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by **V.G. BEREZKIN, V.R. ALISHOYEV** and **I.B. NEMIROVSKAYA,** Institute of Petrochemical Synthesis, Academy of Sciences of the U.S.S.R., Moscow.

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EFFECTS OF SURFACE HETEROGENEITY IN NON-LINEAR AND NON-EQUILIBRIUM GAS-ADSORPTION CHROMATOGRAPHY

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(Received December 13th, 1976)

SUMMARY

It is shown that one can investigate quantitatively the effects of surface heterogeneity in non-linear and non-equilibrium gas–solid chromatography on the basis of elution theories developed for homogeneous surfaces. One such theory, that of Zhitomirskii *et al.*, was accepted for the purpose of this work and a new formulation for the kinetics of adsorption on heterogeneous surfaces was developed and applied. The effect of surface heterogeneity on elution curves is shown, and the possibility of determining quantitatively adsorption and desorption rate coefficients in adsorption systems with heterogeneous surfaces is demonstrated. A parameter that describes quantitatively the degree of surface heterogeneity has been determined.

INTRODUCTION

The theory of gas–solid chromatography (GSC) is an important but difficult subject. Many papers have been published after the pioneering work of Cremer[1], Huber[2], Keulemans[3], Janák[4] and Smolkova and Grubner[5], but even the basic contributions in this field are too numerous to list here. Kiselev and Iashin[6] and Snyder[7] reviewed the literature up to the late 1960s and other fundamental results can be found in the reviews by Huber[8] and Conder and Purnell[9]. In most papers linear adsorption isotherms were considered[10–16], while the very interesting case of non-linear adsorption isotherms has been treated by Zhukhovizkii and co-workers[17,18], Zolotarev[19], Buys and De Clerk[20–22], Acrivos[23], Blauton *et al.*[24] and Thomas[25].

Except for a few workers[26–28], most have adopted the model of a homogeneous adsorbent surface but, apart from graphitized carbons, such ideal surfaces are rarely found in chromatographic practice. The first advanced attempts to describe quantitatively the effects of surface heterogeneity in GSC were those by Dougharty[29], who

* To whom correspondence should be addressed.

developed appropiate expressions for peak moments in GSC systems with heterogeneous surfaces. Because of the great mathematical complexity of his theory, even illustrative model calculations were not performed by the author. We decided to carry out an investigation of surface heterogeneity effects on the basis of the theoretical formulation of GSC by Zhitomirskii *et al.*[30].

THEORETICAL

Zhitomirskii *et al.*[30] succeeded in obtaining an exact analytical solution for the elution function in non-linear and non-equilibrium GSC. The starting point of their theory is the following well known set of partial differential equations:

$$\frac{\partial c}{\partial t} + \frac{\partial a}{\partial t} + u_g \cdot \frac{\partial c}{\partial z} = 0 \tag{1}$$

$$\frac{\partial a}{\partial t} = k_a (a_{\max} - a)\, c - k_d\, a \tag{2}$$

where c and a are the adsorbate concentrations in the free gas phase and on the surface, respectively, k_a and k_d are the adsorption and desorption rate constants, respectively, u_g is the linear velocity of the carrier gas, z is the column length and t is the time. In eqn. 2, $a_{\max}$ is the adsorbate concentration on the surface at monolayer coverage.

The initial conditions for the elution function $c(t)$ were as follows:

$$z = 0,\ c(0, t) = \psi(t) \tag{3}$$

$$t = 0,\ a(z, 0) = 0,\ c(z, 0) = 0 \tag{4}$$

where $\psi(t)$ is the inlet function for which the authors accepted a rectangular inlet pulse:

$$\psi(t) = \begin{cases} c_0, & t \leqslant T \\ 0, & t > T \end{cases} \tag{5}$$

Then, the solutions for $c(t)$ and $a(t)$ are obtained exactly and are expressed by appropriate Bessel functions. When the chromatographic column is sufficiently long, asymptotic expressions can be used for the Bessel functions, and the elution function $c(t)$ takes the following simple form:

$$c(t) = \left(\frac{\sqrt{b_1}}{\sqrt{2\pi}\, t^{\frac{3}{4}}\, 2k_a}\right) \cdot \left\{ \frac{1}{\sqrt{2\pi}} \frac{1}{\sqrt{b_1}\, t^{\frac{1}{4}}} \left[1 - \frac{(k_d\, t/b_2)^{\frac{1}{4}}}{1 + (k_d\, t/b_2)^{\frac{1}{4}}}\right] + \right.$$
$$\left. + \exp\left(b_2 - b_3 - b_1 \sqrt{t} + k_d\, t\right) + \frac{1}{\sqrt{2\pi}\,(\sqrt{b_2} - \sqrt{k_d\, t})} \right\} \tag{6}$$

In this equation

$$b_1 = 2\sqrt{k_d}\, k_a \left(\frac{z}{u_g}\right) a_{\max} \tag{7}$$

$$b_2 = k_a \left(\frac{z}{u_g}\right) a_{\max} \tag{8}$$

and

$$b_3 = k_a \left(\frac{m}{F}\right) \tag{9}$$

where F is the volumetric velocity of the carrier gas and m is the amount of adsorbate introduced into the column. The theory also yields a simple relationship between the concentration at the peak maximum (c_{max}) and the retention time (t_{max}):

$$c_{max} = \frac{-1}{2k_a t_{max}} - \frac{k_d}{k_a} + \left[\left(\frac{z}{u_g}\right)\left(\frac{a_{max}}{t_{max}}\right)\left(\frac{k_d}{k_a}\right)\right]^{\frac{1}{2}} \tag{10}$$

Now, eqn. 2 assumes that the adsorption in the column follows a Langmuir model, leading to monolayer adsorption of fully localized molecules, without lateral interactions. The adsorbent surface is assumed to be homogeneous, *i.e.*, all adsorption sites have the same adsorption energy, ε. The Langmuir isotherm has the following explicit form:

$$\theta(p) = \frac{kp}{1 + kp} \tag{11}$$

where p is the pressure of adsorbate and

$$k = \frac{k_a}{k_d} = k_0 \exp\left(\frac{\varepsilon}{RT}\right) \tag{12}$$

For heterogeneous surfaces, adsorption sites are distributed among various adsorption energies belonging to some interval Ω. For the purpose of mathematical convenience, this interval is often assumed to be $(0, +\infty)$ or $(-\infty, +\infty)$. For the model of localized adsorption without lateral interactions between adsorbed molecules the differential distribution of adsorption energy, $\chi(\varepsilon)$, is usually accepted as the quantitative measure of surface heterogeneity. By means of $\theta(p, \varepsilon)$ and $\chi(\varepsilon)$, the equation for the overall adsorption isotherm $v(p)$ can be written as follows:

$$v(p) = v_0 \int \theta(p,\varepsilon)\, \chi(\varepsilon)\, \mathrm{d}\varepsilon \tag{13}$$

where v_0 is the amount adsorbed at monolayer coverage. Various analytical expressions have been used to represent the function $\chi(\varepsilon)$[31], gaussian distributions probably being most often considered. There are good reasons to assume such distributions as a correct representation of surface heterogeneity at sub-monolayer surface coverages[32]. For these reasons, and on the basis of some other mathematical considerations, we shall further represent $\chi(\varepsilon)$ by the following bell-shaped, gaussian-like distribution:

$$\chi(\varepsilon) = \frac{1}{\varrho} \cdot \frac{\exp\left(\frac{\varepsilon - \varepsilon_0}{\varrho}\right)}{\left[1 + \exp\left(\frac{\varepsilon - \varepsilon_0}{\varrho}\right)\right]^2} \tag{14}$$

which reduces to the Dirac delta function $\sigma(\varepsilon - \varepsilon_0)$ when the heterogeneity parameter ϱ tends to zero. Eqn. 14 has the important advantage that the integral in eqn. 13 can

then be expressed by appropriate derivatives of $\theta(p, \varepsilon)$ with respect to ε, taken at $\varepsilon = \varepsilon_0$. In general, we have[33]

$$\Theta(p,\varepsilon_0) = \int_{-\infty}^{+\infty} \theta(p,\varepsilon) \frac{1}{\varrho} \cdot \frac{\exp\left(\frac{\varepsilon - \varepsilon_0}{\varrho}\right)}{\left[1 + \exp\left(\frac{\varepsilon - \varepsilon_0}{\varrho}\right)\right]^2} d\varepsilon$$

$$= [\theta(p,\varepsilon)]_{\varepsilon=\varepsilon_0} + \frac{\pi^2}{6} \cdot \varrho^2 \left[\frac{\partial^2 \theta}{\partial \varepsilon^2}\right]_{\varepsilon=\varepsilon_0} + \ldots \tag{15}$$

The non-physical part of the integral in eqn. 15 from $-\infty$ to 0 does not introduce a greater contribution, as both $\theta(\varepsilon)$ and $\chi(\varepsilon)$ are then rapidly decreasing functions[34].

In this way, the adsorption isotherm $v(p)$ for a heterogeneous surface can be expressed by a new isotherm equation for a hypothetical homogeneous surface with adsorption sites that have an adsorption energy of ε_0. Let us consider the isotherm $\theta(p)$ in more detail. After performing appropriate differentiations, we obtain

$$\Theta(p,\varepsilon_0) = \left[\theta + \frac{\pi^2}{6}\left(\frac{\varrho}{RT}\right)^2 \theta\,(1-\theta)\,(1-2\theta)\right]_{\varepsilon=\varepsilon_0} \tag{16}$$

or, in terms of pressure p:

$$\Theta(p,\varepsilon_0) = \frac{k'p}{1+k'p}\left[1 + \frac{\pi^2}{6}\left(\frac{\varrho}{RT}\right)^2 \frac{1-k'p}{(1+k'p)^2}\right] \tag{17}$$

where

$$k' = k_0 \exp\left(\frac{\varepsilon_0}{RT}\right) \tag{18}$$

The behaviour of $\Theta(p)$ differs from that of $\theta(p)$. At the initial low pressures, $\Theta(p)$ has higher values than $\theta(p)$, but is smaller at higher relative pressures. Thus, the behaviour of $\Theta(p)$ is typical of adsorption systems with heterogeneous surfaces (*e.g.*, ref. 35). It still remains to establish the highest reasonable value of ϱ for the degree of approximation adopted in eqn. 15. This is carried out by utilizing the condition that the derivative $\partial\Theta/\partial p$ should not be negative at any value of p. A simple analysis yields the following two values of p_1 and p_2 as the possible zero points of the derivative $\partial\Theta/\partial p$:

$$p_1 = \frac{2A - 1 - \sqrt{3A^2 - A}}{k'(1+A)} \tag{19}$$

where $A = \left(\frac{\pi^2}{6}\right)\left(\frac{\varrho}{RT}\right)^2$ and

$$p_2 = \frac{2A - 1 + \sqrt{3A^2 - A}}{k'(1+A)} \tag{20}$$

As the pressure must have real values, then

$$\left(\frac{\pi^2}{6}\right)\left(\frac{\varrho}{RT}\right)^2 \geqslant 2.0 \text{ or } \frac{\varrho}{RT} > 1.1 \tag{21}$$

is the necessary condition such that the zero points for $\partial\Theta/\partial p$ could exist.

In order to use Θa_{max} as the isotherm "a" in the basic differential equations (eqns. 1 and 2), we would have had to know its equivalent kinetic derivation and then to solve the system of these differential equations for the new kinetic mechanism. Both of these problems are very difficult to solve, and we shall adopt here another strategy, as follows.

We shall replace the isotherm $\Theta(p, k')$ by some Langmuir isotherm $\theta(p, k)$ with some effectively changed parameter $k = mk'$, such that $\theta(p, mk')$ approximates best $\Theta(p, k')$ in the pressure range $(0, c_{max})$. This best approximation is found from the condition

$$\frac{\partial}{\partial m}\int_0^{c_{max}} \left\{\frac{m\,k'\,p}{1+m\,k'\,p} - \frac{k'\,p}{1+k'\,p}\left[1+\frac{\pi^2}{6}\left(\frac{\varrho}{RT}\right)^2\frac{1-k'\,p}{(1+k'\,p)^2}\right]\right\} dp = 0 \tag{22}$$

In other words, we shall use for "a" some Langmuir isotherm with the effectively changed parameter mk'. Of course,

$$m = m(\varrho, k', c_{max}) \tag{23}$$

When the velocity of the carrier gas is sufficiently low, *i.e.*, the elution process can be assumed to run at equilibrium, the function $c(t)$ will depend parametrically only upon the ratio $k = k_a/k_d$. Then, the effects of surface heterogeneity can be taken into account directly by replacing k' with mk'. If, however, equilibrium is not attained, then we have to decide the way in which the change in k' is affected by changes in k_a and k_d. This problem leads us to the kinetics of adsorption on heterogeneous surfaces.

Although over 30 years have passed since Roginskii[36,37] considered this problem, his theoretical results are still of major importance. His theory, however, requires much additional information, such as the distribution of adsorption and desorption activation energy. Therefore, we shall adopt here some other means of investigating this problem. Let us consider to this purpose the equation[38]

$$\frac{1}{v_0}\theta_1(p) = \frac{(k_r\,p)^r}{1+(k_r\,p)^r},\quad 0 < r \leqslant 1 \tag{24}$$

which has been found to describe well the adsorption on many heterogeneous surfaces [39,40]. In the limit $r \to 1$, eqn. 24 becomes a Langmuir isotherm. Assuming, as in our theory, that the local adsorption on the energetically homogeneous areas of the surface is governed by the Langmuir equation, Sips[41] evaluated the energy distribution function $\chi_1(\varepsilon)$ corresponding to Bradley's equation[24]. This function has the following form*:

* Eqn. 24 is only a useful empirical relationship. Its related energy distribution function (eqn. 25) was found from the appropriate inverse Stieltjes transform. Honig and Hill[42] formulated the necessary mathematical conditions to be fulfilled by the overall adsorption isotherms, which are Stieltjes transforms of some function $\chi(\varepsilon)$. These conditions are not fulfilled exactly by eqn. 24. Therefore, the function $\chi_1(\varepsilon)$ from eqn. 25 is temperature dependent and does not tend to a delta function as r tends to unity.

$$\chi_1(\varepsilon) = \frac{1}{\pi RT} \cdot \frac{\sin(\pi r) \exp\left[\frac{r(\varepsilon_m - \varepsilon)}{RT}\right]}{1 + 2\cos(\pi r) \exp\left[\frac{r(\varepsilon_m - \varepsilon)}{RT}\right] + \exp\left[\frac{2r(\varepsilon_m - \varepsilon)}{RT}\right]} \tag{25}$$

where ε_m is the most probable energy of adsorption on a given heterogeneous surface. It can be seen that the distribution from eqn. 25 is very similar to the energy distribution from eqn. 14, accepted by us, if $\varepsilon_m = \varepsilon_0$. It can be further shown that

$$k_r = k_0 \exp\left(\frac{\varepsilon_m}{RT}\right) \tag{26}$$

We can formally consider the following set of equations:

$$V_a = k_a p(1 - \theta_1)^{1/r} \tag{27}$$

$$V_d = k_d \theta_1^{1/r} \tag{28}$$

where V_a and V_d denote the adsorption and desorption rates, respectively, as the kinetic derivation of eqn. 24. From these equations, it follows that

$$k_r = \left[\frac{k_a}{k_d}\right]_{\varepsilon = \varepsilon_m} \tag{29}$$

In this way, we have defined the adsorption and desorption rate coefficients for a given heterogeneous surface, as being equal to their values for the areas of surface corresponding to the most probable energy of adsorption ε_m (or ε_0 if the adsorption energy distribution is described by eqn. 14).

The special behaviour of the rates of adsorption and desorption on heterogeneous surfaces is described by the functional relationships $V_a(\theta_1)$ and $V_d(\theta_1)$ given by eqns. 27 and 28. Let us re-write these equations in the form

$$V_a = k_a' p (1 - \theta_1) \tag{30}$$

$$V_d = k_d' \theta_1 \tag{31}$$

where

$$k_a' = (1 - \theta_1)^{1/r - 1} \tag{32}$$

$$k_d' = \theta_1^{1/r - 1} \tag{33}$$

In the kinetic picture of adsorption, our procedure of replacing eqn. 17 with eqn. 11, with the effectively changed parameter mk', is equivalent to replacing eqns. 32 and 33 with the following:

$$k_a' = k_a'' = \frac{k_a}{\theta_1} \int_0^{\theta_1} (1 - x)^{1/r - 1} \, dx = \frac{rk_a}{\theta_1} [1 - (1 - \theta_1)^{1/r}] \tag{34}$$

$$k_d' = k_d'' = \frac{k_d}{\theta_1} \int_0^{\theta_1} x^{1/r - 1} \, dx = \frac{rk_d}{\theta_1} \theta_1^{1/r} \tag{35}$$

Therefore, we can write

$$mk' = \frac{k_a}{k_d} = \frac{k_a}{k_d}\left[\frac{1-(1-\theta_1)^{1/r}}{\theta^{1/r}}\right] \tag{36}$$

If the surface becomes homogeneous with adsorption energy $\varepsilon = \varepsilon_0 = \varepsilon_m$, then $\varrho \to 0$, $r \to 1$ and $m \to 1$. Then, $k' = k_r = k_a/k_d$. The effective constant mk' is calculated here by means of eqn. 22.

In non-equilibrium GSC processes, only in the limiting cases of very low and very high adsorbate concentrations in the column can the problem be solved quantitatively.

With very low concentrations in the column, which correspond to small relative coverages of the surface, the effective adsorption rate constant will still be k_a, while the effective desorption rate constant will be k_d/m. The opposite situation will occur at very high concentrations in the column (very high relative coverages of the surface) *i.e.*, the effective adsorption rate constant will be $k_a m$ while the effective desorption rate constant will be k_d. Of most interest is the region of low adsorbate concentrations in the column, as typical chromatographic processes are carried out by using small doses of adsorbate. For this reason, we performed some illustrative calculations of the effect of surface heterogeneity on the shape of the elution curve, $c(t)$, only in the region of low adsorbate concentrations in the free gas phase, and these calculations are considered in the next section.

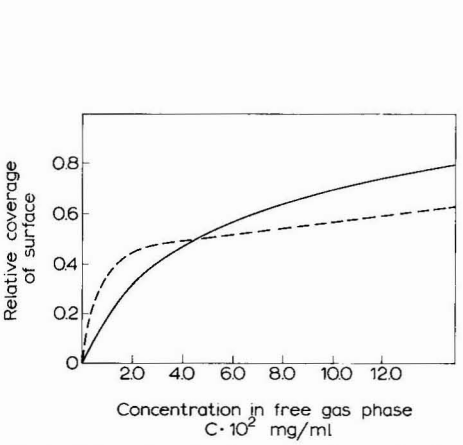
Our conclusions concerning the effect of surface heterogeneity on the rates of adsorption and desorption may be important in catalysis studies, in which the rate of adsorption of substrates and the rate of desorption of products are very important factors governing the effectiveness of catalytic processes.

We should mention that eqns. 32 and 33 provide only basic information on the way in which heterogeneity of the surface affects the rates of adsorption and desorption at very low and very high concentrations of the adsorbate in the free gas phase, *i.e.*, the way in which the surface heterogeneity affects the adsorption and desorption rate constants, when estimated chromatographically by using various sample sizes of the absorbate. Eqns. 32 and 33 are not valid in the limit $p \to 0$, as Bradley's equation (eqn. 24) does not then behave correctly as it does not reduce to Henry's law, which is the limit of all correct adsorption isotherms (see footnote, p. 5). However, eqn. 17 reduces correctly to Henry's law when $p \to 0$ and therefore the parameter m can also be interpreted in this limit.

RESULTS AND DISCUSSION

In our illustrative calculations, we employed parameters similar to those used by Zhitomirskii *et al.*[30]. Thus, we assumed $k_a = 10^5$ ml/mg·min, $k_d = 400$ min^{-1} and $a_{max} = 10$ mg/ml. For $k' = k_a/k_d = 250$ ml/mg, we evaluated both $\theta(p, k')$ and $\Theta(p, k')$, assuming that $\varrho/RT = 1.0$. The results are shown in Fig. 1. The behaviour of $\Theta(p)$ in comparison with $\theta(p)$ is typical for heterogeneous surfaces.

Further, we evaluated m for various concentration ranges $[0, c_{max}]$, taking $k' = 250$ and 100 ml/mg. The results are shown in Fig. 2. It can be seen that m has a tendency to stabilize at very small and very large values of c_{max}. It can also be seen



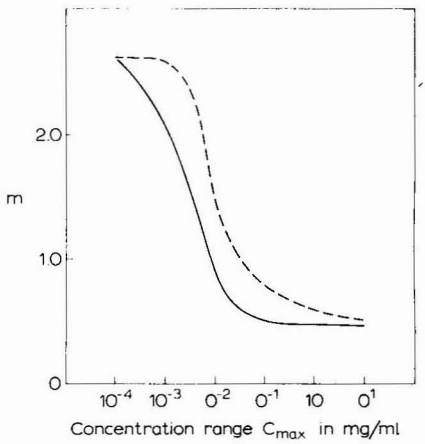


Fig. 1. Effect of surface heterogeneity on the shape of adsorption isotherms. The solid line corresponds to the Langmuir isotherm from eqn. 11 with $k = 250$ ml/mg and $\varrho/RT = 0.0$. The broken line denotes the isotherm from eqn. 17 with $k' = 250$ ml/mg and $\varrho/RT = 1.0$.

Fig. 2. Effect of the concentration range, c_{max}, on the value of the best-fit parameter, m, to be used to approximate the isotherm in eqn. 17 by the isotherm in eqn. 11 in this concentration range. The solid line corresponds to adsorption systems with $k' = 250$ ml/mg and $\varrho/RT = 1.0$ and the broken line is related to adsorption systems with $k' = 100$ ml/mg and $\varrho/RT = 1.0$.

that the greater the adsorption constant k', the greater is the effect of surface heterogeneity at low adsorbate concentrations and the smaller the effect at high adsorbate concentrations in the free gas phase. The adsorption energy ε has only a minor effect on the pre-exponential constant k_0. Thus, the greater the most probable adsorption energy $\varepsilon_0 = \varepsilon_m$, the larger is the decrease in the rate of desorption at low adsorbate concentrations, and the smaller is the decrease in the adsorption rate at high adsorbate concentrations in the free gas phase.

Zhitomirskii *et al.*[30] proposed a method for estimating chromatographically the adsorption and desorption rate constants. The process must be carried out far from equilibrium, and the most convenient way is to use eqn. 10, relating c_{max} to t_{max}. The dependence of c_{max} on t_{max} can be measured experimentally by introducing different amounts of adsorbate into the column at a constant flow-rate, the experiments being repeated for different flow-rates. The dependence of $c_{max} \cdot t_{max}^{1/2}$ on $(z/u_g)^{1/2}$ which, according to eqn. 10, must be a straight line, can be constructed from intersections of the curves obtained with straight lines of equation $t_{max} = \text{constant}$. From the tangent of this straight line one obtains $k_d \cdot a_{max}/k_a$ and from the intercept on the ordinate one obtains $(k_a \cdot l \cdot t_{max})^{-1} + k_d/k_a$. Each of the constants k_a, k_d and a_{max} can be obtained from two such straight lines for two values of t_{max}.

Our considerations show that the above procedure should be applied very carefully. As we already have seen, surface heterogeneity affects both adsorption and desorption rates, although in different ways at different adsorbate concentrations. There may be some balancing of these effects in some concentration regions with the ratio k_a/k_d appearing as a pressure-independent value. This feature, however, is not a sufficient check of surface homogeneity, and the measured rate constants k_a and k_d may have apparent values that differ from their true values. It seems to us that the

following modification of this procedure is necessary in order to estimate reliable values of the adsorption and desorption rate constants:

(1) Firstly, this procedure should be applied in the region of low adsorbate concentrations in the free gas phase, where one should estimate the correct value of the adsorption rate constant, k_a. In other words, experiments should be carried out with very small doses of the adsorbate.

(2) Next, the method should be applied in the region of very high adsorbate concentrations in the column, to estimate reliably the desorption rate constant, k_d. This can be done by using large doses of the adsorbate.

(3) The investigations should be extended to lower and higher ranges of c_{max} until the estimated k_a and k_d values become pressure-independent.

We shall now demonstrate the effect of surface heterogeneity on the shape of the elution function $c(t)$ with small doses of the adsorbate introduced into the column. Let us assume an adsorption system with a homogeneous surface on which $\varepsilon = \varepsilon_0$ and the other parameters are $k_a = 10^5$ ml/mg·min, $k_d = 400$ min^{-1} and $a_{max} = 10$ mg/ml. Let us assume further that the chromatographic process is running at $z/u_g = 0.01$ min and a very small dose of adsorbate is used, corresponding to $m/F = 10^{-5}$ mg/ml·min. The related elution peak is shown as the solid line in Fig. 3.

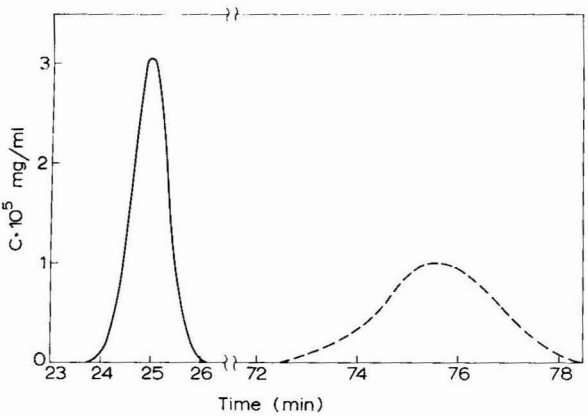


Fig. 3. Effect of surface heterogeneity on the shape of elution curves at low concentrations of adsorbate in the free gas phase (low doses of adsorbate corresponding to $m/F = 10^{-5}$ mg/ml·min). The adsorption system is characterized by the values $k_a = 10^5$ ml/mg·min, $k_d = 400$ min^{-1}, $a_{max} = 10$ mg/ml and $z/u_g = 10^{-2}$ min. The solid line corresponds to the situation when $\varrho/RT = 0.0$, *i.e.*, to a homogeneous surface. The broken line corresponds to the situation when $\varrho/RT = 1.0$. In the latter instance, the apparent value of k_d is, according to our theory, 133 ml/mg.

If we now assume a heterogeneous surface with the most probable adsorption energy equal to ε_0 and a distribution of adsorption energy characterized by $\varrho/RT = 1.0$, at such small concentrations of adsorbate in the free gas phase the best-fit parameter m will be about 3.0. Consequently, the effective desorption rate constant k'_d/m will be about 133 min^{-1}, while the effective adsorption rate constant will still be 10^5 ml/mg·min. With $z/u_g = 10^{-2}$ min and $m/F = 10^{-5}$ mg/ml·min, the evaluated elution curve $c(t)$ has a shape corresponding to the broken line in Fig. 3. It can easily be seen that this latter peak is much flatter, appears at much higher retention times

and some loss of symmetry is observed in comparison with the peak corresponding to a homogeneous surface.

All of the features obtained here by mathematical means have been observed in practice. Our theory may make it possible to estimate quantitatively both the adsorption and desorption rate constants k'_a and k'_d and the heterogeneity parameter ϱ in the same set of experiments. If we assume that we have already estimated k_a and k_d according to the modified procedure recommended here, then we have also estimated the parameter $m(c_{max})$ for the initial region of concentrations in the free phase, usually called the Henry region. According to eqn. 22, in this pressure region we have

$$m\,k'\,p = k'\,p\left[1 + \frac{\pi^2}{6}\left(\frac{\varrho}{RT}\right)^2\right] \tag{37}$$

Thus:

$$\varrho = \frac{RT}{\pi}\,[6\,(m-1)]^{\frac{1}{2}} \tag{38}$$

However, at present we do not have appropriate experimental data at our disposal to illustrate this procedure. This aspect will be considered in future publications.

CONCLUSIONS

It is possible to investigate quantitatively the effects of surface heterogeneity in non-linear and non-equilibrium GSC by using a new formulation for the kinetics of adsorption on heterogeneous surfaces. In the absence of exceptional behaviour of the activation energies of adsorption and desorption on adsorption sites that have different adsorption energies, this new formulation of the kinetics of adsorption can be summarized as follows:

(1) at low adsorbate concentrations in the free gas phase, the surface heterogeneity decreases the rate of desorption, while the rate of adsorption remains unchanged;

(2) at high adsorbate concentrations, the surface heterogeneity decreases the rate of adsorption, but does not affect the rate of desorption;

(3) the adsorption and desorption rate constants are defined as being equal to their values for surface sites with the most probable energy of adsorption.

It is possible to estimate quantitatively these rate constants by using the procedure described here. Simultaneously, a parameter is estimated that describes quantitatively the degree of surface heterogeneity. As the degree of surface heterogeneity increases, the elution peaks corresponding to small sample sizes of adsorbate become increasingly flatter. They appear at increasingly longer elution times, and the peak symmetry is gradually lost.

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EIN SCHNELLER UND EMPFINDLICHER WÄRMELEITFÄHIGKEITS-DETEKTOR ZUR VERWENDUNG MIT KAPILLARSÄULEN

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SUMMARY

Fast and sensitive thermal conductivity detector for use with capillary columns

A thermal conductivity detector is described, that in its time constant equals the flame ionization detector and has the same sensitivity for the measurement of concentration as conventional thermal conductivity detectors used with packed columns. The small time constant and the high sensitivity are the result of a pressure drop being maintained between the column connection and the measuring cell, so that the gas is expanded to a bigger volume and the volume velocity in the measuring cell is increased. A chromatogram, taken with a gas expansion by a factor of 50 is compared with a corresponding chromatogram without gas expansion.

EINLEITUNG

In zunehmendem Masse werden bei der gaschromatographischen Analyse Kapillarsäulen, insbesondere Glas-Kapillarsäulen eingesetzt, die sich durch hohe Trennleistungen auszeichnen, nach verschiedenen Verfahren relativ leicht herstellbar sind und deren Handhabung weit weniger problematisch ist, als es die sprichwörtlich bekannte Zerbrechlichkeit des Glases zunächst vermuten lässt[1–3]. Es sind Eingangsteiler, die über einen grossen Siedebereich der Komponenten hin konstante Teilerverhältnisse einhalten, entwickelt worden[3–5], und bei Anwendung der splitlosen Injektion sind Kapillarsäulen auch für Spurenanalysen einsetzbar[6].

Eines der Hindernisse, das einem noch stärkeren Vordringen von Kapillarsäulen bisher entgegenstand, ist jedoch der Umstand, dass sie nicht in befriedigender Weise mit Wärmeleitfähigkeitsdetektoren (WLD) betrieben werden konnten. Für einen Teil der Analysenaufgaben war daher die Bearbeitung mittels Kapillarsäulen prinzipiell ausgeschlossen, für einen anderen Teil ergaben sich durch die Notwendigkeit einen Flammenionisationsdetektor (FID) verwenden zu müssen, grössere, unter Umständen abschreckend grosse Probleme der Eichung.

Hinsichtlich der Messempfindlichkeit ist, vom Funktionsprinzip der beiden Detektoren aus betrachtet, die Tatsache, dass für Kapillarsäulen nur der FID und nicht auch, oder sogar bevorzugt, der WLD eingesetzt wird, paradox. Der FID misst bekanntlich den Mengenstrom der Komponenten und die Mengenströme sind bei

Kapillarsäulen kleiner als bei gepackten Säulen. Der WLD misst jedoch Konzentrationen und die Konzentrationen der Komponenten im Eluat sind bei Kapillarsäulen eher grösser als bei gepackten Säulen. Deshalb müssten eigentlich alle WLD-Analysen statt mit gepackten Säulen auch mit Kapillarsäulen durchführbar sein.

Schliesst man aber eine Kapillarsäule an eine handelsübliche WLD-Zelle an, so geht die für Kapillarsäulen charakteristische gute Auflösung verloren und die Konzentrations-Messempfindlichkeit des Detektors scheint vermindert. Beides beruht darauf, dass das Volumen des Detektors wesentlich grösser ist als die Peakvolumina*. Infolgedessen ist die Zeit, die eine Komponente benötigt, um durch den Detektor hindurchzuwandern, grösser als die Zeit, in der sie eluiert wird, und ausserdem füllen die Komponenten nur einen Teil der Detektorzelle aus, so dass der Detektor nicht die tatsächliche Konzentration der Komponente im Eluat, sondern einen über das gesamte Zellenvolumen gemittelten und entsprechend kleineren Wert misst.

Zur Anpassung an die kleinen Volumenströme und dementsprechend kleinen Peakvolumina bei Kapillarsäulen sind spezielle Detektoren mit kleinem Messvolumen konstruiert worden[7–11,16,17]. Bei Hitzdrahtdetektoren gelangt man dabei an konstruktive und messtechnische Grenzen, weil einerseits Messvolumina $< 10\,\mu l$ erforderlich sind und andererseits die Messempfindlichkeit von Hitzdrahtdetektoren der Länge des Drahtes proportional ist[12].

Man kann nun aber auch umgekehrt vorgehen und nicht den Detektor an den Gasstrom sondern den Gasstrom an den Detektor anpassen; anstelle der Verkleinerung des Detektorvolumens also einen Vergrösserung des Volumenstroms durch den Detektor vornehmen.

Dies durch Einspeisung von zusätzlichem Trägergas zu tun, ist beim WLD unzweckmässig, weil dadurch das Eluat verdünnt wird. Das physikalische Phänomen der Wärmeleitung fordert jedoch eine andere Massnahme geradezu heraus: Da nämlich die Wärmeleitung bis hinab zu Drucken von 1 mbar nur sehr wenig vom Druck abhängt, liegt es nahe, das Säuleneluat von grossem Druck (1 bar) und kleinem Volumen auf kleinen Druck und grosses Volumen zu bringen. Mittels einer zwischen Säulenausgang und Messzelle eingefügten Drossel in der der Druckabfall von Normaldruck am Säulenausgang auf den erforderlichen Unterdruck in der Messzelle erfolgt, lässt sich diese Zustandsänderung erreichen.

Im Gegensatz zu Verfahren, bei denen die Säule durch direkten Anschluss an einen evakuierten Detektor teilweise mit evakuiert wurde[13–15], können durch den Einbau der Drossel die Parameter der gaschromatographischen Trennung unverändert bleiben.

THEORIE

Das Zeitverhalten des Detektors wird durch die Laufzeit durch den Detektor t_d beschrieben. Es gilt die Beziehung

$$t_d = \frac{V_d}{\dot{V}_d} = \frac{V_d}{\dot{V}_s} \cdot \frac{p_s}{p_d} \tag{1}$$

* Mit Peakvolumen bezeichnet Kaiser[7] das Volumen des Eluats, auf das die Komponente am Säulenausgang verteilt ist.

Die Laufzeit t_d ist gleich dem Quotienten aus dem Detektorvolumen V_d und der Volumengeschwindigkeit im Detektor $\dot{V}_d$, und $\dot{V}_d$ ist gleich der Volumengeschwindigkeit des Trägergases am Ende der Säule $\dot{V}_s$, multipliziert mit dem Druckverhältnis p_s/p_d zwischen Säulenende und Detektorzelle (wenn die Gasmengenströme und die Temperaturen in Säule und Detektor gleich sind). Fordert man, dass die Halbwertszeit eines registrierten Peaks um weniger als 5% von der Halbwertszeit t_e der Elution der Komponente abweicht, so muss $t_d \leqslant 0.5t_e$ sein.

$$t \leqslant 1.05t_e \text{ wenn } t_d \leqslant 0.5t_e$$

Das Messvolumen gebräuchlicher WLD-Zellen liegt bei etwa 0.5 ml, die optimale Trägergasströmung vieler Glas-Kapillarsäulen liegt bei 1 ml/min. Ist $p_s = p_d$, so beträgt die Laufzeit durch den Detektor 30 sec.

Eine Komponente, die mit einer Halbwertszeit $t_e = 1$ sec aus der Säule austritt, erscheint als Peak mit der Halbwertszeit $t = 30$ sec. Um die Verfälschung der Peakform durch den Detektor aufzuheben, wäre ein Drucksprung $p_s/p_d = 60$ auf $p_d = 17$ mbar erforderlich. Alle Peaks mit $t_e > 1$ sec erschienen dann unverfälscht, aber auch ein Peak mit $t_e = 0.5$ sec würde nur auf $t = 0.6$ sec verbreitert werden.

EXPERIMENTELLES

Der normale WLD eines handelsüblichen Gaschromatographen (Modell 417, Packard-Instruments) wurde zu einem Kapillarsäulen-WLD umgebaut, indem vor und hinter der WLD-Zelle je eine Drossel angebracht wurde (Fig. 1). Eingangsseitig diente als Drossel eine zusammengedrückte Stahlkapillare; am Ausgang wurde ein Nadelventil verwendet. Eine Vakuumpumpe hielt hinter der Ausgangsdrossel einen Druck < 0.1 mbar aufrecht. Der Druck im Detektor, der durch den Vordruck und die Strömungswiderstände der beiden Drosseln bestimmt wird, und an einem Manometer abgelesen werden konnte, wurde mit Hilfe des Nadelventils auf 20 mbar eingestellt.

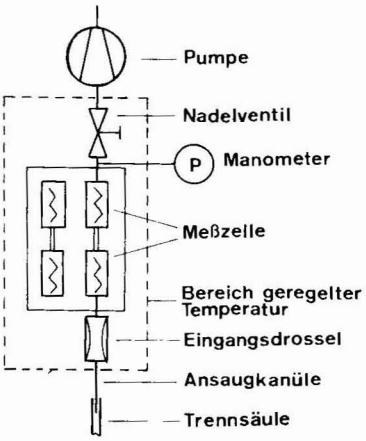

Fig. 1. Prinzip des Wärmeleitfähigkeitsdetektors mit Gas-Expansion.

Die Eingangsdrossel war so zu bemessen, dass bei Atmosphärendruck 1.8 ml Helium/min in sie einströmten. An den Detektor wurde eine 18-m lange Kapillarsäule aus Glas, Innendurchmesser 0.25 mm, angeschlossen. Der Anschluss geschah in sehr einfacher Weise dadurch, dass die sehr dünne Stahlkapillare, die in ihrem hinteren Teil die Eingangsdrossel bildete, auf *ca.* 2 cm Länge in die Trennsäule hineingeschoben wurde. Die Strömung durch die Trennsäule wurde auf 1.85 ml/min eingestellt, so dass 0.05 ml/min an der Ansaugkapillare des Detektors vorbei nach aussen abströmten.

Fig. 2a zeigt ein Chromatogramm, dass mit diesem Detektor aufgenommen wurde. Die Komponenten und die Analysenbedingungen sind in der Bildunterschrift angegeben. Fig. 2b zeigt zum Vergleich ein Chromatogramm, bei dem die Säule direkt an die WLD-Zelle angeschlossen war und sich der Detektor auf Atmosphärendruck befand. Alle anderen Bedingungen waren die gleichen wie bei Fig. 2a. Wie der Vergleich zeigt, sind bei Verwendung des Unterdruck-WLD die Peaks etwa 10 mal so hoch wie bei Normaldruck, während die Halbwertsbreite etwa um den gleichen Faktor kleiner ist.

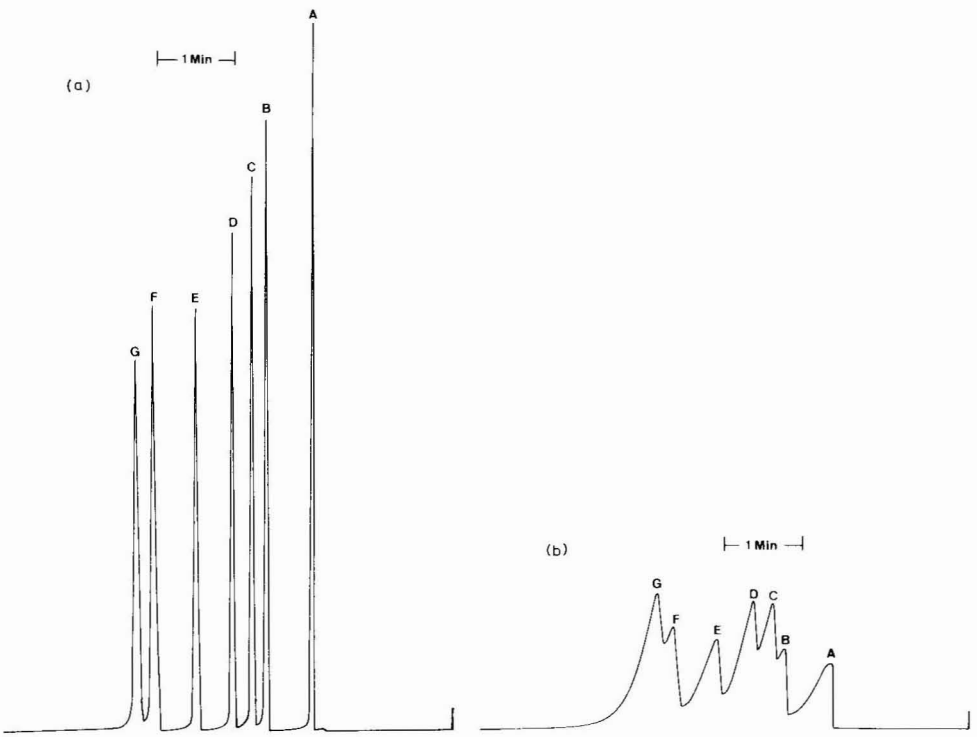

Fig. 2. Kapillarsäulen-Chromatogramm, aufgenommen bei (a) einem Druck von 20 mbar und (b) Atmosphärendruck im Detektor. Gaschromatographische Bedingungen: 18 m Kapillarsäule aus Glas, I.D. 0.25 mm, Belegung OV-101; Trägergas Helium, 1.85 ml/min; Säulentemperatur, 25°; Split, 1:140; Probemenge, 0.3 µl. Komponenten: A = *n*-Pentan, B = 2-Methylpentan, C = 3-Methylpentan, D = *n*-Hexan, E = Methylcyclopentan, F = Benzol, G = Cyclohexan. Detektorstrom 100 mA, Abschwächung 1:8.

DISKUSSION

Die aus den Chromatogrammen ablesbare Verminderung der Halbwertsbreiten und Zunahme der Höhen der Peaks stimmt recht gut mit der Theorie überein. Für ein Messvolumen von 0.5 ml, den Gasstrom von 1.8 ml/min und einen Peak mit der Halbwertsbreite $t_e = 1.5$ sec ergibt sich aus Gleichung 1 $t_d/t_e = 11$. Dabei ist der Vergleich nur für die Komponente A möglich (Fig. 2), weil bei allen anderen Komponenten im Chromatogramm 2b eine teilweise Überlagerung stattfindet und die auf die Basis bezogene Peakhöhe nicht messbar ist. Eine noch bessere Übereinstimmung von Messung und Rechnung ist nicht zu erwarten. Die Annahme eines Messvolumens von 0.5 ml ist nur eine grobe Schätzung, das Zellenvolumen beträgt 0.8 ml, aber die Hitzdrähte erstreckten sich nicht über die volle Länge der Zelle.

Der Trägergasstrom ist mit 1.8 ml/min recht hoch. Der Drucksprung $p_s/p_d = 50$ hätte nach Gleichung 1 offenbar ausgereicht, um auch noch bei kleineren Gasströmen oder schmaleren Peaks die wahre Peakform zu registrieren. Die untere Grenze des Trägergasstroms war jedoch durch die feste Eingangsdrossel vorgegeben.

Die etwas seltsamen Werte von Säulen-, Einspritzblock- und Detektortemperatur haben keine besondere Bedeutung. Das gezeigte Chromatogramm ist eines der ersten, das mit dem neuen Detektor aufgenommen wurde, und die Apparatur wies noch einige Unvollkommenheiten auf. Die bedeutendste ist, dass nur die Messseite des Detektors evakuiert war. Das führt erstens, weil bei 20 mbar doch schon ein gewisser Druckeinfluss auf die Wärmeleitung besteht, zu einer grösseren Brückenverstimmung, die elektrisch kompensiert werden muss und hat zweitens ein durch Druckschwankungen bedingtes Rauschen zur Folge, das bei der Abschwächung 1:8 und 100 mA Brückenstrom allerdings noch nicht sichtbar ist.

Zur Verminderung dieser Störeinflüsse ist es erforderlich, Mess- und Vergleichsseite zu evakuieren. Ausserdem müssen sowohl die eingangsseitigen wie auch die ausgangsseitigen Strömungsdrosseln auf konstanter Temperatur gehalten werden. Es ist deshalb zweckmässig, die Strömungsdrosseln und den eigentlichen WLD konstruktiv zu einer Einheit zusammenzufassen.

ZUSAMMENFASSUNG

Es wird ein Wärmeleitfähigkeitsdetektor beschrieben, der in seinem Zeitverhalten dem Flammenionisationsdetektor gleicht und für Kapillarsäulen die gleiche Konzentrations-Messempfindlichkeit besitzt, wie herkömmliche Wärmeleitfähigkeitsdetektoren bei gepackten Säulen. Die kleine Zeitkonstante und die grosse Messempfindlichkeit des Detektors werden dadurch erreicht, dass zwischen der Anschlussstelle der Säule und der Messzelle ein Druckgefälle aufrechterhalten wird, so dass das Gas auf eine grösseres Volumen expandiert und die Volumengeschwindigkeit in der Messzelle erhöht wird. Ein mit einer Gas-Expansion um den Faktor 50 aufgenommenes Kapillarsäulen-Chromatogramm wird mit einem entsprechenden Chromatogramm ohne Gas-Expansion verglichen.

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DEVELOPMENT AND USE OF CARBON ADSORBENTS IN HIGH-PERFORMANCE LIQUID–SOLID CHROMATOGRAPHY

II. REPRODUCIBILITY OF CARBON ADSORBENTS AND THE INFLUENCE OF GRAPHITIZATION ON THEIR PERFORMANCE

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SUMMARY

The reproducibility of the performance of carbon columns has been studied in terms of retention data and efficiency. The repeatability of the data obtained with one column over several months was excellent, in spite of its use for the analysis of a variety of samples with solvents with a wide range of eluotropic strengths. It was found that the retention data on carbon adsorbents are proportional to the total surface area of the adsorbent in the column, within the limits of error of measurement of this parameter, except for polyaromatics. For these compounds, the carbon adsorbents exhibit active sites and the retention increases faster than the surface area. Graphitization had little effect on the retention data or the eluotropic strengths of the solvents.

The column efficiency for inert peaks is good, showing that excellent packings can be obtained with carbon black particles, either graphitized or not. The column efficiency decreases markedly with increasing retention, which seems to be a property of columns packed with large particles of porous adsorbents. Graphitization has also a small effect on this phenomenon and accordingly is much less useful in liquid than in gas chromatography, except in preparative applications, as the loadability is one order of magnitude greater.

INTRODUCTION

The reproducibility of retention data in liquid chromatography (LC) has been the subject of contradictory reports. Some found it excellent[1], while others found it difficult to obtain comparable retention data on different columns packed with particles of the same batch of adsorbent[2]. In fact, the situation is very different for polar and non-polar adsorbents. In the former instance the reproducibility of the water content of the adsorbent, and hence its degree of activation, is critical and it has been demonstrated that the water contents of alumina and silica control the retention to a large extent[3,4]. Reproducibility of retention data from column to column can be

achieved only if the water content before packing and also the amount of water adsorbed during the packing procedure are controlled. Further, the water concentration in the mobile phase should be kept constant, which is not easy at values below saturation. The reproduction of a given water concentration is even more difficult to achieve. Finally, when not in use, columns should be kept closed, filled with a solvent. All of these steps are critical and often overlooked, which probably explains the poor degree of reproducibility that is often achieved.

When non-polar adsorbents are used in reversed-phase chromatography, the problem is different and simpler. The ubiquitous water is not appreciably adsorbed, and the compounds most strongly adsorbed by non-polar adsorbents do not change appreciably the polarity of the surface. In this instance the reproducibility of retention data depends mainly on the reproducibility of the chemical properties of the surface itself. This is easy with carbon adsorbents derived from carbon black, the surface chemical homogeneity of which is well known.

In this paper we discuss the reproducibility of chromatographic results achieved with carbon adsorbents and the effect of graphitization of the adsorbent. This treatment has a considerable effect on retention data in gas chromatography but the effect is much less important in liquid chromatography.

The preparation and general properties of carbon adsorbents have been described previously[5,6].

REPRODUCIBILITY OF CARBON COLUMNS

Adsorption on carbon surfaces is closely related to the size of the adsorbate molecules, which is the opposite of the effect with polar adsorbents, where the main parameter appears to be the polarity of the adsorbate[5,6]. Of compounds that are liquid at room temperature, water has the smallest molecule and consequently the extent of its adsorption on carbon surfaces is very small. As a result, a column packed with carbon black (CB) particles containing some adsorbed water and eluted with a solvent that has not been adequately dehydrated and the water content of which is not kept constant exhibits the same chromatographic properties as a column with which special care has been taken to keep the water content constant or negligible in both the adsorbent and the eluent.

On the other hand, aromatic impurities have to be carefully removed from the system because of their strong adsorption. However, pollution of either the solvent or the adsorbent by such impurities is less common than pollution by water.

The reproducibility of chromatographic results was studied on the same column used at different times (long-term repeatability) and on columns packed with the same and with different varieties of CB (column-to-column reproducibility). The parameters concerned are the retention data (capacity factors, solvent strengths) and the efficiency (HETP curves and dependence of HETP on the capacity factor).

Long-term repeatability

This property was studied by comparing separations of mixtures carried out at different times, firstly with the column freshly packed (condition I) and secondly after the column had been operated several weeks (condition II), using more than 20 solvents covering a wide range of eluotropic strengths and left open and dry in the

laboratory for 4 months. Plugs of porous PTFE were fitted at both ends of the column so as to prevent any loss of packing material.

The separations of mixtures of methylbenzenes (MB), methylphenols (MP) and diphenols (DP), *i.e.*, weakly polar, polar and very polar solutes, were studied, using as eluents acetonitrile (for MB and MP) and ethyl acetate (for DP). The results are summarized in Table I and Figs. 1 and 2.

TABLE I

REPEATABILITY OF CHROMATOGRAMS ON MODIFIED CARBON BLACKS

*Compound**	*Capacity factor (k')*		*Efficiency (N)*		*Resolution (R_s)*	
	I	*II*	*I*	*II*	*I*	*II*
Benzene	0.04	0.03	2000**	2100**		
					2.80	2.76
o-Xylene	0.35	0.33	1350**	1280**		
					2.40	2.39
1,2,4-Trimethylbenzene	0.81	0.74	1800**	1800**		
					—	—
1,2,4,5-Tetramethylbenzene	2.04	1.90	2100**	2000**		
p-Cresol	0.42	0.43				
					>2	>2
2,4-Xylenol	0.89	0.91	632	751		
					>2	>2
3,4,5-Trimethylphenol	2.96	2.93	345	1050		
MDC	3.61	3.42	1000***	700***		
					0.91	0.90
ME	4.21	4.13	970***	700***		
					0.96	0.74
EE	4.89	4.79	960***	830***		
					>2	>2
MR	8.74	8.87	1500***	1250***		
					1.27	1.25
ER	10.18	10.45	1600***	1350***		

* For abbreviations see Fig. 2.
** Calculated from injection of the pure compound.
*** Calculated from the chromatogram of a mixture.

The resolution, R_s, is given by

$$R_s = 2 \cdot \frac{t_{R1} - t_{R2}}{W_1 + W_2} \quad (1)$$

where t_{Ri} and W_i are the retention time and bandwidth, respectively, of solute i.

In Fig. 1, the separations of MPs (chromatograms A, B and C) were obtained under conditions I, II and II after the column had been washed with ethyl acetate, respectively. It appears that before washing (B) the elution peaks of phenol derivatives are unsymmetrical and broad, whereas the separation of MBs (not shown)

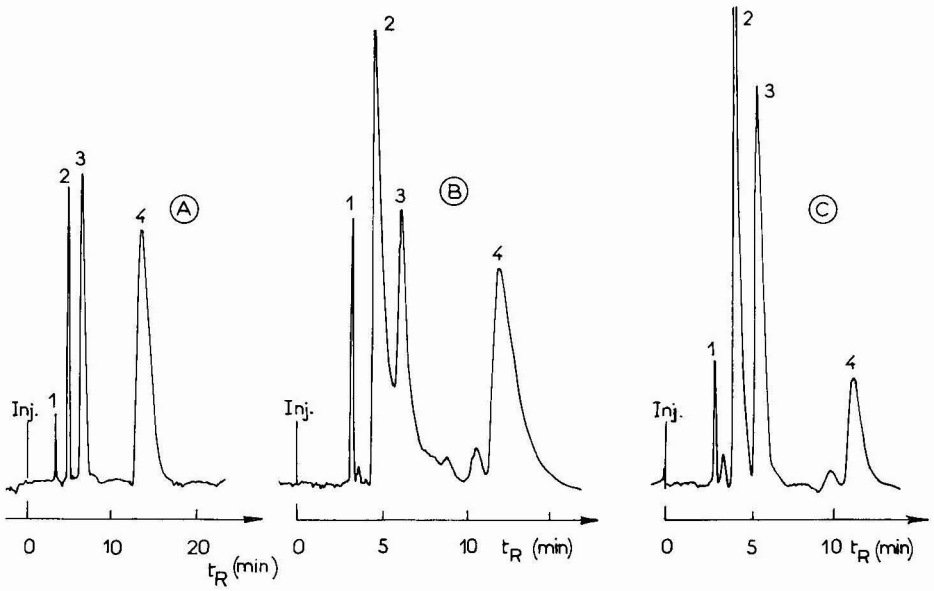

Fig. 1. Reproducibility of the separation of methylphenols. Solvent, acetonitrile; flow-rate, 1 ml/min; Detector, UV (254 nm). column: L = 54 cm; d_c = 2.17 mm; d_p = 25–31.5 μm. Chromatograms, A = freshly packed column (condition I); B = first use of the column under condition II; C = condition II, after flushing with ethyl acetate.

was very good and similar to that obtained under condition I. This suggests that the active sites responsible for the tailing of polar solutes may be provided by polar impurities which are adsorbed on CB and which are soluble in ethyl acetate but not in acetonitrile.

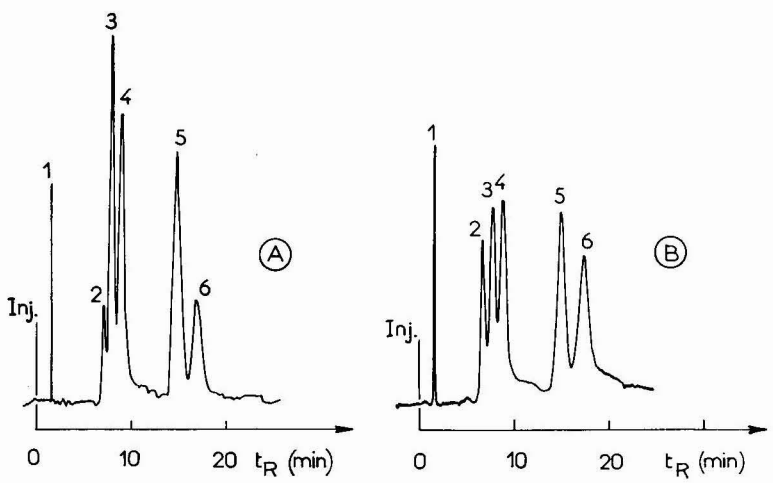

Fig. 2. Separation of diphenol derivatives. Solvent, ethyl acetate; flow-rate, 1 ml/min; detector, UV (254 nm). Peaks: 1 = unretained; 2 = methyl divaricatinate (MDC); 3 = methyl everninate (ME); 4 = ethyl everninate (EE); 5 = Methyl rhizonate (MR); 6 = ethyl rhizonate (ER). For formulae, see ref. 6.

Comparison of chromatograms A and C indicates that excellent repeatability is achieved after cleaning the adsorbent surface; the new peaks in chromatograms B and C are due to a change of sample mixture. The same good repeatability was observed for the separation of diphenols, as shown in Fig. 2, where chromatograms A and B were obtained under conditions I and II, respectively[6].

From the data in Table I, it appears that the long-term repeatability of the capacity factor, k', is generally better than 5%. With regard to column efficiency and resolution, it seems that the analytical properties of columns are constant with time, with only a slight decrease in efficiency.

These results are encouraging for the use of modified CB in high-performance liquid chromatography (HPLC), as they show that there is no problem in keeping columns with constant characteristics. Moreover, because water is not adsorbed on the carbon surface, there is no need for drastic control of the water content of the solvent.

Column-to-column reproducibility

It is of great importance that chromatographic data should be reproducible from one column to another when the columns are packed with the same adsorbent under the same conditions. Not only is such reproducibility necessary when chromatographic measurements are to be used for the determination of physico-chemical constants, but it is also of interest to know if it is possible to predict the retention properties of a column packed with a given carbon adsorbent from the data obtained by using a column packed with another carbon adsorbent.

Both problems were studied in this work, using two kinds of CB: Sterling FT.FF, with a specific surface area (S_{sp}) of about 16 m^2/g, and Black Pearls L, with S_{sp} = 150 m^2/g. Most experiments were carried out using acetonitrile, while in a few ethanol and *n*-heptane were used and gave similar results. The characteristics of the different columns studied are summarized in Table II.

TABLE II

CHARACTERISTICS OF COLUMNS USED IN REPRODUCIBILITY STUDY

Column	*Carbon type**	*Pyrocarbon coating (%)*	*Graphitization*	*Total surface area (m^2)*	*Liquid hold-up (cm^3)*
A	B	55	Yes	47	1.50
B	B	44	Yes	72	1.52
C	B	55	Yes	36	1.09
D	B	43	Yes	193	2.85
E	B	33	Yes	66	1.15
F	B	21	No	79	1.27
G	B	43	No	112	1.77
H	S	14	Yes	18	1.36
I	S	15	No	10.3	1.82
J'	S	15	Yes	20	1.93
K	B	44	No	65	1.55
L	B	44	Yes	72	1.56

* B = Black Pearls L; S = Sterling FT.FF.

The fundamental equation in adsorption chromatography relates the capacity factor, k', the equilibrium distribution coefficient, K, and the characteristics of the column (dead volume V_m and total surface area of the adsorbent A_s):

$$k' = K \cdot \frac{A_s}{V_m} = K \cdot \frac{m\, S_{sp}}{V_m} \tag{2}$$

where m is the amount of adsorbent in the column.

The ratio of the capacity factors of one solute on two different columns, i and j, using the same eluent is

$$R_{i,j} = \frac{k_i}{k_j} = \frac{m_i\, S_{spi}\, V_{Mj}}{m_j\, S_{spj}\, V_{Mi}} \tag{3}$$

Eqn. 3 is valid if we assume that the chemical composition of the liquid–solid interface is the same for columns i and j ($K_i = K_j$), and this assumption should be borne in mind when comparing different adsorbents. Moreover, for identity between K_i and K_j the sample size used must be small enough to ensure a linear adsorption isotherm; the maximum amount that can be injected without producing a change in k' is closely related to the specific surface area of the adsorbent.

Even when using columns packed with particles prepared from the same original adsorbent (*i.e.*, Sterling FT.FF or Black Pearls L), it is likely that $S_{spi} \neq S_{spj}$, as coating with pyrocarbon decreases the specific surface area[6]. Data obtained using such columns are reported in Table III, the theoretical values of $R_{i,j}$ being calculated from eqn. 3. The dispersion of the results is hardly significant, as shown by the small values of the standard deviation; the reproducibility is better than 5%, *i.e.*, within the limits of experimental error. There is also reasonable agreement between the experimental and calculated $R_{i,j}$ values. The deviation is probably due to an imprecise estimation of S_{sp} (S_{sp} values are derived from BET measurements, and it is likely that the gas–solid interface area is slightly different from the liquid–solid interface).

TABLE III

COMPARISON OF RETENTION DATA OBTAINED ON DIFFERENT COLUMNS

Solvent: acetonitrile.

Capacity factor ratio	*Mean value*	*Relative standard deviation*	*Calculated value*
$R_{B,A}$	1.83	0.07	1.55
$R_{A,C}$	1.12	0.03	0.95
$R_{E,C}$	1.88	0.04	1.73
$R_{D,C}$	2.48	0.03	2.05
$R_{D,E}$	1.50	0.08	1.18
$R_{J,H}$	1.12	0.04	0.78
$R_{F,G}$	0.76	0.04	0.98

From eqn. 3 we obtain

$$R_{i,k} = R_{i,j}/R_{k,j} \quad (4)$$

This equation allows a test of the consistency of the results. For instance, the calculated value of $R_{E,C}$ is 1.73, the value derived from direct measurement is 1.88, and $R_{E,D}/R_{C,D}$ is 1.66. All of the columns prepared with the same kind of CB provided very consistent data, regardless of the pyrocarbon coating ratio, provided that account is taken of its effect on the specific surface area.

With regard to carbons of different varieties, significant data are reported in Table IV which suggest that $R_{i,j}$ increases with increasing aromaticity of the solute, being roughly independent of the number of alkyl substituents. The larger the specific surface area before hardening, the larger is the retention of polyaromatic compounds relative to monocyclic compounds. The few results obtained with a carbon with a specific surface area greater than that of Black Pearls L (Black Pearls 800, 254 m^2/g) confirm this trend. This effect must be related to another observation, namely that the peaks of conjugated polyaromatic compounds become increasingly unsymmetrical and subject to tailing with increasing specific surface area, while the capacity factors increase considerably with increasing molecular size ($k'_{naphthalene} = 2.25$ and $k'_{fluorene} = 40$ on Black Pearls L with acetonitrile as solvent).

TABLE IV

COMPARISON OF RETENTION DATA OBTAINED ON BLACK PEARLS L AND STERLING FT.FF IN ACETONITRILE

Solute	$R_{E,H}$ (4.34)*	$R_{E,J}$ (5.54)*
o-Xylene	4.55	—
1,3,5-Trimethylbenzene	4.35	4.85
1,3,5-Triethylbenzene	4.52	—
1,2,4,5-Tetramethylbenzene	4.57	4.72
Pentamethylbenzene	5.00	4.41
Naphthalene	6.00	5.00
2-Methylnaphthalene	6.01	5.08
Acenaphthalene	6.97	5.85
Fluorene	7.96	7.07

* Theoretical values according to eqn. 3.

It seems that the carbon surface exhibits sites where the adsorption of the large and flat molecules of polyaromatics is particularly important. This is similar to the template effect reported by Knox and Pryde[7] for completely different adsorbents. The greater the specific surface area, the larger the number and the greater the energy of these sites. This is also in agreement with the energy distribution on the CB surface: the greater the specific surface area, the broader this distribution. This discussion is continued in the next section.

From the analytical point of view, a consequence of this phenomenon is that CB is not a suitable adsorbent for the separation of large conjugated polyaromatics. If, however, it is necessary to use carbon for particular applications, then the analyst should choose a carbon with a small specific surface area or silica gel particles coated with small amounts of pyrocarbon[8].

EFFECT OF GRAPHITIZATION

When CB is used directly in gas chromatography, without purification after its manufacture, the results are bad, the peaks exhibit pronounced tailing and the column efficiency is poor. This is due (a) to the presence of chemical impurities containing polar groups (–OH, –COOH, –C=O, –SH) on the CB surface and (b) to the broad energy distribution of adsorption sites. This distribution can be explained as follows. Many of the carbon atoms in CB particles belong to graphite crystallites with the well known hexagonal structure, while others belong to aromatic or aliphatic groups bonded to the atoms at the edges of the crystallites. The adsorption energies are different for the atoms in the centre and at the edges of crystallites surfaces, and also for the atoms in the aliphatic groups. The larger the crystallites, the narrower is the energy distribution. The ratio (α) of the number of "edge atoms" to the number of "bulk atoms" is closely related to the energy dispersion.

Graphitization is a thermal process which allows an increase in the size of crystallites and a decrease in the number of crystal defects, including surface defects, and it can thus reduce the value of α. However, as graphitization cannot produce monocrystals, α cannot be zero. Excellent results are obtained in gas chromatography with graphitized thermal carbon blacks (GTCB). Although good results are obtained in liquid chromatography with non-graphitized CB, we studied the influence of graphitization on retention and efficiency.

Graphitization occurs when the carbon sample is heated at very high temperatures (3000°). The degree of graphitization (g) is

$$g = \frac{3.44 - \overline{d_{002}}}{0.086} \tag{5}$$

where $\overline{d_{002}}$ Å is the distance between two layers of carbon atoms. For pure graphite $\overline{d_{002}} = 3.35$ Å and $g = 1$. $\overline{d_{002}}$ is derived from X-ray diffraction measurements (Debye–Scherrer diagrams). There is a close relationship between g and the conditions of thermal treatment. Whatever these experimental conditions, it seems that when the sample has been heated at a temperature higher than 3000–3100°, g is greater than 0.6. The chromatographic results are very similar for samples of GTCB with g between 0.6 and 0.9. We obtained the best results for $g = 0.7$, but the effect was small and no systematic study has yet been made.

Retention and graphitization

The influence of graphitization on the thermodynamic properties of carbon adsorbents can be studied by means of the capacity factor (k') and the solvent strength (ε°).

