel & Co.[13] state that they use the same silica gel for HPTLC as they do for TLC but, for HPTLC, the material "... is very finely fractionated (mean particle size 5–10 μm)". In summary, the particle size difference between HPTLC and conventional TLC silica gel may be smaller than is often assumed.

Cellulose

HPTLC plates pre-coated with cellulose are marketed by Merck. According to our experience, which is based on the separation of four model systems (*cf.*, Table II), similar spot shapes and R_F values (difference 0.00–0.02 R_F unit) are obtained in HPTLC and conventional TLC. More important, the time of development on the HPTLC plates is much smaller than on the TLC plates. Data for two solvent mixtures are presented in Table III; similar results were obtained with various other acidic,

TABLE II

MODEL SYSTEMS USED FOR COMPARISON OF HPTLC- AND TLC-TYPE CELLULOSE

Mobile phase	*Compounds separated**
Acetone–6 *N* HCl (4:1)	Ni, Mn, Co, Cu, Fe
Methanol–6 *N* HCl (4:1)	Ba, Sr, Ca, Mg
Chloroform–glacial acetic acid–water (7:6:1)	Tannin, gallic acid, pyrogallol, pyrocatechol
n-Butanol–glacial acetic acid–water (4:1:1)	Glycine, alanine, valine

* In order of increasing R_F values.

TABLE III

DEPENDENCE OF TIME OF RUN ON TYPE OF CELLULOSE USED FOR TWO MOBILE PHASES

System: TLC- or HPTLC-type cellulose (Merck) with (I) acetone–6 *N* HCl (9:1) or (II) *n*-butanol–glacial acetic acid–water (4:1:1) as mobile phase.

Distance (mm)	*Time of run (min)*			
	I		*II*	
	TLC	*HPTLC*	*TLC*	*HPTLC*
30	3	2	19	12
45	7	4	36	25
60	12	7	60	35
75	16	11	80	50
90	25	16	120	80

neutral and basic mobile phases. Obviously, k_{HPTLC} is about 50% larger than is k_{TLC}. The advantage of the decrease in time of analysis is not offset by a decrease in performance: H_{av} values were generally found to be distinctly smaller on the HPTLC plates, *e.g.*, 40 μm (HPTLC) *vs.* 70 μm (TLC) for cobalt(II) at $z_x = 30$–50 mm. A similar considerable decrease in plate height has been observed by Hauck and Halpaap[2], who recorded minimum values of 15–20 μm (HPTLC) and 30–35 μm (TLC) with leucine as test compound.

"Inactive" silica

Recently, the use of thin-layer plates pre-coated with a synthetic porous silica (Si 50 000) with an extremely high mean pore diameter of about 5000 nm and a very small internal surface area has been reported[2]. According to the authors, this material, which on the basis of their description probably is similar to the material used to prepare concentration zones on other types of HPTLC plates, should best be compared with Kieselguhr. For D-glucose and D-xylose, which are separated with *n*-propanol–water–25% ammonia solution (80:20:1) as the mobile phase, a reduction in plate height of from 100–140 μm (TLC) to 60–80 μm (HPTLC) for a length of run of 5–8 cm is obtained.

Chemically bonded stationary phases

Thin-layer plates pre-coated with LiChrosorb RP-2, RP-8 or RP-18 and containing a fluorescent indicator (or, in the case of RP-18, without the indicator, but with a concentration zone) are manufactured by Merck. Pre-coated KC_{18} plates, which contain C_{18} carbon chains bonded to the silica via Si–O–Si bonds, are supplied by Whatman. With these plates, chemical bonding of the C_{18} chain is followed by a further reaction with C_2 compounds to obtain a fully covered silica gel surface. The total carbon load of the KC_{18} layer is 10%, which allows for a high sample capacity. It has been demonstrated[1] that with methanol–water and acetonitrile–water mixtures containing 0–30% of water as the mobile phase, the four types of bonded stationary phase perform equally well; however, the KC_{18} plates should be favoured because of the relatively short times of run observed for systems involving their use. With mobile phases containing over about 30% of water, the RP-coated plates can no longer be used conveniently owing to the non-wettability of the stationary phase material. No such problem is encountered with the KC_{18} plates, provided that about 3% of sodium chloride or a similar salt is added to the mobile phase in order to prevent dislodgement of the chemically bonded phase from the glass backing.

Recently, Macherey, Nagel & Co. have marketed[13] a series of bonded-phase HPTLC plates. The manufacturers prefer the use of a single (C_{18}) alkylsilane with different percentages of silanization over the use of a range, *e.g.*, C_2–C_{18}, of alkylsilanes. HPTLC silica gel was selected as base material, the reactive groups being totally (100%) or partly (75 or 50%) substituted with alkylsilanes. Results for the separation of chloroanilines and polynuclear aromatic hydrocarbons, carried out with a mobile phase containing a relatively small proportion of water, are shown in Fig. 3. As is to be expected, the retention decreases (slightly) with a decreasing percentage of bonded C_{18} chains. Even with the MN50 material, however, the sorption strength is about equal to that obtained with RP-18-coated layers, while the time of run is considerably shorter. When working with high percentages of water and with 3% of

sodium chloride added, the incompletely substituted MN50 is seen (Fig. 4) to exhibit much better performance than do the 75 and 100% silanized stationary phases. The time of analysis and the separation of the aromatic hydroxy compounds are very similar to those obtained with a Whatman KC_{18} plate. The presence of a substantial percentage of non-reacted silanol groups on the silica gel surface of MN50 apparently does not affect the chromatographic behaviour of such polar solutes as substituted anilines or aromatic hydroxy compounds. In conclusion, MN50 layers appear to be a valuable addition to the list of chemically bonded stationary phases for HPTLC.

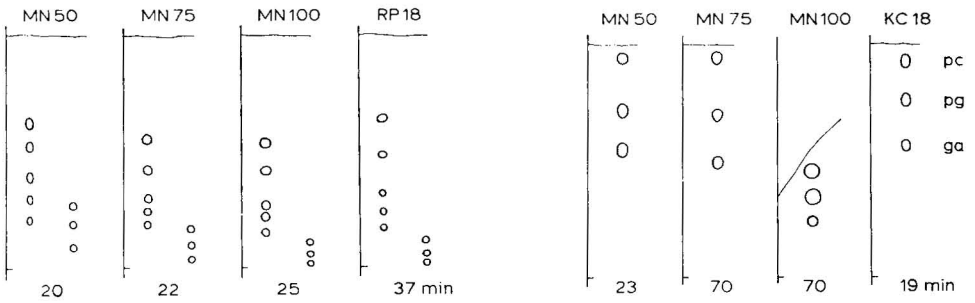

Fig. 3. HPTLC separation of chloroanilines (left) and polynuclear aromatic hydrocarbons (right) on four different chemically bonded stationary phases (for details, see text) using methanol–water (9:1) as mobile phase. Run, 5 cm. Model compounds (in order of decreasing R_F): 4-mono-, 3,4-di-, 2,3,4-tri-, 2,4,6-tri- and 2,3,5,6-tetrachloroaniline; phenanthrene, pyrene and perylene.

Fig. 4. HPTLC separation of aromatic hydroxy compounds on four different chemically bonded stationary phases (for details, see text) using methanol–water (1:1) plus 3% NaCl as mobile phase. Symbols: ga = gallic acid; pg = pyrogallol; and pc = pyrocatechol.

Polyamide

Plastic sheets coated on both sides with polyamide-6, without the use of a binder, are offered[13] by Macherey, Nagel & Co. under the name "polyamide sheets according to Wang". The separation of series of hydroxy compounds with two mobile phases is illustrated in Fig. 5. Spot size and resolution are seen to be slightly better on the HPTLC than conventional TLC polyamide-6 sheets. However, the time of

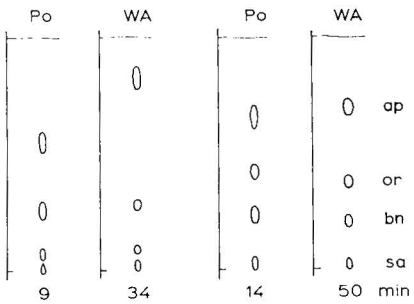

Fig. 5. Separation of salicylic acid (sa), β-naphthol (bn), orcinol (or) and *m*-aminophenol (ap) on polyamide-6 thin layers (Po) and HPTLC polyamide sheets according to Wang (WA), using acetone–water (3:7) and methanol–methyl ethyl ketone–water (1:1:2) as mobile phases.

analysis in HPTLC is 3–4-fold longer, *i.e.*, in an equal time of separation the migration distance in TLC will be about double that in HPTLC. It appears that modification of the process for manufacturing the Wang-type sheets in order to increase considerably their velocity constant would be necessary before they could become useful for analytical purposes.

CONCLUSION

A wide variety of thin-layer plates pre-coated with HPTLC-quality stationary phases are commercially available. As concerns plates pre-coated with silica gel and, even more so, polyamide, the relatively low permeability (small velocity constant) of the HPTLC compared with the conventional TLC plates detracts from their usefulness. With silica gel, this disadvantage has already been partly remedied by the introduction, by Merck, of a newer type of plates that display improved permeability. The use of HPTLC cellulose plates can be recommended on account of their favourable velocity constants and plate heights. The major problem noted so far with chemically bonded reversed phases, *i.e.*, the extremely slow, if any, development of the plates when using mobile phases containing a high proportion of water, can be solved for at least two stationary phase materials by the simple addition of a small amount of sodium chloride to the mobile phase mixture.

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GEL CHROMATOGRAPHIC STUDY OF THE POLYMERIZATION OF SILICIC ACID IN ACID SOLUTIONS

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SUMMARY

The polymerization of silicic acid in hydrochloric, nitric, perchloric and sulphuric acid solutions has been studied by gel chromatography. The rate of polymerization of silicic acid is in the order of $HClO_4 > HCl \approx HNO_3 > H_2SO_4$. A mechanism for the growth of particles of polysilicic acids is proposed. The growth in 1 *N* acid solutions is due mainly to the reaction between monosilicic and polysilicic acids, whereas that in 3 *N* and 5 *N* acid solutions is due mainly to the reaction between polymer species.

INTRODUCTION

The rate of polymerization of silicic acid is lowest in aqueous solution of about pH 2. Below this pH, the rate increases with increasing acidity[1,2]. Tarutani[1] studied the polymerization of silicic acid in various acid solutions by colorimetry. Elmer and Nordberg[3] studied the solubility of porous vitreous silica in up to 9 *N* nitric acid solutions.

Gel chromatography has also been used to study the polymerization of silicic acid[4]. The mechanism of the growth of polymer particles was discussed[5] on the basis of changes in the elution curves for polysilicic acids.

In this work, the effect of the concentration of hydrochloric, nitric, perchloric and sulphuric acids on the polymerization of silicic acid was studied by gel chromatography, and a mechanism is proposed for the growth of particles of polysilicic acids.

EXPERIMENTAL

Sample solutions and reagents

All reagents used were of analytical reagent grade. A stock solution of monosilicic acid was prepared by fusing 2.000 g of anhydrous silica with 10.0 g sodium carbonate, dissolving the melt in distilled water and diluting to 1000 ml. A 0.4% Blue Dextran 2000 solution was used. The eluent was 0.1 *M* sodium chloride solution of pH 2.

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Preparation of Sephadex column

Sephadex G-100 (Pharmacia, Uppsala, Sweden) was suspended in the eluent solution and allowed to swell for 3 days. The column (45 × 1.0 cm I.D. or 45 × 1.5 cm I.D.) was a glass tube with a porous polystyrene disc at the bottom. The Sephadex G-100 column was prepared as described in the literature[6].

Procedure

Monosilicic acid solution (500 ppm of SiO_2) of desired acidity was prepared by mixing aliquots of monosilicic acid stock solution and acid. At different times, an aliquot of sample solution was adjusted to pH 2 by adding anion-exchange resin, Dowex 1-X8 (OH^-). The concentration of monosilicic acid was measured by colorimetry and the silicic acid was then chromatographed on a Sephadex column. The volume of sample solution delivered to the column was 2 ml. Using an automatic fraction collector, the effluent was collected in fractions of 1 or 2 ml, with a flow-rate of 8–12 ml/h. The amounts of silicic acid in the fractions were measured by atomic absorption spectrometry using a Nippon-Jarrell Ash Model AA 781 instrument. Blue Dextran 2000 was eluted by using the same procedure. All experiments were carried out at 20 ± 1°C.

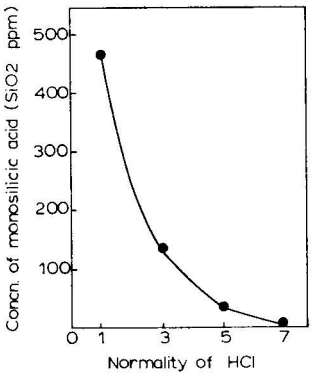
RESULTS AND DISCUSSION

Effect of concentration of hydrochloric acid on the polymerization of silicic acid

Polymerization and depolymerization of silicic acid in solutions of low concentrations of electrolytes do not occur over short periods at pH 2 (ref. 4). Therefore, silicic acid is usually chromatographed on a Sephadex column after the sample solution has been adjusted to pH 2. However, when monosilicic acid solution containing a high concentration of hydrochloric acid was allowed to stand for a desired period and then adjusted to pH 2 with sodium hydroxide, gel was formed rapidly. It is suggested that the concentration of sodium chloride is so high as to accelerate the polymerization reaction. Therefore, as it is necessary to minimize the concentration of electrolytes after adjustment of pH, an anion-exchange resin (OH^-) was used for adjustment of pH. Addition of the anion-exchange resin had no effect on the polymerization of silicic acid.

The rate of polymerization of silicic acid changes with changing acidity. In Fig. 1, the concentration of monosilicic acid in hydrochloric acid solutions, as measured by colorimetry after 3 h, is plotted against normality of hydrochloric acid. The concentration of monosilicic acid decreases in proportion to the acidity. The decrease in the concentration is due to polymerization of monosilicic acids and of monosilicic and polysilicic acids.

The variation with time of the elution curves for silicic acid in 1 *N* hydrochloric acid solution is shown in Fig. 2. The point V_0 is the elution volume of Blue Dextran 2000. It can be assumed that the elution volume of Blue Dextran 2000 is equal to the void volume of the bed. The peaks on the right are due to monosilicic acid and those on the left to polysilicic acid. The concentrations of monosilicic acid at the given times are shown in parentheses. The elution curves for the polymers after 50 h indicate a symmetrical particle-size distribution.



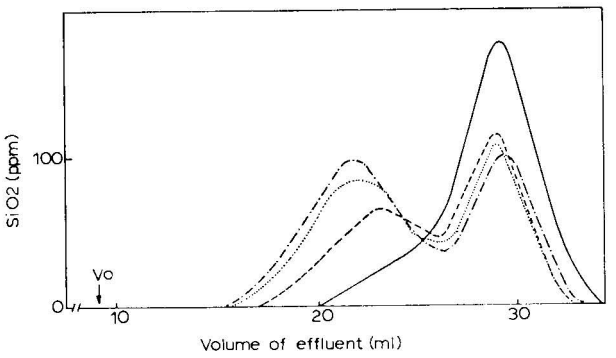


Fig. 1. Plot of the concentration of monosilicic acid after 3 h against normality of hydrochloric acid.

Fig. 2. Variation with time of the elution curves for silicic acid in 1 *N* hydrochloric acid solution. Column, 45 × 1.0 cm I.D. ———, 24 h [M(monosilicic acid concentration) = 367 ppm; — — —, 50 h (M = 278 ppm); . . . . ., 75 h (M = 227 ppm); —·—·—, 100 h (M = 204 ppm).

The distribution coefficient, K_{av}, for a given gel in gel chromatography is defined[7] as

$$K_{av} = (V_e - V_0)/(V_t - V_0) \tag{1}$$

where V_0 is the void volume, V_t the total bed volume and V_e the elution volume. K_{av} is a measure of molecular size and decreases with increasing particle size. When the elution curve for polysilicic acid indicates a broad particle-size distribution, the mean elution volume, V_e, must be calculated, using the relationship

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

where C_i is the concentration in the ith fraction and V_i is the volume of the ith fraction. In Fig. 3, K_{av} values, calculated from eqns. 1 and 2, are plotted against time.

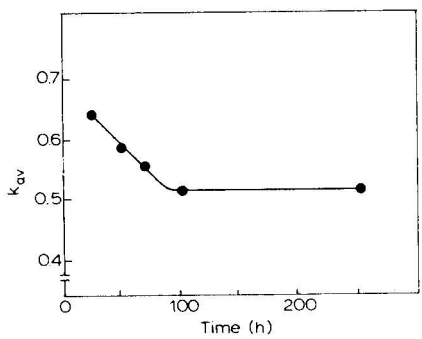


Fig. 3. Plot of K_{av} values against time.

The elution curves for the polysilicic acids indicate a symmetrical particle-size distribution after 50 h (Fig. 2), and the K_{av} value of the polymers obtained after 250 h was almost the same as that obtained after 100 h (Fig. 3). These results indicate that, when the concentration of monosilicic acid is close to the solubility of amorphous silica, the growth rate of the polymer particles is very slow. It is concluded that the growth of the polymer particles is due mainly to polymerization between monomer and polymer, and there is little polymerization between polymer species.

Solutions of 3 *N* hydrochloric acid containing different concentrations of monosilicic acid were allowed to stand for 3 h and then chromatographed. The results are shown in Fig. 4. The elution curves for the polymers are symmetrical, and the K_{av} values calculated are almost same in spite of differences in the initial concentration of monosilicic acid. This means that the growth of the polymer particles under these conditions is due mainly to polymerization between monomer and polymer.

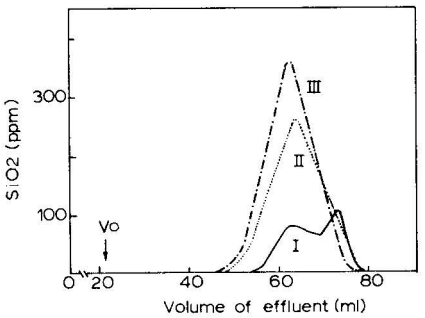


Fig. 4. Elution curves after 3 h for silicic acid in 3 *N* hydrochloric acid solutions with different initial concentrations of monosilicic acid. Initial monosilicic acid concentration: I, 500 ppm; II, 1000 ppm; III, 1500 ppm. Column, 45 × 1.5 cm I. D. ———, I (M = 136 ppm); . . . . ., II (M = 136 ppm); —.—.—, III (M = 163 ppm).

Polymerization of silicic acid in various acid solutions

The variation with time of the concentration of monosilicic acid in 3 *N* solutions of hydrochloric, nitric, perchloric and sulphuric acids is shown in Fig. 5.

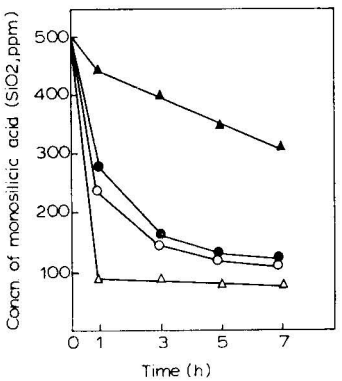


Fig. 5. Variation with time of the concentration of monosilicic acid in 3 *N* solutions of hydrochloric (○), nitric (●), perchloric (△) and sulphuric (▲) acids.

The rate of decrease in the concentration of monosilicic acid is in the order $HClO_4 > HCl \approx HNO_3 > H_2SO_4$.

The elution curves for silicic acid in 1 N solutions of these acids after 250 h are shown in Fig. 6. A symmetrical particle-size distribution in all the solutions is indicated, and the K_{av} values are almost identical in spite of the differences in the concentration of monosilicic acid after 250 h. From the results shown in Figs. 2 and 6, it is concluded that the increase in size of the polymer particles is due mainly to the reaction between monomer and polymer in 1 N acid solutions, and that there is little polymerization between polymer species. The behaviour of silicic acid in nitric acid solution seems to be identical with that in hydrochloric acid solution (Figs. 5 and 6).

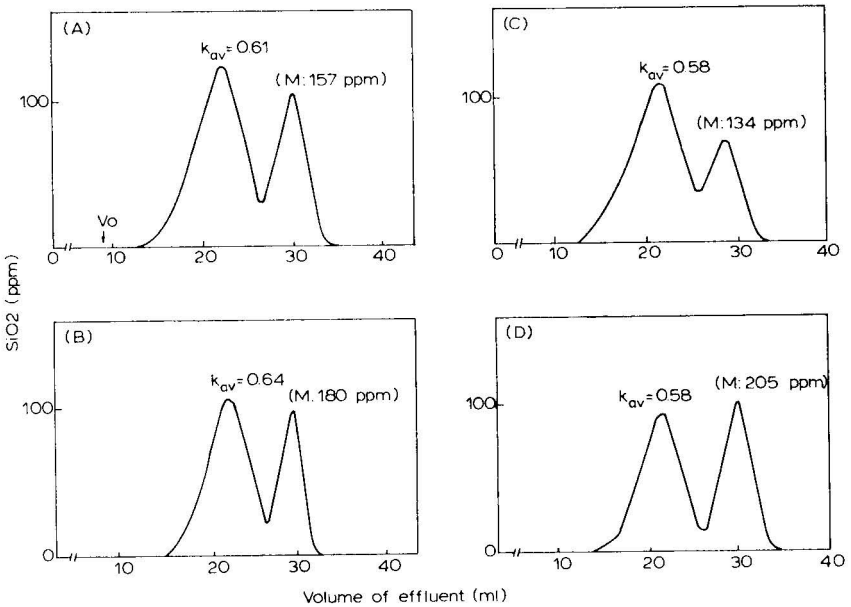
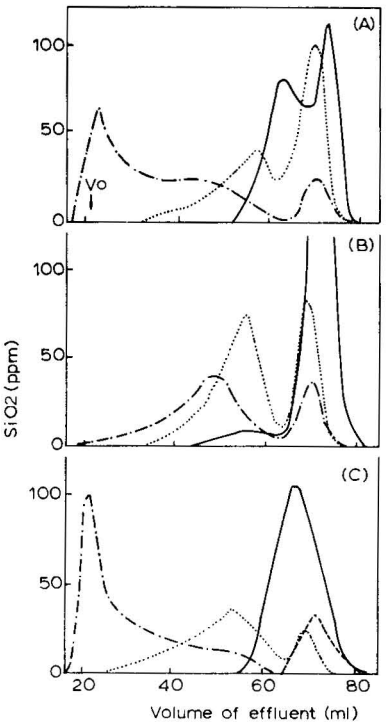


Fig. 6. Elution curves after 250 h for silicic acid in 1 N solutions of hydrochloric (A), nitric (B), perchloric (C) and sulphuric acids (D). Column, 45 × 1.0 cm I.D.

The variation with time of the elution curves for silicic acid in 3 N solutions of hydrochloric, perchloric and sulphuric acids is shown in Fig. 7. In 3 N perchloric acid solution only one peak was obtained for silicic acid after 3 h (Fig. 7C) because the peak for polymer was superposed on that for monomer. The elution curve for the polymers after 24 h indicates a broad particle-size distribution. The highest peak was obtained at the point V_0 after 100 h. All polymers that have larger sizes than the possible separation range for Sephadex G-100 are eluted at V_0. The results indicate that the growth of the polymer particles proceeds by the polymerization between polymer species, because the decrease in the concentration of monosilicic acid was very slow after 1 h (Fig. 5). The mechanism of the growth of the polymer particles in 3 N acid solutions is different from that in 1 N acid solutions (Figs. 2 and 6). Although the rate of polymerization of silicic acid in hydrochloric and sulphuric acid



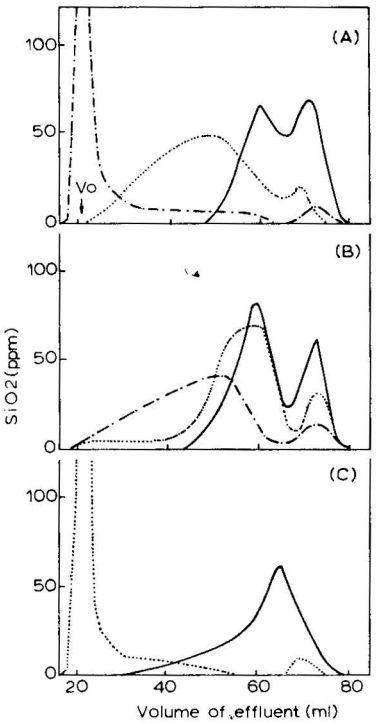


Fig. 7. Variation with time of the elution curves for silicic acid in 3 *N* solutions of hydrochloric, perchloric and sulphuric acids. Column, 45 × 1.5 cm I.D. A, HCl, ———, 3 h (M = 154 ppm); . . . . ., 24 h (M = 115 ppm); ·—·—·, 100 h (M = 52 ppm). B, H_2SO_4, ———, 3 h (M = 403 ppm); . . . . ., 24 h (M = 162 ppm); ·—·—·, 100 h (M = 134 ppm). C, $HClO_4$, ———, 3 h (M = 92 ppm); — — —, 3 h monomer; . . . . ., 24 h (M = 59 ppm); ·—·—·, 100 h (M = 39 ppm).

Fig. 8. Variation with time of the elution curves for silicic acid in 5 *N* solutions of hydrochloric, perchloric and sulphuric acids. Column, 45 × 1.5 cm I.D. A, HCl, ———, 3 h (M = 103 ppm); . . . . ., 24 h (M = 14 ppm); ·—·—·, 100 h (M = 11 ppm). B, H_2SO_4, ———, 3 h (M = 47 ppm); . . . . ., 24 h (M = 41 ppm); ·—·—·, 100 h (M = 39 ppm). C, $HClO_4$, ———, 3 h (M = 16 ppm); . . . . ., 24 h (M = 9 ppm).

solutions was slow compared with that in perchloric acid solution, similar results were obtained.

The elution curves for silicic acid in 5 *N* solutions of hydrochloric, perchloric and sulphuric acids are shown in Fig. 8. The rate of polymerization of silicic acid in these solutions is faster than that in the corresponding 3 *N* solutions, and the mechanism of growth of the polymer particles is similar to that in the latter solutions.

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CHROM. 13,009

ANALYSIS OF PURINE RIBONUCLEOTIDES AND DEOXYRIBONUCLEOTIDES IN CELL EXTRACTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic (HPLC) assay for the fourteen major cellular purine ribonucleotides and 2′-deoxyribonucleotides is presented. Following an initial separation by anion-exchange HPLC, the nucleotides are hydrolyzed to their respective nucleosides by alkaline phosphatase and quantified by reversed-phase HPLC and UV absorbance detection. The assay is reproducible, specific, and has a detection limit of 10 pmol/sample. The recovery of nucleosides derived from nucleotides is 85%. Purine nucleotide pool sizes have been measured in cultured mouse T lymphoma (S-49) cells before and after treatment with 2.0 μM mycophenolic acid, an inhibitor of the enzyme IMP dehydrogenase, for 3 h. Control nucleotide levels obtained by this method are consistent with those reported for S-49 cells using other methods, and the observed decrease in guanine nucleotides and increase in IMP after treatment with mycophenolic acid agree with previous reports.

INTRODUCTION

The measurement of the acid-soluble purine and pyrimidine mononucleotide pools is important in the study of the regulation of cellular function and its alteration by enzyme abnormalities or antimetabolite chemotherapeutic treatment. Current assay procedures for the measurement of the purine and pyrimidine mononucleotides are not capable of detecting all of the major ribonucleotides and 2′-deoxyribonucleotides at physiological concentrations[1–4]. High-performance liquid chromatographic (HPLC) assays are usually limited to the analysis of ribonucleotides by the incomplete separation of 2′-deoxyribonucleotides from the more abundant ribonucleotides[1,2]. HPLC analysis of ribonucleotide monophosphates is often hindered by their low intracellular concentrations and interfering endogenous compounds such as dinucleotide cofactors. The selective chemical degradation of ribonucleotides by periodate oxidation has been used to measure 2′-deoxyribonucleotide triphosphates, but not the corresponding mono- or di-phosphates[3]. The *in vitro* DNA template assay is also limited to quantitation of 2′-deoxyribonucleotide triphosphates[4].

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We now report an HPLC–UV assay procedure for the determination of all the major purine mononucleotides. The assay consists of an initial separation of the nucleotides by anion-exchange HPLC followed by hydrolysis of the collected nucleotides to nucleosides with alkaline phosphatase and quantitation by reversed-phase HPLC with UV detection. Our laboratory has previously reported an assay procedure for the measurement of the pyrimidine mononucleotides[5], based on the same analytical principles, which can be combined with the purine assay presented here to allow a comprehensive analysis of cellular mononucleotide pools.

MATERIALS AND METHODS

Reagents

Nucleosides and nucleotides of analytical grade and alkaline phosphatase (orthophosphoric monoester phosphohydrolase; E.C. 3.1.3.2) type III, from *Escherichia coli*, at an activity of 22 I.U./mg protein, were obtained from Sigma, St. Louis, MO, U.S.A. [2-^{3}H]Adenosine 5′-monophosphate (^{3}H-AMP), specific activity 15.8 Ci/mmol, 1 mCi/ml ethanol–water (1 : 1) was purchased as the ammonium salt from Amersham, Arlington, IL, U.S.A.

Apparatus

HPLC analysis was performed on a Model ALC/GPC 204 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) with an additional Model 6000A solvent delivery system and a Model 660 solvent programmer.

Cell culture

The growth and lymphocytic properties of wild type mouse T lymphoma (S-49) cells have been described in detail previously[5]. Cells were grown to a density of 10^6 cells/ml in Dulbeco's modified Eagle's medium with 10% horse serum at 37°C. A 100-ml volume of the cell suspensions was then incubated in the presence or absence of 2.0 μM mycophenolic acid for 3 h. An acid-soluble extract was obtained from the cells by centrifugation, PBS wash of the cell pellet, perchloric acid extraction of the cell pellet and neutralization of the extract by ion pair extraction as previously reported[5,7].

HPLC separation of nucleotides

Nucleotides were separated by anion-exchange HPLC on an Aminex A-29 column (30 cm × 4.0 mm I.D.; resin supplied by Bio-Rad Labs., Richmond, CA, U.S.A.) using the chromatographic conditions previously reported by this laboratory for the analysis of pyrimidine nucleotides[5]. Briefly, a 30 min isocratic elution using 0.025 M sodium citrate, pH 8.2, was followed by a 2-h linear gradient to a final eluent concentration of 0.500 M sodium citrate, pH 8.2. The flow-rate was maintained at 0.3 ml/min throughout the procedure and the column temperature was 50°C. Nine effluent fractions were collected (Fig. 1) and concentrated by lyophilization.

Enzymatic conversion of nucleotides into nucleosides

Lyophilized nucleotide fractions 1–7 were reconstituted with 1 ml of water. Fractions 8 and 9 were reconstituted with 1.5 ml of warm water (*ca.* 60°C) to dissolve

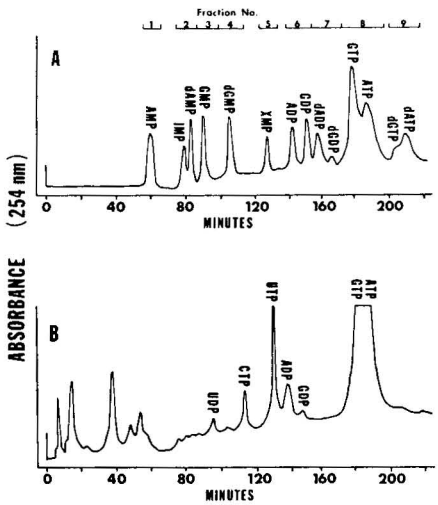

Fig. 1. Separation of purine nucleotides by Aminex A-29 anion-exchange HPLC. (A) Chromatogram of purine nucleotide standards and fraction collection intervals. (B) Chromatogram of an acid-soluble extract from mouse T lymphoma (S-49) cells. The detector sensitivity is 0.5 absorbance units full scale.

the larger amount of citrate present. One unit of *E. coli* alkaline phosphatase was added and the fractions were incubated at 37°C for 30 min. This enzyme preparation replaces the potato acid phosphatase previously used for this procedure[5]. *E. coli* alkaline phosphatase has the following advantages over potato acid phosphatase: (1) the commercially available enzyme has a sufficiently high activity that protein denaturation and centrifugation are no longer required before HPLC analysis; rather the incubation mixture can be directly subjected to HPLC analysis, with a resultant increase in analytical recovery; (2) no pH adjustment is necessary since the pH of the anion-exchange HPLC eluent is close to the pH optimum of the enzyme; (3) a component of the potato acid phosphatase preparation degrades adenosine and 2′-deoxyadenosine to the base adenine, which precludes the separate analysis of adenine ribonucleotides and 2′-deoxyribonucleotides with this enzyme.

Analysis of resultant nucleotides

A 10-μl volume of 99.7% glacial acetic acid was added to samples containing inosine, guanosine, or 2′-deoxyguanosine after phosphate hydrolysis in order to obtain reproducible retention times during reversed-phase chromatography. Fraction 4, containing xanthosine, required the addition of 100 μl of 99.7% glacial acetic acid to obtain reproducible retention times. An aliquot of each sample up to the entire volume was injected onto a LiChrosorb RP-18 reversed-phase column (25 cm × 10 mm I.D.; Altex Scientific, Berkeley, CA, U.S.A.). Samples were eluted with acetonitrile–water (3.5:96.5) at a flow-rate of 7.0 ml/min. Absorbances at 254 nm and 280 nm were monitored. Peaks were quantitated by peak height measurement and comparison with an external standard solution.

High sensitivity analysis of nucleotides

Five- to ten-fold higher sensitivity was attained, when necessary, by collecting the appropriate effluent fraction from the 10 mm I.D. reversed-phase column, concentrating it to *ca.* 250 μl under a stream of nitrogen at 70°C, and reinjecting it onto a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D.; Waters Assoc.) using an optimized eluent at a flow-rate of 2.5 ml/min. Guanosine, 2′-deoxyguanosine, inosine, and xanthosine were eluted with acetonitrile–water (2.5:97.5) buffered at pH 4.7 with 0.01 *M* sodium acetate. Adenosine and 2′-deoxyadenosine were eluted with acetonitrile–water (2.0:98.0) buffered at pH 3.0 with 0.01 *M* potassium phosphate.

RESULTS AND DISCUSSION

HPLC analysis of nucleotides

Separation. Fig. 1 shows (A) the separation of a solution of pure purine nucleotides and (B) the separation of nucleotides from an extract of S-49 cells on Aminex A-29. Weak anion-exchange chromatography with citrate buffer at pH 8.2 was chosen over strong anion-exchange methods with phosphate buffer at acidic pH values[2] because phosphate buffer inhibits alkaline phosphatase, an integral part of this assay. The fully porous anion-exchange resin Aminex A-29 was chosen over pellicular resins because of its higher sample capacity. Moreover, Aminex A-29 has a high separation efficiency for mononucleotides, including the partial or complete separation of ribo- from 2′-deoxyribonucleotides. This porous resin does, however, require lower flow-rates for proper performance and therefore a longer total analysis time of 4 h. Fig. 2 shows the nucleosides derived from nucleotides in an extract of S-49 cells on the 10 mm I.D. reversed-phase column. This high-capacity column was

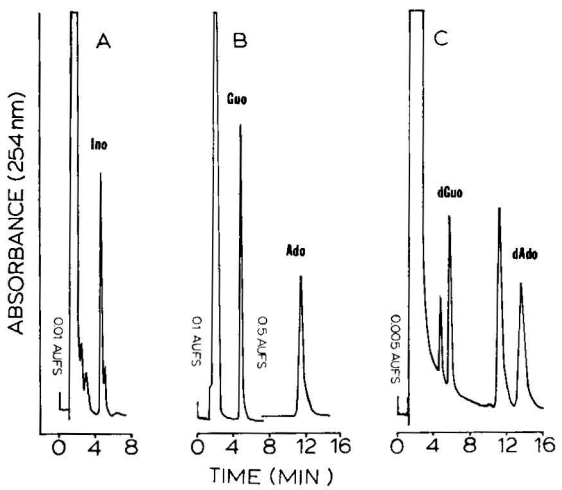

Fig. 2. Chromatography of nucleosides derived from the nucleotides in a biological sample consisting of the extract of S-49 cells on LiChrosorb RP-18 reversed-phase column (10 mm I.D.). (A) 1.48 nmol of inosine derived from IMP). (B) 13.3 nmol of guanosine derived from GTP and 74.3 nmol of adenosine derived from ATP. (C) 0.59 nmol of deoxyguanosine derived from dGTP and 1.08 nmol of deoxyadenosine derived from dATP. The peaks immediately preceding each deoxyribonucleoside are small amounts of the respective ribonucleoside derived from GTP and ATP.

used because of the large amount of citrate in samples after anion exchange, which can overload a smaller column. Fig. 3 shows the increased sensitivity attainable when the nucleosides eluted from the 10 mm I.D. column are collected, concentrated, and reinjected onto an analytical reversed-phase column.

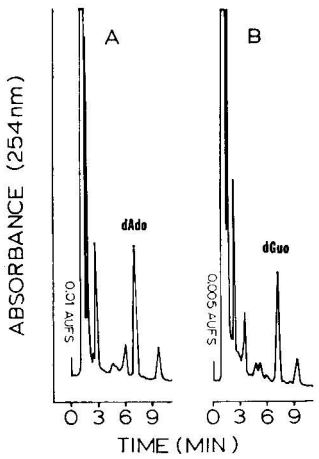

Fig. 3. Chromatography of nucleosides derived from the nucleotides in a biological sample consisting of the extract of S-49 cells which were collected from the 10 mm I.D. column and reanalyzed on a μBondapak C_{18} reversed-phase column (3.9 mm I.D.). (A) 0.103 nmol of deoxyguanosine derived from dGDP. (B) 0.218 nmol of deoxyadenosine derived from dADP.

Minimum detection limit. The detection limit for nucleosides isolated from cell extracts on the 10 mm I.D. reversed-phase column ranges from 40 pmol/sample for guanosine to 100 pmol/sample for 2'-deoxyadenosine. An ultimate sensitivity of at least 10 pmol per sample is attainable by reanalysis on the analytical reversed-phase column. In a sample of 10^8 cells only dGMP and dAMP are present at levels that require the higher sensitivity of the final analytical step. XMP was not present in detectable quantities ($<$ 10 pmol/*ca.* 10^8 cells) in the S-49 cell line.

Analytical recovery. The analytical recovery of the method was determined by the addition of trace amounts of ^{3}H-AMP to samples at the beginning of the procedure. ^{3}H-AMP recovery after quantitation on the 10 mm I.D. reversed-phase column was 85 $\pm$ 3%. The effect of the different citrate concentrations of mono-, di-, and triphosphate fractions on quantitation by reversed-phase HPLC was studied by analyzing nucleoside standards in solutions of citrate ranging from 0.1 to 2.5 *M*. No difference was observed. There was also no difference ($<$ 5%) observed between quantitation on the 10 mm I.D. reversed-phase column and upon collection, concentration, and reanalysis of nucleosides by analytical reversed-phase column, suggesting complete analytical recovery of nucleosides during this step. Alkaline phosphatase quantitatively hydrolyzes nucleotides present in amounts far in excess of those found in biological samples in 30 min. Leaving samples in solution with the active enzyme longer than this does not result in any degradation of the nucleosides.

Precision. The precision of the assay was studied by the measurement of the

nucleotide pools of three separate aliquots of the same S-49 cell extract. The standard deviations of these measurements for each nucleotide, given in Table I, are below 10% except for the dNMPs, which are present in levels approaching the sensitivity limit of the assay.

TABLE I

PRECISION OF NUCLEOTIDE ANALYSIS

The nucleotide contents of three aliquots from the same extract of S-49 cells were separately determined.

	nmol/10⁸ cells	*S.D. (%)*		*nmol/10⁸ cells*	*S.D. (%)*
AMP	3.66	6	dAMP	0.023	12
ADP	12.4	6	dADP	0.12	3
ATP	141	6	dATP	1.93	10
GMP	0.18	*	dGMP	0.009	19
GDP	2.27	1	dGDP	0.062	5
GTP	24.8	5	dGTP	0.80	9
IMP	0.42	*			
XMP	**				

* S.D. not calculated because of sample mishandling.
** Nucleotide not present in detectable amounts.

Specificity. The specificity of the method for the desired nucleosides has been confirmed by retention times on both anion-exchange and reversed-phase HPLC, peak symmetry, and the ratio of their absorbances at 254 nm to those at 280 nm. dADP cannot be quantitated under the conditions used for the high capacity reversed-phase HPLC step because of an interfering compound with a UV absorbance ratio different from that of authentic 2′-deoxyadenosine; however, the interference is separated from 2′-deoxyadenosine under the conditions used for high sensitively analysis, pH 3.0. Dinucleotide cofactors are resistant to treatment by the phosphatase enzyme, *i.e.*, NAD is not converted to adenosine + P_i + NMN by alkaline phosphatase and therefore do not interfere with the assay[8].

Comparison with other methods. Table II shows the nucleotide levels in S-49 cells determined by this method and those determined by other methods. There is consistency between the different methods.

TABLE II

NUCLEOTIDE CONTENT OF S-49 CELLS AS DETERMINED BY THIS AND OTHER METHODS

	Nucleotide content (nmol/10⁸ cells)				
	IMP	*ATP*	*GTP*	*dATP*	*dGTP*
This method*	0.59	130	22.7	2.02	0.78
Other methods	—	185**	24.2**	1.44***	0.98***

* Values are the mean obtained from six separate S-49 cell extracts.
** Determined by anion exchange with UV absorbance[12].
*** Determined by DNA template[13].

Nucleotide pool size changes in S-49 cells following treatment with mycophenolic acid.

Mycophenolic acid is a potent inhibitor of the enzyme inosinate dehydrogenase, an essential enzyme in the biosynthesis of guanine ribo- and deoxyribonucleotides from inosinate[9]. We are studying the toxic effects resulting from depletion of either guanine ribonucleotides or guanine deoxyribonucleotides in S-49 cells. As a part of this study, we treated wild type S-49 cells with 2.0 μM mycophenolic acid for 3 h and observed the resulting changes in purine nucleotide pool sizes. Mycophenolic acid reduced the concentrations of GTP and dGTP to 30 and 55% of control values, respectively, while the IMP level increased to *ca* 170% of control values. These changes can be attributed to inosinate dehydrogenase inhibition. In addition, the level of ATP was relatively unaffected while the level of dATP increased slightly to 130% of control values. Similar purine nucleotide pool size changes caused by mycophenolic acid have been observed in the murine lymphoma L5178Y cell line[10,11].

CONCLUSION

The presented assay for purine mononucleotides yields reproducible results that are consistent with previous methods for the analysis of selected nucleotides. Moreover, the analytical approach combining high-resolution anion-exchange and reversed-phase HPLC with one enzymatic step allows the flexibility to determine any or all of the endogenous purine or pyrimidine mononucleotides as well as nucleotide derivatives of administered antimetabolite drugs. The analytical features of the method make it a more generally applicable tool than previous procedures in the study of the mononucleotide metabolism of cells.

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CARBOXYMETHYL-CELLULOSE MICROCHROMATOGRAPHY FOR THE QUANTITATION OF HEMOGLOBIN BART'S (γ_4) AND ITS USE IN THE DETECTION OF THE α-THALASSEMIA CONDITIONS

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SUMMARY

A modification of an existing (micro) CM-cellulose chromatographic procedure is introduced for the quantitation of hemoglobin Bart's (or γ_4) in blood samples of newborn babies. Normal newborn with four active α chain genes ($\alpha\alpha/\alpha\alpha$) have small amounts (average 0.55%) of this abnormal hemoglobin while increased percentages are present in newborn with an α-thalassemia-2 heterozygosity ($\alpha^0\alpha/\alpha\alpha$; average 1.55%) or an α-thalassemia-2 homozygosity ($\alpha^0\alpha/\alpha^0\alpha$; average 4.65%). The identification of hemoglobin Bart's in normal newborn was made by high-performance liquid chromatography, and the absence of contaminating non-hemoglobin proteins was confirmed by electrophoresis and additional chromatographic experiments. This rapid procedure is useful for the detection and differentiation at time of birth of the different α chain deficiencies which are common among various populations in the world.

INTRODUCTION

Hemoglobin (Hb) α chain deficiencies are often difficult to detect in the adult. Two of these, the α-thalassemia-2 homozygosity ($\alpha^0\alpha/\alpha^0\alpha$; β/β) and the α-thalassemia-1 heterozygosity ($\alpha^0\alpha^0/\alpha\alpha$; β/β), are characterized by the presence of only two functional α chain genes (instead of four) although their organization on chromosomes differs. The two conditions are characterized by a distinct microcytosis (mean corpuscular volume less than 72 fl; mean corpuscular Hb less than 23 pg) and an occasional Heinz body in the red cells; Hb H (β_4) is not detectable (refs. 1–4 and references therein). The diagnosis is usually based on data from chain synthesis analyses, on quantitation of the α-mRNA and β-mRNA ratio, on data from restriction endonuclease analyses and on family studies[1–6]. Hematological values in persons with the α-thalassemia-2 heterozygosity ($\alpha^0\alpha/\alpha\alpha$; β/β) are (nearly) indistinguishable from those of normal adults. Hb H disease ($\alpha^0\alpha^0/\alpha^0\alpha$; β/β), however, exhibits distinct character-

* Contribution No. 0602.

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istics, such as microcytosis, hypochromia, poikilocytosis, numerous Heinz bodies, moderate anemia, 10–30% Hb H (β_4) and thus its diagnosis can be readily made. The complete absence of functional α chain genes (hydrops fetalis or $\alpha^0\alpha^0/\alpha^0\alpha^0$; β/β) is incompatible with life.

Wasi and co-workers (refs. 7 and 8; see also refs. 1 and 2) were among the first to show that the amount of Hb Bart's (or γ_4) at birth was related to the degree of α chain deficiency. Thus, in hydrops fetalis over 80% of the total Hb is Hb Bart's, in newborn with Hb H disease up to 30% Hb Bart's is present, while α-thal-1 heterozygosity and α-thal-2 homozygosity are characterized at birth by the presence of *ca.* 5% of this abnormal Hb. Only a minute amount is observed in blood samples from newborn with the α-thal-2 heterozygosity.

Hb Bart's is usually quantitated after elution of the hemoglobin components from unstained cellulose acetate strips, electrophoresed at either acidic or alkaline pH[1,2,7,8], or by carboxymethyl (CM)-cellulose chromatography[2,9–11]. A microchromatographic procedure has been developed [10,11] and this procedure forms the basis of the modification which is described in this communication. The present method which is commercially available allows the rapid quantitation of Hb Bart's (γ_4) with an accuracy of 5–10% and appears most useful for the diagnosis of Hb H disease, α-thal-2 heterozygosity and α-thal-2 homozygosity (and perhaps even α-thal-1 heterozygosity) at birth.

MATERIALS AND METHODS

Cord blood samples from 139, partially preselected, Black newborn and a few normal adults were collected in vacutainers with EDTA as anticoagulant. Red cell lysates, containing *ca.* 10 g Hb/dl, were prepared by standard procedures[2]. In some instances whole blood was hemolyzed with hemolyzing solution provided by the manufacturer (Isolab, Akron, OH, U.S.A.).

Microchromatography

CM-Cellulose microcolumns (10 × 0.8 cm I.D.) were provided by Isolab. The columns were equilibrated with 0.03 *M* Bis-Tris, 0.01% KCN, HCl; pH 6.2. Prior to the chromatographic experiment, the caps are removed from the top and bottom of the column, the top disc is pushed down gently with a Pasteur pipette to touch the resin bed, and the supernatant is removed and allowed to drain into the resin. Two drops (*ca.* 0.1 ml) of red cell lysate, mixed with four drops of water and two drops of 0.004 *M* maleic acid, are pipetted on top of the disc. A 4-ml volume of the "Hb Bart's elution buffer" (*i.e.* 0.03 *M* Bis–Tris, 0.001 *M* NaCl, 0.01% KCN, HCl; pH 6.2) is added and the effluent collected in one tube. The microcolumn is next placed on top of a second collection tube able to hold at least 20 ml, the remaining Hb is removed with 4 ml of the "other Hb elution buffer" (*i.e.* 0.03 *M* Bis–Tris, 0.2 *M* NaCl, 0.01% KCN, HCl; pH 6.2) and this effluent is mixed with 16 ml of deionized water. The absorbances at 415 nm of the two Hb solutions (X = absorbance of the Hb Bart's fraction; Y = absorbance of the other Hb fraction) are determined and the amount of Hb Bart's calculated with the formula % Hb Bart's = $100X/[X + 5Y]$. The entire procedure is run at room temperature.

Other procedures

All red cell lysates were analyzed by starch gel electrophoresis at pH 8.9[2] to evaluate the possible presence of no Hb Bart's, small amounts of Hb Bart's (1–2%) and larger amounts of Hb Bart's (up to 5%). The identity of the protein in the first effluent was evaluated by starch gel electrophoresis[2] and by high-performance liquid chromatography (HPLC)[12–14]. Effluents of several runs from the same sample were combined and the protein concentrated by ultrafiltration under reduced pressure using Amicon Diaflo ultrafiltration membrane PM10. The concentrate was used as such in the electrophoretic experiment. About 0.01–0.1 ml of the same solution containing perhaps 10–30 μg of Hb Bart's was analyzed by the HPLC procedure, which allows the detection of heme and the isolated α, β, $^{G}\gamma$, and $^{A}\gamma$ chains of adult and fetal hemoglobins[12–14].

RESULTS AND DISCUSSION

Reproducibility

The low absorbance reading of the Hb Bart's fraction of the cord blood sample can result in a considerable variability of the percentages. Table I lists data for 20 cord blood red cell lysates as well as average values, standard deviations and ranges for two samples with a low and a higher percentage of Hb Bart's. Despite this limitation, the method appears more than adequate for the quantitation of Hb Bart's at the 0.1% level (*i.e.* percentage of total Hb) particularly when the determination is made in duplicate or triplicate. It is not advisable to load the microcolumn with more than 15–20 mg of Hb (or 0.15–0.2 ml of red cell lysate) because overloading results in contamination of the Hb Bart's fraction with Hb F_1. The red cell lysate can be replaced by packed red cells, collected in a micro hematocrit tube, and hemolyzed with ten drops of hemolyzing solution (Isolab) and two drops of 0.004 *M* maleic acid.

TABLE I

ACCURACY OF THE METHOD (VALUES IN %)

Sample No.	*Analysis 1*	*Analysis 2*	*Sample No.*	*Analysis 1*	*Analysis 2*
1	1.00	0.91	11	4.80	4.82
2	0.81	0.68	12	5.50	3.95
3	0.49	0.33	13	1.90	1.90
4	0.56	1.01	14	0.44	0.51
5	3.94	3.28	15	0.63	0.62
6	1.11	0.78	16	1.55	1.20
7	1.00	0.83	17	1.86	1.87
8	1.22	1.30	18	2.10	2.12
9	1.34	1.23	19	1.61	1.60
10	1.25	1.64	20	0.56	0.68

"Small Hb Bart's" sample: 1.78 $\pm$ 0.08 (S.D., $n = 10$); range 1.72–1.88.
"Large Hb Bart's" sample: 4.36 $\pm$ 0.48 (S.D., $n = 10$); range 3.90–5.50.

Identification of the Hb Bart's fraction

It has been known for several years that non-hemoglobin proteins (NHP) with slight absorbance at 415 nm are eluted rapidly from a CM-cellulose column[9,10]. A

major contributor to this NHP fraction is carbonic anhydrase, which is adsorbed rather tightly to the anion exchanger DEAE-cellulose[15,16]. Thus, addition of a small segment of DEAE-cellulose on top of the CM-cellulose column should eliminate the "Hb Bart's" fraction provided it is mainly composed of NHP. Data from such experiments are presented in Table II. Indeed, the absorbance readings were significantly decreased for adult red cell lysates, but such an effect was not observed for the six normal cord blood red cell lysates and the three with higher Hb Bart's values. These results suggest that an NHP fraction in cord blood red cells does not contribute to any great extent to the absorbance readings of the Hb Bart's zone.

TABLE II

THE VALUES OF Hb BART'S WITH AND WITHOUT ADDITION OF DEAE-CELLULOSE
DEAE-cellulose, equilibrated with 0.03 *M* Bis–Tris, 0.01% KCN, pH 6.2, was layered on top of the micro CM-cellulose column to form a 0.8 × 0.5 cm addition which is known to absorb (most of the) non-Hb protein[15,16].

Sample No.	*With*	*Without*	*Sample No.*	*With*	*Without*
Cord 1	0.82	0.66	Cord 7	1.68	1.82
Cord 2	0.38	0.25	Cord 8	5.42	5.30
Cord 3	0.32	0.39	Cord 9	4.90	5.50
Cord 4	0.39	0.52	Adult 1	0.07	0.22
Cord 5	0.44	0.29	Adult 2	0.07	0.22
Cord 6	0.48	0.73	Adult 3	0.07	0.20

The nature of the Hb Bart's zone was evaluated by electrophoresis on starch gels and cellulose acetate strips at alkaline pH. The results were not satisfactory; the Hb zone appeared as a smeary band moving in front of Hb A. Other proteins, such as carbonic anhydrase which has a much slower electrophoretic mobility in these systems, were not detected.

Identification of the Hb Bart's zone by HPLC was more successful. The system used identifies at least four Hb chains in addition to the heme component. The chromatogram of a normal cord blood red cell lysate, shown in the top panel of Fig. 1, indicates that heme is eluted first followed by the α and β chains and next by

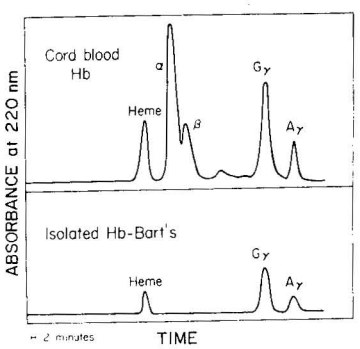

Fig. 1. Identification of heme and hemoglobin chains by HPLC. The chromatogram of normal cord blood Hb is compared with that of the material isolated as Hb Bart's from a normal cord blood red cell lysate by micro CM-cellulose chromatography.

the two γ chains ($^{G}\gamma$ and $^{A}\gamma$). The method is most useful for the quantitation of these two γ chains which occur in cord blood Hb F in a ratio of 7:3[13,14]. The same two γ chains were present in the isolated Hb Bart's zone and in about the same ratio. The absence of any α or β chains suggests that the Hb in this zone consists exclusively of γ chains, *i.e.* Hb Bart's or γ_4.

The amount of Hb Bart's at birth

Fig. 2 compares the amounts of Hb Bart's in 139 Black newborn. Electrophoretic examination identified nine with large amounts (up to 5%) of Hb Bart's while Hb Bart's was also demonstrable in 27 additional cases but in considerably smaller amounts. These results are in good agreement with the quantitative microchromatographic data except for the overlap between the large group of babies without Hb Bart's (average value: 0.55%; $n = 103$) and that of the babies with small amounts of Hb Bart's (average value: 1.55%; $n = 27$). The average value of Hb

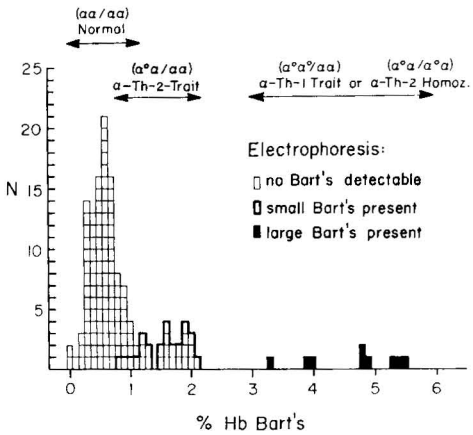

Fig. 2. Distribution of the percentages of Hb Bart's in cord blood samples from 139 newborn.

Bart's in the nine additional babies was 4.65%. Several newborn had a β chain abnormal Hb (six with Hb S; three with Hb C). The presence of these variants did not interfere with the quantitation of Hb Bart's, and probably did not influence the amount of Hb Bart's that was present (average value: 0.35; $n = 9$, range 0–1.05). Partial preselection of the samples prevents the use of these data for the calculation of gene frequencies of the α chain deficiencies.

CONCLUSIONS

(1) CM-Cellulose microchromatography is a rapid and inexpensive method for quantitating Hb Bart's (γ_4) in red cell lysates of newborn babies. The accuracy of the method is in the order of 5–10%. Hb Bart's, eluted from the column, is not contaminated with a significant amount of hemeproteins or other components which could interfere with its quantitation. It is advisable to remove insoluble material (cell debris, a.o.) from the sample prior to application. The presence of common β or α chain Hb variants does not interfere with the determination of Hb Bart's.

(2) Hb Bart's (γ_4) is detectable in (nearly) all cord blood samples. Babies without an apparent inherited α chain deficiency have only a few tenths of one percent of this Hb, which is composed of $^{G}\gamma$ and $^{A}\gamma$ chains in a ratio comparable with that of Hb F of the newborn.

(3) Increased amounts of Hb Bart's (γ_4) are readily detectable, and the presence of an α-thalassemia-1 heterozygosity ($\alpha^0\alpha^0/\alpha\alpha$) or an α-thalassemia-2 homozygosity ($\alpha^0\alpha/\alpha^0\alpha$) is characterized by the presence of 3–6% of this Hb type. The level of Hb Bart's is also increased in babies with an α-thalassemia-2 heterozygosity ($\alpha^0\alpha/\alpha\alpha$) but the values of 0.8–2% may overlap to some extent with those of normal newborn. Higher amounts of Hb Bart's (γ_4), such as the 20–30% found in newborn with Hb H disease ($\alpha^0\alpha^0/\alpha^0\alpha$), will readily be quantitated with this chromatographic procedure although none were found in this study.

(4) CM-Cellulose microchromatography can be used either as a primary screening procedure for the α-thalassemia conditions in newborn or as a confirmatory test when the original testing is done by starch gel or cellulose acetate electrophoresis. Since the genetic α chain deficiencies are wide spread among the populations of the world (Blacks, Indians, East Asians, Chinese, Greeks, Italians, a.o.), the method could be helpful in various laboratories involved in testing programs for hemoglobinopathies around the world.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF GIBBERELLINS

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SUMMARY

The analysis of different gibberellins (GAs) by high-performance liquid chromatography (HPLC) was carried out with two types of columns: μBondapak C_{18} and the recently developed Radial-Pak-A. A reversed-phase HPLC procedure has been developed which enables the separation of different GAs with high resolution by means of ionic suppression, including the separation of double bond isomers such as GA_1 and GA_3, GA_4 and GA_7, GA_5 and GA_{20}, without the necessity for derivatization. A combined HPLC–bioassay procedure proved to be suitable for rapid routine assay of GA-activities in plant extracts.

INTRODUCTION

The most extensive and rigorous analysis of gibberellins (GAs) in plant extracts has been carried out by MacMillan and co-workers[1,2] who used combined gas chromatography–mass spectrometry (GC–MS). Their techniques required, however, a relatively high degree of purity of extracts and the availability of sophisticated GC–MS facilities for signal processing[2], and are thus not suitable for routine analyses of GAs in plant extracts.

Reeve and Crozier[3] have described the analysis of GAs by means of preparative and subsequent analytical high-performance liquid chromatography (HPLC). One of the problems in using HPLC for GA analyses is detection of these compounds. Reeve and Crozier[3] chose to derivatize the GAs by esterification of the carboxyl group at C-7; the resulting GA-benzyl esters have an absorption maximum at 256 nm and can thus be detected with a standard UV monitor at 254 nm. Morris and Zaerr[4] described the 4-bromophenacyl esters of gibberellins as useful derivatives for separations of GAs by HPLC. The separation of isomers of GAs containing double bonds, such as GA_1 and GA_3, GA_4 and GA_7, GA_5 and GA_{20}, by HPLC was achieved by Heftmann *et al.*[5] in the form of their *p*-nitrobenzyl esters. Yamaguchi *et al.*[6] have used HPLC to separate conjugated GAs. Finally, Jones *et al.*[7] recently reported the fractionation of GAs in plant extracts by reversed-phase HPLC.

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The studies in refs. 1–7 required derivatization of the GAs, particularly in order to separate the above pairs of double bond isomers. Since we have found the derivatization of GAs, particularly in extracts, to be rather troublesome and unreliable, we used the wavelength of 203 or 206 nm for detection of GAs.

The GAs are weak acids. Therefore, the ionic suppression technique, which involves the regulation of pH, was considered suitable for the separation of GAs. A reversed-phase HPLC procedure has been developed which enables the separation of the GA double bond isomers without derivatization, as well as a combined HPLC–bioassay procedure which enables rapid assay of GA activities in plant extracts without the necessity of extensive and time-consuming purification.

EXPERIMENTAL

High-performance liquid chromatography

The HPLC system consisted of the following components from Waters Assoc. (Milford, MA, U.S.A.): two sequentially connected solvent-delivery systems (Model 6000 A); a universal liquid chromatography injector (Model U6K); a solvent programmer (Model 660); a variable-wavelength detector (Model 450); an Omniscribe strip-chart recorder and a LKB 7000 Ultrorac fraction collector. Two types of columns (Waters Assoc.) were employed, *i.e.*, μBondapak C_{18} (30 cm × 3.9 mm I.D.) and Radial-Pak-A liquid chromatography cartridges. The latter were used in a Radial compression system (RCM-100). The mobile phase for the reversed phase HPLC consisted of double distilled water–methanol (LiChrosolv; E. Merck, Darmstadt, G.F.R.) mixtures or gradients, containing 0.01 *M* H_3PO_4 and adjusted to the appropriate pH with KOH. The detection took place at either 203 nm or 206 nm.

Gibberellins

GA_3 was purchased from Sigma (St. Louis, MO, U.S.A.) and a mixture of GA_4 and GA_7 from United States Biochemical Corp. (Cleveland, OH, U.S.A.); the other samples of relatively pure GAs, *e.g.*, GA_1, GA_4, GA_5, GA_7, GA_9 and GA_{20}, were obtained from the laboratory of Professor N. Takahashi. The GA samples were dissolved in methanol. Except for GA_3, the exact amounts of injected GAs were not known, but are estimated as 1–5 μg GA per injection.

Extraction and purification

Immature seeds of *Pharbitis nil* Choisy cv. Violet (5 g) were homogenized in methanol (50 ml) in a Sorvall omnimixer at maximum speed, filtered and the residue reextracted in methanol (50 ml) for another 24 h at 5°C. The combined filtrate was evaporated to a small volume (40 ml) under vacuum at 40°C (Rotavapor), and subsequently purified by forcing the solution through a Sep-Pak C_{18} cartridge (Waters Assoc.) with the aid of a 10-ml syringe, which removes the non-polar compounds from the solution. The filtrate is then further reduced in volume (3 ml) and once again forced through a Sep-Pak C_{18} cartridge. The filtrate is subsequently evaporated to dryness and taken up in 1 ml of the starting mobile phase for reversed-phase HPLC. It is essential that the pH of the sample to be injected is checked and if necessary adjusted to a value, *e.g.*, pH = 2.5, at which the GAs are retained on the column. The sample is injected in portions of 200 μl. The corresponding collected

fractions of 2.5 ml (1 min) were pooled, evaporated to dryness, taken up again in 0.5 ml of distilled water and used for bioassay.

Bioassay

The bioassay was carried out with dwarf corn d_1 seedlings. Seeds were soaked for 24 h in tap-water, sown in vermiculite and after 5–6 days selected for homogeneity and transplanted. The plants were treated with 0.1 ml of the above fractions, four plants per treatment. The elongation of the leaf sheaths of the first and second leaf were measured 7 days after treatment and compared with a standard concentration range of GA_3 solutions. The biological activity is expressed as the percentage increase over the control (water) treatment.

RESULTS AND DISCUSSION

There are two main problems associated with the analysis of the many naturally occurring GAs, *i.e.*, detection and separation, particularly the separation of double bond isomers. Because GAs do not absorb at the standard UV monitoring wavelength of 254 nm they were monitored at 203 or 206 nm.

The first efforts were aimed at the separation of the double bond isomers GA_1 and GA_3. This separation was finally achieved by reversed-phase chromatography on a μBondapak C_{18} column with 20% methanol, containing 0.01 *M* H_3PO_4 adjusted to pH = 2.3 as mobile phase (Fig. 1). It was established that the presence of 0.01 *M* H_3PO_4 and a low pH were essential for a clear separation of the double bond isomers. Several non-isomeric GAs, *e.g.*, GA_3, GA_5, GA_4 and GA_9, can be separated by an increasing methanol gradient without the presence of H_3PO_4. Subsequently, other

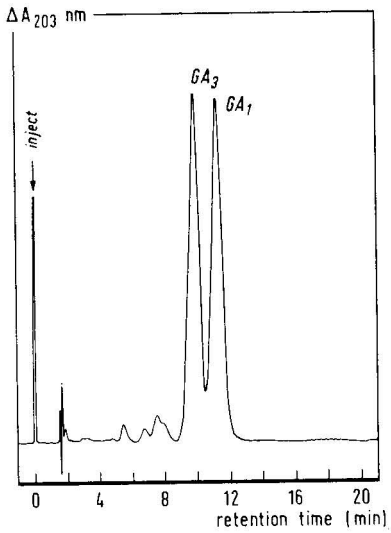

Fig. 1. Reversed-phase HPLC of GA_1 and GA_3 (2 μl, 1 μg/μl). Column: μBondapak C_{18}, 30 cm × 3.9 mm I.D. Mobile phase: 20% methanol, containing 0.01 *M* H_3PO_4 adjusted with KOH to pH = 2.3; flow-rate 2 ml/min. Detection: 0.1 absorption units full scale (a.u.f.s.) at 203 nm. Chart speed: 0.25 in./min.

double bond isomers, *i.e.*, GA_5 and GA_{20}, GA_4 and GA_7, were separated by increasing the methanol concentration at the low pH of 2.3. Finally, a methanol gradient was developed whereby the available three pairs of double bond isomers and GA_9 could be separated in one run (Fig. 2). The baseline shift caused by the increasing concentration of methanol was suppressed by adding a small amount of acetone to the lowest methanol concentration. The gradient is easily adapted for the separation of particular GAs.

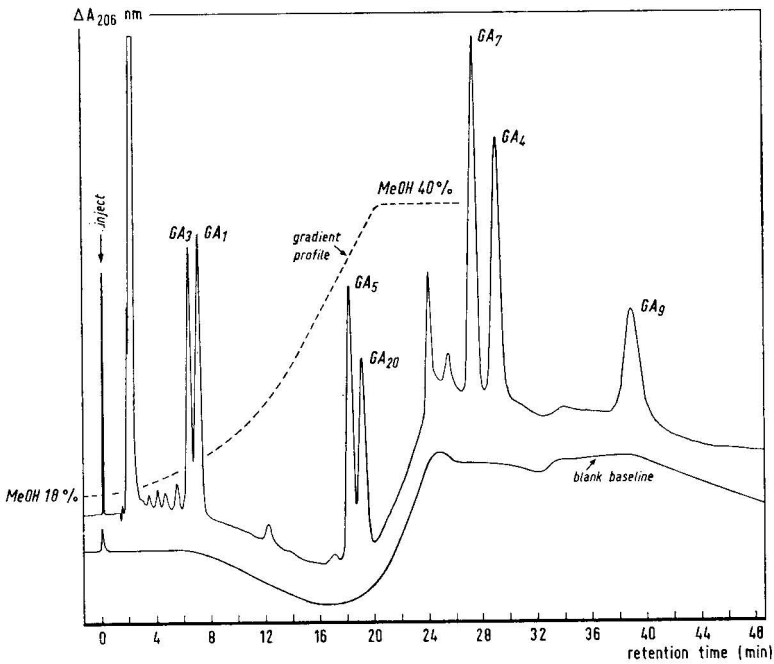

Fig. 2. Reversed phase HPLC of GA_1, GA_3, GA_4, GA_5, GA_7, GA_9 and GA_{20}. Mobile phase: gradient (curve 7) from 18% methanol, containing 0.01 *M* H_3PO_4 and acetone (10 ml/l) to 40% methanol, 0.01 *M* H_3PO_4 at pH = 2.2 in 20 min. Flow rate: 1.8 ml/min. Detection at 206 nm. Other details as in Fig. 1.