The differences between capacity factors measured on graphitized and non-graphitized carbon blacks (GCB and NGCB) may depend on the solutes and solvents used. Experiments were carried out with four series of compounds, *n*-alkylbenzenes (AB and MB), methylphenols (MP) and polyaromatics (PA), the molecules of which have different sizes, polarities and aromaticities. We used ethanol, acetonitrile and *n*-heptane as solvents. The results for three pairs of columns are reported in Table V, which gives the average values of the ratio $R_{i,j}$. The two columns of each pair were

packed with the same carbons, one of them being graphitized and the other not. Data for $R_{D,G}$ are missing because column *G* was accidentally destroyed during the experiments. Data for alkylbenzenes in *n*-heptane are not given because of their very small retentions.

TABLE V

COMPARISON OF RETENTION DATA OBTAINED ON GCB AND NGCB

Solvent	*Solute**	$R_{D,G}$ *(1.07)***	$R_{L,K}$ *(1.10)***	$R_{J,I}$ *(1.83)***
Ethanol	AB	0.8	—	—
	MB	1.1	1.2	2.2
	MP	1.9	2.6	2.4
	PA	1.1	1.3	1.7
Acetonitrile	AB	0.9		2.3
	MB	1.1	1.3	2.3
	MP	1.1	2.5	2.4
	PA	0.8 1.3	—	1.9
n-Heptane	AB		—	
	MB	—	2.7	2
	MP	-	0.5	0.9
	PA	—	1.3	1.4–1.8

* AB = alkylbenzenes; MB = methylbenzenes; MP = methylphenols; PA = polyaromatics.
** Theoretical values according to eqn. 3.

It appears from Table V that in polar solvents (ethanol and acetonitrile) the retention is generally larger for GCB than for NGCB. The increase in k' seems to be the most important for the polar solutes (MP). The smaller the specific surface area (columns *J* and *I*), the less scattered were the $R_{i,j}$ values, which is in agreement with the previous comments about the distribution of adsorption energy. The results were very similar in ethanol and acetonitrile.

When using *n*-heptane as the solvent, the retention of phenols is less important on GCB than on NGCB. This decrease in k' is probably due to the removal of polar impurities from the surface of CB, such impurities acting as strong adsorption sites for polar solutes. The very small values of $R_{L,K}$ for PA in ethanol and *n*-heptane are surprising.

It seems that, in general, graphitization increases the adsorption energy of solutes. On the other hand, it also produces a more homogeneous surface, decreasing the number and energy of active sites. These two phenomena have opposite effects on the retention of compounds such as PAs, as previously mentioned; consequently, it is difficult to predict the variation of $R_{i,j}$.

As graphitization changes the retention data, it may also affect the eluotropic strength of solvents. This aspect was studied using mixtures of water and ethanol, where ε^0 decreases from 0 (reference value for pure acetonitrile) to −0.5 (in pure water). According to the following equation, changing the composition of the mixture from pure water to pure acetonitrile produces a 10^4-fold decrease in k' for a solute with a molecular area of 8.0 units (*i.e.*, 68 Å^2), which is typical:

$$\log (k'_j/k'_i) = A\,(\varepsilon^0_i - \varepsilon^0_j) \qquad (6)$$

where k'_j is the capacity factor of the solute of molecular area A in solvent j of eluotropic strength ε_j°. By convention A is the molecular area in Å^2 divided by 8.5 (ref. 9). The terms related to the solvent effect and activity coefficients[6] are neglected. Different solutes are used to measure ε°, depending on the water content of the solvent (1,2,4-trimethylbenzene, *o*-xylene and benzene). Polar solutes are not used because neglecting the term for the activity coefficients would lead to a large error because of the strong solute–solvent interactions[6]. Data are reported in Fig. 3 for GCB and NGCB, and there is excellent agreement between results obtained with the two adsorbents. Note that the dependence of ε° on the acetonitrile content is almost linear.

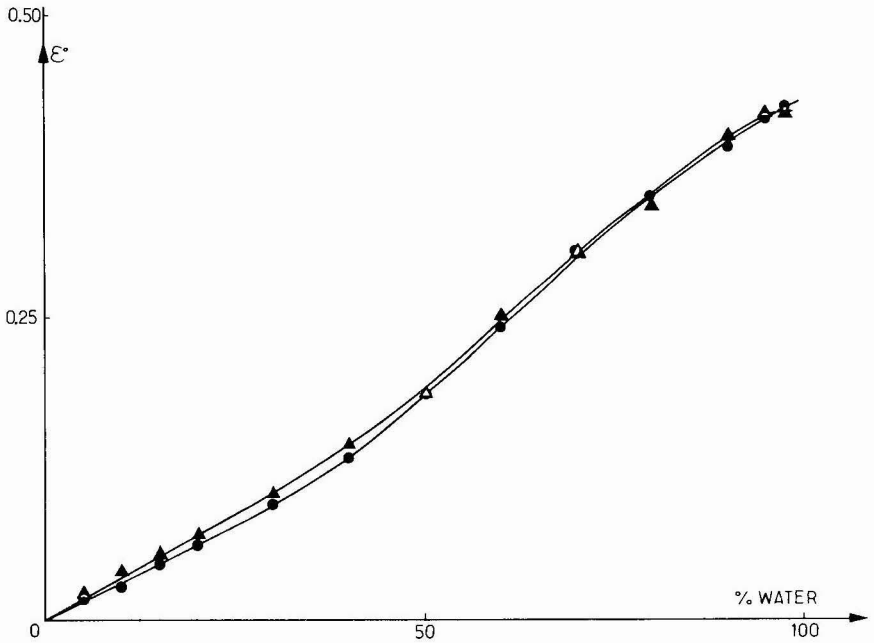

Fig. 3. Solvent strength of ethanol–water mixtures. ●, Graphitized carbon black; ▲, non-graphitized carbon black. Solvent composition is given in volume %.

Let k'^0 and k'^1 be the capacity factors on the graphitized and the non-graphitized adsorbent, respectively. If we assume that the molar area of the solute is the same when adsorbed on GCB or NGCB, eqn. 6 gives

$$\log (k'^0_j/k'^1_j) - \log (k'^0_i/k'^1_i) = A\,[(\varepsilon^{\circ,0}_i - \varepsilon^{\circ,1}_i) - (\varepsilon^{\circ,0}_j - \varepsilon^{\circ,1}_j)] \quad (7)$$

where $\varepsilon^{\circ,0}$ and $\varepsilon^{\circ,1}$ are the solvent strength when the adsorbent is graphitized and non-graphitized, respectively. If $\varepsilon^{\circ,0} = \varepsilon^{\circ,1}$, then

$$k'^0_j/k'^1_j = k'^0_i/k'^1_i \quad (8)$$

Eqn. 8 is in good agreement with the data in Table V for polar solvents. The larger deviation for *n*-heptane is probably explained by the activity coefficient term, which is not taken into account in eqn. 6 because of the lack of data in the literature[6].

In conclusion, it seems that the ε° values are almost identical for GCB and NGCB, which is not surprising as ε° is a relative parameter.

Graphitization of carbon black can also modify its adsorption capacity, and we studied this aspect by comparing the linear capacities of different columns packed with NGCB and GCB. We have chosen as definition of the linear capacity of a chromatographic column the amount of solute per gram of adsorbent which produces a 10% change in the capacity factor (generally a decrease). This capacity is closely related to the capacity factor of the solute, *i.e.*, to the solute, the solvent and the adsorbent. The comparison is tedious as the solute adsorption is different when measured on GCB and NGCB, but some measurements are reported in Table VI. Data are given only for ethanol as reversed-phase chromatography is associated with polar solvents. It appears from Table VI that graphitization generally improves the column loadability. It is not possible to give a general equation that would account for this improvement as too many parameters must be taken into account.

TABLE VI

LINEAR CAPACITIES OF DIFFERENT COLUMNS IN ETHANOL

Column	*Solute**	*Capacity factor*	*Linear capacity*	
			μg/g	*μg/m²*
G	1,3,5-TMB	0.55	12	0.11
D	1,3,5-TMB	0.63	260	1.35
G	3,4,5-TMP	0.77	37	0.33
D	3,4,5-TMP	1.67	115	0.60
K	3,4,5-TMP	0.42	24	0.37
L	3,4,5-TMP	1.23	26	0.36
K	2,3,4,6-TeMP	1.17	22	0.34
L	2,3,4,6-TeMP	3.32	21	0.29
I	1-MMN	0.72	3	0.29
J	1-MMN	1.26	9	0.45
I	1,2-DMN	2.02	2.5	0.24
J	1,2-DMN	3.60	8.5	0.43

* TMB = trimethylbenzene; TMP = trimethylphenol; TeMP = tetramethylphenol; MMN = monomethylnaphthalene; DMN = dimethylnaphthalene.

This effect is important for preparative applications and it is preferable to use GCB in such cases. It is worth noting that for all of the solutes we used, the linear capacities are similar, being between 0.1 and 0.6 $\mu g/m^2$ (with the exception of 1,3,5-trimethylbenzene on column *D*). These values are somewhat smaller than those obtained with silica gel (0.5–5 $\mu g/m^2$).

Column efficiency and graphitization

From the previous discussion, it appears that graphitization does not markedly affect the retention. It would be useless in LC if it does not improve column efficiency. During graphitization the carbon layers move, mainly by rotation, the crystallite size increases, and the structure of CB becomes more similar to the graphite structure. It is well known that the graphite planes can slip easily one over the other, thus providing

its lubricating properties. We would therefore expect that graphitization would produce a decrease in the hardness, porosity and specific surface area of the particles. Our method of measurement of hardness[6] is not sensitive enough to detect small changes in hardness and we did not observe any difference between GCB and NGCB. As far as we are concerned, packings with GCB are as stable as those with NGCB.

Conversely, S_{sp} decreases markedly upon graphitization. For instance, for Black Pearls 46 $S_{sp} = 650$ m^2/g before and 150 m^2/g after graphitization. This effect, however, is less important when CB is coated with a large amount of pyrocarbon, and when the specific surface area of the original NGCB is small. For example, graphitization of Sterling FTFF modified with 30% pyrocarbon produces a decrease in S_{sp} of less than 3%.

Precise porosity measurements have not yet been made, but the different columns packed in the laboratory with samples of CB differing only in graphitization treatment have similar permeabilities and hold-up volumes, suggesting constant porosities (both internal and external).

The packing performances were tested by measuring the variation of the HETP for an inert compound at various flow velocities and fitting the data with the equation

$$h = \frac{B}{v} + Av^{0.33} + Cv \tag{9}$$

were B is assumed to be 2 (refs. 5 and 6). Data obtained with different columns (*D* and *G*, *L* and *K* and *J* and *I*) are similar and results are given in Table VII for the last two columns only. The results in Table VII suggest that the packings are good in both instances (for "good" columns A is between 1 and 2.5 and C between $0.5 \cdot 10^{-2}$ and $5 \cdot 10^{-2}$) and that the efficiency is slightly better for GCB. This result should be connected with the observation that the mechanical stability of particles is unchanged or decreased very slightly by graphitization. A characteristic feature of CB columns is the relationship between efficiency and retention. The C term in eqn. 9 is a function of k'.

TABLE VII

COEFFICIENTS OF THE HETP EQUATION ($k' = 0$)

Column	*A*	*C*	h_{min}	v_{opt}
J	1.79	$0.8 \cdot 10^{-2}$	3.25	2.50
I	2.77	$1.9 \cdot 10^{-2}$	4.50	1.80

Different expressions have been derived for this coefficient[10,11], but they do not agree. In fact, nearly any type of C *vs.* k' plot can be found experimentally from HETP curves.

The decrease in HETP with increasing k' is much less important for LC columns packed with fine silica particles than that found here for CB, but Loheac *et al.*[12] found a comparable decrease in efficiency when using silica particles as large as the CB particles used here.

Experiments were performed to determine whether graphitization can improve the efficiency for retained compounds. Plots of N/N_0 against k' are shown in Figs. 4 and 5, with acetonitrile and *n*-heptane as solvents, N_0 and N being the number of theoretical plates for unretained and retained solutes, respectively. N_0 is independent of the solute and is merely a function of the flow velocity of the mobile phase.

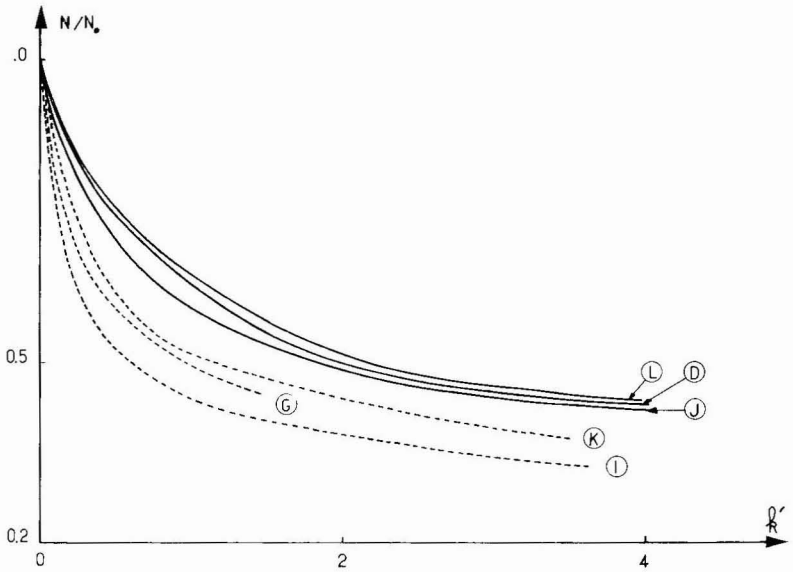

Fig. 4. Variation of efficiency with capacity factor for several columns, using acetonitrile as solvent (*cf.*, Table II). Solid lines refer to graphitized and broken lines to non-graphitized carbon black.

Using acetonitrile as solvent, the efficiency for GCB (solid lines in Fig. 4) is slightly better than that for NGCB (broken lines). For the sake of clarity, the points for the various solutes are not reported but it should be pointed out that the experimental points that correspond to compounds of very different polarities are all very near the curves shown, which indicates that there is a strong correlation between N and k'. Few measurements were carried out at large values of the capacity factor, but it seems that, at least for GCB, HETP decreases only very slowly for $k' > 7$.

The situation is different when the solvent is apolar (*n*-heptane, Fig. 5). It is not possible to use polar solutes because of the large peak asymmetry. The behaviour of GCB columns is different when dealing with monoaromatics (solid lines 1, 3 and 4) and polyaromatics (solid lines 2 and 7). In the former instance the plots show a minimum at k' values which increase with increasing specific surface area. On the other hand, when using NGCB the efficiency decreases steadily with increasing k' in all instances, which suggests that graphitization is important mainly for the use of non-polar solvents because it cleans the surface, removing polar impurities whose effect is small with polar solvents. However, as we have shown above, CB does not seem to be a good adsorbent for the separation of polyaromatics, especially with a non-polar solvent. The results are better with polar solvents, but then the retention of these solutes become important.

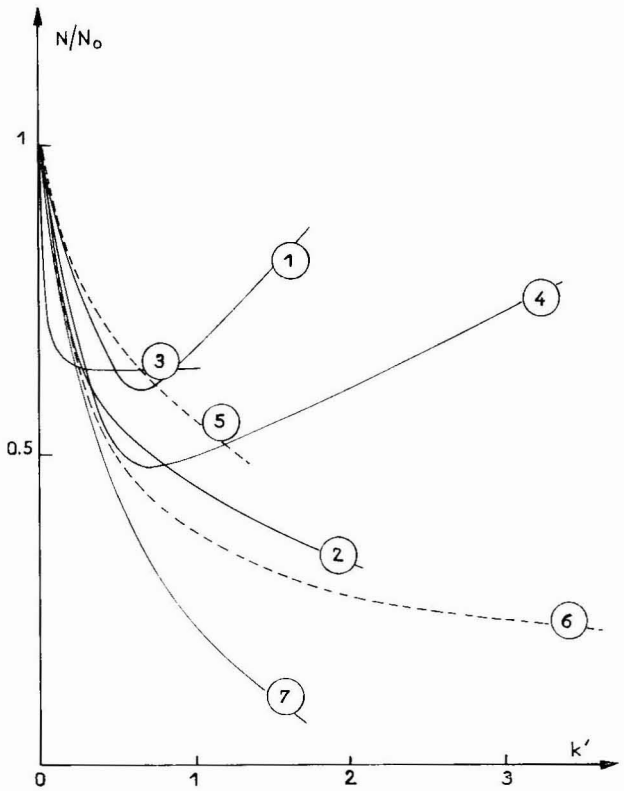

Fig. 5. Variation of efficiency with capacity factor for several columns, using *n*-heptane as solvent (*cf.*, Table II). 1 = MB on column *D*; 2 = PA on column *J*; 3 = MB on column *J*; 4 = MB on column *L*; 5 = MB on column *K*; 6 = MB and PA on column *I*; 7 = PA on column *L*.

In conclusion, it must be pointed out that as reversed-phase chromatography is associated with the use of polar solvents, the influence of graphitization on the performance of carbon adsorbents is small in liquid chromatography.

CONCLUSION

The influence of graphitization on the chromatographic properties of carbon black is of moderate importance in liquid chromatography. The decrease in retention, for instance, can easily be obviated by using a weaker solvent. The greater efficiency of graphitized carbon is interesting, especially when using apolar solvents such as *n*-heptane. The possibility of packing columns with fine particles (5–10 μm) will partially offset the importance of this effect. Further, it is possible that the decrease in the number of theoretical plates with increasing capacity factor will be less critical for smaller particles that for the larger particles used here (30–50 μm).

It is really of interest to use graphitized carbon only in preparative chromatography, as the loadibility of the columns is greater and the efficiency is less sensitive to the amount of solute injected. With regard to physical measurements, it is essential

to use graphitized carbon black because its structure is well known and the experimental conditions for hardening have no influence on the thermodynamic properties.

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ESSAI DE TRANSPOSITION À LA CHROMATOGRAPHIE EN PHASE LIQUIDE SUR COLONNE DES RÉSULTATS DE LA CHROMATOGRAPHIE EN COUCHE MINCE

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SUMMARY

An attempt to use HPTLC data for prediction of HPLC retention coefficients

Both HPLC and TLC may be carried out on silica particles of similar size, specific surface area and activity; problems encountered when trying to correlate the observed retention data are due to different wetting of the adsorbent in the two techniques. Whenever an adequate pre-saturation of the thin-layer plates is achieved and a proper value of the coefficient in Snyder's equation is selected an excellent correlation between log k' and R'_M is observed. This transposition has been successfully applied to aromatic hydrocarbons and to a group of phenyl triazenes.

INTRODUCTION

La chromatographie en phase liquide sur colonne (CPL) et la chromatographie en couche mince (CCM) possèdent bien des analogies. Toutefois, les utilisateurs de la CPL sont toujours frappés de voir la rapidité avec laquelle on peut déterminer le solvent adéquat à la migration d'un soluté en CCM alors que ce choix est plus long et plus délicat en CPL.

Il est tentant de chercher à extrapoler les résultats de la CCM pour savoir très rapidement quel liquide vecteur employer pour avoir en CPL un valeur k' d'un ordre de grandeur donné.

Schlitt et Geiss[1] ont établi des équations permettant la transposition des données de la CCM à la CPL mais il est nécessaire de déterminer soigneusement certains paramètres et les calculs sont longs ce qui rebute l'utilisateur qui cherche quelque chose de simple pour effectuer d'éventuels changements assez rapidement d'autant plus que la précision de tels calculs est souvent illusoire et que l'on cherche

plutôt des ordres de grandeur que des relations exactes. Coq *et al.*[2] ont déterminé un coefficient de transposition K_{tr} entre R_F et k'.

En CCM, si l'on utilise habituellement le R_F pour déterminer la rétention d'un soluté, il est plus sûr afin d'obtenir des valeurs reproductibles d'utiliser la grandeur R_M:

$$R_M = \log\left(\frac{1}{R_F} - 1\right) \tag{1}$$

En première approximation et sous les réserves indiquées plus loin, le facteur de capacité k' qui caractérise la rétention d'un soluté en CPL est lié au R_F par la relation

$$R_F = \frac{1}{1 + k'} \tag{2}$$

La substitution de la valeur de R_F dans l'expression de R_M conduit à

$$R_M = \log k' \tag{3}$$

Cependant en CPL l'adsorbant est complètement mouillé par la phase mobile et le problème de la saturation ne se pose pas; en CCM cet état de complète saturation de toute la plaque est plus difficile et exige des précautions expérimentales délicates qui sont souvent oubliées. Il en résulte d'importantes variations de R_F. La différence de vitesse entre le front du solvant et le reste du solvant sur la couche a conduit à considérer que le R_F observé n'est pas le R_F vrai, mais que

$$R_{F\text{obs.}} = \xi \cdot R_{F\text{vrai}} \tag{4}$$

et Snyder a déterminé des valeurs de R_M à partir des valeurs de R_F observées[3].

Disposant d'une importante quantité de données de rétention mesurées en CPL sur des hydrocarbures aromatiques élués à l'heptane[4,5] ou calculées à partir de chromatogrammes de CPL publiés[6,7], nous avons étudié essentiellement la transposition CCM et CPL pour ces produits. Notons que les valeurs de Coq *et al.*[2] sont obtenues avec les mêmes particules de silice que celles utilisées pour nos plaques et que ces valeurs sont en bon accord avec celles que nous avons mesurées sur un appareil de chromatographie en phase liquide.

PARTIE EXPÉRIMENTALE ET RÉSULTATS

Nous avons utilisé les composés suivants: naphtalène, acénaphtène, anthracène, fluoranthène, pyrène, chrysène, pérylène.

Nous avons tracé sur la Fig. 1 le graphe $\log k' = f(R_M)$, analogue à celui publié par Soczewinski et Golkiewicz[8]. La droite obtenue ne passe pas par l'origine, ce qu'avaient signalé ces auteurs; sa pente est supérieure à 1 (1.13) et il est nécessaire d'utiliser la méthode des moindres carrés pour obtenir cette pente. En CCM les R_F sont déterminés par la valeur du rapport de la distance parcourue par le soluté à la distance parcourue par le solvent sur une couche initialement sèche, mais en équilibre avec la

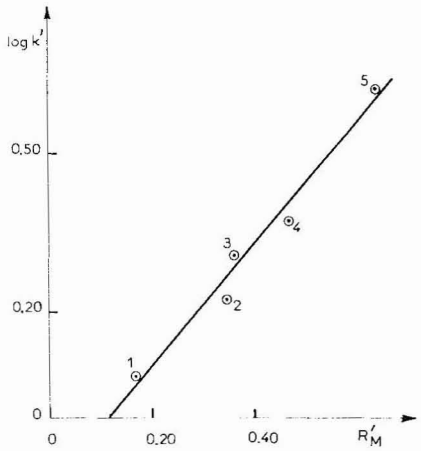

Fig. 1. Corrélation entre données de rétention pour les hydrocarbures aromatiques polynucléaires obtenues par chromatographie sur colonne (log k') et en couche mince (R'_M). 1 = naphtalène, 2 = acénaphtène, 3 anthracène, 4 pyrène, 5 chrysène.

vapeur de solvant sous une certaine pression partielle, plus ou moins inférieure à la pression de vapeur saturante, alors qu'en CPL la détermination des k' s'opère sur une couche préalablement imbibée par le solvant et en équilibre avec lui. Les conditions de saturation de l'adsorbant influent donc sur la transposition des données de CCM en CPL.

Il est donc souhaitable de "mouiller" d'une façon ou d'une autre l'adsorbant utilisé en CCM pour se rapprocher des conditions de la CPL. La technique d'"overrunning" de Dallas[9] consiste à faire monter le solvant sur la plaque jusqu'à l'extrémité de celle-ci et attendre qu'il y ait homogénéité de la transparence. Cette technique ne nous a pas permis d'obtenir des R_F très reproductibles.

On peut aussi faire monter le solvant le long de la plaque, la sortir de la chambre à développement, déposer rapidement un soluté puis effectuer le développement mais on constate alors que le front du solvant est difficile à repérer avec précision. On peut également, laisser la plaque s'évaporer pendant 2 à 3 min, le temps de déposer les solutés. En fait, le solvant s'évapore peu car l'heptane a une tension de vapeur assez faible à la température ambiante. Lorsqu'on met ensuite la plaque à développer on peut suivre aisément la montée du solvant car dans ces conditions le front est net. Cette technique est cependant "acrobatique" et difficilement reproductible.

Nous avons utilisé la méthode suivante qui nous a donné des résultats reproductibles. Dans le réservoir on dispose une plaque de silica gel que l'on laisse imprégner totalement puis la plaque à utiliser est déposée parallèlement, mais au-dessus du solvant, soutenue par un petit support de façon que le liquide vecteur ne puisse pas monter. On laisse le système se mettre en équilibre pendant une nuit, puis le dépôt des solutés se fait le plus rapidement possible et on effectue la chromatographie.

Le solvant monte plus vite que sur une couche sèche (400 sec au lieu de 460 sec pour une avance de 5 cm avec une plaque d'épaisseur 0.10 mm). Les valeurs de R_F observées sont plus faibles que celles observées sur couche sèche, comme on le voit sur les Tableaux I et II.

TABLEAU I

COMPARAISON ENTRE LES RÉTENTIONS D'HYDROCARBURES AROMATIQUES EN CCM SUR COUCHES SÈCHE ET PRÉSATURÉE

Soluté	R_F *sur couche sèche*	R_F *sur couche saturée*
Naphtalène	0.40	0.36
Anthracène	0.31	0.26
Chrysène	0.22	0.15

TABLEAU II

RÉTENTION DE CHLOROTRIAZÈNES EN CPL ET CCM

CCM		*CPL*		
R_F*	R'_M*	R_F**	k'	*Log k'*
0.05	1.16	0.10	12.75	1.11
0.14	0.65	0.28	4.42	0.65
0.32	0.14	0.58	1.42	0.15
0.37	0.03	0.67	1.17	0.07
0.42	−0.08	0.78	0.83	−0.07

* Couche préalablement imprégnée.
** Couche simple.

La plaque ayant été saturée au préalable on ne peut utiliser l'éqn. 1 mais la relation dérivée de l'éqn. 4:

$$R'_M = \log\left(\frac{1}{\xi \cdot R_F} - 1\right) \tag{5}$$

La choix de la valeur de ξ est important. Lorsque la plaque est complètement saturée Snyder[10] recommande de prendre $\xi = 1.5$ mais la sortie de la plaque de l'atmosphère saturée pour y déposer le soluté provoque une certaine évaporation et nous avons pris $\xi = 1.3$. En traçant le graphe $\log k' = f(R'_M)$ pour $\xi = 1.3$ on obtient une bonne corrélation entre k' et R_F comme le montre le Tableau III et la Fig. 2. Si l'on utilise $\xi = 1.4$, on obtient une droite qui ne passe pas par l'origine mais dont l'ordonnée à l'origine est négative et égale à −0.05.

TABLEAU III

RÉTENTION D'HYDROCARBURES AROMATIQUES EN CCM ET EN CPL

Soluté	R_F	R'_M	*Log k'*
Naphtalène	0.36	0.05	0.0 8
Acénaphtène	0.27	0.26	0.2 5
Anthracène	0.26	0.29	0.3 1
Pyrène	0.23	0.37	0.3 8
Fluoranthène	0.20	0.45	0.4 9
Chrysène	0.15	0. 62	0.64
Pérylène	0.14	0 .65	0.71

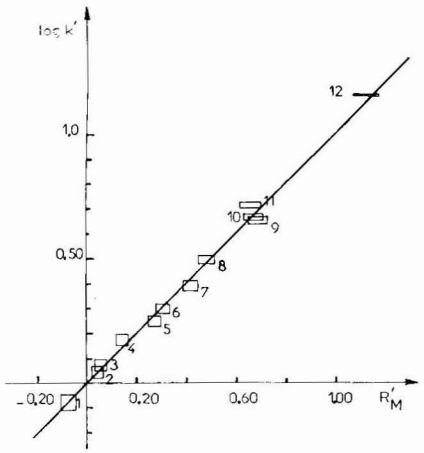

Fig. 2. Corrélation entre données de rétention obtenues pour des chlorotriazènes (cf. Tableau II) et des hydrocarbures aromatiques (cf. Tableau III) par chromatographie sur colonne et en couche mince, sur silice. Les dimensions des rectangles sont égales aux erreurs de mesure. 1 = bis(2,3,5-trichloro) 1,3-phényl triazène, 2 = bis(2,3,4-trichloro) 1,3-phényl triazène, 3 = naphtalène, 4 = bis(2,4-dichloro) 1,3-phényl triazène, 5 = acénaphtène, 6 = anthracène, 7 = pyrène, 8 = fluoranthène, 9 = chrysène, 10 = bis(3,5-dichloro) phényl triazène, 11 = pérylène, 12 = bis(4-chloro) 1,3-phényl triazène.

Nous avons alors cherché à vérifier si la corrélation obtenue est valable pour d'autres solutés. Nous avons utilisé pour cela les triazènes étudiés ci-dessus (cf. Tableau II). Le tétrachlorure de carbone n'a pu être employé comme liquide vecteur en CPL car il n'est pas compatible avec l'emploi du détecteur UV dont nous disposons. L'emploi d'un mélange d'heptane et de dichlorométhane (80:20, v/v) malgré les difficultés liées à l'emploi des mélanges de solvants en CCM[11] nous a permis de rassembler les données de rétention du Tableau II.

On voit sur le Tableau II que les R_F sont plus faibles que ceux obtenus avec des plaques sèches. On peut surtout constater un excellent accord entre valeurs de R'_M et log k'. L'erreur est la plus importante sur le triazène de R_F 0.05, mais il faut tenir compte du fait que les R_F sont déterminés avec une précision de 0.01 unité, au mieux. Une telle erreur conduit pour les faibles valeurs de R_F à une erreur sur R'_M beaucoup plus grande ($R'_M = 0.09$ pour $R_F = 0.05$) alors que la détermination de k' peut être beaucoup plus précise puisque l'on peut jouer sur la vitesse de déroulement du papier de l'enregistreur. L'erreur sur les R_F très faibles est donc trop grande pour que l'on puisse prévoir k' avec précision et l'utilisateur devra se limiter au domaine des R_F permettant une plus grande précision, R_F étant avantageusement supérieur à 0.10. On voit sur la Fig. 2 que la corrélation est excellente si l'on tient compte des erreurs de mesure propres aux deux méthodes. Le résultat est d'autant plus remarquable si l'on tient compte de ce qu'il est obtenu avec un mélange de solvants.

CONCLUSION

Dans la mesure où l'on prend des précautions suffisantes pour travailler avec une plaque présaturée ou pratiquement présaturée, on obtient une excellente corréla-

tion entre valeurs du facteur de capacité de la colonne en chromatographie sur colonne et R_F en chromatographie en couche mince, en utilisant l'éqn. 5 et une valeur du coefficient ξ comprise entre 1.3 et 1.5 suivant les conditions expérimentales.

Les précautions dont on doit entourer la réalisation des mesures faites en chromatographie en couche mince sont cependant sévères et réduisent sensiblement la simplicité et la rapidité qui sont généralement considérées comme les avantages de cette méthode. C'est pourquoi on ne cherchera en général à employer la chromatographie en couche mince que pour obtenir une idée approchée des systèmes chromatographiques convenables, sans chercher une corrélation exacte, difficile, voire illusoire à atteindre. Enfin la méthode est délicate d'emploi avec des mélanges de solvants de polarité nettement différente[11].

RÉSUMÉ

On montre qu'il est possible d'obtenir une corrélation acceptable, compte-tenu des erreurs de mesures courantes dans les deux méthodes, entre données de rétention observées en chromatographie en couche mince et sur colonne, lorsque l'on utilise le même système chromatographique. Il faut pour cela veiller à utiliser des plaques présaturées.

Il convient cependant d'émettre des réserves lorsque la phase mobile est un mélange de solvants de polarités très différentes.

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USE OF THIN-LAYER CHROMATOGRAPHIC SYSTEMS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS

PROCEDURE FOR SYSTEMATIZATION AND DESIGN OF THE SEPARATION PROCESS IN SYNTHETIC CHEMISTRY*

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SUMMARY

A procedure is described for the simple scale-up of thin-layer chromatographic (TLC) systems to high-performance liquid chromatography (HPLC), which was developed in order to improve the separations involved in the work-up of synthetic reaction mixtures. The semi-quantitative compositional evaluation of the reaction products was performed by TLC, employing specially developed solvent systems. The retention of solutes was controlled by using TLC as a pilot technique, based on the correlation between TLC and HPLC mobilities. HPLC achieved complete removal of the reaction medium and reagents in addition to isolating the required products. According to the scheme described, single-step isolation and purification of synthetic products was accomplished without the need for any pre-treatment of the sample.

INTRODUCTION

Various techniques, such as thin-layer chromatography (TLC), gas chromatography, ultraviolet absorption spectroscopy and nuclear magnetic resonance spectroscopy, have been employed to trace the course of chemical reactions. TLC has been the most widely utilized because of its speed, its applicability to a wide range of compound types, from non-polar hydrocarbons to polar inorganic salts[1], and its ability to give detailed information on the component distribution in a crude reaction mixture.

The constituents of a crude synthetic reaction mixture, *i.e.*, substrates, reagents and products, are often routinely determined semi-quantitatively by TLC, and this procedure readily provides a means for the optimization of the reaction conditions. However, subsequent processes, including separation and isolation of the compounds of interest, generally involve time-consuming, tedious operations. The solvent is

* Presented at the *11th International Symposium on Advances in Chromatography, Houston, Texas, November 1–5, 1976*; the majority of the papers presented at this symposium has been published in *J. Chromatogr.*, Vol. 126 (1976).

generally removed by evaporation, subsequent solvent extraction transfers inorganic reagents into the aqueous layer and finally vacuum distillation, recrystallization, sublimation and/or other procedures are used to isolate a pure product.

Such classical treatment requires modernization if substantial improvements in ease of operation and resolution are to be achieved. The thin-layer chromatogram can be used to develop a high-performance liquid chromatographic (HPLC) separation that will enable one to obtain the desired reaction products while removing the reaction medium and reagents in a single operation. This paper presents a simple and efficient approach to the development of a laboratory-scale method for the separation and subsequent isolation of synthetic reaction products.

Diverse complex mixtures are encountered in modern synthetic research. In order to establish criteria for the classification of reaction products, a series of synthetic reactions can be considered as a model. The sequential reactions shown in Fig. 1 were utilized by Hara and co-workers[2–6] in the total synthesis of salamander alkaloids. All synthetic steps were monitored by TLC so as to provide a check on product composition and to help optimize the reaction conditions. Liquid chromatography was also needed in order to isolate and help in the characterization of intermediates, this information being required for the determination of subsequent synthetic pathways. Some of the thin-layer chromatograms are illustrated in Fig. 1.

It should be noted that, although the chromatographic patterns of the crude reaction mixtures are relatively simple, the actual procedures for product isolation are complicated, and that a single operation involving HPLC elution for the isolation of the compounds of interest would appear to be desirable.

Non-polar organic solvents are readily eluted from a silica column without any retention, and polar solvents are retained longer than the organic product fraction; however, they can be eluted from the column by employing a stepwise solvent gradient. Most inorganic reagents are strongly retained on a silica column and are therefore removed with a silica pre-column connected ahead of the main column. On the other hand, if a reversed-phase packing material, *e.g.*, ODS or C-18, is employed, polar solvents and inorganic reagents are eluted rapidly from the column and are easily removed.

Complete removal of an associating solvent such as dimethyl sulphoxide, dimethylformamide, higher alcohols, pyridine and acetic acid from a reaction mixture often presents a severe problem to the synthetic chemist. However, HPLC fractionation can readily solve this problem.

EXPERIMENTAL

TLC procedure

The adsorbent was standard TLC-grade silica (Wakogel B; Wako, Osaka, Japan) with an average pore size of 60 Å. TLC was carried out by the usual ascending procedures described previously[7].

HPLC procedure

The adsorbent was irregularly shaped, totally porous silica (Wakogel LC-10) with a mean particle size of 10 μm, of the same quality as the standard TLC adsorbent. Silica was deactivated by equilibration with ambient moisture.

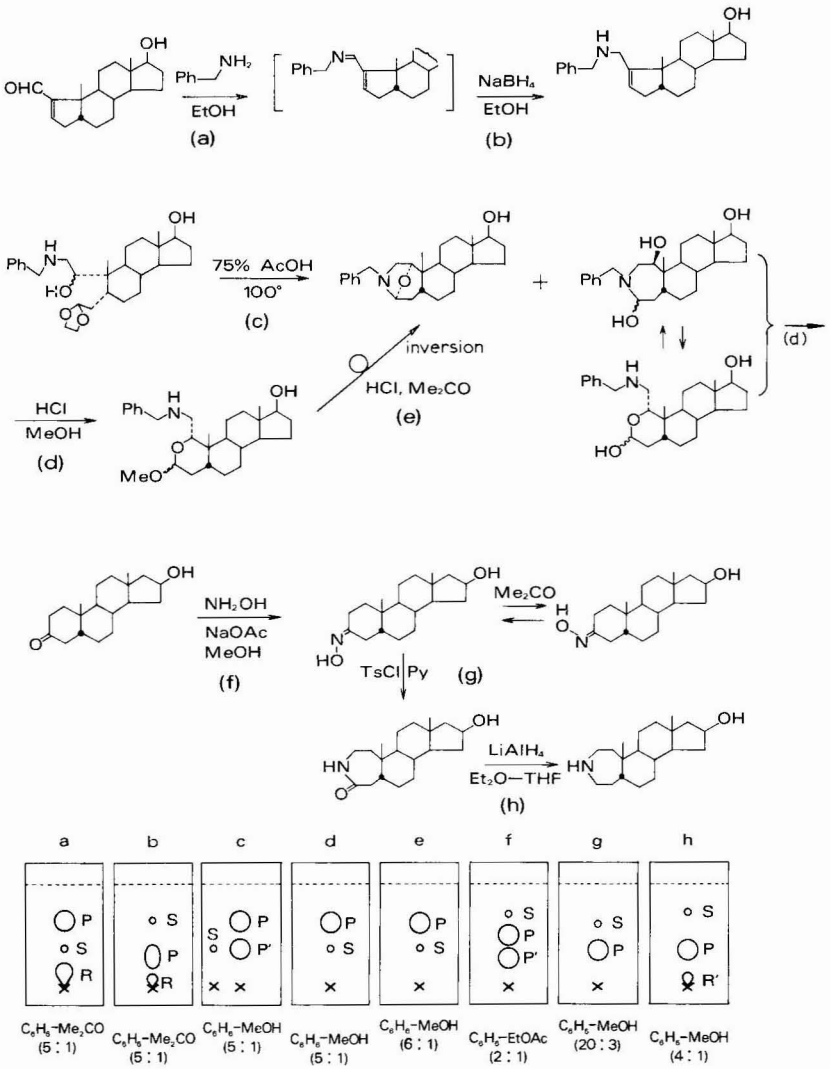

Fig. 1. Examples of thin-layer chromatograms in synthetic studies of salamander alkaloids. S = Substrate; P, P′ = products; R = reagent.

Columns and apparatus

A new column system, shown in Fig. 2, was developed for the isolation of pure products from a crude reaction mixture. A conical-inlet glass (CIG) column, having a fan-shaped inlet* that was adopted for the effective introduction of the sample into a large-diameter column, was used. Various features of this system can be summarized as follows:

(i) A large number of theoretical plates was obtained, possibly owing to the

* A silica pre-packed disposable glass column and a stainless-steel column, having similarly shaped inlets, have been developed by E. Merck and Varian Aerograph, respectively.

smooth inside wall of the CIG column and its inlet shape. The number of theoretical plates (N) for 4-, 5- and 8-mm I.D. silica dry-packed columns was 1700 per 30 cm using 5α-cholestan-3-one as the solute and n-hexane–ethyl acetate (20:1, v/v) as the solvent.

(ii) A PTFE plug was designed to fit CIG columns of various sizes. The procedure for fitting and removing the column plug with a metal clip is extremely simple.

(iii) The upper pressure limit of the CIG column is sufficiently high for our preparative programme. Columns of length (excluding conical inlet parts) 30 cm and I.D. 4, 5, 8, 15 and 30 mm were prepared. For example, 4- and 8-mm I.D. columns were used with pressures up to 50 and 30 kg/cm^2, respectively.

(iv) A pre-column can be connected directly to the main column. The preferred length of the pre-column was 5 cm.

More detailed data for the CIG column system will be reported elsewhere.

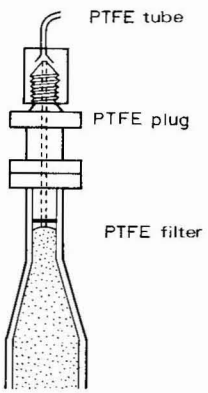

Fig. 2. CIG column.

HPLC was performed using a reciprocating piston pump (SF-0396, Milton Roy, Philadelphia, Pa., U.S.A.), an RI detector (RI-401, Waters Assoc., Milford, Mass., U.S.A.), a recorder (DR-1S, Ohkura Electric Co., Tokyo, Japan) and 10-, 100- and 500-μl microsyringes (Kusano Scientific Co., Tokyo, Japan). The experiments were carried out at room temperature (15 $\pm$ 5°).

Examples of applications

(a) 5α-Cholestan-3β-ol (366 mg) and p-toluenesulphonyl chloride (1.3 g) were dissolved in 3 ml of pyridine and the mixture was allowed to stand overnight at room temperature. The crude reaction mixture was injected into the silica column. Evaporation of the solvent from the tosylate fraction gave 500 mg of crystalline product, which was identified with an authentic sample by measuring the infrared absorption.

(b) α-Phenylpropionic acid (288 mg) and dicyclohexylcarbodiimide (DCC) (198 mg) were dissolved in 3.0 ml of tetrahydrofuran (THF), then cholesterol (389 mg) and triethylamine (97 mg in 2.0 ml of THF) were added. The crude reaction mixture was allowed to stand overnight and then injected directly into the silica column. A crystalline product (370 mg) which was identified with an authentic sample was obtained.

(c) Dihydrocholesteryl acetate, which was obtained from 550 mg of cholesteryl acetate by catalytic hydrogenation, was hydrolyzed with sodium hydroxide in methanol–water[8]. The reaction mixture was pumped through the reversed-phase column in methanol–water as the mobile phase. The residual dihydrocholesterol was injected into the silica column, and 5β- and 5α-cholestanol (55 and 430 mg, respectively) were isolated.

RESULTS AND DISCUSSION

Design of a solvent system

The mobile phases employed in liquid chromatography are often selected by trial and error in a non-systematic manner. Current schemes for the mechanism of liquid–solid chromatography (LSC) stress the contribution of hydrogen bonding provided by the solvent as an acceptor or a donor[9–11]. Based on this consideration, a new classification of a solvent system for LSC has been attempted.

Solvents with non-bonded electron-pair donors, including hetero atoms such as nitrogen and oxygen, and solvents with proton donors have been classified as "class B" and "class AB", respectively[12]. Solvents that do not take part in hydrogen bond formation are now designated as "class O", which is further subdivided into three categories, *viz.*, O (aliphatic hydrocarbons), P (aromatic hydrocarbons) and N (haloalkanes). For preparative work, the solvents used are usually limited to commonly available volatile substances, which can be listed as follows:

"class O": hydrocarbons, halides —*n*-hexane (O-type), benzene (P-type), methylene chloride, chloroform (N-type);

"class B": *n*-donors (base) —diethyl ether, ethyl acetate, acetone;

"class AB": H-donors (acid) —methanol, ethanol, 2-propanol, acetic acid.

For moderately polar compounds, binary solvent systems and silica as the adsorbent[13] have usually been preferred, as in the examples of the synthetic reactions shown in Fig. 1. Depending on the character of the solute, a binary solvent system can be readily formulated by choosing a pair of solvents, *i.e.*, O + O, O + B, O + AB, B + B or B + AB.

Solvent combinations. To find a suitable solvent, consideration of the solvent selectivity is necessary[9,10,14] and for this purpose the relationship between mobility and solvent system was examined by using several steroid compounds. $R_m [= \log(1/R_F - 1)]$ values for pairs of compounds that differ only by the presence of a characteristic functional group were obtained by employing TLC R_F values[15]. ΔR_m values for particular functional groups using various binary solvents were calculated according to Martin's additive rule. The results are illustrated in Fig. 3.

Even though the non-polar component was changed, similar ΔR_m values were obtained if the polar component remained the same. The selectivity of the solvent system is controlled by the more polar solvent.

Ethyl acetate (class B) systems afforded comparatively small ΔR_m values for carbonyl and hydroxyl groups, especially for the acidic phenolic hydroxyl group. Açetone (class B) systems gave smaller ΔR_m values than others, particularly for the keto group. Methanol (class AB) systems afforded large positive ΔR_m values for the phenolic hydroxyl group, and affected the alcoholic hydroxyl ΔR_m values in a non-systematic manner.

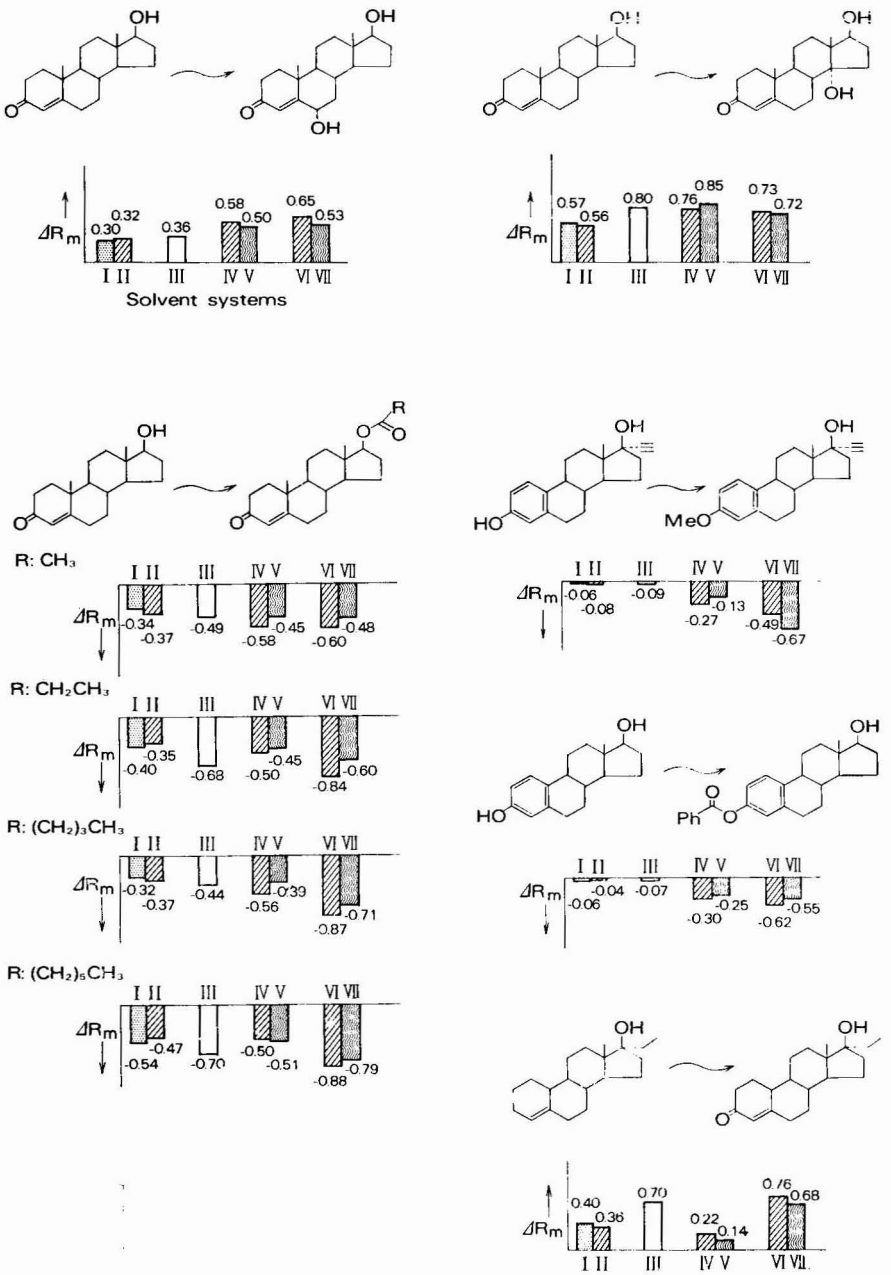

Fig. 3. ΔR_m values for functional groups of steroids in several solvent systems. The values represent the arithmetic means of results from 10 runs[15]. Solvent systems: I = *n*-hexane–ethyl acetate (2:8, v/v); II = benzene–ethyl acetate (3:7, v/v); III = diethyl ether; IV = benzene–acetone (4:1, v/v); V = chloroform–acetone (3:1, v/v); VI = benzene–methanol (9:1, v/v); VII = chloroform–methanol (97:3, v/v).

These observations indicate that a "strong" solvent specifically affects the ΔR_m values of a solute with a functional group which is similar to that contained in the "strong" solvent. Of course, this conclusion is based upon the limited material presented here; additional data will be reported in a later paper.

Solvent composition. With binary solvent systems, the solvent strength for a given solute can be optimized by adjusting the strong solvent composition. Soczewiński[11] described an effect of strong solvent composition on retention in LSC, which can be expressed by

$$R_m = \text{constant} - n \log N_B \tag{1}$$

where N_B denotes the molar fraction of polar component B and $n > 1$. Eqn. 1 was supported by TLC data for some phenols and amines[16–18]. To confirm this relationship, the effect of solvent composition on the retention of some steroids was examined by employing binary solvent HPLC*, because accurate measurements of the mobility require HPLC rather than TLC[10].

Data obtained with mono- and difunctional steroids and two types of solvent systems, O + B and O + AB, are illustrated in Fig. 4. Here the log k' values in HPLC are analogous to the R_m values in TLC. The actual correlations between mobilities in TLC and HPLC will be discussed later.

In the graphs of log k' *versus* N_B, the slope (n) increased from acyloxy to keto groups and then to hydroxyl groups in steroids from mono- to difunctional compounds. For a particular solute, the slope decreased from the O + B type of solvent system to the O + AB type.

The intercept on the abscissa, corresponding to the constant in eqn. 1, increased from acyloxyl to keto and then hydroxyl groups and from mono- to difunctional steroids, and decreased from the O + B type of solvent system to the O + AB type.

There is an additive tendency in the slopes and intercepts for difunctional compounds relative to the values for monofunctional compounds. Therefore, an appropriate binary solvent composition for analogous compounds with similar functional groups can be predicted.

Correlation between TLC and HPLC mobilities

The use of TLC data to provide an insight into HPLC has often been suggested[21–25], but no simple and reliable relationship has been reported. Some characteristic phenomena that have been observed in TLC are the volume of mobile phase, which varies with respect to the height of the thin-layer plate, pre-adsorption of solvent vapour and solvent demixing[26]. Considering these effects, extensive investigations finally led to rather simple conclusions[15]. A simple relationship has also been observed by Soczewiński and Gołkiewicz[27].

At first, the TLC R_F values and HPLC mobilities (R) of some steroids were directly compared by applying the same binary solvents as mobile phases. Irregularly shaped, totally porous silica, with properties similar to those of TLC-grade silica,

* A similar relationship has been observed for some binary solvent systems in HPLC by Scott and Kucera[19] and Gołkiewicz[20].

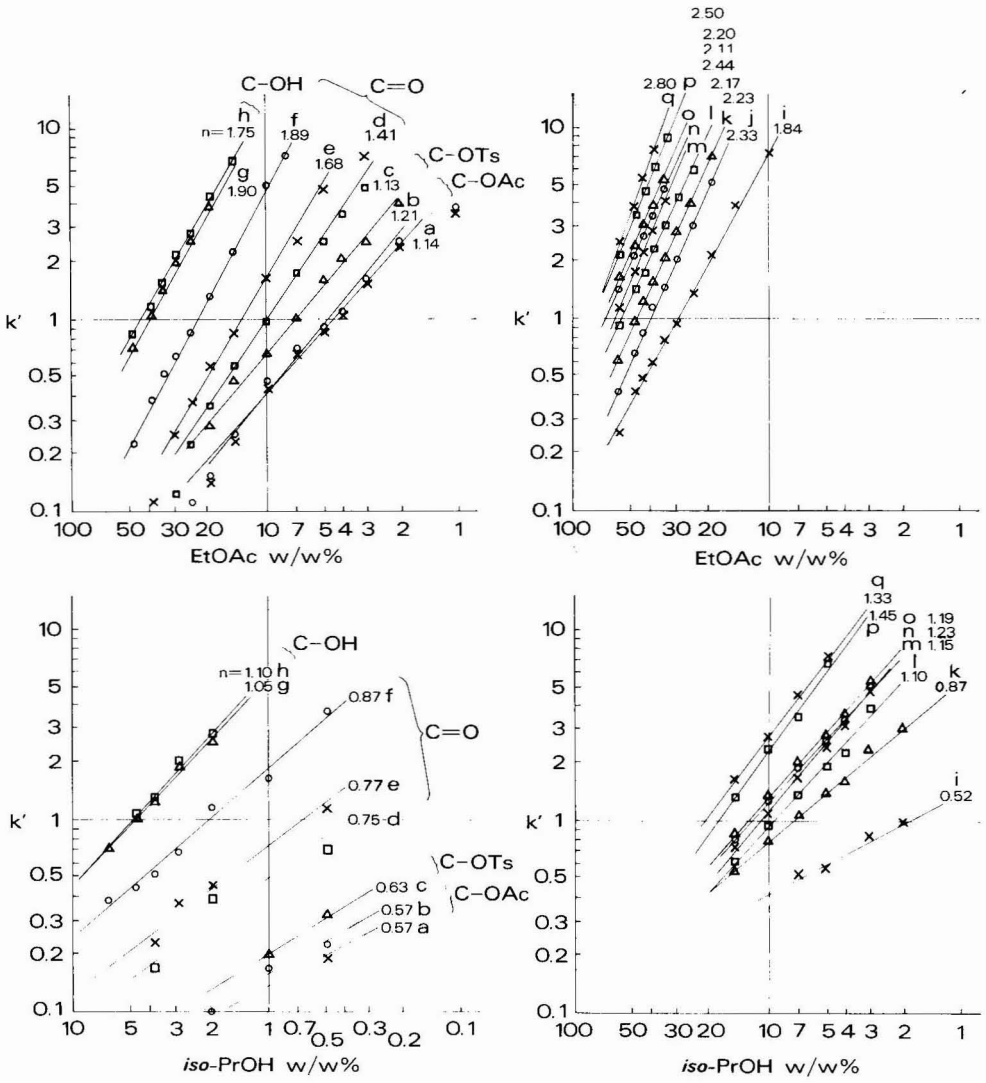

Fig. 4. Correlation between capacity factor and polar solvent composition using a silica column. Packing: particle size 10 μm, 4.4 g. CIG column, 300 × 5 mm I.D. Solvent systems: *n*-hexane–ethyl acetate and *n*-hexane–2-propanol. Flow-rate: 1 ml/min. Void volume, V_0: 6.5 ml (cyclohexane). Samples: 0.1 mg in 5 μl of dichloromethane. Steroids: a = 3β-acetoxy-5α-cholestane; b = 3β-acetoxy-5-cholestene; c = 3β-tosyloxy-5-cholestene; d = 5β-cholestan-3-one; e = 5α-cholestan-3-one; f = 4-cholesten-3-one; g = 5α-cholestan-3β-ol; h = 5-cholesten-3β-ol; i = 3β-acetoxy-5α-androstan-17-one; j = 3-hydroxy-1,3,5(10)-estratrien-17-one; k = 17β-acetoxy-4-androsten-3-one; l = 17β-hydroxy-17α-methyl-5α-androstan-3-one; m = 17β-hydroxy-5α-androstan-3-one; n = 3β-hydroxy-5-androsten-17-one; o = 3β-hydroxy-5α-androstan-17-one; p = 17β-hydroxy-4-androsten-3-one; q = 17β-hydroxy-19-nor-4-androsten-3-one.

was packed by a mechanical dry-tapping procedure. The results in Table I indicate that the R/R_F ratio fell into the range 1.18–1.81 with an average of *ca.* 1.5, except for the methanol-containing solvent (system VII), which gave extremely high values. In

TABLE I

R/R_F RATIO OBSERVED IN TLC AND HPLC OF STEROIDS BY EMPLOYING BINARY SOLVENTS

The R/R_F values were obtained by using as steroid samples 17β-hydroxy-4-estren-3-one, 17β-hydroxy-17α-methyl-4-estren-3-one, 17β-hydroxy-17α-ethyl-4-estren-3-one, 17β-hydroxy-17α-ethynyl-4-estren-3-one and 17β-hydroxy-4-androsten-3-one.

No.	*Solvent system*	R/R_F
I	*n*-Hexane–ethyl acetate (2:8, v/v)	1.27, 1.27, 1.28, 1.30, 1.43
II	Benzene–ethyl acetate (3:7, v/v)	1.46, 1.50, 1.51, 1.55, 1.58
III	Diethyl ether	1.18, 1.44, 1.50, 1.51, 1.53
IV	Benzene–acetone (4:1, v/v)	1.29, 1.47, 1.51, 1.53, 1.55
V	Chloroform–acetone (3:1, v/v)	1.46, 1.65, 1.66, 1.76, 1.81
VII	Chloroform–methanol (97:3, v/v)	2.60, 2.66, 3.00, 3.03, 3.25

this instance, the R/R_F ratio can be adjusted when the methanol content in HPLC eluents is decreased[15].

These results and other relevant data can be summarized as follows:

(i) with O + O or O + B class binary solvents,

$$R_F \text{ (TLC)} \times 1.5 \approx R \text{ (HPLC)}$$

$$R \text{ (mobility)} = \frac{1}{k' + 1}$$

where k' = capacity factor;

(ii) with O + AB class binary solvents, the AB (*e.g.*, methanol) proportion in TLC should be decreased from 1/5 to 1/10 on transfer to HPLC systems.

In the first relationship, the approximate coefficient 1.5 can be interpreted as the ratio of the volumes of mobile and stationary phases within a column or thin-layer bed. This ratio seems to be derived from the difference in the mobile phase profile on the stationary phase between the TLC and HPLC systems[22].

The decrease in the proportion of the AB solvent from 1/5 to 1/10 on transfer to HPLC systems signifies the ratio of the strong component in the HPLC wet column eluent to the original TLC solvent in the chamber; this is so because AB solvent on the thin-layer plate should be pre-adsorbed if de-mixing occurs.

Although the simple scaling procedure described above seems to be an extremely rough approximation, it shows unusually good agreement with several independent data, as follows:

	R_F	k'	$k'_{calc.}$
(i) *Solute*	R_F*	k'**	$k'_{calc.}$**
Cholesteryl benzoate	0.36	0.82	0.85
Cholesteryl phenylacetate	0.21	2.06	2.17
(ii) *Solute*	R_F***	k'§	
15-Epiprostaglandin $F_{2\alpha}$	0.38	3.0	
Prostaglandin $F_{2\alpha}$	0.22	6.6	

* Waters Assoc. data given by Prep LC/System 500. Solvent: benzene–hexane (1:1).

** $k'_{calc.} = \frac{1}{R_F \cdot 1.5} - 1$.

*** Solvent: ethyl acetate–acetic acid (98:2).

§ Solvent: ethyl acetate–acetic acid (98.8:0.2). Data were provided by Waters Assoc., brochure AN 146.

TABLE II

RETENTION DATA GIVEN BY SYNTHETIC REACTION (a)

Solute	*n-Hexane–ethyl acetate (4:1)*			*n-Hexane–ethyl acetate (20:1): HPLC*	
	TLC		*HPLC: k'_{found}*	$k'_{pred.}$*	k'_{found}
	$R_{F\ pred.}$*	$R_{F\ found}$			
Tosylate	0.57	0.68		1.60	1.45
Pyridine			2.68		
Cholestanol	0.18	0.23		ca. 60	

* Predicted values from the data for cholesterol derivatives.

Examples of applications

The scheme described above was applied to several synthetic reactions, as described under Experimental.

(a) 5α-Cholestan-3β-ol + TsCl $\xrightarrow[C_5H_5N]{}$ Tosylate + TsOH

5α-Cholestan-3β-ol as a substrate and its tosylate as a product were characterized on the basis of their structures as monohydroxy and monoacyloxy derivatives of cholestane, respectively. When the silica *n*-hexane–ethyl acetate system is chosen, the correct ethyl acetate composition can be found directly by referring to Fig. 4. Optimization of the TLC solvent was then easily accomplished. The observed R_F values are shown in Table II.

The eluotropic behaviour of pyridine as the solvent was examined by using a silica HPLC column. Pyridine was eluted from the column long after the tosylate.

The crude reaction mixture was injected directly on to the silica column fitted with a short pre-column for retaining *p*-toluenesulphonic acid, which was derived from tosyl chloride. The tosylate fraction was collected with the aid of a differential refractometer (RI detector). Unreacted cholestanol and pyridine were removed by stepwise elution.

(b) Cholesterol + α-Phenylpropionic acid + DCC $\xrightarrow[Et_3N,\ THF]{}$ Propionate + + Dicyclohexylurea

TLC binary solvent systems were formulated and utilized in pilot separations, making an initial guess concerning the chromatographic behaviour of the expected products in this reaction mixture. Some of the results are shown in Table III. The O + O system was preferred for HPLC isolation of the phenylpropionate fraction because there was no overlapping with the DCC peak. In contrast, the O + B system did result in some overlapping.

The propionate fraction was collected and DCC and phenylpropionic acid were removed by stepwise elution. Cholesterol and dicyclohexylurea remained on the pre-column.

(c) Cholesteryl acetate $\xrightarrow[HOAc]{H_2/Pt}$ Dihydrocholesteryl acetate (5α- and 5β-) $\xrightarrow[H_2O]{NaOH}$ Dihydrocholesterol (5α- and 5β-)

TABLE III

RETENTION DATA GIVEN BY SYNTHETIC REACTION (b)

Solute	*TLC R_F value*		*HPLC k′ value*	
	O + B: n-hexane–ethyl acetate (5:1)	*O + O: n-hexane–benzene (1:1)*	*O + O: n-hexane–benzene (1:1)*	
			$k'_{calc.}$	k'_{found}
Cholesteryl phenylpropionate	0.56	0.44	0.51	0.70
DCC	0.54	0.29 (t*)		
Phenylpropionic acid	0.28 (t*)	0.21 (t*)		
Cholesterol	0.15	0.05		
Dicyclohexylurea	0	0		

* Tailing.

TABLE IV

RETENTION DATA GIVEN BY SYNTHETIC REACTION (c)

Solute	*n-Hexane–ethyl acetate (4:1)*		
	TLC R_F value	*HPLC*	
		$k_{calc.}$	k_{found}
5β-Cholestan-3β-ol	0.35	0.91	0.94
5α-Cholestan-3β-ol	0.27	1.47	1.71

Dihydrocholesteryl acetate, which was obtained from cholesteryl acetate by catalytic reduction, was hydrolyzed in the presence of alkali.

The eluotropic behaviour of the pair of stereoisomeric dihydrocholesterols was then studied by TLC and the retention data are shown in Table IV. When a silica column was used, both stereoisomers were quantitatively separated.

According to the scheme described above, single-step isolation and purification of the synthetic products without any pre-treatment was effectively accomplished.

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SEPARATION OF ISOMERIC ALKYLPHENOLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND GAS–LIQUID CHROMATOGRAPHIC TECHNIQUES

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SUMMARY

The separation of 13 isomeric alkylphenols has been studied by high-performance liquid (HPLC), gas–liquid (GLC) and high-performance thin-layer chromatographic (HPTLC) techniques. It has been shown that the separation of isomeric alkylphenols is dependent upon the adsorption, the polarity of the solvent systems and the configuration of the compounds when using HPLC technique. The separation of all the 13 isomeric alkylphenols by GLC is also possible but the long analysis times and instability of the stationary phase at higher temperatures makes it inferior to HPLC. It has also been shown that HPLC is more powerful than HPTLC. A modification of the injection port for increasing the life of HPLC septa is suggested.

INTRODUCTION

The analysis of isomeric alkylphenols is of interest because of their presence in tar acids[1] which may be used as the starting material for phenol–formaldehyde polycondensates as well as for analysis[2,3] of final resins by pyrolysis, giving phenols of composition related to that of the raw material used. This class of compounds has also been recognized[4] as a major source of pollutants. Phenols are introduced into the environment through the discharge of industrial wastes and the decomposition of pesticides and herbicides. Further, the principal simple phenols have been reported[5] to be present in human urine. These phenols may be derived not only from the dietary intake of proteins, fats, smoked foods such as meat and water, but also from a wide variety of exogenous materials. A rapid, simple and effective method of analysis for phenolic compounds would be useful.