In the following experiments the new Waters Assoc. column involving a Radial-Pak-A column in a Radial compression module was used for the analysis of the different GAs. With this type of column the resolution of double bond isomers is even better than with the μBondapak C_{18} column, *cf.*, Figs. 2 and 3. With the Radial-Pak-A column one does not have to resort to a methanol gradient which causes large baseline shifts, but merely to apply a pH gradient (Fig. 3). A change in pH results in a baseline shift caused by differences in the potassium concentration which is smaller and acceptable at 206 nm. By choosing a particular methanol concentration, containing 0.01 *M* H_3PO_4, and an appropriate pH a particular set of GAs can be analyzed with good resolution. An example is given in Fig. 4 which shows the separation of the the double bond isomers GA_4 and GA_7 and of GA_9 with a mobile phase consisting of 35% methanol at pH = 7.2.

The GAs separate according to their degree of hydroxylation. Those having

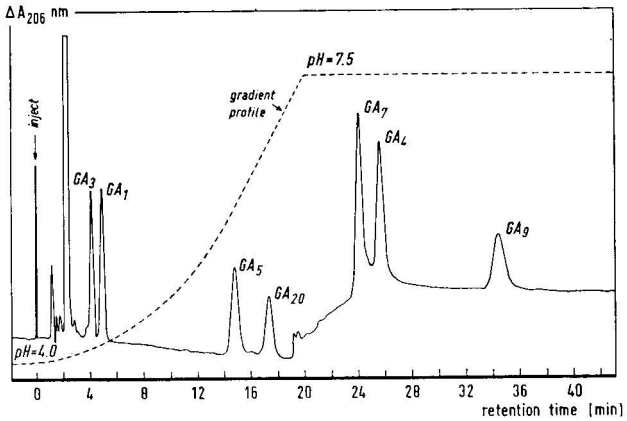

Fig. 3. Reversed phase HPLC of GA_1, GA_3, GA_4, GA_5, GA_7, GA_9 and GA_{20}. Column: Radial-Pak-A. Mobile phase: gradient (curve 9) from 35% methanol, 0.01 *M* H_3PO_4 at pH = 4.0 to 35% methanol, 0.01 *M* H_3PO_4 at pH = 7.5 in 20 min. Flow-rate: 2 ml/min. Detection: 0.1 a.u.f.s at 206 nm. Chart speed: 0.25 in./min.

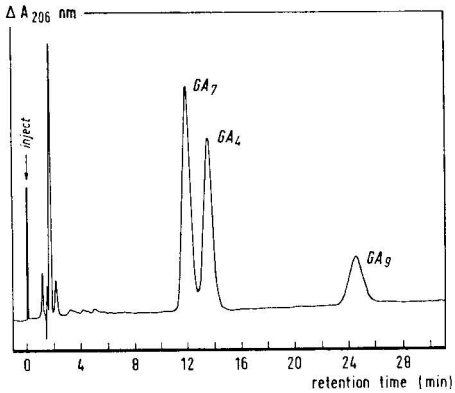

Fig. 4. Reversed-phase HPLC of GA_4, GA_7 and GA_9. Column: Radial-Pak-A. Mobile phase: 35% methanol, 0.01 *M* H_3PO_4 at pH = 7.2. Other details as in Fig. 3.

no hydroxyl groups such as GA_9 have the greatest retention, and they are preceded by the monohydroxylated GAs, *e.g.*, GA_4 and GA_7, GA_{20} and GA_5, which in turn are preceded by the dihydroxylated GA_1 and GA_3. Taking this into account, the Radial-Pak-A column will be more suitable than the μBondapak C_{18} column for reversed-phase HPLC for the separation of GAs with three, *e.g.*, GA_8, or even with four hydroxyl groups, *e.g.*, GA_{32}, because with μBondapak C_{18} columns GA_1 and GA_3 show retention at 18% methanol and below this methanol concentration both retention as well as resolution cannot be improved, and also the lower limit of pH (2.3) has been reached. In contrast, with the Radial-Pak-A column, GA_1 and GA_3 already show retention at 35% methanol and pH = 4.0. Thus with this column both the methanol concentration and the pH can be further manipulated in order to separate the GAs having more hydroxyl groups, such as GA_8 and GA_{32}. In addition

the resolution of GAs on Radial-Pak-A columns appears also to be somewhat better than with μBondapak C_{18} columns.

Finally, the crucial test for the practical usefulness of a particular method lies in its suitability for analyzing GAs in plant extracts. In spite of the great advances in analytical instrumentation, the bioassay remains an effective means of detecting minute quantities of GAs, *e.g.*, 1–10 ng, in plant extracts. In comparison, the detection limit of the above HPLC techniques is *ca.* 100 ng for relatively pure GA samples. However, the quantitative accuracy of a bioassay depends greatly upon the purity of the extract as the bioassay response reflects the interaction of GA-activity and inhibitory compounds present in the samples. Therefore, laborious purification procedures, including partitioning, thin-layer chromatography (TLC) and/or column chromatography, of extracts are employed before the sample is ready for bioassay. In this respect reversed-phase HPLC combined with a bioassay offers potential advantages with regard to speed as well as degree of purification compared with the conventional methods.

We have developed a procedure which involves extraction of the plant material with methanol, followed by a two-step purification with a Sep-Pak C_{18} cartridge as described under Experimental, whereafter the sample can be directly injected into the HPLC under the appropriate conditions, followed by bioassay of the collected fractions. An example of the results of such a procedure is presented in Fig. 5, which

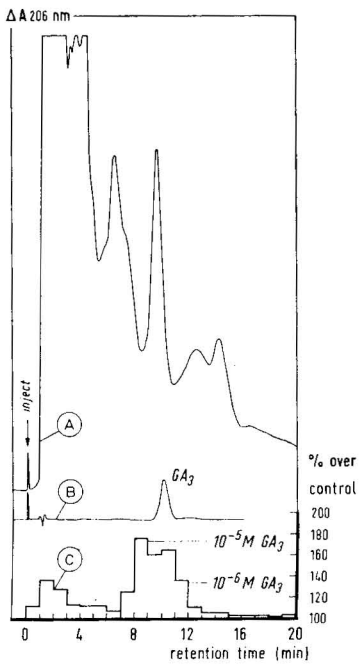

Fig. 5. Reversed-phase HPLC of a plant extract. Column: Radial-Pak-A. Mobile phase: 35% methanol, 0.01 *M* H_3PO_4 at pH = 2.5. Flow-rate: 2 ml/min. Detection: 1 a.u.f.s. at 206 nm. A, Profile of an injection with 200 μl of extract; B, reference chromatogram of GA_3 (15 μg); C. Biological activity of the collected 2-ml (1-min) fractions on the dwarf corn d_1 assay compared with standards of 10^{-6} *M* and 10^{-5} *M* GA_3, respectively.

gives the elution profile of the sample (A), the GA_3 reference chromatogram (B) and the biological activities of the collected fractions (C).

A disadvantage of monitoring at 206 nm is that at this wavelength many other compounds present in the plant extract have a relatively large absorption, as shown in the profile A of Fig. 5. At 254 nm this extract looked "clean" since, there were hardly any peaks beyond 4 min after injection (not shown). However, monitoring of derivatized extracts at 254 nm results also in new unknown absorption peaks. The reversed-phase HPLC results in considerable purification of the extract which compares favourably with conventional partitioning and TLC procedures (see also ref. 7). Consequently, the total biological activity obtained after HPLC is higher than with conventional purification procedures. The total calculated GA-activity, after extraction of equal amounts of plant material, was 1.5 μg GA_3-equivalent per 10 seeds for the Sep-Pak-HPLC procedure and 0.7 μg for the conventional method, *i.e.*, liquid–liquid partitioning followed by TLC. This Sep-Pak-HPLC purification and separation procedure is now routinely and successfully used in our laboratory either to investigate endogenous GA-activities or to analyse metabolites of exogenously applied radioactive GAs in plant extracts. Moreover, this reversed-phase HPLC procedure is promising for the relatively rapid purification of extracts by recycling, to a degree that the samples become suitable for further analyses by GC–MS techniques.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ADAMANTANOLS AND OTHER CYCLIC ALCOHOLS

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SUMMARY

Chromatographic elution data for 28 monocyclic and polycyclic alcohols of the adamantane type have been measured, using silica gel as the stationary phase and mixed mobile phases in different concentration ratios.

The position of the hydroxyl group has the largest effect on the chromatographic behaviour of polycyclic alcohols. The introduction of an alkyl group into alcohols such as adamantanol results in a decrease in retention time. This decrease is larger when the adamantanol is substituted by several small alkyl substituents ($-CH_3$, $-C_2H_5$) than by one large alkyl group.

For polycyclic alcohols having the same formal substitution type, the elution time decreases with increasing size of the basic skeleton. The rate of this decrease depends on the composition of the mobile phase used. The effect of the mobile phase composition on the elution data for the compounds studied is discussed.

INTRODUCTION

Several thousand adamantane derivatives have been prepared at different laboratories. These compounds are important in basic research, and they also have practical applications in pharmacy, biochemistry, medicine, the chemistry of plastics and in the synthesis of special lubricants, etc.

The aim of the present work was to describe the liquid chromatographic behaviour of some polycyclic alcohols, using silica gel as stationary phase and different mobile phases based on *n*-heptane in combination with 2-propanol, chloroform and diethyl ether. The objectives may be summarized as follows: (i) to find the optimum conditions for the separation of some adamantane and diamantane alcohols by high-performance liquid chromatography (HPLC); (ii) to investigate the fundamental relationships between molecular structure and chromatographic behaviour of polycyclic alcohols in adsorption liquid chromatography; (iii) to utilize the data from the analytical study for separations using preparative scale liquid chromatography.

In adsorption liquid chromatography, the retention time of a compound on a column with a given geometrical arrangement and at a constant mobile phase flow-

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rate is determined by the type of adsorbent, the composition of the mobile phase and the constitution of the sample. The relationship between the chromatographic behaviour of a substance and its molecular structure represents one of the most important problems[1].

Two contributions of the sample character to the sample adsorption energy may be differentiated: (i) the type of functional groups present, *i.e.*, the "primary effect"; (ii) the arrangement of these groups, *i.e.*, the "secondary effect". With the steadily increasing separation efficiency of columns, it has become possible to separate compounds having very similar molecular structures.

EXPERIMENTAL

Apparatus

A Varian 8500 liquid chromatograph with a syringe pump was used, connected with a RI detector and A25 dual-channel strip-chart recorder (Varian, Palo Alto, CA, U.S.A.). Sample injection was performed with the stop-flow technique; 5- and 10-μl syringes (Hamilton, Bonaduz, Switzerland) were used. The column was a Micropak Si-10 (50 cm $\times$ 2 mm I.D.; Varian), packed with 10-μm silica gel LiChrosorb Si 60.

Retention data were calculated on an HP 9830A calculator connected with an HP 9866A thermal printer (Hewlett-Packard, Avondale, PA, U.S.A.). Graphical processing of data was carried out on the same calculator equipped with an HP 9862A plotter.

Reagents

Nearly all of the standard compounds used for measurements were prepared in our laboratory. Diamantan-3-ol and 1-hydroxymethyldiamantane were kindly provided by Professor M. A. McKervey, University College, Cork, Ireland. 2-Propanol, analytical grade (Lachema, Brno, Czechoslovakia), was used without further treatment. *n*-Heptane (Reakhim, Moscow, U.S.S.R.) and diethyl ether (Lachema) were dried over sodium before use, distilled and stored over Nalsit A4 molecular sieves (CHZJD, Bratislava, Czechoslovakia). Chloroform, analytical grade (Lachema), was shaken with a 20% solution of NaOH, then distilled water, dried over phosphorus pentoxide and distilled on a glass perforated-plate column with exclusion of moisture.

Mobile phase

The mobile phases were prepared by weight from the degassed components. The following mobile phases were used: *n*-heptane–2-propanol; *n*-heptane–diethyl ether; *n*-heptane–diethyl ether–2-propanol; and *n*-heptane–chloroform–2-propanol. The compositions are given in Table I.

Procedure

Retention data were measured at laboratory temperature (18–22°C). The flow-rate of the mobile phase was 30 ml/h. Before the measurements, the column was stabilized by washing with fresh mobile phase (flow-rate, 30 ml/h) for 12 h. Column activity was checked once before the beginning of a measurement, then several times during the analyses and again after the completion of the measurement, by injecting a solution of cyclohexanol in the mobile phase. The dead volume of the column was

TABLE I

MOBILE PHASE COMPOSITIONS (%)

No. 1	*n-Heptane*	*2-Propanol*
a	99	1
b	98	2
c	97	3
No. 2	*n-Heptane*	*Diethyl ether*
a	65	35
b	50	50
c	35	65
No. 3	*n-Heptane–diethyl ether (65:35)*	*2-Propanol*
a	99.9	0.1
b	99.5	0.5
c	99.0	1.0
No. 4	*n-Heptane–chloroform*	*2-Propanol*
a	99.5 (80:20)	0.5
b	99.5 (65:35)	0.5
c	99.5 (50:50)	0.5

determined by measuring the retention time of an unretained compound, *viz.*, isooctane.

Retention data were measured on chromatograms obtained by injecting solutions of compounds in the mobile phase.

RESULTS AND DISCUSSION

Retention times t_R and capacity factors k' are given in Tables II–V.

It was found that three main factors affect the adsorption of adamantanols and diamantanols:

(1) the position of the OH group on the adamantane or diamantane skeleton
(2) the type of alkyl substitution
(3) the size of the basic skeleton

Effect of the OH group position

For adamantane compounds containing only one OH group, the position of this group has a predominant effect on the chromatographic behaviour. Compounds in which the OH group is attached to a tertiary carbon atom where it is easily accessible to the adsorption centres of the silica gel surface, *e.g.*, adamantan-1-ol and diamantan-4-ol (see Fig. 1), are characterized by the strongest interaction with the adsorbent and their elution times are the longest. Shorter elution times are observed for compounds in which the OH group is on a secondary carbon atom, *e.g.*, adamantan-2-ol and diamantan-3-ol. The fastest elution was found for diamantan-1-ol, where the interaction of the OH group with the silica gel adsorption centres is probably diminished by steric effects involving the *syn* axial hydrogen atoms on the carbon atoms in the vicinity of the hydroxyl group[2]. Chromatograms of the adamantanols, diamantanols and of cyclohexanol are shown in Fig. 2. Change of mobile phase

composition has a relatively greater effect on the elution time of cyclohexanol compared with the polycyclic alcohols (Tables II–V).

TABLE II

RETENTION DATA

Mobile phase: $a\%$ *n*-heptane–$b\%$ 2-propanol. C = Number of carbon atoms; t_R = Retention time (sec); k' = capacity factor.

Compound	C	$a = 99, b = 1$		$a = 98, b = 2$		$a = 97, b = 3$	
		t_R	k'	t_R	k'	t_R	k'
Cyclohexanol	6	1606	8.91	943	4.82	660	3.07
Adamantan-1-ol	10	1334	7.24	773	3.77	550	2.39
3-Methyladamantan-1-ol	11	1225	6.56	710	3.39	504	2.11
3,5-Dimethyladamantan-1-ol	12	1106	5.83	648	3.00	466	1.87
3,5,7-Trimethyladamantan-1-ol	13	996	5.15	590	2.64	428	1.64
3-Ethyladamantan-1-ol	12	1130	5.98	668	3.13	474	1.93
3-Ethyl-5-methyladamantan-1-ol	13	1013	5.25	607	2.75	434	1.68
3-Ethyl-5,7-dimethyladamantan-1-ol	14	924	4.70	580	2.58	406	1.50
3-Propyladamantan-1-ol	13	1058	5.53	635	2.92	463	1.86
3-Isopropyladamantan-1-ol	13	1068	5.59	624	2.85	446	1.76
3-Butyladamantan-1-ol	14	1002	5.19	602	2.72	431	1.66
2-Methyladamantan-1-ol	11	872	4.39	544	2.36	408	1.52
Adamantan-2-ol	10	948	4.85	588	2.63	444	1.74
1-Methyladamantan-2-ol	11	616	2.80	385	1.38	310	0.91
2-Methyladamantan-2-ol	11	666	3.11	420	1.59	336	1.07
2-Ethyladamantan-2-ol	12	468	1.89	310	0.91	262	0.61
2-Propyladamantan-2-ol	13	376	1.32	256	0.58	228	0.41
2-Butyladamantan-2-ol	14	358	1.21	234	0.44	214	0.32
2-Isobutyladamantan-2-ol	14	313	0.93	222	0.37	162	0.07
Diamantan-1-ol	14	610	2.76	418	1.58	324	1.00
Diamantan-3-ol	14	860	4.31	548	2.39	404	1.50
Diamantan-4-ol	14	1246	6.69	728	3.50	510	2.15
1-Hydroxymethylbicyclo[3,3,1]nonane	10	1007	5.21	612	2.78	450	1.78
1-Hydroxymethyladamantane	11	1114	5.87	630	2.89	455	1.81
2-Hydroxymethyladamantane	11	1290	6.96	766	3.73	536	2.31
3,5-Dimethyl-1-hydroxymethyladamantane	13	847	4.23	539	2.33	406	1.50
2-(Adamant-1-yl)propan-2-ol	13	516	2.19	358	1.21	300	0.85
1-Hydroxymethyldiamantane	15	876	4.41	527	2.25	406	1.50

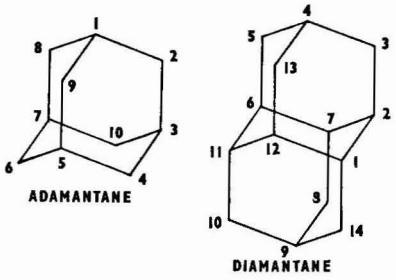


Fig. 1. Adamantane and diamantane.

TABLE III

RETENTION DATA

Mobile phase: a% n-heptane–b% diethyl ether.

Compound	*C*	$a = 65, b = 35$		$a = 50, b = 50$		$a = 35, b = 65$	
		t_R	k'	t_R	k'	t_R	k'
Cyclohexanol	6	1356	7.37	814	4.02	697	3.30
Adamantan-1-ol	10	1686	9.41	1094	5.76	842	4.20
3-Methyladamantan-1-ol	11	1524	8.41	1030	5.36	780	3.81
3,5-Dimethyladamantan-1-ol	12	1350	7.33	938	4.79	709	3.38
3,5,7-Trimethyladamantan-1-ol	13	1126	5.95	816	4.04	622	2.84
3-Ethyladamantan-1-ol	12	1326	7.19	791	3.88	618	2.81
3-Ethyl-5-methyladamantan-1-ol	13	1296	7.00	865	4.34	574	2.54
3-Ethyl-5,7-dimethyladamantan-1-ol	14	1126	5.95	739	3.56	490	2.02
3-Propyladamantan-1-ol	13	1326	7.19	841	4.19	684	3.22
3-Isopropyladamantan-1-ol	13	1398	7.63	827	4.10	671	3.14
3-Butyladamantan-1-ol	14	1302	7.04	858	4.30	670	3.13
2-Methyladamantan-1-ol	11	942	4.81	596	2.68	488	2.01
Adamantan-2-ol	10	894	4.52	586	2.61	467	1.88
1-Methyladamantan-2-ol	11	462	1.85	341	1.10	282	0.74
2-Methyladamantan-2-ol	11	570	2.52	418	1.58	334	1.06
2-Ethyladamantan-2-ol	12	346	1.13	278	0.72	240	0.48
2-Propyladamantan-2-ol	13	300	0.85	252	0.56	218	0.35
2-Butyladamantan-2-ol	14	270	0.67	228	0.41	202	0.24
2-Isobutyladamantan-2-ol	14	240	0.48	206	0.27	192	0.19
Diamantan-1-ol	14	648	3.00	458	1.83	370	1.28
Diamantan-3-ol	14	656	4.28	577	2.56	455	1.81
Diamantan-4-ol	14	1626	9.04	1055	5.51	840	4.19
1-Hydroxymethylbicyclo[3,3,1]nonane	10	786	3.85	500	2.09	406	1.50
1-Hydroxymethyladamantane	11	846	4.22	553	2.41	434	1.68
2-Hydroxymethyladamantane	11	1150	6.10	714	3.41	552	2.41
3,5-Dimethyl-1-hydroxymethyladamantane	13	690	3.26	577	2.56	360	1.22
2-(Adamant-1-yl)propan-2-ol	13	532	2.28	382	1.36	311	0.92
1-Hydroxymethyldiamantane	15	766	3.73	526	2.24	408	1.52

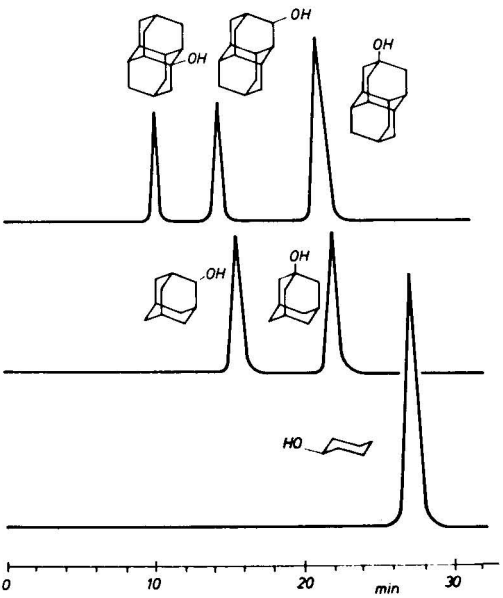


Fig. 2. Separation of diamantanols, adamantanols and cyclohexanol. Column, MicroPak Si-10; mobile phase, 99% n-heptane–1% 2-propanol; flow-rate, 30 ml/h.

TABLE IV

RETENTION DATA

Mobile phase: a% mixture of (65% n-heptane–35% diethyl ether)–b% 2-propanol.

Compound	*C*	*a* = 99.9, *b* = 0.1		*a* = 99.5, *b* = 0.5		*a* = 99.0, *b* = 1.0	
		t_R	k'	t_R	k'	t_R	k'
Cyclohexanol	6	1385	7.55	918	4.67	752	3.64
Adamantan-1-ol	10	1394	7.61	937	4.79	744	3.59
3-Methyladamantan-1-ol	11	1320	7.15	874	4.39	686	3.24
3,5-Dimethyladamantan-1-ol	12	1032	5.37	818	4.05	643	2.97
3,5,7-Trimethyladamantan-1-ol	13	886	4.47	683	3.21	606	2.74
3-Ethyladamantan-1-ol	12	1128	5.96	828	4.11	638	2.94
3-Ethyl-5-methyladamantan-1-ol	13	1020	5.30	780	3.81	594	2.67
3-Ethyl-5,7-dimethyladamantan-1-ol	14	880	4.43	630	2.89	564	2.48
3-Propyladamantan-1-ol	13	1080	5.67	774	3.78	600	2.70
3-Isopropyladamantan-1-ol	13	1092	5.74	766	3.73	598	2.69
3-Butyladamantan-1-ol	14	1073	5.62	742	3.58	588	2.63
2-Methyladamantan-1-ol	11	791	3.88	583	2.59	468	1.89
Adamantan-2-ol	10	784	3.84	574	2.54	478	1.95
1-Methyladamantan-2-ol	11	421	1.60	326	1.01	302	0.87
2-Methyladamantan-2-ol	11	518	2.20	406	1.50	346	1.13
2-Ethyladamantan-2-ol	12	325	1.01	274	0.69	254	0.57
2-Propyladamantan-2-ol	13	286	0.76	253	0.56	230	0.42
2-Butyladamantan-2-ol	14	252	0.56	228	0.41	216	0.33
2-Isobutyladamantan-2-ol	14	228	0.41	205	0.27	202	0.24
Diamantan-1-ol	14	608	2.76	444	1.74	371	1.29
Diamantan-3-ol	14	778	3.80	541	2.34	444	1.74
Diamantan-4-ol	14	1426	7.80	898	4.54	695	3.29
1-Hydroxymethylbicyclo[3,3,1]nonane	10	676	3.17	1150	6.10	420	1.59
1-Hydroxymethyladamantane	11	731	3.51	536	2.31	458	1.83
2-Hydroxymethyladamantane	11	948	4.85	674	3.16	588	2.63
3,5-Dimethyl-1-hydroxymethyladamantane	13	614	2.79	439	1.71	386	1.39
2-(Adamant-1-yl)propan-2-ol	13	469	1.90	360	1.22	322	0.99
1-Hydroxymethyldiamantane	15	690	3.26	503	2.10	422	1.61

Effect of alkyl substitution of adamantanols

Introduction of an alkyl group into adamantan-1-ol or -2-ol lowers the retention time relative to that of the parent molecule. Fig. 3 illustrates the dependence of log k' on the number of carbon atoms for 3-alkyladamantan-1-ols (both substituents on bridgehead positions) and for 2-alkyladamantan-2-ols (both substituents on one non-bridgehead position). In both cases, there is a linear relationship between the total number of carbon atoms in the molecule, the number of carbon atoms in the alkyl group and log k'. The differences in retention times ($\Delta\ t_R$) between the individual members of these homologous series are higher for 2-alkyladamantan-2-ols than for 3-alkyladamantan-1-ols, but even in the latter case the $\Delta\ t_R$ value is sufficient to permit efficient separation. Similarly, there is a linear dependence of log k' on the number of carbon atoms for polymethyladamantan-1-ols having methyl groups on tertiary carbon atoms and for ethylmethyladamantan-1-ols with one ethyl group in position 3 and methyl groups in positions 5 and 7 (Fig. 4). Fig. 5 illustrates the separation of the polymethyladamantanols.

When comparing the change in the capacity factor of adamantan-1-ol substi-

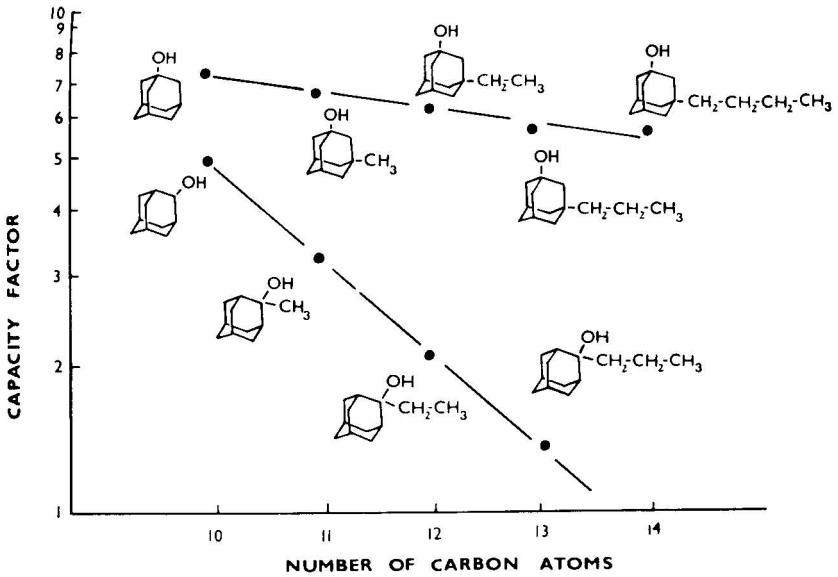


Fig. 3. Plot of capacity factor k' *vs.* the number of carbon atoms for 3-alkyladamantan-1-ols and 2-alkyladamantan-2-ols.

TABLE V

RETENTION DATA

Mobile phase: 99.5% (*a*% *n*-heptane–*b*% chloroform)–0.5% 2-propanol.

Compound	*C*	*a* = 80, *b* = 20		*a* = 65, *b* = 35		*a* = 50, *b* = 50	
		t_R	k'	t_R	k'	t_R	k'
Cyclohexanol	6	2004	11.37	1924	10.87	1772	9.94
Adamantan-1-ol	10	1560	8.63	1500	8.26	1410	7.70
3-Methyladamantan-1-ol	11	1378	7.50	1318	7.13	1264	6.80
3,5-Dimethyladamantan-1-ol	12	1248	6.70	1204	6.43	1141	6.04
3,5,7-Trimethyladamantan-1-ol	13	1150	6.10	1120	5.91	1054	5.50
3-Ethyladamantan-1-ol	12	1356	7.37	1284	6.93	1166	6.20
3-Ethyl-5-methyladamantan-1-ol	13	1298	7.01	1234	6.61	1030	5.36
3-Ethyl-5,7-dimethyladamantan-1-ol	14	1152	6.11	1098	5.78	960	4.93
3-Propyladamantan-1-ol	13	1195	6.38	1168	6.21	1068	5.59
3-Isopropyladamantan-1-ol	13	1202	6.42	1164	6.19	1038	5.41
3-Butyladamantan-1-ol	14	1146	6.07	1098	5.78	971	4.99
2-Methyladamantan-1-ol	11	1036	5.39	966	4.96	936	4.78
Adamantan-2-ol	10	1169	6.21	1084	5.69	1050	5.48
1-Methyladamantan-2-ol	11	638	2.94	546	2.37	576	2.56
2-Methyladamantan-2-ol	11	815	4.03	750	3.63	682	3.21
2-Ethyladamantan-2-ol	12	684	3.22	630	2.89	355	1.19
2-Propyladamantan-2-ol	13	655	3.04	606	2.74	329	1.03
2-Butyladamantan-2-ol	14	613	2.79	576	2.56	288	0.78
2-Isobutyladamantan-2-ol	14	582	2.59	544	2.36	262	0.61
Diamantan-1-ol	14	738	3.56	676	3.17	690	3.26
Diamantan-3-ol	14	1046	5.46	960	4.93	894	4.52
Diamantan-4-ol	14	1494	8.22	1350	7.33	1282	6.91
1-Hydroxymethylbicyclo[3.3.1]nonane	10	1410	7.70	1236	6.63	1032	5.37
1-Hydroxymethyladamantane	11	1387	7.56	1210	6.47	1069	5.60
2-Hydroxymethyladamantane	11	1685	9.40	1469	8.07	1298	7.01
3,5-Dimethyl-1-hydroxymethyladamantane	13	928	4.73	871	4.38	804	3.96
2-(Adamant-1-yl)propan-2-ol	13	686	3.24	672	3.15	612	2.78
1-Hydroxymethyldiamantane	15	1140	6.04	1036	5.39	886	4.47

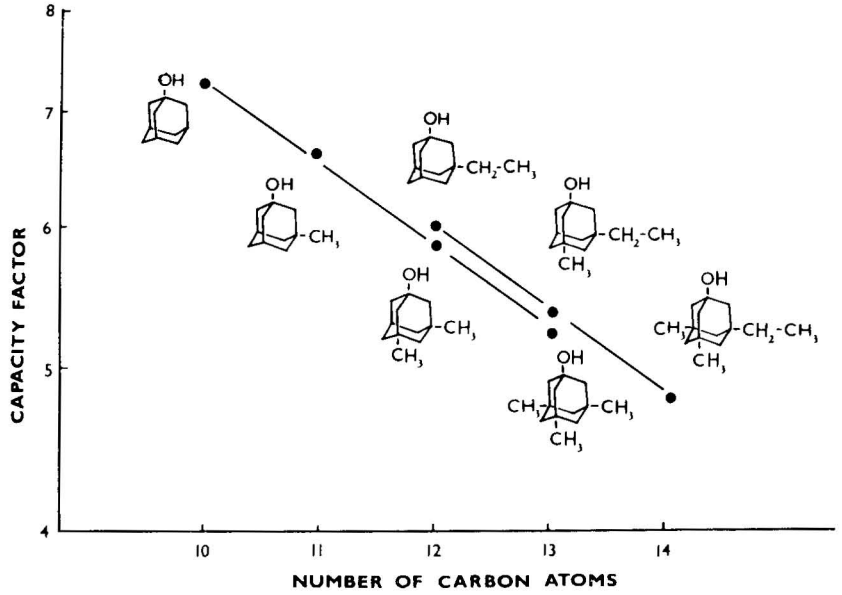


Fig. 4. Plot of capacity factor *k' vs.* the number of carbon atoms for polymethyladamantan-1-ols and ethylmethyladamantan-1-ols.

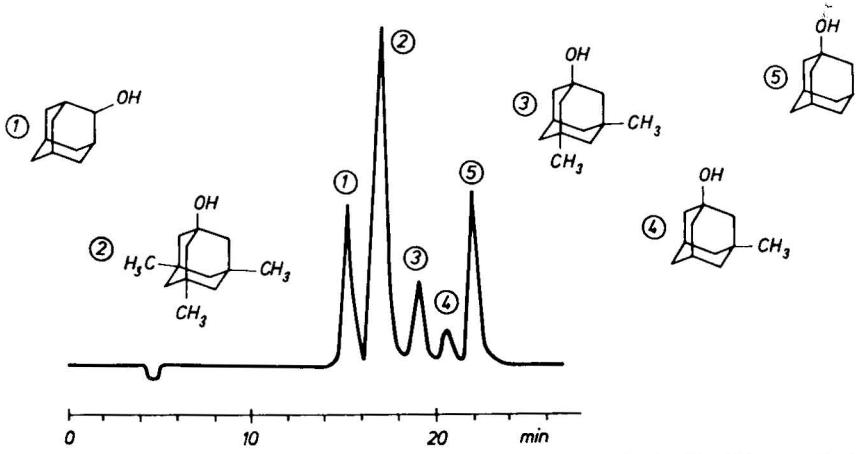


Fig. 5. Separation of adamantanols and polymethyladamantan-1-ols. Conditions as in Fig. 2.

tuted by several lower alkyls (*e.g.*, three methyls) with the change caused by substitution with a larger alkyl group giving the same overall number of carbon atoms (*e.g.*, propyl), it is evident that a greater change is achieved by introducing a higher number of lower alkyls. Fig. 6 shows two examples of this (polymethyladamantan-1-ols and 3-alkyladamantan-1-ols).

The largest change in retention times for substitution with one methyl group was found when this group is situated on the carbon atom adjacent to the carbon

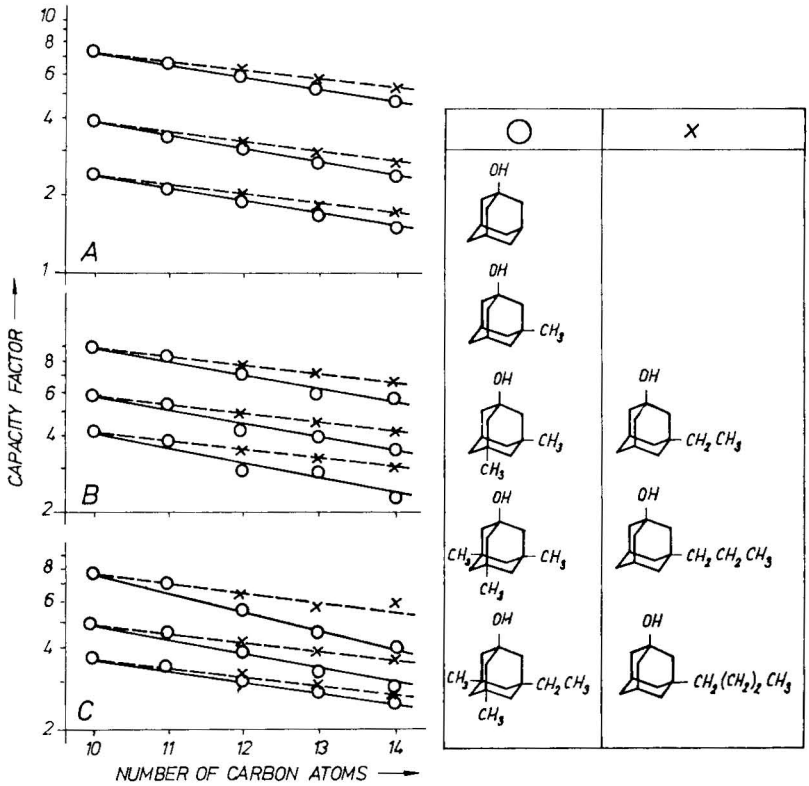
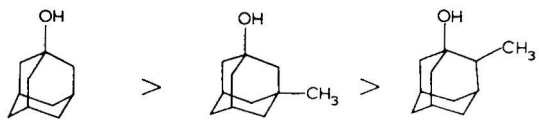


Fig. 6. Plots of log k' *versus* the number of carbon atoms for polymethyladamantan-1-ols and 3-alkyladamantan-1-ols. Mobile phases: A = *n*-heptane–2-propanol; B = *n*-heptane–diethyl ether; C = *n*-heptane–diethyl ether–2-propanol.

atom bearing the OH group. For alkyladamantan-1-ols, retention times decrease in the following order:



In the case of methyl substitution in adamantan-2-ol the situation is similar:

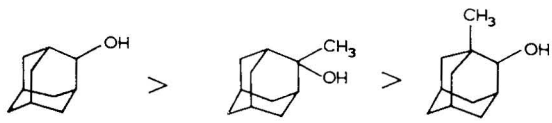


Fig. 7 summarizes the separations achieved with these adamantanol derivatives.

Comparison of the retention times of adamantanols having methyl or other alkyl substituents at the bridgehead (tertiary) positions with that of the parent alcohol reveals that successive replacement of bridgehead hydrogen atoms leads to gradual

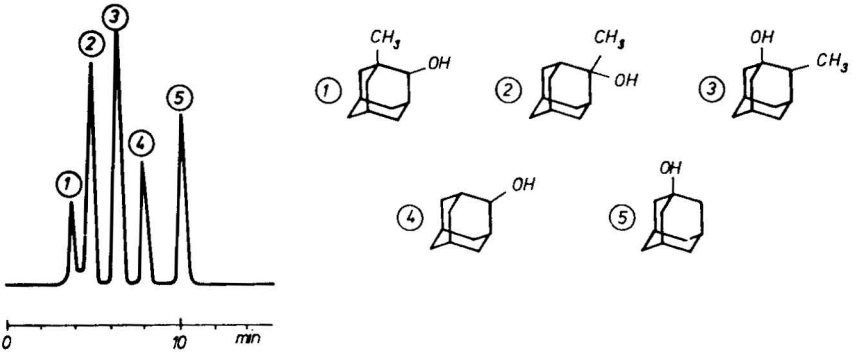


Fig. 7. Separation of adamantanols and methyladamantanols. Conditions as in Fig. 2.

reduction in retention times. This pattern of behaviour has also been observed in the gas chromatographic behaviour of polymethyladamantanols[3]. Increasing the length of the alkyl chain in 3-alkyladamantan-1-ols does not decrease the total molecular polarity to the same extent as does adding further bridgehead alkyl substituents. These effects may be associated with structural features of the adamantane skeleton. It follows from these observations that the increasing solubility of alkylated adamantan-1-ols in the mobile phase (interaction between solvent molecules and sample molecules in solution[1]) has a more significant effect on their chromatographic behaviour than the decreasing molecular polarity.

For methyl-substituted adamantan-1-ols, there is steric hindrance in the neighbourhood of the OH group if the methyl group is located at the 2 (vicinal) position. However, if the methyl group is at a tertiary carbon atom (positions 3, 5 or 7), there is practically no steric hindrance to the OH group. In the case of substituted adamantan-2-ols, there is steric hindrance at the OH group both for 2-substitution (geminal substitution) and 1-substitution (vicinal substitution). From the viewpoint of the chromatographic behaviour of the methyladamantan-2-ols, greater changes were found for the case of vicinal than for geminal substitution. Thus it can be concluded that steric hindrance has only a complementary effect on the change in k' with alkylated adamantanols.

Effect of the basic skeleton size

In the absence of additional steric effects, polycyclic alcohols substituted in a formally identical manner, *e.g.*, when the OH group is located on a tertiary carbon atom, exhibit retention times which decrease with increasing size of the basic skeleton of the molecule. The rate of this decrease is also dependent upon the composition of the mobile phase. In the case of cyclohexanol two different kinds of behaviour can be observed. When 2-propanol is present in the mobile phase the behaviour of cyclohexanol is similar to that of a polycyclic alcohol which has the OH group located on a tertiary carbon atom but without additional steric hindrance. Thus the cyclohexanol retention time is the longest, in agreement with the smaller size of the basic skeleton. On the other hand, in the absence of 2-propanol (for example in the *n*-heptane–diethyl ether system), the chromatographic behaviour of cyclohexanol is similar to that of the group of compounds in which the OH group is located at a secondary carbon

atom, the elution order being: diamantan-3-ol, adamantan-2-ol, cyclohexanol (see Fig. 8).

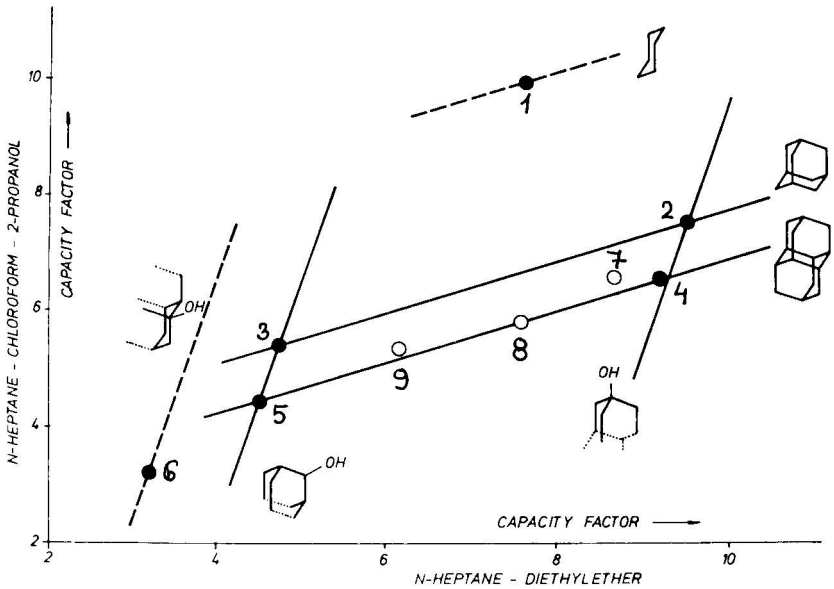


Fig. 8. Capacity factors of some adamantanols and diamantanols for two mobile phases. Compounds: 1 = cyclohexanol; 2 = adamantan-1-ol; 3 = adamantan-2-ol; 4 = diamantan-4-ol; 5 = diamantan-3-ol; 6 = diamantan-1-ol; 7 = 3-methyladamantan-1-ol; 8 = 3,5-dimethyladamantan-1-ol; 9 = 3,5,7-trimethyladamantan-1-ol.

Dependence of the capacity factor (k') on the mobile phase elution strength

When using two-component mobile phases, *i.e.*, *n*-heptane–2-propanol and *n*-heptane–diethyl ether, the change in the capacity factor k' with the change in elution strength of the mobile phase is determined by an approximate relation derived by Jandera and Churáček[4]

$$\log k' = A - n \log c \tag{1}$$

where c is the concentration of the more polar component in the two-component mobile phase and n and A are constants.

In our case c corresponds to the concentration of 2-propanol. Good agreement has been found between this equation and the experimental results, even for a three-component mobile phase (*n*-heptane–diethyl ether–2-propanol with the *n*-heptane: diethyl ether ratio held constant). Fig. 9 illustrates the dependence of the k' value on the concentration of 2-propanol in *n*-heptane; Fig. 10 shows the same dependence for 2-alkyladamantan-2-ols on the 2-propanol concentration when using the three-component (*n*-heptane–diethyl ether–2-propanol) mobile phase. In the case of the *n*-heptane–chloroform–2-propanol mobile phase, a linear dependence of $\log k'$ on $\log c$ was found for most compounds, and this was the result with all the other mobile phases used.

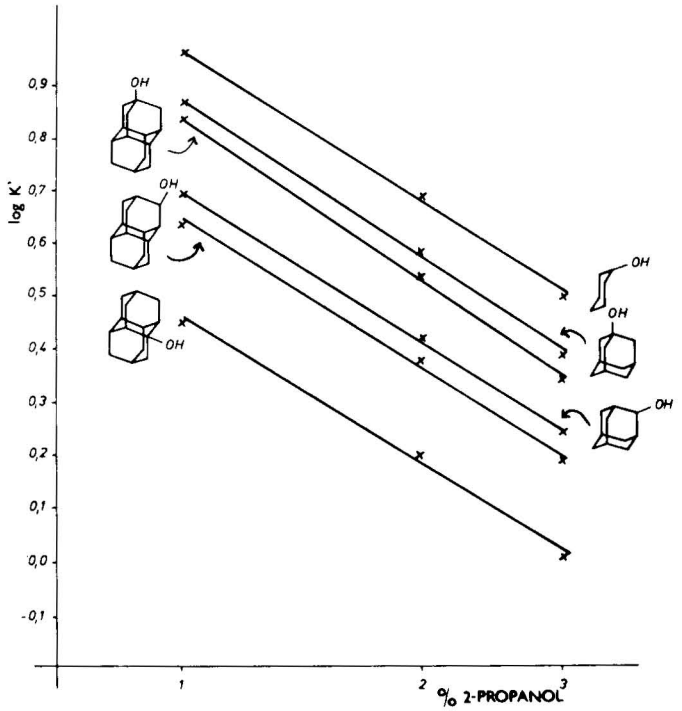


Fig. 9. Variation of the logarithm of capacity factors with the concentration of 2-propanol in the mobile phase (*n*-heptane–2-propanol) for adamantanols and diamantanols.

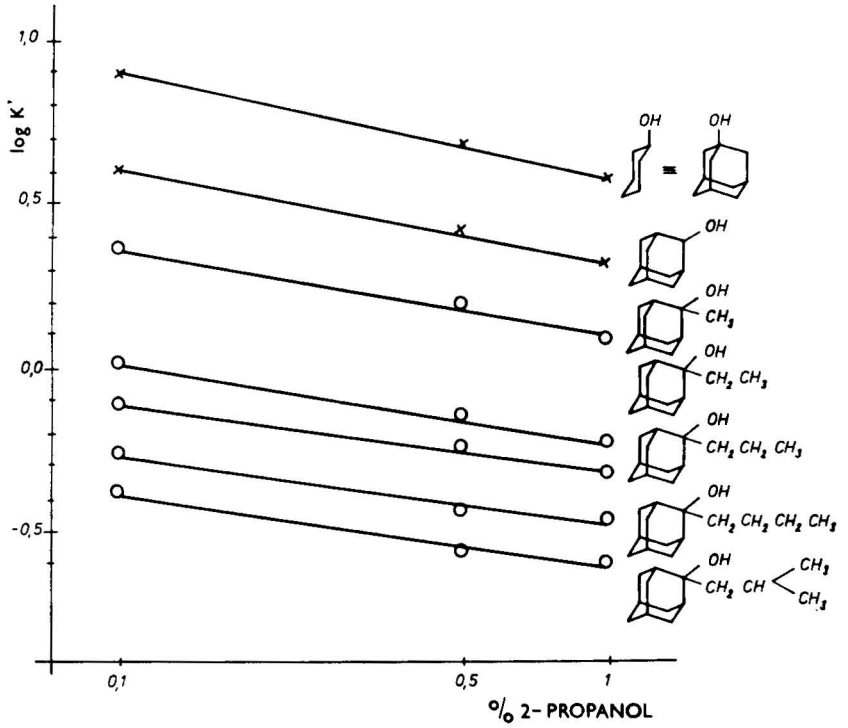


Fig. 10. Variation of the logarithm of capacity factors with the concentration of 2-propanol in the mobile phase (*n*-heptane–2-propanol) for 2-alkyladamantan-2-ols.

Effect of the mobile phase composition

The twelve mobile phases used in the present study may be divided into two basic groups according to their effect on the chromatographic behaviour of the compounds studied. The first group comprises nine systems containing 2-propanol and the second group includes mobile phases containing *n*-heptane and diethyl ether in various ratios. The difference found when using the two types of mobile phases is illustrated in Fig. 8, which gives k' values in the *n*-heptane–chloroform–2-propanol and *n*-heptane–diethyl ether systems.

Further small changes in the chromatographic behaviour of polycyclic alcohols may be achieved by changing the composition of the mobile phase used. The dependence of k' on the number of carbon atoms for alkyladamantan-1-ols is demonstrated in Fig. 6. When using the *n*-heptane-2-propanol mobile phase, the difference between the k' values for 3-alkyladamantan-1-ols (dashed line) and polymethyladamantan-1-ols (solid line) is insignificant and does not increase with decreasing elution strength. When using *n*-heptane–diethyl ether, the difference in k' values is larger and again it does not change greatly with the elution strength. However, in case of the *n*-heptane–diethyl ether–2-propanol system, the difference between the k' values for polymethyladamantan-1-ols and 3-alkyladamantan-1-ols increases with decreasing concentration of 2-propanol in the mobile phase.

Another mobile phase, containing only *n*-heptane–chloroform, has been tested. However, this system proved to be unsuitable in chromatographic analyses of polycyclic alcohols, as some of the compounds yielded broadened peaks, while others were not eluted from the column under these conditions.

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CHROM. 13,065

SEPARATION OF ENANTIOMERIC IODINATED THYRONINES BY LIQUID CHROMATOGRAPHY OF DIASTEREOMERS

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SUMMARY

A method for the separation of D,L-triiodothyronine and D,L-tetraiodothyronine optical isomers is described. The iodinated thyronines are coupled with L-leucine and the resulting diastereomers are separated by reversed-phase liquid chromatography. The derivatives are detected in the UV region at 230 nm. The technique can be used for the determination of the optical purity of thyroid hormones. It is possible to determine 0.2% of the L-isomer in D-tetraiodothyronine with a relative standard deviation of 8%. One complete analysis takes about 2 h. The results are in good agreement with those of an enzymatic method.

INTRODUCTION

The hormones triiodothyronine (T_3) and tetraiodothyronine (T_4) exist as enantiomers that have very different pharmacological effects. The chromatographic techniques used so far permit only a separation of the different thyronines without differentiation between the optical isomers. Such separations are possible by means of gas chromatography following derivatization[1–3], gel chromatography[4,5], ion-exchange chromatography[6,7] and high-performance liquid chromatography (HPLC) on reversed phases[8,9]. The HPLC determinations are fast and simple and applicable to routine determinations. For this reason, we tried to use the same technique for the separation of the optical isomers.

With chiral stationary phases separations by direct injection of the test substances can be performed. Most chiral stationary phases are polymers with proline or hydroxyproline as active centres. After complexation with Cu(II) ions a high stereo selectivity is attained[10–13]. High-speed separations have not proved possible with this technique. Recently, the synthesis of a similar chiral stationary phase based on silica was described[14]. For all of these separations Cu(II) ions must be present as complexing agent in the mobile phase. Therefore, it can be expected that problems

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will arise with iodinated compounds. Another possibility is the use of chiral eluents[15–17]. In all instances metal complexes are necessary for separation, and problems similar to those described for the chiral stationary phases can be expected. A review of these direct resolution techniques was published by Audebert[18].

An alternative approach is the synthesis of diastereomers and their separation by HPLC. The most important methods were reviewed recently[19]. *tert.*-Butyloxy-L-leucine-N-hydroxysuccinimide ester (BOC-L-Leu-SU) has proved to be well suited as a reagent for preparing diastereomeric peptides from racemic amino acids[20,21]. Racemization under the mild conditions of this reaction is below 0.1 %[20,22]. The L-leucine derivatives prepared can be easily separated by reversed-phase liquid chromatography, as shown by Nachtmann[21]. In this paper the application of this technique to the determination of D,L-triiodothyronine (T_3) and D,L-tetraiodothyronine (T_4) enantiomers is demonstrated.

EXPERIMENTAL

Materials

The solvents used were of analytical-reagent grade (E. Merck, Darmstadt, G.F.R.). The iodinated thyronines were synthetized by Sanabo (Vienna/Schaftenau, Austria) and BOC-L-Leu-SU (puriss CHR) was provided by Fluka (Buchs, Switzerland). For the HPLC separations LiChrosorb RP-8 and RP-18 (Merck), particle size 7 μm, were used, packed in stainless-steel columns (150 × 3.2 or 250 × 3.2 mm I.D.) by a slurry technique[23].

Apparatus

A Waters 1000 A pump combined with a U6K injection system was used for the chromatographic separations. Detection was performed at 230 nm using a Schoeffel SF 770 spectrophotometric detector. Gradient elution and thermostating were not necessary, and the separations were performed isocratically at room temperature (20–22°C). For integration of the peak areas a Hewlett-Packard 3380 integrator and a Hewlett-Packard Laboratory Data System 3353 were used.

Derivatization

As a result of the optimization studies the following procedure can be recommended: 1.5 mg of T_3 or T_4 is weighed into a 25-ml flask and 200 μl of a 0.1 *M* solution of sodium hydrogen carbonate and 400 μl of methanolic reagent solution (7 mg/ml BOC-L-Leu-SU in methanol) are added. The reagent solution must be prepared immediately before use. The reaction mixture is placed in an ice-bath for 30 min and stirred well, then the solution is evaporated to dryness (the temperature must not exceed 30°C). Trifluoroacetic acid (100 μl) is added to the dry residue and, after standing for 30 min at room temperature it is neutralized by the addition of 2 ml of 1 *M* sodium hydrogen carbonate solution. The precipitate is centrifuged, the supernatant liquid removed and the precipitate dissolved in 600 μl of methanol–0.02 *M* sodium hydroxide solution (1:1). The solution obtained is injected into the chromatograph.

RESULTS AND DISCUSSION

Derivatization

The D,L-T_3 and D,L-T_4 enantiomers react with BOC-L-Leu-SU and give diastereomeric peptides. For the detection of small amounts of one isomer in a large excess of the other, pure derivatives must be obtained. As the iodinated thyronines are relatively unstable, the reaction was optimized with respect to mild reaction conditions. The formation of the peptides is not only possible in tetrahydrofuran–sodium hydrogen carbonate solution, as described by Mitchell et al.[20], as methanolic solutions have also proved to be suitable. The basicity of the reaction solution is of great importance. Solutions of sodium hydrogen carbonate, sodium hydroxide and sodium carbonate of different molarity were tested. The best results were obtained with 0.1 *M* sodium hydrogen carbonate solution. In methanol–0.1 *M* sodium hydrogen carbonate solution (1:1) the derivatization is completed within 10 min at 0°C. For quantitative determinations the best reproducibility was achieved with a reaction time of 30 min.

Recently, Nachtmann[21] found that the quantitative determination of small amounts of L-penicillamine in the D-form was not possible using the BOC-protected L-Leu derivatives, and the same is true for the iodinated thyronines. The deprotection step must be carried out under very mild conditions in order to avoid the formation of degradation products. Mixtures of trifluoroacetic acid with solvents previously used[20,21] could not be used owing to the formation of by-products.

With 100 µl trifluoroacetic acid, added to the evaporated dry residue of the derivatization reaction, deprotection of the peptides succeeds within a few minutes. Owing to the low solubility of the residue, a reaction time of 30 min is recommended. The evaporation of the acid is not possible without the formation of by-products. An alternative is the addition of 2 ml of 1 *M* sodium hydrogen carbonate solution, which causes precipitation of the peptides. After centrifugation the precipitate can be dissolved in methanol–0.02 *M* sodium hydroxide solution (1:1) and injected into the chromatograph.

Using the optimal conditions, the excess of reagent for quantitative derivatization of D,L-T_3 and D,L-T_4 was investigated (Table I). To 200 µl of the substrate solutions in 0.1 *M* sodium hydrogen carbonate solution (7,5 mg/ml) various amounts of BOC-L-Leu-SU (7 mg/ml in methanol) were added. The chromatographic determination was performed as shown in Fig. 3. For L-T_3 and L-T_4 200 µl of BOC-L-Leu-SU

TABLE I

OPTIMIZATION OF THE REACTION OF BOC-L-LEU-SU WITH D,L-T_3 AND D,L-T_4

Reaction conditions: see Experimental. Chromatographic conditions: see Fig. 3.

Volume of BOC-L-Leu-SU (µl)	*Relative peak height of L-Leu-derivatives (mm)*			
	L-T_3	L-T_4	D-T_3	D-T_4
50	16	12	7	7
100	45	32	22	23
200	65	36	27	44
400	62	38	45	64
600	62	38	43	65

solution are sufficient for derivatization, whereas for the D-forms a larger excess of reagent is necessary.

Optimization of the chromatographic conditions

For the separation of iodinated thyronines reversed-phase materials with

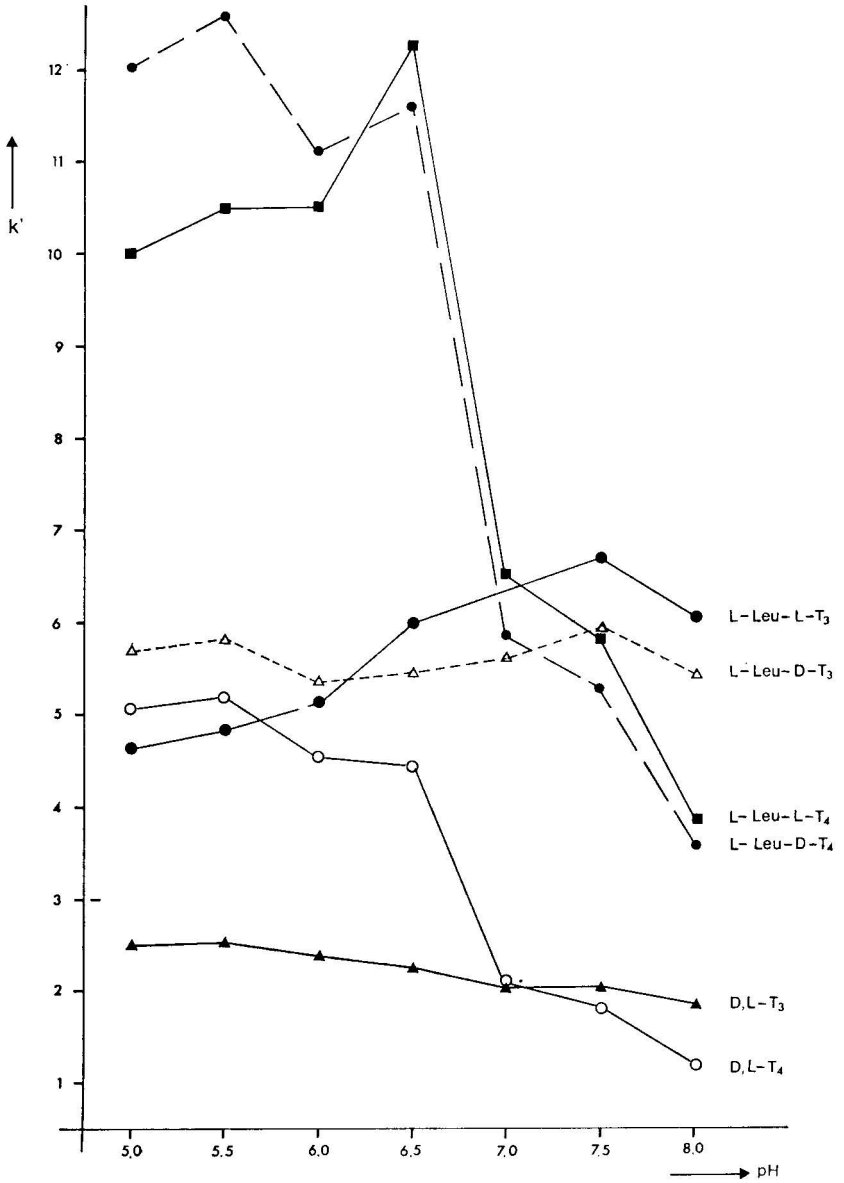

Fig. 1. Chromatographic characteristics of D,L-T_3, D,L-T_4 and their L-Leu derivatives. Column: LiChrosorb RP-18 (7 μm), 150 × 3.2 mm I.D. Mobile phase: methanol–phosphate buffer (0.033 *M*) of various pH (60:40). Flow-rate: 1 ml/min.

mixtures of phosphate buffer and methanol have been described[8]. Systems of this type were studied for the separation of the diastereomeric L-Leu derivatives of D,L-T_3 and D,L-T_4 (Fig. 1). At pH below 6.0 the L-form of the derivatives of T_3 and T_4 elute before the D-form. At higher pH the order of elution is reversed. For quantitative determinations of small amounts of the L-isomer in the D-form, pH values lower than

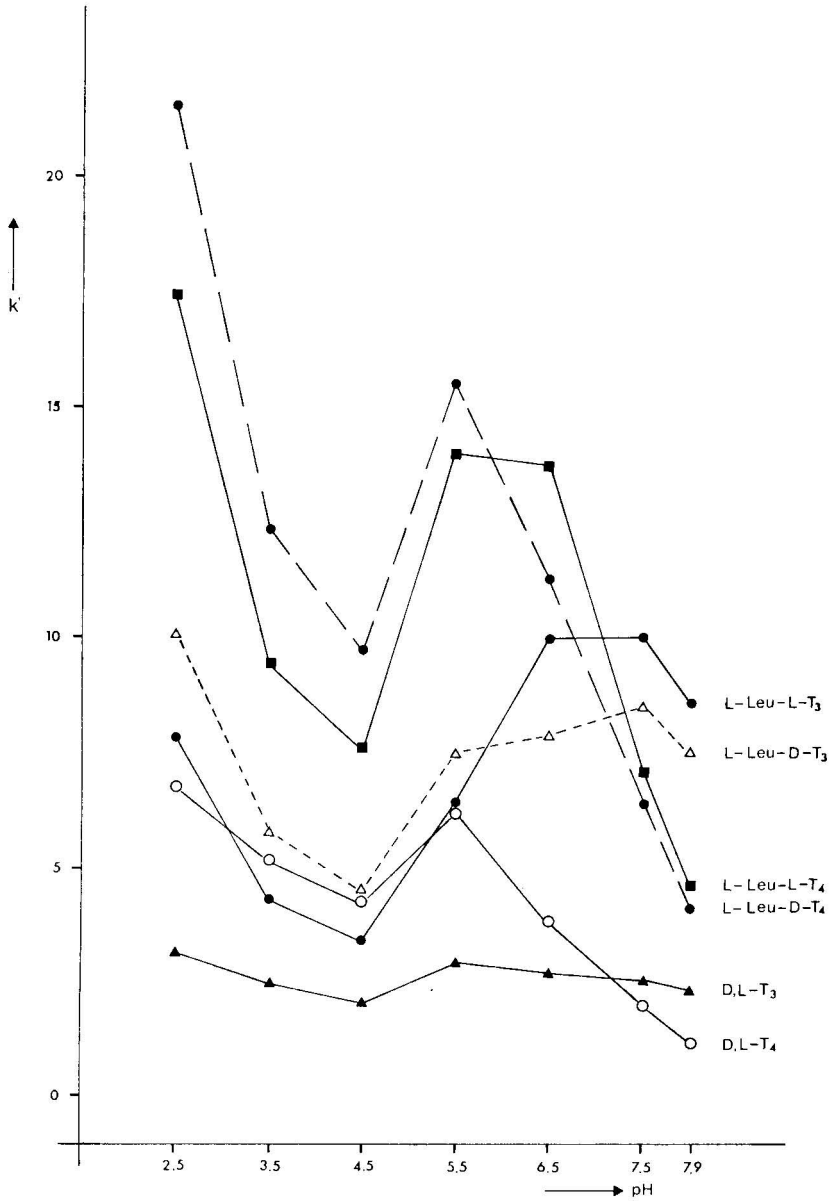

Fig. 2. Chromatographic characteristics of D,L-T_3, D,L-T_4 and their L-Leu derivatives. Column: LiChrosorb RP-18 (7 μm), 150 × 3.2 mm I.D. Mobile phase: methanol–phosphate–citrate buffer of various pH (60:40). Flow-rate: 1 ml/min.

6.0 should be chosen. It is also interesting that in the pH range 7.0–7.5 the order of elution of T_3 and T_4 is changed. In acetic media T_3 is always eluted before T_4, and in alkaline phosphate buffer T_4 is eluted before T_3. The same is true for the L-Leu derivatives of the iodinated thyronines.

A baseline separation of all compounds was not possible with the solvent system tested. The addition of citrate buffer results in an improvement in the separation selectivity (Fig. 2). The various pH values were reached by mixing 0.2 *M* phosphate buffer (pH 8.0) with 0.1 *M* citrate buffer (pH 2.2) to give the desired pH value. The change in the order of elution of T_3 and T_4 and the derivatives of the D- and L-forms occurs at similar pH values to that in the phosphate buffer system. A better separation between the six substances is obtained. At pH 6.4 a baseline separation is easily possible, as shown in Fig. 3. This chromatographic system is very useful for the analysis of mixtures of D,L-T_3 and D,L-T_4.

The quantitative determination of small residues of the L-isomer in D-T_4 for

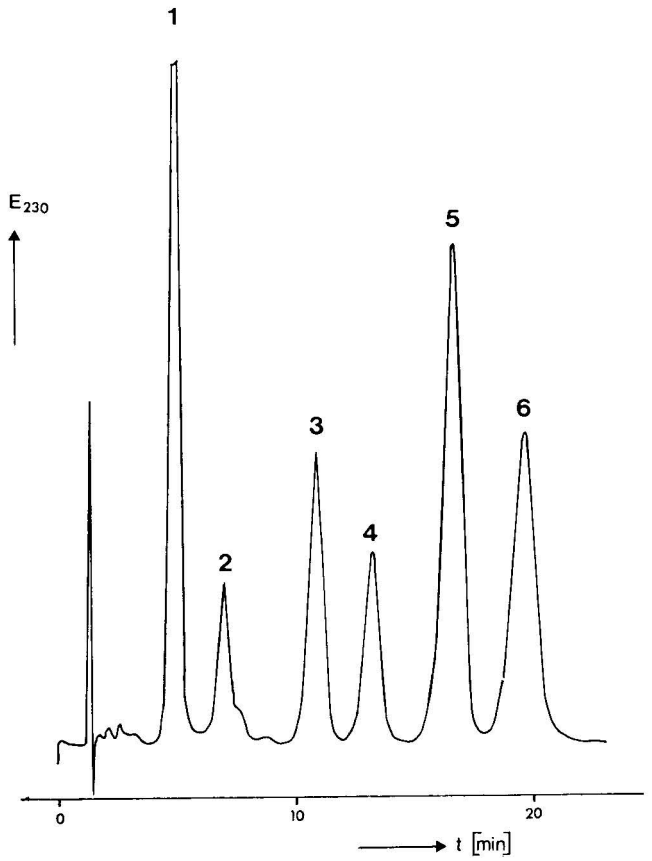

Fig. 3. Separation of D,L-T_3 (1), D,L-T_4 (2), L-Leu-D-T_3 (3), L-Leu-L-T_3 (4), L-Leu-D-T_4 (5) and L-Leu-L-T_4 (6). Column: LiChrosorb RP-18 (7 μm), 250 × 3.2 mm I.D. Mobile phase: methanol–phosphate–citrate buffer (0.085 *M*), pH 6.4 (60:40). Flow-rate: 1 ml/min. Injection volume: 30 μl. The unmarked peaks are unknown impurities.

purity tests is not possible with the mobile phases discussed because of the formation of by-products during the derivatization steps that cannot be separated from the peak of L-Leu-L-T_4. Such separations can be carried out with methanesulphonic acid as ion-pairing agent in the mobile phase. The calculation of the percentage of the L-isomer in the D-form is easily possible by comparison of the peak areas of the corresponding L-Leu derivatives. A presupposition for reproducible results is a good integration system such that the slope sensitivity can be optimized in small steps.

Quantitative determinations of as little as 0.2% of the L-form in D-T_4 are possible with a relative standard deviation of 8%. The detection limit is 0.05% of L-T_4 in D-T_4. Recovery studies were carried out by adding known amounts of L-T_4 to D-T_4. The concentration range was 0.1–1% of L-T_4 and the recoveries were 90–104%.

Chromatograms showing the enantiomeric purity of commercial L-T_4 and D-T_4 are given in Figs. 4 and 5. In L-T_4 the D-form is not detectable (<0.05%); the content of the L-isomer in the D-T_4 tested (Fig. 5) was 0.2%. Three different batches of D-T_4 were analysed in parallel by using the described HPLC method and an enzymatic method based on the work of Neudecker and co-workers[24,25]. For the final colour reaction 4-aminodiphenylamine-2-sulphonic acid was used instead of *o*-dianisidine. The results are summarized in Table II. The results of the two methods showed a good correlation.