The complete analysis of a mixture of phenol, the three methylphenols, the three ethylphenols and the six dimethylphenols, which are generally present in a tar acid cut (b.p. 180–226°), is very difficult on conventional packed columns[2] using polar or non-polar stationary phases. These compounds have a high polarity and low

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vapour pressure at moderate temperatures. In addition, certain pairs of these compounds have nearly the same vapour pressures, *e.g.*, 3-methylphenol and 4-methylphenol; 2,4-dimethylphenol and 2,5-dimethylphenol; 3-ethylphenol and 2,3-dimethylphenol; and 4-ethylphenol and 3,5-dimethylphenol.

A comprehensive review[2] of phenol analysis, covering the literature from 1956 to 1965, was published. The use of high-efficiency capillary columns[6–8] using specific selective stationary phases is necessary to obtain a good resolution of these compounds. This procedure leads to long analysis times because such columns are long and the best stationary phases are not stable at high temperatures. Graphitized carbon black[9] deposited as a thin layer on the walls of a glass capillary column has been shown[10,11] to be a very effective absorbent for the separation of phenolic isomers. A much better analysis[12,13] is obtained with derivatives rather than with the free phenols, but this technique has some limitations[14]. Sterically hindered phenols can react slowly and incompletely and hydrolysis should also be taken in account.

The direct thin-layer chromatographic (TLC) separation of isomeric alkylphenols using silica gel as adsorbent has not been very successful owing to the limited resolution power of the adsorbent. It is believed[15] that gas–liquid chromatography (GLC) is superior to most other methods employed for the separation of isomeric phenols. However, derivatives of some isomeric alkylphenols have been reported[16] to be separated on silica gel plates. Detection limits and the semi-quantitative determination of phenol, the three methylphenols and the six dimethylphenols on ready-made impregnated sheets of silica gel have been reported[17]. Recently, a method for the identification of phenolic substances by means of six one-dimensional TLC systems and four spray reagents has been recommended[18].

Literature on the separation of isomeric alkylphenols using high-performance liquid chromatography (HPLC) is scanty. Some alkylphenols, whenever available with other classes of compounds, have been reported to be separated by HPLC. A mixture containing five phenols (phenol and 4-methyl-, 2,6-dimethyl-, 2,4-dimethyl- and 3,4-dimethylphenol) has recently been separated on a Chromosorb G (5–10-μm) column using HPLC[19].

This paper describes what we believe to be the first separation of almost all of the isomeric alkylphenols by HPLC. A modification to the injection port has been employed in order to avoid frequent rupture of the septa. The separation of these alkylphenols using two stationary phases on packed columns by GLC is also reported.

EXPERIMENTAL

Alkylphenols were obtained from Fluka (Buchs, Switzerland) and were of technical grade. Compounds with purity less than 98% were recrystallized from light petroleum (b.p. 60–70°) before use. 2,4-Dimethylphenol was the only compound to be used without purification and its composition was *ca.* 90% 2,4-dimethylphenol + 5–7% 2,5-dimethylphenol + methylphenol. Cyclohexane and methylene dichloride, used as solvents, were pure and did not show any impurities in the 250–400-nm UV range.

HPLC

A DuPont Model 830 high-performance liquid chromatograph equipped with

a DuPont Model 835 UV detector was used. The elution was monitored at 254 nm. The pumps were capable of operating at pressures up to 4500 psi. The following three columns were used: (1) a stainless-steel column (1000 × 3 mm I.D.), dry packed in the laboratory using 30-μm LiChrosorb SI 60 (Merck, Darmstadt, G.F.R.), as stationary phase; (2) a stainless-steel column (300 × 3 mm I.D.), packed in the laboratory with 10-μm LiChrosorb SI 60 (Merck) using the balanced-density slurry method[20]; (3) a pre-packed column of Zorbax Sil (250 × 2.1 mm I.D.), particle size 5 μm, obtained from DuPont (Wilmington, Del., U.S.A.).

Solutions (0.05%) of the alkylphenols were prepared in cyclohexane and 1.0-μl samples were injected below the surface of the column packing with a 5-μl Hamilton syringe for HPLC. Whenever the pressure was more than 1000 psi, the stop–flow injection method was employed.

Septa made of perfluorelastomer supplied by DuPont were used. A life of 20 or more injections at room temperature for these septa has been claimed. It was impossible to use these septa above 40° as they frequently ruptured. In order to overcome this difficulty, a packing of PTFE was provided at the injection port so that a minimal area of the septum would come into contact with the solvent. An excellent improvement in the life of the septa was achieved with this modification. Fig. 1 illustrates this PTFE packing.

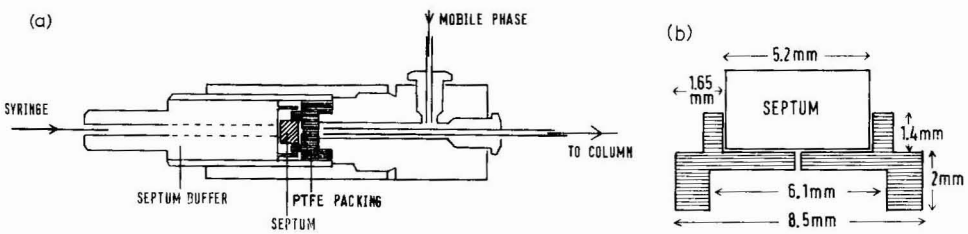

Fig. 1. (a) Schematic diagram of the injection port (Dimensions as in DuPont Accessories and Parts Catalogue) with PTFE packing. (b) Schematic diagram of the PTFE packing and septum.

HPTLC

The pre-coated fluorescence silica gel high-performance thin-layer chromatographic (HPTLC) plates used in this investigation were obtained from Merck[21] and were dried at 105–110° for 30 min before use. The compounds were dissolved in methylene dichloride and nanogram amounts were applied with a glass capillary 10 mm from the edge of the plates. The plate was then placed inside the chamber for development. Pure cyclohexane, pure methylene dichloride and their mixtures in different proportions were used as solvent systems. The spots were observed under UV illumination.

GLC

A Varian Aerograph Model 1800 gas chromatograph equipped with a flame-ionization detector was used with nitrogen as the carrier gas.

Di-(3,3,5-trimethylcyclohexyl) phthalate (DTCHP), 2.5% on Chromosorb W, 80–100 mesh, and trimethylolpropane tripelargonate (Celanese ester No. 9), 15% on Celite 545, 60, 80–100 mesh, liquid phases were obtained from Perkin-Elmer

(Norwalk, Conn., U.S.A.) and were packed in glass columns (4 m × 3 mm I.D.) by the conventional method. These columns were conditioned before use as described in the instructions supplied by Perkin-Elmer. The other conditions were: oven temperature, see Table IV; injector temperature, 250°; and carrier gas (nitrogen) flow-rate, 25 ml/min.

RESULTS AND DISCUSSION

HPLC

The stainless-steel column (1000 × 3 mm I.D.) packed with LiChrosorb SI 60 of particle size 30 μm was first used for the HPLC separation of isomeric alkylphenols using different proportions of cyclohexane and methylene dichloride. Broad peaks were obtained for most of the phenols as their adsorption on this column was very strong. A mixture of 13 isomeric alkylphenols gave no more than four peaks. Known amounts of water were added to the methylene dichloride before mixing with cyclohexane in order to study the influence[21] of water on the separation of these alkylphenols, but no improvement in the separation was observed.

Table I gives the retention times of the 13 isomeric alkylphenols on LiChrosorb SI 60 (300 × 3 mm I.D.) of particle size 10 μm and on Zorbax Sil (250 × 2.1 mm I.D.) of particle size 5 μm using HPLC under different operating conditions. It can be seen that the performances of both columns are similar, provided that the ratio of cyclohexane to methylene dichloride is 1:1. The following compounds were not separated: 2-methyl-, 2,5-dimethyl- and 2,3-dimethylphenols from each other; 3-ethyl-

TABLE I

RETENTION TIMES OF ISOMERIC ALKYLPHENOLS BY HPLC USING SILICA GEL COLUMNS OF PARTICLE SIZE 30 AND 5 μm

(A) 300 × 3 mm I.D. column of LiChrosorb SI 60 (30 μm); pressure, 2500 psi; eluent, cyclohexane–methylene dichloride (1:1); flow-rate, 1.4 ml/min; temperature, 25°. (B) 250 × 2.1 mm I.D. column of Zorbax Sil (5 μm); pressure, 3300 psi; eluent, cyclohexane–methylene dichloride (1:1); flow-rate, 0.8 ml/min; temperature, 25°. (C) Column as B; pressure, 3800 psi; eluent, cyclohexane–methylene dichloride (2:1); flow-rate, 0.8 ml/min; temperature, 25°. (D) As C, except flow-rate, 0.95 ml/min; temperature, 40°.

Compound	*Retention time (min)*			
	A	*B*	*C*	*D*
2,6-Dimethylphenol	2.2	2.4	3.6	2.8
2-Ethylphenol	3.6	3.6	5.9	4.2
2-Methylphenol	3.8	4.5	7.4	5.3
2,5-Dimethylphenol	3.8	4.5	7.4	5.3
2,3-Dimethylphenol	4.0	4.5	7.4	5.3
2,4-Dimethylphenol	4.2	4.8	8.2	6.0
3-Ethylphenol	6.4	6.8	12.0	8.7
Phenol	6.4	6.8	12.0	8.7
4-Ethylphenol	7.0	7.6	13.4	9.6
3-Methylphenol	7.0	7.6	13.4	9.6
4-Methylphenol	7.0	7.6	14.0	10.2
3,5-Dimethylphenol	7.0	7.6	14.0	10.2
3,4-Dimethylphenol	—	—	14.8	11.0

TABLE II

RETENTION TIMES OF ISOMERIC ALKYLPHENOLS BY HPLC AT DIFFERENT TEMPERATURES

(E) 250 × 2.1 mm I.D. column of Zorbax Sil (5 μm); pressure 2200 psi; eluent, cyclohexane–methylene dichloride (3:1); flow-rate, 0.56 ml/min; temperature 46°. (F) As E, except pressure, 2000 psi; flow-rate, 0.54 ml/min; temperature 53°. (G) As E, except pressure, 2000 psi; flow-rate, 0.61 ml/min; temperature, 63°.

Compound	*Retention time (min)*		
	E	*F*	*G*
2,6-Dimethylphenol	5.7	5.0	3.6
2-Ethylphenol	9.2	8.0	5.2
2-Methylphenol	11.7	10.0	6.6
2,5-Dimethylphenol	11.7	10.0	6.6
2,3-Dimethylphenol	12.2	10.5	6.8
2,4-Dimethylphenol	13.3	11.5	7.3
3-Ethylphenol	18.9	15.8	10.0
Phenol	19.6	16.5	10.6
4-Ethylphenol	20.8	17.3	10.9
3-Methylphenol	20.8	17.3	10.9
4-Methylphenol	21.8	18.4	10.9
3,5-Dimethylphenol	21.8	18.4	10.9
3,4-Dimethylphenol	24.3	19.8	12.6

TABLE III

RETENTION TIMES OF ISOMERIC ALKYLPHENOLS BY HPLC USING DIFFERENT PROPORTIONS OF THE COMPONENTS OF THE SOLVENT SYSTEM

(H) 250 × 2.1 mm I.D. column of Zorbax Sil (5 μm); pressure 2200 psi; eluent, cyclohexane–methylene dichloride (4:1); flow-rate, 0.53 ml/min; temperature, 42°. (I) Column as H; pressure, 2000 psi; eluent, cyclohexane–methylene dichloride (4:1); flow-rate, 0.5 ml/min; temperature, 50°. (J) Column as H; pressure, 2100 psi; eluent, cyclohexane–methylene dichloride (6:1); flow-rate, 0.5 ml/min; temperature, 48°. (K) Column as H; pressure, 2500 psi; eluent, cyclohexane–methylene dichloride (7.5:1); flow-rate, 0.6 ml/min; temperature, 48°.

Compound	*Retention time (min)*				*Peak No. (see Fig. 2)*
	H	*I*	*J*	*K*	
2,6-Dimethylphenol	6.4	6.4	8.6	8.8	3
2-Ethylphenol	10.8	10.2	13.9	14.3	6
2-Methylphenol	14.2	12.8	17.8	18.2	2
2,5-Dimethylphenol	14.2	12.8	18.1	19.0	8
2,3-Dimethylphenol	14.9	13.3	18.9	20.7	9
2,4-Dimethylphenol	16.0	15.8	20.3	21.4	7
3-Ethylphenol	23.5	20.8	29.2	30.4	11
Phenol	24.2	21.3	32.0	33.8	1
4-Ethylphenol	25.7	22.8	33.5	35.1	10
3-Methylphenol	25.7	22.8	33.5	35.1	5
4-Methylphenol	27.1	23.8	34.2	37.0	4
3,5-Dimethylphenol	27.1	23.8	34.2	37.0	12
3,4-Dimethylphenol	30.0	26.4	37.6	40.6	13

phenol from phenol; and 4-ethyl-, 3-methyl-, 4-methyl- and 3,5-dimethylphenol from each other.

When the cyclohexane to methylene dichloride ratio was changed to 2:1, an extra peak was obtained (Table I, C) and 4-ethyl- and 3-methylphenol were separated from 4-methyl- and 3,5-dimethylphenol.

Table II gives the retention times of these alkylphenols at different temperatures using a cyclohexane to methylene dichloride ratio of 3:1. It can be seen that with an increase in the non-polar content in the solvent system, it was possible to separate 2,5-dimethyl- from 2,3-dimethylphenol and 2-ethyl- from phenol at 46° and 53°. Poor separations were found at 63° (Table II, G).

Table III gives the retention times of these isomeric alkylphenols using different proportions of the components of the solvent system. By further increasing the cyclohexane content, it was possible to separate 2-methylphenol from 2,5-dimethylphenol.

A typical separation of 11 isomeric phenols from a mixture containing 13 compounds is shown in Fig. 2. The separation of 4-ethyl- from 3-methylphenol and 4-methyl- from 3,5-dimethylphenol could not be achieved.

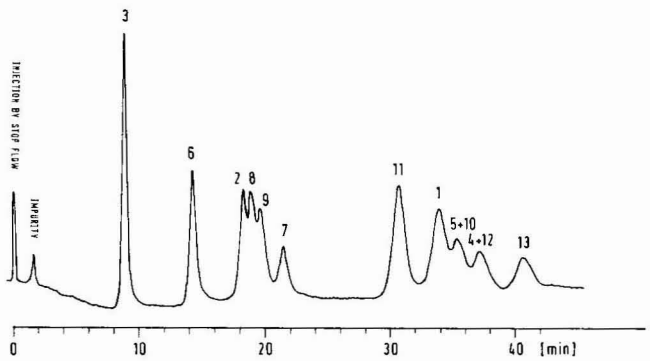

Fig. 2. Separation of isomeric alkylphenols by HPLC. For conditions and identification of peaks, see Table III, K.

It was found that the separation of these isomeric alkylphenols depends upon the adsorption phenomenon, the polarity of the solvent system and the configuration of the compounds. The resolving power of the solvent system could be increased by decreasing its polarity. *Ortho*-substituted compounds were eluted earlier than the corresponding *meta*-isomers, which is a result of the *ortho*-effect[22].

HPTLC

The compounds studied did not move from the starting point when pure cyclohexane was used as the solvent in HPTLC. By increasing the polarity of the solvent systems by addition of methylene dichloride, it was possible to move these compounds from the starting line. When pure methylene dichloride was used as the solvent in HPTLC, the maximum R_F value (0.39) was obtained for 2,6-dimethylphenol. However, the overall resolution of these phenols was very poor. When a mixture containing 13 compounds was spotted on an HPTLC plate using methylene dichloride as the solvent, only three spots were obtained. The ranges of the R_F values

TABLE IV

BOILING POINTS AND RELATIVE RETENTION TIMES OF ISOMERIC ALKYLPHENOLS BY GLC

Peak No. (see Fig. 3)	*Compound*	*Boiling point (°C)*	*DTCHP (2.5%)*			*Celanese ester No. 9 (15%) at 175°*
			155°	*135°*	*125°*	
1	Phenol	181.75	1 (16.9 min)	1 (38.3 min)	1 (47 min)	1 (28.5 min)
2	2-Methylphenol	190.95	1.30	1.34	1.34	1.25
3	2,6-Dimethylphenol	201.00	1.54	1.43	1.42	1.34
4	4-Methylphenol	202.30	1.56	1.67	1.72	1.55
5	3-Methylphenol	202.60	1.89	1.8	1.85	1.55
6	2-Ethylphenol	207.00	2.14	2.15	2.23	1.80
7	2,4-Dimethylphenol	210.00	2.18	2.27	2.36	1.94
8	2,5-Dimethylphenol	210.00	2.19	2.41	2.51	1.94
9	2,3-Dimethylphenol	218.00	2.59	2.85	2.98	2.35
10	4-Ethylphenol	218.20	2.85	2.93	3.11	2.35
11	3-Ethylphenol	219.00	2.88	3.15	3.34	2.45
12	3,5-Dimethylphenol	219.50	2.91	3.27	3.45	2.45
13	3,4-Dimethylphenol	225.00	3.10	3.59	3.81	2.81

of these three groups are as follows: phenol, 3-methyl-, 4-methyl-, 3,4-dimethyl-, 3,5-dimethyl-, 3-ethyl- and 4-ethylphenol, 0.16–0.177; 2-methyl-, 2,5-dimethyl-, 2,3-dimethyl-, 2,4-dimethyl and 2-ethylphenol, 0.24–0.27; 2,6-dimethylphenol, 0.39.

From these results, it appears that the resolving power of HPLC is superior to that of HPTLC for the compounds and conditions studied in this work.

GLC

Relative retention times of the 13 isomeric alkylphenols on two liquid phases using different temperatures are listed in Table IV. The elution sequence of these phenols follows the order of their boiling points. It was possible to separate all 13 alkylphenols using DTCHP as the stationary phase (Fig. 3), whereas only nine compounds were separated when Celanese ester No. 9 was used (Table IV). By employing

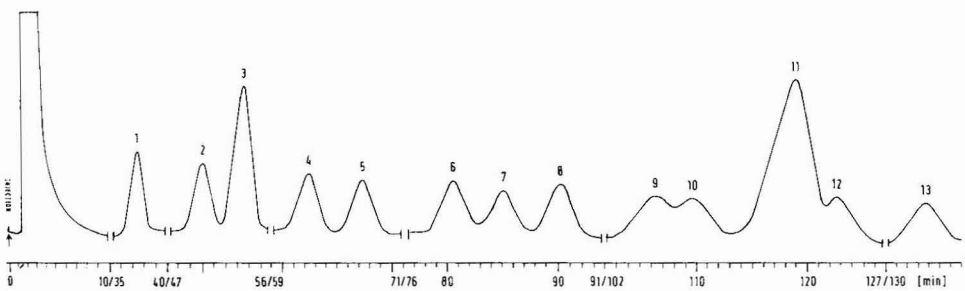

Fig. 3. Separation of isomeric alkylphenols on a glass packed column using DTCHP as stationary phase at 125°. For other conditions, see Experimental; for identification of peaks, see Table IV.

temperatures higher than 125°, the separation was found to be better on DTCHP. Unfortunately, DTCHP is unstable at higher temperatures.

CONCLUSIONS

This study established the chromatographic conditions for separating 13 isomeric alkylphenols by HPLC and GLC. These compounds were separated by HPLC on silica gels using cyclohexane and methylene dichloride as solvents. The adsorbent surface area and mobile phase polarity were varied so as to optimize the separations.

The complete GLC separation of a mixture of isomeric alkylphenols on a packed column using DTCHP as the stationary phase could be achieved. However, the long analysis times and the instability of the stationary phase at temperatures higher than 125° make this method inferior to HPLC.

ACKNOWLEDGEMENTS

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CHROM. 9931

THE QUANTITATIVE DETERMINATION OF METABOLITES OF 6-MERCAPTOPURINE IN BIOLOGICAL MATERIALS

II. ADVANTAGES OF A VARIABLE-WAVELENGTH HPLC SPECTROPHOTOMETRIC DETECTOR FOR THE DETERMINATION OF 6-THIOPURINES

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SUMMARY

The advantages of a variable-wavelength spectrophotometric detector for use in high-performance liquid chromatography are demonstrated with the detection of 6-mercaptopurine metabolites in cell extracts. 6-Methylthiopurines, unsubstituted 6-thiopurines and 6-thioguanines are most sensitively detected at 291, 322 and 342 nm, respectively. Compared with detection at 254 nm, the sensitivity at these wavelengths is about one to two orders of magnitude greater. Furthermore, in the 291–355-nm range, common purines, which are normally contained in cell extracts as free bases, nucleosides and nucleotides exhibit only minute absorbances and so do not interfere in quantitative determinations of 6-thiopurine compounds.

INTRODUCTION

The separation of 6-mercaptopurine (6MP) metabolites on the base and (deoxy-)ribonucleoside level can easily be achieved by high-performance liquid cation-exchange chromatography[1]. However, the quantitative determination of these compounds, which are contained in neutralized, enzymatically digested perchloric acid (PCA)-extracts of biological materials, proved impossible when using UV detectors with a fixed wavelength of 254 or 280 nm, owing to the low molar absorbances ($\lambda_{max.}$) of the 6-thiopurines at 254 and 280 nm and to the minute 6-thiopurine concentrations in the digests. In addition, the common purine and pyrimidine derivatives which are contained in the cell extracts also exhibit high absorbances at these wavelengths.

In cultured cells grown with radioactively labelled 6MP, the 6MP metabolites can be detected and identified in the eluent by means of their label and elution volumes. Quantitative determinations can be effected according to the specific radioactivity of the 6MP batch[2].

If a radioactive label cannot be used, however, *e.g.*, in experiments with humans, qualitative and quantitative determinations of 6MP metabolites have to be carried out spectrophotometrically at wavelengths between 291 and 355 nm. In this region the 6-methylthiopurines, the unsubstituted 6-thiopurines, the 6-thioguanines and some oxidized 6-thiopurines, including 6-thiouric acid, exhibit their $\lambda_{max.}$ values[3–5]. The common purine and pyrimidine bases and ribonucleosides hardly show any absorbance above 290 nm[6,7].

In this paper, we report the determination of 6-thiopurine compounds in the 291–355-nm region with a variable-wavelength spectrophotometer designed for use in high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

The 6-thiopurine bases and (deoxy-)ribonucleosides were purchased from Papierwerke Waldhof-Aschaffenburg (Mannheim, G.F.R.), P.L-Biochemicals (Milwaukee, Wisc., U.S.A.), and Deutsche Wellcome (Grossburgwedel, G.F.R.). Common oxidized purines and their ribonucleosides were obtained from E. Merck (Darmstadt, G.F.R.). All reagents used were of the highest available purity.

About 30 mg of dry material of each purine or 6-thiopurine compound were dissolved in 1.0–2.5 l of the eluent. The amount of buffer thus depended on the

TABLE I

UV SPECTRAL DATA OF NORMALLY OCCURRING PURINES AND OF VARIOUS 6-THIOPURINES

Molar absorbances expressed as $\varepsilon a_M \cdot 10^{-3}$. For abbreviations of compounds, see Figs. 2 and 3.

Compound	$\lambda_{max.1}$ *(nm)*	$\varepsilon_{max.1}$* *(l·mole^{-1}·cm^{-1})*	$\lambda_{max.2}$ *(nm)*	$\varepsilon_{max.2}$ *(l·mole^{-1}·cm^{-1})*	*pH*	*Ref.*
Adenine	260.5	13.4	—	—	7.0	7
Guanine	276	8.15	246	10.7	7.0	7
Hypoxanthine	249.5	10.7	—	—	6.0	7
Xanthine	267	10.25	—	—	6.0	7
Uric acid	284	11.6	231	8.4	2.0	6
Inosine	248.5	12.3	—	—	6.0	7
Xanthosine	248	10.2	278	8.9	8.0	7
6MP	327	21.3	—	—	1.0	5
	311	19.7	—	—	12.0	5
	322	21.5	—	—	4.6	— **
6MPdR	322	26.1	—	—	4.6	— **
6MPR	322	27.3	—	—	4.6	— **
6MeMP	291	17.9	—	—	4.6	— **
6MeMPR	291	18.9	—	—	4.6	— **
2A6MP	342	25.6	255	7.2	4.6	— **
2A6MPR	342	26.7	258	9.7	4.6	— **
2A6MeMP	311	23.0	242	6.4	4.6	— **
6MNIMP	280	16.8	—	—	1.0	5
	280	18.2	—	—	4.6	— **
6TUA	355	28.65	263	9.4	1.0	3
	~347	~29	—	—	4.6	— **

* If two ε_{max} values are reported, $\varepsilon_{max.1}$ represents the higher value.

** Calculated by us.

solubility of each substance, which, for the 6-thiopurine compounds, is extremely low.

The UV spectra of the compounds were measured in a 1-cm cuvette both manually (5-nm steps, except for 0.5-nm steps in the maximum and minimum regions of each spectrum) and with a self-recording Zeiss Type PMQII spectrophotometer (for more detailed information, see ref. 1). A Varian LCS 1000 high-performance liquid chromatograph was used, equipped with a Zeiss Type PM2DLC variable-wavelength spectrophotometric HPLC detector with 8-μl cuvettes. The 1-m stainless-steel column (0.18 cm I.D.) was filled with strongly acidic cation-exchange resin, Type M71, particle diameter 10–12 μm, obtained from Beckman (Munich, G.F.R.), according to the slurry method of Scott and Lee[8]. The column was eluted with 0.4 *M* (with respect to the NH_4^+ concentration) ammonium formate solution, pH 4.6. At a constant pressure of 2,800 psi (200 bar), the flow-rate was adjusted at 8.0 ml·h^{-1} (flow velocity 5.2 cm·min^{-1}). The column oven temperature was 50°. The samples were injected on to the column at intermittant flow with a 10- or 50-μl Hamilton syringe with a 7-cm needle.

RESULTS AND DISCUSSION

According to the spectral data (Table I) that were obtained from our spectral analyses (see Fig. 1) and from reports on 6-thiopurines and common purines[3–7], four groups of compounds were distinguished: (1) the common purines and their oxidized

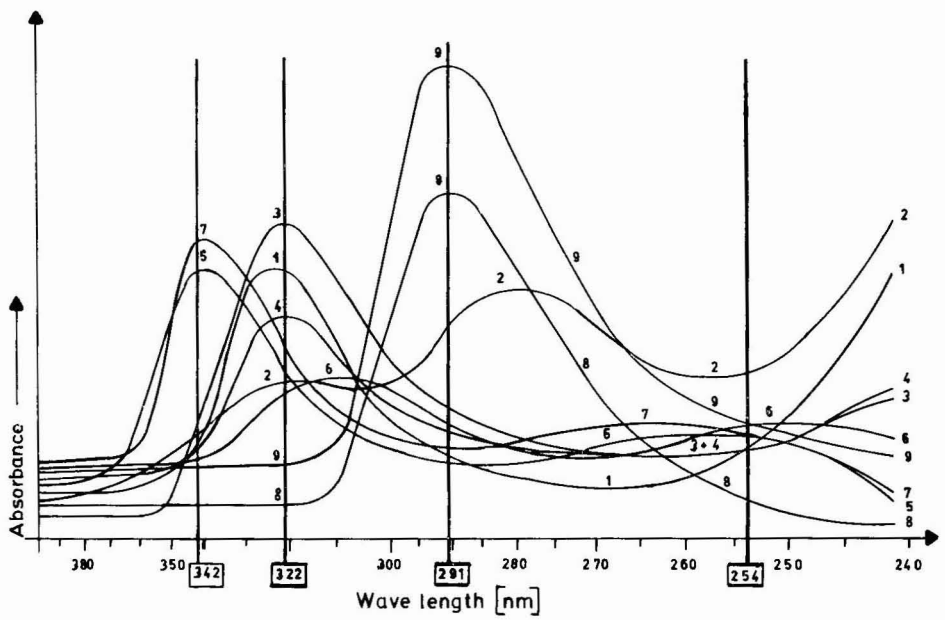

Fig. 1. UV spectra of 6-thiopurine compounds. 1 = 6-Mercaptopurine; 2 = 6-(1-methyl-4-nitro-imidazole)mercaptopurine (Azathioprine–Wellcome); 3 = 6-mercaptopurine ribonucleoside; 4 = 6-mercaptopurine 2′-deoxyribonucleoside; 5 = 6-thioguanine; 6 = 6-methylthioguanine; 7 = 6-thioguanine ribonucleoside; 8 = 6-methylmercaptopurine; 9 = 6-methylmercaptopurine ribonucleoside. The vertical lines indicate the $\lambda_{max.}$ values of 6-thiopurine compounds and of the normally used 254-nm detection.

end-products, which exhibit maximum absorbance ($\lambda_{max.}$) in the range 240–280 nm; (2) the 6-methylthiopurines with $\lambda_{max.}$ at 291 nm; (3) the unsubstituted 6-thiopurines with $\lambda_{max.}$ at 322 nm; and (4) the 6-thioguanines and some 6-thioxopurines with $\lambda_{max.}$ in the range 342–355 nm. Additionally, the 6-thioguanines exhibit a second $\lambda_{max.}$ between 240 and 260 nm. All values given here were measured in 0.4 *M* ammonium formate solution at pH 4.6.

Using these wavelengths for the quantitative determination of the various 6-thiopurines, each compound can be detected with high sensitivity. For this purpose, however, a variable-wavelength spectrophotometric HPLC detector must be available.

Fig. 2 gives an example of the increase in sensitivity obtained when using the spectrophotometric HPLC detector. Five common purines and three 6-thiopurines

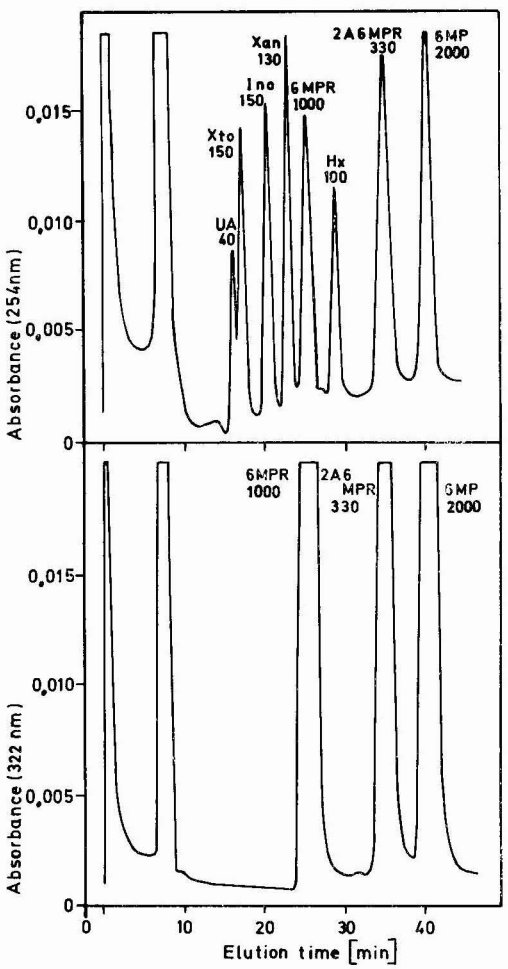

Fig. 2. Elution profiles of a separation of some common purines and of three 6-thiopurines as detected at 254 and 322 nm. UA = Uric acid; Xto = xanthosine; Ino = inosine; Xan = xanthine; 6MPR = 6-mercaptopurine ribonucleoside; 2A6MPR = 6-thioguanine ribonucleoside; 6MP = 6-mercaptopurine. The numbers on the peaks indicate the amount of each compound (in picomoles).

are separated from each other. Detection is performed at 254 and 322 nm. Amounts less than 300 pmole of each common purine are not detected at 322 nm. Relatively large amounts of the 6-thiopurines had to be injected on to the column in order to obtain peak heights comparable to those of the common purines at 254 nm. These amounts, however, cause huge peak heights and full-scale deflections of the recorder pen at 322 nm.

At 322 and 342 nm, about 30 pmole of each unsubstituted 6-thiopurine and of each 6-thioguanine are detected quantitatively. The sensitivity of detection is greater by a factor of 70 for the 6-thiopurines and 10 for the 6-thioguanines compared with detection at 254 nm.

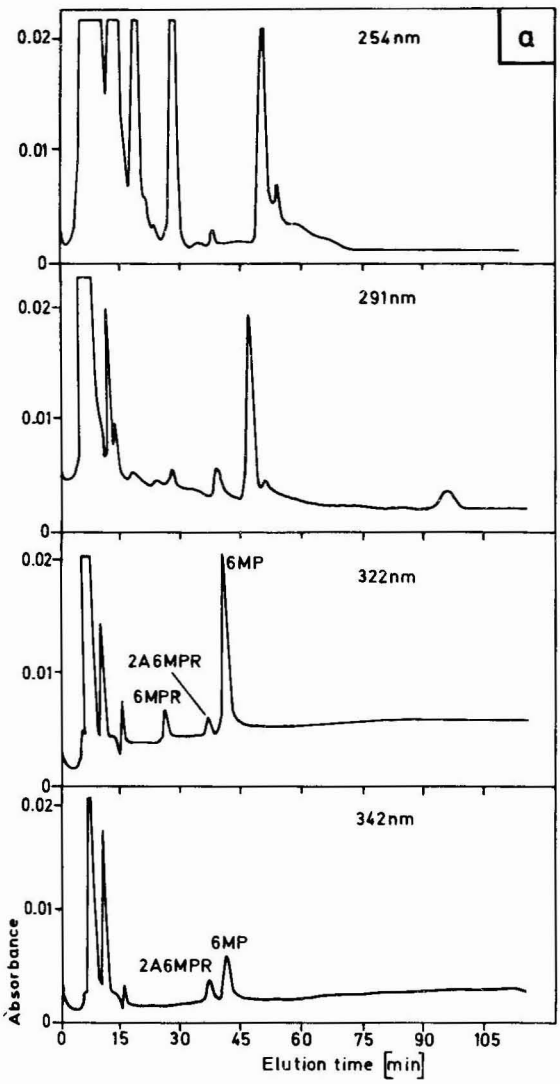

Fig. 3 (a).

(Continued on p. 66)

Fig. 3 shows a series of chromatograms of a PCA-extract of cells that were incubated with 6MP: (a) extract with no enzymatic digestion, (b) after phosphatase digestion and (c) after phosphatase plus purine nucleoside phosphorylase digestion. The common purine and pyrimidine free bases and ribonucleosides and some related compounds that exhibit $\lambda_{max.}$ in the 240–280-nm range are eluted at the beginning of the chromatograms. Detection at 254 nm hardly gives any resolution between the different compounds and 6-mercaptopurine metabolites are not detected. On switching, however, to 291, 322 and 342 nm, the chromatograms become increasingly simple. Common purines are no longer detected, but 6-methylthiopurines, unsubstituted 6-thiopurines and 6-thioguanines are recorded as sharp peaks, which can serve

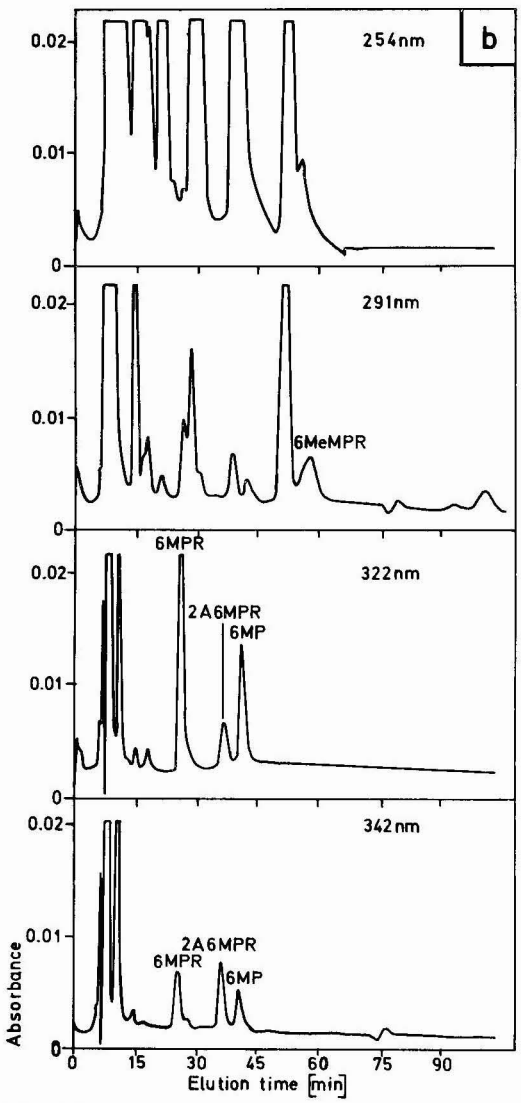

Fig. 3 (b).

for quantitative evaluation. More detailed information will be given in Part III[2].

The results clearly demonstrate that a variable-wavelength spectrophotometric detector is essential for the qualitative and quantitative determination of minor compounds that differ in their spectral characteristics from the major constituents of a given sample.

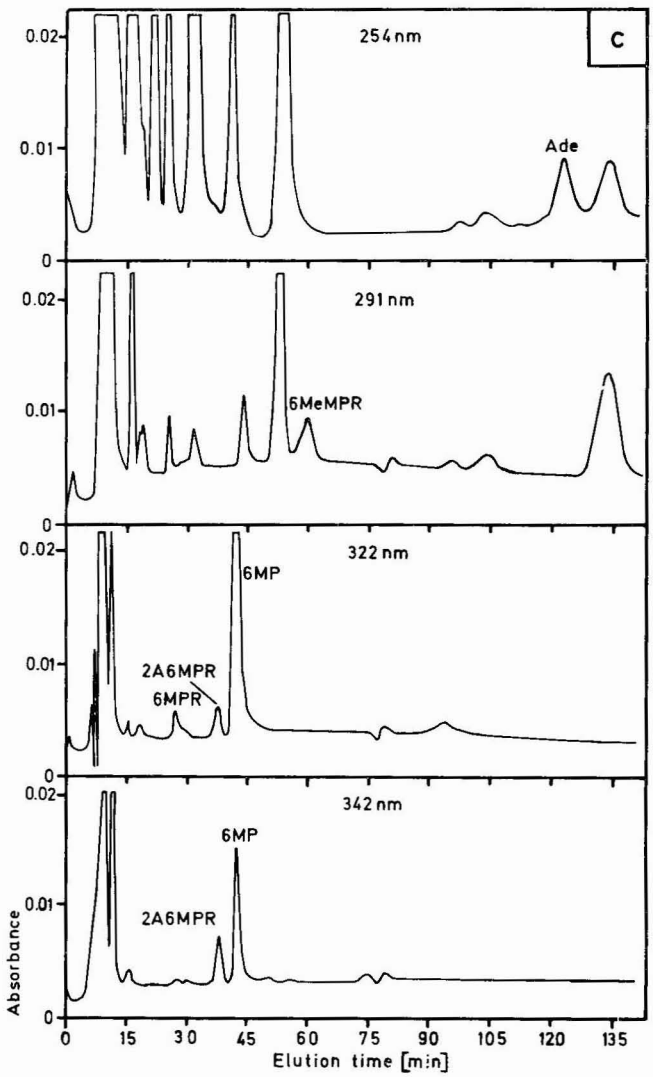

Fig. 3. Elution profiles of the separation of a PCA-extract of L5178Y mouse lymphoma cells grown in 6MP-containing medium as detected at the wavelengths indicated. (a) PCA-extract without enzymatic digestion; (b) PCA-extract digested with phosphatase, which liberates the ribonucleosides; (c) PCA-extract digested with phosphatase + purine nucleoside phosphorylase, which liberates the free bases except 6MeMP. 6MPR = 6-Mercaptopurine ribonucleoside; 2A6MPR = 6-thioguanine ribonucleoside; 6MP = 6-mercaptopurine; 6MeMPR = 6-methylthioguanine ribonucleoside; Ade = adenine. For details, see ref. 2.

HPLC, with its high-efficiency columns, proved superior to other chromatographic methods for the resolution of multi-component samples and, by use of the spectrophotometric detector, this advantage can even be enhanced.

A further application of the variable-wavelength spectrophotometric HPLC detector has recently been reported[9]. With intermittant stop–flow of the eluent, the absorbance of a substance that just passes the flow cell of the detector can be checked at several wavelengths. The identity and purity of the substance peak can then be established from its characteristic absorbance ratios which, throughout the peak, should be constant and of given values for a specified compound.

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POLYACRYLAMIDE GRADIENT ELECTROPHORESIS FOR PROTEIN PURIFICATION ON THE MILLIGRAM SCALE

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SUMMARY

A preparative-scale electrophoretic technique for protein fractionation and elution on a discontinuous gradient of acrylamide is described, which permits the separation and elution of a pure protein from a mixture containing 4–20 electrophoretically different proteins. The sharpness of the gradient electrophoretic resolution is demonstrated by the separation of proteins consisting of bovine serum albumin polymers and lactate dehydrogenase and enzymes such as acid phosphatase. The compositions of various discontinuous gradients of acrylamide and their application to enzyme purification are discussed. It was found that 60% of the enzyme activity loaded on the gel is recovered after gel fractionation and elution.

INTRODUCTION

Polyacrylamide gel electrophoresis has been widely used for both analytical and preparative purposes[1–30]. The technique described here, involving the use of polyacrylamide gel gradients, was developed in order to purify labile enzyme preparations in view of the shortcomings of classical methods of protein fractionation.

Several types of apparatus for electrophoresis or elution for large-scale preparative gel electrophoresis have been described. Lewis and Clark[1] were the first to adapt the Ornstein[2] and Davis[3] disc electrophoresis method to a preparative scale and to develop a system for collecting the bands of proteins, as they emerged from the end of the column, by a perpendicular flow of buffer. However, the tapered shape of the elution column caused heating during elution. Jovin *et al.*[4] tried to solve this problem by designing an apparatus for preparative, temperature-regulated, polyacrylamide gel electrophoresis, which required a complicated cooling installation.

A review of different elution processes was given by Hjerten *et al.*[30]. Several papers since 1963 have described various types of elution apparatus that involved continuous electrophoresis and collection of the proteins. As they emerged from

the bottom of the gel, the proteins were carried to a fraction collector by one or several perpendicular flows of buffer[4,6,17]. This method has some disadvantages as far as protein recovery is concerned because of dissipation of proteins present in low concentrations by thermal convection, mixing of two bands of proteins, well separated on the acrylamide column, in the eluting chamber owing to the convexity of the bottom of the gel, and dilution of the eluted fraction owing to the continuous flow of eluting buffer.

Other elution systems that have been proposed require the application of electrophoresis following homogenization of gel sections[11] or electrodialysis of excised pieces of gel[12]. The excision process used by Lewis and Clark[1], involving homogenization in buffer and centrifugation of the pieces of gel, has the disadvantage of a large recovery volume.

With these considerations in mind, we attempted to devise a simple technique for preparative polyacrylamide gel electrophoresis, with an elution system that would minimize the inactivation of unstable enzymes and ensure the recovery of proteins in concentrated solutions with a good yield. These aims were achieved by the introduction of a discontinuous gradient of acrylamide, whose resolution greatly exceeds that observed with the classical acrylamide technique, and an electrophoretic elution of the gel sections in 1-ml collecting tubes.

MATERIALS AND METHODS

Chemicals

Acrylamide, N,N′-methylenebisacrylamide, β-diethylaminopropionitrile, potassium hexacyanoferrate(III), ammonium persulphate, Tris and glycine were purchased from Merck (Darmstadt, G.F.R.). Coomassie Brilliant Blue R 250 and bovine serum albumin (BSA) were obtained from Mann Labs. (New York, N.Y., U.S.A.), trypsin from Armour Labs. (London, Great Britain) and pepsin and phosphorylase from Worthington (Freehold, N.J., U.S.A.). Amido-Schwartz was purchased from Apelab (Bagneux, France), lyophilized acid phosphatase, grade II, from potato from Boehringer (Mannheim, G.F.R.) and lactate dehydrogenase (LDH), band 1 native (4H), from pig heart from Boehringer (ref. 15378).

Phosphatase assay

The reaction mixture contained (in a total volume of 1.1 ml) 1 μmole of *p*-nitrophenyl disodium phosphate, 150 μmole of acetate buffer (pH 5.0), 10 μmole of EDTA and the enzyme solution. After 10 min of incubation at 37°, the reaction was stopped by adding 0.2 ml of 2 *N* aqueous ammonia. The absorption at 400 nm was measured against a suitable blank.

Protein determination

Protein was measured using a micro-biuret technique[14].

Electrophoresis apparatus

The apparatus (Fig. 1) was constructed on the same principle as the commercially available analytical model from Pleuger (S. A. Wijnegem, Belgium) and adapted to give a large-scale model. It is made from a Plexiglass cylinder 14 cm in diameter and

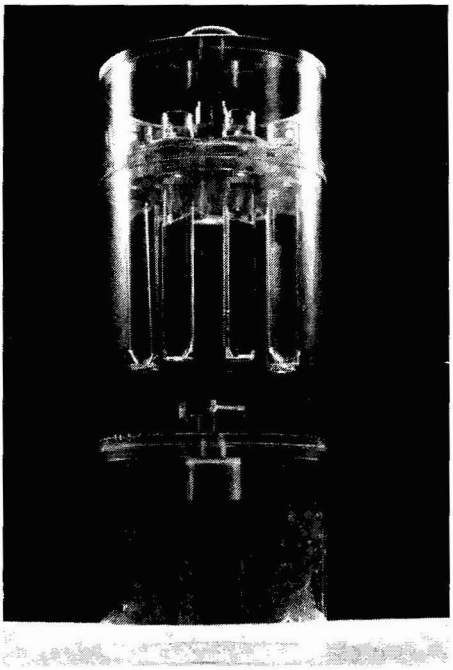

Fig. 1. Electrophoresis apparatus.

includes two tanks, the upper tank, 6.4 cm high, having eight holes of I.D. 30 mm arranged at 45° intervals around the centre. Each hole is fitted with a plastic screw system (Fig. 2) which allows the glass columns, previously filled with polyacrylamide gel, to fit in easily and safely. The lower tank, having the same diameter, is 15 cm high. One of the two circular Plexiglass electrodes is fixed in the lower chamber while the other is part of the apparatus cover.

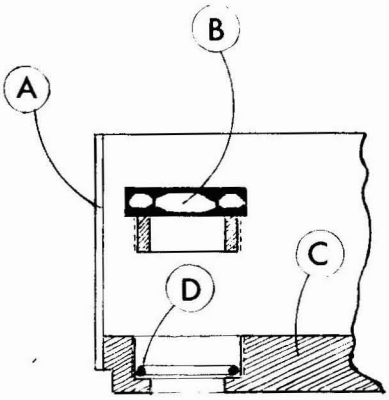

Fig. 2. Cross-section of the system for fixing the electrophoresis glass column in the upper tank (A). B, Plastic ring; C, bottom of the upper tank of the electrophoresis apparatus; D, O-ring.

Gel slicer

A Plexiglass gel slicer (Fig. 3) was adapted from the model described by Lewis and Clark[1]. It has 0.2-cm spaced slots and a central hole of 2.0 cm diameter, allowing the gel to be held in position while being sectioned with a steel wire.

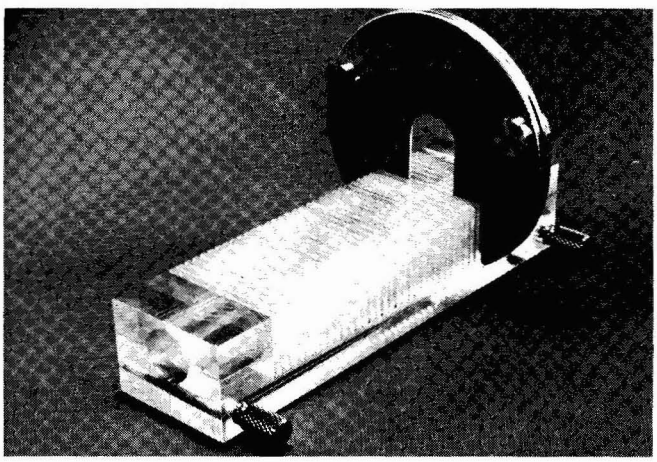

Fig. 3. Gel slicer.

Elution apparatus

The system (Fig. 4) is made of a wide Plexiglass cylinder 29 cm in diameter and 40 cm high. It is composed of two sections placed one on the top of the other, the upper one having eight holes to allow the insertion (Fig. 5, I) of the elution columns (Fig. 5, F) of 5.2 cm diameter, in which squashed gel sections are placed.

The columns are closed at the top end with a nylon diaphragm and at the bottom end with a glass-wool plug (Fig. 5, G and H). At the bottom of the elution column (Fig. 5, L) collecting tubes are fitted (1-ml volume) which are closed off with a dialysis membrane (Fig. 4) held in place by a rubber-band. The collecting tubes are immersed in the buffer contained in the lower section to which one electrode is attached, the upper electrode being part of the apparatus cover.

Preparation of reagents

The following were prepared.

(1) A solution of acrylamide (40 g per 100 ml) and bisacrylamide (1.06 g per 100 ml) was used for forming gels containing acrylamide in the range 3–10%. This solution, after appropriate dilution, was mixed with an equal volume of reagents (2), (3) and (4) described below.

(2) A 1.6-g amount of β-diethylaminopropionitrile was dissolved in the Tris–glycine stock buffer diluted 2.5 times (30.3 g/l of Tris and 144 g/l of glycine).

(3) Potassium hexacyanoferrate(III) solution, 0.03%.

(4) Ammonium persulphate solution, 0.48%.

(5) The electrophoresis buffer consisted of 25 m*M* Tris–glycine (pH 8.3), prepared by dilution of 0.25 *M* Tris–glycine concentrate (30.3 g/l of Tris and 144 g/l of glycine).

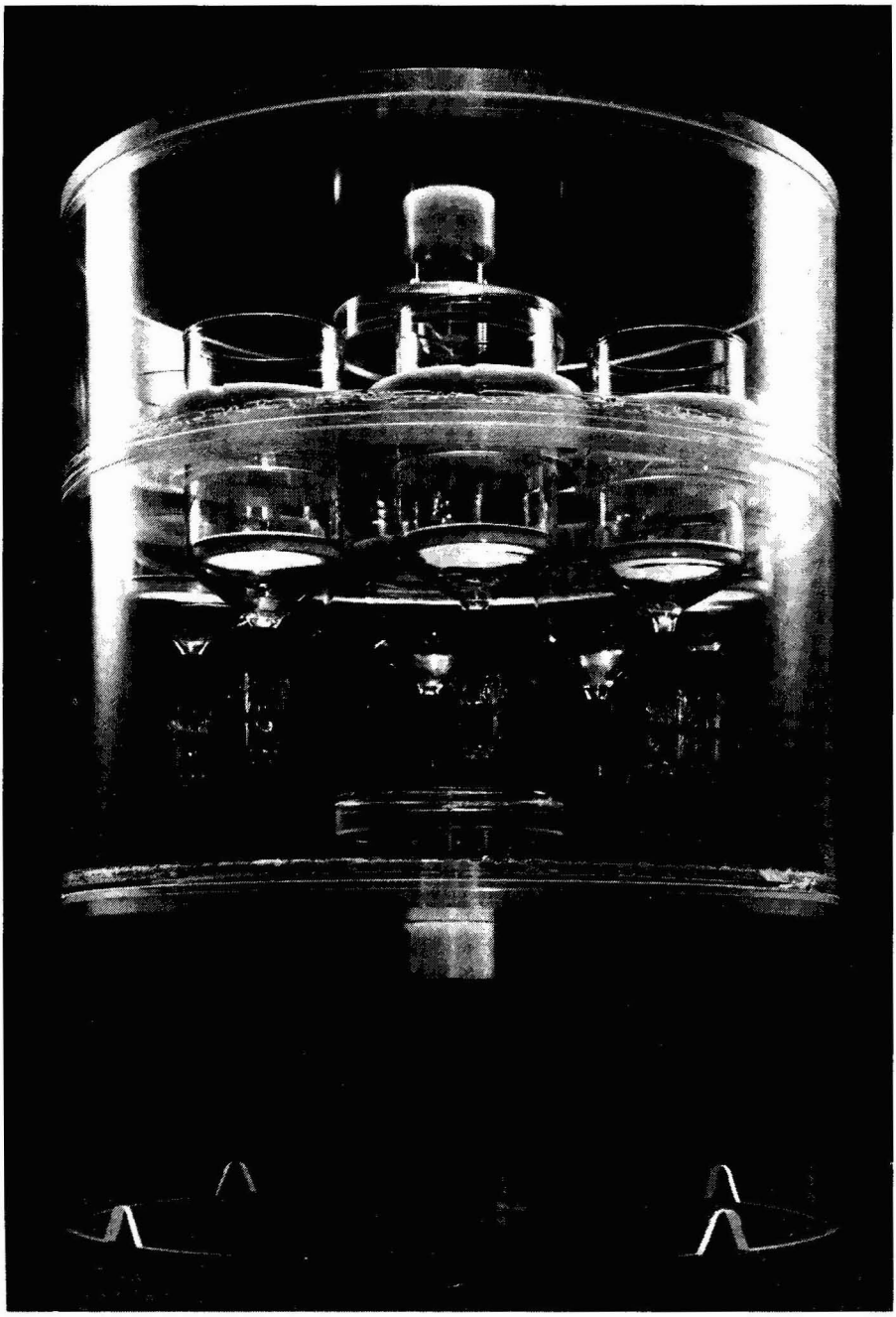

Fig. 4. Elution apparatus.

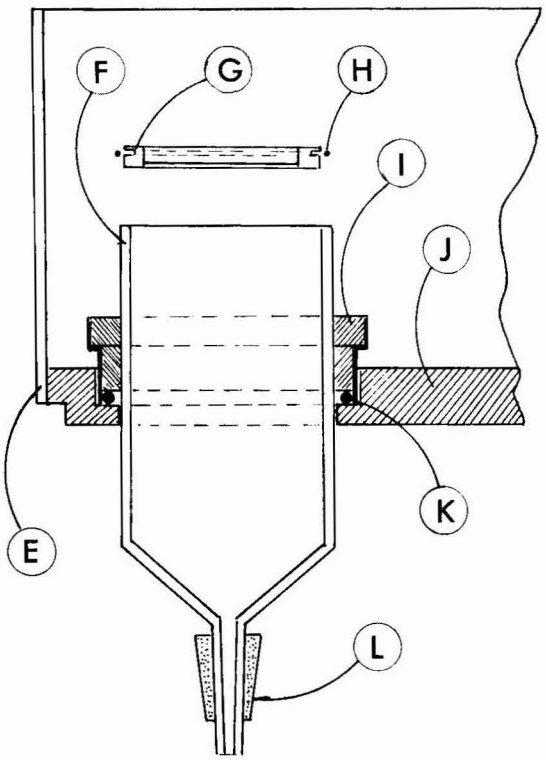

Fig. 5. Cross-section of an elution glass vessel (F). J, Plastic bottom of the upper tank (E) of the elution apparatus; I, plastic screw; K, O-ring; G, nylon cover tightened by a plastic ring (H).

(6) Amido-Schwartz stain: 50 mg of Amido-Schwartz dissolved in a mixture of 45 ml of methanol, 45 ml of water and 10 ml acetic acid.

(7) Coomassie Blue stain: 0.25 g of Coomassie Brilliant Blue R 250 dissolved in a mixture of 45 ml of methanol, 45 ml of water and 10 ml of acetic acid.

(8) De-staining solvent: 200 ml of methanol plus 70 ml acetic acid diluted to 1000 ml with distilled water.

Preparation of gels

The gels were formed by mixing the appropriate reagents in solution and allowing them to polymerize directly in the tubes (15 × 1.6 cm) in which the electrophoresis was to be carried out. In order to obtain a flat surface the gel was prepared "downwards", with a rubber stopper inserted in the glass tube. Thus, the gradient of acrylamide was set as follows: 5 ml of 5.5% acrylamide solution was first poured and allowed to set until polymerization occurred (about 20 min), the buffer remaining above the gel layer was removed and then 5 ml of 6.5% acrylamide was poured; when this layer had polymerized, 7.5, 8, 8.5 and 9% acrylamide solutions were poured in the same way.

Electrophoresis

In this procedure, designed for thermolabile enzymatic proteins, electrophoresis

was performed at 4° with a constant voltage of 80 V. No heating of the gel occurred under those conditions. The duration of electrophoresis depended on the gradient gel system chosen and the protein material used; it may vary from 4 to 16 h. After electrophoresis, the gels were removed from the glass tubes by directing a fine jet of buffer between the tube and the gel and were stained either in Amido-Schwartz for 30 min or Coomassie Blue for 4 h and then de-stained with solution (8) at 37°. Gels to be eluted were not stained but were cut into slices 0.2 cm thick, the slices being pulped in a syringe and injected with electrophoresis buffer into the eluting tubes (Fig. 5). A current of potential 200 V (≡ 1 mA per column) was passed through the apparatus for 5–10 h and the proteins eluted were recovered in the lower part of the elution system closed with a dialysis membrane. In some instances the protein became partly adsorbed on to the dialysis membrane, but reversal of the current for 3 min served to return it into solution in the eluting buffer.

Loading of protein

Protein samples were made denser than the overlying buffer by addition of an inert solute (10% glycerol or sucrose) before being layered into the gel. About 5–10 mg of protein were loaded per gel.

RESULTS

BSA polymers and LDH isozymes were subjected to different experiments in which either the composition of the polyacrylamide gradient or the time of electrophoresis was changed.

Fig. 6 shows a 7.5% polyacrylamide gel reference (A) and three gradients (B–D). B corresponds to a gradient of 5.5–8.5% polyacrylamide while C and D are identical gradients of 8.5–5.5% polyacrylamide with different electrophoresis times. The four gels were 15 cm in length and 1.6 cm in diameter.

For the gels A, B and C, the electrophoresis was carried out at 4° and 80 V until the bromophenol blue arrived at the bottom of the gels (usually 5 h), while electrophoresis in gel D was maintained for a further 6 h. As shown in Table I, where migration values of BSA monomer, dimer and trimer and LDH are reported, the best results were obtained with polyacrylamide gel of type D.

The measured distances between monomer and dimer protein bands, which are approximately the same in gels A, B and C (16, 13.5 and 17 mm), are more than twice as large in gel D (40 mm). This result also applies to the observed distance in gel D between monomer and trimer (57 mm), monomer and LDH (34 mm) and monomer and tetramer (70 mm).

To test the possible range of the polyacrylamide gradient technique for protein separation the LDH band, which migrates very close to the BSA dimer, was examined using these different gel gradients. A comparison of their migrations in the four gels demonstrates an improvement in their separation: in A 2 mm, in B 3 mm, in C 3.5 mm and in D 6 mm. It should be noted that an increase in the time of electrophoresis increases the separation of the proteins only with polyacrylamide gradient type D; when the time of electrophoresis is increased when using 7.5% polyacrylamide (gel type A), diffusion and mixing of the protein bands occur.

An impure sample of acid phosphatase from potato was chosen in order to

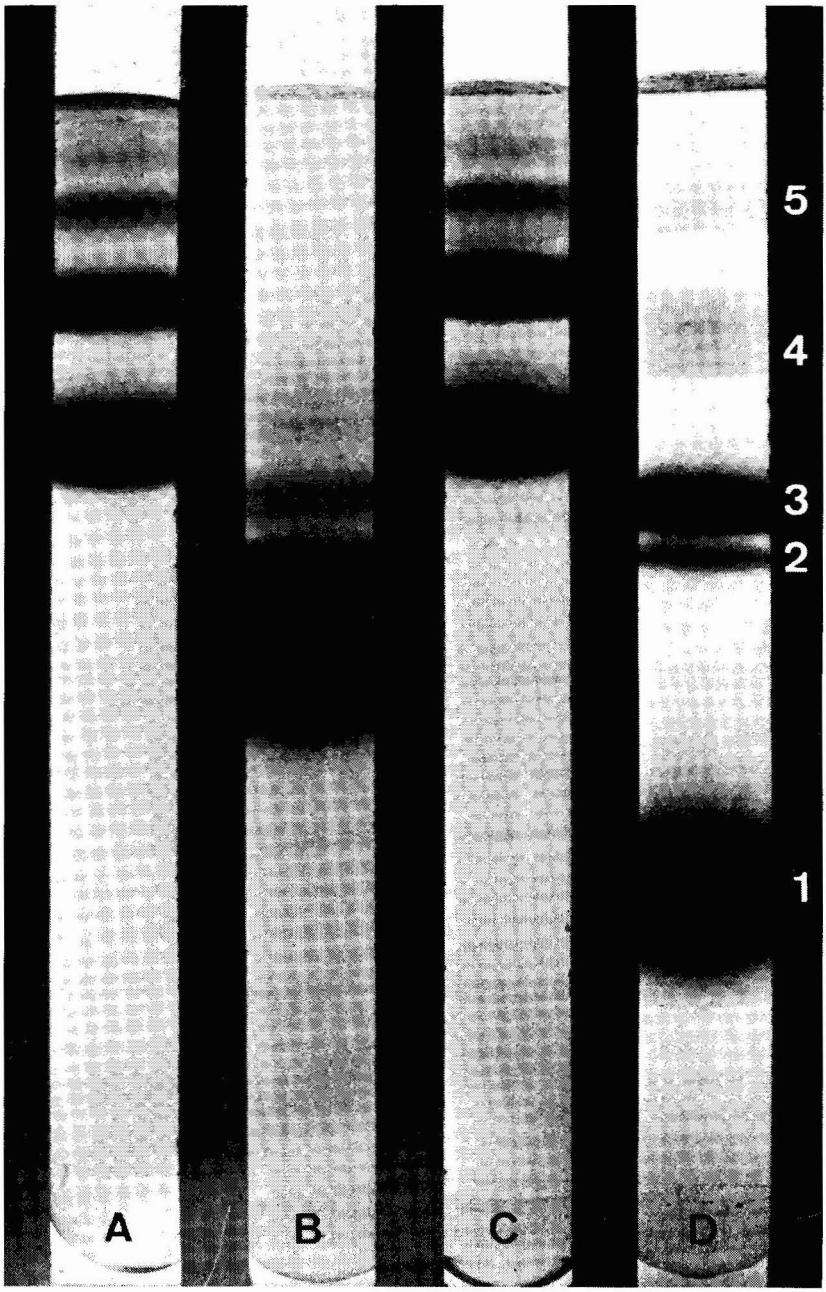

Fig. 6. Separation of BSA polymers and LDH on three different polyacrylamide gradients: A, 7.5% polyacrylamide gel control; B, gradient of 5.5–8.5% polyacrylamide; C and D, gradients of 8.5–5.5% polyacrylamide. 1 – BSA monomer; 2 – LDH; 3 – BSA dimer; 4 – BSA trimer; 5 – BSA tetramer.

TABLE I

MIGRATION OF PROTEINS ON DIFFERENT POLYACRYLAMIDE GELS

The values given represent the migrations in mm. A, B, C and D correspond to the gel shown in Fig. 6.

Protein	*Polyacrylamide gel*			
	A	*B*	*C*	*D*
BSA monomer	36	62	36	83
LDH	22	51.5	22.5	49
BSA dimer	20	48.5	19	43
BSA trimer	11	42	11	26
BSA tetramer	6	33	6	13

develop this method for enzyme separation, purification and elution. The enzyme preparation (acid phosphatase grade II) was examined on three types of gel: a 7.5% polyacrylamide gel (type A), a 5.5–8.5% gradient (type B) and an 8.5–5.5% gradient (type C). Its activity and the corresponding stained protein appeared as a wide and diffused zone in the 7.5% gel (A) containing contaminating proteins, while phosphatase activity was found in a narrower fraction in the two polyacrylamide gradients distinctly separated from the contaminants. It was also found that the acid phosphatase did not migrate further than the 6.5% acrylamide stage in gradient B. Therefore, as shown in Fig. 7, we reduced the number of polyacrylamide stages to four, *viz.*, 5.5, 6, 6.5 and 7%, and also doubled their length. Unlike LDH and BSA polymers, the phosphatase separation was better in gel gradient type B than type C.

As shown in Fig. 8, by using this procedure the phosphatase was separated and eluted as one pure protein.

To study the recovery of proteins, trypsin, pepsin, lactate dehydrogenase, phosphorylase a and purified acid phosphatase were submitted to a type B gradient and a standard 7.5% polyacrylamide gel. The recoveries from these two types of gel were similar, showing that the proteins were delayed at each surface in the polyacrylamide gradient but were not adsorbed on to these surfaces.

Table II shows the proportions of protein recovered and the recovery of phosphatase activity in the elution system described above. The proportion of protein recovery varied between 60 and 80%. A recovery of 60% was obtained for the acid phosphatase activity.