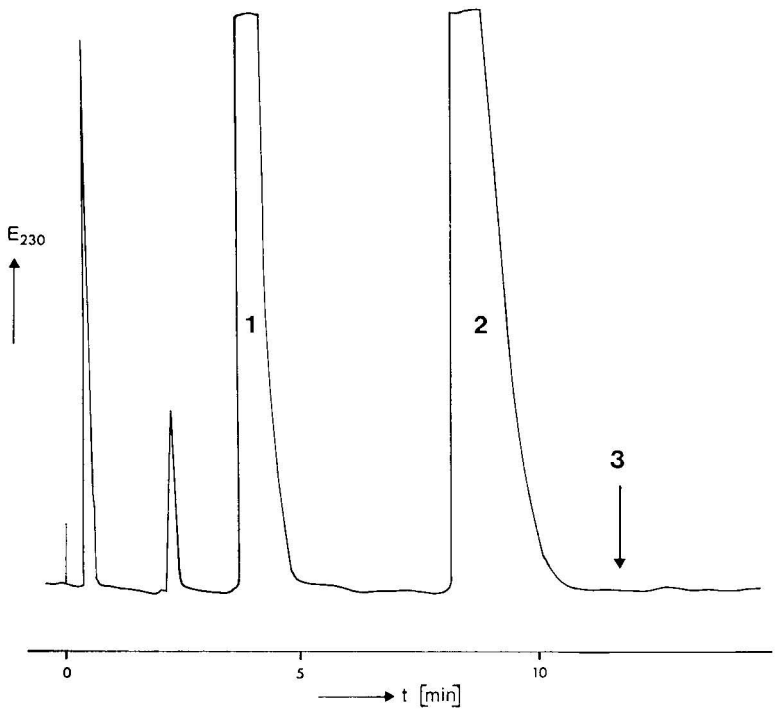

Fig. 4. Determination of the D-isomer in L-T_4. Column: LiChrosorb RP-18 (7 μm), 150 × 3.2 mm I.D. Mobile phase: methanol–water (60:40) + 0.05% of methanesulphonic acid. Flow-rate: 1 ml/min. Injection volume: 15 μl. Peaks: 1 = D,L-T_4 (underivatized); 2 = L-Leu-L-T_4; 3 = L-Leu-D-T_4. The other peaks are unknown impurities.

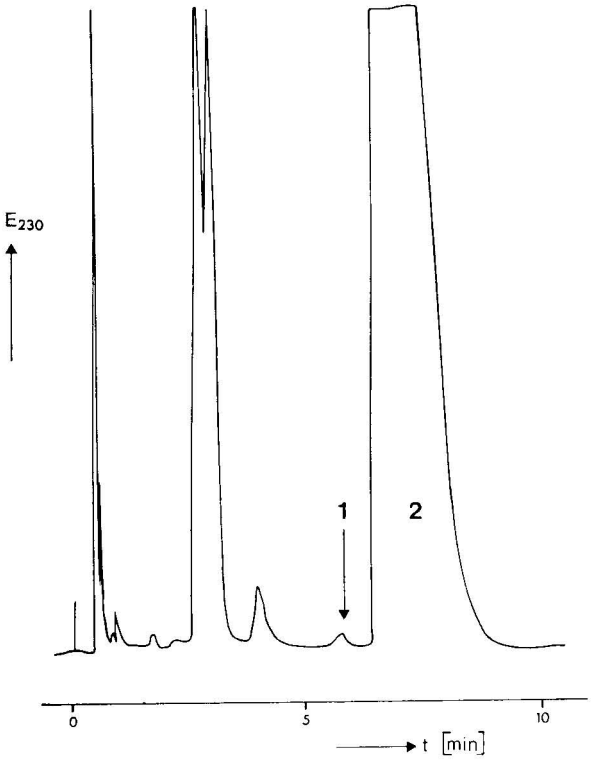

Fig. 5. Determination of the L-isomer in D-T_4. Column: LiChrosorb RP-18 (7 μm), 150 × 3.2 mm I.D. Mobile phase: methanol–water (63:37) + 0.05% of methanesulphonic acid. Flow-rate: 1 ml/min. Injection volume: 15 μl. Peaks: 1 = L-Leu-L-T_4; 2 = L-Leu-D-T_4.

TABLE II

COMPARISON OF THE DETERMINATION OF THE L-ISOMER IN D-T_4 USING HPLC AND AN ENZYMATIC METHOD

Batch	*L-Isomer content (%)*	
	HPLC	*Enzymatic analysis*
1	0.19	0.22
2	0.20	0.21
3	0.24	0.23

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DOSAGE PHOTODENSITOMÉTRIQUE DE LA "PR-TOXINE" MÉTABOLITE DE *PENICILLIUM ROQUEFORTI*

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(Reçu le 19 juin 1980)

SUMMARY

Photodensitometric determination of "PR-toxin", metabolite of Penicillium roqueforti

Two methods are proposed for the determination of PR-toxin. One allows a direct quantitative estimation of the mycotoxin, the other utilizes an imine formation after reaction with ammonium hydroxide. In both cases a fluorodensitometric assay on thin-layer chromatographic plates is carried out after spraying with *p*-dimethylaminobenzaldehyde reagent. The two procedures can be applied to estimate the toxin in foodstuffs at levels as low as 10 and 1 μg/kg, respectively.

INTRODUCTION

L'étude de la toxinogénèse de *Penicillium roqueforti* a été abordée à la suite d'observations mettant en évidence une relation entre l'apparition d'accidents pathologiques chez des bovins et la consommation de grain moisi[1]. L'un des métabolites secondaires de ce micromycète, qui présentent une toxicité dans des conditions expérimentales, a été désigné "PR-toxine"[2]. C'est un sesquiterpénoïde de type érémophilane[3]. Ultérieurement, divers expérimentateurs ont étudié les conditions de production de la PR-toxine par différentes souches de *P. roqueforti*[4–7] et montré que, dans le fromage, le métabolite est instable du fait de la formation de composés avec des acides aminés, des amines, vraisemblablement d'autres produits de dégradation de la caséine, et d'une imination par l'ammoniaque[8]. Les méthodes de détection et de dosage, employées au cours de ces travaux, apparaissent insuffisantes; elles utilisent soit une émission de fluorescence très instable, soit une révélation, par l'acide sulfurique, peu sensible et non spécifique. Celles que nous proposons dans cette note pourraient permettre de préciser certains résultats.

MATERIEL ET MÉTHODES

Préparation de la PR-toxine et de la PR-imine de référence

La PR-toxine est obtenue selon la méthode proposée par Wei *et al.*[2], à partir

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de cultures de *P. roqueforti* CBS 221-30 (NRRL 849) et 596A (cette dernière souche appartient à la collection de notre laboratoire). La nature du produit est confirmée par mesure du point de fusion (155–156°C) et de sa masse moléculaire en spectrométrie de haute résolution ($M = 320.126$; $C_{17}H_{20}O_6$). La "PR-imine"[9], résultat de la réaction entre la PR-toxine et l'ammoniaque, est obtenue en ajoutant à une solution chloroformique de la toxine un mélange méthanol–NH_4OH (90:10). La solution est ensuite séchée sous courant d'azote à température ambiante, reprise par le chloroforme puis appliquée à une colonne de chromatographie (gel de silice Si 60, Merck, Darmstadt, R.F.A.). L'élution par le mélange méthanol–chloroforme en proportions variant selon un gradient linéaire de 1:99 à 5:95 permet d'isoler l'imine qui cristallise en chloroforme–tétrachlorure de carbone. L'identité de la PR-imine est confirmée par la mesure de son point de fusion (190–192°C) et de sa masse ($M = 319.142$; $C_{17}H_{21}NO_5$).

Chromatographie sur couche mince (CCM)

Dans tous les essais, les chromatogrammes sont développés sur des plaques finies Merck, en verre (20 × 20 cm; silice Si 60 sans additif fluorescent; 0.25 mm d'épaisseur). Les couches minces sont soit utilisées telles quelles, soit acidifées par l'acide oxalique à 2% (ref. 10), avant d'être activées par chauffage à 110°C durant 30 min. Sauf cas particuliers précisés dans l'exposé des résultats, tous les échantillons sont déposés sous 5 μl, en solution chloroformique, à l'aide d'une microseringue. L'élution est effectuée à température ambiante, en cuves à atmosphère saturée, par les systèmes suivants: I, benzène–*n*-hexane (40:60); II, toluène–acétate d'éthyle–acide formique (50:40:10); III, toluène–acétate d'éthyle (30:70) saturé d'eau; IV, chloroforme–isopropanol (10:1); V, chloroforme–méthanol (96:4). Après développement, les chromatogrammes sont laissés 30 min à température ambiante, à l'obscurité. Les composés sont révélés soit (R1) par une exposition aux rayonnements ultra-violets (UV) de courte longueur d'onde durant 30 sec (lampe Hanovia, 253.7 nm), soit par vaporisation d'un des réactifs suivants: (R2) acide sulfurique à 50% (les plaques sont ensuite chauffées à 150°C durant 20 min); (R3) *p*-diméthylaminobenzaldéhyde (*p*-DMAB) à 1% en solution éthanolique, les plaques étant ensuite exposées aux vapeurs d'acide chlorhydrique (HCl) durant 10 min; (R4) *p*-DMAB en solution dans HCl concentré (1 g dans 10 ml; cette solution est diluée 10 fois par l'acétone au moment de l'emploi). Les taches fluorescentes sont repérées sous UV de grande longueur d'onde (lampe Philips HPW 125).

Photodensitométrie en fluorimétrie d'émission

Les mesures sont effectuées sur un photomètre Vernon PHI-5 (Vernon, Paris, France) équipé d'une source "Mercure" haute pression (longueur d'onde d'excitation: 365 nm) et des filtres supplémentaires Wratten-Kodak No. 4 pour la PR-imine, 2A et 47B pour la PR-toxine. Chaque série de mesures réclame un étalonnage de l'appareil réalisable en faisant migrer parallèlement les échantillons à doser et les dilutions sériées d'une solution standard. La quantité de toxine est rapportée à la surface des pics enregistrés.

Introduction de la PR-toxine dans les extraits de fromages

100 g de pâte de fromages du commerce (Bleu de Bresse et Roquefort) sont

soumis à une extraction selon une méthode proposée par Scott et Kanhere[8] (méthode II de ces auteurs, utilisant l'acétate d'étyle). En fin d'opération, on ajoute à ces extraits la PR-toxine cristallisée en solution chloroformique pour réaliser des concentrations allant de $1 \cdot 10^{-6}$ à $0.1 \cdot 10^{-9}$.

Imination des échantillons

Une fraction aliquote (100 μl) de l'échantillon à analyser, en solution chloroformique, est déposée dans un microtube et additionnée de 20 μl de méthanol–NH_4OH (90:10); on mélange à l'aide d'une microseringue. Après quelques minutes, le contenu du tube est séché sous un courant d'azote, à température ambiante; le résidu est repris par 100 μl de chloroforme. Des dilutions sériées sont développées sur couche mince d'abord en phase I puis, après séchage, dans la même direction en phases II, IV ou V. Ces manipulations sont effectuées à l'abri de la lumière. La PR-imine formée est dosée par photodensitométrie.

RÉSULTATS ET DISCUSSION

Choix des conditions de CCM

Les valeurs de R_F de la PR-toxine et de la PR-imine dans les divers systèmes utilisés sont rassemblées dans le Tableau I. L'acidifcation des plaques par l'acide oxalique ne modifie pas sensiblement les valeurs de R_F de ces deux composés, mais elle permet d'employer des conditions chromatographiques identiques à celles préconisées pour d'autres mycotoxines, susceptibles de contaminer des fromages, telles que l'acide cyclopiazonique, l'acide mycophénolique, et des toxines neurotropes produites par divers *Penicillium*: les "penitrems"[10,11]. La phase I peut servir à un premier développement nécessaire dans le cas d'extraits de fromages; l'utilisation de la phase II doit être retenue pour la PR-toxine, celle des phases IV et V pour la PR-imine.

TABLEAU I

CARACTERISTIQUES CHROMATOGRAPHIQUES DE LA PR-TOXINE ET DE LA PR-IMINE

A: Plaques acidifiées par l'acide oxalique; B: plaques non acidifiées.

Système	R_F			
	PR-toxine		*PR-imine*	
	A	*B*	*A*	*B*
Benzène–*n*-hexane (40:60)	0.00	0.00	0.00	0.00
Toluène–acétate d'éthyle–acide formique (50:40:10)	0.42	0.43	0.05	0.05
Toluène–acétate d'éthyle (30:70) (saturé d'eau)	0.42	0.57	0.08	0.09
Chloroforme–propanol-2 (10:1)	0.45	0.58	0.15	0.18
Chloroforme–méthanol (96:4)	0.60	0.61	0.16	0.18

Comparaison des modes de détection de la PR-toxine et de la PR-imine

Cette comparaison fait intervenir d'une part la définition de la plus petite quantité de produit décelable en CCM, d'autre part la possibilité d'effectuer des mesures photodensitométriques. L'aspect de la PR-toxine et de son dérivé iminé, sous UV ou après traitement par H_2SO_4 a déjà été décrit[2]. Nous avons défini les limites d'application des deux méthodes à la détection des deux substances (*cf.* Tableau II). Dans le cas de la PR-toxine, l'induction d'une fluorescence réclame une brève exposition aux UV; ultérieurement, quelles que soient les conditions dans lesquelles sont conservés les chromatogrammes, l'intensité de la fluorescence décroit rapidement; des séries de mesures photodensitométriques que nous avons effectuées à ce stade, 15 et 30 min après la première exposition aux UV, ont fourni des résultats très dispersés, l'erreur atteignant 100%. En ce qui concerne la PR-imine, l'intensité de la fluorescence étant instable, elle ne peut, de même, être employée en matière de dosage. L'état des chromatogrammes après traitement par l'acide sulfurique est incompatible avec l'application d'une méthode densitométrique.

TABLEAU II

DÉTECTION DE LA PR-TOXINE ET DE LA PR-IMINE SOUS UV EN CCM

R_1: Révélation par les UV de courte longueur d'onde; R_2: révélation par H_2SO_4 à 50% et chauffage; R_3: révélation par la *p*-DMAB à 1% dans l'éthanol–vapeurs d'HCl; R_4: révélation par la *p*-DMAB en solution dans HCl (1 g dans 10 ml, dilué 10 fois par l'acétone).

Toxine	*Quantité minimum visible (ng)* *			
	R_1**	R_2	R_3	R_4
PR-toxine	10	10	1	1
PR-imine	50	50	0.2	0.2

* Ces mesures sont des moyennes d'au moins 15 essais; elles ont été effectuées à l'oeil nu, ce qui empêche de préciser les valeurs des écarts-types.

** L'appréciation de l'intensité de fluorescence a été définie immédiatement après l'exposition aux UV.

Sous l'effet de la *p*-DMAB–HCl, la PR-toxine demeure invisible en lumière du jour mais elle émet une fluorescence bleue, intense, sous UV de grande longueur d'onde, stable durant plusieurs heures. En prenant la simple précaution de conserver les plaques empilées les unes sur les autres, on évite un jaunissement de la couche mince; les variations constatées en matière de mesure de l'intensité de la fluoresecence ont été de 2% en 4 h, de 6% en 48 h. Les deux formules (R3 et R4) du réactif donnent des résultats en moyenne identiques mais sensiblement moins dispersés lorsque les chromatogrammes sont exposés aux vapeurs d'HCl. Ceci peut être dû à l'instabilité du réactif R4.

Après traitement par la *p*-DMAB et les vapeurs d'HCl, la PR-imine émet une fluorescence bleue, intense, plus sombre que celle de la PR-toxine. L'exposition aux UV provoque une évolution de la fluorescence du bleu vers le jaune. Cette transformation peut être induite aussitôt après l'action des vapeurs d'acide par 2 min d.exposition aux UV. La fluorescence jaune a atteint alors son maximum; ensuite elle reste stable; on peut ainsi détecter en vision directe 0.2 ng du composé. Les chromatogrammes doivent être conservés comme il a été indiqué ci-dessus.

Dosages fluorimétriques —courbes de référence

La courbe d'émission de fluorescence de la PR-toxine révélée par la *p*-DMAB (R3) est représentée sur la Fig. 1. Les mesures densitométriques suivent la loi de Beer pour des quantités de composé comprises entre 100 et 4000 ng.

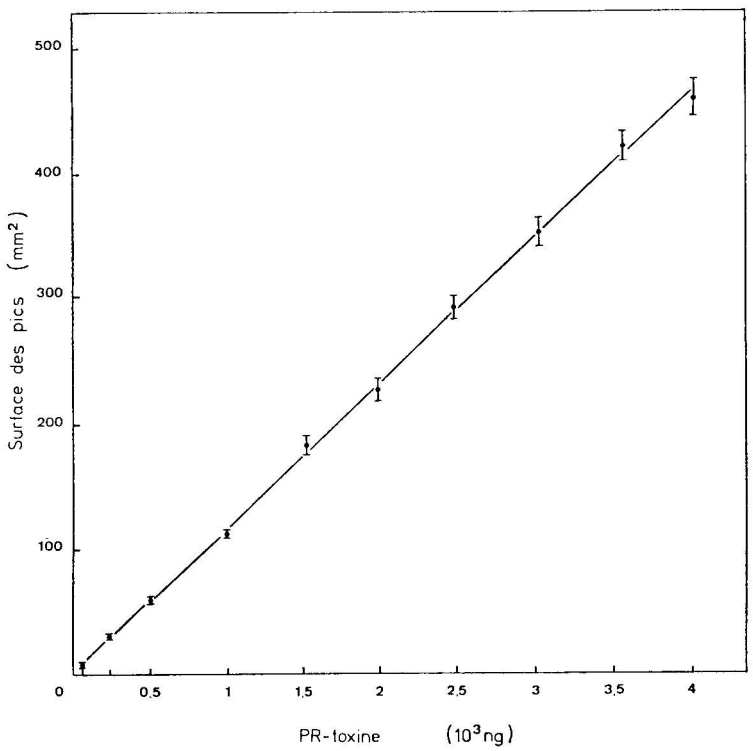

Fig. 1. Dosage fluorimétrique de la PR-toxine —courbe de référence. Excitation à 365 nm; filtres Wratten 47B et 2A; chromatogrammes développés en toluène–acétate d'éthyle–acide formique (50:40:10); révélation par la *p*-DMAB et les vapeurs d'HCl; chaque point représente la moyenne de 12 mesures; les segments verticaux figurent les écarts-types.

La courbe correspondant à la PR-imine, traitée par le réactif R3 puis exposée aux UV de grande longueur d'onde, est représentée sur la Fig. 2. La loi de Beer est respectée pour des valeurs comprises entre 5 et 300 ng. L'écart de sensibilité entre les deux dosages ne s'explique pas seulement par la différence d'intensité des émissions de fluorescence respectives de la PR-toxine et de son dérivé. Le pourcentage de transmission du filtre jaune (Wratten No. 4) utilisé pour doser la PR-imine (région d'émission à 570–580 nm) est de l'ordre de 90% tandis que celui de l'association des filtres 2A et 47B (bleu) employé dans le cas de la PR-toxine (région d'émission: 430–440 nm) n'est que de 40%.

Les résultats concernant l'application de la méthode à l'étude d'extraits de fromages sont présentés dans le Tableau III. Le dosage de la PR-toxine apparait satisfaisant pour des concentrations du métabolite supérieures ou égales à 10 μg/kg.

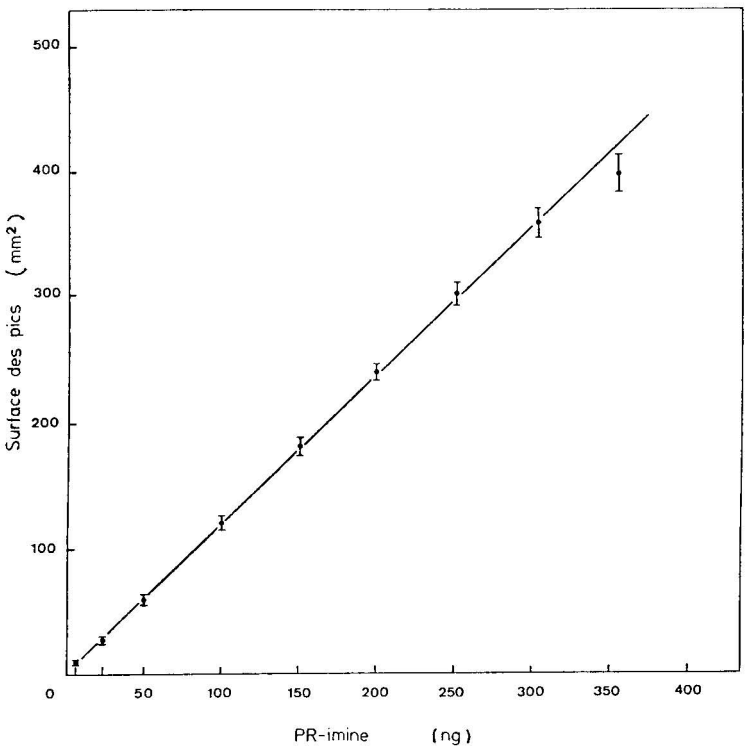

Fig. 2. Dosage fluorimétrique de la PR-imine —courbe de référence. Excitation à 365 nm; filtre Wratten No. 4; chromatogrammes développés en chloroforme–méthanol (96:4); révélation par la *p*-DMAB et les vapeurs d'HCl puis exposition des plaques aux UV (365 nm) durant 2 min; chaque point représente la moyenne de 10 mesures; les segments verticaux figurent les écarts-types.

TABLEAU III

DOSAGE DE LA PR-TOXINE DANS DES EXTRAITS ARTIFICIELLEMENT CONTAMINÉS DE FROMAGES

A: Roquefort; B: Bleu de Bresse. Volume d'échantillon déposé: 5 μl pour les concentrations supérieures à $2 \cdot 10^{-9}$, 20 μl pour les autres. Moyennes et écarts-types pour $n = 6$.

Concn. de PR-toxine incorporée (10^{-9})	*Concn. mesurée et écart-type* (10^{-9})		*Erreur maximum* (%)
	A	*B*	
1000	983.0 ± 1.6	989.1 ± 1.2	1.9
200	189.2 ± 0.6	193.3 ± 0.6	5.7
50	51.0 ± 0.6	49.7 ± 0.6	3.0
10	9.3 ± 0.2	10.3 ± 0.2	9.0
2	1.2 ± 0.3	1.5 ± 0.4	55.0
0.5	non dosable	—	—

Utilisation de l'imination comme moyen de confirmation et de dosage de la PR-toxine

Le traitement par le mélange MeOH–NH_4OH a été réalisé simultanément sur la PR-toxine pure en solution chloroformique, à diverses concentrations, et sur des

TABLEAU IV

DOSAGE DE LA PR-IMINE, APRÈS IMINATION D'EXTRAITS DE FROMAGE ARTIFICIELLEMENT CONTAMINÉS PAR LA PR-TOXINE

A: Roquefort; B: Bleu de Bresse. Volume d'échantillon déposé: 5 μl pour les concentrations supérieures à $1 \cdot 10^{-9}$, 10 μl pour les autres. Moyennes et écarts-types pour $n = 8$.

Concn. de PR-toxine incorporée (10^{-9})	*Concn. de PR-imine mesurés et écart-type (10^{-9})*		*Erreur maximum (%)*
	A	*B*	
1000	1011.2 ± 20.9	1017.3 ± 24.5	4.18
200	204.5 ± 5.7	201.2 ± 10.3	5.57
50	49.8 ± 3.3	50.4 ± 4.0	8.80
10	10.2 ± 0.4	10.0 ± 0.5	6.00
2	2.0 ± 0.2	2.0 ± 0.2	10.0
1	1.0 ± 0.1	1.0 ± 0.1	10
0.5	0.48 ± 0.1	0.51 ± 0.1	25

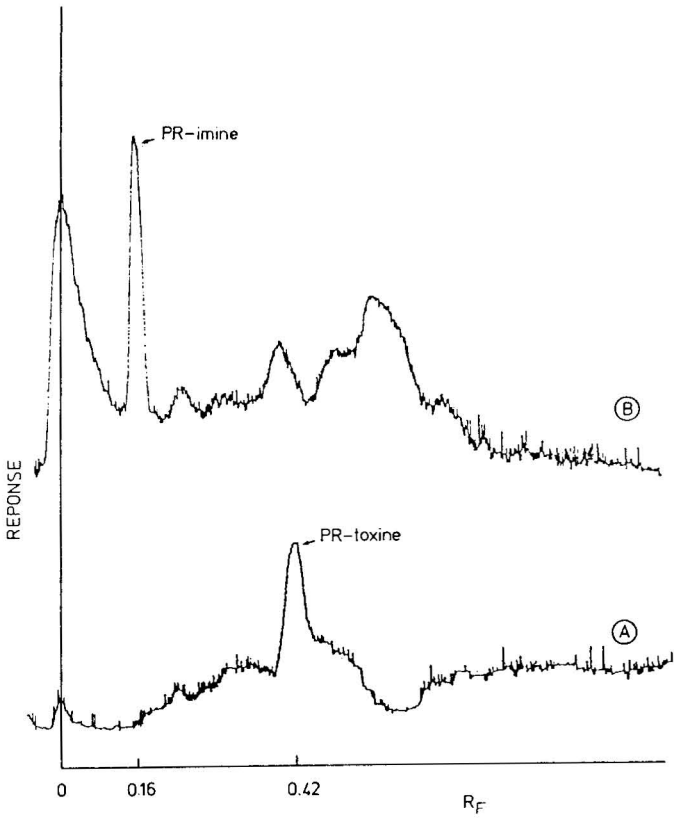

Fig. 3. Courbes photodensitométriques des chromatogrammes de séparations en CCM de la PR-toxine dans un extrait de fromage (A) et de la PR-imine après imination du même extrait (B). Plaques de gel de silice Si 60 de 0.25 mm d'épaisseur. Révélation par la *p*-DMAB et les vapeurs d'HCl. Concentration en PR-toxine: 10^{-7}. (A): développement en toluène–acétate d'éthyl–acide formique (5:4:1); filtres Wratten 47B et 2A. (B): développement en chloroforme–méthanol (96:4); filtre Wratten No. 4; la PR-imine a été exposée aux UV (365 nm) pendant 2 min après révélation.

dilutions des extraits chloroformiques de fromages artificiellement contaminés, des solutions titrées préparées à partir de PR-imine cristallisée servant de témoins. Le dosage photodensitométrique de la PR-imine formée à partir de la PR-toxine pure a montré que la transformation est complète, dans les conditions expérimentales précisées, pour des quantités de la mycotoxine allant de 100 à 0.1 μg. Dans les extraits de fromages, le dosage de la PR-imine est possible pour des concentrations aussi faibles que $1 \cdot 10^{-9}$ avec une erreur de l'ordre de 10% (Tableau IV). Il faut cependant remarquer que le filtre jaune (Wratten No. 4) étant très peu sélectif, la qualité de la séparation en CCM joue un rôle déterminant dans la reproductibilité des mesures (Fig. 3). D'autre part l'imination constitue un moyen pratique et simple de confirmation de la présence de PR-toxine; en effet, nous n'avons pas observé, sous l'action de l'ammoniaque sur les extraits de fromages ou de cultures *in vitro* de *P. roqueforti*, la formation de substances qui puissent interférer.

Cette remarque montre que les deux méthodes proposées de dosage de la PR-toxine peuvent, indifféremment, être utilisées dans l'étude des contaminations de certains aliments par ce métabolite fongique.

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Note

Formulation of multicomponent mobile solvents for liquid chromatography

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One approach to the optimization of the chromatography of a multicomponent mixture is to select the strength of the mobile solvent to elute the components of the mixture in a suitable retention-time range, and then to adjust resolution by changing the selectivity of the mobile solvent. Snyder[1,2] has devised a set of solvent characterization parameters, based on the work of Rohrschneider[3], and has proposed the use of these parameters for formulating binary mixtures of different selectivity, but the same overall solvent strength.

The use of three or more components in the mobile solvent offers the possibility of improving the effectiveness of this approach[4,5], since the selectivity of a multicomponent mobile solvent can be varied continuously throughout a range. We present here a scheme for efficiently and systematically varying the composition of a multicomponent mobile solvent, while keeping the overall solvent strength constant; this scheme may be generally useful for optimizing separations in high-performance liquid chromatography (HPLC). We used this method to optimize the reversed-phase HPLC separation of a mixture of four closely related corticosteroids (cortisone, hydrocortisone, prednisolone and prednisone) by means of a three-component mobile solvent consisting of water, methanol and tetrahydrofuran.

THEORY

We assumed that the overall eluting strength of a mixture of several miscible liquids is simply the volume-weighted mean of the strengths of the components[1] and derived (see Appendix) a set of formulae that gives the proportions in which the components are to be combined to yield a mixture with a given eluting strength. If the eluting-strength parameters of a set of pure liquids are $P_1, P_2, \ldots, P_n$ (where $P_1 > P_2 > \ldots > P_n$), then the respective volume fractions, $X_1, X_2, \ldots, X_n$, of these liquids to be combined to give a mixture of eluting strength, P, (where $P_1 \geqslant P \geqslant P_n$) can be deduced as set out below.

Three-component mixtures

A mixture ($X_1 + X_2 + X_3 = 1$) has the required eluting strength if, and only if, X_1 is non-negative and in the range

$$\frac{P - P_3}{P_1 - P_3} \geqslant X_1 \geqslant \frac{P - P_2}{P_1 - P_2} \tag{1}$$

and

$$X_2 = \frac{P - P_3}{P_2 - P_3} - \left(\frac{P_1 - P_3}{P_2 - P_3}\right) X_1 \tag{2}$$

and

$$X_3 = 1 - X_1 - X_2 \tag{3}$$

The entire selectivity range of the mobile solvent can be spanned without repetition by varying X_1 through its range expressed in eqn. 1. One arrives at the optimum solvent composition by trial and error, but this procedure eliminates all those compositions that would cause large shifts to longer or shorter retention times.

There is an alternative method for formulating these three-component mixtures, which is equivalent to the above method (see Appendix) if the volume changes on mixing are neglected. Further, this alternative method may be simpler in practice because it can be used without performing calculations with eluting-strength parameters, and it allows the use of a two-channel solvent programmer for the actual mixing (Fig. 1).

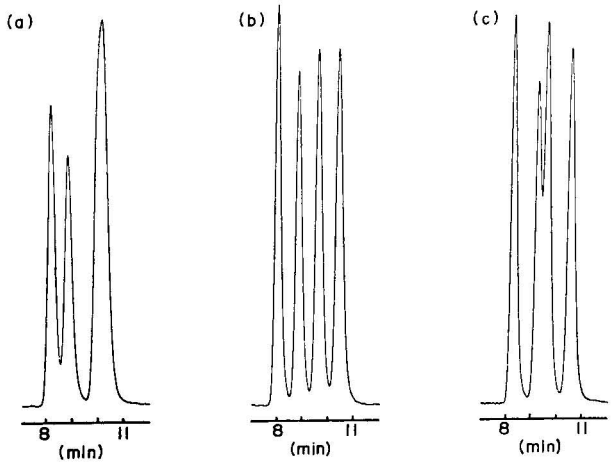

Fig. 1. Optimization of the selectivity of the ternary mobile phase water–methanol–tetrahydrofuran by combining the binary mixtures A (water–methanol, 42:58) and B (water–tetrahydrofuran, 73:27). (a) Mobile phase A; (b) mobile phase 0.14 A + 0.86 B (water–methanol–tetrahydrofuran, *ca.* 69:8:23); (c) mobile phase B. The order of elution is prednisone, cortisone, prednisolone, hydrocortisone. Mobile phase B was formulated by trial and error to have the same average eluting strength as phase A.

One formulates the two binary mixtures A (components 1 and 3) and B (either components 1 and 2 or components 2 and 3) that have the desired eluting strength, P; this can be done by calculation from a set of solvent-strength parameters or by experimentation. In the former instance, one calculates the unique mixtures of A and B that each simultaneously satisfy the equations $P = P_i X_i + P_j X_j$ and $X_i + X_j = 1$. In the latter instance, one arrives by trial and error at the compositions of A and B that give the same desired retention time for either a single chosen peak in the chro-

matogram or the average retention time of all the peaks. In either instance, combining A and B in varying proportions from 100% A to 100% B yields, without repetition, all and only those three-component mixtures that have the given eluting strength. Therefore, if no combination of A and B produces the required resolution, then no ternary mixture of these solvents at this strength will be effective.

Four-component mixtures

A mixture ($X_1 + X_2 + X_3 + X_4 = 1$) has the required eluting strength if, and only if, X_1 is non-negative and in the range

$$\frac{P - P_4}{P_1 - P_4} \geqslant X_1 \geqslant \frac{P - P_2}{P_1 - P_2} \tag{4}$$

and X_2 is non-negative and in the range

$$\frac{P - P_4}{P_2 - P_4} - \left(\frac{P_1 - P_4}{P_2 - P_4}\right) X_1 \geqslant X_2 \geqslant \frac{P - P_3}{P_2 - P_3} - \left(\frac{P_1 - P_3}{P_2 - P_3}\right) X_1 \tag{5}$$

and

$$X_3 = \frac{P - P_4}{P_3 - P_4} - \left(\frac{P_1 - P_4}{P_3 - P_4}\right) X_1 - \left(\frac{P_2 - P_4}{P_3 - P_4}\right) X_2 \tag{6}$$

and

$$X_4 = 1 - X_1 - X_2 - X_3 \tag{7}$$

These four-component mixtures can also be obtained by combining three binary mixtures (see Appendix) —A, B and C— that each have the required eluting strength, P. If $P > P_2$, which is usual, then these binary mixtures are A (components 1 and 4), B (1 and 3), and C (1 and 2). If $P_2 > P > P_3$, then the binary mixtures are A (1 and 4), B (2 and 3), and C (either one of 1 and 3 or 2 and 4). If $P_3 > P$, then the binary mixtures are A (1 and 4), B (2 and 4), and C (3 and 4).

EXPERIMENTAL

Chromatography was carried out with a DuPont Zorbax ODS column (4.9 × 250 mm) at ambient temperature, using a Waters Model 204 liquid chromatograph with the absorbance detector at a wavelength of 254 nm and a sensitivity of 0.05 ABS. The flow-rate was 1.0 ml/min, and the pressure was *ca.* 1800 p.s.i., depending on the mobile-solvent composition. Injections, all 15 μl, were made with a Waters WISP 710A sample processor. The absorbance was recorded with a Varian A-25 10-inch strip-chart recorder set at a sensitivity of 10 mV full-scale and a chart speed of 50 in./h. Retention times and peak widths (full width at half maximum) were determined from the chart.

Mobile solvents were formulated by volume using house-distilled water, methanol (Burdick and Jackson, Muskegon, MI, U.S.A.; suitable for liquid chromatography) and tetrahydrofuran (Burdick and Jackson, UV grade). Tetrahydrofuran was purified just before mixing by passing it through a column (2 × 15 cm) of alumina (ICN, Irwine, CA, U.S.A.; alumina acid, activity grade I). The solvent mixtures were filtered (Millipore, type LS, 5.0 μm) under vacuum before use.

Cortisone (Research Plus Steroid Laboratories), hydrocortisone, prednisolone and prednisone (all USP Reference Standard) were used as received.

RESULTS AND DISCUSSION

Mixtures of cortisone (22 μg/ml) and hydrocortisone (17 μg/ml), and of prednisolone (20 μg/ml) and prednisone (15 μg/ml), each dissolved in water–methanol (42:58), were separately chromatographed with each different mobile solvent. These solutions were first chromatographed using a series of water–methanol mixtures of different solvent strengths to determine a solvent-strength parameter, P, for calculating water–methanol–tetrahydrofuran mixtures of equal strength; water–methanol–tetrahydrofuran mixtures of strength $P = 7.61$ were formulated because this strength was judged to give a good balance between resolution and elution time.

The retention times and peak widths determined from these chromatograms are given in Table I. The average retention time of the chromatograms is insensitive to changes in solvent composition in the middle of the range (from 48 to 66 parts of water). The variation of the average retention time is larger in the extremes of the range (from 42 to 48 and from 66 to 71 parts of water), but even there the variation is smaller than that resulting from changing the overall solvent strength. Drift in retention times during data collection was less than 3%, determined by chromatography using water–methanol (42:58) as mobile solvent at intervals during data collection. Thus, Snyder's solvent-strength parameters were useful for controlling the eluting strength of the solvent in the system studied here.

TABLE I

RETENTION TIMES AND PEAK WIDTHS DETERMINED FROM CHROMATOGRAMS

THF = Tetrahydrofuran; t_R = retention time (min); t_W = full width at half maximum (min).

Mobile phase composition (%)			*Elution strength*	*Prednisone*		*Cortisone*		*Prednisolone*		*Hydrocortisone*	
H_2O	CH_3OH	THF	P^*	t_r	t_W	t_r	t_W	t_r	t_W	t_r	t_W
38	62	0	7.51	6.47	0.26	6.90	0.27	7.78	0.29	7.94	0.28
40	60	0	7.56	7.62	0.30	8.26	0.33	9.33	0.35	9.62	0.35
42	58	0	7.61	8.90	0.37	9.73	0.38	11.08	0.40	11.40	0.42
44	56	0	7.66	10.00	0.38	10.82	0.40	12.47	0.43	12.63	0.43
43	56	1	7.61	8.15	0.29	8.82	0.33	10.36	0.37	10.60	0.36
45	52	3	7.61	7.84	0.27	8.45	0.29	10.22	0.34	10.51	0.34
48	46	6	7.61	7.30	0.26	7.86	0.27	9.67	0.33	9.97	0.33
54	34	12	7.61	7.19	0.25	7.73	0.26	9.46	0.33	9.81	0.32
60	22	18	7.61	7.30	0.25	8.07	0.26	9.25	0.29	10.05	0.32
66	10	24	7.61	7.54	0.24	8.34	0.26	9.11	0.29	9.89	0.31
71	0	29	7.61	8.07	0.26	9.01	0.28	9.35	0.29	10.23	0.29

* $P = \Sigma X_i P_i$; $P_{H_2O} = 9$; $P_{CH_3OH} = 6.6$; $P_{THF} = 4.2$.

The resolution, capacity factors, and separation factors calculated from the data in Table I are shown in Table II. The resolution values reveal an optimum in the overall resolution in the composition region 60 to 66 parts of water. The values of the separation factors show that the changes in resolution are due mainly to changes in the selectivity of the mobile solvent, rather than to changes in efficiency.

TABLE II

PARAMETERS CALCULATED FROM RESULTS IN TABLE I

Mobile phase composition (%)			*Resolution**			*Capacity factors***				*Separation factors*		
H_2O	CH_3OH	THF	R_{12}	R_{23}	R_{34}	k'_1	k'_2	k'_3	k'_4	k'_2/k'_1	k'_3/k'_2	k'_4/k'_3
38	62	0	1.0	1.9	0.3	1.61	1.78	2.14	2.20	1.11	1.20	1.03
40	60	0	1.2	1.9	0.5	2.07	2.33	2.76	2.88	1.13	1.18	1.04
42	58	0	1.3	2.1	0.5	2.59	2.92	3.47	3.60	1.13	1.19	1.04
44	56	0	1.3	2.4	0.2	3.03	3.36	4.03	4.09	1.11	1.20	1.01
43	56	1	1.3	2.6	0.4	2.29	2.56	3.18	3.27	1.12	1.24	1.03
45	52	3	1.3	3.4	0.5	2.16	2.41	3.12	3.24	1.12	1.29	1.04
48	46	6	1.3	3.6	0.5	1.94	2.17	2.90	3.02	1.12	1.34	1.04
54	34	12	1.3	3.5	0.6	1.90	2.12	2.81	2.96	1.12	1.33	1.05
60	22	18	1.8	2.6	1.6	1.94	2.25	2.73	3.05	1.16	1.21	1.12
66	10	24	1.9	1.7	1.6	2.04	2.36	2.67	2.99	1.16	1.13	1.12
71	0	29	2.1	0.7	1.8	2.25	2.63	2.77	3.13	1.17	1.05	1.13

* $R = \dfrac{2(t_r - t'_r)}{1.67(t_w + t'_w)}$

** $k' = \dfrac{t_r - t_0}{t_0}$; $t_0 = 2.48$ min (determined by injection of water).

APPENDIX

Derivation of formulae

Eqns. 1–7 are obtained as follows. Given that

$$P = \sum_{i=1}^{n} X_i P_i \tag{8}$$

$$1 = \sum_{i=1}^{n} X_i \tag{9}$$

$$1 \geqslant X_i \pm 0 \qquad i = 1, 2, \ldots, n \tag{10}$$

$$P_1 > P_2 > \ldots > P_n \tag{11}$$

$$P_1 \geqslant P \geqslant P_n \tag{12}$$

then eqns. 2 and 6 are obtained by combining eqns. 8 and 9. Expression 1 is obtained by combining expressions 2, 3, 9, 10, 11 and 12. First, the left-hand side of expression 10 is discarded as redundant using eqn. 9. Then eqns. 2 and 3 are combined in turn with expression 10 to yield a pair of inequalities, which are rearranged into the form of expression 1 using expressions 11 and 12. Expressions 4 and 5 are obtained in similar fashion. Eqns. 3 and 7 are simply rearrangements of eqn. 9. None of these manipulations is of the kind that introduces extraneous solutions; therefore, all the compositions consistent with expressions 1–3 and 4–7 are consistent with eqns. 8 and 9.

The validity of obtaining all the mixtures consistent with expressions 8–12 by combining various binary mixtures is demonstrated as follows[6]. Consider the n-dimensional vector space over the real field in which the vectors are the ordered sets $(X_1, X_2, \ldots, X_n)$. The $n-1$ dimensional sub-space of this space, for which $\sum_{i=1}^{n} (P - P_i) \cdot X_i = 0$, contains a unique vector representing each solvent composition consistent with expressions 8–12. The sets of vectors corresponding to the binary mixtures A and B for $n = 3$ (and A, B and C for $n = 4$) are linearly independent and hence are bases of the sub-space. Further, these basis sets are the ones of practical usefulness, since these sets (and only these sets) insure the existence of positive coefficients for all vectors for which $X_i \geqslant 0$, $i = 1, 2, \ldots, n$.

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Note

Study of the interactions between substituted phenols and alcohols and dibenzyl sulphoxide by means of gas chromatography

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It is well established that gas chromatography is useful for the study of the molecular interactions and properties of dilute solutions. Also, in a chromatographic system with mixed stationary phases the molecular interactions can be used to improve the separation efficiency of the column. However, independently of whether gas chromatography is competitive with the more popular spectroscopic methods, it is obvious that the standard states must be very carefuly defined[1–6].

The aim of this work was to study specific interactions in the column with benzyldiphenyl and dibenzyl sulphoxide as the stationary phase and different phenols and alcohols as injected samples. The thermodynamic studies are based on the direct correlation between retention times and partition coefficients[7–10]. For a simple 1:1 association

$$A + B \rightleftharpoons AB \tag{1}$$

the partition coefficient, K_R, of a solute between a mixed stationary phase in which the concentration of the active component (A) is X_A may be written as

$$v^0_{A,S}K_R = v^0_S K^0_R\,[1 + (\psi + K^*)\,X_A] \tag{2}$$

where K^0_R is the partition coefficient of B in the pure solvent S. $v_{A,S}$ is the molar volume of the solution A and S, so that the molar volume varies linearly with the molar fraction. ψ is a factor comprising different molar volumes of a solvent, a solute and an additive, A, and is given by the equation

$$\psi = \frac{v^0_A}{v^0_S} \cdot \frac{\exp v^0_B/v^0_A}{\exp v^0_B/v^0_S} - 1 \tag{3}$$

where v^0_S and v^0_A are molar volumes of pure S and A, respectively. The equilibrium constant, K^*, in eqn. 2 depends on the solvent and can therefore be defined as

$$K^* = \frac{X_{AB}}{X_A X_B} \cdot \frac{\gamma^*_{AB}}{\gamma^*_A \gamma^*_B} \tag{4}$$

The activity coefficient, γ^*_i, of the ith component is defined as $\gamma^*_i \to 1$ as $X_i \to 0$.

From the linear relationship between $v^0_{A,S}K_R$ and X_A (eqn. 2), the equilibrium constant, K^*, can be determined.

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EXPERIMENTAL

All measurements were performed on a Varian 1860 gas chromatograph with a flame-ionization detector. Stainless-steel columns loaded with packing containing 20% of liquid phase were used. The exact amount of liquid phase was determined by a combustion method. The concentration (molar fraction) of dibenzyl sulphoxide (DBSO) in benzyldiphenyl (BDP) varied from 0.00 to 0.25. Measurements were carried out at 363, 373, 383 and 393°K. The true temperature of the column was measured with a precision of ± 0.2°K. A thermocouple (copper–constantan) was introduced into the oven and the temperature was measured with a potentiometer.

The sample sizes were in the range 0.1–0.2 mm³. Within this limit we could not observe any dependence of retention time on the amount of sample. The peaks were symmetrical, so the well known methods were used for the evaluation of specific retention data. As the solutes were solid at room temperature, they were first dissolved in diethyl ether. In all instances on-column injection was employed. Argon was used as the carrier gas at a flow-rate of 60 cm³/min. The flow-rate was measured with a bubble flow meter at the working temperature.

TABLE I

$K_R v^0_{A,S}$ VALUES OF PHENOLS WITH THE SYSTEM OF DBSO IN BDP (dm³)

Solute	X_A											
	363°K						*373°K*					
	0.00	*0.05*	*0.10*	*0.15*	*0.20*	*0.25*	*0.00*	*0.05*	*0.10*	*0.15*	*0.20*	*0.25*
Phenol	376	648	—	904	—	1213	217	308	—	683	—	680
2-Methylphenol	684	988	—	1796	—	2190	356	440	—	898	—	933
4-Methylphenol	766	1245	—	1847	—	2720	451	608	—	1228	—	1342
2-Ethylphenol	1534	2662	3329	4362	5201	6101	1050	1707	2202	2782	3436	3902
3-Ethylphenol	—	—	—	—	—	—	1558	2710	3626	4526	6125	7518
2-Chlorophenol	485	705	1076	1262	1555	1857	359	520	786	831	1095	1260
2-Fluorophenol	169	307	471	663	808	920	124	231	358	455	573	703
3-Fluorophenol	519	1570	2600	4025	5118	6017	374	1019	1674	2606	3326	4148

TABLE II

$K_R v^0_{A,S}$ VALUES OF *n*-ALCOHOLS WITH THE SYSTEM OF DBSO IN BDP (*dm*³)

Solute	X_A											
	363°K						*373°K*					
	0.00	*0.05*	*0.10*	*0.15*	*0.20*	*0.25*	*0.00*	*0.05*	*0.10*	*0.15*	*0.20*	*0.25*
Ethanol	6.6	7.3	7.5	8.2	8.4	9.0	6.8	7.4	7.7	7.9	8.6	9.0
n-Propanol	14	15	16	17	19	20	13	14	14	16	16	18
n-Butanol	27	28	32	34	39	36	23	28	26	28	31	35
n-Pentanol	44	48	53	57	63	66	37	42	43	44	50	55
n-Hexanol	94	86	119	130	159	149	76	86	85	89	110	116
n-Heptanol	234	247	259	281	300	313	168	177	189	183	208	219
n-Octanol	556	604	597	663	688	720	372	426	382	406	486	478
n-Nonanol	1200	1239	1305	1333	1451	1465	777	863	860	825	963	934
n-Decanol	2577	2737	2854	3002	3166	3344	1713	1815	1869	1985	2051	2125

RESULTS AND DISCUSSION

The specific retention volumes of phenols and *n*-alcohols on the column of benzyldiphenyl and on the column of benzyldiphenyl plus different concentrations of dibenzyl sulphoxide were measured at four temperatures. Also, the products of the molar volumes and partition coefficients, $K_R v_{A,S}$, for all solutes were determined. Tables I and II show the results for phenols and *n*-alcohols. It can be seen that $K_R v_{A,S}$ increases with increasing concentration of the active additive.

The data in Table I show that specific interactions between the polar sample and the stationary phase have a considerable influence on the retention volumes. Thus, for a sample with known composition the separation of components can be improved by using a combined stationary phase. In this instance specific interactions have to exist only with components of immediate interest.

However, the data given in Table II demonstrate that the main reason for the greater specific retention volumes at greater molar fraction of the active additive under the conditions given is the chain length of alcohols and not the specific interaction.

383°K						*393°K*					
0.00	*0.05*	*0.10*	*0.15*	*0.20*	*0.25*	*0.00*	*0.05*	*0.10*	*0.15*	*0.20*	*0.25*
160	177	—	364	—	481	63	122	—	—	—	183
184	259	—	441	—	533	99	105	—	—	—	306
224	349	—	479	—	690	118	212	—	—	—	346
723	932	1505	1804	2188	2545	428	580	870	1057	1557	1629
1025	1670	2579	2829	3753	4326	603	861	1328	1615	1951	2301
246	343	516	580	685	843	164	202	287	381	475	570
95	158	249	307	362	480	65	94	154	213	267	328
275	604	1255	1532	2032	2678	171	326	780	788	1412	1535

383°K						*393°K*					
0.00	*0.05*	*0.10*	*0.15*	*0.20*	*0.25*	*0.00*	*0.05*	*0.10*	*0.15*	*0.20*	*0.25*
6.7	7.1	7.0	8.0	8.5	8.6	6.8	7.2	7.4	8.0	8.1	8.5
10	11	11	12	13	13	9.9	11	11	11	12	13
18	20	22	22	23	25	13	15	15	15	18	18
27	30	32	32	36	37	22	24	25	26	30	31
52	62	58	65	80	77	41	40	46	47	59	58
102	102	114	127	127	130	90	100	90	97	110	115
255	271	289	262	325	320	181	184	173	213	216	216
427	470	495	531	491	528	353	407	345	346	421	410
955	1022	998	1056	1160	1170	703	737	779	769	817	820

The plots of $K_R v^0_{A,S}$ *versus* X_A for 2-fluorophenol are shown in Fig. 1, and are straight lines, in agreement with our prediction that only the 1:1 complex can be formed.

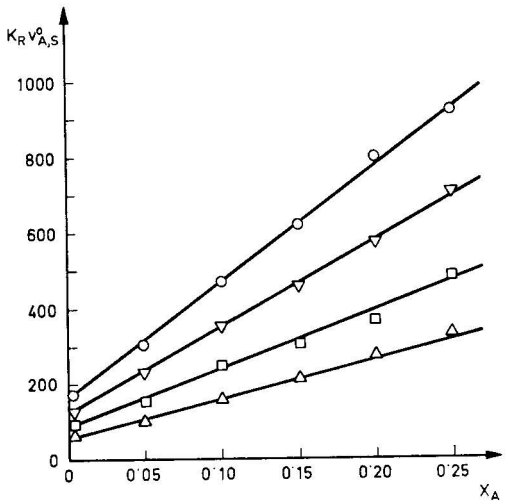

Fig. 1. Graph of $K_R v^0_{A,S}$ *versus* X_A for 2-fluorophenol on the column of DBSO in BDP. ○, 363°K; △, 373°K; □, 383°K; △, 393°K.

From these lines, the equilibrium constants, K^*, are derived, and the results for *n*-alcohols and phenols are given in Tables III and IV. The values in Table III are of the same order of magnitude and as the relationships between the retention values of two neighbours are also virtually identical, it can be concluded that specific interactions between the alcohols and dibenzyl sulphoxide have no significant influence on retention values.

It follows from Table IV that the equilibrium constants for phenols are higher than those for *n*-alcohols. The equilibrium constant demonstrates the influence of the additive added to the stationary phase on the retention value. It is instructive to

TABLE III

VALUES OF K^* FOR *n*-ALCOHOLS WITH THE SYSTEM OF DBSO IN BDP

Sample	K^*			
	363°K	*373°K*	*383°K*	*393°K*
Ethanol	1.61	1.45	1.31	1.18
n-Propanol	1.94	1.69	1.50	1.33
n-Butanol	2.08	1.88	1.75	1.68
n-Pentanol	2.15	1.93	1.80	1.66
n-Hexanol	2.43	2.20	2.03	1.83
n-Heptanol	1.44	1.31	1.20	1.10
n-Octanol	1.25	1.21	1.10	1.04
n-Nonanol	0.94	0.86	0.76	0.71
n-Decanol	1.18	1.05	0.94	0.87

TABLE IV

VALUES OF K^* FOR PHENOLS WITH THE SYSTEM OF DBSO IN BDP

Sample	K^*			
	363°K	*373°K*	*383°K*	*393°K*
Phenol	9.25	8.67	8.16	7.78
2-Methylphenol	8.94	8.02	7.70	7.02
4-Methylphenol	10.32	9.26	8.46	7.82
2-Ethylphenol	11.62	11.10	10.39	9.93
3-Ethylphenol	—	12.81	11.84	11.29
2-Chlorophenol	11.15	10.37	9.54	8.92
2-Fluorophenol	20.22	18.29	16.37	15.23
3-Fluorophenol	44.41	39.55	35.12	32.02

compare *o*-chlorophenol and *m*-fluorophenol, the $K_R V^0_{A,S}$ values of which on pure benzyldiphenyl are virtually identical, but the difference in the equilibrium constant at 363°K is 33.26. The reason for the lower values for *ortho* substituents is that they can form intramolecular hydrogen bonds, whereas *meta* and *para* substituents cannot.

In conclusion, it has been shown that the specific interactions between a polar sample and the stationary phase can influence the retention parameters. The equilibrium constants can be determined from partition coefficients. The greater the equilibrium constant, the greater is the effect of specific interactions on the resolution of the components.

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Note

Operational parameters of anion-exchange chromatography using AG MP-1 resin for rapid assay of adenine nucleotides

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A conventional anion-exchange chromatography for adenine nucleotides using AG MP-1 resin has been described previously[1]. This system is not only simple but also has many potential applications. Among the applications are assays for enzymatic reactions involving adenine nucleotides[2], the determination of adenylate charge[3] and further extension for the analysis of other nucleotides. The operational parameters which may modify and optimize this system are described in the report.

EXPERIMENTAL

Materials

AG MP-1 anion-exchange resin (Cl^-, 200–400 mesh) was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). It contained 55–65% of water and had a total capacity of 4.2 mequiv./g dry weight. Adenine nucleotides were obtained from ICN Pharmaceuticals (Irwine, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.).

Determination of apparent affinity

An exact amount of the resin (100 mg) was weighed and packed into a column. A neutral nucleotide solution containing 2 m*M* was then slowly pumped through the column until the effluent solution contained the same concentration of the nucleotide as the original solution. The column was then washed with water to remove the free nucleotide. The total nucleotide bound to the resin was eluted out with 5 ml of 0.3 *N* HCl and was measured by reading the absorbance at 257 nm.

Determination of distribution coefficient

The chromatography was performed essentially according to the procedure described previously[1], except that the scanning was done on Gilson Holochrome fitted with a 40-μl flow cuvette of 10-mm light path. A 2-g amount of the resin was packed with water in a small column without any pretreatment. It gave a packed volume of 1.7 ml/g of the resin. After washing the column with water, 0.1 μmole of each nucleotide was absorbed into the bed. The column was then eluted with water at a flow-rate of 2 ml/min using a peristaltic pump. After 10 min, the upper column water

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was replaced with different concentrations of HCl and eluted with the same. The total elution volume from the start of elution to the peak of each nucleotide was determined from the recorded chromatogram and the flow-rate.

The distribution coefficient was calculated and the operational parameters were evaluated[4–6].

RESULTS

Apparent exchange capacity for adenine nucleotides

The apparent affinities for AMP, ADP and ATP were 2.1, 2.5, and 1.8 mmoles/g dry resin. Approximately 1 mole of the adenosine nucleotide could exchange with 2 moles of Cl^-.

Distribution coefficients and HCl concentrations

The distribution coefficient (K_d) was calculated from the retention volume (V_R) of each peak and void volume (V_0) according to the equation, $V_R = V_0 + mK_d$, where m is the weight of resin. The void volume was determined by the retention volume of adenosine which was not retained by the resin, and the volume did not change with the concentration of HCl. It was 2.1 ml for the 2-g resin column used. The distribution coefficients at different HCl concentrations are shown in Fig. 1. It is clear that adenosine nucleotides can be separately eluted out from the column at different HCl concentrations. Therefore, it is suggested that proper selection of stepwise elution mode is more advantageous than the gradient elution as previously described[1].

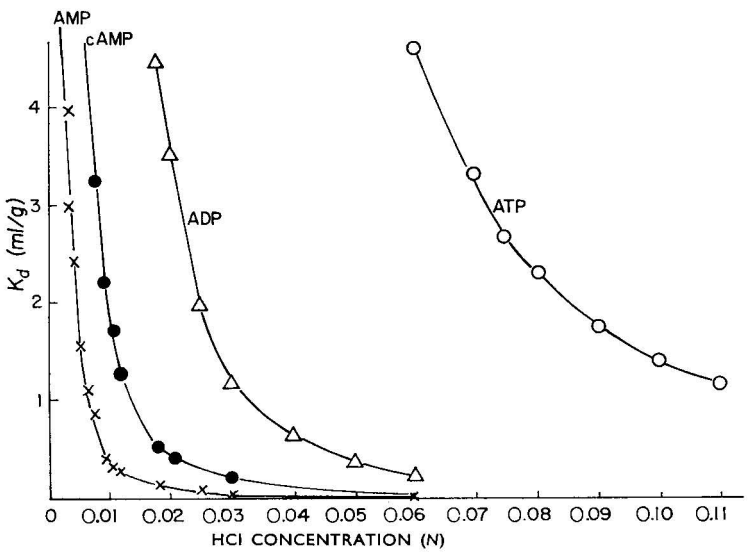

Fig. 1. The distribution coefficient (K_d) *versus* HCl concentrations.

Distribution coefficient and band broadening

The broadening of each nucleotide band measured as the peak width at half height was proportional to the distribution coefficient. The results are shown in Fig. 2. At the operational range of distribution coefficients 0.2–8, the relationship

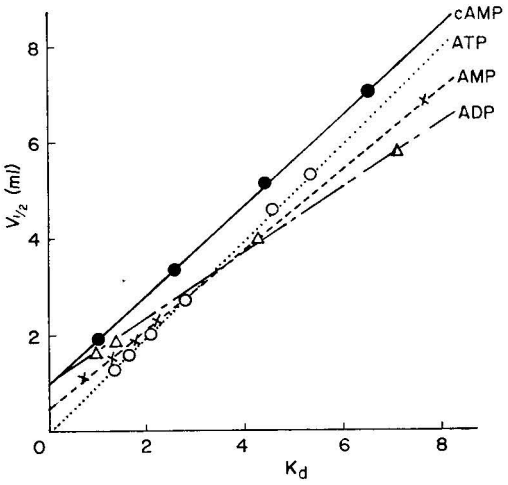

Fig. 2. The width at half height peak *versus* distribution coefficient.

between the volume at half height of peak ($V_{\frac{1}{2}}$) and the distribution coefficient for each nucleotide can be expressed by the following equations:

$$V_{\frac{1}{2}} \text{ for AMP} = 0.4 + 0.8\,K_d$$
$$V_{\frac{1}{2}} \text{ for cAMP} = 0.94 + 0.92\,K_d$$
$$V_{\frac{1}{2}} \text{ for ADP} = 0.9 + 0.68\,K_d$$
$$V_{\frac{1}{2}} \text{ for ATP} = K_d$$

If K_d is known the maximum absorbance at the peak ($A_{\text{max.}}$) can be estimated from the equation as previously described[1].

$$A_{\text{max.}} = \frac{\text{nmoles nucleotide} \times \varepsilon_{257}}{V_{1/2} \times 1066}$$

Since the Gaussian peak width expressed in volume (V_b) is related to the variance of the peak (σ), $V_b = 4\sigma$, and $V_{\frac{1}{2}} = 2\sigma\sqrt{2 \ln 2}$, the total volume of each peak is equivalent to $1.7\,V_{\frac{1}{2}}$.

The performance of the column is therefore calculated from the above relationship according to the equation:

$$N = \frac{16V_R^2}{V_b^2} = \frac{5.545V_R^2}{V_{1/2}^2}$$

At $K_d = 1$, the numbers of theoretical plates (N) for AMP, cAMP, ADP and ATP are 65, 25, 37 and 93 at a flow-rate of 2 ml/min, respectively; corresponding to 1640, 632, 935 and 2340 plates per meter, respectively.

Resolution

In a previous paper[1] the separation of AMP and cAMP and cAMP was accomplished by a very mild HCl gradient. Fig. 3 shows that they were easily separated by an isocratic elution at various HCl concentrations. The experimental results were compiled in Table I. The resolution (R_s) of two peaks was calculated from the equation $R_s = \Delta V_R / \overline{V}_b$, where ΔV_R is the retention volume difference, and $\overline{V}_b$ is the mean peak width. At HCl concentrations higher than 0.012 N, $R_s < 1$, the resolution was incomplete using a column bed of 40 × 9 mm. By increasing the bed height to 60 mm, using 3 g resin, they were completely separated.

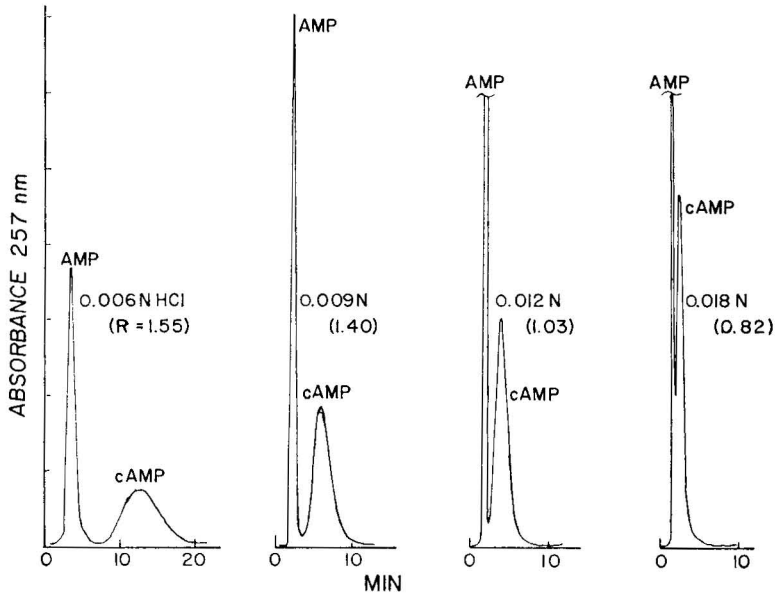

Fig. 3. Chromatographic profile for the separation of AMP and cAMP by isocratic elution at different concentrations of HCl. The column size was 40 × 9 mm.

The numbers of theoretical plates required to give $R_s = 1$ are also given in Table I. They were calculated according to eqn. 1:

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{K'}{1 + K'}\right)\sqrt{N} \qquad (1)$$

where R_s is the separation factor of the two peaks. α is the ratio of the two K_d values, and K' is the average of the two K_d values.

TABLE I

COLUMN PARAMETERS OBTAINED FOR AMP AND cAMP

HCl (N)	*Nucleotide*	$V_R(ml)$	$K_d(_{ml/g})$	$V_{\frac{1}{2}}(ml)$	N	R_s	$N_{req.}$*
0.006	AMP	6.4	2.15	2.13	50	1.55	35
	cAMP	23.8	10.85	11.10	26		
0.0075	AMP	4.70	1.30	1.45	58	1.48	39
	cAMP	15.30	6.55	7.00	26		
0.009	AMP	3.58	0.74	1.16	53	1.40	44
	cAMP	11.00	4.45	5.11	26		
0.012	AMP	3.30	0.55	0.86	70	1.09	69
	cAMP	7.23	2.57	3.37	26		
0.018	AMP	2.48	0.19	0.55	112	0.82	92
	cAMP	4.19	1.05	1.91	26		

*$N_{req.}$ = the number of theoretical plates calculated according to eqn. 1 by assuming $R_s = 1$.

DISCUSSION

Because the exchange capacity of the resin is high, and K_d values of AMP, ADP and ATP vary greatly with HCl concentration, these three nucleotides can be easily separated on a small column by a stepwise elution using different concentrations of HCl. The HCl concentration determines the retention time and the spreading of the peak. Thus, the speed and the sensitivity of the analysis increase. The mode of elution can be therefore favorably selected according to the relative contents of the three nucleotides in a solution to be analysed. Several examples are illustrated as follows.

If the relative amounts of AMP, ADP and ATP of a solution do not vary greatly, the analysis is better accomplished by a three-step elution. Choosing HCl at higher concentration to give a lower K_d at each step not only facilitates the analysis but also increases the sensitivity. For instance, if each HCl concentration is selected at a K_d smaller than 1, the retention volume for each nucleotide is less than 4 ml, and the peak width at half height of the peak is less than 2 ml for a column size of 40 × 9 mm. The values of these parameters can be estimated from Figs. 1 and 2, or calculated from the equations given in the text. At a flow-rate of 2 ml/min, the total analysis can be accomplished in less than 10 min. At the same time as little as 100 pmoles of each nucleotide can be determined by the system described. Thus, the sensitivity and speed can be comparable with that of high-performance liquid chromatography. However, this system is much more convenient and the sensitivity may be higher because the loading capacity is much higher in this system.

For the analysis of a solution containing the nucleotides in high disproportion, a two-step elution mode can be considered. For instance, if the amount of AMP is very small, AMP and ADP can be eluted out in one step with an HCl concentration

which elutes AMP out at near void volume (low K_d), and ATP at high K_d. By the same token, if the ADP content is much smaller than that of ATP, after AMP is eluted from the column, ADP and ATP are eluted out in one step by the similar principle. This one- step elution principle is, therefore, favorable for the assay of the initial rate of many enzyme reactions involving ATPase and many kinases which yield ADP or AMP as a product. For a reaction which produces ATP, after ADP or AMP is eluted at a relatively low HCl concentration, ATP is eluted at a high HCl concentration.

AMP and cAMP have close K_d values; however, their difference is still large enough to favor an isocratic elution. As shown in Table I, the ratio of the K_d values (α) did not vary with HCl concentration. The resolution is improved by either using a lower HCl concentration to give a large K value or a longer column to increase the number of theoretical plates. It is clear from Fig. 3 that using a column resin bed of 40 $\times$ 9 mm and 0.009 M HCl as eluent is suitable for the assay of cAMP phosphodiesterase.

In short, this system can be easily modified and optimized by proper selection of HCl concentration, mode of elution and column size for assays of nucleotides.

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Note

Separation of tunicamycin homologues by reversed-phase high-performance liquid chromatography

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Tunicamycin is a naturally occurring antibiotic which inhibits N-acetylglucosamine-1-phosphate transferase and thereby blocks the production of N-acetylglucosamine-pyrophosphoryldolichol[1]. This event culminates in the synthesis of underglycosylated glycoproteins by preventing the formation of intermediates necessary for the synthesis of N-glycosidic linkages[2,3]. As such, tunicamycin has become a valuable experimental tool for the study of the biological role of the carbohydrate moiety (of the N-glycosidic type) of glycoproteins and the pathway leading to the formation of lipid-linked oligosaccharides[3,4].

It has been suggested that tunicamycin acts as a substrate analogue of UDP-N-acetylglucosamine and functions as either a competitive inhibitor[5,6] or a non-competitive inhibitor of N-acetylglucosamine-1-phosphate transferase[7]. An appealing argument has also been put forth suggesting that tunicamycin may act as a multi-substrate analogue at the transition state[6]. In addition, tunicamycin has been reported to inhibit protein synthesis to a variable degree depending upon the system of study[8–11]. At times this duality has caused difficulty in the interpretation of results[8].

Recently, we reported that tunicamycin was not a single compound but consists of at least 10 homologues which can be separated by reversed-phase high-performance liquid chromatography (HPLC)[12]. When two of these homologues were tested for biological activity one exhibited very little influence on protein synthesis while the other inhibited protein synthesis by 50% when both displayed their maximum degree of inhibition of protein glycosylation. This difference in biological activities, although not found universally with each cell line or type examined[13,14], has demonstrated the desirability of obtaining pure homologues of tunicamycin.

This paper describes the separation of the tunicamycin homologues by reversed-phase HPLC using volztile solvents to simplify the recovery of individual components. We also propose a simple system to designate the homologues based upon the combination of their molecular weights and the order of their elution from a reversed-phase resin.

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MATERIALS AND METHODS

Chemicals

Glass-distilled methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Purified tunicamycin was a generous gift from Dr. R. Hamill, Eli Lilly, Indianapolis, IN, U.S.A. (Lot 361-26E-79A).