TABLE II

PROTEIN RECOVERY

Protein	*Molecular weight*	*Protein (% eluted)*	*Enzyme activity (% eluted)*
Trypsin	23,000	80	–
Pepsin	35,000	80	
LDH	140,000	60	–
Phosphorylase *a*	94,000	70	–
Acid phosphatase	–		60

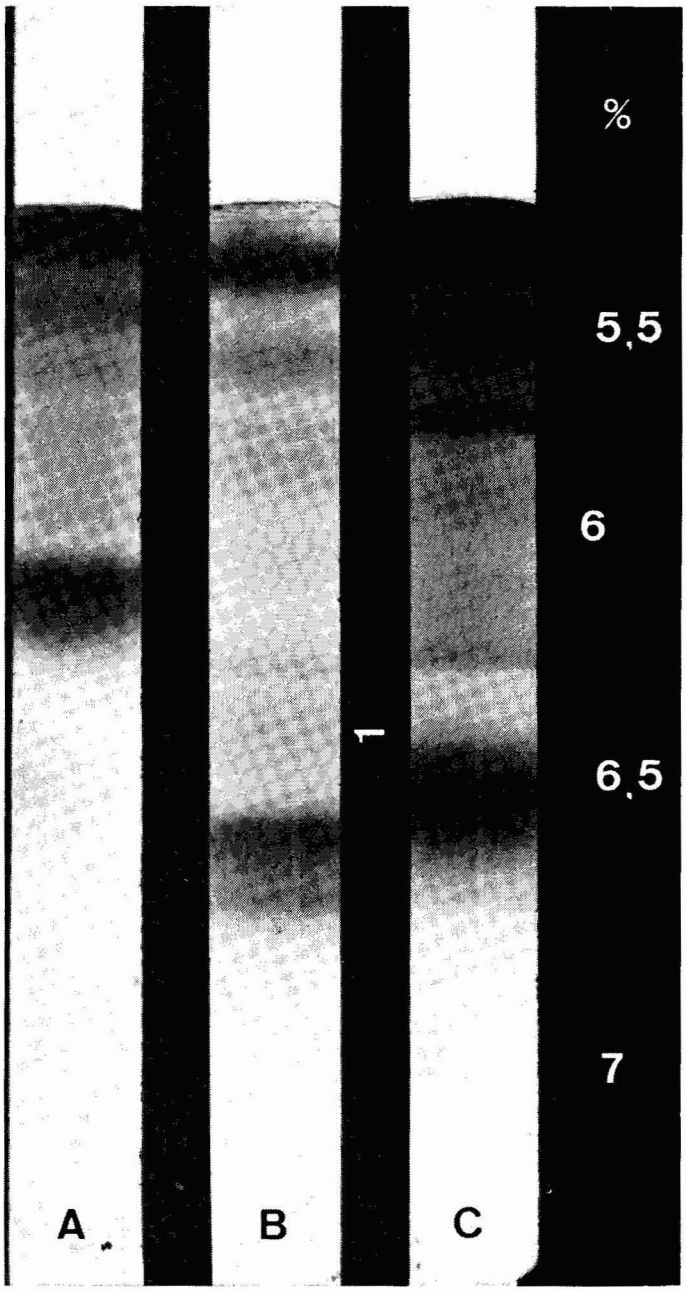

Fig. 7. Separation of acid phosphatase from potato on polyacrylamide gels: A, 7.5% polyacrylamide gel; B, polyacrylamide gradient of 5.5–7%; C, gradient of 7–5.5% polyacrylamide. The arrow shows the position of the acid phosphatase protein band.

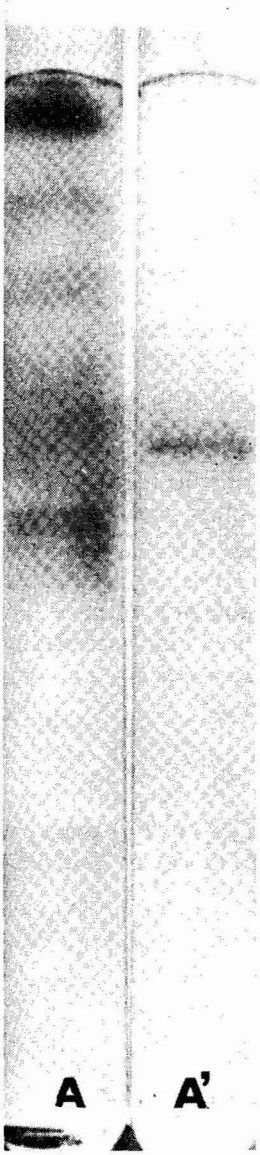

Fig. 8. Disc electrophoresis of the acid phosphatase on 7.5% polyacrylamide gels: A, before separation on polyacrylamide gradient (type B); A', after elution of a 5.5–7% polyacrylamide gradient (type B).

DISCUSSION

In order to enhance the resolution in polyacrylamide gel electrophoresis, the use of techniques such as pH gradients[19], isoelectric focusing[22,33,34] and linear pore gradient electrophoresis[23,25] for analytical purposes has been proposed. Better resolution was observed with electrophoresis on polyacrylamide gel in a continuous molecular sieve

gradient than on homogeneous gel[26]. This result was confirmed and even improved with the introduction of differential disc electrophoresis for the qualitative separation of serum proteins[20,24] and preparative enzymatic protein fractionation as described here. The most important aspect of this discontinuous polyacrylamide gradient technique is the number and length of the different acrylamide concentration stages.

A greater use of molecular sieving is obtained with electrophoresis through gels with different pore sizes; gels with increasing (from 9 to 5%) or decreasing (from 5 to 9%) pore size permitted the separation of large molecules from the smaller components and the separation of molecules with similar dimensions but slightly different charges.

Low recoveries of proteins in preparative polyacrylamide gel electrophoresis may be related to adsorption of the proteins on the membrane of the elution chamber or during concentration of the too dilute eluate (when continuous elution is carried out)[21]. In the present system, elution in a small volume at 4° prevents dilution and denaturation of the purified enzymatic proteins. Moreover, this method requires simple and inexpensive apparatus that can be constructed in most laboratories.

The reproducibility and reliability of this protein fractionation and elution method, the amount of protein that could be applied on the gels (5 mg/cm^2) and the percentage of protein and enzymatic activity recovered (Table II) demonstrated that this method can be used as a purification step, with a comparable or even better recovery than that obtained in column chromatography. It has been used successfully for the purification of an ATP-dependent deoxyribonuclease from *Bacillus subtilis*[31], a trehalase from pig kidney[32] and glycosidases from alfalfa seeds[18].

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USE OF COMPLEX FORMATION EQUILIBRIA IN THE ANALYTICAL ISOTACHOPHORESIS OF STRONG ELECTROLYTE IONS*

SEPARATION OF HALIDES AND SULPHATES

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SUMMARY

The possibility of effecting isotachophoretic separations by means of complex formation equilibria between a counter ion and the components being separated was investigated. This procedure was shown to be of potential significance mainly for the separation of ionic species of strong electrolytes, and the separation of sulphates and halides was examined. The use of Cd(II) as a a counter ion and NO_3^- as a leading ion is suggested. The migration of sulphate and nitrate zones is considered theoretically, and data calculated for actual operating conditions are verified experimentally.

INTRODUCTION

An isotachophoretic separation is based on the differences in the effective mobilities of ionic species. Separations have been described in which effective mobilities were altered, with the aim of obtaining the required separations, by changing the solvation[1] and by selecting an appropriate pH of the leading electrolyte[2,3]. In isotachophoresis, sufficient attention has not been devoted to the possibility of influencing the effective mobilities of the ionic species being separated by means of complex formation equilibria. Unlike the effect of pH on the effective mobilities of ionic species, complex formation may be applicable even with anions of strong acids or with a number of cations where the degree of dissociation is not affected substantially by pH.

The significance of this possibility is evident when one considers, for example, the separation of halides and sulphates, which is an important analytical problem that can hardly be solved by classical isotachophoresis using water. The general

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problem can be treated by considering such a practically important model. It follows from the principle of isotachophoresis that the only ionic species in the sample that can be separated are those with mobilities lower than that of the leading ion and greater than that of the terminating ion. There is no problem in finding a suitable terminating anion in a sample containing sulphates and chlorides because, under the conditions used in practice, *i.e.*, room temperature and with concentrations of the solutions varying from 10^{-2} to 10^{-4} *M*, both sulphates and halides are among the most mobile anions. However, finding a suitable leading electrolyte with which sulphates and halides will form isotachophoretic zones represents a problem.

A possible solution to the last problem is to find suitable anions with mobilities greater than those of sulphate and chloride. Such leading ionic species could be, for example, chromates, hexacyanoferrates(II) and hexacyanoferrates(III), the limiting mobilities of which are greater than those of sulphate and chloride. Experiments showed[4], however, that under the above conditions the mobilities of these ionic species are not sufficiently high, owing either to incomplete dissociation or to a considerably greater retarding effect of the ionic strength on multivalent ions in accordance with the Debye–Hückel–Onsager relationship. Also, the use of OH^-, which is the most mobile anion of all in water, is not suitable as the leading electrolyte would acquire a pH of 11–12 and an interference with the separation could be expected from carbonates present in the solution or from possible hydrolytic products in the injection port where the leading electrolyte comes into contact with the sample. In addition, this pH value lies outside the so-called[5] "safe region" for aqueous media.

Another possibility is to utilize selective depression of the mobilities of sulphate and halide. Such effects on the mobilities of strong ions were obtained by using non-aqueous media, *e.g.*, methanol, instead of water[1] and by isotachophoresis on paper[6,7], the isotachophoretic migration being combined with chromatographic effects. The mobilities of strong acid anions can also be altered by using complex formation equilibria.

If we respect the fundamental requirement of isotachophoresis, *i.e.*, that any electric current in a given zone may be transferred only by the ionic species under separation and by a counter ion, then only the complex formation equilibria between the counter ion and the ionic species being separated can be utilized. Hence the use of complex formation equilibria in isotachophoresis differs from that in zone electrophoresis, where use is made of the equilibria between the ionic species being separated and suitable complex-forming ionic species present in a background electrolyte[8,9].

Another important requirement is that the time of existence of each ionic species involved in the complex equilibria must be small in comparison with the time of migration, *i.e.*, the equilibrium adjustments must be very rapid (*cf.*, Tiselius[10]). The use of complex formation equilibria in isotachophoresis therefore differs from the isotachophoresis of kinetically inert complexes[11–13].

The selection of a suitable central cation that would serve as a counter ion in the isotachophoretic migration of halides and sulphates, a description of the system and an experimental verification are the subjects of this paper.

THEORETICAL

When selecting an operational electrolyte system such that sulphates and

halides are affected by complex formation in such a way that they form isotachophoretic zones, the following requirements apply:

(1) The establishment of the complex formation equilibria of halides and sulphates with a counter ion must be sufficiently rapid. The composition of the system should be such that the effective mobilities of halide and sulphate may decrease to such an extent that a suitable leading component can be found and, at the same time, the decrease should not be too great so that finding a suitable terminator would not be difficult. If some of the limiting mobilities at 25° are compared (*cf.*, ref. 14) *e.g.*, 80.0, 76.4, 71.5, 67.0, 55.0, 41.4 and $30.4 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$ for sulphate, chloride, nitrate, chlorate, fluoride, acetate and picrate anions, respectively, and if the concentration dependence is assumed to be approximately constant, it is evident that the decrease in the mobilities of sulphate and chloride anions due to the complex formation equilibrium should be more than *ca.* 10%. From the viewpoint of the selection of the terminator, the decrease should not exceed *ca.* 70%. Thus, for example, with sulphate the effective mobility of sulphates, $\bar{u}$, should be between 30 and 90% of the ionic mobility, $u_{SO_4^{2-}}$.

(2) The counter ion must not have a disturbing effect in combination with any of the anions assumed to be present in the sample or with the terminating electrolyte. Hence there must be no risk of the formation of precipitates or of products that are only slightly dissociated.

(3) A suitable anion must be available that virtually does not form a complex with the selected counter ion and with a sufficiently high mobility that it may serve as a leading component.

(4) It is advantageous if the complexes of halides and sulphates with the selected counter ion are well known, *i.e.*, their compositions, stability constants and temperature dependences and, moreover, if the mobilities of the individual particles involved in complex formation equilibria are also known.

Under such conditions, for a given composition of the leading electrolyte used, a mathematical description of the isotachophoretic zones can be given and the resulting net mobilities of halides and sulphates can be calculated, as will be shown. Such a case is analogous to the calculation of concentrations in isotachophoretic zones where acid–base equilibria are taken into account[15].

On the basis of the above considerations, the cadmium(II)-ion was selected as a suitable counter ion as it forms in aqueous media both sulphato and halogeno complexes, the compositions and stability constants of which are known. The nitrate anion, which virtually does not form complexes with the cadmium(II)-ion in *ca.* 0.01 *M* solutions[16] and the mobility of which is sufficiently great, was selected as the leading ionic species.

Citrate and/or tartrate can be used as terminator as both of them have sufficiently low mobilities.

A mathematical description of the zones, numerical calculation of net mobilities and experimental verification of the calculated values were carried out for sulphates and nitrates. The assumed compositions of the zones are as follows: leading zone, Cd^{2+}, NO_3^-; and sulphate zone, Cd^{2+}, $CdSO_4$, SO_4^{2-}. Data on the limiting ionic mobilities of the ions involved are available (see Experimental) and the mobility of $CdSO_4$ may be considered to be zero.

In all instances, the limiting ionic mobility, $u_{i,0}$, of an ion i is corrected for

the given concentration, c, in its zone according to the Debye–Hückel–Onsager relationship as follows[17]:

$$u_{i,c} = u_{i,0} - \left(\frac{29.14\, z_i}{\eta\,(\varepsilon T)^{1/2}} - \frac{9.90\cdot 10^{-5}}{(\varepsilon T)^{3/2}}\cdot u_{i,0}\,\omega\right)\cdot I^{1/2} \tag{1}$$

$$\omega = z_+\, z_-\cdot\frac{2q}{1+q^{1/2}} \tag{2}$$

$$q = \frac{z_+\, z_-}{z_+ + z_-}\cdot\frac{u_+ + u_-}{z_+\, u_- + z_-\, u_+} \tag{3}$$

$$I = 0.5\,(c_+\, z_+^2 + c_-\, z_-^2) \tag{4}$$

where $u_{i,c}$ indicates the ionic mobility at concentration c and c, z and u indicate the concentration, the valency of the ion (absolute value) and the mobility, respectively. The subscripts indicate the appropriate cation or anion. ε is relative permittivity and η the viscosity.

Further, net mobilities are calculated by using definition equations[10,18]. For the net mobility of sulphate in its zone, $\bar{u}_{SO_4,c_S}$, where the total concentration of sulphates in their zone is c_S, the following relationship holds:

$$\bar{u}_{SO_4,cS} = \frac{[SO_4^{2-}]}{c_S}\cdot u_{SO_4^{2-},cS} \tag{5}$$

$\bar{u}_{Cd,cS}$ represents the net mobility of Cd ionic species, where

$$\bar{u}_{Cd,cS} = \frac{[Cd^{2+}]}{c_{Cd}}\cdot u_{Cd^{2+},c} = \frac{[SO_4^{2-}]}{c_S}\cdot u_{Cd^{2+},cS} \tag{6}$$

as the equivalence of the total concentrations, $c_{SO_4} = c_{Cd} = c_S$, and of the electric neutrality, $[SO_4^{2-}] = [Cd^{2+}]$, obviously holds. The value of $[SO_4^{2-}]$ can be expressed by means of the expression for the total concentration of the ionic species, *i.e.*, $c_S = [SO_4^{2-}] + [CdSO_4]$ and the definition for the thermodynamic complex stability constant of $CdSO_4$:

$$K = \frac{a_{CdSO_4}}{a_{Cd^{2+}}\cdot a_{SO_4^{2-}}} = \frac{[CdSO_4]}{[Cd^{2+}]\cdot[SO_4^{2-}]\cdot y^2} \tag{7}$$

The following equation[19] is used in order to determine the activity coefficient, y:

$$-\log y = 0.5\, z_{Cd}\cdot z_{SO_4}\cdot\left(\frac{I_S^{1/2}}{1+I_S^{1/2}} - 0.3\, I_S\right) \tag{8}$$

For the ionic strength in the sulphate zone, $I_S = 4[SO_4^{2-}]$.

The total concentration of sulphates in their zone, c_S, is given by a moving-boundary equation, modified for isotachophoretic migration into the following form[20]:

$$\frac{\bar{u}_{SO_4,c_S}}{\kappa_{SO_4}} = \frac{u_{NO_3^-,c_N}}{\kappa_{NO_3}} \tag{9}$$

where κ is the specific conductivity of the sulphate or nitrate zone and $u_{NO_3^-,c_N}$ is the net mobility of nitrates in their zone, their total concentration being c_N. After expressing κ explicitly, eqn. 9 can be rearranged into the following form[21]:

$$\frac{c_S}{c_N} = \frac{\bar{u}_{SO_4,c_S}}{\bar{u}_{SO_4,c_S} + \bar{u}_{Cd,c_S}} \cdot \frac{u_{NO_3^-,c_N} + u_{Cd^{2+},c_N}}{u_{NO_3^-,c_N}} \cdot \frac{z_{NO_3}}{z_{SO_4}} \tag{10}$$

By applying eqns. 1–4 to nitrate, sulphate and cadmium ionic species in their respective zones and by combining them with the eqns. 5–8 and 10, we obtain the system of equations that describes the isotachophoretic migration of sulphates, *i.e.*, the system of equations that describes the concentrations and the mobilities in the zones of sulphates as a function of the concentration of the leading anion, NO_3^-, in the leading electrolyte, $Cd(NO_3)_2$.

Such a system of equations can be solved by iteration in such a way that for a given concentration, c_N, a zeroth approximation, $[SO_4^{2-}]_0 = 0.5\, c_N$ is selected. By substituting $[SO_4^{2-}]_0$ into eqn. 8, the first approximation, y_1, is obtained.

By substituting c_N and $[SO_4^{2-}]_0$ into a combination of eqns. 5, 6 and 10, the first approximation, $(c_S)_1$, is obtained. Using y_1, $(c_S)_1$ in eqn. 7 yields the first approximation $[SO_4^{2-}]_1$. The whole procedure is repeated with the approximation so obtained until two subsequent approximations are in agreement, corresponding to the required accuracy; an accuracy of better than 1% is sufficient in this instance. The required mobility of sulphate, $\bar{u}_{SO_4,c_S}$, is then calculated from eqn. 5.

EXPERIMENTAL

The equipment used for isotachophoresis consisted of a block of organic glass into which were built electrode chambers, control cocks, connecting channels and a separation capillary. The separations were performed in a separation capillary of tangular cross-section, with dimensions 200 × 1.0 × 0.2 mm, created by a groove in the organic block, covered with PTFE foil pressed on the block with a thermostatted plate. Detection was carried out by measuring the electric gradient by means of platinum contacts (two wires of diameter 0.05 mm, *ca.* 0.05 mm apart), penetrating into the groove a distance of *ca.* 16 cm from the injection port. A detailed description was published earlier[22].

A stabilized current of up to 400 μA at the maximum of up to 16 kV served as a high-voltage source. Detection was effected with a voltmeter with a high input resistance, simultaneously insulating galvanically a high-voltage section of the measuring circuits from the section connected to a recorder. The high-voltage source and the detection device were of our own design and have been described earlier[23].

A Perkin-Elmer Model 196 recorder was used. The chemicals used were of analytical-reagent grade (Lachema, Brno, Czechoslovakia). The thermostatted metal plate was maintained at 25° by means of circulating water.

The mobilities of the individual ionic species being investigated were calculated and measured as relative mobilities, where the leading NO_3^- ion was used as a reference ionic species; hence the calculated values represented the ratio $\bar{u}_i/u_{NO_3^-}$. Experimental measurements of the relative mobilities were carried out by using a procedure described earlier[21]. An inverse ratio of the step heights, $h_{NO_3^-}/h_i$, was measured. The ionic species being investigated were injected separately; the concentrations of standards were $5\text{–}8 \cdot 10^{-4}$ *M* and the sample size was *ca.* 1 μl. A solution of $Cd(NO_3)_2$ was used as the leading electrolyte with concentrations selected in the range 0.002–0.010 *M*; 0.010 *M* citric acid was used as the terminating electrolyte.

In the device used[22], at a driving current of approximately 300 μA and a thermostat temperature of 25°, the temperature of the solution in which migration occurred was 35° (ref. 24). The operating parameters were as follows: Temperature, 35°; relative permittivity, $\varepsilon = 82.3$; viscosity, $\eta = 0.00716$ P; limiting ionic mobilities at the given temperature, $u_{SO_4^{2-},0}$, $u_{NO_3^-,0}$ and $u_{Cd^{2+},0}$, 96.0, 85.8 and $64.8 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$, respectively. The thermodynamic complex stability constant of $CdSO_4$ is, under the conditions given, $\log K = 2.11$ (ref. 16). The valencies of the ions are obvious: $z_{SO_4} = 2$, $z_{Cd} = 2$ and $z_{NO_3} = 1$.

RESULTS AND DISCUSSION

Using the above relationships, data were calculated for the isotachophoretic migration of sulphate and nitrate with cadmium as counter ion, and a comparison of the experimental values of the relative mobility of sulphate with the calculated values is shown in Table I. Good agreement was found.

The effect of the complex formation equilibria on the relative mobilities of halides was evaluated experimentally in a similar manner. The calculations were not performed in these instances as the ionic mobilities of the ions of the type CdX^+ are not available. It is evident that the reverse procedure, *i.e.*, experimental determination of the mobility of the ionic species with a known composition of the equilibrium

TABLE I

COMPARISON OF EXPERIMENTAL VALUES (AVERAGE FROM THREE DETERMINATIONS) OF THE RELATIVE STEP HEIGHTS, $h_{NO_3^-}/h_{SO_4}$, WITH THE CALCULATED VALUES OF THE RELATIVE MOBILITIES, $\bar{u}_{SO_4,c_S}/u_{NO_3^-}$.

Concentration of leading electrolyte, $Cd(NO_3)_2$ (M)	$h_{NO_3^-}/h_{SO_4}$	$\bar{u}_{SO_4,c_S}/u_{NO_3^-}$	*Difference*	*Difference (%)*
0.002	0.917 ± 0.018	0.934	0.017	1.8
0.004	0.872 ± 0.018	0.870	0.002	0.2
0.005	0.847 ± 0.018	0.840	0.007	0.8
0.006	0.806 ± 0.018	0.813	0.007	0.9
0.008	0.763 ± 0.018	0.781	0.018	2.3
0.010	0.741 ± 0.018	0.752	0.011	1.5

TABLE II

EXPERIMENTAL VALUES OF THE RELATIVE MOBILITIES OF HALIDES, $u_i/u_{NO_3^-}$

The data represent averages from three measurements and the confidence interval is ± 0.018.

Concentration of leading electrolyte, $Cd(NO_3)_2$ (M)	*F*	*Cl*	*Br*	*I*
0.002	0.794	0.952	0.917	0.800
0.004	0.769	0.892	0.862	0.752
0.005	0.769	0.862	0.840	0.714
0.006	0.769	0.847	0.806	0.694
0.008	0.756	0.820	0.775	0.671
0.010	0.746	0.794	0.756	0.637

system in the zone can provide data on the ionic mobilities of complexes, which can be obtained by other means only with difficulty.

The experimental values of the relative mobilities of halides are given in Table II, and confirm the possibility of affecting the mobilities of strong electrolyte anions by means of a complex-forming cationic species. If the limiting mobilities[14] tabulated are compared, *e.g.*, fluoride, chloride, bromide, iodide and nitrate have limiting mobilities of 55.4, 76.4, 78.1, 76.8 and 71.5 $\cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$, respectively, it can be seen that except for fluoride, the halides are more mobile than nitrate and, moreover, the difference between the mobilities of chloride and iodide is small. On using the complex formation equilibrium with cadmium, the mobilities of halides not only become smaller than that of nitrate but they also differ sufficiently from one another and their isotachophoretic separation is then easy, as shown in Fig. 1, where the separation of a mixture of chlorides, bromides and iodides is shown.

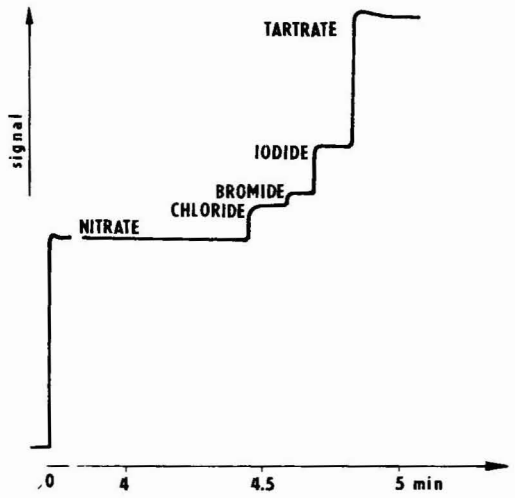

Fig. 1. Analysis of a mixture of chloride, bromide and iodide; 0.006 *M* $Cd(NO_3)_2$ served as the leading electrolyte. The volume injected was about 2 μl and the concentration of each component was about 0.01 *M*. The driving current was 400 μA.

A practical analysis of 10 μl of IDA mineral water from Spa Běloves, Czechoslovakia, containing 119.0 mg of sulphate, 17.60 mg of chloride, 0.30 mg of fluoride, 6.08 mg of nitrate and 1.05 mg of arsenate in 1 l, is shown in Fig. 2.

Chloride and sulphate provide zones that can be evaluated analytically. A quantitative analysis was carried out by direct comparison with a standard mixture and the results, representing average values from three determinations, are given in Table III.

Fluoride and arsenate zones are, under the operating conditions used, below the sensitivity of the apparatus, which was about $1 \cdot 10^{-8}$ g of halides.

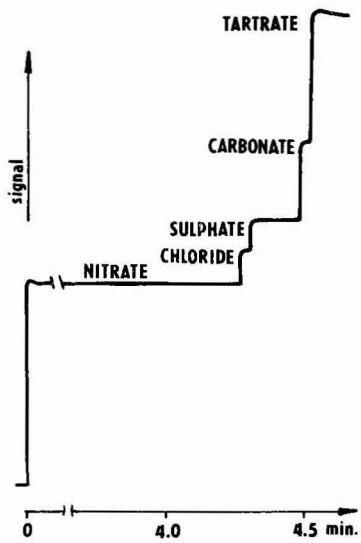

Fig. 2. Analysis of 10 μl of IDA mineral water. The leading electrolyte was 0.006 *M* $Cd(NO_3)_2$ and driving current was 400 μA. The carbonate zone is unstable. A; the sample is injected, with tartrate as terminator, it migrates but it vanishes during the migration and the quantitative information is lost.

TABLE III

DETERMINATION OF SULPHATE AND CHLORIDE IN IDA MINERAL WATER

Species	*True concentration (mg/l)*	*Concentration determined (mg/l)*	*Standard deviation*		*Deviation from true value*	
			mg/l	*%*	*mg/l*	*%*
Sulphate	119.0	117.1	2.1	1.8	1.9	−1.6
Chloride	17.60	17.70	0.16	0.9	+0.1	+0.6

CONCLUSIONS

Complex formation equilibria are a significant factor by means of which the effective mobilities and thus also the possibility of the isotachophoretic separation of ionic species of strong electrolytes can be influenced. A complex-forming agent acts

as the counter ion; hence complex formation between the counter ion and the component being separated is involved. In the separation of the anions of strong acids, an appropriately selected cation acts as the complex-forming agent.

If the stability constants and the mobilities of all of the particles involved in the complex formation equilibrium of the component being separated with the counter ion are known, the total concentration of the ionic species being separated in its zone and its net mobility can be calculated for a given composition of the leading electrolyte. Conversely, the mobilities of complex particles can be determined from the experimental data.

The calculation was carried out for the zone of sulphate migrating isotachophoretically behind the zone of nitrate with Cd(II) as the counter ion, for concentrations of cadmium nitrate in the leading electrolyte varying over the range 0.002–0.010 *M*. The calculated effective mobilities of sulphate relative to nitrate were compared with the data measured experimentally and the agreement was found to be good.

The system formed by the leading nitrate anion and the cadmium counter ion appeared to be suitable for the separation of halides and sulphates. The suggested system of electrolytes was applied to the analysis of sulphates and chlorides in mineral waters. The determination took about 5 min.

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INFLUENCE OF MODIFIED NUCLEOSIDES IN *E. coli* TRANSFER RIBONUCLEIC ACIDS ON CHROMATOGRAPHIC MOBILITIES OF TRANSFER RNA

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SUMMARY

The relationship between structure and partition coefficient K of 23 *E. coli* tRNA species has been investigated. Fractionation was performed by counter-current distribution in two salt–solvent systems containing phosphate buffer and two organic components: 2-methoxy ethanol and 2-butoxy ethanol (PMB system), formamide and isopropanol (PFI system). For the tRNAs studied, dependence of K on nucleoside composition is described by the relationship: $\log K = c(A/Y) + d$. The values for d vary over an interval Δd, which is a function of the polarity of the anticodon loop. The modified nucleosides are contained mainly in this highly exposed region of the tRNA molecule.

The tRNAs fall into three groups according to the anticodon loop polarity: group 1 with the lowest d value (hydrophilic anticodon loop) includes $tRNA_{1A}^{Ala}$, $tRNA^{Ile}$, $tRNA^{Lys}$, $tRNA_{M}^{Met}$, $tRNA_{1,3}^{Ser}$, $tRNA^{Thr}$ and $tRNA_{1}^{Val}$, each containing a polar N-(purin-6-yl carbamoyl)-threonine riboside, or a 5-oxyacetic uridine acid in the anticodon loop; group 2 with neutral anticodon loop containing 2-methyl adenosine and/or a modified 2-thio uridine is composed with $tRNA_{1,2}^{Arg}$, $tRNA_{1}^{Asp}$, $tRNA_{1,2}^{GlX}$, $tRNA_{1-3}^{Gly}$, $tRNA_{1}^{His}$, $tRNA_{1,2}^{Leu}$, $tRNA_{F}^{Met}$ and $tRNA_{2A}^{Val}$; group 3 with the highest d value (lipophilic anticodon loop) comprises $tRNA^{Phe}$, $tRNA^{Trp}$ and $tRNA^{Tyr}$, characterized by a lipophilic 2-methylthio N^6-isopentenyl adenosine at the 3′-end of the anticodon. For similar overall composition, the order of increasing mobility of tRNAs leads to an increase in Δd, which depends on the decreasing polarity of the modified nucleosides located in the anticodon loop.

INTRODUCTION

Analytical or preparative methods used in the fractionation of tRNA species

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by liquid–liquid continuous separation (partition chromatography) and discontinuous separation (counter-current distribution, CCD) are based on different partition coefficients (K) for different tRNA species. The relation between partition coefficient and the tRNA structure has been studied by Garel and Mandel[1] for yeast tRNAs. Within a homogenous family of polynucleotides like the tRNA species (80 $\pm$ 5 nucleotides, helix content 54 $\pm$ 3%), the partition coefficient is mainly a function of the total nucleotide composition of the tRNA, expressed by the relationship $\log K = A/Y$, where A and Y represent the number of adenosine and pyrimidine nucleoside residues, respectiely. This relation has been checked for oligoribonucleotides[2] and fragments of rRNA[3].

For prokaryotic tRNA species, one expects a comparable mobility relationship. Nevertheless, it must be noted that modified nucleosides are less numerous and mainly located in the anticodon loop. Several experimental approaches —associations with codons and oligonucleotides[4], chemical reactivity[5–7], enzymic degradability[8]— have shown that this loop is exposed. The tertiary structure of $tRNA^{Phe}$ confirms the accessibility of the anticodon loop (see reviews[9,10]). We have therefore considered the influence of the degree of polarity of the anticodon loop on the chromatographic mobility of tRNA species fractionated by CCD.

MATERIALS AND METHODS

tRNA and L-*amino acid:tRNA ligases (EC 6.1.1.)*

E. coli B tRNA was purchased from Calbiochem (San Diego, Calif., U.S.A.). [^{14}C]amino acids used for their *in vitro* acylation assays were provided by CEA (Saclay, France). tRNA ligases were prepared as described by Chavancy *et al.*[11] from a post-ribosomal supernatant of *E. coli* culture generously given by G. Dretzen (Strasbourg, France) and used in standard conditions (Mg^{++}/ATP = 1.5).

Counter-current distribution

160 Transfers were made with 600 A_{260} units of *E. coli* B tRNA at 15° in the PMB solvent system containing 18.6% 2-butoxy ethanol as previously described[1] (1,200 ml of 1.50 M potassium phosphate buffer (pH 7.0), 400 ml 2-methoxy ethanol, 365 ml 2-butoxy ethanol and 1.6 ml 1 M $MgCl_2$). In these conditions the average partition coefficient is about 1. Extraction of tRNA fractions, acylations and calculations of the partition coefficient of specific isoaccepting tRNA species have been described[12]. Statistical studies of the base distribution in the whole molecule of tRNA and the distribution of purine bases in the anticodon region (stem and loop) were carried out by J. L. Chasse (Laboratoire de Biométrie, Université Claude Bernard, Lyon I, France) with a Wang 700 calculator.

RESULTS

Structural data

The 28 tRNA sequences of *E. coli* B and K count 37 homologous positions for 90% of the molecules studied (Fig. 1). The most marked structural modification is found in the arms with paired bases and in the three bases of the anticodon. The numbering used (1–98) is based on the principle of maximum recovery of primary

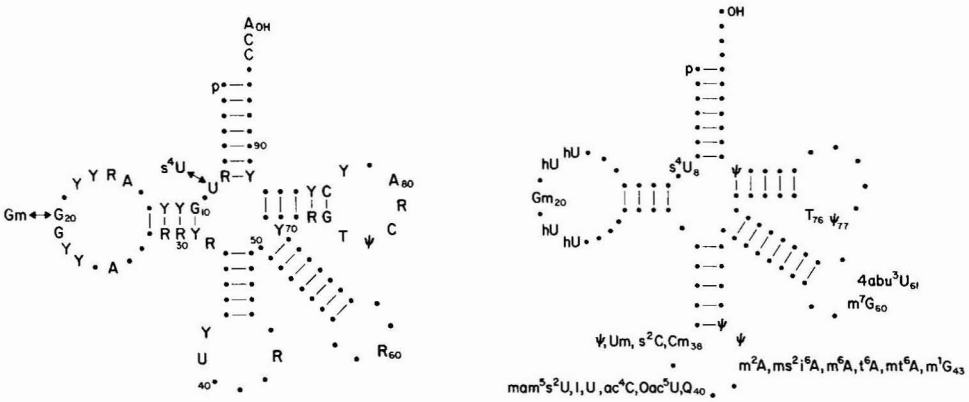


Fig. 1. Common nucleotides in *E. coli* tRNA species. The numbering used (1–98) is based on the principle of maximum overlapping of the primary structures of 28 *E. coli* tRNAs. The letters indicate the nature and position of nucleotides common to more than 90% of the tRNAs studied. A = adenosine-5′-phosphate; C = cytidine-5′-phosphate; G = guanosine-5′-phosphate; U = uridine-5′-phosphate; R = purine riboside-5′-phosphate; Y = pyrimidine riboside-5′-phosphate; T = ribothymidine-5′-phosphate; ψ = pseudouridine-5′-phosphate. For modified nucleotides, see legend of Table II.

Fig. 2. Modified nucleosides in *E. coli* tRNA species. The numbering is as in Fig. 1. m^1G = 1-methyl guanosine; m^2G = 2-methyl guanosine. For other abbreviations see legend of Table II.

TABLE I

STRUCTURAL DATA FOR 28 *E. COLI* tRNA SPECIES

tRNA	*A*	*G*	*C*	*U*	*hU*	*ψ*	*T*	*N*	*Total*	*R/Y*	*A/A + G* (%)	*A/Y* (%)	*Refs.*
Ala_{1A}	11	27	24	11		1	1		75	1.03	29.0	29.8	13
Arg_1	15	23	23	12		1	1	I	76	1.05	39.5	40.5	14, 15, 16
Arg_2	16	23	23	12		1	1	I	77	1.02	41.0	41.8	17
Asp_1	11	27	22	14		2	1		77	0.97	29.0	28.2	18
Gln_1	15	21	22	14		2	1		75	0.92	41.6	38.4	19, 20
Gln_2	15	21	23	12		3	1		75	0.92	41.6	38.4	19
Glu_1	13	23	27	10		2	1		76	0.90	36.1	32.5	21
Glu_2	14	22	27	10		2	1		76	0.90	39.0	35.0	22
Gly_1	15	20	22	15		1	1		74	0.90	42.8	38.5	23
Gly_2	13	19	24	17		1	1		75	0.74	40.6	30.2	24, 25
Gly_3	13	25	21	15		1	1		76	1.00	34.2	34.2	26
His	14	22	19	18		3	1		77	0.88	38.9	34.2	27, 28
Ile_1	16	25	19	14		2	1		77	1.14	39.0	44.5	29, 16
Leu_1	15	29	24	15		3	1		87	1.02	34.1	34.9	30, 31
Leu_2	17	29	21	17		2	1		87	1.12	37.0	41.5	30, 31
Lys	17	20	18	17		2	1	X	76	0.95	46.0	43.6	32
$Met^F_{1,2}$	15	24	26	10		1	1		77	1.03	38.5	39.5	33, 34
Met^M	19	20	20	15		2	1		77	1.02	48.7	50.0	35, 36
Phe	15	24	21	12		3	1		76	1.05	38.5	40.5	37, 16
Ser_1	19	29	24	14		1	1		88	1.20	39.6	47.5	38, 39
Ser_3	18	32	28	13		1	1		93	1.16	36.0	41.9	40, 41
Thr	17	23	18	16		1	1		76	1.11	42.5	47.0	42
Trp_{4u}	15	23	21	15		1	1		76	1.00	39.5	39.5	43
$Tyr_{1,3u}$	19	23	27	13		2	1		85	0.98	45.2	44.1	44
Tyr_2	20	25	27	12		2	1		87	1.07	44.4	47.6	45
Val_1	15	24	23	12		1	1		76	1.05	38.5	40.5	46, 47, 48
Val_{2A}	13	26	21	15		1	1		77	1.02	33.3	34.2	49, 16
Val_{2B}	16	23	18	18		1	1		77	1.02	41.0	42.0	49, 16

structures. The chain length varies mostly in the extra arm (positions 50–70), whereas it is constant for the dihydrouridine region (positions 10–31).

Table I lists some structural data. The molar ratio of purines to pyrimidines (R/Y) changes from 0.88 for $tRNA_1^{His}$ to 1.20 for $tRNA_1^{Ser}$ while the relative proportion of A to G, expressed by the ratio A/(A + G), varies from 0.29 for $tRNA_1^{Asp}$ or $tRNA_{1A}^{Ala}$ to 0.487 for $tRNA_M^{Met}$. The chain lengths are very close for 22 tRNA species (76 ± 1 nucleotides) and higher for 6 species: $tRNA^{Leu}$, $tRNA^{Ser}$ and $tRNA^{Tyr}$ (89 ± 4 nucleotides).

Fig. 2 shows the positions generally occupied by modified nucleosides and Table II indicates the nature and location of these modified nucleosides clustered mainly in the anticodon loop.

TABLE II

ALKYLATED BASES AND MODIFIED NUCLEOSIDES IN *E. COLI* tRNA SPECIES

tRNAs are arranged according to the three groups shown in the semi-logarithmic plots based upon the polarity of the anticodon loop (Figs. 6 and 7). U* = unknown modification; 4abu^{3}U = 3-(3-amino 3-carboxypropyl) uridine[15,16]; Q = 7-(4,5-*cis*-dihydroxy-1-cyclopenten-3-yl aminomethyl) 7-deazaguanosine[57]; m^{2}A = 2-methyl adenosine; m^{6}A = N^6-methyl adenosine; ms^{2}i^{6}A = 2-methylthio N^6-isopentenyl adenosine; t^{6}A = N-(purine-6-ylcarbamoyl)threonine riboside; mt^{6}A = N-methyl N-(purine-6-ylcarbamoyl) threonine riboside; ac^{4}C = N^4-acetyl cytidine; s^{2}C = 2-thio cytidine; Cm = 2′-O-methyl cytidine; m^{7}G = N^7-methyl guanosine; Gm = 2′-O-methyl guanosine; I = inosine; oac^{5}U = 5-oxyacetic uridine acid; s^{4}U = 4-thio uridine; mam^{5}s^{2}U = 5-methylaminomethyl 2-thio uridine; hU = 5,6-dihydrouridine; Um = 2′-O-methyl uridine; ψ = pseudouridine.

tRNA	*Position 8*	*hU loop*	*Anticodon loop*			*Extra arm*
			Position 38	*Wobble base, position 40*	*Position 43*	
Ala_{1A}	s^{4}U			oac^{5}U		m^{7}G
Ile					t^{6}A	m^{7}G, 4abu^{3}U
Lys				mam^{5}s^{2}U	t^{6}A	m^{7}G, X
Met^M	s^{4}U	Gm		ac^{4}C	t^{6}A	m^{7}G
Ser_1	s^{4}U	m^{2}G	Cm	oac^{5}U	ms^{2}i^{6}A	
Ser_3	s^{4}U		s^{2}C		t^{6}A	
Thr					mt^{6}A	
Val_1	s^{4}U			oac^{5}U	m^{6}A	m^{7}G
Arg_1	s^{4}U		s^{2}C	I	m^{2}A	m^{7}G, 4abu^{3}U
Arg_2	s^{4}U			I	m^{2}A	m^{7}G, 4abu^{3}U
Asp_1	s^{4}U			Q	m^{2}A	m^{7}G
Gln_1	s^{4}U	Gm	Um	mam^{5}s^{2}U	m^{2}A	
Gln_2	s^{4}U	Gm	Um		m^{2}A	
Glu_1				mam^{5}s^{2}U	m^{2}A	
Glu_2	s^{4}U			mam^{5}s^{2}U	m^{2}A	
Gly_1	s^{4}U					
Gly_2				U*		
Gly_3						m^{7}G
His_1	(s^{4}U)$_9$			Q	m^{2}A	m^{7}G
$Leu_{1,2}$		Gm			m^{1}G	
Met^F	s^{4}U		Cm			
Val_{2A}	s^{4}U					m^{7}G, 4abu^{3}U
Phe	s^{4}U		ψ		ms^{2}i^{6}A	m^{7}G, 4abu^{3}U
Trp	s^{4}U		Cm		ms^{2}i^{6}A	m^{7}G
$Tyr_{1,2}$	s^{4}U	Gm		Q	ms^{2}i^{6}A	

TABLE III

OBSERVED AND THEORETICAL DISTRIBUTION OF IDENTICAL BASE SEQUENCES FOR *E. COLI* tRNA SPECIES

The upper lines give the observed distribution, the lower lines that theoretical distribution[1]. n_A is the number of nucleosides A in the tRNA, n_G the number of nucleosides G, I, etc.; r is the number of consecutive sequences in the tRNA; for instance, r_3 indicates the frequency of NNN in the sequence, N is successively A, G, C, U (including ψ and T); for $r_{>3}$, the number of consecutive sequences is shown in parentheses after the figure giving the observed distribution.

tRNA	*A*					*G, I*				
	n_A	r_1	r_2	r_3	$r_{>3}$	n_G	r_1	r_2	r_3	$r_{>3}$
Ala_{1A}	10	10	0	0	0	26	13	4	0	1 (5)
		7.61	0.99	0.12	0.01		10.83	3.92	1.38	0.71
Arg_1	14	10	2	0	0	24	12	6	0	0
		9.37	1.74	0.30	0.06		11.04	3.63	1.16	0.51
Asp_1	10	8	1	0	0	27	13	3	1	1 (5)
		7.59	0.95	0.11	0.01		11.05	3.99	1.40	0.72
Gln_1	14	8	3	0	0	21	5	6	0	1 (4)
		9.37	1.74	0.30	0.06		10.89	3.11	0.86	0.28
Glu_2	13	9	2	0	0	22	6	4	0	2 (4)
		9.05	1.53	0.24	0.04		11.10	3.28	0.94	0.35
Gly_3	12	8	2	0	0	25	14	2	1	1 (4)
		9.00	1.51	0.21	0.04		11.19	3.78	1.24	0.57
His_1	13	11	1	0	0	22	11	4	1	0
		9.13	1.50	0.23	0.04		11.34	3.26	0.91	0.32
Ile	15	11	2	0	0	25	8	7	1	0
		9.83	1.91	0.35	0.07		11.34	3.78	1.22	0.55
Leu_1	14	8	3	0	0	29	12	6	0	1 (5)
		9.99	1.58	0.23	0.04		12.81	4.37	1.46	0.69
Leu_2	16	10	3	0	0	29	9	7	2	0
		10.77	1.97	0.34	0.06		12.81	4.37	1.46	0.69
Met_1^F	14	5	3	1	0	24	9	3	1	1 (4)
		9.48	1.71	0.29	0.05		11.33	3.62	1.12	0.47
Met^M	18	14	2	0	0	20	10	3	0	1 (4)
		10.64	2.51	0.56	0.15		10.99	2.90	0.73	0.23
Phe	14	10	0	0	1 (4)	24	11	3	1	1 (4)
		9.43	1.73	0.30	0.06		11.19	3.62	1.14	0.49
Ser_1	18	5	3	1	1 (4)	29	14	6	1	0
		11.48	2.35	0.46	0.10		12.96	4.37	1.44	0.67
Ser_3	17	7	3	0	1 (4)	32	13	6	1	1 (4)
		11.32	2.06	0.35	0.07		13.45	4.74	1.63	0.82
Thr	17	12	2	0	0	23	12	4	1	0
		10.06	2.13	0.43	0.10		11.16	3.46	1.04	0.41
Trp	14	6	2	0	1 (4)	23	9	2	2	1 (4)
		9.43	1.73	0.30	0.06		11.16	3.46	1.04	0.41
Tyr_1	18	8	2	2	0	23	10	3	1	1
		11.27	2.40	0.49	0.11		12.26	3.37	0.90	0.30
Tyr_2*	19	9	2	2	0	24	9	3	3	0
		11.69	2.57	0.54	0.13		12.60	3.53	0.96	0.33
Val_1	14	12	1	0	0	24	7	3	2	1 (5)
		9.43	1.73	0.30	0.06		11.19	3.62	1.14	0.49
Val_{2A}	12	12	0	0	0	26	11	5	0	1 (5)
		8.68	1.32	0.19	0.03		11.32	3.93	1.33	0.64
Val_{2B}	15	11	2	0	0	23	12	6	0	1 (5)
		9.83	1.91	0.35	0.07		11.29	3.45	1.02	0.40

* Without the modified pyrimidine ($tRNA_2^{Tyr}$).

TABLE IV

OBSERVED AND THEORETICAL DISTRIBUTION OF IDENTICAL BASE SEQUENCES FOR *E. COLI* tRNA SPECIES

For explanation of symbols see text to Table III.

tRNA	*C*					*U*, *ψ*, *T*				
	n_C	r_1	r_2	r_3	$r_{>3}$	n_U	r_1	r_2	r_3	$r_{>3}$
Ala_{1A}	23	15	1	2	0	13	7	1	1	0
		10.88	3.37	1.07	0.45		8.55	1.36	0.20	0.03
Arg_1	21	12	3	1	0	14	12	1	0	0
		11.01	3.10	0.84	0.29		9.43	1.73	0.30	0.06
Asp_1	20	9	4	1	0	17	9	4	0	0
		10.99	2.90	0.73	0.23		10.40	2.41	0.49	0.12
Gln_1	20	5	4	1	1 (4)	17	8	2	0	1 (5)
		10.78	2.94	0.76	0.25		10.24	2.34	0.51	0.13
Glu_2	25	9	1	2	2 (4)	13	6	2	1	0
		11.19	3.78	1.24	0.57		9.05	1.53	0.24	0.04
Gly_3	19	12	2	1	0	17	8	3	1	0
		10.74	2.72	0.66	0.20		10.32	2.33	0.50	0.12
His_1	17	5	3	2	0	22	10	6	0	0
		10.40	2.31	0.49	0.12		11.22	3.27	0.92	0.34
Ile	17	9	2	0	1 (4)	17	11	3	0	0
		10.40	2.31	0.49	0.12		10.40	2.31	0.49	0.12
Leu_1	22	12	1	0	1 (8)	19	9	5	0	0
		12.33	3.16	0.78	0.24		11.69	2.57	0.54	0.13
Leu_2	19	7	3	2	0	20	12	4	0	0
		11.69	2.57	0.54	0.13		11.93	2.76	0.61	0.16
Met^F_1	24	12	2	1	1 (5)	12	10	1	0	0
		11.33	3.62	1.12	0.47		8.68	1.32	0.19	0.03
Met^M	18	13	1	1	0	18	12	3	0	0
		10.64	2.51	0.56	0.15		10.64	2.51	0.56	0.15
Phe	19	6	3	1	1 (4)	16	8	4	0	0
		10.74	2.72	0.66	0.20		10.06	2.13	0.42	0.10
Ser_1	22	9	5	1	0	16	6	5	0	0
		12.23	2.95	0.68	0.19		10.82	1.96	0.33	0.06
Ser_3	26	10	4	0	2 (4)	15	13	1	0	0
		13.30	2.78	1.04	0.37		10.54	1.68	0.25	0.04
Thr	16	11	1	1	0	18	12	3	0	0
		10.06	2.13	0.43	0.10		10.55	2.53	0.58	0.16
Trp	19	7	4	0	1 (4)	17	9	4	0	0
		10.74	2.72	0.66	0.20		10.32	2.33	0.50	0.12
Tyr_1	25	9	4	1	1 (5)	16	8	4	0	0
		12.44	3.73	1.09	0.42		10.65	2.00	0.35	0.07
Tyr_2*	25	10	2	1	2 (4)	15	7	4	0	0
		12.69	3.71	1.05	0.39		10.39	1.77	0.28	0.05
Val_1	21	10	1	3	0	14	8	3	0	0
		11.01	3.10	0.84	0.29		9.43	1.73	0.30	0.06
Val_{2A}	19	13	3	0	0	17	9	4	0	0
		10.83	2.40	0.65	0.19		10.40	2.31	0.49	0.12
Val_{2B}	16	10	3	0	0	20	6	7	0	0
		10.13	2.11	0.42	0.09		10.99	2.90	0.73	0.23

* Without the modified pyrimidine ($tRNA_2^{Tyr}$).

TABLE V

DISTRIBUTION OF PURINE BASES IN THE ANTICODON ARM OF *E. COLI* tRNA SPECIES

The upper lines give the observed distribution of purine bases in the anticodon arm (positions 33–48 according to Fig. 1). The lower lines indicate the calculated or theoretical distribution of purine bases assuming a random base distribution in the whole molecule of tRNA (ref. 1).

tRNA	*Anticodon*		*Total number or residues*		
	A	*G*	*A*	*G, I*	*n (−CCA)*
Ala_{1A}	2	5	10	27	72
	2.39	6.22			
Arg_1	3	5	14	24	73
	3.26	5.59			
Asp_1	2	5	10	27	74
	2.30	6.20			
$Gln_{1,2}$	2	5	14	20	72
	3.30	4.72			
Glu_2	1	5	13	22	73
	3.03	5.12			
Gly_3	3	5	12	25	73
	3.07	5.67			
His_1	3	5	13	22	74
	2.99	5.05			
Ile	4	5	15	25	74
	3.45	5.74			
Leu_1	3	5	14	29	84
	2.83	5.87			
Leu_2	3	6	16	29	84
	3.36	5.87			
Met_1^F	4	4	14	25	75
	3.17	5.67			
Met^M	6	2	18	20	72
	4.25	4.72			
Phe	5	5	14	24	72
	3.30	5.67			
Ser_1	3	5	18	29	85
	3.60	5.80			
Ser_3	3	5	17	32	90
	3.21	6.04			
Thr	3	6	16	23	73
	3.73	5.36			
Trp	4	4	14	23	73
	3.26	5.36			
Tyr_1	4	4	18	23	82
	3.73	4.77			
Tyr_2	5	4	19	24	84
	3.85	4.86			
Val_1	4	4	14	24	73
	3.26	5.59			
Val_{2A}	3	5	12	26	74
	2.76	5.97			
Val_{2B}	3	5	15	23	74
	3.45	5.28			

Tables III and IV present the distribution of identical base sequences observed (upper line) and calculated (lower line) without considering the common 3′-terminal trinucleotide. The hypothesis of a random distribution of bases is acceptable (χ^2 test) for the great majority of *E. coli* tRNAs except for $tRNA_F^{Met}$ which has an excess of G and C compared to A and U. There is a random sequence distribution of identical bases, except for $tRNA_1^{Ser}$, characterized by a significant cluster of A, and for $tRNA_1^{Gln}$ with regard to C. Table V notes the distribution of purine bases in the anticodon arm. Contrary to some yeast tRNAs[1], *E. coli* tRNAs do not differ significantly in purine distribution in this region compared with their average distribution in the whole molecule.

Counter-current distribution

Fig. 3 shows the absorbance profile of *E. coli* tRNA species fractionated by CCD, over 160 transfers at 15°, in the solvent system PMB (18.6% 2-butoxy ethanol,

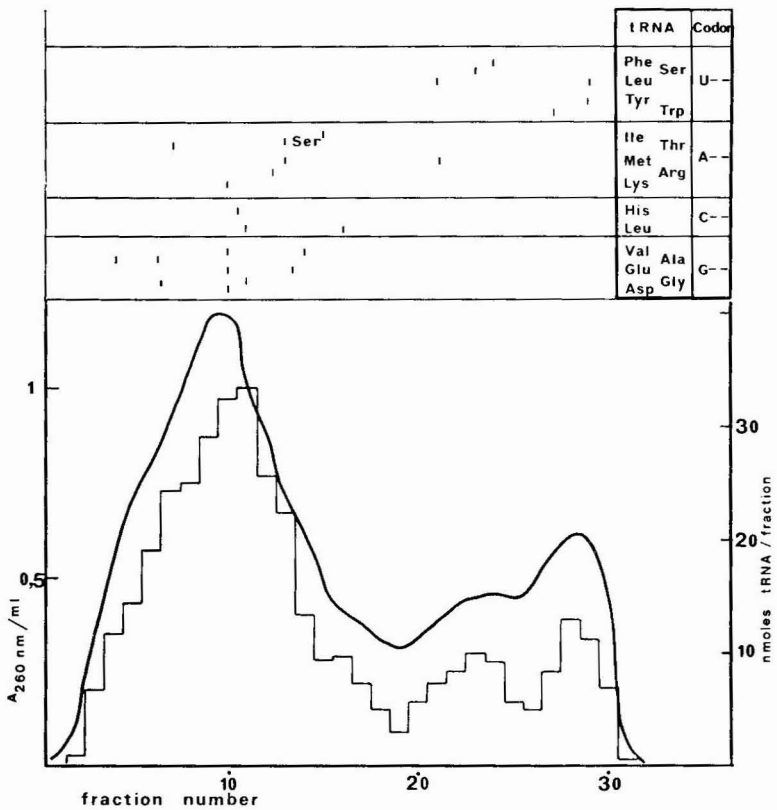
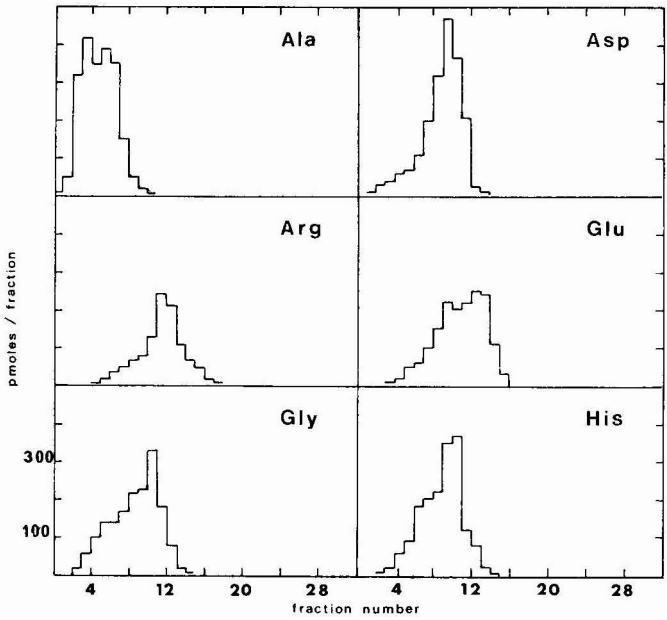


Fig. 3. CCD of *E. coli* tRNA. 600 $A_{260\ nm}$ units of *E. coli* B tRNA are distributed over 160 transfers at 15° in the salt–solvent system PMB (1.50 potassium phosphate buffer pH 7.0, 2-methoxy ethanol and 2-butoxy ethanol) with 18.6% 2-butoxy ethanol and 0.8 m*M* $MgCl_2$. —, Absorbance of tRNA at 260 nm in the upper phase; —, distribution curve of 16 individual tRNA species titrated by acylation in nmoles per fraction (one fraction is composed of the tRNA content of five distribution elements); ı, indication of the position of the maximum concentration of the isoacceptor species arranged according to their corresponding codons[50].



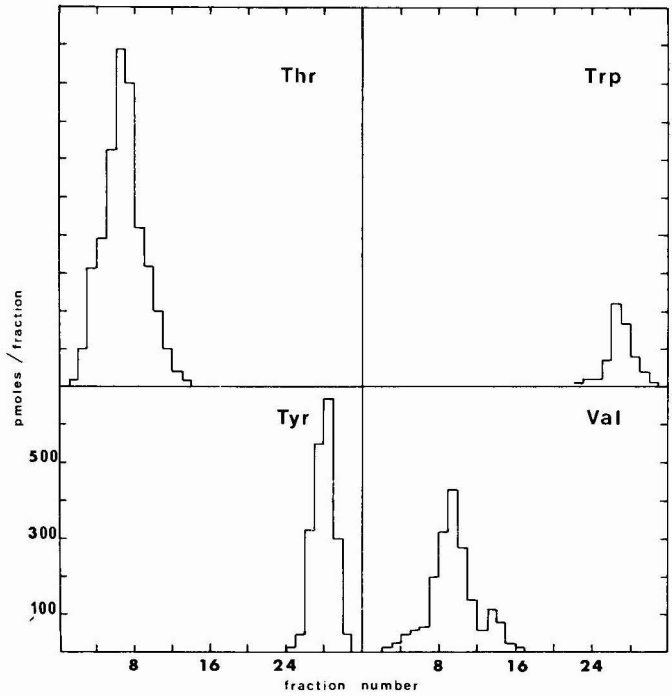


Fig. 4.

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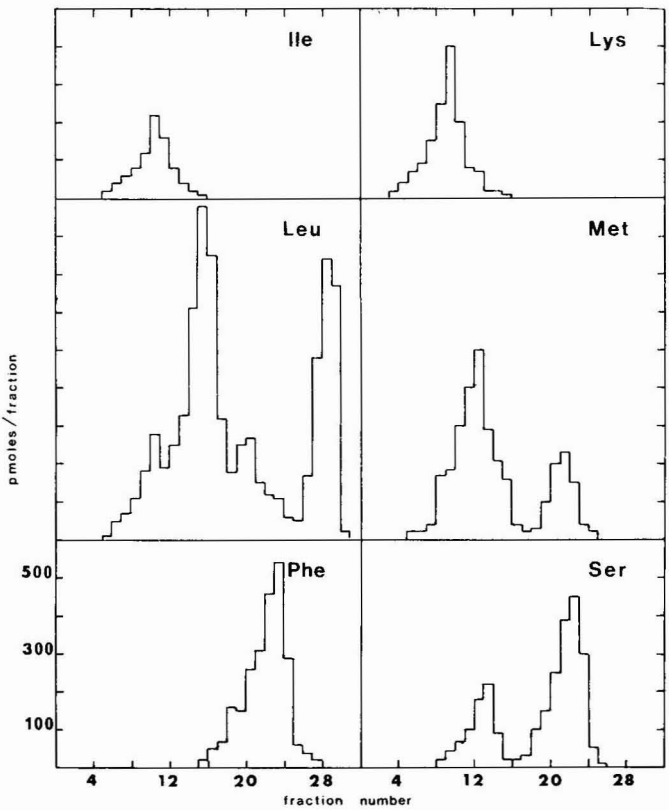

Fig. 4. Analysis of *E. coli* tRNA species fractionated by CCD. Conditions of the CCD in the PMB solvent system are indicated on Fig. 3. After 160 transfers, the contents of five distribution tubes are pooled, 5 ml of 2-methoxy ethanol is added; the solution is equilibrated in the cold and the lower phase discarded whereas the organic top phase is extracted with diethylether, dialyzed and lyophilized. tRNA from each of the 32 fractions was dissolved in 2.0 ml of 5 m*M* sodium acetate buffer (pH 4.7), 1 m*M* $MgCl_2$ and acylated according to Chavancy *et al.*[11] with homologous enzymes. The order of mobility of isoaccepting species is given according to the nature of the first base of the codon, indicated in decreasing order of polarity: A, U, G and C according to Wehrli and Staehelin[50].

0.8 m*M* $MgCl_2$). The mobility order of individual tRNA species is indicated on the diagram of Fig. 4. It is analogous to the elution order found by Wehrli and Staehelin[50], who did partition chromatography with the PEB solvent system described by Muench and Berg[51]: 1.25 *M* potassium phosphate buffer (pH 6.88), 2-ethoxy ethanol and 2-butoxy ethanol containing 1 % of triethylamine. Figs. 3 and 5 suggest a direct relationship between the polarity of tRNA and its coding properties. Fig. 5 shows the mobility of *E. coli* tRNA fractionated by CCD over 970 transfers in the PFI solvent system[52] as used by Goldstein *et al.*[53] (1.7 *M* sodium phosphate buffer (pH 6.0), formamide and isopropanol). Formamide plays an analogous role to that of 2-methoxy ethanol in the PMB system; its addition increases the partition coefficient of nucleic acid. Isopropanol, less polar, has a similar effect to that of 2-butoxy ethanol, which decreases partition coefficient[12,54]. Table VI summarizes partition coefficients values available and based on CCDs in the solvent systems PMB and PFI.

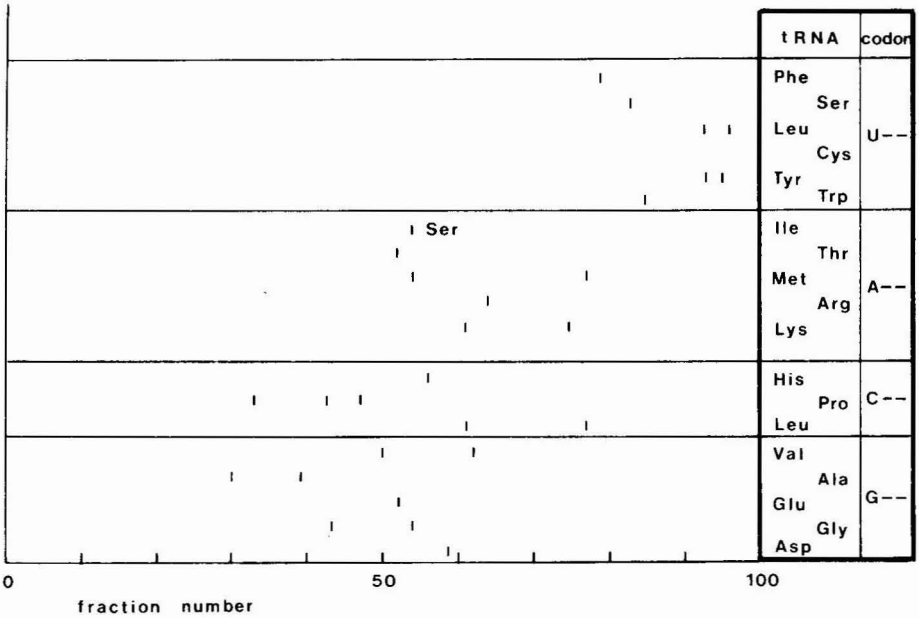

Fig. 5. Order of mobility of *E. coli* tRNA species fractionated by CCD over 970 transfers in the salt–solvent system PFI (phosphate buffer, formamide and isopropanol) according to Goldstein *et al.*[53].

Mobility law of E. coli tRNA species

As we have shown for oligoribonucleotides[2], yeast tRNAs[1] and ribosomal RNA fragments[3], the partition coefficient K increases with an enrichment in adenosine residues expressed as A/(A + G) or A/R and also with an increase in the ratio of purine/pyrimidine nucleotides, R/Y. For a salt–solvent system S studied at temperature T, one may write the following relationship:

$$\log K_{(S,T)} = c\,\mathrm{A}/(\mathrm{A} + \mathrm{G})\cdot \mathrm{R}/\mathrm{Y} + d$$

where c and d are constants dependent on the solvent system used and the characteristics of the nucleic compound (anticodon loop polarity, helix content, chain length and conformation). By neglecting the presence of inosine, the mobility of which is close to that of guanosine, and by considering the pyrimidine nucleotides as a whole (see Table VII for cytidine and uridine values of K), this relationship is reduced to

$$\log K_{(S,T)} = c\,\mathrm{A}/\mathrm{Y} + d$$

This formula is only valid for a similar total number of nucleotides.

The semi-logarithmic plot of the partition coefficients of *E. coli* tRNAs as a function of A/Y nucleotide composition (Fig. 6 for the PMB system and Fig. 7 for the PFI system) shows a linear relationship for tRNAs according to the polarity of their anticodon loops. The position of each point appears to be the result of two structural factors: A/Y composition for tRNA species having closely similar anti-

TABLE VI

PARTITION COEFFICIENTS OF *E. COLI* tRNA SPECIES FRACTIONATED BY COUNTER-CURRENT DISTRIBUTION IN SALT-SOLVENT SYSTEMS

Partition coefficients have been calculated using the formula $K = r/n - r$ where $r \leq t$ (r = element of the distribution apparatus corresponding to the maximum concentration of an isoacceptor species, n = total of transfers for a distributor with t elements or tubes).

tRNA	*PFI (ref. 52)*	*PFI (ref. 59)*	*PMB (ref. 54)*	*Suggested fit with a tRNA of known sequence*
Alanine	0.39		0.13	
	0.58		0.22	Ala_{1A}
Arginine	1.50		0.69	$Arg_{1,2}$
Aspartate	0.50			
	1.22		0.46	Asp_1
Glutamate			0.49	Glu_1
	0.96		0.73	Glu_2
Glycine	0.72		0.25	Gly_2
	1.00		0.52	$Gly_{1,3}$
Histidine	1.13		0.50	His_1
Isoleucine		0.47		
		0.79		
		1.00	0.88	Ile_1
Leucine	1.32		0.52	
	2.58	1.35	1.00	Leu_1
			1.91	Leu_2
	9.0	9.0	7.1	
Lysine	1.32			
	2.31		0.46	Lys
Methionine	0.96		0.68	Met^F
	2.58		1.90	Met^M
Phenylalanine	2.70		3.00	Phe
Proline	0.45			
	0.66			
	0.79			
Serine	1.04	0.61	0.74	Ser_3
	3.54	1.44	2.60	Ser_1
Threonine	0.92		0.31	Thr
Tryptophane	4.00		5.4	Trp
Tyrosine	6.7			Tyr_1
	7.9		9.7	Tyr_2
Valine	0.89		0.46	Val_{2A}
	1.38		0.76	Val_1

codon loop polarity (neighbouring d values) and, for a nearly identical overall composition, variable polarity of the anticodon loop (d variable over an interval Δd).

Group 1 (hydrophilic anticodon loop) comprises $tRNA^{Ile}$, $tRNA^{Lys}$, $tRNA_M^{Met}$, $tRNA_3^{Ser}$ containing in the 3′-position of the anticodon a polar N-(purin-6-yl carbamoyl)-threonine riboside (t^6A), $tRNA^{Thr}$ having a methylated t^6A or mt^6A, $tRNA_{1A}^{Ala}$, $tRNA_1^{Ser}$ and $tRNA_1^{Val}$ carrying a 5-oxyacetic uridine acid in the wobble position[55]. In a semi-logarithmic diagram, correlation lines can be drawn as shown in Figs. 6 and 7. The partition coefficient value for $tRNA^{Thr}$ has not been taken into account. It should be noted that because of the presence at the 3′-end of the anticodon of the

TABLE VII

PARTITION COEFFICIENTS OF SOME MODIFIED NUCLEOSIDES

Determinations are done at 20° in the PMB solvent system with 20% 2-butoxy ethanol[60].

Nucleoside	*K*	*log K*
N^6-Dimethyl adenosine	13.8*	1.14*
Adenosine	3.75	0.57
N^1-Methyl adenosine	0.70	−0.15
N^2-Dimethyl guanosine	11.5	1.06
1-Methyl guanosine	1.75	0.24
Guanosine	1.40	0.15
Inosine	1.10	0.04
N^7-Methyl guanosine	0.37	−0.43
Thymidine	1.90	0.28
Uridine	1.15	0.06
5-Methyl cytidine	1.08	0.03
Cytidine	1.04	0.02
Pseudouridine	0.66	−0.19
N^3-Methyl cytidine	0.30	−0.52

* Estimated according to the log *K* of corresponding modified base and the negative contribution of ribose residue (ΔF_{rib} = −0.09).

lipophilic nucleoside ms^2i^6A, $tRNA_1^{Ser}$ might be expected to be located in the group 3 with apolar anticodon loops, but the strong polarity of the nucleoside oac^5U prevails over the lipophilic effect of the modified adenosine.

Group 2 (neutral anticodon loop) contains a 2-methyl adenosine in the 3′-position of the anticodon except a 1-methyl guanosine for $tRNA_{1,2}^{Leu}$. It should be noted that $tRNA_{1,3}^{Gly}$ and $tRNA_{2A}^{Val}$ do not have a modified nucleoside in that loop.

Group 3 (lipophilic anticodon loop) consists of $tRNA^{Phe}$, $tRNA^{Trp}$ and $tRNA^{Tyr}$,

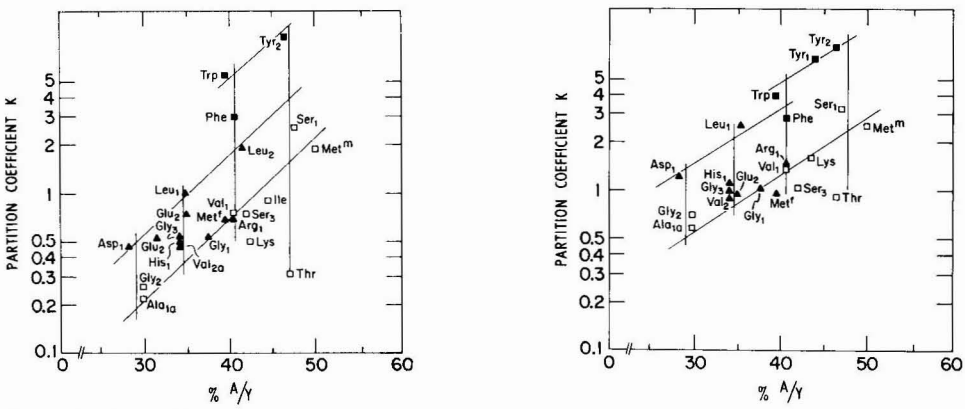

Fig. 6. Semi-logarithmic plot indicating the change of partition coefficients as a function of the total nucleotide composition of *E. coli* tRNA species distributed by CCD in the salt–solvent system PMB (see Fig. 3 and Tables I and VI). □, tRNA group with polar anticodon loop; ▲, tRNA group with neutral anticodon loop; ■, tRNA group with lipophilic anticodon loop.

Fig. 7. Semi-logarithmic plot giving the change of the partition coefficients as a function of the total nucleotide composition of *E. coli* tRNA species distributed by CCD in the salt–solvent system PFI according to Goldstein *et al.*[53] (see Fig. 5 and Tables I and VI). For legend see Fig. 6.

and is characterized by a 2-methylthio N^6-isopentenyl adenosine at 3'-end of the anticodon.

The strong effect exerted by the modified nucleosides of the exposed anticodon loop on the mobility of tRNA macromolecules can be shown by considering the polarity order of modified purine and pyrimidine nucleosides. From data given in Table VII and from the chromatographic mobilities of modified adenosines in the partition solvent system used by Rogg *et al.*[56], we suggest the following decreasing order of polarity: t^6A, mt^6A, m^7G, m^1A, I, G, m^1I, m^1G, m^2G, A, m^2A, m^6A, m_2^2G, m_2^6A, i^6A, ms^2i^6A. The mobility order of m^1I and m^2G with respect to m^1G is uncertain. Most of the modified purine nucleosides are located in the wobble position and from the 3'-end to the anticodon (positions 40 and 43 of the generalized cloverleaf structure of Fig. 2). For modified pyrimidine nucleosides mainly located in positions 38 and 40, we propose the order: oac^5U, m^3C, ψ, C, m^5C, U, ac^4C, s^4U, mam^5s^2U, Cm, Um. The contribution of a positive charge carried by the methyl group in the nucleosides m^1A, m^3C and m^7G can be quantitated: $\log K = -0.70 \pm 0.02$ for purine and -0.54 for cytidine nucleoside in the PMB solvent system. For extreme polarities of purine nucleosides K values could change by a factor 30. Our series includes some modified residues (m^1A, m^3C, m_2^2G) present only in eukaryotic tRNA species.

For similar overall composition of tRNAs expressed as A/Y ratio, the increasing order of K values can be correlated with the variable polarity of the anticodon loop expressed as $\Delta d = \log K_1 - \log K_2$ ($K_1 > K_2$). The three *E. coli* tRNA species (Ala_{1A}, Gly_2 and Asp_1) with the lowest A/Y ratio ($29.4 \pm 0.8\%$) have anticodon loops with different polarities: a polar oac^5U nucleoside for $tRNA_{1A}^{Ala}$ and an unknown modified uridine for $tRNA_2^{Glu}$ in contrast to the non-polar loop of $tRNA_1^{Asp}$ containing a modified guanosine, the Q nucleoside having a 7-deazaguanosine nucleus with a cyclopentenediol side chain (Kasai *et al.*[57]), and a m^2A residue ($\Delta d = 0.32$ in both solvent systems).

For tRNA species having the composition $A/Y = 34.5 \pm 0.4\%$ (Val_{2A}, Gly_3, His_1, Glu_2 and Leu_1) one observes an analogous difference in partition coefficients ($\Delta d = 0.34$ in PMB and 0.45 in PFI systems).

The remarkable set of eight tRNA species with a close overall composition ($A/Y = 40.7 \pm 1.2\%$) is arranged in order of increasing mobility: $tRNA^{Lys}$, $tRNA_F^{Met}$, $tRNA_1^{Arg}$, $tRNA_3^{Ser}$, $tRNA_1^{Val}$, $tRNA_2^{Leu}$, $tRNA^{Phe}$ and $tRNA^{Trp}$ ($\Delta d = 0.90$ in PMB and 0.62 in PFI systems). Their mobility differences depend upon the differences in lipophilicity of their anticodon loops: anticodon loops with polar residues such as oac^5U in $tRNA_1^{Val}$ or t^6A in $tRNA^{Lys}$ and $tRNA_3^{Ser}$ and the lipophilic residue ms^2i^6A found in $tRNA^{Phe}$ and $tRNA^{Trp}$. The presence of an apolar 2'-O-methyl cytidine (Cm) in the anticodon loop of $tRNA^{Trp}$, in contrast to the relatively polar pseudouridine (ψ in position 38) in $tRNA^{Phe}$, might play a role in the higher mobility of $tRNA^{Trp}$. In addition, we may consider that $tRNA_2^{Leu}$ is 15% longer than $tRNA_1^{Arg}$ with comparable anticodon polarity. According to Brönsted relationship (Brönsted[58]), one may expect that an increase in molecular weight of a macromolecule induces an increase in the partition coefficient.

The mobility differences are much greater for the last five tRNA species: $A/Y = 47.1 \pm 2.9\%$ for $tRNA^{Thr}$, $tRNA^{Ile}$, $tRNA_M^{Met}$, $tRNA_1^{Ser}$ and $tRNA^{Tyr}$ ($\Delta d = 1.51$ in PMB and 0.94 in PFI systems). The lower K value of $tRNA_1^{Ser}$ results from the antagonistic effects of polar oac^5U and lipophilic ms^2i^6A. In the same way

we can explain the higher mobility of $tRNA_M^{Met}$ relative to $tRNA^{Ile}$ by substitution in the wobble position of a non-polar ac^4C with a guanosine. The peculiar mobility of $tRNA^{Thr}$ in both salt–solvent systems remains much more difficult to interpret.

DISCUSSION

In a given salt–solvent system S at the temperature T, the partition coefficient K is a function of five structural parameters: the nucleotide composition, the sequence, the molecular weight or the chain length, the helix content and the conformation.

Nucleotide composition. The nucleotide composition indicated by the ratio A/Y for nucleic acids of similar chain length. Our general formula does not take into account the particular contribution of modified nucleosides or their location in a region available to the solvent. As we have shown, the presence or absence of lipophilic or hydrophilic residues in the anticodon loop greatly changes the partition coefficient of tRNA species having close overall compositions. On the other hand, tRNAs with different compositions can have similar chromatographic mobilities if their anticodon loop polarities compensate the effect of the whole composition on the K value. An increasing lipophilicity of tRNA (increasing A/Y ratio) corresponds to an increasing polarity of the anticodon loop for the following couples: $tRNA_2^{Glu}$–$tRNA_3^{Ser}$, $tRNA_3^{Gly}$–$tRNA^{Lys}$, $tRNA_1^{Leu}$–$tRNA^{Ile}$ in the PMB system and $tRNA_F^{Met}$–$tRNA^{Thr}$ in the PFI system for intermediate K values; $tRNA^{Phe}$–$tRNA_t^{Ser}$ and $tRNA_3^{Leu}$–$tRNA_M^{Met}$ for high K values.

Sequence. Garel *et al.*[2] have shown that different sequences have no measurable effect on K, except when there is a non-random distribution of bases and clustering of purines or pyrimidines in an accessible region of the macromolecule (Garel and Mandel[1]). Tables III–V show that bases in *E. coli* tRNA species so far studied, including the bases in the anticodon arm, are randomly distributed.

Molecular weight or the chain length. According to the Brönsted relationship (Brönsted[58]), an increase in molecular weight brings about an increase in the partition coefficient for a similar nucleotide composition. tRNA species with higher chain length ($tRNA^{Leu}$, $tRNA^{Ser}$ and $tRNA^{Tyr}$) but with differing anticodon loop polarities are found among the most lipophilic.

Helix content. Double strandedness strongly increases the partition coefficient, as has been shown by Albertsson[61] with native and denatured DNA and various polynucleotides. Since the helix content of *E. coli* tRNA species varies only within very narrow limits ($54 \pm 3\%$), we may consider its contribution to tRNA mobility nearly identical for all species.

Conformation. It is known that in high ionic strength, the tertiary structure of tRNA is more compact (Fresco *et al.*[62]). Melting temperature or Tm values of various fractions, determined in presence of both high ionic strength (potassium phosphate buffer 2.6 M for the lower phase, 0.21 M for the upper phase in the PMB solvent system with 18.6% 2-butoxy ethanol at 15° according to Garel *et al.*[60]) and 2-methoxy ethanol, are much higher than in low ionic strength medium[63]. The presence of hydrophilic organic solvents and their interactions with nucleotides located at the surface of a tRNA macromolecule do not produce noticeable denaturation. Hanlon and Major[64] observed that the polyadenylic acid solubilized in slightly acidic polyethylene glycol maintains its secondary structure. Our results show that the main factor affecting

the chromatographic mobility of tRNA species is the polar or non-polar character of the anticodon loop. Our analysis indicates that this region remains accessible to the solvent in all tRNA species. This conformation in salt–solvent systems is consistent with that determined by X-ray crystallographic data[9,10].

When both the partition coefficient K value and overall composition A/Y ratio are determined for a tRNA of unknown sequence, its position on our semi-logarithmic diagram allows predictions for modified nucleosides in the anticodon loop, as suggested for $tRNA^{Ala}$ and $tRNA^{Tyr}$ species from silk gland of *Bombyx mori* L.[12].

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RELATIVE ELECTRON CAPTURE RESPONSE OF THE 2-CHLOROETHYL DERIVATIVES OF SOME BARBITURIC ACIDS AND ANTICONVULSANT DRUGS

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SUMMARY

Data are given for the relative electron capture response of the 2-chloroethyl derivatives of ten barbituric acids and five common anticonvulsant drugs. Results indicate that these new derivatives significantly improve the detection limits of the barbituric acids but lead to no advantages for the anticonvulsant compounds. Structural features in the two classes of compounds are believed to explain the differences in the sensitivity of detection. Linear calibration plots exist for the concentration ranges 0.1–1.0 and 1.0–10.0 μg/ml of amobarbital.

INTRODUCTION

Whereas barbiturate analyses by gas chromatography have been based almost exclusively on flame ionisation detection, study of the electron capture detection of barbiturates has been confined to some free barbituric acids[1], and more recently, their pentafluorobenzyl derivatives[2,3]. Yet, analytical methods based on the electron capture of the 2-chloroethyl esters of short-chain fatty acids[4] were investigated many years ago. More recently, analytical methods utilizing the 2-chloroethylation of the herbicides 2-methyl-4-chlorophenoxyacetic acid[5] and 2,4-dichlorophenoxyacetic acid[6], and bis(4-chlorophenyl) acetic acid, a metabolite[7] of DDT, have been reported.

Following the synthesis[8,9] of the 2-chloroethyl derivatives as a prerequisite to the quantification of barbiturates and some common anticonvulsant drugs, we report here the electron capture response of the 2-chloroethyl derivatives of ten barbituric acids and several ethyl and methyl derivatives, and their detection limits. Included also are the detection limits of the 2-chloroethyl derivatives of a number of common anticonvulsant drugs. All the derivatives were prepared by reaction of the barbituric acid or anticonvulsant drug with the appropriate dialkyl sulphate in a mildly alkaline medium rather than with the Claisen-type reaction employed for the pentafluorobenzyl[2,3], benzyl, allyl and methoxymethyl derivatives[10,11].

TABLE I

GAS CHROMATOGRAPHIC PROPERTIES OF BARBITURIC ACIDS AND THEIR N-ALKYLATED DERIVATIVES

R_1 and R_2 are substituents at C-5 of the pyrimidine ring.

Barbiturate	*Detection limit (ng)*						*Column temp. (°C)*	*Retention time (min)*
	Free acid	*Dimethyl*	*Monoethyl*	*Diethyl*	*Monochloroethyl*	*Bischloroethyl*		
Amobarbital	8.5						200	1.0
(R_1 = ethyl,		111					155	1.8
R_2 = 3-methylbutyl				136			155	2.4
						0.16	225	1.1
Pentobarbital	4.0						200	1.1
(R_1 = ethyl,		20					155	1.9
R_2 = 1-methylbutyl)				26			155	2.7
						0.10	225	1.2
Phenobarbital								
(R_1 = ethyl,		28					185	1.7
R_2 = phenyl)				14			185	2.0
						0.14	235	1.6
Mephobarbital	9.3						200	1.9
(R_1 = ethyl,			13				185	1.8
R_2 = phenyl)					0.36		205	2.1
Barbital								
(R_1 = ethyl,				115			155	1.1
R_2 = ethyl)						0.12	205	1.3
Secobarbital	0.07						200	1.9
(R_1 = 1-methylbutyl,				0.88			155	3.6
R_2 = prop-2-enyl)						0.12	225	1.3
Secbutobarbital								
(R_1 = ethyl,						0.07	205	1.9
R_2 = 1-methylpropyl)								
Butobarbital								
(R_1 = ethyl,						0.12	205	2.0
R_2 = butyl)								
Cyclobarbital	2.4						200	2.0
(R_1 = ethyl,				1.3			215	1.9
R_2 = cyclohex-1-enyl)						0.34	235	1.8
Hexobarbital	31						200	1.6
(R_1 = methyl,			36				185	1.8
R_2 = cyclohex-1-enyl)					1.9		205	1.9

EXPERIMENTAL

A Hewlett-Packard 5750 gas chromatograph was used with a 2-mC ^{63}Ni electron capture detector (ECD) operated at 240°. A coiled borosilicate glass column (1.63 m × 6.4 mm O.D.) packed with 3% SE-30 (w/w) on Chromosorb 750 (100–120 mesh) and re-silanized with hexamethyldisilazane prior to use, was employed throughout this work. The carrier gas [argon–methane (95:5)] flow-rate was maintained at 72–75 ml/min except for the 5,5′-diphenylhydantoin derivatives (136 ml/min). A pulsed voltage was applied to the detector (amplitude 30 V, period 50 μsec, width 0.75 μsec).

The ECD response was determined by injection of known quantities (1.5–2.5 μl) of pure derivatives[8,9] dissolved in hexane or ethyl acetate. Minimum detectable quantities in nanograms, based on a peak height signal of three times the background noise level, were determined by duplicate injections of the individual compounds. Retention times for the derivatives were adjusted to 1–2.5 min by alteration of the column temperature. In contrast, the free barbituric acids were chromatographed isothermally at 200° after saturation of the active sites in the column with repeated injections of the free acids. This procedure was adopted in order to obtain reproducible[12–15] data for the acids and to avoid the variation[1,16,17] in retention times caused by the adsorption of submicrogram amounts of barbituric acids on the column.

RESULTS AND DISCUSSION

Detection limits for the derivatives of the barbituric acids and the anticonvulsant compounds are given in Tables I and II respectively. To facilitate comparison, the detection limits of the free-acid forms of these compounds are also included. However, interpretation of the data for the free barbituric acids is complicated by their typical chromatographic behaviour of displaying considerable tailing on the silanized, non-polar column. Because of this characteristic, the detection limits for the free barbituric acids were approximated by measuring the areas of the distorted peaks and are expressed as the quantity of acid in nanograms per unit area (here, 50 mm^2). The free acids of the anticonvulsant drugs, with the exception of ethosuximide and diphenylhydantoin, gave reasonably symmetrical peaks so that detection limits could be obtained by the measurement of peak heights. All alkylated derivatives exhibited symmetrical chromatographic peaks and showed no evidence of adsorption.

Since an adequate understanding of the mechanism of electron capture is important if further reduction of detection limits is to be achieved, attempts to explain differences in response have endeavoured to identify the structural region of a molecule associated with the electron capture process. Thus, Landowne and Lipsky[18] proposed that the carbonyl carbon atom, and not the halogenated α-carbon atom of the introduced group, was responsible for the initial electron capture in a series of cholesteryl haloacetates. Clarke *et al.*[19] made a further important distinction between O-monochloroacetyl and N-monochloroacetyl derivatives to account for the reduced sensitivity of the amine derivatives towards electron capture. In the amine derivative, delocalization of the positive charge on the carbonyl carbon atom, through a resonance mechanism involving the lone-pair of the nitrogen atom, was seen as the influence opposing the effect of the particular halogenated group. Although it was recognised

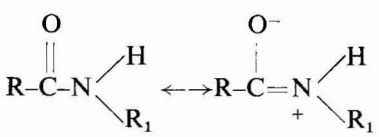
TABLE II

GAS CHROMATOGRAPHIC PROPERTIES OF ANTICONVULSANT COMPOUNDS AND THEIR N-ALKYLATED DERIVATIVES

Anticonvulsant	*Detection limit (ng)*				*Column temp. (°C)*	*Retention time (min)*
	Free acid	*Monoethyl*	*Mono-chloroethyl*	*Bis-chloroethyl*		
Peganone (3-ethyl-5-phenyl-imidazolidine-2,4-dione)	49				185	1.6
			55		185	2.6
Mephenytoin (5-ethyl-3-methyl-5-phenylimidazolidine-2,4-dione)	20				185	1.5
			51		185	3.2
Glutethimide (3-ethyl-3-phenyl-piperidine-2,6-dione)	27				185	1.8
		652			185	1.8
			86		205	2.1
Ethosuximide (3-ethyl-3-methyl-pyrrolidine-2,5-dione)	36				125	1.3
			71		155	0.9
Diphenylhydantoin (5,5-diphenylimida-zolidine-2,4-dione)		111*			235	1.2
			20**		235	1.5
				1.3	235	2.2

* Ethylated at N-3.
** Chloroethylated at N-3.

that electron capture could possibly occur in a polyhalogenated chain, such as in N-pentafluoropropionamide and N-heptafluorobutyramide derivatives of amines, the explanation was consistent even for the least sensitive N-trifluoroacetylated amine derivative because of the same counteractive mechanism. Again, a detailed study of various derivatives of primary and secondary amines by Matin and Rowland[20] supported the concept that an amide functionality, where the groups C=O and C=N were present simultaneously, provides the electron-deficient centre necessary for good ECD response. Furthermore, in the general representation:



it was shown that the greater the electron-withdrawing inductive effect of the substituent R_1, the greater was the polarizability of the carbonyl group and, consequently, its electron-capturing capacity.

Although it is a unique cyclic amide, a resonance mechanism such as that outlined above can be visualized as operating in the barbiturate nucleus also. The magnitude of the electron-deficiency of the electrophore would be influenced by the nature

of a substituent on one or both nitrogen atoms. Replacement of an imino hydrogen by an ethyl or methyl group could be expected to lead to a reduction in electron-deficiency at the nitrogen atom(s) due to an electron-releasing inductive effect of the alkyl groups. In this way, polarization of the adjacent carbonyl groups would be diminished and lead to reduced ECD response. Introduction of an electron-withdrawing group, such as a chloroethyl substituent, would reverse the effect and enhance the ECD response. The results presented in Table I lend support to this hypothesis, particularly for the derivative compounds. However, because of adsorption of the barbituric acids on the column, direct comparison of the detection limits of acids and derivatives may not be valid. Of interest too is the wide range of responses obtained for the methyl and ethyl derivatives in contrast to the chloroethylated barbiturate derivatives (with the exception of hexobarbital) which show a uniform and significantly higher ECD response falling within a relatively small range of detection limits. A similarly narrow (albeit, considerably lower) range of detection limits has been observed[2] for the pentafluorobenzyl derivatives. Both of these separate findings suggest that a limiting effect may be conferred upon the overall molecule by the electrophilic N-substituent.

As a final comment on the results in Table I, attention may be drawn to the paucity of evidence regarding the influence of the C-5 substituents. Gudzinowicz and Clark[1] suggested that the ability of a particular barbituric acid to capture electrons depends to some extent on the substituent and decreases in the order: phenyl > cyclohex-1-enyl > alkyl. Although the narrow range of detection limits does restrict interpretation of the results for the chloroethyl derivatives, taking the five barbituric acids differing only in one of the C-5 substituents (that is, amobarbital, barbital, cyclobarbital, pentobarbital and phenobarbital) and their methylated and ethylated derivatives, the response decreases in the order: cyclohex-1-enyl > phenyl, secondary alkyl > primary alkyl. The effect of the different C-5 substituents on the overall ECD response of the molecule cannot be explained however, and other factors[21] such as the stability of the resultant negative ion or electron capture by the products of a dissociative step may influence the ultimate ECD response. The unusually high response for secobarbital, and to a lesser extent its ethyl derivative, is probably due to the prop-2-enyl substituent, in agreement with the finding[22] that a doubly-bonded alkyl moiety makes a substantial contribution to the electron-capturing capability of a molecule.

When contrasted with the chloroethylated barbituric acids, only a weak ECD response was observed for the chloroethyl derivatives of the anticonvulsant compounds. This result is attributed to the different molecular structure comprising only two polarizable carbonyl groups, and with the exception of diphenylhydantoin, only one nitrogen available for alkylation. The polarizability of the carbonyl groups in these molecules is thus limited, in comparison with the barbiturates, by the reduced number of possible resonance forms. Moderate ECD response is seen only in the bis-(chloroethyl)derivative of diphenylhydantoin. It appears that the electron-releasing inductive effect of the ethyl group again contributes to the low response of the ethyl derivative of glutethimide relative to that of the parent compound and that of the N-3 ethylated diphenylhydantoin compared with its chloroethylated analogue.

The value of the chloroethyl derivatives can be seen in the linearity of the ECD response for the bis(chloroethyl)amobarbital derivative. A linear response was found for the range 0.1–1.0 μg/ml at a pulse period of 50 μsec (see Fig. 1), and has been demonstrated for the range 1.0–10.0 μg/ml when the pulse period was reduced to

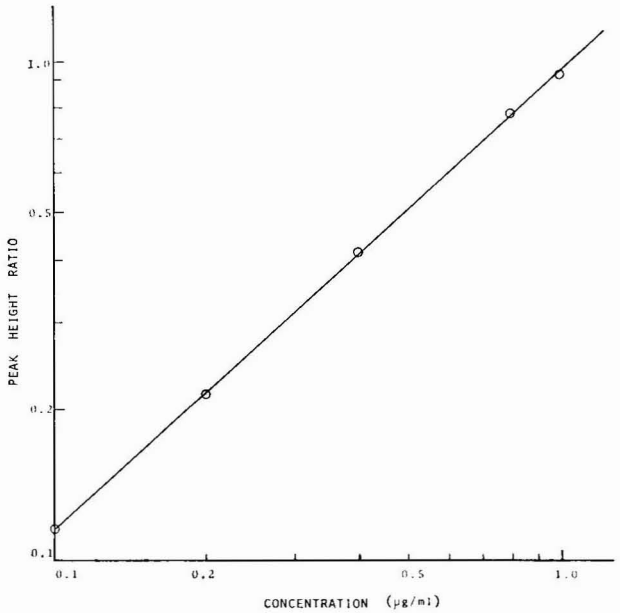


Fig. 1. Calibration curve for bis(chloroethyl)amobarbital using bis(chloroethyl)cyclobarbital (at 1.0 μg/ml) as internal standard. Each point on the curve is a mean value from two determinations.

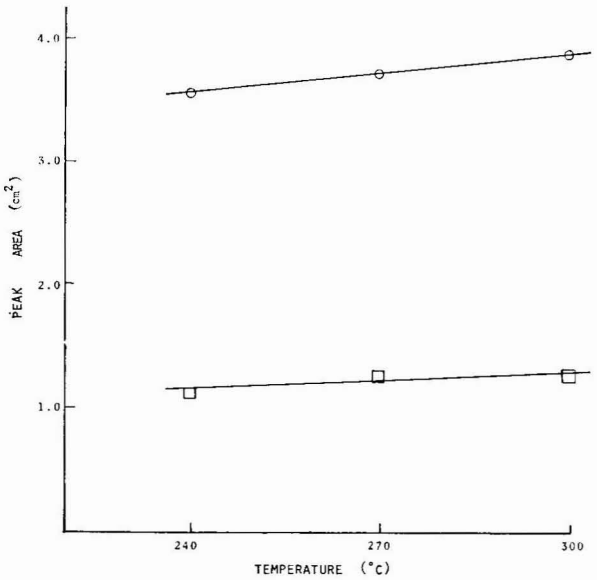


Fig. 2. The relationship between ECD response and detector temperature for bis(chloroethyl)secobarbital (○) and bis(chloroethyl)amobarbital (□). Each point on the curves is a mean value from two determinations at concentrations of 1 μg/ml. Column temperature, 225°; electrometer sensitivity, 128 × 1.

5 μsec. Although the electron affinity of many compounds has been shown[23] to be strongly dependent on the detector cell temperature, little variation in response is evident for the chloroethyl derivatives of amobarbital and secobarbital (see Fig. 2) when the cell temperature was varied between 240° and 300°.

It may be concluded that the 2-chloroethylation of barbituric acids leads to a significant improvement in their sensitivity to electron capture gas chromatography. However, with the exception of diphenylhydantoin, chloroethylation of the anticonvulsant compounds considered here confers no enhancement upon their response so that for these compounds the main advantage in the derivatives lies in possible qualitative applications. The development of a quantitative analytical scheme for the barbiturates is being explored. Currently, the near-complete conversion of the acid form of amobarbital at the 0.5 μg/ml level has been successful.

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RAPID MICRO-METHOD FOR THE MEASUREMENT OF ETHCHLORVYNOL IN BLOOD PLASMA AND IN URINE BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid gas–liquid chromatographic method has been developed for use in the measurement of the hypnotic drug ethchlorvynol in small (50 μl) volumes of either blood plasma or urine. Neither solvent transfer nor evaporation steps are used in the procedure and sources of interference have proved to be minimal. The method has been applied primarily to the analysis of specimens obtained from patients who had ingested an overdose of this drug. However with slight modification, the technique may be used in the measurement of the plasma concentrations of ethchlorvynol attained during therapy.

INTRODUCTION

Ethchlorvynol (1-chloro-3-ethyl-1-penten-4-yn-3-ol) is used as both a sedative and hypnotic agent. Overdosage with this drug is infrequent[1], but fatal cases of self-poisoning have been reported[2,3] and the treatment of such patients by either haemodialysis or charcoal haemoperfusion has been discussed[1,4,5].

The presence of ethchlorvynol in a urine specimen can be detected easily by means of the chromogenic reaction of this compound with diphenylamine sulphate[6,7]. However, even a urine sample obtained from a volunteer who had ingested a normal therapeutic dose of ethchlorvynol was found to yield a strongly positive reaction in this test[7]. Therefore, in order to establish that overdosage with this drug has occurred and also to assess the value of any treatment applied, an accurate, rapid and reproducible method for use in the measurement of plasma ethchlorvynol concentrations is required.

Some of the techniques already available have been discussed by Cravey and Jain[8]. The spectrophotometric methods, including that of Wallace *et al.*[9], require relatively large sample volumes, involve derivative formation and may measure metabolites in addition to the parent drug. A liquid chromatographic procedure has been advocated[10], but again the formation of a derivative is required. Indeed, the use of this technique in the measurement of ethchlorvynol is difficult to justify since the volatility, ease of detection, pH-independent solubility in organic solvents[9] and concentrations of this compound encountered in overdose facilitate a straight forward anal-

ysis by the more widely available technique of gas–liquid chromatography (GLC). The analysis of ethchlorvynol by this latter method has been described as "unrewarding"[9,10], but this applies only if solvent evaporation procedures are used following the extraction of the drug from the biological fluid since such procedures will often result in the simultaneous loss of ethchlorvynol. The GLC analysis of solvent extracts of plasma without prior concentration has been used for some time in the assay of ethchlorvynol[8,11,12], but large volumes (1–5 ml) of both sample and solvent, together with long extraction times, were employed. Furthermore, only one procedure which has been applied to the analysis of biological specimens incorporated an internal standard[11].

The method described in this paper is based upon the principle of rapid extraction of a small (50 μl) plasma volume with an equal volume of solvent containing an internal standard, followed by the direct GLC analysis of this extract. This principle has been applied previously to the plasma analysis of barbiturates and allied hypnotics[13,14], diazepam and desmethyldiazepam[15] and acetanilide[16]. The use of a sample–solvent ratio of 1:1 enables the measurement of ethchlorvynol concentrations down to 2 mg/l. However, as shown for both diazepam and desmethyldiazepam[15], an increase in this ratio to 4:1 yields a concomitant increase in the sensitivity of the method. Thus the plasma concentrations of ethchlorvynol reported after therapeutic dosage can be measured without resort to electron capture detection[17]. In addition, the method described here has been shown to be applicable to the analysis of ethchlorvynol in urine specimens.

EXPERIMENTAL

Gas–liquid chromatography

A Pye 104 Model 24 dual-column gas chromatograph fitted with flame ionisation detectors was used throughout. The column and detector oven temperatures were 140° and 200°, respectively, and the injection port setting was 2. The carrier gas (nitrogen) flow-rate was 60 ml/min and the hydrogen and oxygen inlet pressures were 15 and 10 p.s.i., respectively, giving flow-rates of approximately 45 and 200 ml/min.

The column, a coiled glass tube 1.5 m × 4 mm I.D., was treated with a solution of 5% dichlorodimethylsilane in toluene for 1 h. It was then washed with methanol, dried at 100° and subsequently packed with 2% (w/w) Carbowax 20M (Field Instruments, Richmond, Great Britain) and 5% (w/w) KOH on HP Chromosorb W (80–100 mesh). The column packing was prepared using the rotating evaporator technique, the KOH loading being obtained initially by the evaporation of a methanolic solution of this compound in the presence of the support. The final loading was then obtained using this pretreated material and a solution of Carbowax 20M in chloroform. The packed column was conditioned at 200° with a nitrogen flow-rate of 60 ml/min for approximately 12 h prior to use.

On this system, ethchlorvynol had a retention time of 0.69 relative to the internal standard, 2-methylnaphthalene. The chromatography of a chloroform solution of both ethchlorvynol and 2-methylnaphthalene is illustrated in Fig. 1.

Extraction procedure

The extraction solvent was a 25 mg/l solution of 2-methylnaphthalene (Hopkin

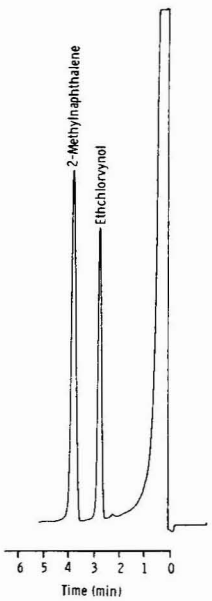

Fig. 1. The analysis of a chloroform solution containing ethchlorvynol (30 mg/l) and 2-methylnaphthalene (25 mg/l) on the Carbowax 20M–KOH column system. Injection, 3 μl.

& Williams, Chadwell Heath, Great Britain) in chloroform (analytical reagent grade). Ethchlorvynol was obtained as a light yellow oil from Abbott Labs., Queenborough, Great Britain; such material is said to be greater than 98% pure[18].

Plasma or urine (50 μl) was introduced into a Dreyer tube (Poulten, Selfe & Lee, Wickford, Great Britain) by means of a 50-μl semi-automatic pipette. Subsequently, 50 μl of the internal standard solution were added via a 2.5-ml Hamilton gas-tight luer-fitting glass syringe fitted with a Hamilton repeating mechanism (both available from Field Instruments, Richmond, Great Britain). An Everett stainless-steel needle (No. II serum) was affixed to this syringe.

The contents of the tube were mixed thoroughly on a vortex mixer for 30 sec and the tube was centrifuged for 2–3 min at approximately 2750 *g*. A 3–5-μl portion of the chloroform phase was subsequently obtained by (1) drawing 5 μl of air into a gas chromatographic syringe, (2) passing the syringe needle through the lipoprotein interface into the chloroform phase, (3) expelling the air and drawing-up the required volume of chloroform and (4) withdrawing the syringe and wiping the needle with a tissue. This extract was then injected onto the column of the gas chromatograph.

The extraction was performed in duplicate and a mean result obtained; if the difference between the duplicates was greater than 10% both the extractions and analysis were repeated.

Instrument calibration and calculation of results

Standard solutions containing ethchlorvynol at concentrations of from 10 to 100 mg/l in increments of 10 mg/l were prepared in chloroform by dilution of a 1-g/l

stock solution in this same solvent. Each standard also contained 2-methylnaphthalene at a concentration of 25 mg/l, obtained from a separate stock source. The ratio of the peak height of ethchlorvynol to the peak height of 2-methylnaphthalene bore a linear relationship to the ethchlorvynol concentration over the range studied; the normal calibration gradient obtained (*i.e.*, peak height ratio/drug concentration) was 0.028 l/mg. The results of plasma analyses were multiplied by a "recovery factor" of 1.08 to compensate for the incomplete extraction of the drug; the factor of 1.03 was used for urine analyses.

RESULTS AND DISCUSSION

Recovery studies

Standard ethchlorvynol solutions were prepared in 10.0 ml of either heparinised bovine plasma or drug-free human urine by dilution of from 50 to 250 μl of a 2-g/l solution of the drug in ethanol; the range of concentrations thus obtained was from 10 to 50 mg/l, in increments of 10 mg/l. The triplicate analysis of the plasma solutions by the present method revealed a mean ethchlorvynol recovery of 94 $\pm$ 3% (S.D.); this recovery was uniform over the range studied. The duplicate analysis of the urine standards revealed a similar uniform recovery of 97 $\pm$ 2% (S.D.).

Specificity

No interference from either endogenous sample constituents or other drugs has been encountered and extract contamination from other sources has not been found to occur. However, some possible sources of non-drug interference in direct-extract analyses have been discussed[14]. The analysis of a chloroform extract of drug-free human plasma is illustrated in Fig. 2. The chromatography of similar extracts performed in the absence of the internal standard has not revealed the presence of compounds which could elute with 2-methylnaphthalene. The analysis of a chloroform extract of plasma obtained from a patient who had ingested an overdose of ethchlorvynol is illustrated in Fig. 3. Entirely analogous results have been obtained on the analysis of urine specimens.

Two compounds which were found both to extract from plasma and to elute on the column system under the conditions of this assay are trichloroethanol and chlormethiazole. The retention times of these drugs were, respectively, 0.52 and 1.28 relative to 2-methylnaphthalene and thus they did not interfere in the analyses; the chromatography of a chloroform solution containing all of these compounds is illustrated in Fig. 4.

Alternative column systems

Although the Carbowax 20M–KOH column system has proved the system of choice for use in this assay (*cf.*, Figs. 1–4), additional systems were investigated. One was a 0.9-m coiled glass tube packed with 3% (w/w) Poly A 103 (Field Instruments) on HP Chromosorb W (80–100 mesh). The GLC conditions were identical to those described for the Carbowax 20M–KOH system, except that the column oven temperature was 120°. Ethchlorvynol had a retention time of 0.54 relative to 2-methylnaphthalene on this system and the calibration gradient obtained was 0.031 l/mg. Since this same column system formed an integral part of the procedure routinely used

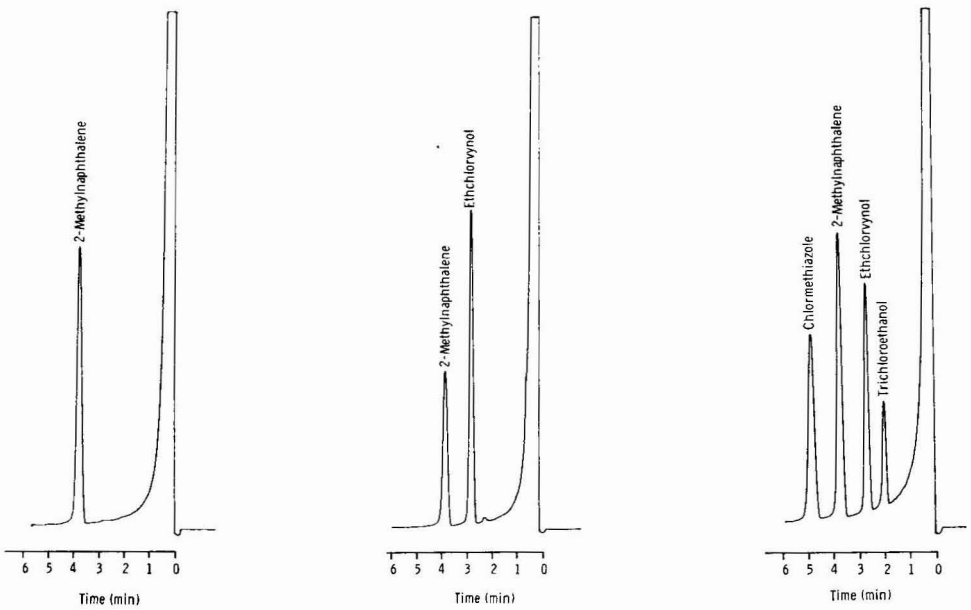

Fig. 2. The analysis of an extract of drug-free human plasma on the Carbowax 20M–KOH column system. The concentration of 2-methylnaphthalene was 25 mg/l; 3-μl injection.

Fig. 3. The analysis of an extract of plasma obtained from an ethchlorvynol overdose patient on the Carbowax 20M–KOH column system; 3-μl injection. The plasma ethchlorvynol concentration was found to be 78 mg/l.

Fig. 4. The analysis of a chloroform solution containing ethchlorvynol (30 mg/l), 2-methylnaphthalene (25 mg/l), trichloroethanol and chlormethiazole (both 50 mg/l) on the Carbowax 20M–KOH column system; 3-μl injection.

in our laboratory for the analysis of barbiturates and other hypnotics[14], a chromatograph containing such a system was always available, and was used in some emergency ethchlorvynol analyses. However, trichloroethanol was found to co-chromatograph with ethchlorvynol on this system and thus could interfere; chlormethiazole had a retention time of 0.85 relative to 2-methylnaphthalene and was resolved from the compounds of interest.

Columns based upon the stationary phase CDMS have proved less satisfactory than the systems discussed above since the ethchlorvynol peak was found to "tail". Non-polar phases, such as OV-1, were found to elute ethchlorvynol much more rapidly than 2-methylnaphthalene, *e.g.*, the relative retention time of ethchlorvynol on a 2.1-m 3% OV-1 system was 0.19, and thus a different internal standard would have been required if such columns had been used in the assay. Conversely, ethchlorvynol was found to have a retention time of 0.83 relative to 2-methylnaphthalene upon a 2.1-m 3% Carbowax 20M column system. Therefore, the Carbowax 20M–KOH system was preferred since the resolution of ethchlorvynol from the internal standard was greater. However, the retention times relative to 2-methylnaphthalene of trichloroethanol and chlormethiazole on the Carbowax 20M system were 0.55 and 1.55, respectively, and thus these compounds would not interefere in analyses performed using this sytem.

Limits of sensitivity

The use of a 1:1 sample–solvent ratio in the extraction procedure resulted in a limit of sensitivity of the technique to ethchlorvynol of 2 mg/l. However, it was found that by the use of a sample–solvent ratio of 4:1, plasma drug concentrations down to 0.5 mg/l could be measured easily. The formation of emulsions during the extraction was found to present no more of a problem when using this high sample–solvent ratio than in the normal extraction procedure; similar findings have been reported when using *n*-butyl acetate as an extraction solvent[15].

To illustrate this point, standard ethchlorvynol solutions in the range 2.0–10.0 mg/l were prepared in chloroform, in increments of 2.0 mg/l; each solution also contained 5.0 mg/l of 2-methylnaphthalene. The calibration gradient given upon analysis of these solutions was 0.148 l/mg. A further set of solutions containing from 0.5 to 2.0 mg/l of ethchlorvynol in increments of 0.5 mg/l were prepared in heparinised bovine plasma; the triplicate analysis of these solutions using 200 μl of plasma and 50 μl of internal standard solution (5 mg/l 2-methylnaphthalene in chloroform) revealed a mean ethchlorvynol recovery of 77 $\pm$ 2% (S.D.).

Applicability to urine analyses

Although the present method was designed for use with plasma specimens, it has been found to be equally applicable to urine; the qualitative analysis of urine specimens by the plasma hypnotic assay[13,14] has given similar results (R. J. Flanagan, unpublished results).

The measurement of urinary ethchlorvynol concentrations could prove of use in the measurement of the severity of overdosage if a plasma specimen is not available. As has been emphasised, the diphenylamine sulphate test[7] is not suitable for this purpose. The analysis of a urine specimen which gave a strongly positive reaction in this test following the ingestion of 500 mg of the drug[7] revealed an ethchlorvynol concentration of only 2 mg/l. In contrast, the urinary concentrations measured in six "spot" specimens which were obtained at approximately the same time as plasma samples from ethchlorvynol overdose patients were found to be from 34 to 63% of the plasma values; the lowest urinary concentration observed in these samples, the results from which are summarised in Table I, was 17 mg/l. Thus, although inevitably subject to a large degree of variability when compared to plasma concentrations, the measurement of urinary ethchlorvynol concentrations is likely to give more infor-

TABLE I

THE PLASMA AND URINARY CONCENTRATIONS OF ETHCHLORVYNOL MEASURED IN SPECIMENS FROM OVERDOSE PATIENTS

Specimen No.	*Ethchlorvynol (mg/l)*		*Urine–plasma ratio (%)*
	Plasma	*Urine*	
1	47	18	38
2	45	19	42
3	27	17	63
4	44	25	57
5	38	21	55
6	50	17	34

mation than that available from the diphenylamine sulphate test, which takes almost as long to complete. It should be noted that no advantage would be gained from an attempt to relate urinary ethchlorvynol and creatinine concentrations; the utility of this type of measurement has been both generally criticised[19] and shown not to be of use in the measurement of the urinary excretion of either α-amino nitrogen[19] or D-glucarate (R. J. Flanagan, unpublished results).

CONCLUSIONS

The results described in this paper provide further evidence of the advantages of micro-extraction techniques over bulk-extraction methods in drug analysis. Such considerations, *i.e.* the small sample requirement, speed of performance and enhanced accuracy and reproducibility, together with the wide applicability of these techniques, have been discussed in detail elsewhere[14,15].

The procedure described in the present paper represents an improvement over previously published methods for the measurement of both plasma and urinary ethchlorvynol concentrations and has been in routine use for over three years. The duplicate analysis of sample extracts can be accomplished within 15 min and both the qualitative and the quantitative measurements are performed by reference to an internal standard. The investigation of potential sources of interference in this assay has shown not only that both trichloroethanol and chlormethiazole do not interfere in this procedure, but also that this same technique may be of use in the simultaneous measurement of these drugs at the plasma concentrations attained in overdose.

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GAS CHROMATOGRAPHIC AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF AMPICILLIN*

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(Received December 21st, 1976)

SUMMARY

A method was developed for the quantitative gas chromatographic (GC) determination of ampicillin. The procedure requires silylation with hexamethyldisilazane, trimethylchlorosilane, trimethylsilylimidazole and N,O-bis(trimethylsilyl)acetamide in pyridine and subsequent GC on an OV-17 column, using 5α-cholestane as an internal standard. This method was applied to the determination of ampicillin in some pharmaceutical products. The characteristics of the mass spectra and the derivatization GC of ampicillin are also discussed.

INTRODUCTION

Numerous approaches have been described for the analysis of ampicillin. Among these, chemical[1–5], spectrophotometric[6–18] thin-layer and column chromatographic[19–23], microbiological[24–27], and high-performance liquid chromatographic[28,29] methods have been applied to the determination of ampicillin in pharmaceutical preparations and biological fluids.

Several gas chromatographic (GC) methods for the analysis of β-lactam antibiotics[30–33] have been established. However, a literature survey indicated that there was no GC method for the determination of ampicillin. In this work, an attempt was made to determine ampicillin by GC, and the method developed was shown to be sensitive and selective for its separation and quantitation.

EXPERIMENTAL

Chromatographic conditions

A Shimadzu GC-6A gas chromatograph equipped with a flame-ionization detector and a 2 m × 3 mm I.D. glass column containing 1.5% (w/w) OV-17 on Chromosorb W AW DMCS, 60–80 mesh, was used. The injection port and detector were kept at 270° .The column temperature was maintained at 230° for 5 min after

* This work was presented in part at the 96th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1976.

injection and then programmed at 4°/min to 270°, at which temperature it was kept for 5 min. Helium was used as the carrier gas at a flow-rate of 115 ml/min. An injection of 20–30 μl of monotrimethylsilylated sample solution was made before each day's run in order to minimize column adsorption. Determinations were made by plotting the peak-height ratio against weight ratio of ampicillin and 5α-cholestane.

Mass spectrometric conditions

A Hitachi RMU-6 mass spectrometer was combined with a gas chromatograph (Hitachi K-053). The following operating condition were used: separator temperature, 290°; ionization source temperature, 270°; ionization energy, 70 eV; acceleration voltage, 3.5 kV; and trap current, 60 μA.

Chemicals and reagents

Ampicillin, sodium ampicillin and α-aminobenzylpenicilloic acid were gifts from Shionogi Co. (Osaka, Japan), Meiji Co. (Tokyo, Japan) and Bristol Labs. (Syracuse, N.Y., U.S.A.), respectively. 5α-Cholestane, benzene, pyridine, acetonitrile and absolute methanol were of reagent grade. Phenylmethylsilicone (OV-17, Nishio Kogyo Co., Tokyo, Japan), hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS) (Wako, Osaka, Japan), trimethylsilylimidazole (TMSI) and N,O-bis(trimethylsilyl)acetamide (BSA) (Ohio Valley, Marietta, Ohio, U.S.A.) were used without further treatment.

Internal standard solution

Weigh accurately about 10 mg of 5α-cholestane into a 20-ml volumetric flask, and dissolve it in and dilute to volume with benzene.

Reference standard solution

Four reference standard solutions were prepared by dissolving accurately weighed amounts of about (1) 20 mg of ampicillin, (2) 20 mg of ampicillin plus 5% of α-aminobenzylpenicilloic acid, (3) 20 mg of sodium ampicillin and (4) 20 mg of sodium ampicillin plus 5% of α-aminobenzylpenicilloic acid in absolute methanol in a series of 20-ml volumetric flasks, and diluted to volume with absolute methanol.

Calibration graph

Transfer aliquots from 0.5 to 3.0 ml of each reference standard solution into successive glass-stoppered flasks. Add 0.4 ml of the internal standard solution to each flask, evaporate just to dryness at 35 $\pm$ 2° with a rotary evaporator and submit the residue to vacuum desiccation over P_2O_5 overnight. Construct calibration graphs by plotting the weight ratios of ampicillin to 5α-cholestane on the abscissa against their peak-height ratios on the ordinate.

Sample preparation

Capsule. Weigh accurately each of the capsule contents and transfer a suitable amount, equivalent to about 20 mg of ampicillin, into a 20-ml flask, mix well and dilute to volume with absolute methanol.

Vial. Transfer quantitatively the contents of the vial into a 100-ml volumetric flask and dilute to volume with absolute methanol. Pipette an aliquot equivalent to

about 20 mg of sodium ampicillin into a 20-ml volumetric flask and dilute to volume with absolute methanol. Proceed as described under *Calibration graph.*

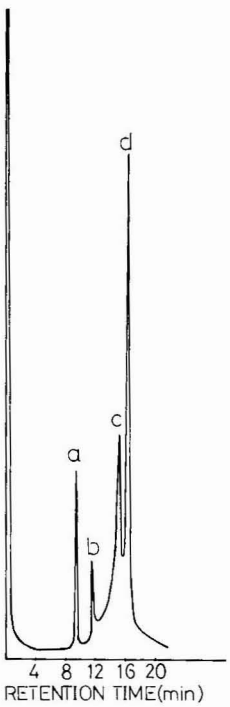
Silylation procedure

To each dried sample, add 0.3 ml of pyridine, 0.3 ml of HMDS, 0.3 ml of TMCS, 0.1 ml of TMSI and 0.05 ml of BSA. The reaction mixture is allowed to stand for 2 h at room temperature before injection of 0.6–1.6 μl of the silylated solution into the gas chromatograph.

RESULTS AND DISCUSSION

Gas chromatography

Several combinations of silylating agents were tried for the derivatization of amplicillin at room temperature. The silylation of ampicillin with pyridine—BSA (0.2 +0.2 ml) resulted in the formation of multiple peaks, as shown in Fig. 1(b,c,d). The same phenomenon also occurred on using acetonitrile–BSA (0.2 + 0.2 ml). The peak-height ratios of these triple peaks changed continuously for 5 hours, so that these conditions were therefore unsuitable for the determination of ampicillin.



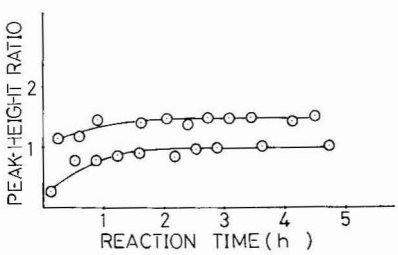


Fig. 1. Gas chromatogram of mixtures of silylated ampicillin. (a) 5α-Cholestane (internal standard); (b) tris-TMS-ampicillin; (c) bis-TMS-ampicillin; (d) mono-TMS-ampicillin. Conditions: 1 m × 3 mm I.D. column containing 1.5% (w/w) OV-17; column temperature, 190° (5 min) then programmed at 5°/min to 250° held at 250° for 5 min; carrier gas, helium at a flow-rate of 65 ml/min. These conditions were employed only to characterize the TMS derivatives.

Fig. 2. Effect of reaction time on formation of mono-TMS-ampicillin. Peak-height ratios of ampicillin (above) and sodium ampicillin (below) to 5α-cholestane (internal standard).

Pyridine–HMDS–TMCS (0.3 + 0.3 + 0.3 ml) was then tried as the silylation species, but no peak was obtained after 3 h. The use of pyridine–HMDS–TMCS–TMSI (0.3 + 0.3 + 0.3 + 0.05 ml) as silylating agent resulted in poor derivatization, even though a single peak was obtained. Silylation of ampicillin with pyridine–HMDS–TMSI (0.3 + 0.5 + 0.05 ml) gave good results with a single peak, but sodium ampicillin was not derivatized, possibly owing to the charge on the carboxyl group, which Shaw[34] considered to resist silylation.

For derivatization of both ampicillin and sodium ampicillin under the same conditions, silylation with pyridine–HMDS–TMCS–TMSI–BSA (0.3 + 0.3 + 0.3 + 0.1 + 0.05 ml) gave satisfactory results. Single peaks were obtained for both ampicillin and sodium ampicillin, as shown in Fig. 3(d). The relationship between reaction time and peak-height ratio is indicated in Fig. 2. The peak-height ratio of ampicillin or sodium ampicillin to 5α-cholestane became constant after 2 h. Therefore, the GC determination was carried out 2 h after silylation.

α-Aminobenzylpenicilloic acid, one of the degradation or metabolic products of ampicillin, was derivatized under the same conditions and gave double peaks, as shown in Fig. 3(b,c), completely separated from the ampicillin peak. Hence this substance does not interfere in the determination of ampicillin.

The calibration graphs for ampicillin and sodium ampicillin were linear over the range 0.5–3.0 mg, as shown in Figs. 4 and 5. The results in Table I indicate good precision.

Further applications to other pharmaceutical products should be possible.

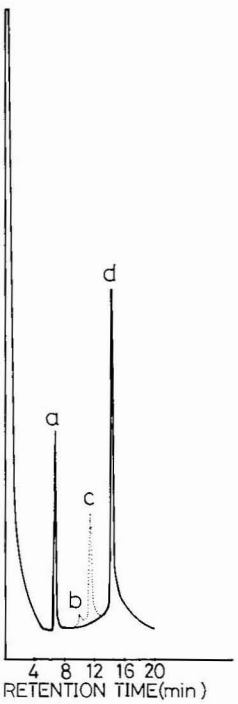


Fig. 3. Composite gas chromatogram of (a) 5α-cholestane and (b and c) α-aminobenzylpenicilloic acid, with unknown characteristic (d) mono-TMS-ampicillin.

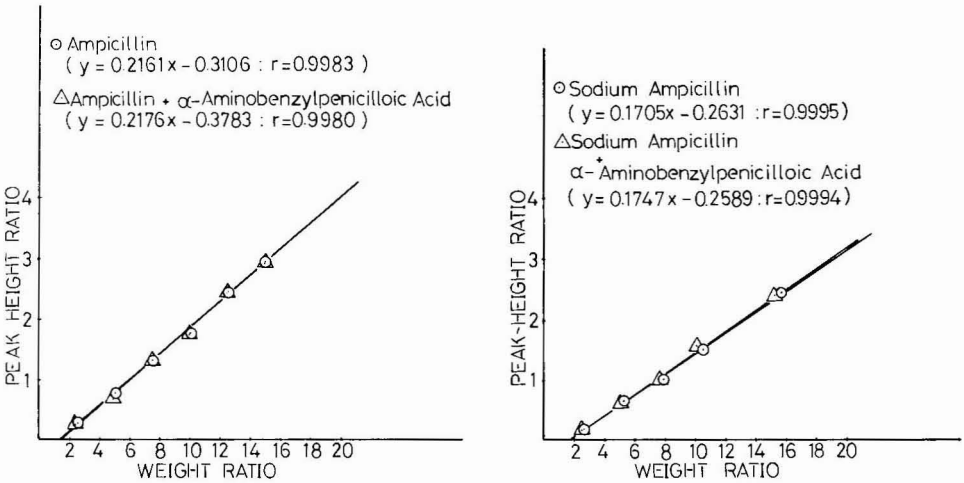


Fig. 4. Calibration graphs for ampicillin (○) and ampicillin plus 5.0% of α-aminobenzylpenicilloic acid (△).

Fig. 5. Calibration graphs for sodium ampicillin (○) and sodium ampicillin plus 5.5% of α-aminobenzylpenicilloic acid (△).

TABLE I

RESULTS OF ASSAY OF AMPICILLIN PRODUCTS

Product	*Label claim** (*mg*)	*Batch*	*Found*** (*mg*)	*Recovery* (%)
Capsule: 1	250	A	245.37	98.15
2	250	A	241.45	96.58
3	250	A	242.60	97.04
4	250	A	244.18	97.67
5	250	B	243.77	97.51
Vial: 1	250	A	250.66	100.26
2	250	A	241.83	96.73
3	500	B	480.71	96.14

* The ampicillin content is expressed in units of potency.
** Average of duplicate determinations calculated as anhydrous ampicillin.

Mass spectrometry (MS)

GC–MS was performed in order to elucidate the peak characteristics of ampicillin under different silylation conditions. The mass spectra B, C and D in Fig. 6 correspond to peaks b, c and d, respectively, in Fig. 1, for ampicillin silylated with pyridine–BSA (0.2 + 0.2 ml).

Spectrum B contains a molecular ion at *m/e* 565 (M), an M$-CH_3$ ion at *m/e* 550, and decomposition of the parent ion by elimination of trimethylsilanol led to a peak of *m/e* 475 [M$-(CH_3)_3SiOH$], which was further degraded by cleavage of the methyl group to give a peak of *m/e* 460. Scission of the β-lactam ring gave an ion at *m/e* 232 after gaining one hydrogen atom and another ion at *m/e* 334. The ion at *m/e* 232 is assumed to be due to the mono-TMS fragment silylated at the carboxyl

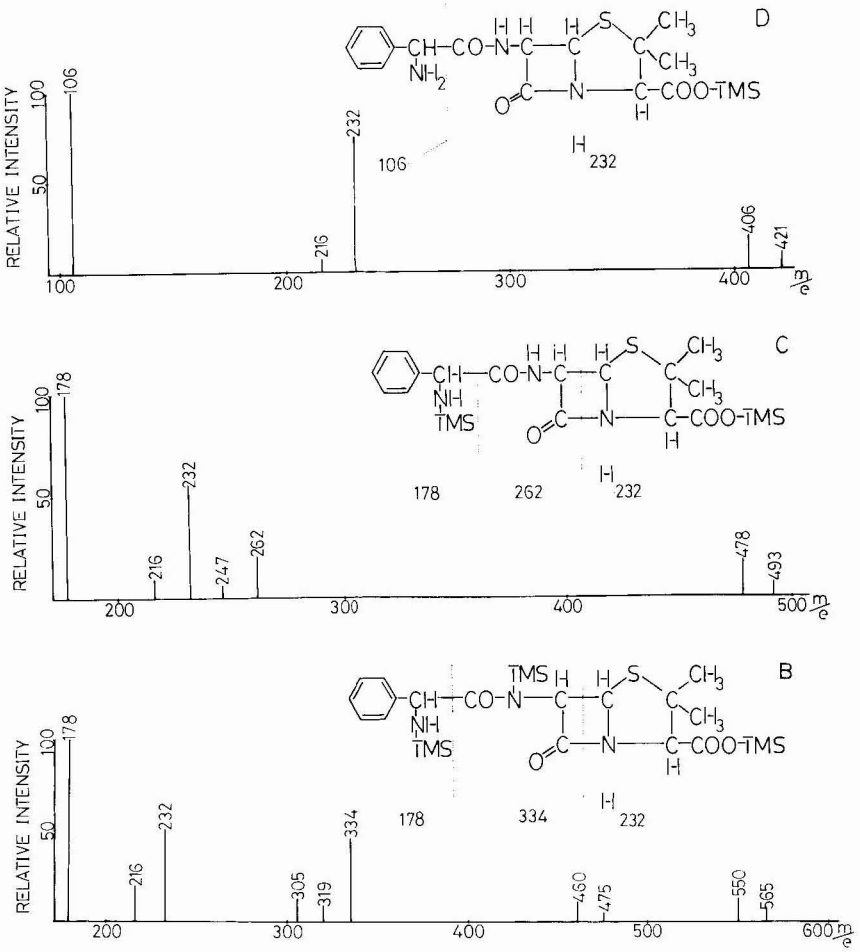


Fig. 6. Mass spectra of mono-TMS-ampicillin (above), bis-TMS-ampicillin (centre) and tris-TMS-ampicillin (below), corresponding to peaks d, c and b, respectively, in Fig. 1.

function, and the ion at *m/e* 334 is assumed to be the bis-TMS fragment silylated at the amide and amino functions. Cleavage between the benzyl and amide carbon atoms is thought to induce the ion at *m/e* 178, probably due to the N-trimethylsilyl-aminobenzylimmonium fragment.

In spectrum C, the molecular ion occurs at *m/e* 493 (M), elimination of the methyl group gave a peak of *m/e* 478 (M$-CH_3$), β-lactam scission evolved an ion at *m/e* 232 (the same as that in spectrum B) and an ion peak at *m/e* 262 represents the mono-TMS fragment. An ion peak appeared at *m/e* 178, as in spectrum B.

In spectrum D, the ion at *m/e* 421 (M) corresponds to the parent ion, removal of a methyl group led to *m/e* 406 (M$-CH_3$), β-lactam cleavage gave the ion at *m/e* 232 common to spectra B and C, and an ion at *m/e* 106 represents the aminobenzylimmonium fragment.

The above ion peak profile leads to the conclusion that the triple peaks b, c and

d shown in Fig. 1 represent tris-TMS-ampicillin, bis-TMS-ampicillin and mono-TMS-ampicillin, respectively, rather than the degradation products of ampicillin under the silylation conditions used. On the other hand, peak d found in Fig. 3, obtained by silylation of ampicillin with pyridine–HMDS–TMCS–TMSI–BSA (0.3 + 0.3 + 0.3 + 0.1 + 0.05 ml) has a mass spectrum identical with spectrum D in Fig. 6, indicating that peak d is mono-TMS-ampicillin. The peak characteristic of α-aminobenzylpenicilloic acid as shown in Fig. 3(b,c) was monitored by GC–MS, but no definite conclusion could be drawn.

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SEPARATION AND DETECTION OF PHENCYCLIDINE IN URINE BY GAS CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and selective analytical procedure for phencyclidine and one of its metabolites in urine has been developed. Three techniques have been studied for extraction of the drug from the biological matrix: (*a*) reversed-phase XAD resin, (*b*) charcoal absorption, and (*c*) solvent extraction using chloroform. Temperature-programmed gas chromatography was used to quantitate the illicit drug. Solvent extraction appears to offer the most efficient separation of the drug and its metabolite, as the recovery was 94% and the technique required only 7–8 min per sample. Reversed-phase column extraction is also quite useful; although more time-consuming for an individual sample, it would be useful for screening purposes.