High-performance liquid chromatography

Separation of tunicamycin components was carried out using reversed-phase HPLC[12]. HPLC was performed using a Varian Model 5000 high-performance liquid chromatograph equipped with a Rheodyne Model 7125 sample injector with a 100-μl sample loop, a Vari-Chrom detector operating at a wavelength of 260 nm, a Linear Model 260/MM chart recorder, and a 25 $\times$ 0.46 cm I.D. LiChrosorb C- 5-μm column (Brownlee Labs., Santa Clara, CA, U.S.A.). The HPLC separation was achieved using an isocratic solvent system of 68 or 80% methanol in twice glass-distilled water. Tunicamycin components were quantitated using a Hewlett-Packard 3380 S recording integrator.

Thin-layer chromatography

Analytical C_{18} reversed-phase thin-layer chromatography (TLC) plates ($MKC_{18}F$) containing a fluorescent label (F-254) were purchased from Whatman (Clifton, NJ, U.S.A.; Lot 000444). Chromatograms were developed at room temperature using an eluent consisting of methanol–water (3:1).

RESULTS

The separation of the naturally occurring homologues of tunicamycin was achieved by reversed-phase chromatography on either TLC plates or by HPLC. On TLC plates, using 10 μg of tunicamycin, four spots could be identified by absorbance of fluorescence (chromatogram not shown). The four factors corresponding to the four major molecular weight classes of tunicamycin were designated by the letters A, B, C, and D, with D being the closest to the origin. Table I presents the R_F values and the molecular weights and formulae for the tunicamycin factors.

TABLE I

PHYSICAL CHEMISTRY AND TLC OF TUNICAMYCIN FACTORS

Molecular weights and formulae are provided for the unsaturated form of each tunicamycin factor.

Factor	R_F*	*Molecular formulae***	*Molecular weight***
A_0	—	$C_{36}H_{59}N_4O_{16}$	804
A	0.31	$C_{37}H_{61}N_4O_{16}$	818
B	0.24	$C_{38}H_{63}N_4O_{16}$	832
C	0.16	$C_{39}H_{65}N_4O_{16}$	846
D	0.10	$C_{40}H_{67}N_4O_{16}$	860

* R_F values were determined on C_{18} thin-layer plates developed using methanol–water (3:1).

** Physical data provided by Dr. R. Hamill and subsequently confirmed (data not shown).

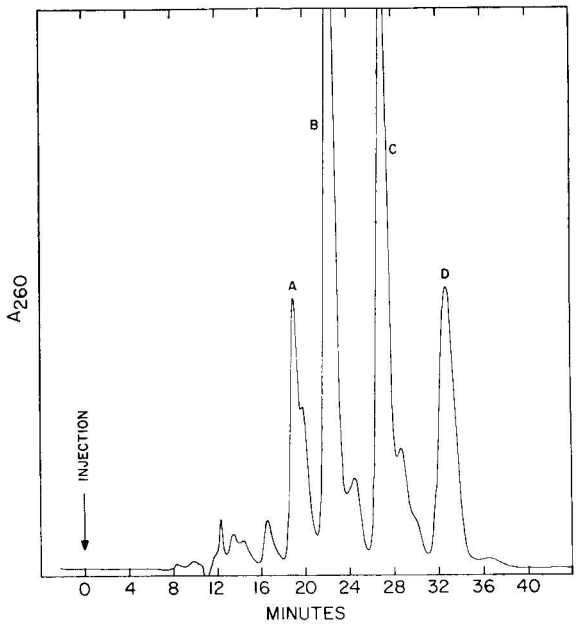

Fig. 1. HPLC chromatogram illustrating the resolution of the homologues of tunicamycin. Sample, 100 μg of tunicamycin dissolved in methanol–water (7:3); chart speed, 1 cm/min; pressure, 80 atm; temperature, 40°C; flow-rate, 1 ml/min. The chromatogram was developed isocratically using methanol–water (4:1).

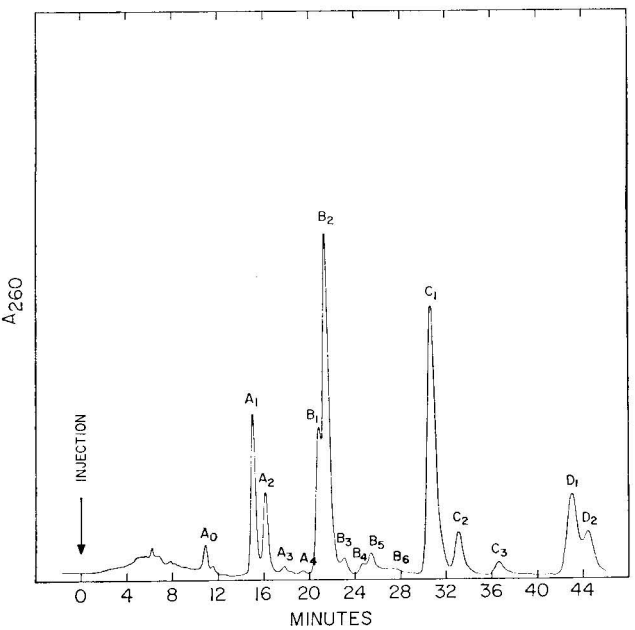

Fig. 2. HPLC chromatogram illustrating the typical resolution of the homologues of tunicamycin using methanol–water (68:32). Sample, 100 μg of tunicamycin dissolved in methanol–water (7:3). Pressure, 68 atm; chart speed, 1 cm/min, temperature, 40°C; flow-rate, 1 ml/min.

Upon analysis by HPLC using a solvent system consisting of methanol–water (4:1), four major factors could again be identified. However, there was clear additional heterogeneity within each factor (Fig. 1). When this analysis was repeated using only 68% methanol several components within each factor were identified. Fig. 2 shows a typical chromatogram. Table II presents the chromatographic data and the yield of each homologue. Increasing the temperature above 40°C decreases the separation of A_1 from A_2 and B_1 from B_2 although it speeds the analysis. By collecting B_1, B_2, D_1, and D_2 and resubjecting this mixture to HPLC using 55–60% methanol these individual components can be isolated in pure form.

TABLE II

CHROMATOGRAOHIC AND YIELD DATA FOR TUNICAMYCIN HOMOLOGUES ON C_8 REVERSED-PHASE HPLC

Flow-rate, 1 ml/min at 40°C.

Homologue	*Relative movement*	*Capacity factor (k')* *	*Yield (μg)* **	*Previous nomenclature*[12]
A_0***	1.00	2.6	0.20	
A_1	1.36	4.0	0.95	I
A_2	1.43	4.4	0.56	II
A_3	1.54	4.9	0.08	
A_4	1.65	5.5	0.05	
B_1	1.74	5.9	11.22	
B_2	1.78	6.1	33.09	IV
B_3	1.89	6.7	2.53	
B_4	2.00	7.2	1.76	
B_5	2.05	7.4	3.63	V
B_6	2.18	8.2	0.01	
C_1	2.39	9.2	33.28	VI
C_2	2.56	10.0	0.59	VII
C_3	2.80	11.2	0.24	VIII
D_1	3.21	13.3	11.26	IX
D_2	3.30	13.8	0.65	X

* Capacity factor: $k' = (t_R - t_0)/t_0$, where t_R is the sample retention time and t_0 is the time required for non-retained material to pass through the column.

** Yield of individual homologues using a starting injection of 100 μg of purified tunicamycin.

*** See Discussion for details of nomenclature.

DISCUSSION

We previously identified ten homologues within the tunicamycin family by HPLC and found each to be active in inhibiting N-acetylglucosamine-1-phosphate transferase, although differences were found when the biological activities of A_1 and A_2 were compared[12]. The method presented here provides higher yields of each homologue and allows easy recovery from volatile solvents using lyophilization, with no loss of biological activity. The relative amounts of the individual factors and their respective components appear to vary with lot number and thus apparently with the growth conditions of the producing strains of Streptomyces. So rather than having two major components as previously reported[12], in most preparations of tunicamycin

there are four major molecular weight classes consisting of between two and six minor components of variable amount.

Since the tunicamycin factors elute from a reversed-phase column in the order of increasing molecular weight, we propose that each factor (molecular weight class) be identified with the letters A, B, C, and D in the order of elution. We also suggest that components within the same general molecular weight class be identified by the subscript 1, 2, 3, etc., also in the order of elution from reversed phase (Table II).

The differences between factors A, B, C, and D have been explained by the presence of four different carbon chain lengths within the lipid moiety of the antibiotic (Table I)[15]. Recently, Takatsuki *et al.*[16] have shown that more than ten different fatty acids can be identified as components of tunicamycin. Although it is not yet known what the structure of the lipid moiety is from each of the homologues isolated by HPLC, we are currently working toward this goal in addition to further investigating the biological differences between tunicamycin homologues.

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Note

Rapid and sensitive assay of tyrosine 3-monooxygenase activity by high-performance liquid chromatography using the native fluorescence of DOPA

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Recent studies on the biosynthesis of catecholamines in the nervous system and chromaffin cells of the adrenal medulla have called for a sensitive assay of tyrosine 3-monooxygenase (tyrosine hydroxylase, E.C. 1.14.16.2) activity, *i.e.*, the first and probably rate-limiting step of the pathway [for a review, see ref. 1]. Radiochemical methods, using either ^{14}C- or ^{3}H-labelled L-tyrosine as the substrate, have generally been considered the most accurate[2], but high-performance liquid chromatography (HPLC) with electrochemical detection has more recently been found convenient for the assay of dihydroxyphenylalanine (DOPA)[3,4]. Owing to the inherent problems of lifetime and maintenance of the electrochemical detector[5], we have based our detection of biogenic amines on measurement of their native fluorescence using a sensitive spectrofluorimeter equipped with a 20-μl flow-through cell[6–9]. Although the native fluorescence of DOPA has generally been considered too weak for the assay of tyrosine 3-monooxygenase activity in biological material[10], we have found it very useful in combination with HPLC. In the present study it is shown that the assay of DOPA by HPLC and fluorescence detection gives a sensitivity comparable to that of the electrochemical detector[3,4] and, owing to its simpler experimental approach, our method can more easily be applied to automated analyses to meet the special requirements of multiple analyses. Further, the method requires no particular maintenance, as is the case with the electrochemical detector. Finally, the published HPLC procedures for the assay of tyrosine hydroxylase activity have been found to be unsatisfactory in one or more of the following respects: (1) the procedures are laborious and time consuming; (2) they cannot be applied to crude biological materials owing to interference from endogenous substances of that material, notably catecholamines; and (3) they require preliminary clean-up of the sample before HPLC analysis can be performed.

EXPERIMENTAL

Materials

L-Tyrosine was obtained from Koch-Light (Colnbrook, Great Britain) and benzyloxyamine (*o*-benzylhydroxylamine hydrochloride), 6,7-dimethyltetrahydropterin and 2-(N-morpholinoethane)sulfonic acid (MES) from Sigma (St. Louis, MO, U.S.A.). Other reagents (analytical-reagent grade) were supplied by E. Merck (Darmstadt, G.F.R.).

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Subcellular fractions of bovine adrenal homogenates were prepared by centrifugation in 0.25 *M* sucrose at 0–5°C[11].

HPLC analysis

The high-performance liquid chromatograph with a fluorescence detector and integrator (Hewlett-Packard Model 3380A) has previously been described in detail[6–8]. The chromatographic separation was achieved at ambient temperature on a sulphonated fluorocarbon polymer coated on a pellicular silica support (Zipax SCX from DuPont, Wilmington, DE, U.S.A., dry-packed in a 50.0 cm × 3 mm I.D. stainless-steel tube) with a theoretical plate number of about 1000/m. A short precolumn (40 × 2 mm I.D. stainless-steel tube) packed with pellicular silica (HC Pellosil from Whatman, Maidstone, Great Britain) was used to protect the cation-exchange column. The mobile phase, consisting of 10 m*M* acetate buffer (pH 3.70) with 1% (v/v) of propanol, was pumped at a flow-rate of 1.5 ml/min (890 p.s.i.).

Assay of tyrosine 3-monooxygenase activity

Tyrosine 3-monooxygenase activity was assayed at 30°C as described by Nagatsu[2], with the following modifications. The total volume of the incubation mixture was 100 μl, containing 40 m*M* MES buffer (pH 6.0), 0.3 m*M* L-tyrosine and 0.1 m*M* benzyloxyamine in order to inhibit any aromatic-L-amino-acid decarboxylase (DOPA decarboxylase, E.C. 4.1.1.28) activity present in the crude enzyme preparation[12].

The reaction was stopped after 10–20 min by the addition of an equal volume of ice–cold ethanol, containing glacial acetic acid to pH 4.1. This mixture was allowed to stand for 30 min in an ice–water bath, after which it was centrifuged at 10,000 rpm for 5 min (Eppendorf Model 5412 Microfuge). Samples (10–50 μl) of the supernatant were injected directly into the liquid chromatograph.

Preparation of standards for HPLC analyses

Standards of L-tyrosine and DOPA were dissolved in 10 m*M* acetate buffer (pH 3.70) (mobile phase solvent), and their concentrations were determined spectrophotometrically using the known molar absorptivities, *i.e.*, $\varepsilon = 1.42$ mmol l^{-1} cm^{-1} at 274.5 nm[13] and $\varepsilon = 2.63$ mmol l^{-1} cm^{-1} at 280.0 nm[14] for tyrosine and DOPA, respectively.

Protein determination

Protein was determined according to the procedure described by Bradford[15].

RESULTS

Fluorescence properties of L-tyrosine and DOPA

The uncorrected fluorescence excitation and emission spectra of DOPA and L-tyrosine were obtained by injection of the compounds directly into the flow-through cell of the spectrofluorimeter. The spectrum of DOPA ($\lambda_{ex} = 281$ nm and $\lambda_{em} = 314$ nm) and of tyrosine ($\lambda_{ex} = 274$ nm and $\lambda_{em} = 304$ nm) were obtained with very little contribution from the solvent.

Chromatographic conditions

A sulphonated fluorocarbon polymer coated on pellicular silica support was selected for the separation of L-tyrosine and DOPA using 10 m*M* sodium acetate buffer (pH 3.70) with 1% (v/v) of propanol as the mobile phase. Fig. 1a shows that the chromatographic conditions selected allow the complete separation of DOPA (retention time, t_R = 1.03 min) from L-tyrosine (t_R = 1.55 min), and this separation was obtained even in the presence of a 100-fold higher concentration of L-tyrosine. Noradrenaline and adrenaline were eluted in this system with long retention times of *ca.* 10 and 40 min, respectively.

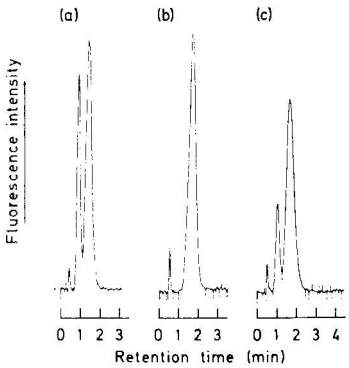

Fig. 1. (a) Chromatogram of 384 pmol of DOPA (t_R = 1.03 min) and 1.92 nmol of L-tyrosine (t_R = 1.55 min). (b) and (c) chromatograms of an acidic ethanol extract of an incubation mixture in the assay of tyrosine 3-monooxygenase activity of bovine adrenal medulla microsomes; (b) zero-time control with a single peak of L-tyrosine (t_R = 1.55 min), and (c) the formation of DOPA (t_R = 1.03 min) following a reaction period of 20 min. Volumes of 20 μl of the diluted (twice) incubation mixture were injected into the liquid chromatograph; λ_{ex} = 281 nm and λ_{em} = 314 nm.

A linear relationship was obtained between the amount of DOPA injected and the integrated peak area (r = 0.99) or the peak height (data not shown). The limit of detection was about 5 pmol of DOPA (signal-to-noise ratio = 3).

Assay of tyrosine hydroxylase activity in subcellular fractions of the bovine adrenal medulla

The useful application of HPLC to the assay of DOPA in crude biological material is shown by the assay of tyrosine 3-monooxygenase (tyrosine hydroxylase E.C. 1.14.16.2) activity in subcellular fractions of the bovine adrenal medulla. From Fig. 1c it can be seen that about 3.48 nmol min^{-1} of DOPA per milligram of protein is formed when the microsomal fraction is the enzyme source. All subcellular fractions revealed tyrosine 3-monooxygenase activity, but the specific activity was found to be highest in the microsomal fraction (data not shown).

DISCUSSION

HPLC with electrochemical detection has recently been introduced for the rapid and sensitive assay of DOPA formed in the tyrosine 3-monooxygenase (tyrosine

hydroxylase, E.C. 1.14.16.2) reaction[3,4]. However, owing to the non-selectivity of this detector, working with crude extracts of biological materials, as well as the rapid deterioration of the electrode and problems with its maintenance[5], an alternative method for the detection of biogenic amines has been developed in this laboratory[6–9]. In this study it was found that the native fluorescence of DOPA is sufficiently strong for its detection by HPLC at acidic pH, in good agreement with our recent finding for catecholamines[6–8].

The main advantages of HPLC with fluorometric detection are its sensitivity, higher selectivity, reliability and precision[6–9]. With DOPA, the limit of detection is about 5 pmol at a signal-to-noise ratio of 3, which is comparable to that of electrochemical detection with a reported lower limit of about 5 pmol of DOPA formed enzymically[4]. Fluorescence detection is, however, more specific than electrochemical detection, as DOPA has unique fluorescence properties in the UV region. As already stressed[6–9], the fluorimetric detector has no problems with maintenance and is particularly useful in combination with an automatic injector. As in most HPLC techniques, the present method for the assay of DOPA has a high precision (better than $\pm 1\%$ relative standard deviation). The short time required for analysis and the simple procedure generally followed make the method very convenient for routine analysis and automation.

The chromatographic system selected is a modification of that described by Blank and Pike[3]. Using the original perchlorate solvent system of Blank and Pike[3] and a flow-rate of 1.5 ml/min, we found considerable interference in the assay of DOPA (t_R = 1.01 min) by catecholamines [noradrenaline (NA) and adrenaline (A)] present in the biological material being analysed [t_R (NA) = 0.89 min and t_R (A) = 2.13 min]. However, by using the acetate buffer as the solvent system, the retention times were 1.03 min (DOPA), *ca.* 10 min (NA) and *ca.* 40 min (A). Further, the acetate solvent system also increases the lifetime of the column.

It should be mentioned that the sensitivity of this HPLC assay of DOPA may be increased even further, *e.g.*, by using a flow cell of slightly larger volume and surface area, with only a slight loss in chromatographic resolution. Finally, the cost of this method, in terms of reagents and work, is lower than that for any assay of tyrosine 3-monooxygenase activity published so far.

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CHROM. 13,051

Note

Rapid high-performance liquid chromatographic determination of amino acids in synaptosomal extracts

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(First received May 12th, 1980; revised manuscript received June 18th, 1980)

Amino acids can be analyzed by high-performance liquid chromatography (HPLC) using a variety of systems and derivatization methods[1–10].

The purpose of the present investigation was to develop a simple and rapid assay for determination of amino acids that are substrates and products of enzymes engaged in glutamate and γ-aminobutyric acid (GABA) metabolism, such as aspartate amino transferase (E.C. 2.6.1.1), alanine amino transferase (E.C. 2.6.1.2), glutaminase (E.C. 3.5.1.2), glutamate dehydrogenase (E.C. 1.4.1.2), glutamate decarboxylase (E.C. 4.1.1.15) and GABA transferase (E.C. 2.6.1.19).

Earlier studies have focused on samples containing all amino acids and the analysis was completed approximately within 1-h intervals. Here we report a more rapid determination of glutamic acid (Glu), glutamine (Gln), aspartic acid (Asp), alanine (Ala) and GABA in the presence of the amino acids usually found in synaptosomes[11]. The time of determination was lowered to 10 min and, thus, four analyses can be performed per hour.

Separation and detection of the amino acid *o*-phthalaldehyde (OPA) derivatives can be achieved with sufficient resolution and sensitivity to follow uptake and formation of Glu, Gln, Asp, Ala and GABA by isolated synaptosomes. The separation was carried out on a reversed-phase (C_{18}) column using a gradient-elution (methanol–potassium acetate buffer) procedure.

EXPERIMENTAL

HPLC was carried out on a Spectra-Physics Model 8000 liquid chromatograph with a gradient accessory and helium degassing system. An autoinjector (CV-6-UHPa-N60); Valco Instrument, Houston, TX, U.S.A.) with 10-μl loop was used. The column effluent was monitored by an Aminco Fluoro-Colorimeter (American Instrument, Silver Spring, MD, U.S.A.) equipped with a 9-μl flow-through cell operated at an excitation wavelength of 340 nm (7-60 Corning filter) and an emission wavelength of 455 nm (2A Wratten cut-off filter). The detector was fitted with a Supergrator 2 computing integrator (Columbia Scientific Industries, Austin, TX, U.S.A.) and a Varian G-4000 one-pen chart recorder. A Normaton Spherosil XOA 600, C_{18} column (10 cm $\times$ 4 mm I.D.), particle size 5 μm (Prolabo, Paris, France), was used.

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Reagents and chemicals

The mobile phase was composed of 0.1 *M* potassium acetate, pH 5.50, and methanol (HPLC grade; Rathburn, Walkerburn, Great Britain). Individual amino acid standards were obtained through Sigma (St. Louis, MO, U.S.A.). A stock solution of each amino acid was prepared by dissolving it in 10 m*M* HCl to provide a concentration of 100 μmol/ml. Standard solutions containing 200 nmol/ml of each amino acid were prepared from the stock solutions by dilution with water. Gln stock solution was freshly prepared every day. The solutions were stored at 4°C. All other reagents were of analytical reagent grade and used without further purification.

The derivatization reagent was prepared by dissolving 10 mg *o*-phthalaldehyde (Fluoropa; Durrum, Palo Alto, CA, U.S.A.) in 500 μl absolute ethanol. To this solution 500 μl of 2-mercaptoethanol were added and then diluted up to 10 ml with 0.4 *M* boric acid (adjusted to pH 10.4). To maintain the reagent strength, 50 μl 2-mercaptoethanol were added every 2–3 days[10], and the solution was kept under nitrogen.

An ethanol extract of rat brain synaptosomes[12], which was incubated for 2 min at 37°C with 2 m*M* Gln[13], was used for the determination of amino acids.

Derivatization

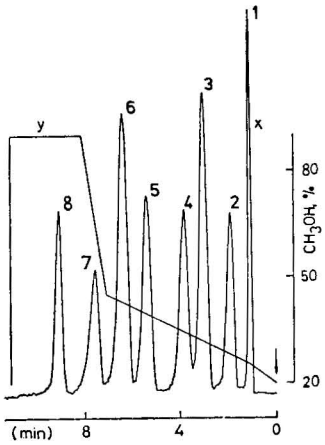
One volume (5 μl) of amino acid standard solution or synaptosomal extract was mixed with two volumes (10 μl) of the derivatization reagent solution in 100-μl Reacti-vials (Pierce, Rockford, IL, U.S.A.). The contents were mixed and injected after 90 sec at room temperature.

Chromatography

The mobile phase gradient was run from 20% to 45% methanol in two linear steps (Fig. 1) at a flow-rate of 2 ml/min. The gradient elution program was followed by a 3-min washing step (90% methanol) and, finally, the column was equilibrated with 20% methanol. The column temperature was maintained at 40°C. The relative retention times were measured from retention times which had been corrected for the void volume of the column.

RESULTS AND DISCUSSION

A typical separation of OPA derivatives of a standard solution of selected amino acids is shown in Fig. 1. In Fig. 2 is seen a chromatogram of an ethanolic extract from incubated synaptosomes[13]. The day-to-day reproducibility of the relative retention times (Table I) expressed as the coefficient of variation (C.V.) was less than 2%. The method can be used for identification of other amino acids than those listed above. The amino acids asparagine, serine, histidine, threonine, methionine sulphone, glycine, arginine and tyrosine are present in very low concentrations in the synaptosomes[11] and are completely separated from the amino acids listed in Table I (data not shown). β-Ala however overlaps to some extent with Ala. This presents no problem due to the very low concentration of β-Ala in synaptosomal extracts[11]. The OPA derivatives of tryptophan, methionine, valine, isoleucine, leucine, lysine and taurine required more than 10 min for elution and were removed from the column during the washing step.



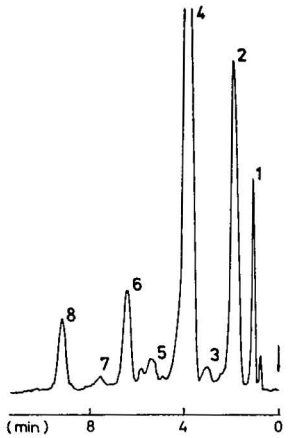


Fig. 1. Chromatogram (×) of OPA-derivatized amino acids (*ca.* 300 pmol of each). Peaks: 1 = aspartic acid; 2 = glutamic acid; 3 = serine; 4 = glutamine; 5 = glycine; 6 = alanine; 7 = histidine; 8 = GABA. Mobile phase: methanol (y)–potassium acetate, pH = 5.50. Flow-rate, 2.0 ml/min. Column temperature, 40°C.

Fig. 2. Chromatogram of an ethanolic extract (30 mg brain tissue per ml) of synaptosomes which were incubated for 2 min at 37°C with 2 m*M* glutamine and the proteins precipitated with ethanol (70% final concentration). For peaks and chromatographic conditions, see Fig. 1.

TABLE I

RELATIVE RETENTION TIMES, PEAK HEIGHTS AND COEFFICIENTS OF VARIATION (C.V.)

Concentration of each compound, 140 pmol; number of determinations, 5.

Compound	*Relative retention time**	*C.V. (%)*	*Peak height ratio***	*C.V. (%)*
Asp	0.12	1.0	1.68	3.0
Glu	0.39	0.7	0.89	2.7
Gln	1.00	0.5	1.00	0.9
Ala	1.80	1.4	0.79	3.6
Gaba	2.66	1.2	1.22	1.4

* Glutamine = 1.00.

** Defined as the ratio between peak height and amount of the compound in question, relative to that of glutamine.

The peak heights in the chromatogram were directly proportional to the amount of the OPA derivatives of the Glu, Gln, Asp, Ala and GABA in the range 20 pmol to 5000 nmol. Estimations of amino acids in synaptosomal extracts were made by the external standard method. Individual run-to-run peak-height variation, due to different degrees of derivatization, can be corrected using an internal standard (*e.g.*, asparagine) that is not present in synaptosomal extracts. Under these chromatographic conditions the coefficient of variation of the heights estimated with samples containing 140 pmol and 100 nmol of each amino acid (Glu, Gln, Asp, Ala and

GABA) was less than 5% (five determinations at each concentration). The sensitivity of the method was better than 10 pmol for all the amino acids investigated.

CONCLUSION

The HPLC method described is well suited for the determination of the amino acids present in synaptosomal extracts and is thus an excellent tool for general studies of the glutamine, glutamate and GABA metabolism. The OPA fluorimetric derivatization provides high sensitivity and the described method gives reproducible results for minimal sample preparation.

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Note

High-performance liquid chromatography of diglyceride *p*-nitrobenzoates

An approach to molecular analysis of phospholipids

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As part of a program of structural studies of the outer membrane of Gram negative bacteria[1], it became necessary to devise a method for characterisation of the molecular species of phospholipids present.

Although phosphatidyl ethanolamine and phosphatidyl serine have been studied as the 4-biphenylcarbonyl derivatives[2], other reported separations of phospholipids by high-performance liquid chromatography (HPLC) have employed the unmodified lipids[3–5]. This has necessitated ultraviolet detection at short wavelengths[3,4] or insensitive refractive index detection[5].

The most complete structural studies of phospholipids have involved degradation to 1,2-diglycerides by means of phospholipase C from *Bacillus cereus*. These diglycerides are fractionated, on the basis of degree of unsaturation, using silver nitrate-impregnated silica gel plates[6,7]. The positional distribution of the fatty acids in the fractions so obtained is then determined by means of a lipase[8]. The individual combinations of fatty acyl substituents cannot, in general, be determined by this method.

We now report HPLC separation of the *p*-nitrobenzoates of mono- and diglycerides which enables complete molecular analysis of phospholipids.

EXPERIMENTAL

Materials

Diglycerides were purchased from Nu Chek Prep (Elysian, MN, U.S.A.), *p*-nitrobenzoyl chloride and 4-dimethylaminopyridine from Aldrich (Milwaukee, WI, U.S.A.), bovine serum albumin (A grade) from Calbiochem (San Diego, CA, U.S.A.), phosphatidyl choline from Applied Science Labs. (State College, PA, U.S.A.), phospholipase C from *B. cereus* (E.C. 3.1.4.3) and lipase from *Rhizopus arrhizus* (E.C. 3.1.1.3) from Boehringer (Mannheim, G.F.R.) and Unichrom HPLC solvents from Ajax Chemicals (Sydney, Australia).

High-performance liquid chromatography

Two LDC Constametric pumps (Models I and IIG) with a dynamic gradient mixer were operated in conjunction with an LDC Gradient Master. A Rheodyne

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Model 7120 syringe-loading injector was used with a 20-μl loop and detection was by means of an LDC UV III monitor operating at 254 nm. A 25-cm Brownlee RP-18 column (10 μm) was used. All samples were dissolved in acetonitrile for injection. All chromatographic manipulations were carried out at 25 $\pm$ 1°C.

Calculation of effective average chain length (EACL)

Retention times (t_R) are expressed relative to that of 1,2-dipalmitin-3-*p*-nitrobenzoate. For the series of saturated 1,2-diglyceride derivatives, a plot of log (relative t_R) *vs.* average fatty acid chain length is linear with equation:

$$\log (\text{rel. } t_R) = 0.145 \text{ (chain length)} - 2.31.$$

Substitution into this expression permits calculation of EACL values.

Preparation of diglyceride p-nitrobenzoates

Diglyceride (1 mg or less) was dissolved in dry pyridine (1 ml) containing 4-dimethylaminopyridine (1 mg), *p*-nitrobenzoyl chloride (4 mg) added and the mixture heated in a sealed tube at 60°C for 2 h. One drop of water was then added and the solution heated for a further 5 min before evaporation to dryness in a stream of nitrogen. The mixture was then partitioned between benzene and water (each 2 ml) and the organic layer washed in turn with equal volumes of 0.1 *M* hydrochloric acid, 0.1 *M* sodium hydroxide, water and saturated sodium chloride solution. Evaporation of the benzene gave the diglyceride nitrobenzoate, which was immediately dissolved in acetonitrile for storage.

Bromination of diglyceride p-nitrobenzoates

Diglyceride *p*-nitrobenzoate, dissolved in chloroform (0.5 ml), was treated with sufficient 5% (w/v) solution of bromine in chloroform to give a persistent colour. After 5 min at room temperature, the sample was carefully evaporated to dryness in a stream of dry nitrogen.

Treatment of phospholipid with phospholipase C

Phospholipid (0.1–2 mg) was dispersed in water (0.2 ml) by sonication before addition of 0.1 *M* triethanolamine HCl buffer, pH 8.0 (0.2 ml). Phospholipase C suspension (3 μl) was added and the mixture incubated at 37°C for 1 h. The mixture was diluted with water (1 ml) and the diglyceride extracted into benzene (2 ml).

Lipase treatment of diglyceride nitrobenzoate

Diglyceride nitrobenzoate (2 mg or less) was dispersed by means of sonication in 0.2 *M* Tris–HCl buffer, pH 8.0 (1 ml) containing bovine serum albumin (4 mg) and 5 m*M* calcium chloride. Lipase suspension (3 μl) was added and the mixture incubated at 37°C for 1 h. After extraction with benzene (2 ml) the organic layer was evaporated in a stream of nitrogen.

Isomerisation of monoglyceride p-nitrobenzoate

2-Palmitoyl-3-*p*-nitrobenzoyl-*sn*-glycerol (approx. 100 μg) was dispersed in 0.2 *M* Tris–HCl buffer, pH 8.0 (1 ml) and incubated overnight at 37°C. The resultant 1-palmitoyl-3-*p*-nitrobenzoyl-*sn*-glycerol was extracted with benzene (2 ml).

RESULTS AND DISCUSSION

Introduction of a *p*-nitrobenzoate chromophore into a diglyceride by acylation of the free hydroxyl group permits sensitive ultraviolet detection at 254 nm. Acylation in the presence of 4-dimethylaminopyridine[10] proceeds more rapidly than acyl migration. Only trace amounts of the nitrobenzoates of the 1,3-diglycerides were detected after derivatisation of the 1,2-isomers.

Chromatography of a mixture of diglyceride *p*-nitrobenzoates shows good separation and peak shape (Fig. 1a). For convenience, a flow-rate of 1 ml/min was normally used. Significant improvement in chromatographic performance is, however, obtained using a flow-rate of 0.5 ml/min.

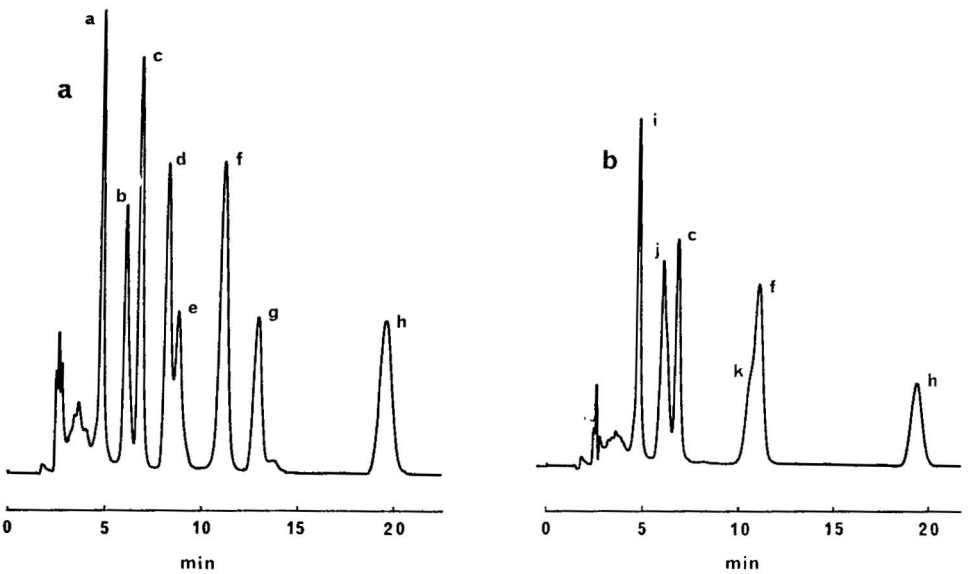

Fig. 1. Separation of diglyceride *p*-nitrobenzoates by HPLC. Operating conditions: column, 25 cm RP-18 (10 μm); mobile phase, 2-propanol–acetonitrile (35:65); column temperature, 25 $\pm$ 1°C; flow-rate 1 ml/min. Peaks: a = 1,2-di-12:0; b = 1,2-di-16:1(9); c = 1,2-di-14:0; d = 1,3-di-18:1(11); e = 1,2-di-18:1(11); f = 1,2-di-16:0; g = 1-18:0-2-18:1; h = 1,2-di-18:0; i = a + bromo-b; j = bromo-d + bromo-e; k = bromo-g.