INTRODUCTION

Phencyclidine, 1-(1-phenylcyclohexyl)piperidine (PCP) was developed as an anaesthetic in the 1950s and marketed as Sernyl in 1958. Although the drug appeared to be useful in certain types of surgery[1–8] and as a possible remedy for some psychological disorders[9], its use was limited by observations that some patients exhibited severe degrees of manic behaviour[1], hallucinations and delirium[2] after its administration. It was noted that the psychotic reaction was most frequently encountered in young and middle-aged males, the most violent forms following 20-mg doses, and that the excitation was not noted in elderly patients[2]. As a result of these adverse reactions to PCP, the drug was not approved for general usage, and is now used only for animals.

In recent years, PCP has become a serious drug of abuse, often being sold under such names as LBJ, Angel Dust and the Peace Pill[10]. Several instances of the use of PCP have led to overdose intoxication and death, and PCP is frequently sold in combination with (or in place of) other illicit drugs such as mescaline, LSD, THC and amphetamines[11–13]. The common use of PCP may be related to the relative ease

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of preparation[14] of the drug; to exemplify this point, the Royal Canadian Mounted Police seized more than 100 kg of the drug and 900 kg of precursors from five underground laboratories in Canada during the first six months of 1976 (see ref. 15).

The serious consequences of ingestion of PCP would suggest that an analytical procedure for determining the drug in body fluids would be of great value for emergency-room personnel; since the victim may be unaware that the abused drug is PCP, such an analytical procedure would be even more useful. To date, several procedures (all based on gas chromatography) have been reported for determining the drug in dosage forms. Lin *et al.*[16] have developed an analytical procedure for determining the drug in urine or blood by gas chromatography–chemical ionization mass spectrometry and a recent report indicates that the drug may be quantified by gas chromatography after extraction from urine[17].

We report here the development of a rapid analytical procedure for determining PCP and one of its metabolites in urine. Urine was chosen because the levels therein are notably higher than those in blood or serum. The fact that a metabolite, 1-(1-phenylcyclohexyl)piperidin-4-ol (PCP·OH) is readily observable should provide useful confirmation of the presence of PCP. An additional advantage of the detection of the metabolite is the possibility of determining the presence of the drug in those cases where much of it has already been metabolized.

EXPERIMENTAL

Chemicals

Phencyclidine (Lot CI-395, Parke Davis & Co., Brockville, Canada), ketamine hydrochloride (Lot C430738, Parke Davis & Co.) 1-(1-phenylcyclohexyl)piperidin-4-ol (Lot Q, Parke Davis & Co., Detroit, Mich., U.S.A.), caffeine and 1-phenylcyclohexene (Aldrich, Milwaukee, Wisc., U.S.A.) were used as received.

All solvents used were Fisher certified-reagent grade (Fisher Scientific, Pittsburgh, Pa., U.S.A.). Norit A (Fisher) was used as a decolorizing charcoal. Amberlite resins were obtained from Mallinkrodt [St. Louis, Mo., U.S.A. (XAD-2]) and BDH [Toronto, Canada (XAD-4 and XAD-7)]. Pre-packaged XAD-2 columns were obtained from a Drug-Skreen Kit (Brinkman Instruments, Rexdale, Canada).

Urine was pooled from healthy individuals and stored at 4° for periods up to 7 days.

Ketamine hydrochloride (KET·HCl) was used as internal standard.

Drug extraction from urine

Using a macro-reticular resin. Resins were cleaned by stirring four times with four bed-volumes of acetone, three times with three bed-volumes of methanol and three times with three bed-volumes of distilled water as previously recommended[18,19]. The resins were stored for at least a week at 4° under distilled water to ensure optimum hydration and then packed into glass chromatography columns (25 × 0.7 cm I.D.) to a height of 10 cm (approximately 1.3 g of dry resin).

A 100-μl sample of PCP and internal standard was mixed with 10 ml of urine, the pH was adjusted to 9 with 5 *N* sodium hydroxide, and the sample was mixed and centrifuged for 2 min. The supernatant was then placed on the XAD column and the flow was controlled; when the flow ceased, suction was applied to the tip to remove

residual urine. The column was then eluted with 20 ml of methanol–chloroform (1:9), the eluate was mixed with 2 ml of 0.5 *N* sodium hydroxide (back-extraction), and the separated organic layer was made acidic with 1 drop of concentrated hydrochloric acid–methanol (1:1) and evaporated to dryness under nitrogen in a water bath at 55°. The residue from the methanol–chloroform extraction was reconstituted in 100 μl of acetone, and 1 μl was injected into the chromatograph.

Using a universal absorbent (charcoal). Approximately 100 mg of charcoal were mixed with 1 ml of buffer solution (0.4 *M* Na_2CO_3 and 0.1 *M* $NaHCO_3$, pH 11) to ensure that it was completely wetted; 10 ml of distilled water were added to the residue. The charcoal–water mixture was shaken vigorously and centrifuged, and the aqueous layer was discarded. Then 2.5 ml of methanol–chloroform (1:9) were added, and the sample was placed on a vortex-type mixer for approximately 30 sec. The organic layer was removed, and filtered through glass wool to remove traces of charcoal (the glass wool was washed with an additional 0.5 ml of elution solvent, which was added to the filtrate). The solvent system was removed in the same way as described above.

Using micro-phase solvent extraction. Solid potassium chloride (5%, w/v) was added to 5 ml of urine containing PCP and internal standard, and the pH was adjusted to 9 with 0.5 *N* sodium hydroxide, then 100 μl of chloroform were added to the tube, which was shaken and centrifuged. A 1-μl portion of the organic phase was withdrawn through the aqueous layer and injected into the gas chromatograph.

Gas chromatography

A Tracor model 550 gas chromatograph (Tracor, Austin, Texas, U.S.A.) equipped with a flame ionization detector was used; it was fitted with a column (1.7 m × 2.0 mm I.D.) containing 5% of SE-30 plus 1% of Carbowax 20M on Chromosorb W HP (80–100 mesh). The column temperature was programmed from 150 to 230° at 7.5°/min, the injection-port temperature was held at 210° and the detector was kept at 270°.

RESULTS AND DISCUSSION

The development of an analytical scheme for PCP and its metabolites in body fluids involves separation of the compounds of interest from the matrix before quantitation. We have considered three common procedures for the separation of the drug and its metabolites from urine: (a) reversed-phase extraction using a macro-particulate resin[18,19], (b) charcoal absorption[20] and (c) solvent extraction[21]. Several criteria were considered in the choice of suitable pre-concentration procedures, including the fraction recovered, the presence of compounds that would interfere with the detection step, the simplicity of the procedure, and the amount of time required.

Reversed-phase solvent extraction with macro-reticular resin

The use of micro-reticular resins such as XAD has become popular for the separation of weakly acidic, weakly basic and neutral drugs in urine[18,19,22–24]. Various factors were studied in order to maximize the efficiency of this method, including flow-rate, the use of back-extraction procedures, and the method of solvent removal

TABLE I

EFFECT OF FLOW-RATE AND BACK-EXTRACTION ON RECOVERY OF PCP·HCl AND KET·HCl FROM XAD-2 RESIN

Type of extraction	*Recovery* (%)*		*Flow-rate (ml/min)*
	PCP·HCl	*Ket·HCl*	
Simple	60 ± 10	93 ± 4	5.6–8.5
Simple	68 ± 5	106 ± 5	4.0–6.6
Simple	58**	89	2
Back-extraction	58 ± 2	92 ± 3	5.6–8.5
Back-extraction	84 ± 7	101 ± 3	2

* Based on peak-height comparisons with a drug standard.

** Average of three determinations (all other values are the average ± S.D. of results for six samples).

before injection into the gas chromatograph. The importance of control of the flow-rate can be seen from Table I and Fig. 1. Use of gravitational flow (4–8 ml/min) leads to poor recovery of PCP and a high background. Incorporation of back-extraction into the procedure reduced the urine background considerably, but did not increase the rate of recovery. Control of the urine flow (2 ml/min) through the resin resulted in chromatograms having a high background, which again resulted in poor quanti-

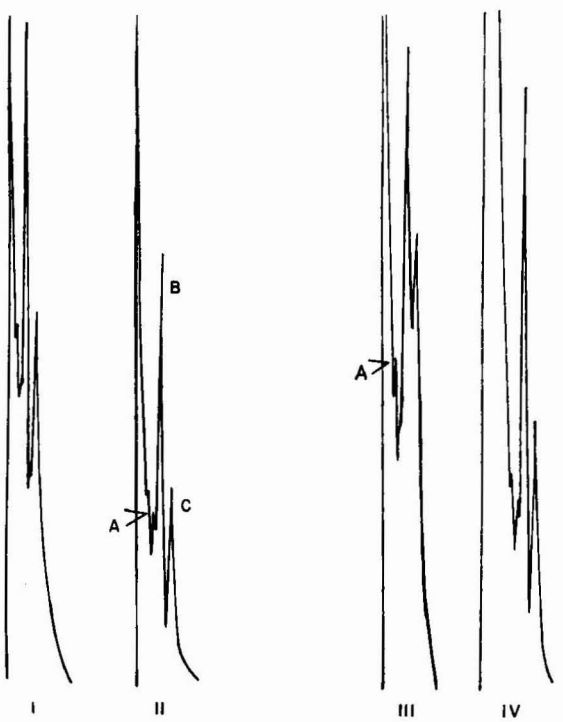

Fig. 1. Effect of flow-rate and extraction on drug recovery: I, gravitational flow, no back-extraction; II, gravitational flow, back-extraction; III, flow controlled (2 ml/min), no back-extraction; IV, flow controlled (2 ml/min), back-extraction. A = Caffeine; B = ketamine; C = PCP.

tation. The recovery could be significantly improved by including the back-extraction. This is not to suggest that the extraction actually increased the recovery; what happens is that the background in the chromatogram (see Fig. 1) is reduced, so that the quantitation is improved upon back-extraction.

The effect of the choice of solvent may be seen from Table II. In this respect, it might be pointed out that, although we used 20 ml of the methanol–chloroform (1:9), about 80% of the PCP and more than 98% of the ketamine used as standard were eluted within the first 10 ml.

TABLE II

EFFECT OF VARIOUS ELUTION SOLVENTS ON RECOVERY OF PCP·HCl AND KET·HCl FROM XAD-2 RESIN

Elution solvent	*Recovery (%)*		*Comments*
	PCP·HCl	*Ket·HCl*	
Chloroform	63*	100	Low background; good drug-peak shape and separation
Methanol–chloroform (1:9)	94	104	As above; somewhat higher background
Methanol–chloroform (1:3)	26	36	Poor drug separation from high background; unsuitable for use
Hexane	—	—	No apparent recovery
Isopropanol–chloroform (1:9)	54	96	Background similar to use of CH_3OH–$CHCl_3$ (1:9); good peak profiles
Isopropanol–chloroform (1:3)	24	32	Relatively low background, but small broad drug peaks; unsuitable for use
Isopropanol–ethyl acetate–dichloroethane (5:9:6)	12	30	Highly coloured residues; little drug recovery; unsuitable for use

* Values are the average for duplicate samples.

A water bath was used to remove solvent from the eluted sample. We found that the recovery of both PCP and standard was maximized by the addition of one drop of concentrated hydrochloric acid–methanol (1:1) before evaporation of the methanol–chloroform solvent system. The drug recovery fell if the tube containing the residue was not removed shortly after the solvent was completely evaporated; for example, recovery of the drug was 43% if the sample was removed from the heat 5 min after dryness was achieved; this value fell to 21% if the interval was 30 min. In the presence of the hydrochloric acid, quantitative recoveries of both PCP and standard were obtained, even with a 30-min period of heating following total evaporation of solvent.

The resin technique was extended by including the drug metabolite PCP·OH to the urine; the recovery of metabolite was maximized by increasing the pH of the urine from 6 to 9 before application to the resin bed[23].

The flow-rate of the methanol–chloroform eluent was fairly slow; it could be improved by adding 1 ml of acetone to the column before the eluent or by the use of pressure by placing the palm of the hand over the top of the column reservoir. The use of XAD-7 resin led to a flow-rate that had to be controlled at 2 ml/min.

To determine the useful range of the method, 4 μg of PCP, 5 μg of PCP·OH and 1.6 μg of ketamine were extracted from 10, 20, 30 and 40 ml of urine; recovery of both drugs was greater than 90% at all four concentrations. A sample chromatogram is shown in Fig. 2; the PCP·OH is resolved only poorly, and a total loss of peak separation for PCP·OH was noted at 0.17 ppm due to the presence of unknown impurities. For PCP, the technique is suitable to the 0.1-ppm level, or lower, and appears to be limited only by the inability to obtain a suitably clean extract*.

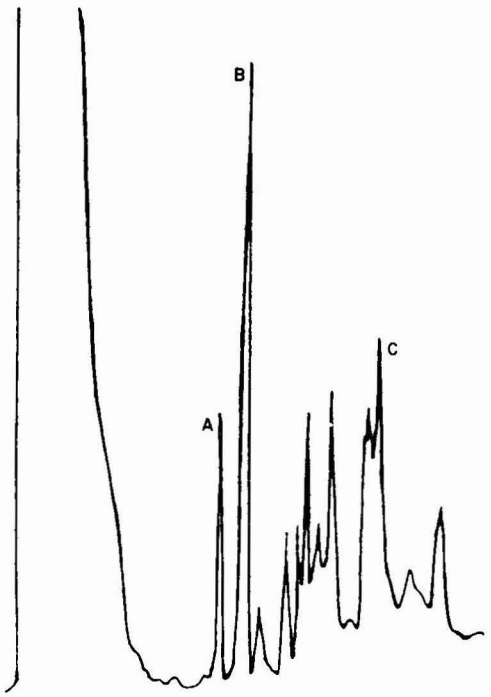

Fig. 2. Chromatogram of residue from resin extraction: A = 0.4 ppm of PCP; B = 1.6 ppm of ketamine; C = 0.5 ppm of PCP·OH.

The use of pre-packaged absorbent cartridges containing XAD-2 resin was considered. Flow-rates approaching that of XAD-7 could be achieved if the cotton plugs used for stabilization and support of the column were replaced by glass-wool. Although urine flow-rates were not controlled, the extraction efficiency for PCP was 100% and the corresponding efficiency for ketamine was 96%; recovery of PCP·OH was 89%. We observed a generally higher background at the temperature required for elution of PCP·OH on the chromatogram; this we attribute to extraction of plasticizers from the column (no additional peaks were observed). It is possible that some other solvent system would overcome the problem.

* PCP·OH would be found as the glucuronide in the urine of individuals who have ingested PCP. Lin *et al.*[16] have detected the complexed metabolite in urine and hydrolyze it to PCP·OH with a β-glucuronidase preparation at pH 5. Following hydrolysis, the pH would be adjusted to 9, and PCP·OH would be extracted into the organic phase.

Extraction with a universal absorbent

We observed that the use of wetted charcoal led to extremely clean residues if the solvent system suggested by Cordova and Banford[20] [chloroform–diethyl ether–isopropanol (50:54:10)] was used; unfortunately, recovery of the compounds of interest was low (PCP 22 ± 5%, PCP·OH 35 ± 8% and ketamine 47 ± 7%). When the elution solvent that was successful for the resin-extraction procedure was used with charcoal, recoveries were increased (PCP 72 ± 11%, PCP·OH 75 ± 7% and ketamine 30 ± 3%), but the background increased to a point at which the procedure was useless for quantitation.

Microphase solvent extraction

The recovery of PCP, PCP·OH and the standard by solvent-extraction procedures depends on the relative solubility of the compounds in the two solvents. At the natural pH of urine (approximately 6), chloroform recovered about 74% of the added PCP, whereas methanol–chloroform (1:9) extracted about 85%. If chloroform was used to extract the compounds of interest, it was found that an increase in the pH of the urine to 9 enhanced recovery of PCP to 94%, with quantitative recovery of PCP·OH and the standard. Fig. 3 shows a chromatogram derived from the extraction of urine containing PCP, its metabolite and the standard. The lower limit of sensitivity for PCP is about 0.3 ppm. Addition of the salt to the urine was found to eliminate formation of emulsions, which led to large deviations in drug recovery.

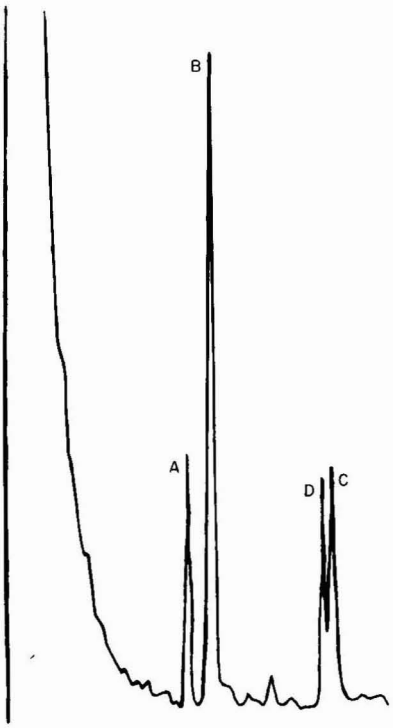

Fig. 3. Chromatogram of chloroform extract: peaks A, B and C as in Fig. 2; D = unknown.

Gas chromatography

It was determined that isothermal operation would permit detection of PCP at 155° with ease; the metabolite was not observable under such circumstances, probably because of the higher polarity of the hydroxy-piperidine ring.

CONCLUSIONS

We have developed a fairly rapid and sensitive procedure for determining PCP and one of its metabolites in urine. Although several assays for the drug in dosage forms have been reported[25–30], only a few useful procedures have been reported for the drug in biological samples. Gupta *et al.*[17] have developed a procedure involving chloroform extraction, but their assay takes longer than 30 min and does not include the metabolite.

A gas chromatography–chemical ionization mass spectroscopy technique that is more sensitive than our procedure has been reported[16]; however, we believe that the use of this procedure is limited by the availability of the instrument and the internal standard (pentadeuterated PCP).

We believe that the analyst would be best served by either the XAD resin extraction procedure or the chloroform extraction procedure. Although the reversed-phase resin procedure is lengthier and slightly less sensitive for the metabolite, it permits a large number of samples to be processed at the same time, which would be useful in a screening programme. For individual samples, the chloroform extraction procedure is recommended as being the fastest and providing a very clean background.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS IN BIOLOGICAL FLUIDS

IV. DETERMINATION OF CLOFIBRINIC ACID

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SUMMARY

A rapid, sensitive and specific high-pressure liquid chromatographic method is described for the quantitative analysis of clofibrinic acid in plasma, saliva and urine. In contrast to previously reported gas–liquid chromatographic methods, which require derivatization of clofibrinic acid before chromatography, the present method involves a simple two-step extraction procedure and chromatographic determination of the underivatized clofibrinic acid. Concentrations between 1.0 and 25.0 μg per sample can be measured with a coefficient of variation from 1 to 6%.

INTRODUCTION

A number of methods have been reported for the analysis of clofibrinic acid, the active metabolite of the hypolipidaemic drug clofibrate, in plasma and urine (Table I). The most widely used method has been a spectrophotometric assay[1,2] that involves solvent extraction of clofibrinic acid from acidified plasma or urine, and subsequent measurement of the ultraviolet absorbance at 226 nm. Although this method is rapid and convenient, it suffers from the lack of specificity inherent in spectrophotometric assays.

Several gas–liquid chromatographic (GLC) methods have been described for the determination of clofibrinic acid. Some of these methods have time-consuming preparative steps before GLC, such as column[3] or thin-layer chromatography[4,5]; another involves several extraction steps[6]. Such techniques of sample preparation are time-consuming and render these methods unsuitable for use with large numbers of samples. Four additional GLC methods[7–10] have a number of common features;

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TABLE I

COMPARISON OF METHODS OF ANALYSIS FOR CLOFIBRINIC ACID IN BIOLOGICAL FLUIDS

References	*Analytical method*	*Sample*	*Sensitivity (μg/ml)*	*Reproducibility (coefficient of variation)*	*Comments**
1, 2	Spectrophotometric	Plasma, urine	Not given	Not given	Lacks specificity
3	Column chromatography–GLC	Serum	Not given	Not given	Few details; prior chromatography
4	Thin-layer chromatography–GLC	Plasma, urine, bile, faeces	0.1	6.4% (Replicates between 53.68 and 322 μg/ml)	Prior thin-layer chromatography
5	Thin-layer chromatography–GLC	Plasma	Not given	Not given (correlation coefficient 0.999 for calibration curve)	No internal standard; prior thin-layer chromatography
6	GLC	Plasma	1	4.8% (replicates at 2.5, 10.1 and 50.5 μg/ml)	Multiple extractions; diazomethane used***
7	GLC	Serum	Not given	Not given	Few details
8	GLC	Plasma	0.25	Not given (correlation coefficient 0.999 for calibration curve)	Diazomethane used***
9	GLC	Plasma, urine	3	9%**	Time consuming
10	GLC	Plasma, urine	1	10% (Replicates at 1 μg/ml)	Diazomethane used***
11	GLC	Plasma, urine	1	3.0% (Calibration curves of 10–200 μg/ml from plasma and urina	Two-step extraction
This paper	HPLC	Plasma, urine, saliva	0.5	4.9% (Calibration curves of 1–25 μg/sample from plasma, urine and saliva	Two-step extraction without derivatization

* All the GLC methods include a derivatization step.
** Quoted in ref. 11.
*** See Results and discussion.

each involves solvent extraction, evaporation and derivatization before GLC. The most recently reported GLC method[11] appears to be rapid and convenient.

This paper reports the application of high-pressure liquid chromatography (HPLC) to the analysis of clofibrinic acid in plasma, saliva and urine. The method

is rapid, specific, sensitive and accurate, and, in contrast to GLC methods, does not involve derivatization.

EXPERIMENTAL

Reagents and materials

Clofibrinic acid [2-(4-chlorophenoxy)-2-methylpropionic acid] was a gift from Ayerst Laboratories (Montreal, Canada) and 2-(4-chloro-3-methylphenoxy)-2-methylpropionic acid, the internal standard, was a gift from Astra Pharmaceuticals (Södertälja, Sweden). The acetonitrile was of "distilled in glass" quality and was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.); all other solvents and reagents were of reagent grade.

Sample preparation

A schematic representation of the procedure is shown in Fig. 1. Plasma (0.1–1.0 ml), saliva (1.0 ml) or urine diluted 1:100 with distilled water (1.0 ml), is placed in a PTFE-lined screw-capped culture tube, and 100 μl of internal standard solution (containing 6.7 μg of the internal standard), 0.5 ml of 0.5 *N* sulphuric acid and 5 ml of toluene are added. The samples are extracted by mixing (using a Labquake® automatic shaker) for 10 min, followed by centrifugation at 1200 *g* for 10 min to separate the organic and aqueous phases. The lower aqueous phase is frozen by immersing the tube in a dry ice–acetone bath, and the organic phase is poured into another tube, which has an elongated cone (capacity approx. 50 μl) at its base. Then 50 μl of 0.2 *N* NaOH are added, and the mixture is extracted on a Vortex mixer for 2 min. After brief centrifugation, the aqueous phase is drawn into a syringe that already contains 10 μl of a solution of 5% glacial acetic acid in water, and this mixture is injected into the chromatograph.

For the analysis of the glucuronide conjugate of clofibrinic acid in urine, 5 ml

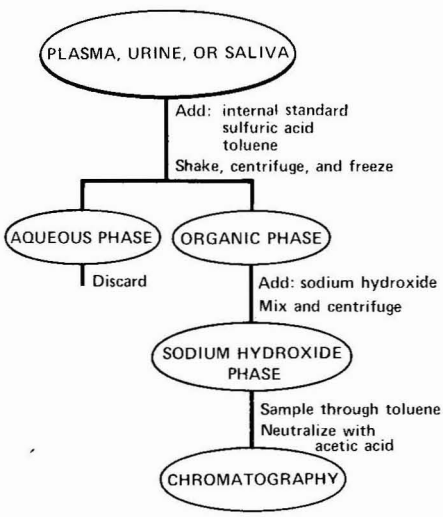

Fig. 1. Flow diagram of sample preparation.

of 6 *N* hydrochloric acid were added to each sample, and the solutions were heated at 98° for 30 min before the extraction. The samples were then cooled and analysed as described above, except that the addition of dilute sulfuric acid in the first step was omitted.

Chromatography

A Varian (model 8500) high-pressure liquid chromatograph fitted with a Varian MicroPak CH-10 reverse-phase column (25 cm × 6.3 mm O.D. × 2.2 mm I.D.) was used for the analysis. The absorbance was measured at 235 nm, with 0.5 absorbance unit full-scale deflection and a slit width of 2 nm, using a Varian Variscan variable-wavelength spectrophotometer. One pump of the dual-pump gradient-elution chromatograph contained acetonitrile and the other 0.5% acetic acid in distilled water; an isocratic 42% acetonitrile mixture of the two solvents was used. With a dual-pump chromatograph, it was convenient to use two pumps operating under isocratic conditions. However, an acceptable alternative was to use the desired mixture of the two solvents in a single pump. The flow-rate of the solvent mixture was 70 ml/h with a column-input pressure of 197 atm (2900 p.s.i.). Chromatograms were recorded on a Varian A-25 dual-pen recorder with 0–50 and 0–200 mV spans.

Calibration and accuracy

Calibration curves were constructed by adding known amounts of clofibrinic acid and internal standard to control plasma, saliva or urine. The peak-height ratio of clofibrinic acid to internal standard was plotted against the amount of clofibrinic acid added. In order to calibrate the method and determine its accuracy for each batch of unknown samples, standards of 1, 2, 5, 10, 15, 20 and 25 μg of clofibrinic acid were added to the control samples, which were assayed concurrently with the unknown samples. The peak-height ratio of each standard was divided by the amount of clofibrinic acid added to give normalized peak-height ratios. The mean normalized peak-height ratio was used to calculate the amount of clofibrinic acid in unknown samples, and the standard deviation of the normalized peak-height ratios was used to determine the accuracy of the method over the range of clofibrinic acid standards employed. The reproducibility of the method was also studied by submitting replicate plasma samples containing 1, 5, 10 and 20 μg of clofibrinic acid to the entire procedure. The effect of sample size on the method was investigated by adding 10 μg of clofibrinic acid to tubes containing different volumes of plasma (between 0.1 and 1.0 ml), which were then assayed for clofibrinic acid. The volumes of the internal-standard solution, sulphuric acid and toluene were kept constant.

To estimate the recovery for the analytical procedure, five control-plasma samples with 10 μg of clofibrinic acid added were analysed, and the mean height of the clofibrinic acid peaks was compared to the mean height of five peaks obtained by injecting 10 μg of clofibrinic acid directly into the chromatograph.

Application of the method to measure plasma and saliva concentrations

A healthy male volunteer received five doses of 1 g of clofibrate (2 capsules of Atromid-S®, Ayerst) every 12 h for 3 days. Samples of venous blood and saliva were collected at 0, 1, 2, 4, 6, 8, 13, 24 and 28 h after administration of the fifth and last dose. The blood (5 ml) was collected in heparinized Venoject® tubes, and, after cen-

trifugation, the plasma was transferred to glass containers, which were stored at −15° until analyzed.

Saliva samples were obtained by having the subject chew on a small PTFE disc; all the saliva produced during approx. 4 min just before blood sampling was collected in a glass vial. The saliva samples were immediately frozen and stored at −15° until analyzed.

RESULTS AND DISCUSSION

In order to be sufficiently volatile for GLC determination, clofibrinic acid must be derivatized, usually by methyl esterification of the carboxyl group. Methods of derivatization in which hydrochloric acid–methanol[4] or boron trifluoride–methanol[5] is used are time consuming, and other methods involve use of toxic and unstable reagents such as ethereal solutions of diazomethane[6,8,10]. The use of HPLC allows the determination of underivatized clofibrinic acid, thus shortening the sample preparation and avoiding use of toxic reagents.

The use of a reverse-phase column permits direct injection of an aqueous solution of the sample on to the column. Because of this, a simple procedure for sample preparation can be used (Fig. 1), which selectively extracts and concentrates acidic compounds. Such a technique is both more rapid and more selective than are those involving evaporation of solvent. The efficiency of this method of extraction is also high, as shown by the observation that 82% ± 8% (S.D.) of the clofibrinic acid added to plasma was actually injected into the chromatograph (see Experimental).

In order to achieve efficient separation of compounds with ionizable functions on reverse-phase columns, either the ionization must be suppressed, or a large counter-ion must be added and the compounds of interest chromatographed as ion-pairs[12]. For carboxylic acids such as clofibrinic acid, it is convenient to suppress ionization by adding acetic acid to the solvent. Acetic acid has appreciable ultraviolet absorbance, and therefore it would be desirable to keep its concentration low in order to reduce background absorbance and thus achieve a high signal-to-noise ratio. However, the need for rapid neutralization of the aqueous sodium hydroxide solution that is injected on to the column with each sample requires the use of an adequate amount of acetic acid. Failure to neutralize the sodium hydroxide gives rise to peak tailing and double peaks and results in deterioration of the column. For these reasons, the sodium hydroxide solution containing the sample is neutralized before injection on to the column, by drawing the sample into a syringe that contains 10 μl of aqueous 5% acetic acid.

Although clofibrinic acid has an absorption peak at 226 nm[1,2], the absorbance is measured at 235 nm in this method. This is because of the high background absorbance encountered at lower wavelengths, primarily a function of the acetic acid concentration. Since it is necessary to suppress the ionization of the clofibrinic acid with an acetic acid concentration in the eluting solvent mixture of about 0.3%, this wavelength was chosen as a compromise between sensitivity and the need to reduce the background absorbance. At 235 nm, good reproducibility is still attainable with 1 μg of clofibrinic acid, and concentrations as low as 0.5 μg/ml can be measured.

None of the plasma, saliva or urine control samples showed peaks interfering with the peaks of clofibrinic acid or the internal standard (Fig. 2a); a typical chro-

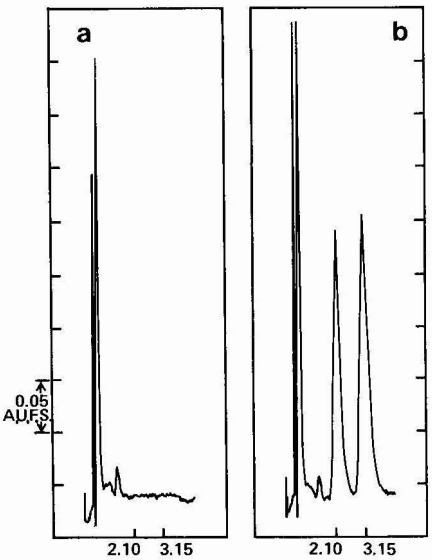

Fig. 2. Chromatograms of (a) control plasma and (b) plasma containing 5.0 μg of clofibrinic acid and the internal standard. The marks on the chromatograms correspond to the retention times of clofibrinic acid (2.10 min) and the internal standard (3.15 min). In order to improve visual clarity, only one tracing of the dual pen recorder is shown.

matogram for a plasma sample is shown in Fig. 2b. With the chromatographic conditions previously described, the retention times for clofibrinic acid and the internal standard are 2.10 and 3.15 min, respectively, allowing a sample injection to be made approximately every 5 min.

Estimates of accuracy for the method are shown in Table II. The average normalized peak-height ratio obtained from calibration curves from plasma, saliva

TABLE II

ESTIMATES OF ACCURACY FOR THE METHOD

Biological fluid	*Concentration, µg/sample*	*Number of samples*	*Mean normalized peak-height ratio*	*Coefficient of variation, %*
Calibration curve data				
Plasma	1–25	7	0.19935	2.9
Plasma	1–25	7	0.19102	5.0
Saliva	1–25	7	0.21027	6.3
Urine (untreated)	1–25	7	0.20114	4.8
Urine (acid hydrolysis)	1–25	7	0.18763	5.4
		Average:	0.19788	4.9
Reproducibility at a given concentration				
Plasma	1	7	0.21764	5.5
Plasma	5	3	0.20048	1.9
Plasma	10	5	0.19532	1.4
Plasma	20	5	0.21475	0.7
		Average:	0.20705	2.4

and urine had a mean coefficient of variation of 4.9% for 5 such calibration curves. This estimate of accuracy covers the entire range of the assay procedure, from 1 to 25 μg of clofibrinic acid per sample. Reproducibility studies on replicates containing 1, 5, 10 or 20 μg of clofibrinic acid per sample had a mean coefficient of variation of 2.4% (Table II). As it may be necessary to use variable volumes of plasma for clofibrinic acid measurements, the effect of plasma volume on the peak-height ratio of clofibrinic acid to internal standard was examined; the peak-height ratios were independent of the volume of plasma used between 0.1 and 1.0 ml.

Clofibrinic acid is excreted in the urine, some 70–95% of the dose being excreted as a glucuronide, and the rest as unconjugated acid[8,9,13]. On a typical dose regimen of 2 g per day, and assuming a daily urine output of 1–2 l, the total concentration in the urine is of the order of 1–2 mg/ml. By using 1 ml of 1:100 dilution of urine, the concentrations of both the unconjugated (untreated sample) and the conjugated clofibrinic acid (acid hydrolysed) in the urine fall within the concentration range of the analysis.

Application of the method to determination of clofibrinic acid in plasma and saliva from a healthy male volunteer is demonstrated in Fig. 3. Although the concentrations in saliva are only about 1% of those in plasma, the method is able to measure clofibrinic acid in both body fluids in a subject receiving a commonly employed dosing regimen. These results suggest the potential usefulness of saliva analysis for monitoring clofibrinic acid concentrations in patients.

The method described here for the quantitative determination of clofibrinic acid in plasma, saliva and urine by HPLC has the advantage over most other published methods of being simple and rapid (Table I). By using the techniques described, 40–50 samples can easily be assayed in a day. The sample preparation is a simple

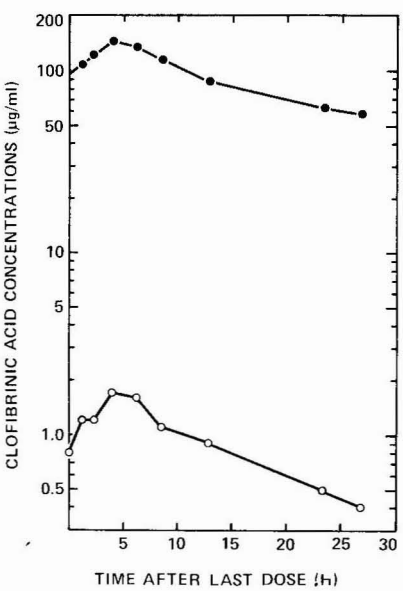

Fig. 3. Semi-logarithmic plot of plasma (●) and saliva (○) concentrations of clofibrinic acid in a healthy volunteer after the last dose of a multiple-dosing regimen of 2 g of clofibrate per day.

two-stage procedure (Fig. 1), which does not require the prior chromatographic preparation[3–5] or multiple extractions[6] described in published GLC methods (Table I). The use of a reverse-phase HPLC system permits the determination of underivatized clofibrinic acid and the internal standard and allows a simple method of sample preparation. Of the previously published methods, only the GLC analysis described by Gugler and Jensen[11] offers similar advantages of convenience (Table I). However, the present HPLC method offers a simpler approach to the analysis of clofibrinic acid than GLC methods by eliminating the need to form volatile derivatives before chromatographic separation.

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CHROMATOGRAPHY OF METAL CHELATES

VII*. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF METAL 1,2-DIKETOBISTHIOBENZHYDRAZONES, METAL DIALKYLDITHIOCARBAMATES AND METAL 1,2-DIKETOBISTHIOSEMICARBAZONES

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SUMMARY

High-performance liquid chromatographic separations of Pb(II), Zn(II), Cd(II), Hg(II), Cu(II), Ni(II) and Co(III) chelates at nanogram levels by adsorption chromatography on silica gel are reported. 1,2-Diketobisthiobenzhydrazones, dialkyldithiocarbamates and 1,2-diketobisthiosemicarbazones were used as chelate-forming ligands.

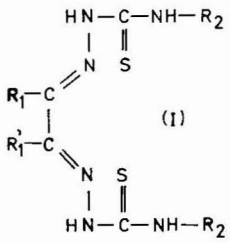
INTRODUCTION

In trace metal analysis, the use of chelate-forming ligands for the chromatographic separation and determination of the elements is of great importance. The separation and determination of chelated metal ions by gas chromatography (GC) has often been described[1–9], but this method is limited because of the low volatility and the low thermal stability of most of the metal chelates.

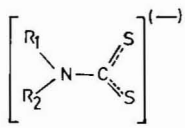
In liquid chromatography, these problems do not arise and this method often therefore appears to be more suitable for the separation and determination of extracted metal chelates[10,11]. Ion-exchange chromatography offers an other possibility for the separation of metal ions[12–14]. Separations and determinations of metal ions by forced-flow ion-exchange chromatography have been described[15,16] but, compared with this method, the use of chelating ligands for the determination of metal ions after solvent extraction permits enrichment of the components in the organic solvent before the separation step.

This paper demonstrates the possibilities of separating metal chelates by high-performance liquid chromatography (HPLC). The following ligands were used: 1,2-diketobisthiosemicarbazones (I)[17], dialkyldithiocarbamates (II)[18–21] and 1,2-diketobisthiobenzhydrazones (III)[22,23] (Fig. 1).

* Part VI: P. Heizmann and K. Ballschmiter, *Z. Anal. Chem.*, 266 (1973) 206.



(I)



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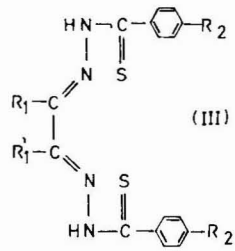


(III)

Fig. 1. Structures of ligands.

EXPERIMENTAL AND RESULTS

Adsorbents

Perisorb A (30–40-μm) silica gel, LiChrosorb SI 60 (20, 30 and 40-μm) silica gel and Alox T (20, 30 and 40-μm) alumina were obtained from E. Merck, Darmstadt, G.F.R.

Solvents

Benzene, *n*-hexane, chloroform, acetonitrile, cyclohexane and *n*-heptane (all p.a. quality) were purchased from E. Merck.

Liquid chromatographs

The equipment mainly used consisted of the following: Labotron LDP 13 A pumps (1.5–80 and 0.5–20 ml/h) (Kontron Technik, Eching/München, G.F.R.); PTFE capillary tubing, 0.7 mm I.D. (Kontron Technik); glass columns, 2 mm I.D., 6 mm O.D. (according to Fig. 2); PTFE injector (Fig. 2); and a Zeiss PM 2D detector with a micro-flow attachment and a 2-mV recorder.

Some experiments were carried out with HPLC equipment obtained from Waters Assoc., Königstein/Ts., G.F.R., which consisted of M 6000 pumps (0.1–10 ml/min), a Model U 6 K injector, an M 660 gradient programmer and a Perkin-Elmer LC 55 detector with a 2-mV recorder.

General aspects of the chromatographic system used

Chelates of diacetylbisthiobenzhydrazone were used to study the chromatographic system.

Column filling. The columns were filled according to the Kirkland method[24]. Using irregularly shaped particles, 20 μm was found to be the lowest limit for dry filling of the columns with satisfactory separation characteristics.

Linearity. With Hg(II) and Cu(II) within the range 20–2000 ng and with an injected volume of 1–100 μl, no change in the retention time and no deviation from the linear adsorption isotherm were found.

Detection limit. An amount of 10^{-9} g of Hg(II) and Cu(II) in an injected volume of chelate solution of 50 μl (signal to noise ratio 10:1, resolution $R_{s\ Hg/Cu} = 1.5$) could be determined. On extracting deionized water with a benzene solution of the ligand, no blank values were found.

Using standard micro-techniques for the enrichment of extracted metal

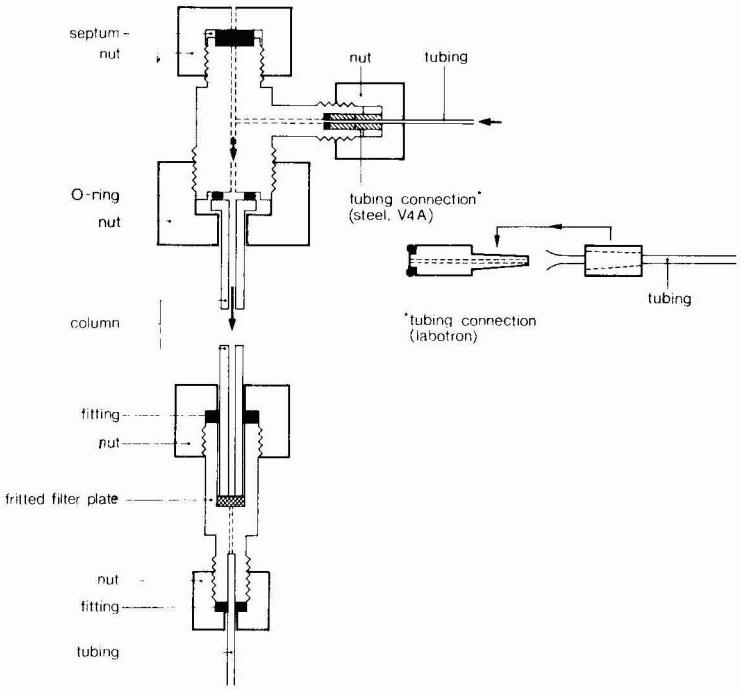


Fig. 2. Scheme of injection port and column termination (PTFE).

chelates, quantitative determinations of trace elements in aqueous solutions at the picogram level should be possible.

Reproducibility. Using Hg(II) and Ni(II) chelates as examples, the reproducibility was determined. Portions of 10 μl of chelate solution were injected (septum injection) and the resulting peak areas integrated. The standard deviation was found to be 1.6% for the Hg(II) chelate (20 injections) and 2.2% for the Ni(II) chelate (15 injections). The reproducibility can be improved by using a septumless injector (standard deviation *ca.* 1%).

To avoid any contact between the chelates and metal parts, which might lead to decomposition, all experiments were run in glass columns, although commercial steel columns (5-μm silica gel) nowadays have a much better separation efficiency.

The same peak areas were obtained when the same amounts of chelate solution [Hg(II), Cu(II), Ni(II)] were injected once into a column filled with silica gel and once into a column that contained no adsorbent (ten injections of each chelate on each type of column).

These results indicated that chromatography on LiChrosorb SI 60 does not result in decomposition or irreversible adsorption of the chelates.

HPLC of metal diacetylbisthiobenzhydrazones[10]

Hg(II), Ni(II), Cu(II), Zn(II) and Pb(II) diacetylbisthiobenzhydrazones can be separated, using silica as the adsorbent.

Isocratic elution. The influence of column length, particle diameter and flow-

TABLE I

HPLC SEPARATIONS OF Hg(II) AND Cu(II) DIACETYLBISTHIOBENZHYDRAZONES
LiChrosorb SI 60 (40 μm); glass column, 35 cm; solvent, benzene; temperature, 23°.

Solvent flow-rate (ml/h)	*Resolution, R_s [Hg(II)/Cu(II)]*	*Plate height, Hg(II) chelate (mm)*	*Analysis time, t_A (min)*
3	1.8	0.9	50
6	1.6	1.3	25
15	1.1	2.2	10
30	0.9	2.6	5
60	0.7	2.8	4

rate of the eluent on the separation conditions for the Hg(II), Cu(II) and Pb(II) chelates were studied (Tables I and II). Because of its excessive retention on LiChrosorb SI 60, the determination of the Zn(II) chelate of diacetylbisthiobenzhydrazone is not possible when benzene is used as the eluent, and gradient elution is required.

Tables III and IV show the separations obtained with LiChrosorb SI 60 and Perisorb A as adsorbents and *n*-heptane–benzene solvent mixtures as the eluent. Although the eluent flow-rate is increased by adding more *n*-heptane while the flow-

TABLE II

HPLC SEPARATIONS OF Hg(II) AND Pb(II) DIACETYLBISTHIOBENZHYDRAZONES
LiChrosorb SI 60 (20 μm); glass column; solvent, benzene; temperature, 23°.

Column length (cm)	*Solvent flow-rate (ml/h)*	*Resolution, R_s [Hg(II)/Pb(II)]*	*Analysis time, t_A (min)*
15	6	2.3	19
15	15	1.9	11
10	15	1.5	7
15	30	1.3	5

TABLE III

INFLUENCE OF THE SOLVENT GRADIENT ON THE RESOLUTION (R_s) OF METAL DIACETYLBISTHIOBENZHYDRAZONES
LiChrosorb SI 60 (30 μm); glass column, 15 cm; solvent, benzene–*n*-heptane; temperature, 23° (t_A = analysis time).

Benzene–n-heptane ratio	*Flow-rate (ml/h)*	*R_s*				*t_A (min)*	
		Hg/Cu	*Hg/Ni*	*Ni/Cu*	*Cu/Pb*	*Hg/Cu*	*Hg/Pb*
15:0	15	0.8	0.5	0.4	1.5	6	10
15:1	16	0.9	0.5	0.4	1.7	6	10
15:2	17	1.2	0.5	0.5	2.2	6	16
15:5	20	1.4	0.7	0.5	2.3	10	20
15:10	25	1.8	1.3	0.9	2.6	13	28
15:12.5	11	3.2	1.8	1.5	–	32	–

TABLE IV

INFLUENCE OF THE SOLVENT GRADIENT ON THE RESOLUTION (R_s) OF METAL DIACETYLBISTHIOBENZHYDRAZONES

Perisorb A (30–40 μm); glass column, 35 cm; solvent, benzene–*n*-heptane; temperature, 23° (t_A = analysis time).

Benzene–n-heptane ratio	*Flow-rate (ml/h)*	R_s				t_A	
		Hg/Cu	*Hg/Ni*	*Ni/Cu*	*Cu/Pb*	*Hg/Cu*	*Cu/Pb*
15:12.5	11	1.8	1.0	0.7	2.3	8	14
15:20	16	2.2	1.5	0.8	2.2	6	12
15:25	35	2.6	2.3	1.0		14	—

rate of benzene remains constant, the separation (R_s) is increased because of the reduced solvent strength. Fig. 3 shows the separation of Hg(II), Ni(II) and Cu(II) diacetylbisthiobenzhydrazones, while Figs. 4 and 5 show the separations of the Hg(II), Cu(II) and Pb(II) chelates. In comparison with Fig. 4, Fig. 5 demonstrates the much better separation efficiency of a slurry-packed 10-μm-column; better separations within shorter analysis times are achieved. Using Perisorb A as the adsorbent, good separations can be achieved at lower solvent strengths.

Gradient elution. Gradient elution improves poor separations and reduces otherwise large capacity ratios (k' values). Using solvent mixtures of low solvent strength, the columns could be reconditioned to their initial state by running the

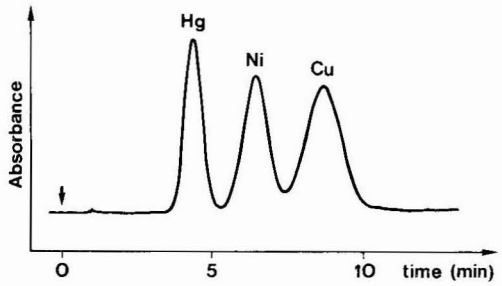


Fig. 3. HPLC separation of metal diacetylbisthiobenzhydrazones (isocratic elution). Packing, LiChrosorb SI 60 (30 μm); glass column, 500 × 2 mm; solvent, *n*-heptane–benzene (1:1); flow-rate, 1.5 ml/min; detection, 360 nm (Perkin-Elmer LC 55); pump, Waters M 6000; temperature, 23°.

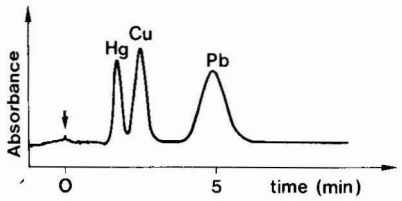


Fig. 4. HPLC separation of metal diacetylbisthiobenzhydrazones (isocratic elution). Packing, LiChrosorb SI 60 (30 μm); glass column, 500 × 2 mm; solvent, *n*-heptane–benzene (3:7); flow-rate, 2.0 ml/min; detection, 360 nm (Perkin-Elmer LC 55); pump, Waters M 6000; temperature, 23°.

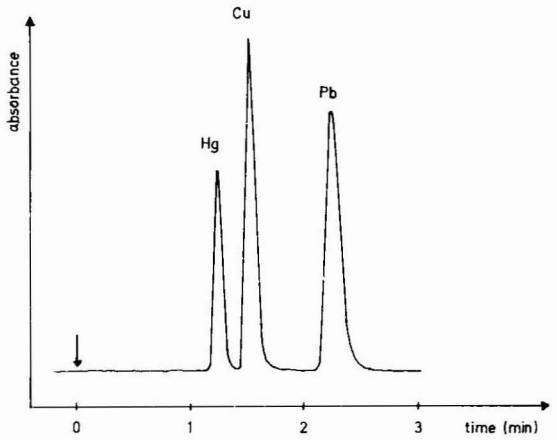


Fig. 5. HPLC separation of metal diacetylbisthiobenzhydrazones (isocratic elution). Packing, Nucleosil 100-10; glass column, 300 × 2.3 mm (pre-packed column from Macherey, Nagel & Co., Düren, G.F.R.); solvent, benzene; flow-rate, 1.5 ml/min; detection, 360 nm (Perkin-Elmer LC 55); pump, Waters M 6000; temperature, 23°.

reverse programme. By varying the gradient programme of the components of the *n*-heptane–benzene eluent system, good separations of Hg(II), Ni(II), Cu(II) and Pb(II) chelates are possible (Figs. 6 and 7).

Gradient programmes using *n*-heptane–chloroform as eluent also give good results (Fig. 8). By using this solvent mixture, a separation of the otherwise strongly adsorbed Zn(II) chelate from the other components is possible. The programme consisted of a linear gradient (10 min), the solvent being 10–60% of chloroform in *n*-heptane with a flow-rate of 4 ml/min, using a 60-cm LiChrosorb SI 60 (20 μm) column. The $R_{s\ Cu/Zn}$ value was 2.5 and the analysis time (t_A) was 5 min.

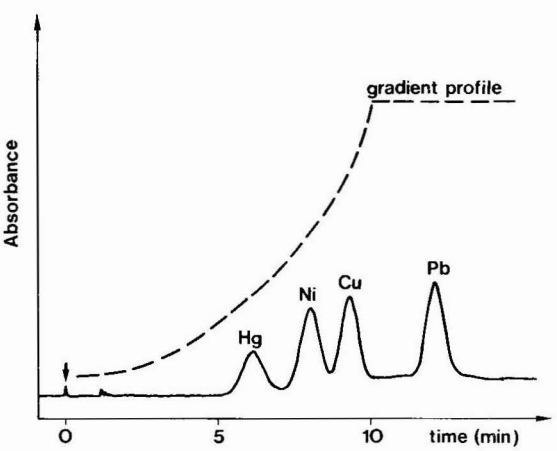


Fig. 6. HPLC separation of metal diacetylbisthiobenzhydrazones (gradient elution). Packing, LiChrosorb SI 60 (30 μm); glass column, 500 × 2 mm; solvent, *n*-heptane–benzene; flow-rate, 1.5 ml/min; programme, curve 7 on Waters M 660 (running time 10 min); conditions, from 40% benzene in *n*-heptane to 100% benzene; detection, 360 nm (Perkin-Elmer LC 55); pumps, Waters M 6000; temperature, 23°.

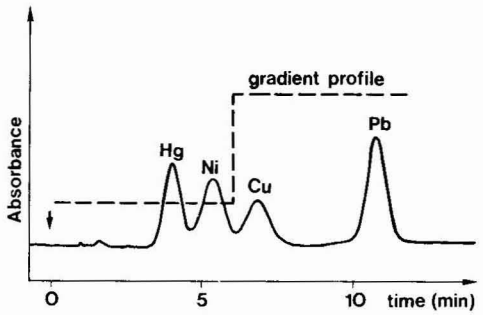


Fig. 7. HPLC separation of metal diacetylbisthiobenzhydrazones (gradient elution). Packing, LiChrosorb SI 60 (30 μm); glass column, 500 × 2 mm; solvent, *n*-heptane–benzene; flow-rate, 1.5 ml/min; programme, curve 11 on Waters M 660 (running time 6 min); conditions, from 50% benzene in *n*-heptane to 100% benzene; detection, 360 nm (Perkin-Elmer LC 55), pumps, Waters M 6000; temperature, 23°.

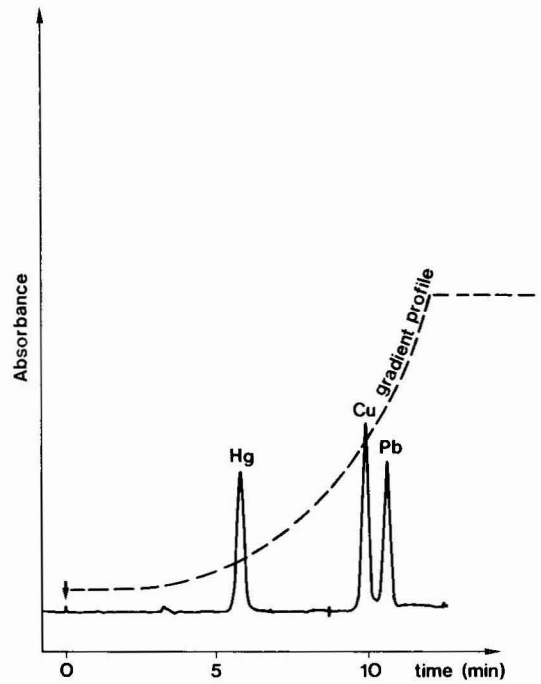


Fig. 8. HPLC separation of metal diacetylbisthiobenzhydrazones (gradient elution). Packing, LiChrosorb SI 60 (30 μm); glass column, 500 × 2.2 mm; solvent, *n*-hexane–chloroform; flow-rate, 3.0 ml/min; programme, curve 8 on Waters M 660 (running time 12 min); conditions, from 20% chloroform in *n*-hexane to 70% chloroform in *n*-heptane; detection, 360 nm (Perkin-Elmer LC 55); pumps, Waters M 6000; temperature, 23°.

HPLC of chelates of disubstituted dithiocarbamates (DTC)

Previously described TLC separations of metal dithiocarbamates[18–21] were attempted by using HPLC. Dithiocarbamates with different substituents can easily be prepared and the influence of different substituents on the separation can be studied.

HPLC of metal diethyldithiocarbamates. Separations of the Cu(II), Hg(II), Ni(II) and Co(III) diethyldithiocarbamates are possible within a reasonable time using silica gel as adsorbent and benzene as eluent (Table V).

TABLE V

HPLC SEPARATIONS OF METAL DIETHYLDITHIOCARBAMATES

LiChrosorb SI 60 (30 μm); glass column, 15 cm; solvent, benzene; detection, 330 nm. Elution sequence: Cu > Hg > Ni > Co.

Pair	R_s	*Solvent flow-rate (ml/h)*	*Analysis time, t_A (min)*
Cu/Hg	0.4	4	15
Cu/Ni	1.1	4	18
Ni/Co	1.6	8	15
Cu/Co	1.9	20	6
Ni/Co	1.3	20	6

The separation of the Cu(II) and Ni(II) chelates is possible on a short column (15 cm), although only at low solvent flow-rates. On the other hand, the separation of the Hg(II) and Cu(II) chelates is difficult, but can be achieved by using a 50-cm column. Separations of the Cu(II) and Co(III) chelates and the Ni(II) and Co(III) chelates pose no problems. Again, increasing the solvent flow-rate reduces analysis time. The Zn(II) chelate is eluted between the Cu(II) and the Hg(II) chelates, the Cd(II) chelate is eluted before the Cu(II) chelate and the Pb(II) chelate is eluted after the Ni(II) chelate. However, under these conditions, the three chelates show considerable tailing on silica gel.

Further HPLC results will be published later[25].

HPLC of metal benzylmethyldithiocarbamates. As the results obtained by TLC[19,21] show, the chelates of the benzylmethyldithiocarbamates are also suitable for liquid chromatographic separations.

For the benzylmethyldithiocarbamates of Cu(II), Zn(II), Ni(II), Cd(II) and Co(III) there was good agreement between the TLC results and HPLC results using LiChrosorb SI 60 and solvent mixtures with low solvent strength (*e.g.*, 3:1 benzene–cyclohexane). Fig. 9 compares the TLC hR_F values with the HPLC retention times (t_R). The sequence of the signals in HPLC is almost identical with those in the separation by TLC.

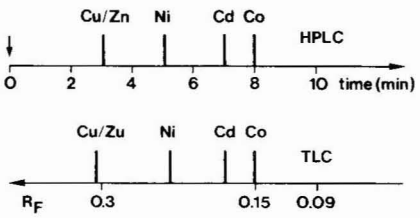
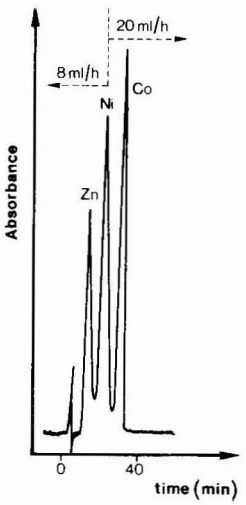


Fig. 9. Comparison between TLC and HPLC separations of metal benzylmethyldithiocarbamates. TLC: DC-Alufolie SiO_2; benzene–cyclohexane (3:1). HPLC: packing, LiChrosorb SI 60 (40 μm); glass column, 350 × 2 mm; solvent, benzene–cyclohexane (3:1).

A flow-programme separation of the Zn(II), Ni(II) and Co(III) benzylmethyldithiocarbamates (Fig. 10) gave good R_s values with an acceptable analysis time. After the peak maximum of the Ni(II) chelate, had been reached, the solvent flow-rate was increased from the initial 8 ml/h to 20 ml/h. The values obtained were $R_{s\ Zn/Ni} = 1.6$, $R_{s\ Ni/Co} = 1.6$ and $t_A = 35$ min. Under these conditions, the Pb(II) chelate will be eluted with the Co(III) chelate.



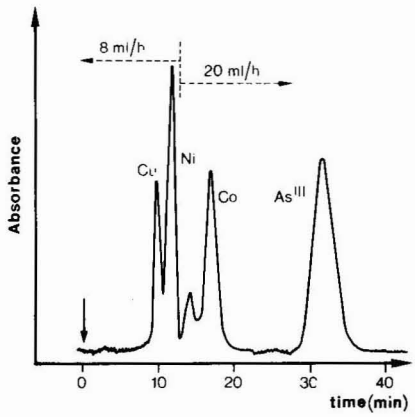


Fig. 10. HPLC separation of metal benzylmethyldithiocarbamates. Packing, LiChrosorb SI 60 (40 μm); glass column, 350 × 2 mm; solvent, benzene–cyclohexane (3:1); flow-rates, 8 and 20 ml/h; detection, 360 nm (Zeiss PM 2 D); pump, Labotron LDP 13 A; temperature, 23°.

Fig. 11. HPLC separation of metal diethoxyethyldithiocarbamates. Packing, LiChrosorb SI 60 (40 μm); glass column, 350 × 2 mm; solvent, carbon tetrachloride–4% acetonitrile; flow-rates, 8 and 20 ml/h; detection, 360 nm (Zeiss PM 2 D); pump, Labotron LDP 13 A; temperature, 23°.

HPLC of metal diethoxyethyldithiocarbamates. Separations of the Hg(II), Cu(II), Ni(II), Co(III) and As(III) diethoxyethyldithiocarbamates were studied. Using binary mixtures, we found small distortions between the TLC and HPLC separations. Separations of the Cu(II) and Ni(II) chelates were possible only at low solvent flow-rates.

Fig. 11 shows the separation of the chelates of Cu(II), Ni(II), Co(III) and As(III). The analysis was started at a solvent flow-rate of 8 ml/h, and was increased to 20 ml/h for the separation of the Co(III) and As(III) chelates. Under these conditions, the Hg(II) chelate is eluted with the Cu(II) chelate. The chelates of Sb(III) and Bi(III) show strong adsorption, resulting in excessive retention times. The chelates of Pb(II), Zn(II) and Cd(II) show large tailing effects, as demonstrated by TLC[19].

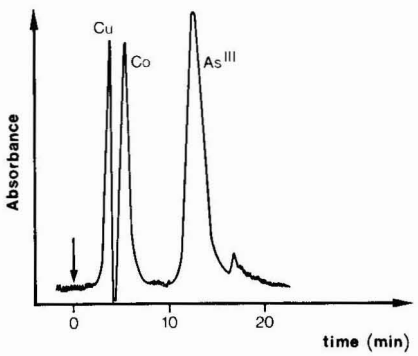
Fig. 12 shows the separation of a three-component mixture at a constant solvent flow-rate of 20 ml/h.

HPLC of chelates of 1,2-diketobis(4-substituted)thiosemicarbazones

The wide analytical use of 1,2-diketobisthiosemicarbazones in trace metal analysis is prevented by the low solubility of most of the chelates in organic solvents.

However, substitution of the ligand molecule in the R_2 position (Fig. 1) leads to chelates that can be extracted into organic solvents such as chloroform and ethyl acetate[17,21,23].

HPLC of metal glyoxalbis(2,2,3,3-tetramethylbutyl)thiosemicarbazones. Using Alox T as the adsorbent and benzene as the eluent, the separation of the Hg(II), Cu(II) and Ni(II) chelates can be achieved (Fig. 13). The Pb(II) chelate decomposes via a catalytic reaction on the adsorbent. The chelates of Cd(II), Zn(II) and Co(III) are strongly adsorbed.



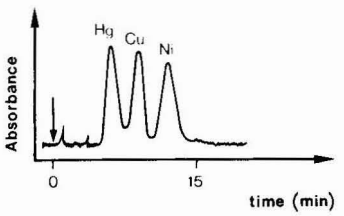


Fig. 12. As Fig. 11; flow-rate, 20 ml/h.

Fig. 13. HPLC separation of metal glyoxalbis(2,2,3,3-tetramethylbutyl)thiosemicarbazones. Packing, Alox T (30 μm); glass column, 350 × 2 mm; solvent, benzene; flow-rate, 8 ml/h; detection, 360 nm (Zeiss PM 2 D); pump, Labotron LDP 13 A; temperature, 23°.

When Perisorb A is used as an adsorbent of lower activity, only a slight separation of the Hg(II), Cu(II) and Ni(II) chelates occurs, but the Pb(II) chelate no longer decomposes and its separation from the other three components is possible ($R_{s\,Hg/Pb} = 1.35$; benzene (20 ml/h) as eluent; column length 35 cm).

HPLC of metal diacetylbis(cyclohexyl)thiosemicarbazones. The separation of the Hg(II) and Cu(II) chelates can be achieved on a silica gel column (LiChrosorb SI 60, 40 μm, length 35 cm) using benzene–3% tetrahydrofuran as eluent. A better separation of these components is possible on Alox T (30 μm) alumina as the adsorbent and benzene as the eluent (column length, 10 cm; flow-rate, 20 ml/h; $R_s = 1.4$; $t_A = 5$ min). Under these conditions, the Pb(II) chelate decomposes. The chelates of Zn(II), Ni(II) and Co(III) are only slightly extracted with benzene. The Cd(II) chelate shows very strong adsorption.

CONCLUSION

The application of HPLC in trace metal analysis offers new possibilities for the separation and determination of metal chelates at nanogram levels. By varying the substituents in the ligand molecule, the separation can be influenced. Substituent effects on the selectivity of liquid chromatographic separations of metal chelates will be discussed elsewhere.

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CHROMATOGRAPHY OF METAL CHELATES

VIII*. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF METAL DITHIZONATES

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(Received December 28th, 1976)

SUMMARY

High-performance liquid chromatographic separations of Pb(II), Zn(II), Cd(II), Hg(II), Cu(II), Ni(II) and Co(II) dithizonates at nanogram levels by adsorption chromatography on silica gel are reported.

INTRODUCTION

In the trace analysis of elements, photometric detection of chelated metal ions is of great importance. Diphenylthiocarbazane (dithizone) (Fig. 1) forms chelates with a large number of metal ions[1] and thus has widespread use in trace metal analysis.

High-performance liquid chromatography (HPLC) appears to be a good method for the separation and determination of extracted metal chelates[2,3] and, in this paper, separations of metal dithizonates by HPLC are reported.

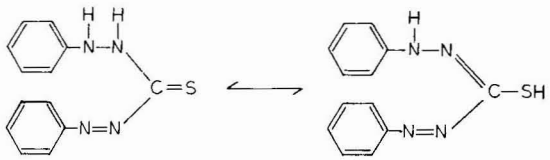


Fig. 1. Structure of dithizone.

EXPERIMENTAL AND RESULTS

Adsorbents

LiChrosorb SI 60 (30 μm) and Perisorb A (30–40 μm) silica gel were used for HPLC and DC-Alufolie Kieselgel 60 and DC-Alufolie Al_2O_3 F_{254} for thin-layer chromatography (TLC). They were all obtained from E. Merck, Darmstadt, G.F.R.

* Part VII: P. Heizmann and K. Ballschmiter, *J. Chromatogr.*, 137 (1977) 153.

TABLE I

THIN-LAYER CHROMATOGRAPHIC hR_F VALUES OF METAL DITHIZONATES

Dithizonate	*SiO_2 adsorbent*														*Al_2O_3 adsorbent*		
	Benzene		*Tetrahydrofuran in benzene (%, v/v)*					*Toluene*		*CH_3CN in CCl_4 (%, v/v)*					*Toluene*	*Benzene*	*$CHCl_3$*
	*A**	*B***	*1*	*2*	*3*	*4*	*5*	*A**	*B***	*1*	*2*	*3*	*4*	*5*			
Hg(II)	41	76	52	57	58	62	63	34	74	29	49	53	58	62	39	50	87
Cu(II)	31	61	46	52	55	59	61	25	55	19	43	47	55	58	0	0	20
Zn(II)	29	69	45	52	54	60	61	22	70	16	42	46	49	57	0	0	30
Ni(II)	29	38	42	51	54	59	61	20	35	14	38	44	50	53	3	5	83
Co(II)	23	48	31	41	45	54	56	11	38	6	27	33	42	47	14	28	85
Pb(II)	16	43	25	34	38	47	51	12	37	10	35	39	44	46	0	0	28
Cd(II)	5	20	—	—	—	—	—	3	18	2	6	11	18	20	0	0	41
Dithizone	12	—	—	—	—	—	—	10	—	—	—	—	—	—	0	0	22

* Our results.

** Results from the literature[4].

Liquid chromatograph

The liquid chromatograph consisted of the following: Labotron LDP 13 A pumps (1.5–80 ml/h) (Kontron Technik, Eching/München, G.F.R.); PTFE capillary tubing, 0.7 mm I.D. (Kontron Technik); glass columns, 2 and 3 mm I.D., 6 mm O.D. (according to Fig. 2 in ref. 3); PTFE injector (Fig. 2 in ref. 3); and a Zeiss PM 2 D detector with a micro-flow attachment and a 2-mV recorder.

Chemicals

The solvents used were benzene, carbon tetrachloride, tetrahydrofuran, acetonitrile, toluene, and chloroform (all p.a. quality from E. Merck).

Dithizone was dissolved in carbon tetrachloride and then cleaned up according to Iwantscheff[1].

The chelates were extracted from 10^{-5} *M* aqueous solutions of the elements. The pH of the aqueous layer was chosen to give only the primary chelates $M(II)Dz_2$ (M = Metal, Dz = dithizone). The pH values used for extraction were as follows: Hg(II), Cu(II) and Cd(II) dithizonates, 2; Co(II), Ni(II) and Pb(II) dithizonates, 7; and Zn(II) dithizonate, 8. TLC was used as a control.

Thin-layer chromatography of metal dithizonates

TLC was used to establish the optimal conditions for the separation in HPLC. Using single-component eluents or binary mixtures of solvents with small polarity differences, transposition of the TLC results to HPLC is possible. Table I summarizes the results of TLC separations on silica gel and alumina. Some of the results are not in accordance with those reported in the literature[4–6]; the reasons for this are not known.

The chelates were eluted in the sequence Hg(II) > Cu(II) > Zn(II) > Ni(II) > Co(II) > Pb(II) > Cd(II). On silica the chelates of Cu(II), Zn(II) and Ni(II) were eluted with small differences in their R_F values and their separation by HPLC would be difficult. The chelate of Cd(II) was strongly adsorbed. Separations of the components Hg(II)/Cu(II)/Zn(II)[Ni(II)]/Co(II)/Pb(II) by HPLC should be possible.

HPLC of metal dithizonates

The columns used were dry filled according to the Kirkland method[7]. When carefully applied, this method results in columns with good separation characteristics. All separations were carried out on silica gel at medium solvent flow-rates (10–40 ml/h) and medium pressures (20–50 bar), and benzene was used as the eluent. The dithizonates were detected at 525 nm.

When 20 μl of chelate solution were injected, the standard deviation of the peak areas was 1.5% for septumless injections and 4% for septum injections. For a given signal to noise ratio of 5:1, 10^{-8} g of Hg(II) and Cu(II) could be determined. By enriching the chelate in the organic layer, determinations of the elements in aqueous solutions at picogram levels should be possible.

The results of the metal-chelate separations are shown in Tables II–IV.

On short columns (Table II), separations between the components of Hg(II)/Ni(II)/Co(II) or Hg(II)/Zn(II)/Co(II) are possible (Figs. 2 and 3), whereas the separations of Hg(II)/Cu(II) and Cu(II)/Ni(II) are difficult.

The separation of Hg(II)/Cu(II) is improved when a longer column with a larger internal diameter is used (Table III). Here, also, the separation of Cu(II)/Ni(II)

TABLE II

HPLC OF METAL DITHIZONATES

Column, 300 × 2 mm LiChrosorb SI 60 (30 μm); eluent, benzene (10 ml/h); detection, 525 nm. t_A = analysis time. Elution sequence and retention times (min) at 10 ml/h: Hg(II) (6.8) > Cu(II) (7.8) > Ni(II) (9.7) > Zn(II) (10.0) > Co(II) (18.4).

Parameter	*Hg(II)/ Cu(II)*	*Hg(II)/ Ni(II)*	*Hg(II)/ Zn(II)*	*Hg(II)/ Co(II)*	*Cu(II)/ Ni(II)*	*Cu(II)/ Zn(II)*	*Cu(II)/ Co(II)*	*Ni(II)/ Co(II)*	*Zn(II)/ Co(II)*
Resolution, R_s	0.5	1.6	1.0	3.7	0.8	0.5	2.9	2.6	1.6
t_A (min)	12	13	15	20	13	15	20	20	20

TABLE III

HPLC OF METAL DITHIZONATES

Column, 600 × 3 mm LiChrosorb SI 60 (30 μm); eluent, benzene; detection, 525 nm. t_A = analysis time. Elution sequence and retention times (min) at 40 ml/h: Hg(II) (7.6) > Cu(II) (9.4) > Ni(II) (11.5) > Co(II) (16.3).

Components	*Flow-rate 40 ml/h*		*Flow-rate 20 ml/h*	
	Resolution, R_s	*t_A (min)*	*Resolution, R_s*	*t_A (min)*
Hg(II)/Cu(II)	0.7	12	1.1	24
Hg(II)/Ni(II)	1.7	14	2.1	28
Hg(II)/Co(II)	3.0	20	3.2	40
Cu(II)/Ni(II)	0.6	14	0.8	28
Cu(II)/Co(II)	1.7	20	2.0	40
Ni(II)/Co(II)	1.4	20	1.6	40

TABLE IV

HPLC OF METAL DITHIZONATES

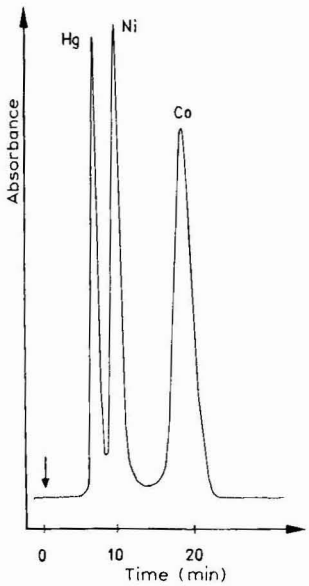
Column, 500 × 2 mm LiChrosorb SI 60 (30 μm); eluent, benzene (20 ml/h); detection, 525 nm. t_A = analysis time. Elution sequence and retention times (min) at 20 ml/h: Hg(II) (3.9) > Cu(II) (4.5) > Ni(II) (5.8) > Zn(II) (7.0) > Co(II) (9.4) > Pb(II) (14.2).

Parameter	*Hg(II)/ Zn(II)*	*Hg(II)/ Pb(II)*	*Cu(II)/ Zn(II)*	*Cu(II)/ Pb(II)*	*Ni(II)/ Zn(II)*	*Zn(II)/ Co(II)*	*Zn(II)/ Pb(II)*	*Co(II)/ Pb(II)*
Resolution, R_s	1.0	2.2	0.6	1.8	0.3	0.5	1.0	0.9
t_A (min)	15	24	15	24	15	13	24	24

is poor. Separations of the other elements are possible, even at high eluent flow-rates (40 ml/h or greater). Fig. 4 illustrates the separation of the Hg(II)/Cu(II)/Ni(II)/Co(II) dithizonates.

The chelates of Zn(II) and Pb(II) show tailing and poor resolutions, R_s (Table IV). Fig. 5 shows the separation of the Hg(II)/Zn(II)/Pb(II) chelates, while Fig. 6 shows the separation of the chelates of Hg(II), Ni(II), Co(II) and Pb(II).

The chelate of Cd(II) is strongly adsorbed. Its separation from the other



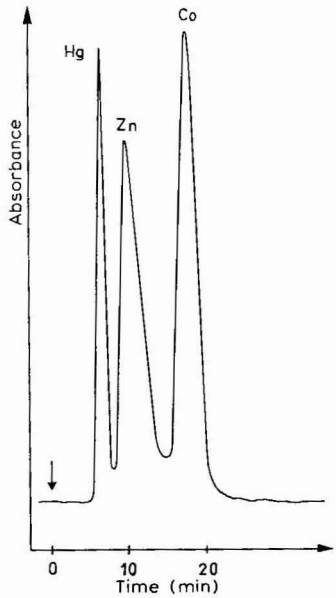


Fig. 2. HPLC separation of metal dithizonates. Packing, LiChrosorb SI 60 (30 μm); glass column, 300 × 2 mm; solvent, benzene; flow-rate, 10 ml/h.

Fig. 3. HPLC separation of metal dithizonates. Packing, LiChrosorb SI 60 (30 μm); glass column, 300 × 2 mm; solvent, benzene; flow-rate, 10 ml/h.

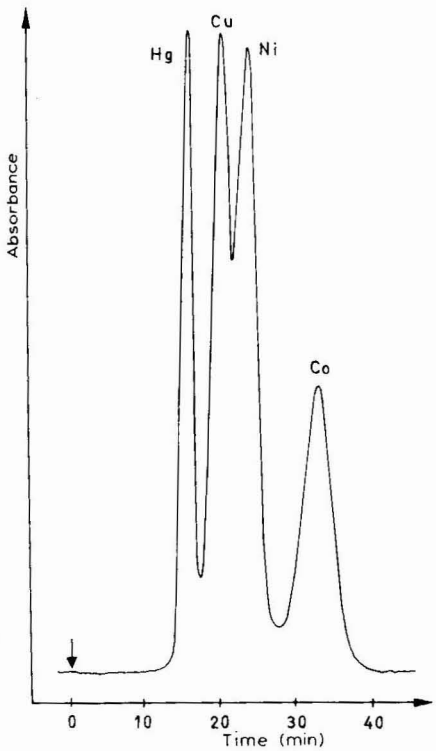
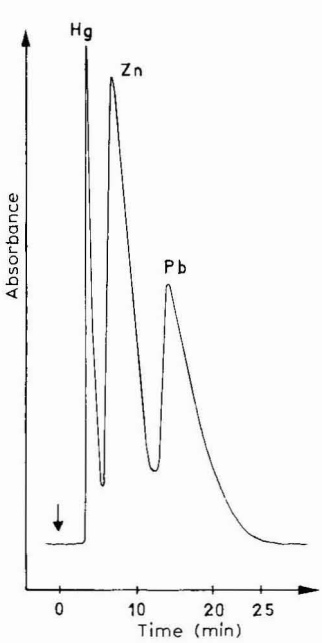
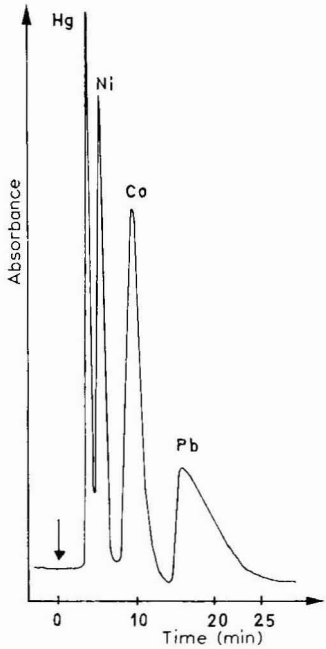


Fig. 4. HPLC separation of metal dithizonates. Packing, LiChrosorb SI 60 (30 μm); glass column, 600 × 2 mm; solvent, benzene; flow-rate, 20 ml/h.





Figs. 5 and 6. HPLC separation of metal dithizonates. Packing, LiChrosorb SI 60 (30 μm); glass column, 500 × 2 mm; solvent, benzene; flow-rate, 20 ml/h.

components can be accomplished by using a more polar eluent or a less polar adsorbent. On Perisorb A, the separation of the Cd(II) chelate from the other chelates is possible, but even here the Cd(II) chelate shows strong tailing.

CONCLUSION

HPLC on silica gel (LiChrosorb SI 60) with benzene as eluent permits the separation and determination of the dithizonates of Hg(II)/Ni(II)/Co(II)/Pb(II)/[Cd(II)], Hg(II)/Cu(II)/Co(II) and Hg(II)/Zn(II)/Pb(II). The separation of Cu(II)/Ni(II)/Zn(II) is difficult, but as the TLC results show, this separation can be improved by using alumina as adsorbent. Glass columns packed with 5-μm material are commercially available and can be used at head pressures up to 3000 p.s.i. Better resolutions and shorter analysis times than those reported here can be obtained.

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ISOELECTRIC FOCUSING AS A PUZZLE

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SUMMARY

The formation of strong complexes of most acidic dyes with Ampholine in isoelectric focusing has been demonstrated by altering the dye to Ampholine ratio, by re-running single dye bands and by measuring the UV–visible spectra of the complexes. These complexes can only be disaggregated by changing the dielectric constant of the solvent or by high temperatures, or both. On an equimolar basis, the order of the dissociating powers of the most commonly used disaggregating agents is dimethylformamide $\approx$ tetramethylurea $>$ dimethyl sulphoxide $>$ urea $>$ formamide. A two-step binding model is postulated: first a strong, polydentate and undissociated Ampholine–dye salt, followed by hydrophobic dye–dye interaction.

INTRODUCTION

In general, estimates of p*I* values determined by electrophoresis are lower than the p*I* values obtained by isoelectric focusing (IEF)[1,2], largely because of interactions of proteins and buffer ions during electrophoresis[3]. Conversely, it has been stated that the p*I* of a protein determined by IEF also represents its isoionic point[4]. This concept implies that there are no interactions among proteins and carrier ampholytes. While this might be generally true, there have nevertheless been reports of the interaction between Ampholine and several classes of amphoteric species, including proteins and nucleic acids.

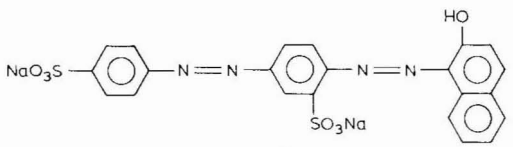
In the course of an extensive screening of dyes for their possible use as pH markers in IEF, some unusual and inexplicable results were obtained. This paper shows how dyes bind strongly to Ampholine and how it is possible to disaggregate these complexes.

EXPERIMENTAL

Materials

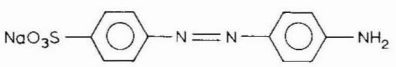
Acrylamide, N,N′-methylenebisacrylamide (Bis), tetramethylurea (TMU), dimethyl sulphoxide (DMSO), dimethylformamide (DMF) and formamide (FA) were obtained from Merck-Schuchardt (Munich, G.F.R.). Bis was recrystallized from acetone and acrylamide from chloroform, as described by Loening[5]. Ammonium persulphate and tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Labs., Richmond, Calif., U.S.A. Urea (ultrapure) was purchased from Mann Labs., New York, N.Y., U.S.A.

Of the several dyes investigated (more than 200, chosen from a list of 1500) we selected the following four: Biebrich Scarlet (I) (BDH, Poole, Dorset, Great Britain),



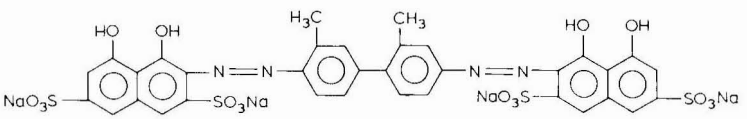
I

Sirius Supra Orange 7GL (Direct Orange 46) (II) (Bayer Italia S.p.A., Milan, Italy)



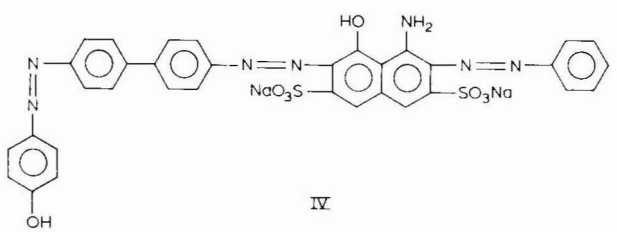
II

Benzo New Blue 5BS (Direct Blue 25) (III) (Bayer Italia) and



III

Benzo Dark Green B (Direct Green 1) (IV) (Bayer Italia).



IV

The rationale for choosing these four dyes is that two of them are amphoteric while the other two are not (and are strong acids). Moreover, two of them (Biebrich Scarlet and Benzo Dark Green B) have a strong affinity for proteins while the other two have a greater affinity for cellulose than for proteins. The criteria for choosing

the original 200 dyes were good solubility in water and absence of a metal as a ligand (because Ampholine binds to some metals[6]). Our results, however, apply to most of the acidic dyes we have investigated (which represent *ca.* 80% of the dyes tested). For the remaining *ca.* 20% (basic dyes), we can give no results at present as it is difficult to obtain good pH gradients in the pH range 9–11 in a gel slab.

Methods

IEF in gel slabs was performed in an LKB Multiphor 2117 chamber, with an ISCO Model 492 constant-wattage power supply[7]. The gels were prepared to contain 7.5% of acrylamide (the ratio of acrylamide to Bis being 25:1), 1% of Ampholine pH 2.5–4, 1% of Ampholine pH 3–6 and 0.1% of Ampholine pH 3.5–10. When additives were used, the gel was polymerized in two halves, as described by Hobart[8]. The additives used, and their final concentrations, were 8 *M* urea or 50% TMU or 50% DMF or 50% DMSO. The mechanical properties and optical clarity of the additive-containing gels were as follows: 8 *M* urea gels, same as control gels; 50% DMSO gels, transparency as good as the control gels, but softer and gluey; 50% DMF gels, completely opaque (milky appearance), very soft and sticky; 50% TMU gels, semi-liquid glue, which keeps losing fluid. With the last gels, the matrix is opaque and the Ampholines themselves are not completely soluble in this medium. Even when 10 or 12% of acrylamide was used, the situation was not much improved.

From the point of view of polymerization time, 8 *M* urea gels behave as control gels, while the 50% DMSO, 50% DMF and 50% TMU gels were poured in as the second layer and left to polymerize overnight (14–16 h). Usually, 1 *N* sodium hydroxide solution was used at the cathode and 1 *M* orthophosphoric acid at the anode. The gel was pre-run at 10 W for 1½ h, then the sample was applied and the run continued for 3½–5½ h at 14–15 W. The coolant temperature was usually 10–12°. Samples of 25 μl were applied to the cathodic site in pockets pre-cast in the gel, either at the original concentration (5 mg/ml in water) or at appropriate dilutions.

The apparent p*I* of each dye was measured by cutting out the coloured zone (*ca.* 60 μl of gel volume) and adding 0.25 ml of 10 m*M* potassium chloride solution. The pH was measured with a combined glass electrode with a Radiometer digital pH meter at 21°. No correction was made for the presence of additives. However, we verified experimentally that the pH increases by 0.05 pH unit per unit of urea molarity in the solution, while in the presence of DMF, TMU or DMSO the pH increases by 0.25 pH unit every 10% increments of concentration of any of the three additives in solution (all measurements at 21°).

UV–visible spectra were measured at room temperature (21°) with a Cary 118 spectrophotometer (Varian, Palo Alto, Calif., U.S.A.) in a 50 m*M* acetate buffer (pH 3.85) at a dye concentration of 30 μg/ml. This pH was the pH of a 1:1 mixture of Ampholines pH 2.5–4 and pH 3–6, so that increasing amounts of Ampholine in the solution did not alter the pH. This pH also happens to be the pH at which two of the dyes (Biebrich Scarlet and Sirius Supra Orange 7GL) show some of their apparent p*I*s in the absence of additives in the gel.

Melting curves (hyperchromic thermal transitions) at 585 nm were measured in a Gilford Model 2400 automatic spectrophotometer equipped with a K2R thermostat (Lauda) and a device programmed to increase the temperature of the cuvettes at a constant rate of 1.0°/min (ref. 9).

RESULTS

Evidence for complex formation

Originally, the 200 dyes selected were run over a wide pH range (pH 3.5–10) in order to be able to select those more suitable as pH markers. When the chosen dyes were subsequently run over narrow pH gradients, we observed non-reproducible and puzzling results, which prompted a more thorough investigation of their behavior in IEF.

As shown in Fig. 1, when the concentration of Biebrich Scarlet was varied over a 50-fold range, the pattern became increasingly complex, starting from one or two bands at low concentrations to six to eight at high concentrations. The apparent p*I* values of the four major components were 3.57, 3.76, 3.85 and 4.11. Similar results were obtained with the other dyes tested. This polydispersity, dependent on the ratio of Ampholine to sample, is currently interpreted in terms of the formation of complexes of the two species[10]. To check this interpretation, the bands of p*I* 3.76, 3.86 and 4.11 were cut out and re-run in a second gel. As shown in Fig. 1 (inset), they all behave as identical species and display a major component that does not occur in a band at any of the above positions, but at p*I* 3.57. Thus, reducing the sample load shifts the pattern towards lower p*I* species and reduces apparent microheterogeneity.

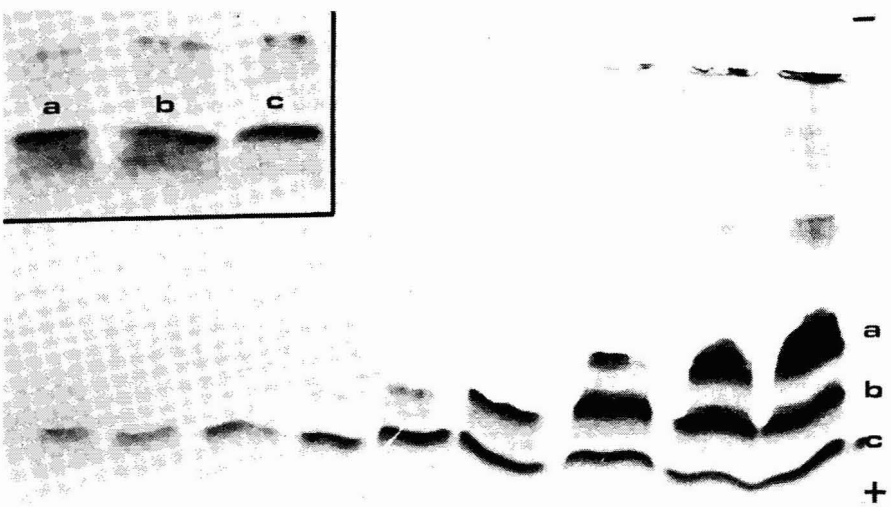


Fig. 1. IEF of Biebrich Scarlet in the pH range 2.5–6. Sample (25 μl) was applied to a pre-focused gel slab in pockets pre-cast in the gel at the cathodic side. The amounts applied were (from left to right) 2.5, 3.1, 4.2, 6.25, 12.5, 25, 50, 62.5 and 125 μg. The inset shows a re-run of bands (a), (b) and (c) in a second gel in the same pH range. The three bands behave identically and form bands in the same positions.

Additional evidence also comes from the detection of focused Ampholine patterns, on the basis of their different refractive indices[11]. As soon as focusing was completed, the gel was photographed against a black background with side illumination. Fig. 2 shows that all of the dye bands run on top of major Ampholine ridges, none being located in the valley between two adjacent species.

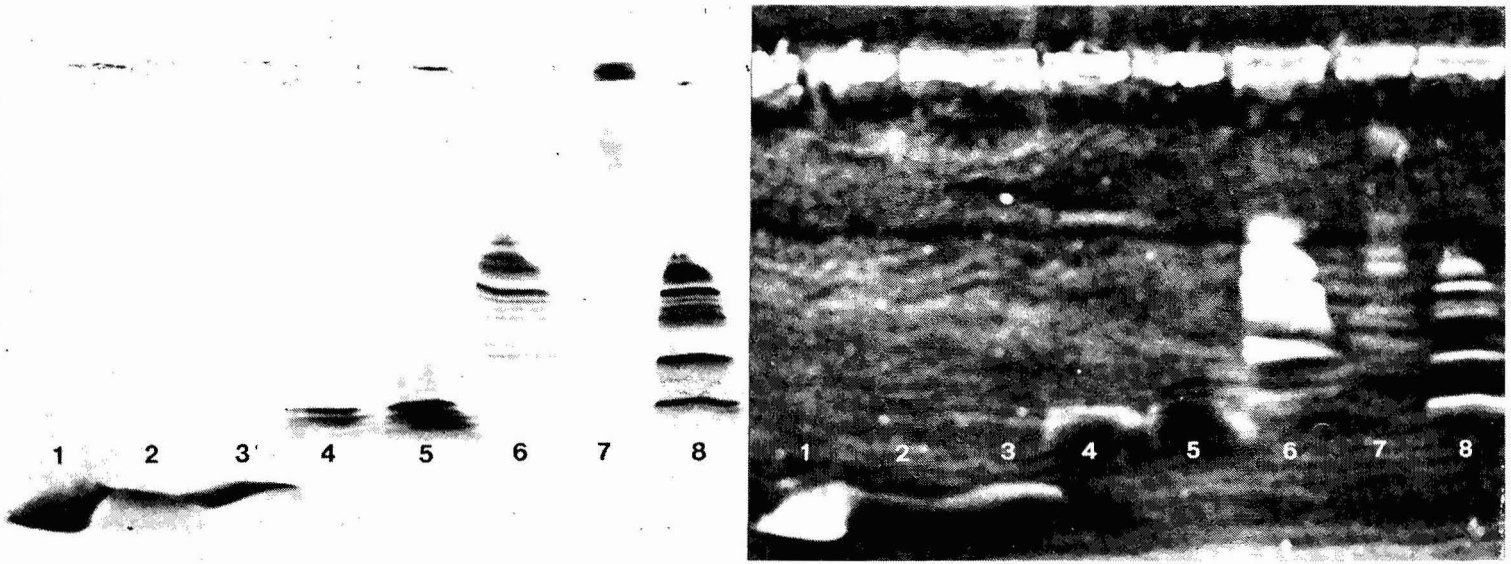


Fig. 2. IEF of dyes. Each dye was a single, major band cut from a previous run and re-run by applying directly the gel-trapped dye band to the surface of the gel slab, near to the cathodic (upper) side. Left: as soon as focusing was completed, the gel was photographed directly in transmitted light. Right: the same gel photographed in reflected light (against a black background with side illumination) to show up the refraction lines of focused Ampholine. The intense white lines are the focused dye bands (they are white because of light scattering and fluorescence, particularly with Nos. 1 and 6). The long, thin, white lines represent focused Ampholine species (Ampholine ridges); the long, thin, dark lines are the valleys in between. The white rectangles at the top are the pieces of gel used to apply the samples to the gel slab. Dyes: 1 = Reactone Red G (Geigy); 2 = Violet Supracen 4BF (BASF); 3 = Violet Supracen 3R (BASF); 4 = Sirius Violet 3B (Bayer); 5 = Diphenyl Brilliant Violet B (Geigy); 6 = Sirius Supra Orange 7GL; 7 = Benzo Red 8BS (Bayer); 8 = Biebrich Scarlet.

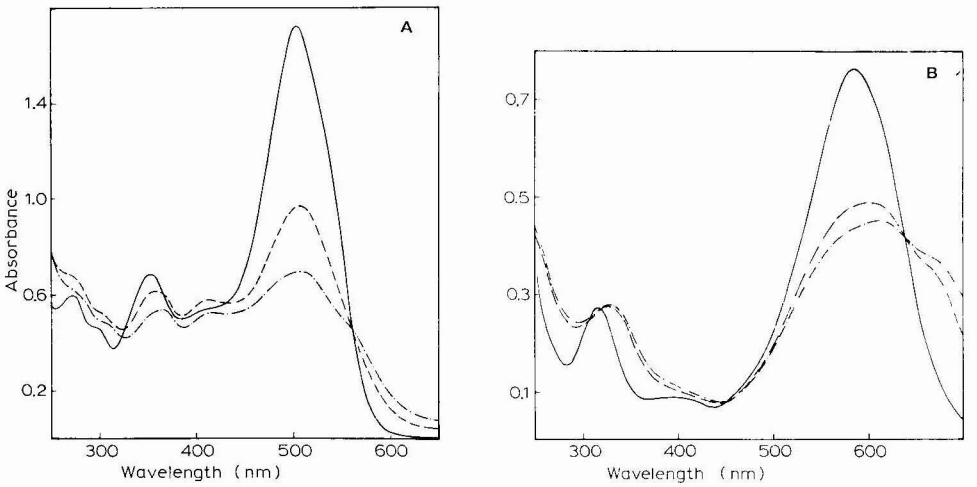


Fig. 3. UV–visible spectra. The spectra were measured with a Cary 118 spectrophotometer in 50 m*M* acetate buffer (pH 3.85) at a dye concentration of 30 μg/ml. ———, Spectrum of the dye alone; — — —, with 1 μl of Ampholine pH 2.5–6 added; –·–·–, with 10 μl of Ampholine pH 2.5–6 added. A, Biebrich Scarlet; B, Benzo New Blue 5BS.

Perhaps the strongest evidence of dye–Ampholine binding comes from UV–visible spectra. When Biebrich Scarlet was analysed in the presence of increasing concentrations of Ampholine, all of the peaks and the minima in its spectrum were shifted towards the red end of the spectrum (bathochromic shift) by 7–10 nm (Fig.3A) and, in addition, a shoulder appeared at 562 nm. The absorption of the 503-nm peak was also extensively reduced; however, part of this reduction might have been due to light scattering by the finely dispersed precipitate formed (only in the case of this dye and at a high Ampholine concentration) by the Ampholine–dye complex. An even more dramatic red shift is apparent in the spectrum of Direct Blue 25, where the main 585-nm peak is shifted to 612 nm and a new chromophore appears at 672 nm (Fig. 3B). As more Ampholine is added to the system, the absorbance of the 612-nm peak decreases in favour of the 672-nm chromophore.

Partial disaggregation in urea

When increasing concentrations of Biebrich Scarlet were run in a control and in an 8 *M* urea gel, the apparent dye microheterogeneity was completely eliminated in the presence of the dissociating agent (Fig. 4). Moreover, the dye ran through to a much lower apparent p*I* value (2.84) compared with the p*I* spectrum in the pH range 3.57–4.11 for the control. This experiment was also followed kinetically. After an exponential rate for the first 15 min, the single dye band in the urea gel travelled at a constant linear rate of 0.66 mm/min up to 60 min. It took 3 h for the dye to reach an apparent p*I* value of 2.84. Conversely, the five major bands of Biebrich Scarlet in the control gel reached their apparent p*I* values (pH range 3.57–4.11) in 30–50 min. Their relative positions remained essentially unaltered for the duration of the experiment (Fig. 5). It is clear that the much slower rate in the absence of urea is due to interaction with Ampholine; were this is not the case, the sample in 8 *M* urea should travel at a slower rate, owing to the higher viscosity of this medium.

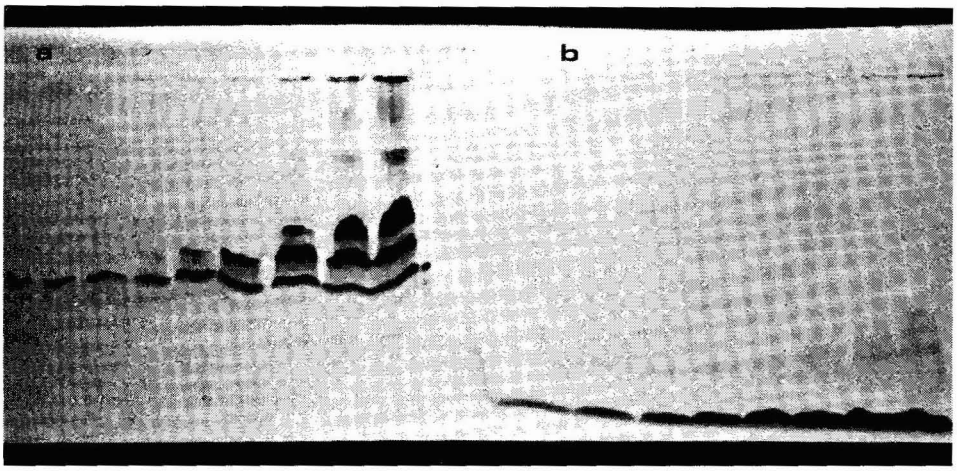


Fig. 4. IEF of Biebrich Scarlet (a) in a control gel and (b) 8 *M* urea gel. Sample loads in the two gels as in Fig. 1. The cathode is uppermost. The two black lines are the electrode filter-paper strips.

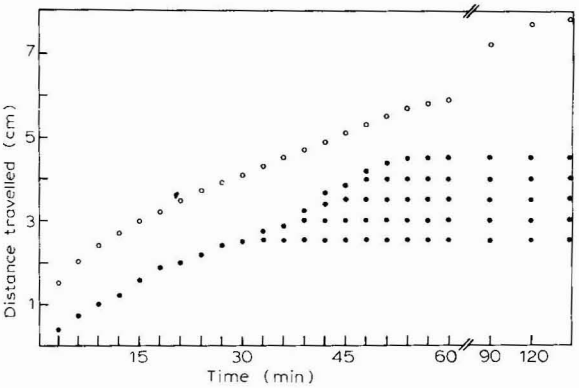


Fig. 5. IEF followed kinetically. The migration rates of the five major bands (closed circles) of Biebrich Scarlet in the control gel and of the single band (open circles) in the 8 *M* urea gel were measured. The migration distance was taken from the edge of the application pocket at the cathodic side.

The experiments were then repeated with all four dyes. The run was performed for a much longer time ($6\frac{1}{2}$ h) under a high voltage gradient (100 V/cm) so as to ensure ample time for equilibrium conditions to be attained. The apparent p*I* values reached were 2.84 (scarlet), 3.25 (orange), 3.53 (blue) and 3.99 (green) (Fig. 6). It is clear that, even under these drastic conditions, no complete disaggregation of the Ampholine–dye complex was achieved in 8 *M* urea.

Complete disaggregation in DMF, TMU and DMSO

When the experiments were run in DMF, TMU or DMSO, the dye–Ampholine complexes seemed to be effectively broken up. In fact, in DMF and TMU all of the dyes ran through the entire length of the gel and escaped in the anodic compartment, being slowly absorbed in the anodic filter-paper strip. With all three reagents, all of

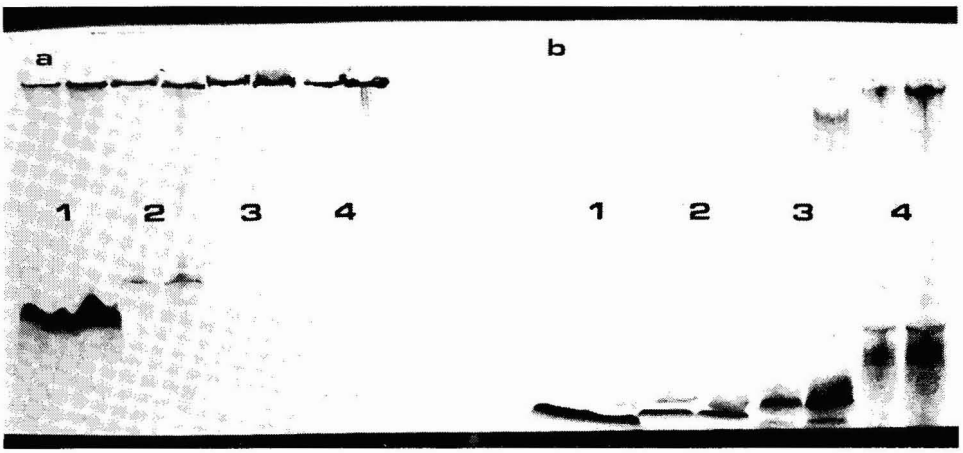


Fig. 6. IEF of dyes (a) in a control gel and (b) 8 *M* urea gel. Each sample was applied at two concentrations (62.5 and 125 μg). Samples: 1 = Biebrich Scarlet; 2 = Sirius Supra Orange 7GL; 3 = Benzo New Blue 5BS; 4 = Benzo Dark Green B.

the dyes reached an apparent p*I* value of *ca.* 2. Judging from the time needed for these conditions to be achieved in IEF and from the apparent p*I* values obtained, the order of the disaggregating power of these four reagents appears to be 50% DMF $\approx$ 50% (TMU > 50% DMSO > 8 *M* urea.

Melting curves

Another means of affecting the Ampholine–dye complexes, apart from altering the dielectric constant of the solvent, should be by changing the solvent temperature. Melting curves were therefore run in the presence of increasing concentrations of each of the four additives used, and the results are summarized in Fig. 7. In the case of Direct Blue 25, the complex has a melting point (T_m) of 87° in the absence of disso-

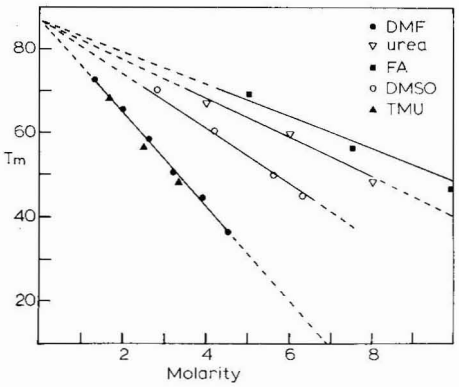


Fig. 7. Hyperchromic thermal transitions. Melting curves were measured with a Gilford Model 2400 automatic spectrophotometer with a programmed temperature increase of 1.0°/min at 585 nm. Benzo New Blue 5BS (30 μg/ml) was dissolved in 50 m*M* acetate buffer (pH 3.85) containing increasing molarities of each of the five dissociating agents tested. The melting point (T_m) of the Ampholine–dye complex, extrapolated to zero additive concentration, is 87°.

ciating agents. The relative slope of each of the curves obtained indicates the dissociating power of the additive concerned: the steeper the curve, the more powerful the dissociating agent. Hence it is clear that in 50% DMF (≡ 6.5 *M*) or 50% TMU (≡ 4.2 *M*) the complex is completely disaggregated at room temperature, while it is not completely inhibited in 50% DMSO (≡ 7.0 *M*). The situation is even worse in the presence of 8 *M* urea. These experiments are in excellent agreement with the empirical order of dissociating power for these four additives as obtained by IEF.

DISCUSSION

The results show that most acidic dyes form strong complexes with Ampholine, which are not dissociated during the process of focusing itself, by high voltages (100 V/cm), relatively high temperatures (15°) (it is customary to run focusing experiments at 2–4°) or prolonged focusing times (up to 8 h). These complexes can be disaggregated only by high temperatures (87° for Direct Blue 25), by drastically changing the dielectric constant of the solvent, or by a combination of the two. With dissociating agents, we have demonstrated that the most effective are DMF and TMU and the least effective are urea and formamide (on an equimolar basis). In IEF experiments we chose the limiting concentrations at which gel focusing can conveniently be carried out (50% TMU is in fact already too much). Higher levels of DMF, TMU and DMSO will either severely hamper gel polymerization or precipitate Ampholine, or do both. Urea gels could also be run in the presence of 10 or even 12 *M* urea[12], but this procedure would require much higher temperatures (55°) than are permissible for gel focusing in slabs.

Our results could be interpreted on the basis of a sequential binding model: first a polydentate salt bridge (with strong $R\text{–}NH_3^+$ $^-O_3S\text{–}R'$ and $R\text{–}NH_3^+$ $^-OOC\text{–}R'$ interactions), followed by hydrophobic dye–dye molecule interaction. Once the polydentate (and undissociated) Ampholine–dye complex is formed, the highly negative charge of the dye is decreased to such an extent that hydrophobic dye–dye interactions are made possible. This in turn could lead to an infinite stack of alternating aromatic rings among dye molecules, as demonstrated, for instance, in the case of riboflavin–adenosine molecules in aqueous solutions[13], reaching such a high molecular weight that the aggregate precipitates (all of the dyes tested are precipitated in the presence of Ampholine at 2–4°). It is symptomatic that these complexes are sensitive to both types of reagents, such as DMF and TMU, which mostly break hydrophobic interactions, and high temperatures, which mostly affect hydrogen bonds.

There have been hints in the literature about Ampholine–dye interaction[14]. Vesterberg[15] and Söderholm *et al.*[16] arrived empirically at a formulation of dissociating conditions by suggesting the staining of gels directly in the presence of Ampholine with Coomassie Brilliant Blue R 250 at high temperatures (60–65°) and at high alcohol concentrations (28% methanol).

Our results could have considerable biological implications. In fact, while Dean and Messer[17] and Baumann and Chrambach[18] excluded any protein–Ampholine interactions, the opposite results have been reported by other workers. Thus Frater[19] reported the binding of Ampholine to wool proteins and Kaplan and Foster[20] and Wallevik[21] complex formation between Ampholine and bovine serum albumin (BSA). These apparent discrepancies can be reconciled as follows: in general, in the pH

range 4–9 there is no Ampholine–protein interaction, but with strongly acidic (and possibily strongly basic) proteins or with unusual structures (BSA in known to bind to a multitude of ligands) interaction with Ampholine is strongly suspected. Another classical example is the focusing of tRNAs. Originally reported by Drysdale and Righetti[22], this was later demonstrated to be an artefact elicited by strong tRNA–Ampholine binding[9]. The two cases have strong common relationships: both tRNAs and the dyes used in this work are strong acids; the microheterogeneity is extensively reduced in both instances by 8 *M* urea; both classes of compounds exhibit apparent p*I*s in the pH range 3.5–4.5.

There are also other puzzling effects that could be interpreted in the light of our results, such as the focusing of soil humic substances[23], which has been challenged by Thornton[24]. Another interesting case is the focusing of heparin[25]. Given the known composition of heparin (a sulphated polysaccharide containing three SO_3^- groups per disaccharide unit), the finding that it is resolved by IEF into about 21 components with p*I* values in the pH range 3.2–4.2 seems to be as good as the finding that Biebrich Scarlet and Benzo New Blue 5BS (which are not even amphoteric) have p*I* values in the same pH range.

In conclusion, whenever complex formation between Ampholine and a substance under investigation is suspected, the experiment should be run at least in 8 *M* urea or, if possible, in 50% DMF and/or at high temperatures, *i.e.*, under conditions that ensure complete dissociation of the complex. We feel that we can now explain some of the puzzling effects that occur in isoelectric focusing.

ACKNOWLEDGEMENTS

We thank Dr. Franco Corbani of I.T.I.S. "C. Facchinetti", Castellanza, Milan, for kindly allowing us to utilize the 1500-dye collection of his Institute. This work was supported by grants Nos. 76.01488.04 and CT 76.00311.06 from the Consiglio Nazionale delle Ricerche (CNR, Rome).

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Note

Selective liquid–liquid partition systems for the chromatographic analysis of some coumarins and phenolic acids (derivatives of cinnamic acid)

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(Received February 28th, 1977)

Phenolic acids that are derivatives of cinnamic acid frequently occur in plants as products of biosynthesis from amino acids (phenylalanine). They play some role in plant physiology as stimulants of plant growth and have antibacterial, antiviral and antifungal activity. It has been reported that some of them accumulate in cancer tissues.

There have been numerous papers on the separation of phenolic acids by paper and thin-layer chromatography, column (gas and liquid) chromatography and electrophoresis[1–11]. However, the methods reported so far do not permit the direct separation of a large number of isomeric phenolic acids and their derivatives (gas chromatography requires derivatization to trimethylsilyl compounds). Therefore, it seemed worthwhile to investigate liquid–liquid systems with minimal mutual solubility of the phases as potential highly selective systems for the separation of the naturally occurring derivatives of cinnamic acid.

EXPERIMENTAL

The "moist paper" technique with controlled impregnation of paper with the aqueous phase was used. Whatman No. 4 paper strips, 7 × 23.5 cm, were impregnated with a 1% aqueous solution of citric acid (to suppress the ionization of the phenolic acids), the excess of the liquid was removed by blotting between two sheets of filter-paper and the phenolic acids and coumarins were spotted as 0.1–0.5% acetone solutions (*ca.* 10 μl). After partial drying to a moisture content corresponding to 0.5 ml of aqueous phase per gram of dry paper, the paper strips were developed in all-glass tanks for descending development[12]. The spots were localized in UV light (at 360 nm) after spraying with a saturated solution sodium of hydrogen carbonate or by coupling with bis-diazotized benzidine.

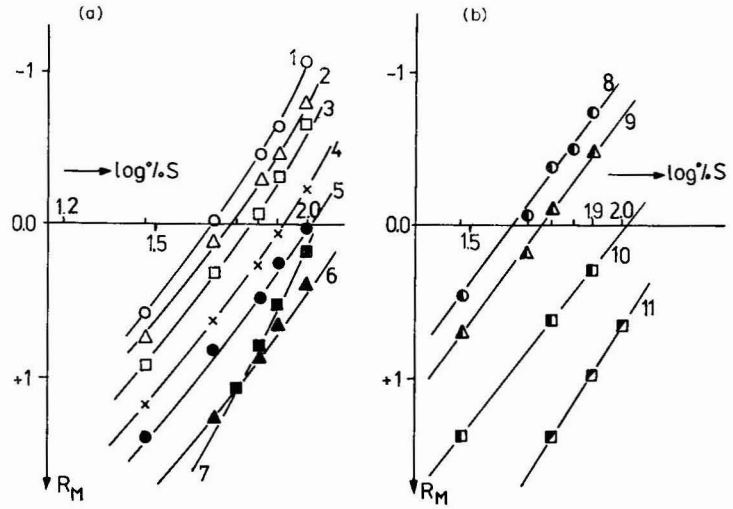


Fig. 1. R_M values of (a) phenolic acids and (b) related coumarins plotted against concentration of diisopropyl ether (S) in the developing solvent. Diluent: cyclohexane. For identification of solutes, see Table I.

RESULTS AND DISCUSSION

The phenolic acids were chromatographed in systems of the type polar solvent diluted with cyclohexane or benzene–1% aqueous solution of citric acid. Electron-donor solvents of various polarities were used as polar components of the mobile phase: diethyl ether, diisopropyl ether, methyl *n*-propyl ketone, diisobutyl ketone and tri-*n*-butyl phosphate. The results are presented as graphs of R_M values of the solutes plotted against the volume-per cent concentration of the polar solvent (on a logarithmic scale); such plots provide a quantitative characterization of the partition

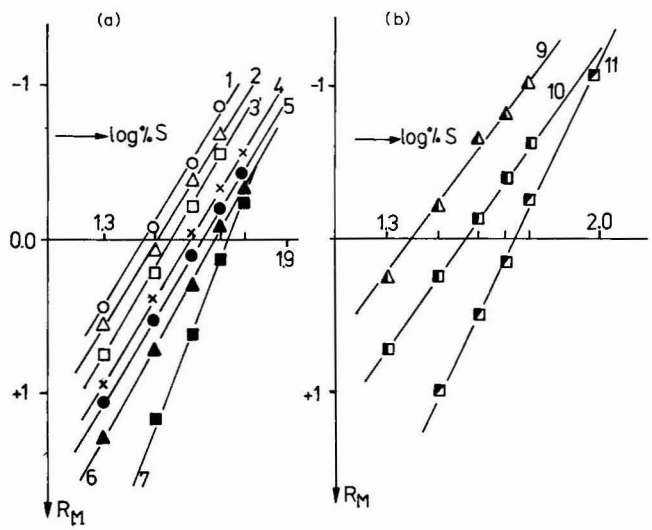


Fig. 2. As Fig. 1; developing solvent, solutions of methyl isopropyl ketone (S) in cyclohexane.

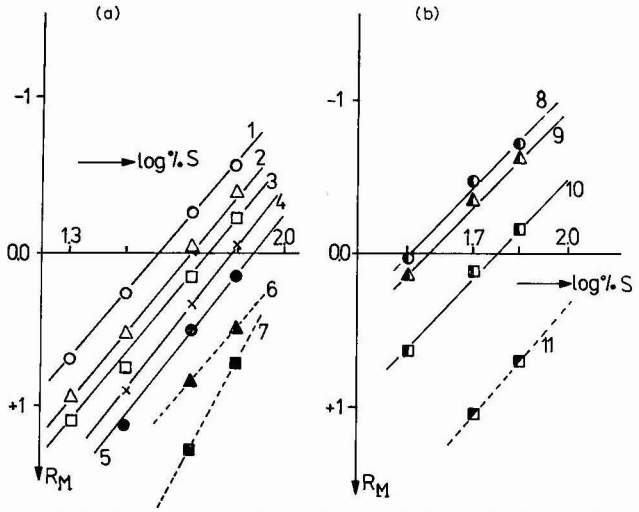


Fig. 3. As Fig. 1; developing solvent, solutions of diisobutyl ketone in cyclohexane.

system and indirect information about the presumed molecular solvation mechanism in the organic phase[13].

Fig. 1 shows that diisopropyl ether is a good extractant of the phenolic acids; a suitable range of k' values is obtained by dilution of the polar solvent with cyclohexane to *ca.* 80% concentration. The R_M *versus* log [iPr_2O] lines are approximately parallel, which indicates similar solvation mechanisms. Analogous results were obtained when diethyl ether was used as the polar solvent.

The slopes (apparent solvation numbers) of the R_M *versus* log [solvent] lines given in Table I seem to indicate that coumaric and ferulic acids form solvation complexes with ethers in the ratio 1:2 or even 1:3; for caffeic acid and aesculetin the plots

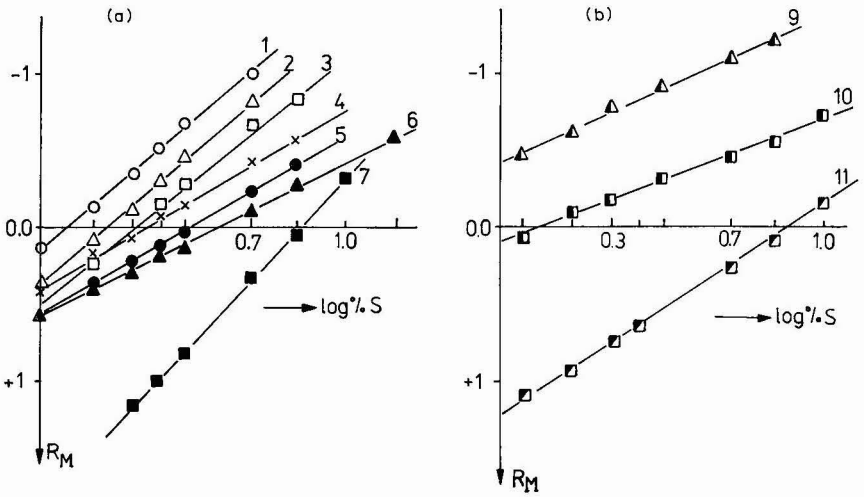


Fig. 4. As Fig. 1; developing solvent, solutions of tri-*n*-butyl phosphate in benzene.

TABLE I

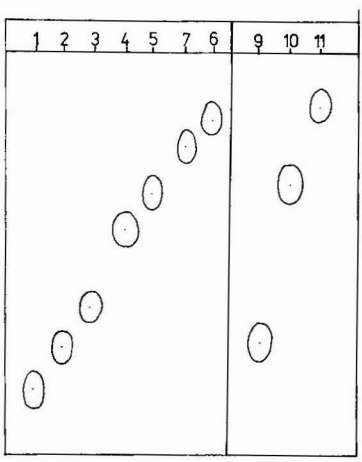
SLOPES (ABSOLUTE VALUES) OF R_M *vs.* LOG % S PLOTS FOR VARIOUS POLAR COMPONENTS OF THE DEVELOPING SOLVENT

No.	*Solute*	Et_2O	iPr_2O	*MePrCO*	iBu_2CO	*TBP*
1	*o*-Coumaric acid	2.8	2.8–3.2	3.3	2.4	1.7
2	*m*-Coumaric acid	2.8	2.7–2.9	3.3	2.5	1.7
3	*p*-Coumaric acid	2.8	2.6–3.0	3.3	2.4	1.6
4	Ferulic acid	2.8	2.5–2.8	3.3	2.4	1.2
5	Isoferulic acid	2.8	2.6	3.4	2.5	1.2
6	Sinapic acid	3.3	3.0	3.5	2.5	1.0
7	Caffeic acid	5.2	4.0	5.0	4.0	2.1
8	4-Hydroxycoumarin	2.8	2.6		2.1	—
9	Umbelliferone	2.8	2.6	2.8	2.0	0.9
10	Scopoletin	2.7	2.5	2.9	2.1	0.8
11	Aesculetin	5.5	3.5	4.0	2.8	1.2

are steeper (3.0–5.5 using diethyl ether, diisopropyl ether and methyl *n*-propyl ketone), presumably because of larger number of unhindered hydroxyl groups and the formation of higher solvates.

Figs. 2 and 3 illustrate the chromatographic behaviour of the phenolic acids and coumarins in solvent systems containing methyl *n*-propyl ketone or diisobutyl ketone. For methyl *n*-propyl ketone the plots are steeper, indicating stronger solvation of the solutes. The solvation ability of the latter solvent is lower owing to its greater molar volume (lower molar concentrations) and steric shielding of the carbonyl group.

Of the five polar solvents investigated, the strongest extraction ability was exhibited by tri-*n*-butyl phosphate (TBP): even low concentrations of the extractant in benzene caused significant increases in the R_F values of the solutes (Fig. 4). The



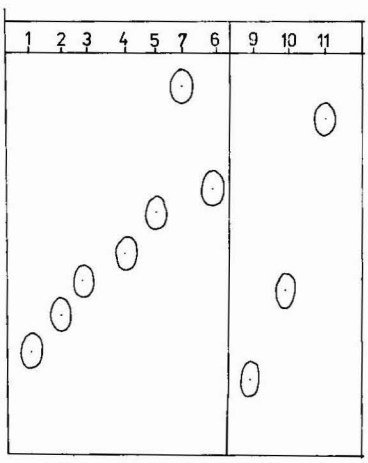


Fig. 5. Chromatogram of the solutes obtained for development with an 80% solution of diisopropyl ether in cyclohexane.

Fig. 6. Chromatogram of the solutes obtained for development with a 2.5% solution of tri-*n*-butyl phosphate in benzene.

slopes of the plots (Table I) seem to indicate that TBP, in the concentration range studied, forms 1:1 solvation complexes with various contributions of 1:2 complexes, which tend to be formed especially by solutes with unhindered hydroxyl groups (*i.e.*, without vicinal methoxy groups).

R_M *versus* solvent composition relationships enable one to choose partition systems with optimal spacing of the spots on the chromatograms; Figs. 5 and 6 illustrate chromatograms obtained for two solvent systems chosen in this way. The duration of analysis is limited owing to the low viscosities of the developing solvents (*ca.* 30 min for a distance of 16 cm). As the two liquid phases are almost immiscible, the selectivity of the systems is relatively high, as can be seen from a comparison of the chromatographic parameters with those reported in the literature (Fig. 7). The systems of the type investigated are therefore also promising in high-performance liquid chromatography with silica impregnated with water, *e.g.*, by the *in situ* technique[14].

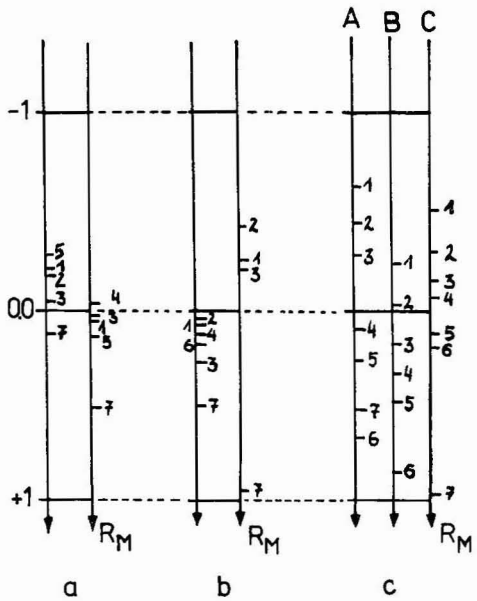


Fig. 7. Comparison of the selectivities of various chromatographic systems: a, ref. 11; b, ref. 2; c, this study; A and C, data from Figs. 5 and 6, respectively; B, chromatogram developed with a 50% solution of diisobutyl ketone in cyclohexane.

The solutes investigated have a wide range of capacity ratios, from the hydrophobic *o*-coumaric acid (a single hydroxyl group tends to form an internal hydrogen bond with the double bond in the side-chain) to the hydrophilic caffeic acid, aesculetin and especially chlorogenic acid. Therefore, the "general elution problem"[15] which may be encountered in the column chromatography of these compounds can be solved by gradient elution based on the R_M ($\log k'$) *versus* solvent composition relationships[16].

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Note

Sensitive gas–liquid chromatographic method for determination of valproic acid in biological fluids

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(Received February 9th, 1977)

Valproic acid (di-*n*-propyl acetate, VPA) has been shown to be effective against petit mal epilepsy and, in combination with phenobarbital, against grand mal epilepsy and other forms of epilepsy with absences and tonic–clonic seizures[1–4]. The exact mode of action is not yet clear, but hypothesis includes the inhibition of an enzymatic system that catalyses the transformation of γ-aminobutyric acid (GABA) in the central nervous system[5]. An elevation of GABA level, however, is suggested to provide protection against a number of forms of epileptic seizures[6]. Little information is available on the pharmacokinetics of VPA, including plasma protein binding behaviour, that can profoundly influence the distribution of the drug in the body[7,8]. All this is essential information, as attempts are made to define a therapeutic concentration range of VPA in plasma, as has been done with a number of drugs, particularly antiepileptic drugs[9].

Methods currently available for the determination of VPA in plasma or urine[10–13] are either time consuming or lack the sensitivity needed for pharmacokinetic studies where concentrations need to be followed for long periods after administration of the dose. Small concentrations may be encountered in particular in cerebrospinal fluid or in saliva if the drug is as highly protein bound as is suggested for VPA[14].

This paper describes a simple and sensitive gas–liquid chromatographic (GLC) method for the determination of VPA in plasma, saliva, spinal fluid and urine.

MATERIALS AND METHODS

Reagents

Sodium valproate was kindly supplied by Desitin-Werk Carl Klinke (Hamburg, G.F.R.). Caprylic acid (Fluka, Buchs, Switzerland) was used as an internal standard; the internal standard solution was prepared by dissolving 4 mg of caprylic acid in 100 ml of distilled water. All solvents used were of reagent grade (Merck, Darmstadt, G.F.R.).

Gas chromatography

A Hewlett-Packard Model 5736 A dual-column gas chromatograph was used, equipped with a flame-ionization detector. The silanized glass column (6 ft. $\times$ 2 mm I.D.) was packed with free fatty acid phase (FFAP, Applied Science Labs., State

College, Pa., U.S.A.) on 80–100-mesh Gas-Chrom Q and operated at 170°. The carrier gas (nitrogen) flow-rate was 30 ml/min and the injection port temperature was 250°. The detector was operated at 250° with a hydrogen flow-rate of 30 ml/min and an air flow-rate of 240 ml/min. Under these conditions, the retention times were 2.8 min for VPA and 4.3 min for caprylic acid. The results were recorded on a Hewlett-Packard Model 7123 A 10-in. recorder at a chart speed of 0.25 in./min. For data analysis, a Hewlett-Packard Model 3380 A integrator was also used.

Procedure

Blood samples (5–6 ml) were drawn by venopuncture from patients receiving VPA and centrifuged within 2 h at 4000 *g* for 10 min. VPA was stable for over 4 months when the plasma was kept at −20° until analysis. A 1-ml volume of plasma was transferred into a 12-ml glass tube and 1 ml of the internal standard solution containing 40 μg of caprylic acid were added. The mixture was acidified with 1 ml of 0.5 *N* hydrochloric acid and the compounds were extracted into 5 ml of organic solvent (*n*-hexane–chloroform, 1:1) containing 2% of methanol by shaking the tubes for 4 min. Following brief centrifugation, 3.5 ml of the organic layer were transferred into a second 12-ml centrifuge tube to which 2 ml of 0.5 *N* sodium hydroxide solution were subsequently added. After shaking and centrifuging, the organic phase was discarded and 1.5 ml of the aqueous layer were pipetted into a pointed tube, together with 1 ml of 3 *N* hydrochloric acid and 50 μl of chloroform. The tubes were vortexed for 30 sec and centrifuged for 2 min. A 1-μl volume of the organic (lower) phase was then injected on to the GLC column.

Determinations of VPA in urine, spinal fluid, saliva and dialysis buffer obtained from protein binding studies were carried out by using the same procedure as described for plasma. For analysis of the glucuronide metabolite of VPA, urine samples were diluted 1:5 with 0.2 *M* sodium acetate buffer of pH 5.0 and the solution was incubated for 24 h at 37° with 2000 Fishman units of a glucuronidase–arylsulphatase solution from *Helix pomatia* (Serva, Heidelberg, G.F.R.). A 1-ml volume of the buffer sample after hydrolysis was assayed as described above.

Calibration graphs for the determination of VPA in plasma or the other biological fluids were obtained by taking blank material to which known amounts of VPA were added. The calibration graphs were tested in the concentration ranges 0.5–150 μg/ml in plasma and 0.5–20 μg/ml in spinal fluid, saliva and buffer. The chromatograms were analyzed according to two different methods: (1) by plotting the peak-height ratio of VPA to the internal standard against the known concentrations; and (2) by using the integrator and plotting the ratio of the area under the curve of VPA to that of the internal standard against the known concentrations.

RESULTS AND DISCUSSION

A typical chromatogram of VPA is shown in Fig. 1. No interfering endogenous compounds are co-extracted with VPA as can be seen when blank plasma is carried through the procedure (C). The peaks of VPA and the internal standard are almost symmetrical and are well separated from each other.

The calibration graphs for VPA in plasma and urine are shown in Figs. 2 and 3. The concentration range covered is 1–150 μg/ml. To our knowledge, this is the

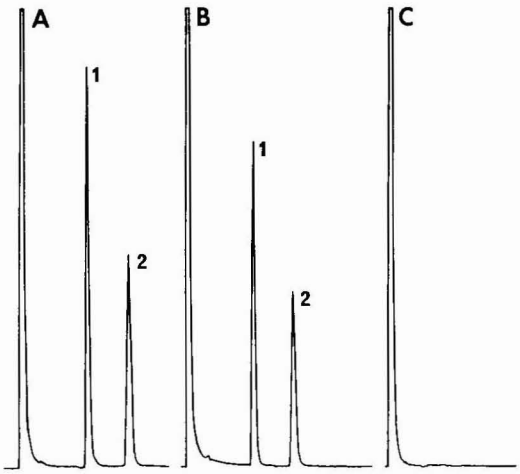

Fig. 1. Gas chromatograms for (A) extraction from blank plasma to which VPA (60 μg) and internal standard (40 μg) were added; (B) material extracted from plasma of an epileptic patient receiving VPA; (C) material extracted from blank plasma.

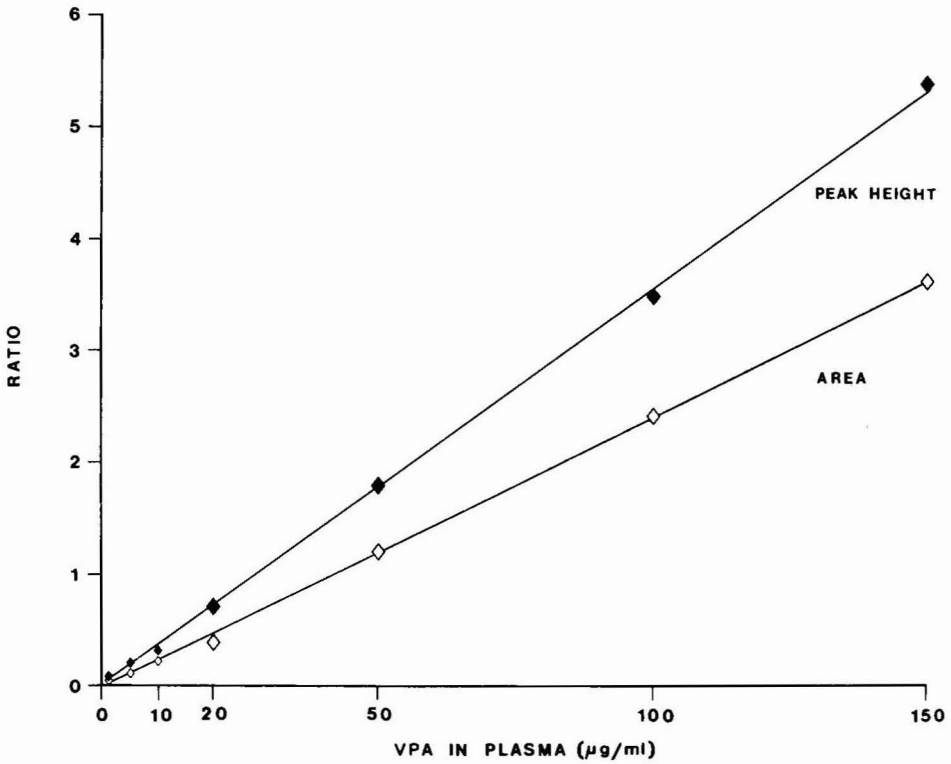

Fig. 2. Calibration graphs for VPA following extraction from plasma. Graphs are shown for analysis with peak-height and peak-area ratios.