The retention behaviour of the diglyceride derivatives is conveniently expressed in terms of the EACL of the fatty acids present (see Experimental). As expected from studies of fatty acid esters[11,12] and phosphatidyl choline[5], unsaturated glycerides have smaller EACL values than the corresponding saturated species (Table I). The 1,2- and 1,3-isomers are well resolved; in each case, the 1,3-diglyceride derivative elutes before the 1,2-isomer (Table I).

Relative peak areas relate directly to the molar ratios of the diglycerides present. Analysis can conveniently be carried out at the microgram level: 0.6 μg (approx. 1 nmol) of 1,2-dipalmitin, as the *p*-nitrobenzoate, gives a full-scale peak at a detector setting of 0.032 a.u.f.s.

TABLE I

HPLC PROPERTIES OF DIGLYCERIDE *p*-NITROBENZOATES ON RP-18

Fatty acyl substituents	*Retention time relative to 1,2-di-16:0*		*Effective average chain length*
	1,2-Isomer	*1,3-Isomer*	
di-12:0	0.27		12.0
		0.25	11.78
di-14:0	0.52		14.0
		0.47	13.67
di-16:0	1.00		16.00
		0.89	15.58
di-18-0	2.00		18.00
		1.67	17.47
di-14:1(9)	—		—
		0.24	11.66
di:16:1(9)	0.43		13.40
		0.40	13.19
di-18:1(11)	0.75		15.07
		0.70	14.86
di-18:2(9, 12)	0.21		11.26
			—
1-18:0-2-18:1(9)	1.23	—	16.55

It is useful to have a simple criterion for judging the presence and degree of unsaturation in each molecular species. Reaction of bromine with unsaturated diglyceride *p*-nitrobenzoates produces bromodiglyceride derivatives which elute even earlier than the unsaturated species (Fig. 1b). The decrease of the EACL values of the brominated species relative to the corresponding unsaturated materials depends on the degree of unsaturation (Table II). The magnitude of this decrease is approximately linear with respect to the number of double bonds present.

TABLE II

EFFECT OF BROMINATION ON HPLC PROPERTIES OF UNSATURATED 1,2-DIGLYCERIDE-*p*-NITROBENZOATES

Substituents before bromination	*Relative retention time of brominated derivatives**	*Effective average chain length (EACL)*	*Bromination shift, Δ EACL*
1-18:0-2-18:1(9)	0.96	15.80	−0.75
di-16:1(9)	0.27	12.01	−1.39
di-18:1(11)	0.43	13.40	−1.67
di-18:2(9,12)	0.11	9.32	−1.94

* Relative to 1,2-di-16:0 *p*-nitrobenzoate.

Treatment of a 1,2-diglyceride nitrobenzoate with lipase leads cleanly to the 2-acyl-3-*p*-nitrobenzoyl-*sn*-glycerol without competing release of *p*-nitrobenzoic acid. The monoglyceride derivatives are readily resolved by HPLC, permitting determination of the 2-acyl substituents (Fig. 2). The slightly alkaline buffer used for the

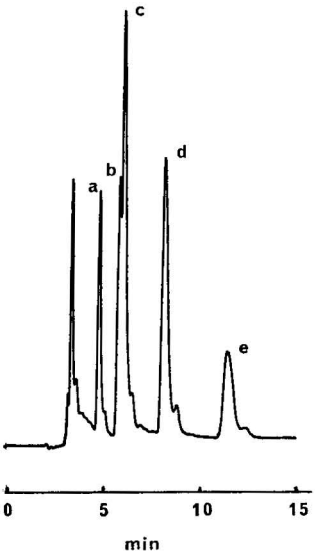

Fig. 2. Separation of 2-acyl-3-*p*-nitrobenzoyl-*sn*-glycerols by HPLC. Eluting solvent, acetonitrile. For other operating conditions, see legend to Fig. 1. Peaks: a = 2-12:0; b = 2-16:1(9); c = 2-14:0; d = 2-16:0; e = 2-18:0.

incubation leads to slow isomerisation of the products to the more stable 1-acyl-3-*p*-nitrobenzoyl-*sn*-glycerols. This is evidenced by the small peaks on the trailing edge of the main peaks (Fig. 2). Complete isomerisation can be achieved by incubation of the 2-acyl derivatives in buffer at pH 8.0.

1,3-Diglyceride-*p*-nitrobenzoates are converted by lipase to 2-*p*-nitrobenzoyl-glycerol with little accumulation of the intermediate monoglyceride derivatives.

Because of the sensitivity and good chromatographic performance in this system, it is possible to isolate a peak fraction from a heavy injection of diglyceride derivatives, treat it with lipase and re-inject to identify the monoglyceride product. We therefore have available an extension of existing methods for molecular analysis of diglycerides and phospholipids which permits characterisation of discrete molecular species.

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Note

High-performance liquid chromatographic analysis of furazolidone in liver and kidney

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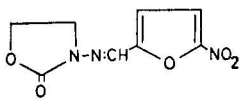
Furazolidone, 3-(5-nitrofurfurylideneamino)-2-oxo-oxazolidine, is a 3-nitrofuran used as an antibacterial agent against livestock diseases. Most nitrofurans are known to be mutagenic and carcinogenic[1–5], and our laboratory has therefore developed a method for the detection of residues of this antibiotic in pig liver and kidney.



FURAZOLIDONE

Cieri[6] described a high-performance liquid chromatographic (HPLC) method for determining residues of furazolidone in feeds. A direct HPLC method for the routine analysis of pig liver and kidney is described here.

EXPERIMENTAL

Chemicals and reagents

Furazolidone (Aesculaap, Boxtel, The Netherlands) was dissolved in acetonitrile; dilution with acetonitrile gave standard solutions containing 10–100 ng of furazolidone per 20 μl. All of the reagents were of analytical-reagent grade. Acetonitrile (Nanograde, Mallinckrodt, St. Louis, MO, U.S.A.) and distilled water, used as eluents in liquid chromatography, were pre-filtered through a G-1 glass filter.

Apparatus

A Hewlett-Packard Model 1084B liquid chromatograph equipped with a variable-wavelength UV detector (Hewlett-Packard Model 79875A) was used. A pre-column (stainless steel, 100 × 2.1 mm I.D.) was packed with Perisorb RP-8 (30–40 μm) and the analytical column (stainless steel, 250 × 4.6 mm I.D.) was packed with Hypersyl SAS. The mobile phase was water–acetonitrile (75:25) at a flow-rate of 1.75 ml/min; the temperature was ambient and the detection wavelength was 360 nm.

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Extraction

Minced samples were extracted by macerating 50 g of sample with 200 ml of ethyl acetate and 50 g of anhydrous sodium sulphate in an Ultra Turrax at moderate speed. The macerate was centrifuged for 5 min at 2500 *g* and the extract was collected. A 100-ml volume of the extract was evaporated to dryness by means of a rotary vacuum evaporator. The residue was dissolved in 2 ml of acetonitrile, warmed to 40°C and immediately cooled to remove fat particles. The solution was filtered through a G-2 glass filter.

Partition clean-up (optional)

A 100-ml volume of the extract was evaporated to dryness by means of a rotary vacuum evaporator. The residue was transferred into a separating funnel, using *ca.* 20 ml of *n*-hexane, and extracted twice with 50 ml of acetonitrile (saturated with *n*-hexane) shaking the funnel well for 3 min each time. The combined acetonitrile phases were evaporated to dryness and the residue was dissolved in 2 ml of acetonitrile. The solution was warmed to 40°C immediately cooled to remove fat and filtered through a G-2 glass filter.

High-performance liquid chromatography

A 20-μl volume of the solution was injected into the liquid chromatograph. Standard solutions containing 10–100 ng of furazolidone per 20 μl were also injected.

RESULTS AND DISCUSSION

Chromatograms of blank and treated samples are shown in Figs. 1 and 2. The partition step had to be used for samples containing more than 2% fat. For routine or screening work the optional clean-up procedure can be omitted.

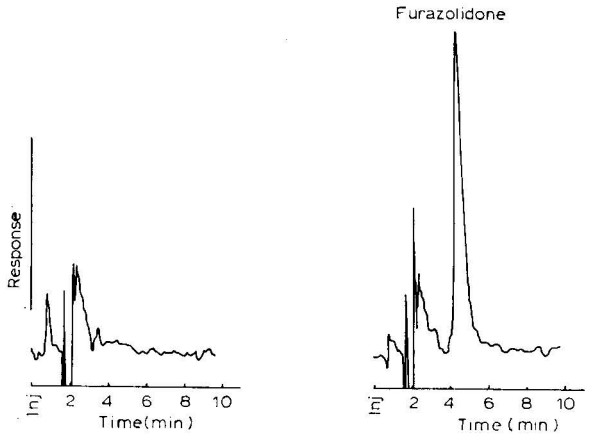

Fig. 1. Chromatogram of extract from blank pig liver after partition. Column, 250 × 4.6 mm I.D., Hypersyl SAS; pre-column, 100 × 2.1 mm I.D., Persisorb RP-8 (30–40 μm); mobile phase, water–acetonitrile (75:25); flow-rate, 1.75 ml/min; temperature, ambient; injection volume, 20 μl; UV detection at 360 nm.

Fig. 2. Chromatogram after partition of blank pig liver treated with furazolidone (0.42 ppm). Conditions as in Fig. 1.

The recovery of furazolidone was determined by analysing pig liver and kidney samples spiked with furazolidone at levels from 0.12 to 1.0 mg/kg. The results are shown in Table I.

TABLE I

RECOVERIES AFTER CLEAN-UP BY PARTITION OF FURAZOLIDONE FROM BLANK PIG LIVER AND KIDNEY TREATED WITH FURAZOLIDONE STANDARD SOLUTIONS

Average values are given for three recovery experiments each carried out with four concentrations of furazolidone.

Furazolidone added (ppm)	*Average recovery (%)*	
	Pig liver	*Pig kidney*
0.12	92.8	92.2
0.25	98.2	96.5
0.33	97.6	96.8
1.0	96.2	89.3

The limit of detection was *ca.* 0.05 mg/kg and a concentration of 0.08 mg/kg gave a peak height of 10% full-scale deflection at a detector sensitivity of 0.1 a.u.f.s.

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Note

Determination of pilocarpine, physostigmine, its degradation product rubreserine and preservatives by high-performance liquid chromatography

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Pilocarpine and physostigmine are used in ophthalmology in the treatment of glaucoma. Their effect is to decrease the intra-ocular pressure in the eye. Many studies have been made of their estimations, degradation patterns and kinetics in aqueous solution.

Eye-drops containing both pilocarpine and physostigmine are produced by Swedish pharmacies. Preservatives are often added. No study has been reported of the separation of pilocarpine from physostigmine, its degradation product rubreserine and from preservatives. The analysis of all these components with conventional spectrophotometric methods after extraction is very time-consuming and therefore a high-performance liquid chromatographic (HPLC) method has been developed.

Pilocarpine alone has been determined colorimetrically by the hydroxamic acid method by Brochmann-Hanssen *et al.*[1] and Murray[2], and its hydrolysis and epimerization kinetics in aqueous solution have been investigated by Chung *et al.*[3] and Nunes and Brochmann-Hanssen[4]. HPLC methods have been used by a number of workers[5–8]. Weber[5] also showed that the stability of pilocarpine was good at pH 3.5–5.5. Noordam *et al.*[8] used the same column as in our study. They separated pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid on a reversed-phase column (RP-18) with a water–methanol mixture at pH 2.5. Degradation of pilocarpine solutions has been studied by Baeschlin and co-workers[9–11] and Neville *et al.*[12].

Physostigmine alone has been determined by Hellberg[13–15] by acidimetric or spectrophotometric methods. Christenson[16] has described its kinetics of hydrolysis, and Parrák and co-workers[17,18] showed by oscillographic polarography that the most stable ophthalmic solution had a pH value between 2 and 4.5. The stability of this compound has also been studied by Fletcher and Davies[19] and Rogers and Smith[20,21].

Analysis of pilocarpine and physostigmine in the same solution has been studied by Fagerström[22]. The alkaloids have not been separated but have been determined by two different spectrophotometric methods.

The degradation product rubreserine was made according to Ehrlén[23]. Fig. 1 shows the preparations presently available.

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EYE DROPS

Pilocarpine: 1%, 2%, 4%
Physostigmine: 0.4%
Pilocarpine + Physostigmine: 4 + 0.4%
2 + 0.2%
4 + 0.2%

Preservatives:
Methyl + Propyl p-hydroxybenzoate 0.04 + 0.02%
Phenethyl Alcohol 0.5%
Phenylmercuric Nitrate 0.001%

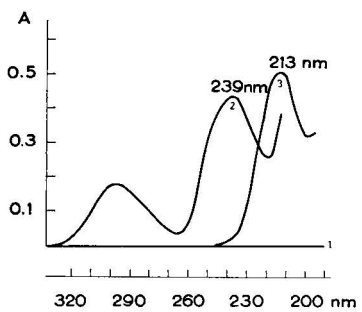
Fig. 1. Different preparations of eye-drops containing pilocarpine, physostigmine and preservatives.

EXPERIMENTAL

Chromatography

A Waters Assoc. pump and injector are used, together with a variable-wavelength detector, LDC Spectromonitor III. The column (30 cm × 3.9 mm) was packed with μBondapak C_{18} (pore size, 10 μm, Waters Assoc.). The mobile phase was 40% methanol with 0.005 *M* heptanesulphonic acid, pH 3.6, filtered through 0.5-μm Celotate®; flow-rate 1 ml/min. Samples of 80 μg pilocarpine and 8 μg physostigimine were injected.

In order to select a suitable wavelength for the HPLC measurements the UV-absorption spectra of the different substances have been recorded (Figs. 2–4). Good results were achieved at 235 nm for the detection of pilocarpine, physostigmine and preservatives. For the detection of the degradation product rubreserine, a wavelength of 292 nm was chosen.



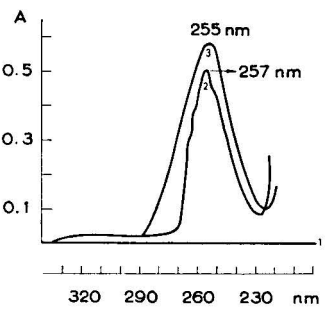


Fig. 2. UV absorption spectra of physostigmine salicylate, 0.001% (2) and pilocarpine hydrochloride, 0.002% (3). 1 = water.

Fig. 3. UV absorption spectra of phenethyl alcohol, 0.3% (2) and methyl + propyl *p*-hydroxybenzoate, 0.004 + 0.002% (3). 1 = water.

As internal standard for unpreserved preparations, methyl *p*-hydroxybenzoate can be used. If the eye-drops are preserved with methyl *p*-hydroxybenzoate for instance, ethyl *p*-hydroxybenzoate can be used.

Fig. 5 shows the chromatographic separation of pilocarpine and phenethyl alcohol. For the detection of phenethyl alcohol the signal from the detector has been amplified ten times.

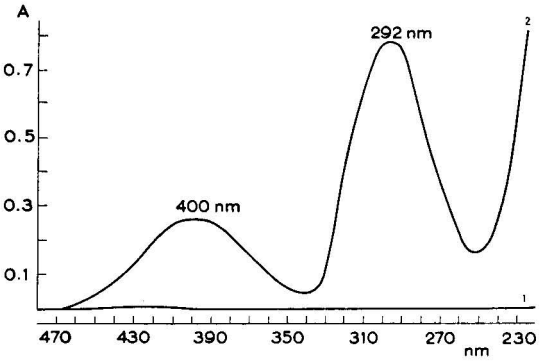


Fig. 4. UV absorption spectrum of rubreserine, 0.17% (2). 1 = water.

Fig. 6 shows the separation of pilocarpine, physostigmine, methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate. The peak from pilocarpine appears about 7 min after injection. Physostigmine salicylate forms two ion pairs and also two peaks, one due to salicylate (2) after 4 min and one due to physostigmine (4) after about 13 min. Methyl *p*-hydroxybenzoate appears after 11 min (3). After *ca.* 15 min the flow is increased to 2 ml/min in order to accelerate the elution of propyl *p*-hydroxybenzoate. The signal from the detector has been amplified twenty times.

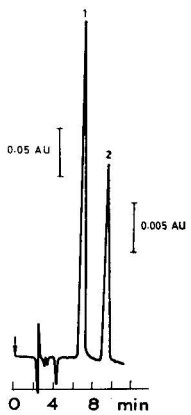


Fig. 5. HPLC of pilocarpine (1) and phenethyl alcohol (2). Detection: UV, 235 nm.

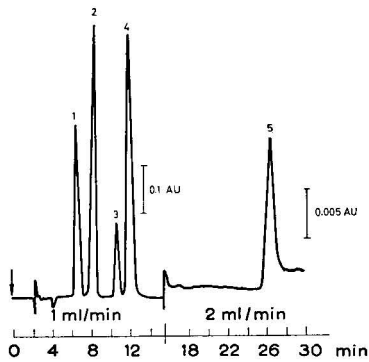


Fig. 6. HPLC of pilocarpine, physostigmine and preservatives. Peaks: 1 = pilocarpine; 2 = salicylate; 3 = methyl *p*-hydroxybenzoate; 4 = physostigmine; 5 = propyl *p*-hydroxybenzoate. Detection: UV, 235 nm.

Stability testing

Degradation of pilocarpine (Fig. 7). Pilocarpine and isopilocarpine have the same retention times and therefore cannot be separated by this method. However, at pH 3–5, which is normally found in the ophthalmic solutions, the stability of pilocarpine is good.

1. Hydrolysis to pilocarpic acid
2. Epemerization to isopilocarpine

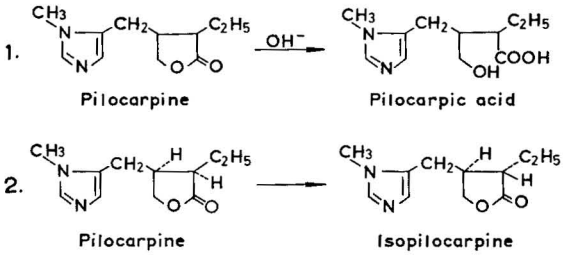
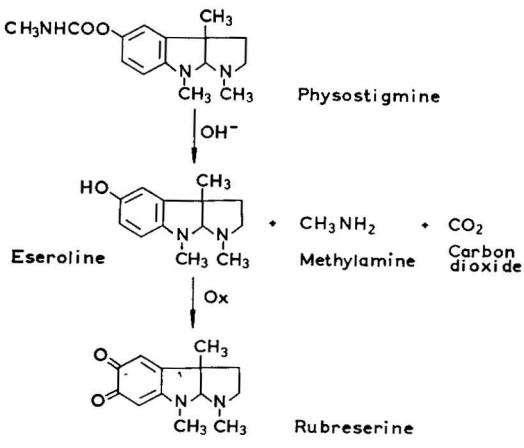


Fig. 7. Degradation of pilocarpine.

Degradation of physostigmine (Fig. 8). Physostigmine is less stable, particularly at pH > 5. During the production of the eye-drops the pH is adjusted to 4.5–5.0.

Physostigmine is hydrolyzed to eseroline, methylamine and carbon dioxide. Eseroline is colourless but is oxidized to rubreserine, which is red. Aged solutions are therefore often red. Fig. 9 shows a chromatogram of an aged physostigmine solution. Rubreserine has been prepared in the laboratory and is used as a reference sample; detection limit, 0.001 μg. A concentration of 0.5 μg/ml rubreserine can be detected by the eye as a very slight discolouration. A sample containing 1 μg/ml is distinctly pink.



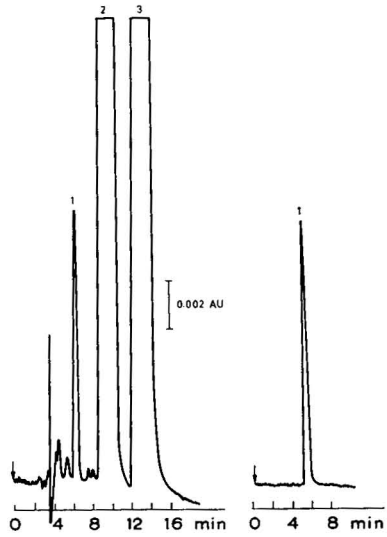


Fig. 8. Degradation of physostigmine.

Fig. 9. HPLC of an aged physostigmine solution (left) and a rubreserine standard solution (right). Peaks: 1 = Rubreserine; 2 = salicylate; 3 = physostigmine. Detection: UV, 292 nm.

RESULTS AND DISCUSSION

Capacity factors, sample concentrations and detection limits have been calculated (Table I). Optimum results are achieved if the capacity factors are between 2

and 6. Rubreserine has the lowest value (1.7) and physostigmine the highest (5.1). The relative standard deviation calculated for the peak height was 1.3% for pilocarpine and 0.3% for physostigmine, and the retention time was for pilocarpine 0.2% and 0.1% for physostigmine.

TABLE I

CAPACITY FACTORS (k'), SAMPLE CONCENTRATIONS AND DETECTION LIMITS

Compound	k'	*Sample conc. (μg)*	*Detection limit (μg)*
Rubreserine	1.7	0.02	0.001
Pilocarpine	2.3	80	0.02
Salicylate	2.9	8	—
Phenethyl alcohol	3.5	10	—
Methyl *p*-hydroxybenzoate	4.2	0.8	—
Physostigmine	5.1	8	0.003

The described method is rapid and accurate. A separation and quantitative analysis of rubreserine, pilocarpine, methyl *p*-hydroxybenzoate and physostigmine is completed in *ca.* 15 min.

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Note

Qualitative differentiation between cocaine, lidocaine and cocaine–lidocaine mixtures ("rock-cocaine") using thin-layer chromatography

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(Received June 19th, 1980)

The abuse of cocaine has steadily increased in recent years. In order to increase the amount available for sale and thus increase profits, cocaine is often diluted with lidocaine (xylocaine). This mixture is referred to as "rock-cocaine". Although gas chromatographic separation of cocaine and lidocaine is easily accomplished, it is not the routine technique in most laboratories. Since thin-layer chromatography (TLC) is the most common identification procedure, it became desirable to determine whether or not lidocaine can be separated from cocaine by this technique.

The plates used were E. Merck pre-coated TLC plates, silica gel 60 F_{254}, 0.25 mm (EM Labs., Elmsford, NY, U.S.A.). The R_F values using chloroform–methanol–conc. ammonia (90:10:1) (solvent system S1) were cocaine, 0.90; and lidocaine, 0.90; the R_F values using ethyl acetate–methanol–water–conc. ammonia (85:10:3:1) (solvent system S2) were cocaine, 0.87; and lidocaine, 0.89.

Although the two drugs were not separated by TLC, the presence of lidocaine can be identified and semi-quantitatively measured by the differences in form and color reaction between the two drugs. The illustration is in black and white and even though the color reaction, which is described in the text, helps in the differentiation of the two compounds, the band which develops above or around the spot is sufficient for the identification of lidocaine.

After developing the TLC plates with solvent systems S1 and S2, they were dried and sprayed with 5% H_2SO_4 followed by iodoplatinate. The immediate reaction resulted in a purple-blue color, the same for both drugs. On standing, however, a ring, formed and the color of the spots changed. The observed shades may vary from run to run, however, one could clearly differentiate the two compounds. The lidocaine spot became brown with a blue-grey ring and the cocaine spot became brown with the presence of a very light purple ring, which continued to fade over time (Fig. 1, B1 and B5). The color difference between the cocaine and lidocaine spots was clearly visible by 4 h after spraying (Fig. 1, B) and it was stable for at least 24 h.

The intensity of the band over the spot could also be used to determine the approximate concentration of a mixture of the two drugs. The TLC plates were spotted using several ratios of cocaine and lidocaine. The amounts were (in μg): 200:0, 150:50, 100:100, 50:150 and 0:200 (Fig. 1, 1–5, respectively). Approximately 200 μg of a "rock-cocaine" sample was also analyzed (Fig. 1, 6). In the "rock-cocaine" sample the cocaine–lidocaine ratio (in %) was 61:39, as determined by gas–liquid

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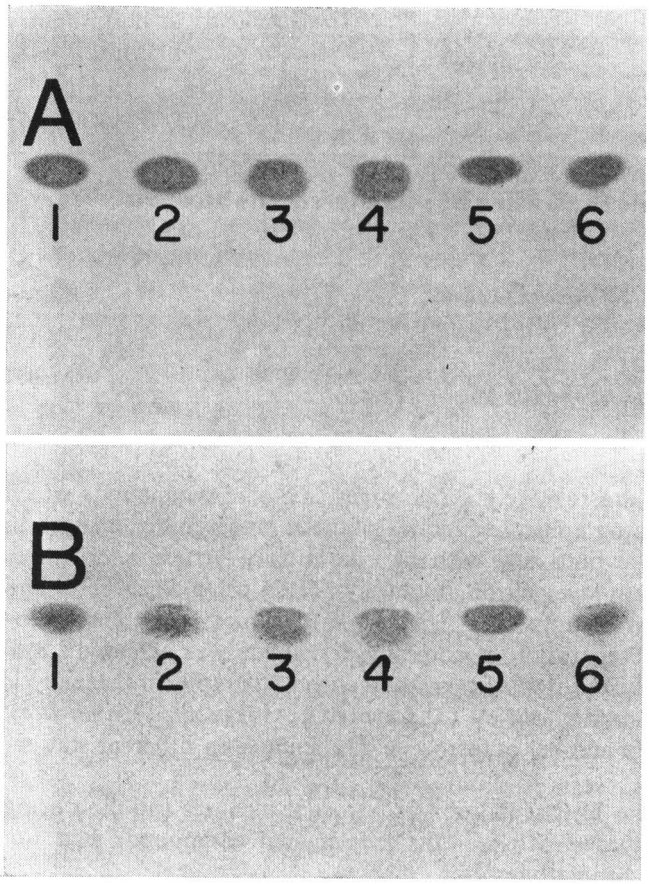

Fig. 1. TLC of cocaine and lidocaine. A = 5 min after spraying; B = 4 h after spraying, using solvent system S2. 1 = 200 μg cocaine (C); 2 = 150 μg C and 50 μg lidocaine (L); 3 = 100 μg C and 100 μg L; 4 = 50 μg C and 150 μg L; 5 = 200 μg L; 6 = 200 μg "rock-cocaine".

chromatography. A separate plate was developed using S1 and S2. The plates were air dried, sprayed, observed at 4 h, and left overnight to allow color stabilization. Photographs were taken at 5 min and 4 h, (Fig. 1, A and B, respectively) after spraying.

The plate developed in S2 showed clearer color and shade differences among the different samples. The lidocaine spot had a blue-grey ring around it and the mixtures had a blue-grey band over the spot. Th thickest band correlated with the highest lidocaine concentrations (Fig. 1, B5). The street sample of "rock-cocaine" most closely resembled the 150:50 cocaine–lidocaine sample. The longer the time elapsed from spraying the clearer the blue-grey ridge became, as the cocaine spot tends to fade on standing. For qualitative determination one need not wait 24 h, since by 1 h after spraying the "rock-cocaine" sample starts to develop the thin blue-grey ridge.

This procedure is simple and inexpensive for the semi-quantitative determination of cocaine–lidocaine mixtures and is especially useful for laboratories where gas–liquid chromatography is not available.

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Note

Thin-layer chromatographic separation of some ferrocene alcohols

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Ferrocene derivatives are relatively stable organometallic compounds which can be analyzed by chromatographic techniques without undergoing changes in chemical structure. Early publications dealt with the separation of ferrocene derivatives from reaction mixtures by thin-layer chromatography (TLC) using silica gel[1,2] and alumina[3], and by column chromatography[4–7]. High-performance liquid chromatography was also used[8,9] and separation of some diastereomers was achieved[9]. Systematic investigations of the application of gas–liquid chromatography in the analysis of ferrocene derivatives were carried out by Tanikawa and Arakawa[10]. Further work yielded the retention data for a number of ferrocene compounds on different stationary phases[11,12].

This paper describes the TLC separation of ferrocene alcohols and diols using silica gel and two mobile phases. Most of the investigated compounds had not previously been analyzed by chromatography.

EXPERIMENTAL

Preparation of ferrocene compounds

Compound I was prepared from ferrocenecarbaldehyde and 2-mercaptoethanol in the presence of *p*-toluenesulphonic acid as a catalyst[13]. Compounds II–XI and XIII–XVII (Table 1) were obtained as described previously[14–25]. Compound XII was prepared by multistep synthesis from 1,1′-ferrocenedicarboxylic acid via 1,1′-di-(hydroxymethyl)ferrocene, 1,1′-di(cyanomethyl)ferrocene and 1,1′-ferrocenediacetic acid[26].

Thin-layer chromatography

Separation of ferrocene compounds was performed using precoated TLC glass plates covered with silica gel 60-F_{254} and having a concentrating zone (E. Merck, Darmstadt, G.F.R.). Plates were heated at 110°C for 30 min immediately before use. Two microlitres of the ferrocene compounds (10 mg/ml) in benzene were developed using the following mobile phases:

(a) *n*-hexane–benzene–acetone (8:1:1)
(b) benzene–chloroform–acetone (4:1:1)

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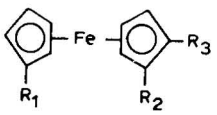
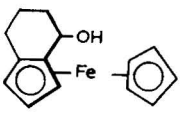
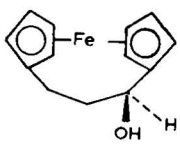
Visualization of the separated substances was accomplished with 3.5% molybdatophosphoric acid spray reagent or UV light at 254 nm.

RESULTS AND DISCUSSION

The R_F values of the examined substances in two mobile phases are given in Table I.

TABLE I

STRUCTURES AND R_F VALUES OF FERROCENE COMPOUNDS

Compound	*Structural formula*	*Ref.*	*Mobile phase*	
			a	*b*
Alcohols				
$R_2 = R_3 = H$,	$R_1 = CH(SCH_2CH_2OH)_2$	13		0.16
	$CH_2SCH_2CH_2OH$	14	0.12	0.48
	CH_2OH	15	0.14	0.52
	$CH(C_6H_5)SCH_2CH_2OH$	14	0.15	0.54
	$CH(CH_3)SCH_2CH_2OH$	14	0.19	0.59
	$CH(OH)CH_3$	16	0.21	0.61
	$CH(C_2H_5)SCH_2CH_2OH$	14	0.22	0.62
	$CH(OH)C_6H_5$	17	0.29	0.68
	$CH(OH)CF_3$	18	0.36	0.74
	$CH(OH)CH(CH_3)_2$	19	0.50	0.81
Diols				
$R_2 = R_3 = CH_2OH$,	$R_1 = H$	20		0.11
$R_3 = H$,	$R_1 = R_2 = CH_2CH_2OH$	26		0.13
	CH_2OH	21		0.16
	$CH(OH)CH_3$	22		0.28
	$CH(OH)C_6H_5$	23		0.34
Other alcohols	 *exo*	24	0.32	0.70
	endo		0.40	0.75
		25	0.83	

The R_M values in mobile phases a and b showed a linear correlation, $R_M^a = f(R_M^b)$, as presented in Fig. 1.

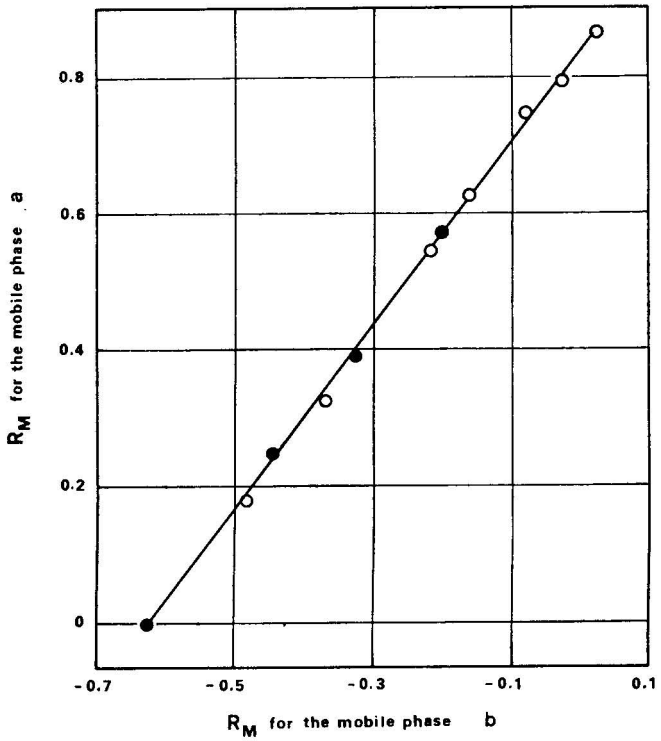


Fig. 1. R_M values for ferrocene compounds in primary alcohols (○) and secondary alcohols (●) as mobile phases.

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Author Index

Erratum

J. Chromatogr., 186 (1979) 249–258
Page 251, eqn. 1a should read:

$$Q = \frac{N_2}{t_R \Delta P_{j'}} = \frac{k_p}{\eta H^2 (1 + k')} = \frac{k_p k'^4}{\eta h^2 (k' + 1)^5} \qquad (1a)$$

PUBLICATION SCHEDULE FOR 1980

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	197/1 197/2 198/1	198/2 198/3 198/4 199	200 201	202/1 202/2 202/3
Chromatographic Reviews			184/1	184/2					184/3			184/4	
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2	183/3	183/4		

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