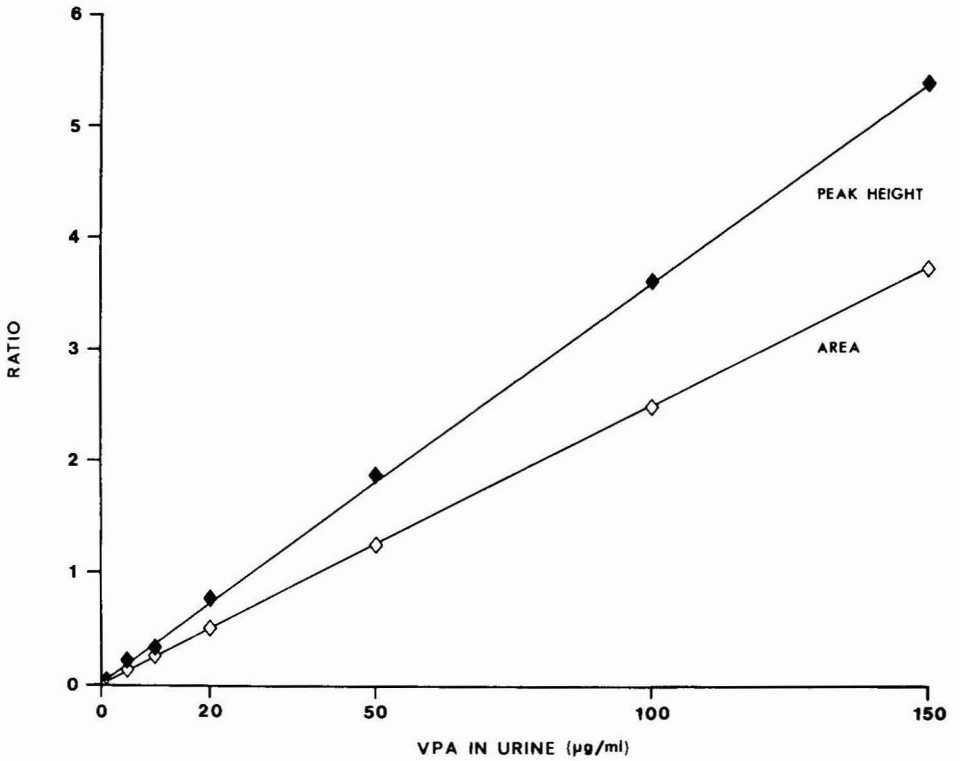

Fig. 3. Calibration graphs for VPA following extraction from urine. Graphs are shown for analysis with peak-height and peak-area ratios.

only method that is sensitive to the 1-μg level or even below. All other reported studies[10–14] are based on methods with sensitivity limits of about 5 μg/ml in plasma. Although under steady-state conditions of treatment with VPA therapeutic concentrations in plasma commonly range between 50 and 120 μg/ml[15–18], we found it necessary to measure concentrations as low as 0.5 μg/ml in pharmacokinetic studies[19] when the time course of the plasma level was followed over 72 h. This is particularly important for VPA, as in one pharmacokinetic study the investigators had calculated half-lives on the basis of plasma concentrations measured over 34 h only[16], although the terminal slope of the plasma level time curve does not begin before 24 h. Therefore, the half-lives reported were shorter than the predominant half-lives of VPA when based on observations over longer time periods.

A sensitive method for the determination of VPA is needed for several reasons: (1) preliminary results indicate that the plasma protein binding of VPA is 90–95%, so that concentrations of 2–4 μg/ml in buffer need to be detected if protein binding is measured by equilibrium dialysis; with highly protein-bound drugs, the degree of binding may be of value for the interpretation of plasma levels in the individual patient[20]; (2) in saliva, VPA concentrations appear to be even lower than the free concentrations in plasma[19]; (3) in spinal fluid, VPA may be present in low concentrations, if concentrations correspond to the free level in plasma as has been shown with phenytoin[21].

All calibration graphs were linear over the concentration range shown (Figs. 2 and 3). If the integrator is used to calculate the results, however, the same amount of internal standard can be used for all concentrations of VPA, whereas with the peak-height comparison a smaller amount of internal standard (2 μg) is appropriate for quantifying adequately concentrations below 2 μg/ml.

The coefficient of variation was different when the integrator and the peak-height ratio were used. In plasma and urine the coefficient of variation was determined by assaying 10 samples each of concentrations 1, 5, 10, 20, 50 and 100 μg/ml. The results were 3.1% in plasma and 2.0% in urine by use of the peak-height ratio but only 2.7% in plasma and 1.6% in urine by use of the integrator system. The reproducibility of the method is good and can even be improved if data are obtained by computerized integration of peak areas.

When identical samples were assayed on 10 different days, the reproducibility was 3.8% in plasma and 3.0% in urine. Although three extraction steps are necessary in order to achieve pure samples for analysis, the recovery is still good, being 91.7% in plasma, 92.2% in urine, 94.9% in spinal fluid and buffer and 94.5% in saliva ($n = 3$).

The accuracy of the method was tested by preparing plasma samples with amounts of VPA added to the plasma that were unknown to the operator. The results show that the concentrations measured by this method agree well with the actual concentrations (Table I). Plasma taken from patients receiving phenobarbital, carbamazepine, phenytoin, primidone, ethosuximide, clonazepam and digoxin was tested and no interfering peaks were found when the drugs were administered in therapeutic doses. Twenty-five plasma samples can easily be assayed in one working day with the proposed method.

TABLE I

ANALYSIS OF PLASMA FOR VPA CONTENT WHEN KNOWN AMOUNTS OF THE DRUG WERE ADDED TO SAMPLES

Valproic acid (μg/ml)	
Added	*Found*
1	0.9
5	5.1
5	5.3
10	9.7
20	20.7
40	40.9
50	48.2
80	79.4

ACKNOWLEDGEMENT

This work was supported by the Sandoz-Stiftung für Therapeutische Forschung.

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CHROM. 9996

Note

Simple preparative and analytical thin-layer chromatographic method for the rapid isolation of phosphatidic acid from tissue lipid extracts*

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The accurate assessment of phosphatidic acid in biological samples is very important, since this compound is a key intermediate in lipid biosynthesis. In addition, certain phosphatidic acid pools are involved, through phosphatidate phosphatase, diglyceride kinase and CDPdiglyceride synthetase, in the "neurotransmitter effect" exerted in the nervous tissue[1]. Moreover, because of the metabolic heterogeneity of phosphatidic acid[2–4], the separation of the molecular species involved and the determination of their fatty acid compositions is of interest.

The available techniques for the preparative isolation of phosphatidic acid from tissue lipid extracts are relatively time-consuming, involving column and then thin-layer chromatography (TLC)[2–5], or alkaline alcoholysis of washed total-lipid extracts followed by isolation of the glycerophosphate by electrophoresis[6,7] or two-dimensional paper chromatography[8]; the last procedure also converts any lysophosphatidic acid in the tissue into glycerophosphate. A rapid analytical procedure useful for the isolation of phosphatidic acid is two-dimensional TLC[9,10], which, although it satisfactorily resolves several phospholipids, implies the use of one plate per sample. Thus, there is need for a rapid procedure for the quantitative isolation of phosphatidic acid on a preparative scale and that will also permit analytical separation of several samples simultaneously. We describe here an oblique-spotting technique used with two-dimensional TLC, which fulfils these requirements.

Extraction of the tissue lipids should be made essentially as previously described[11], with the addition of washings of extracts maintained 0.04 *M* in calcium chloride to avoid loss of phosphatidic acid[6]. Silica gel H layers prepared with 3% of magnesium acetate should be used[6], but the layer thickness may be varied according to the phosphatidic acid concentration in the samples and the amount of total lipids.

A Plexiglas cover[12] modified as follows can conveniently be used to apply the spots obliquely in an atmosphere of nitrogen. The syringe needle or micropipette is introduced through an oblique slit (3 mm wide × 100 mm long) in the top of the Plexiglas cover at an angle of 45° from one corner. Fig. 1 shows the chromatographic

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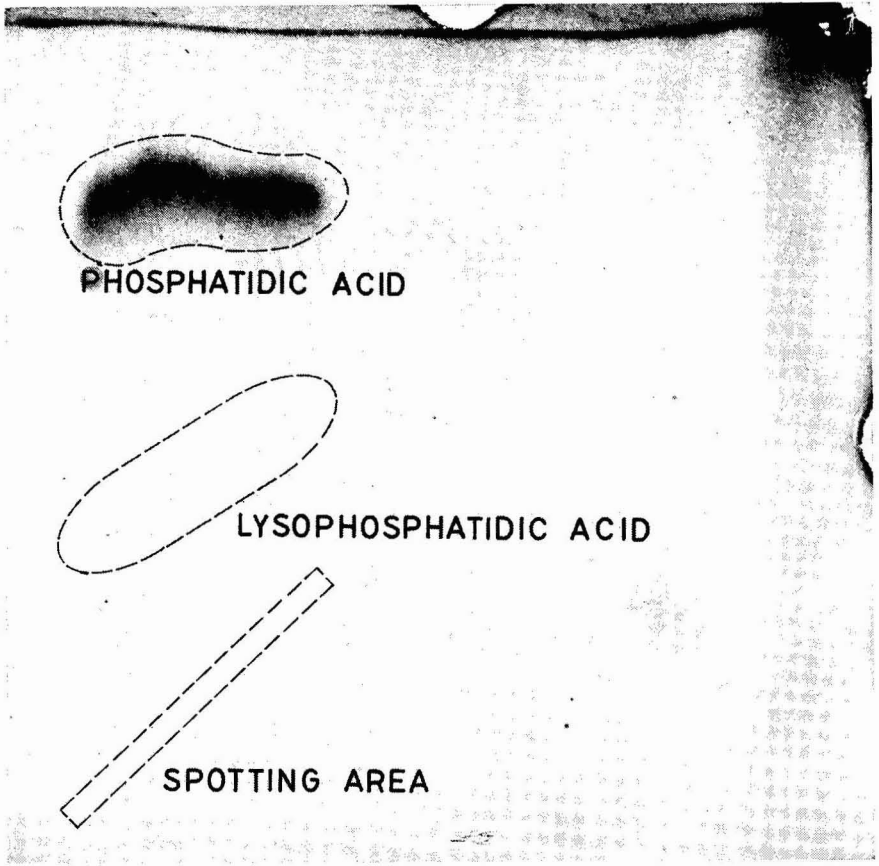
** Research fellow from the Comisión de Investigaciones Científicas of the Provincia de Buenos Aires.



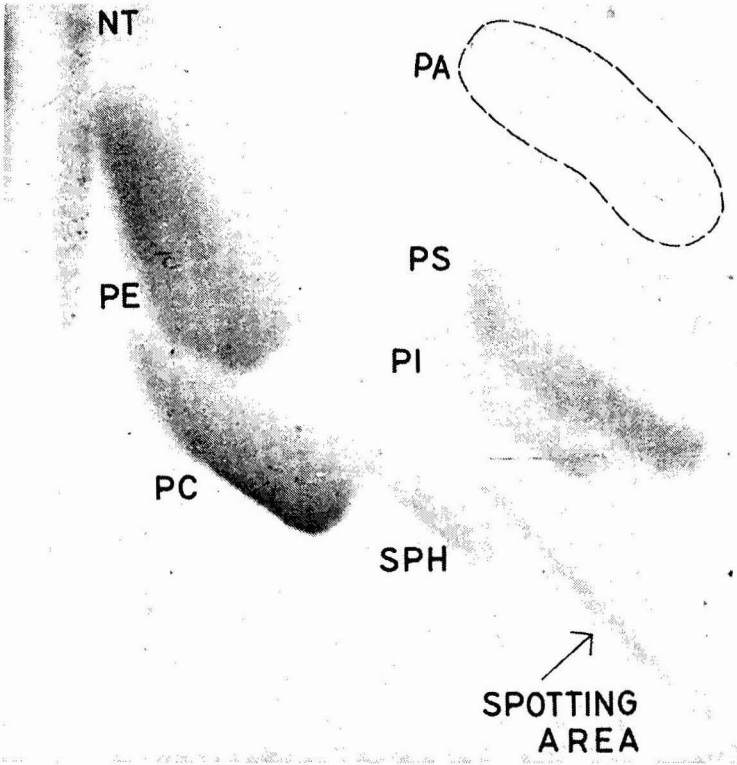


Fig. 1. Separation of standards of phosphatidic and lysophosphatidic acids by two-dimensional development after spotting the lipids along an oblique origin; the spotting area may be located in different positions, so that the directions of development may be as shown here or as in Figs. 2 and 3.

Fig. 2. Preparative two-dimensional TLC with oblique spotting for isolation of phosphatidic acid (PA) from an extract of mouse-brain lipids; the dashed circle indicates the area containing PA. Visualization was made by charring with a copper acetate reagent[13]. The direction of development can be modified, as here, in comparison to Fig. 1, without affecting separation. NT = neutral lipid; PE = phosphatidylethanolamine; PC = phosphatidylcholine; PS = phosphatidylserine; PI = phosphatidylinositol; SPH = sphingomyelin.

behaviour of a phosphatidic acid that contained a minor amount of lysophosphatidic acid. Thus, the sample may be applied as an oblique streak or as several round spots along the line of the slit. By applying streaks, one or two samples per plate may be applied for isolating the phosphatidic acid on the preparative scale (Fig. 2); by applying spots, six or seven samples may be run per plate, so obtaining analytical separations (Fig. 3). The depicted direction of oblique spotting prevents other lipids from over-running the phosphatidic acid during development. In both instances, two-dimensional development is carried out, with chloroform–methanol–ammonia solution (65:25:15) and subsequently chloroform–acetone–methanol–acetic acid–water (6:8:2:2:1) as mobile phase, as described by Rouser *et al.*[9]; it is advisable to overrun for approx.1 h with the former solvent system. When further analyses are intended, such as isolation of molecular species, care must be taken to avoid formation of peroxides; thus, after development in each direction, the surface of the layer should be flushed with dry nitrogen to accelerate evaporation. This can conveniently be done by connecting the nitrogen supply to a plastic box (3 × 5 × 0.5 cm) having several small holes along one of its edges to spread the gas stream. The chromatograms so obtained permit isolation of the phosphatidic acid, whereas other phospholipids are completely or partially overlapped (Figs. 2 and 3).

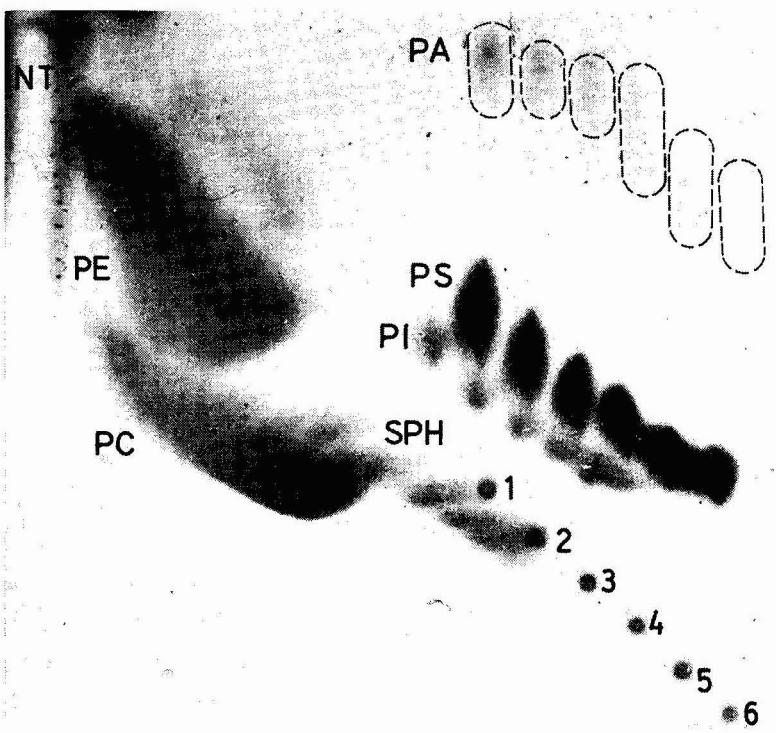


Fig. 3. Analytical two-dimensional TLC after spotting circular areas (1–6) along an oblique line. The dash-encircled areas show phosphatidic acid (PA) separated from mouse-brain-lipid extracts; abbreviations are as in Fig. 2. The direction of development can be modified, as here, in comparison to Fig. 1, without affecting separation.

If it is desired to separate other phospholipid classes, glass plates (40 × 20 cm) can be used. Thus, by suitable choice of development direction and plate size, phosphatidic acid as well as other phosphoglycerides may be isolated on a preparative or analytical scale from complex tissue-lipid extracts by oblique spotting.

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CHROM. 10,002

Note

Gas chromatographic separation and identification of bicyclic aromatic hydrocarbons in kerosene (b.p. 200–280°)

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(Received January 10th, 1977)

The separation and identification of individual bicyclic hydrocarbons in oil fractions (b.p. >200°) is a difficult problem. The usual gas chromatographic techniques are not suitable for the analysis of kerosene (b.p. 200–280°) over such a wide temperature range, because this fraction contains mainly paraffins and naphthenes and about 15–20% of aromatics, of which 3–4% are bicyclics. The naphthalene hydrocarbons consist of many isomers with similar physical and chemical properties, which pose difficulties in their gas chromatographic separation.

The qualitative and quantitative determination of naphthalene hydrocarbons has been studied by a number of workers using chromatographic methods[1–4] or combinations of gas chromatography with spectral methods[5–7]. Most of the work involved investigations of artificial mixtures of naphthalene hydrocarbons[1,2,5,6] with little emphasis on the separation of dimethylnaphthalenes.

In this work, the high-boiling kerosene fraction (b.p. 200–280°) containing mono-, di-, tri- and some tetraalkylnaphthalenes and heteroatomic compounds (mainly sulphur components) has been investigated in order to separate and identify the individual bicyclic hydrocarbons.

EXPERIMENTAL AND RESULTS

We carried out a vacuum distillation of the fraction in a distillation column with 20 theoretical plates and separated five narrow fractions (b.p. 200–240°, 240–250°, 250–260°, 260–270° and 270–280°). Spectrophotometry showed that the proportion of bicyclics in these fractions increased from about 2% bicyclics in the first to about 25% in the last two fractions, *i.e.*, the bicyclic hydrocarbons are concentrated in the latter fractions.

The gas chromatographic separation of bicyclic hydrocarbons from the remainder of the fraction could be achieved by using a selective stationary phase that could separate not only different compounds such as paraffins, naphthenes and aromatics, but also the mono- and bicyclic hydrocarbons. The most suitable stationary phase was polyethylene glycol adipate[3].

We carried out a preparative separation of the bicyclic hydrocarbons from the

narrow fractions, followed by separation of the individual bicyclics in order to identify them by measuring their UV spectra. The apparatus and operating conditions were as follows: gas chromatograph, Fractovap 2400 T (Carlo Erba, Milan, Italy); detector, katharometer; column, stainless steel (6 m × 8 mm I.D.); stationary phase, 15% polyethylene glycol adipate (PEGA) deposited on acid-washed Celite (60–80 mesh) at 180°; injection port temperature, 300°; manifold temperature, 280°; detector temperature, 280°; carrier gas, nitrogen at a flow-rate of 60 ml/min; sample size, 200 μl.

The fraction of b.p. 200–280° was separated into a paraffin-naphthene part, an unsaturated part and an aromatic part using fluorescent indicator adsorption (FIA). These parts were separated chromatographically under conditions as stated above. The aromatics were treated before and after separating of the bicyclic hydrocarbons by preparative thin-layer chromatography. It was shown that the last hydrocarbon of paraffin-naphthenes, unsaturated compounds and monoaromatics is eluted before naphthalene, which was also confirmed by adding hydrocarbons of b.p. > 300°, which were also eluted before naphthalene.

We separated preparatively two fractions, the first containing only bicyclic hydrocarbons and the second the other hydrocarbons. These fractions were checked on an analytical column (7 m × 4 mm I.D. with the same stationary phase) and showed a good separation of bicyclics from the other hydrocarbons.

The individual bicyclic hydrocarbons in the "naphthalene" fraction were separated preparatively with greater precision under the same conditions on a 7 m × 4 mm I.D. column with a sample size of 10 μl. In this instance we used a flame-ionization detector (FID) and a splitting ratio of 1:30. The components for UV spectrophotometry were collected in *n*-heptane (UV grade) with cooling. For the concentration of individual components in *n*-heptane, 5-fold preparative sampling was sufficient. Under these conditions fractions that contained one or two compounds were collected.

The naphthalene hydrocarbons give spectra with a wide absorbance range (210–330 nm) that contain three marked ranges: a sharp maximum at about 200 nm, a wide band with a poorly expressed vibration structure at 230–290 nm and a range with marked fine structure at 290–330 nm[8,9]. The last range is the most suitable because this absorption is specific for the naphthalene compounds.

The spectral analysis was carried out with a Unicam SP-700 UV spectrophotometer and the identification of naphthalenes was carried out by using pure substances and reference UV spectra[8,9]. The UV spectra of the preparatively collected components showed, in some instances, the presence of two compounds in one peak and we achieved complete separations on a capillary column with the same stationary phase in order to observe the elution sequence of these components.

The operating conditions were as follows: detector, FID; column, stainless-steel capillary (100 m × 0.25 mm I.D.); stationary phase, 9% PEGA in benzene at 180°; injection port temperature, 300°; sample size, 1 μl; splitting ratio, 1:100; carrier gas, nitrogen at a flow rate of 100 ml/min; hydrogen flow-rate, 30 ml/min; and oxygen flow-rate, 240 ml/min.

We succeeded in identifying 80% of the bicyclic hydrocarbons that were separated on the capillary column. We determined the Kováts retention indices of all of the identified bicyclic hydrocarbons (b.p. 200–280°) and Table I gives their

TABLE I

KOVÁTS RETENTION INDICES OF BICYCLIC AROMATIC HYDROCARBONS IN THE 200–280° KEROSENE FRACTION ON PEGA AT 180°

No.	*Compound*	*Kováts retention index*	*Identification method*
1	Naphthalene	1886.1	Ethalon
2	2-Methylnaphthalene	1969.8	Ethalon and UV spectrum
3	1-Methylnaphthalene	2015.6	Ethalon and UV spectrum
4	2-Ethylnaphthalene	2053.5	Ethalon and UV spectrum
5	1-Ethylnaphthalene	2058.9	Ethalon and UV spectrum
6	1,6-Dimethylnaphthalene	2070.2	UV spectrum
7	2-Ethenylnaphthalene	2083.6	UV spectrum
8	Dialkylnaphthalene	2101.2	UV spectrum
9	1,3-Dimethylnaphthalene	2110.8	Ethalon and UV spectrum
10	1-*n*-Propylnaphthalene	2120.8	UV spectrum
11	1-*n*-Butylnaphthalene	2130.9	UV spectrum
12	1,4- and 2,3-Dimethylnaphthalene	2139.4	Ethalon and UV spectrum
13	1,5-Dimethylnaphthalene	2145.1	Ethalon and UV spectrum
14	2,6-Dimethylnaphthalene	2158.8	UV spectrum
15	2,7-Dimethylnaphthalene	2169.3	UV spectrum
16	2-Methyl-7-isopropylnaphthalene	2173.9	UV spectrum
17	1,8-Cycloalkenylnaphthalene	2182.6	UV spectrum
18	1,2,7-Trimethylnaphthalene	2192.2	UV spectrum
19	1,4-Dialkylnaphthalene	2204.1	UV spectrum
20	1,3,5-Trimethylnaphthalene	2223.8	UV spectrum
21	2,3-Dihydrophenalene	2231.6	UV spectrum
22	1-Methyl-7-isopropylnaphthalene	2236.5	UV spectrum
23	1,3,6-Trimethylnaphthalene	2248.1	UV spectrum
24	1,6,7-Trimethylnaphthalene	2257.3	UV spectrum
25	1,4,6-Trimethylnaphthalene	2258.1	UV spectrum
26	1,2,4-Trimethylnaphthalene	2273.3	UV spectrum
27	1,2,5-Trimethylnaphthalene	2280.5	UV spectrum
28	Compound of the tetrahydroanthracene type	2292.1	UV spectrum
29	Trialkylnaphthalene	2297.3	UV spectrum
30	1,2,8-Trimethylnaphthalene	2309.5	UV spectrum
31	1-Propyl-2-cyclohexylnaphthalene	2325.6	UV spectrum
32	*d,e*-Cycloalkylnaphthalene	2330.8	UV spectrum
33	Trialkylnaphthalene	2338.5	UV spectrum
34	1,6-Dimethyl-4-isopropylnaphthalene	2345.2	UV spectrum
35	1,4,5-Trimethylnaphthalene	2388.1	UV spectrum
36	3-Alkylbenzo(*b*)thiophene	2992.6	UV spectrum
37	2,3,6,7-Tetramethylnaphthalene	2401.3	UV spectrum
38	1,2,3,4-Tetramethylnaphthalene	2409.2	UV spectrum
39	6-Alkylbenzo(*b*)thiophene	2412.0	UV spectrum
40	1,2-Dimethyl-7-ethylnaphthalene	2417.3	UV spectrum
41	2-Alkenylnaphthalene	2423.8	UV spectrum
42	1,4-Diethyl-6-methylnaphthalene	2425.6	UV spectrum
43	1,6-Diethyl-4-methylnaphthalene	2433.3	UV spectrum
44	2-Alkylnaphthalene	2444.7	UV spectrum
45	1,2,3-Trialkylnaphthalene	2448.6	UV spectrum
46	1,4,6-Trialkylnaphthalene	2480.3	UV spectrum
47	1,2,7-Trialkylnaphthalene	2516.4	UV spectrum

experimental retention indices and the method of identification used. The experimental retention indices were obtained in four replicate experiments and showed good repeatability (0.5–0.6 index unit).

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Note

Steroids and related studies

XL. Thin-layer chromatography of some steroidal ketones, oximes and lactams

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(Received January 18th, 1977)

We reported earlier TLC data for some lactam, tetrazole and basic and quaternary azasteroids[1]. Our continuing programme on azasteroids has yielded a variety of new compounds and in this paper we give mainly the results of TLC studies on several steroidal oximes and lactams; data on some steroidal ketones, including those used as starting materials, are also given.

EXPERIMENTAL

Steroid derivatives

The ketones (Table I), oximes (Table II) and lactams (Tables III and IV) were mostly prepared in our laboratory. Literature references to methods of preparation of the compounds are given in the tables.

Adsorbent and TLC plates

Silica gel G (Merck, Darmstadt, G.F.R.), was mixed with distilled water (30 g of gel per 60 ml of water), and coated on 20 × 20 cm plates with a layer thickness of 0.25 mm. The plates were air dried for 15 min, heated at 110° for 60 min, then stored in a drying cabinet over calcium chloride.

The running distance was 16 cm at a temperature of 22–25°, and the load of steroid derivative applied was 50–100 μg.

Detection

Cerium(IV) sulphate solution [2 g in 100 ml of 10% (v/v) sulphuric acid] was used as the spray reagent, followed by heating at 150° for 30 min, which produced permanent black spots. Exposure to iodine vapour was also used, and produced brown spots in only 2–4 min.

Solvents

All the solvents used were of analytical grade and were used without further treatment. The following solvent systems were used: (1) chloroform–methanol (99:1);

(2) benzene–ethyl acetate (17:3); (3) benzene–methanol (19:1); (4) chloroform–ethyl acetate (9:1); (5) chloroform–methanol (39:1); (6) chloroform–ethyl acetate (8:2); (7) chloroform–methanol (24:1); (8) benzene–methanol–ethyl acetate (16:3:1); (9) ethyl acetate–benzene (9:1); (10) benzene–methanol (17:3); (11) chloroform–ethyl acetate–methanol (12:7:1); (12) chloroform–methanol (9:1); (13) benzene–methanol (3:1); and (14) chloroform–ethyl acetate–methanol (10:7:3).

RESULTS

Table I lists the R_F values of some steroidal ketones, including those which were used as starting materials, Table II of steroidal ketoximes and Tables III and IV of steroid lactams.

TABLE I

THIN-LAYER CHROMATOGRAPHY OF STEROIDAL KETONES IN SOLVENT SYSTEMS 1, 2, 3 AND 4

Compound	R_F *value*			
	1	*2*	*3*	*4*
Cholest-4-ene-3,6-dione[2]	0.66	0.62	0.70	0.62
3-Oxoandrost-4-en-17β-yl acetate[3]	0.40	0.34	0.58	0.38
7-Oxocholest-5-en-3β-yl acetate[4,5]	0.70	0.61	0.70	0.65
7,20-Dioxopregn-5-en-3β-yl acetate[6]	0.41	0.33	0.56	0.37
7,20-Dioxopregna-5,16-dien-3β-yl acetate[7]	0.45	0.35	0.59	0.39
(25*R*)-7-Oxospirost-5-en-3β-yl acetate[7]	0.45	0.34	0.65	0.32
17-Oxo-5α-androstan-3β-yl acetate	0.63	0.56	0.66	0.61
17-Oxoandrost-5-en-3β-yl acetate	0.62	0.55	0.65	0.61
20-Oxopregna-5,16-dien-3β-yl acetate[7]	0.70	0.61	0.68	0.66
Pregna-3,5,16-triene-7,20-dione[7]	0.60	0.52	0.61	0.59
3β-Hydroxy-5α-androstan-17-one	0.23	0.17	0.29	0.18
3β-Hydroxyandrost-5-en-17-one	0.23	0.19	0.29	0.20
3β-Hydroxypregn-5-en-20-one	0.23	0.22	0.34	0.22

TABLE II

THIN-LAYER CHROMATOGRAPHY OF STEROIDAL KETOXIMES IN SOLVENT SYSTEMS 2, 3, 5 and 6

Compound	R_F *value*			
	2	*3*	*5*	*6*
3-Hydroxyiminoandrost-4-en-17β-yl acetate[3]	0.26	0.36	0.39	0.30
3-Hydroxyiminoandrost-4-en-17-one[3]	0.15	0.34	0.38	0.23
17,17-Ethylenedioxy-3-hydroxyiminoandrost-4-ene[3]	0.19	0.38	0.39	0.22
3-Hydroxyiminocholest-4-en-6-one[2]	0.34	0.41	0.39	0.36
3β-Methoxy-17-hydroxyimino-5α-androstane[8]	0.20	0.36	0.45	0.25
7-Hydroxyiminocholest-5-en-3β-yl acetate[4,5]	0.74	0.65	0.88	0.72
(25*R*)-7-Hydroxyiminospirost-5-en-3β-yl acetate[7]	0.46	0.57	0.71	0.55
7-Hydroxyiminopregn-5-ene-3β,20β-diol diacetate[7]	0.50	0.53	0.76	0.62
7,20-Dihydroxyiminopregna-5,16-dien-3β-yl acetate[7]	0.48	0.38	0.57	0.55

TABLE III

THIN-LAYER CHROMATOGRAPHY OF STEROIDAL LACTAMS IN SOLVENT SYSTEMS 7, 8, 9, 10 AND 11

Compound	*R_F value*				
	7	*8*	*9*	*10*	*11*
4-Aza-A-homocholest-4a-ene-3,6-dione[2]	0.57	0.61	0.51	0.60	0.59
3-Aza-A-homocholest-4a-ene-4,6-dione[2]	0.38	0.48	0.22	0.45	0.36
4-Aza-5α-cholestan-3-one[9]	0.49	0.51	0.13	0.55	0.31
(25*R*)-4-Aza-5β-spirostan-3-one[10]	0.51	0.49	0.11	0.58	0.30
(25*R*)-4-Aza-5α-spirostan-3-one[10]	0.47	0.48	0.06	0.53	0.34
4-Aza-5α-pregn-16-ene-3,20-dione[10]	0.46	0.44	0.10	0.50	0.27
4-Oxo-3-aza-A-homoandrost-4a-en-17β-yl acetate[3]	0.33	0.35	0.10	0.40	0.24
3-Aza-A-homoandrost-4a-ene-4,17-dione[11]	0.28	0.32	0.07	0.34	0.18
7-Oxo-7a-aza-B-homocholest-5-en-3β-yl acetate[4,5]	0.70	0.64	0.39	0.65	0.57
7a-Aza-B-homocholest-4-ene-3,7-dione[4,5]	0.74	0.61	0.64	0.63	0.62
3β-Chloro-7a-aza-B-homocholest-5-en-7-one[5]	0.75	0.65	0.47	0.67	0.59
(25*R*)-7-Oxo-7a-aza-B-homospirost-5-en-3β-yl acetate[7]	0.55	0.57	0.25	0.60	0.40
(25*R*)-3β-Hydroxy-7a-aza-B-homospirost-5-en-7-one[7]	0.16	0.37	0.06	0.43	0.15
7-Oxo-7a-aza-B-homopregn-5-ene-3,20-diol diacetate[7]	0.59	0.51	0.20	0.55	0.42
7-Oxo-7a-aza-B-homoandrost-5-ene-3,17-diol diacetate[7]	0.49	0.47	0.20	0.52	0.33
6-Aza-B-homo-5α-cholestane-3,7-dione[12]	0.38	0.47	0.21	0.50	0.31
4α-Bromo-6-aza-B-homo-5α-cholestane-3,7-dione[12]	0.48	0.54	0.54	0.60	0.47
7-Oxo-6-aza-B-homo-5α-cholestan-3β-yl acetate[12]	0.64	0.60	0.31	0.64	0.43
17a-Aza-D-homoandrost-4-ene-3,17-dione[3]	0.25	0.24	0.37	0.31	0.15
3β-Methoxy-17a-aza-D-homo-5α-androstan-17-one[8]	0.41	0.39	0.09	0.44	0.25
7,17-Dioxo-17a-aza-D-homoandrost-5-en-3β-yl acetate[13]	0.27	0.32	0.06	0.34	0.16
17a-Aza-D-homoandrosta-3,5-diene-7,17-dione[13]	0.30	0.36	0.07	0.36	0.20
3,17a-Diacetyl-3,17a-diaza-A,D-bishomoandrost-4a-ene-4,17-dione[3]	0.79	0.57	0.47	0.29	0.11
4-Benzyl-4,17a-diaza-D-homoandrost-5-ene-3,17-dione[14,15]	0.28	0.30	0.04	0.32	0.15

TABLE IV

THIN-LAYER CHROMATOGRAPHY OF STEROIDAL LACTAMS IN SOLVENT SYSTEMS 8, 12, 13 AND 14

Compound	*R_F value*			
	8	*12*	*13*	*14*
17β-Hydroxy-3-aza-A-homoandrost-4a-en-4-one[3]	0.35	0.55	0.53	0.33
17-Hydroxyimino-3-aza-A-homoandrost-4a-en-4-one[3]	0.31	0.50	0.53	0.30
16β-Bromo-17α-hydroxy-3-aza-A-homopregn-4a-ene-4,20-dione[16]	0.39	0.56	0.60	0.40
16β-Chloro-17α-hydroxy-3-aza-A-homopregn-4a-ene-4,20-dione[16]	0.38	0.58	0.61	0.39
17α-Hydroxy-16β-thiocyanato-3-aza-A-homopregn-4a-ene-4,20-dione[16]	0.37	0.54	0.59	0.40
17α-Hydroxy-16β-iodo-3-aza-A-homopregn-4a-ene-4,20-dione[16]	0.39	0.57	0.62	0.42
4,6-Diaza-A,B-bishomocholest-4a-ene-3,7-dione[2]	0.37	0.52	0.67	0.37
3,7a-Diaza-A,B-bishomocholest-4a-ene-4,7-dione[4,5]	0.32	0.53	0.58	0.31
4,6-Diaza-A,B-bishomo-5α-cholestane-3,7-dione[17]	0.25	0.31	0.49	0.21
3,6-Diaza-A,B-bishomo-5α-cholestane-4,7-dione[17]	0.31	0.41	0.54	0.26
3β-Hydroxy-7a-aza-B-homocholest-5-en-7-one[5]	0.41	0.65	0.65	0.48
(25*R*)-3-Hydroxyimino-7a-aza-B-homospirost-4-en-7-one[5]	0.42	0.64	0.64	0.51
3β-Hydroxy-7a-aza-B-homopregna-5,16-diene-7,20-dione[18]	0.32	0.55	0.56	0.34
3β,20β-Dihydroxy-7a-aza-B-homopregn-5-en-7-one[7]	0.27	0.41	0.51	0.32
3β,17β-Dihydroxy-7a-aza-B-homoandrost-5-en-7-one[7]	0.21	0.31	0.47	0.25
3-Hydroxyimino-17a-aza-D-homoandrost-4-en-17-one[3,19]	0.33	0.62	0.56	0.37
3,17a-Diaza-A,D-bishomoandrost-4a-eno-[3,4-*d*]tetrazol-17-one[20]	0.27	0.57	0.49	0.27
7-Hydroxyimino-17-oxo-17a-aza-D-homoandrost-5-en-3β-yl acetate[13]	0.36	0.60	0.55	0.39
3,17a-Diaza-A,D-bishomoandrost-4a-ene-4,17-dione[3,19]	0.16	0.33	0.41	0.16
4,17a-Diaza-D-homo-5α-androstane-3,17-dione[14,15]	0.19	0.43	0.40	0.17
7,17-Dioxo-7a,17a-diaza-B,D-bishomoandrost-5-en-3β-yl acetate[13]	0.19	0.31	0.40	0.16

For steroidal ketones, consistent results were obtained with solvent systems 1, 2, 3 and 4 (Table I), of which systems 1 and 3 are of particular interest. Of the solvent systems useful for the oximes (2, 3, 5 and 6) (Table II), systems 3 and 5 are to be preferred. For the steroidal lactams, several systems (7, 8, 9, 10, 11, 12, 13 and 14) were useful (Tables III and IV), of which solvents 8 and 10 (Table III) and 12 and 13 (Table IV) gave better results for particular compounds.

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Note

Gas–liquid chromatography of eight anticonvulsant drugs in plasma

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The measurement of serum anticonvulsant drug levels greatly assists the management of epileptic patients by enabling a balance to be maintained between the therapeutic and toxic concentrations of these drugs. Several methods have been described for the determination of the anticonvulsant drugs in plasma[1–5]. Most of these, however, describe complicated and time-consuming extraction procedures or a separate analytical procedure for each drug.

This report presents a simple and rapid technique for the simultaneous extraction of eight commonly prescribed anticonvulsant drugs. Seven of these drugs are gas-chromatographed simultaneously using an alkali flame ionization detector. The remaining drug, sodium valproate, is analysed using a flame ionization detector.

EXPERIMENTAL

Reagents

For the extraction step diethyl ether AnalaR grade and 1 *M* hydrochloric acid were used.

As standards were used 1 mg/ml heptabarbitone (internal standard for ethotoin, ethosuximide, carbamazepine, pheneturide and phenobarbitone) in methanol; 1 mg/ml 5-(*p*-methylphenyl)-5-phenylhydantoin (internal standard for primidone and phenytoin) in methanol; 1 mg/ml cyclohexane carboxylic acid (internal standard for sodium valproate) in methanol; and 1 mg/ml ethotoin, ethosuximide, carbamazepine, pheneturide, phenobarbitone, phenytoin, primidone and sodium valproate in methanol.

Methanol AnalaR grade and tetramethylammonium hydroxide (TMAH) 20% in methanol, diluted 1:10 before use, were employed for gas–liquid chromatography.

Extraction

A 1-ml amount of plasma containing 20 μg each of heptabarbitone and 5-(*p*-methylphenyl)-5-phenylhydantoin and 100 μg of cyclohexane carboxylic acid is acidified with two drops of 1 *M* hydrochloric acid and extracted with 5 ml of diethyl ether. The organic phase is evaporated to dryness and dissolved in 100 μl of methanol.

Drug-free plasma containing 5–30 μg/ml phenobarbitone, phenytoin, primi-

done, pheneturide, carbamazepine, ethotoin, 10–50 μg/ml ethosuximide and 40–80 μg/ml sodium valproate is treated similarly to the tests to establish calibration curves.

Gas–liquid chromatography

Phenobarbitone, phenytoin, primidone, pheneturide, carbamazepine, ethosuximide and ethotoin are analysed using a Varian 1400 gas chromatograph equipped with an alkali flame ionization detector. The column used was a 4 ft. $\times$ $\frac{1}{4}$ in. glass tube containing 1% OV-17 on Gas-Chrom Q, 80–120 mesh. The column temperature is programmed from 110 to 240° at 8°/min. The other important gas–liquid chromatographic (GLC) conditions are: injector port temperature, 240°; detector temperature, 280°; carrier gas (nitrogen) flow-rate, 50 ml/min.

A 1-μl aliquot of the final residue in methanol is injected after flash methyl-

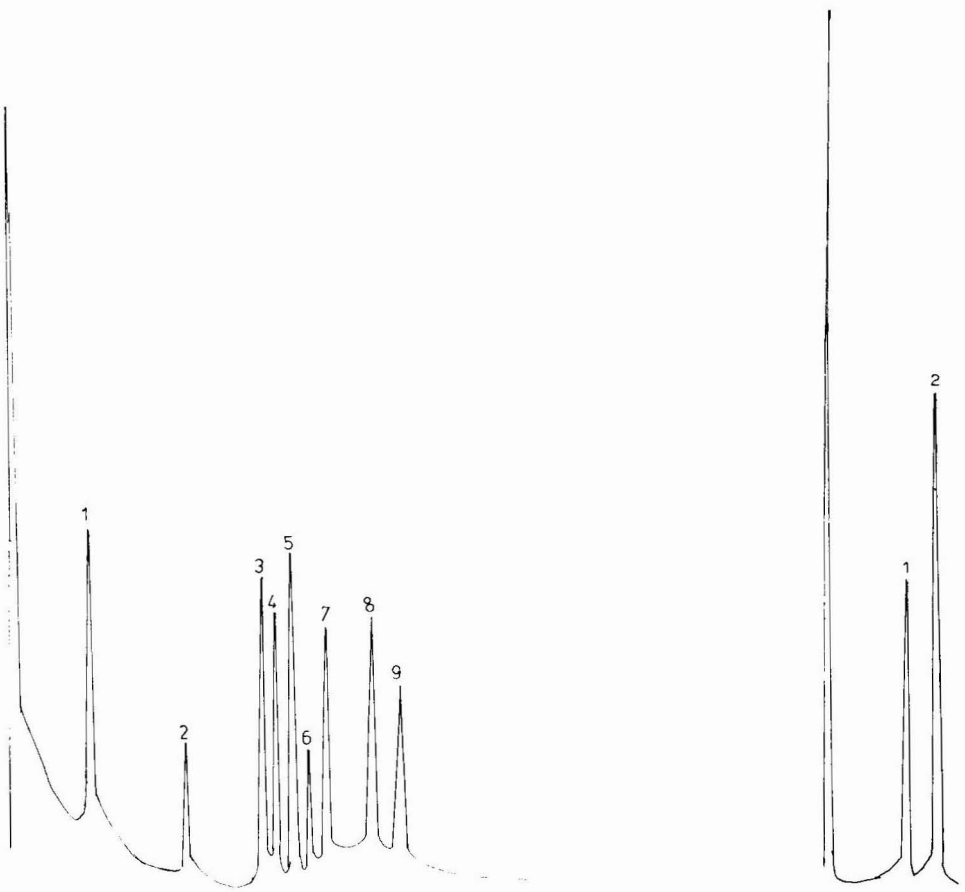

Fig. 1. Chromatogram of seven anti-convulsant drugs in plasma. Peaks: 1 = ethosuximide, 2 = pheneturide, 3 = ethotoin, 4 = phenobarbitone, 5 = heptabarbitone (internal standard), 6 = carbamazepine, 7 = primidone, 8 = phenytoin, 9 = 5-(*p*-methylphenyl)-5-phenylhydantoin (internal standard).

Fig. 2. Chromatogram of sodium valproate in plasma. Peaks: 1 = sodium valproate, 2 = cyclohexane carboxylic acid (internal standard).

ation with 1 μl of the freshly prepared TMAH. A typical chromatogram is presented in Fig. 1.

Sodium valproate is analysed using a Varian 2400 gas chromatograph fitted with flame ionization detectors and a 1 ft. $\times$ $\frac{1}{4}$ in. glass column containing 2% SP-1000 on Universal support, 85–100 mesh. The GLC conditions are: column temperature, 120°; injector port temperature, 200°; detector temperature, 240°; carrier gas (nitrogen) flow-rate, 40 ml/min.

A 2-μl aliquot of the underivatised final extract in methanol is injected on to the column and Fig. 2 shows a chromatogram of sodium valproate plus internal standard extracted from plasma.

In order to test the reproducibility of the method plasma samples spiked with known amounts of the eight anticonvulsant drugs, ranging from 5 to 100 μg/ml, were extracted and analysed by the technique described. Three independent analyses were carried out per sample of plasma and the results are presented in Table I.

TABLE I

ASSESSMENT OF THE REPRODUCIBILITY OF THE METHOD FOR ANALYSING ANTICONVULSANTS IN PLASMA

Drug	*Concentration of anticonvulsant present (μg/ml)*					
	5.5	*15*	*25*	*30*	*60*	*100*
	Recovery (mean ± S.D.)*					
Ethosuximide			25.4 ± 0.49	29.9 ± 0.95	59.0 ± 0.81	
Pheneturide	5.6 ± 0.43	15.5 ± 1.08	24.9 ± 0.68			
Ethotoin		14.7 ± 0.31	24.8 ± 0.61	28.5 ± 0.75		
Phenobarbitone		15.2 ± 0.71	23.8 ± 0.62	29.8 ± 1.5		
Carbamazepine	5.1 ± 0.56	14.6 ± 0.32	24.7 ± 0.9			
Primidone	5.7 ± 0.38	15.5 ± 0.35	25.6 ± 0.47			
Phenytoin	5.2 ± 0.2	15.3 ± 0.21	25.6 ± 0.54			
Sodium valproate			25.0 ± 1.0		61.3 ± 0.94	103.3 ± 1.2

* Mean of 3 values.

RESULTS AND DISCUSSION

The use of an alkali flame ionization detector to monitor seven different anticonvulsants is plasma is described. The method has also been applied to the analysis of several biological media, such as urine and liver homogenate.

The specificity of the alkali flame ionization detector results in a considerable reduction in extraction time as there is no need to remove naturally interfering plasma constituents, such as cholesterol, during the extraction procedure. The other major advantage of this type of detector is the enhanced sensitivity for nitrogen-containing compounds resulting in a lower detection limit than conventional flame ionization detectors and enabling less sample volume to be used if necessary.

Sodium valproate cannot be determined using the alkali flame ionization detector because there are no nitrogen-containing moieties present in the molecule. We found that the SP-1000 column was the most satisfactory column for the valproate analysis because, at the analytical temperature, none of the other commonly en-

TABLE II

DRUG CONCENTRATION IN *POST MORTEM* SPECIMENS FROM A FATAL CASE INVOLVING ANTICONVULSANT DRUGS

Male subject, age 30 years.

Drug	*Peripheral blood (μg/ml)*	*Liver blood (μg/ml)*	*Stomach content (μg/ml)*
Phenobarbitone	83	9	432
Phenytoin	15	23	256
Sodium valproate	32	28	32
Diazepam	0.6	0.4	1.04
Paracetamol	28	45	224
Thioridazine	*	*	0.35

* Not detected.

countered anticonvulsants or naturally occurring compounds interfered with the GLC analysis.

In addition to providing an efficient, reproducible and rapid procedure for the therapeutic monitoring of anticonvulsant drugs in plasma, the technique is used for simultaneous screening and quantitation in cases of suspected poisoning. The analytical results of the toxicological investigations of one such case where death resulted from anticonvulsant drug overdose is presented in Table II.

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Note

Pyrolysis gas chromatographic analysis of some toxic compounds from nitrogen-containing fibres

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There have been few studies on the determination of hydrogen cyanide, benzene and toluene formed during thermal degradation of polymers in air, in atmospheres of inert gases or in an atmosphere containing a small amount of oxygen. For the determination of pyrolysis products, several methods have been used[1–4]. When burning or similar thermal processes are carried out, the degradation of polymers and the formation of products takes place rapidly, and pyrolysis gas chromatography offers the best means for studying such degradation products.

Ingham[5] studied the thermal degradation of untreated wool and wool treated with flame retardants using pyrolysis gas chromatography. The pyrolysis took place at 550° for 60 sec and methane, hydrogen sulphide, carbon monoxide and hydrogen cyanide were determined. Our study covered the range 625–925° and three nitrogen-containing fibres, namely wool, a polyacrylic (Acrilan) and a polyamide (Rilsan) were pyrolysed.

The problem was to determine the small amount of hydrogen cyanide formed by thermal destruction and to establish suitable conditions for the gas chromatographic separation of hydrogen cyanide, benzene and toluene.

HYDROGEN CYANIDE SEPARATION AND DETERMINATION

Previously a hot-wire detector was used for the gas chromatographic separation of hydrogen cyanide[6], while it has been stated that under normal conditions the flame-ionization detector (FID) does not respond to hydrogen cyanide[7]. In the present time, both detectors are used[8].

For the determination of the sensitivity of the hot-wire and FID detectors towards hydrogen cyanide a standard sample of hydrogen cyanide was synthesized according to Wöhler. Pure hydrogen cyanide was mixed with air to give a concentration of 3500–0.35 mg/m^3.

A sample of size 0.1–2.0 ml was introduced into the chromatograph using a Hamilton 1001 gas syringe. The gas chromatograph used was a Perkin-Elmer Model 900 instrument with hot-wire and FID detectors connected with a Hewlett-Packard 3370 A integrator. A set of glass column was used: 2 m × 1 mm I.D. packed with 7% Carbowax 20M on Chromosorb W (80–100 mesh), plus 4 m × 4 mm I.D. packed

TABLE I

OPERATING CONDITIONS

Condition	*FID*	*Hot-wire detector*
Carrier gas (He) flow-rate (ml/min)	30	30
Hydrogen flow-rate (ml/min)	24	—
Air flow-rate (ml/min)	400	—
Oven temperature (°C)	40	40
Injector temperature (°C)	30	30
Detector temperature (°C)	150	150
Detector current (mA)	—	225

with 7% triacetin on Chromosorb W (80–100 mesh) (column I). The operating conditions used are given in Table I.

The results obtained show that the sensitivity of the hot-wire detector was 500 times lower than that of the FID; the detection limits were 0.35 μg for the hot-wire detector and 0.0007 μg for the FID.

For the determination of hydrogen cyanide, common supports such as Porapak Q, Porapak T, 1,2,3-tris(cyanoethoxy)propane, Carbowax 20M and triacetin can also be used[9]. In this study the problem of separating hydrogen cyanide from other light pyrolysis products arose, and for this purpose we used column I and a stainless-steel column (2 m × 2 mm I.D.) packed with 5% dimethylsulpholane on Inerton AW (0.125–0.160 mm) (column II). Two pyrograms of wool obtained by using the above two columns are shown in Fig. 1.

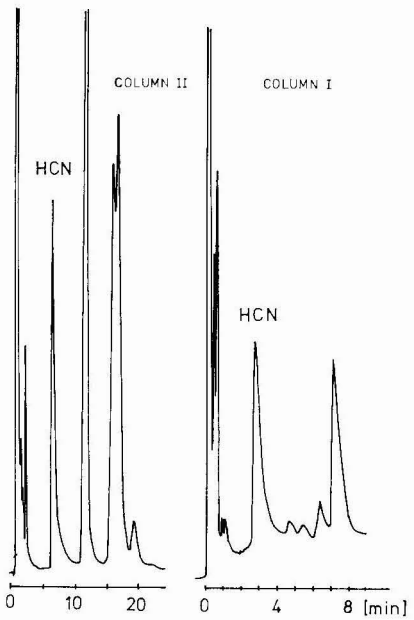


Fig. 1. Pyrograms of wool obtained with the use of columns I and II.

TABLE II

RESULTS OF ELEMENTAL ANALYSIS OF FIBRES

Fibre	*N (%)*	*C (%)*	*H (%)*
Polyacrylonitrile (Acrilan)	3.4	65.6	5.5
Polyamide (Rilsan)	7.6	73.3	11.2
Wool	14.7	46.0	6.2

QUANTITATIVE ANALYSIS OF PYROLYSIS PRODUCTS

For quantitative analysis a dimethylsulpholane column was used because it also separated benzene and toluene and other light compounds formed during the pyrolysis. Benzene and toluene peaks on the chromatogram were compared by using standard substances. In addition, the peaks of interest were separated and their UV spectra measured.

In order to gain some insight into the structures of the three types of fibres under study, the hydrogen, nitrogen and carbon composition was determined with a Hewlett-Packard Model 185 CHN Elemental Analyser, and the results are shown in Table II. The results are average values from triplicate determinations. Polyacrylonitrile has the highest nitrogen and the lowest hydrogen contents compared with the other polymers.

Gas chromatograms of fibre pyrolysis products at 850° are shown in Fig. 2. Although wool has a more complex structure than the other fibres, the three chromatograms of the lighter components are virtually identical, the only difference being in the peaks areas.

A furnace-type pyrolyser with a quartz tube was attached to the inlet port of the gas chromatograph with an FID detector, and the operating conditions used are given in Table III.

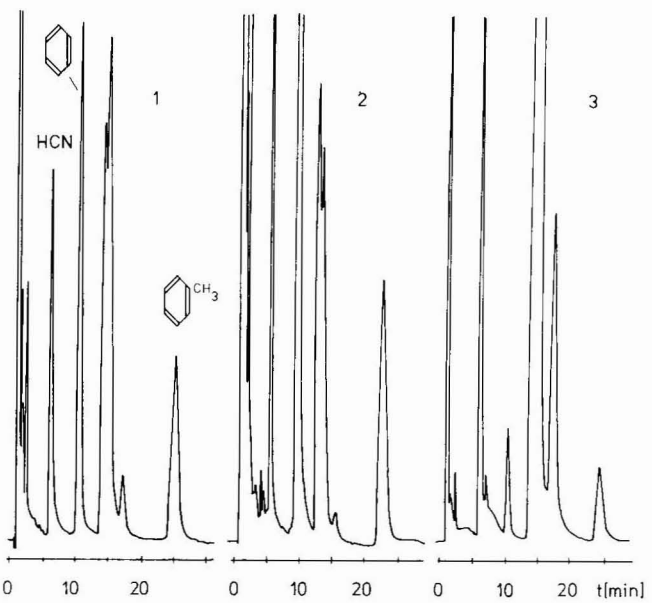


Fig. 2. Pyrograms of fibres obtained with the use of column II. 1, Wool; 2, Rilsan; 3, Acrilan.

TABLE III

OPERATING CONDITIONS WITH COLUMN II

Condition	*Value*
Oven temperature (°C)	20
Carrier gas (He) flow-rate (ml/min)	30
Hydrogen flow-rate (ml/min)	27
Air flow-rate (ml/min)	400
Injector temperature (°C)	150
Detector temperature (°C)	200
Pyrolysis temperature range (°C)	625–925
Time (sec)	10
Sample size (mg)	0.4–0.6

Fig. 3 shows curves for the evolution of hydrogen cyanide from the fibres studied, and it can be seen that the amount of hydrogen cyanide evolved is not proportional to the nitrogen contents of the fibres, as wool, which contains more nitrogen than Rilsan, produces less hydrogen cyanide.

The evolution of hydrogen cyanide from all three fibres is also not proportional to the temperature of pyrolysis and the curves show minima and maxima. The amounts of benzene formed are proportional to the nitrogen contents of the fibres, and not proportional to the carbon and hydrogen contents. The evolution curves also show minima and maxima (Fig. 4).

The amount of toluene formed is lower at higher temperatures, which indicates the formation of lighter products (Fig. 5).

The amounts of hydrogen cyanide, benzene and toluene evolved per gram of fibre are given in Table IV. The results indicate that the amounts evolved are high and potentially dangerous.

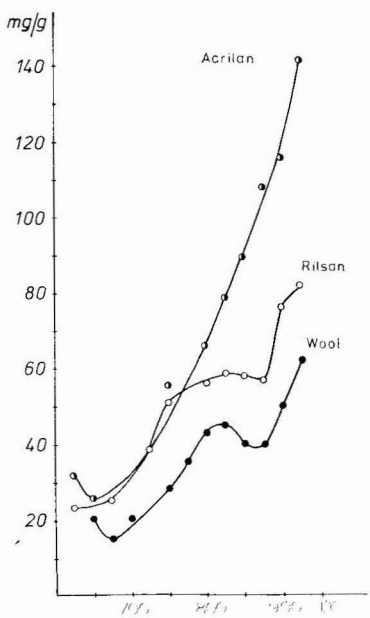
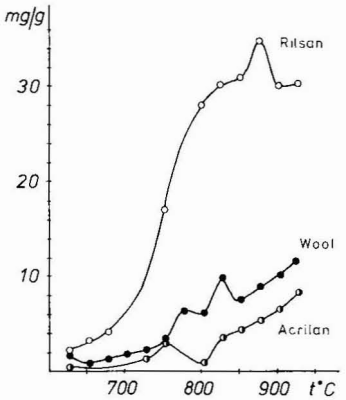


Fig. 3. HCN evolution curves for wool, Rilsan and Acrilan.



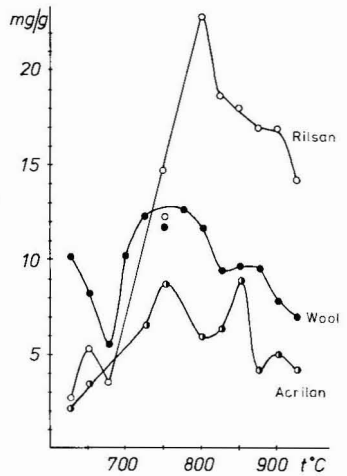


Fig. 4. Benzene evolution curves for wool, Rilsan and Acrilan.

Fig. 5. Toluene evolution curves for wool, Rilsan and Acrilan.

TABLE IV

EVOLUTION OF HYDROGEN CYANIDE, BENZENE AND TOLUENE PER GRAM OF FIBRE

Pyrolysis temperature: 625–925°.

Fibre	*HCN (mg/g)*	*Benzene (mg/g)*	*Toluene (mg/g)*
Acrilan	32–140	0.4–8	2–8.8
Rilsan	32–82	2–35	2.6–22.8
Wool	22–62	1–11.5	5.5–12.5

The use of pyrolysis gas chromatography may introduce some errors derived from the accuracy and stability of the operating conditions, such as the stability of the pyrolysis temperature to within $\pm 10°$, a small sample size and sample inhomogeneity. On the other hand, pyrolysis gas chromatography is one of the best possibilities for the selective determination of thermal degradation products using a small sample.

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CHROM. 10,055

Note

Analysis of nitrazepam and its metabolites by high-pressure liquid chromatography

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The benzodiazepines are a widely used class of drugs possessing sedative and muscle-relaxant properties. The most generally applicable procedure for the analysis of benzodiazepines in biological fluids, such as blood and urine, involves a multi-stage solvent extraction, followed by gas–liquid chromatographic assay[1–3].

Gas chromatography of diazepam, medazepam, oxazepam and their metabolites can be satisfactorily performed on silicone phases such as OV-1, OV-17 and OV-225[3,4]. Nitrazepam and its metabolites, however, fail to chromatograph satisfactorily, giving rise to severely tailing peaks. It is therefore necessary to hydrolyse these compounds to their corresponding benzophenones[5] or to convert nitrazepam to its methyl derivative[6] prior to chromatography. These methods suffer from the drawback that neither of the major metabolites of nitrazepam, 7-aminonitrazepam and 7-acetamidonitrazepam can be separately determined.

Analyses of benzodiazepine mixtures by high-pressure liquid chromatography (HPLC) have been reported by several authors. Mixtures of intact benzodiazepines have been separated on silica[7], cation-exchange resins[8], Durapak OPN[9] and Carbowax 400 coated support[10].

Although many procedures have been developed for the separation of nitrazepam from other benzodiazepines, no technique has been reported for the chromatographic resolution of nitrazepam and its metabolites by gas or liquid chromatography. This note describes such an analysis performed by HPLC on an anion-exchange packing.

EXPERIMENTAL

The liquid chromatograph consisted of twin Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 reciprocating piston pumps, controlled by a Waters Model

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660 solvent programmer. Column effluent was monitored by a Varian (Walnut Creek, Calif., U.S.A.) Model 635 M spectrophotometer, equipped with twin 8-μl flow cells. The detector was operated at 260 nm with a slit width of 2 mm.

The column consisted of a 50 cm $\times$ 2 mm I.D. stainless-steel tube, dry packed with the strong anion-exchange material, Zipax SAX (30 μm mean particle diameter; DuPont, Wilmington, Del., U.S.A.). Sample injection was made directly onto the column via a septum injection port.

Reagents

Reagent-grade ethyl acetate and hexane were obtained from BDH (Poole, Great Britain). These were redistilled and degassed by boiling prior to use. Benzodiazepines were obtained from Roche Products (Welwyn Garden City, Great Britain).

METHOD AND RESULTS

Extraction

An amount of 10 ml of human urine, adjusted to pH 7 with 1 *M* acetate buffer,

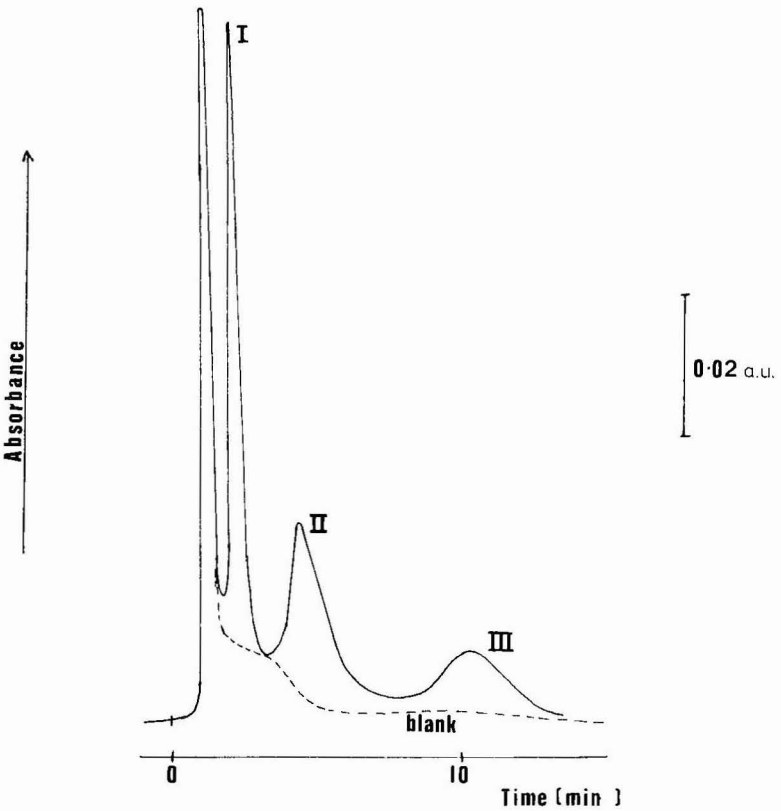

Fig. 1. HPLC separation of nitrazepam and metabolites on Zipax SAX. – – –, Urine blank (5 μl of extract injected onto column); ———, extract from urine spiked with 10 μg/ml nitrazepam and metabolites (5 μl injected onto column). Peaks: I – nitrazepam; II – 7-aminonitrazepam; III – 7-acetamidonitrazepam.

was extracted three times with 10-ml portions of ethyl acetate. The combined extracts were evaporated to dryness and redissolved in 1 ml of ethyl acetate. Recovery of spiked nitrazepam and metabolites from urine was 80 ± 4% at concentrations of 10 μg/ml.

Chromatography

Benzodiazepine samples were injected onto a column of Zipax SAX and eluted with a solvent mixture consisting of ethyl acetate–hexane (3:7) at a flow-rate of 1 ml/min. A typical chromatogram of an extract obtained from urine spiked to a level of 10 μg/ml with nitrazepam and its metabolites is given in Fig. 1. Detector response was found to be linear for all three compounds over the range 0–700 ng. Detection limits were in the range 20–100 ng (peak height = 2 × noise signal), corresponding to 0.4–2.0 μg of benzodiazepine per millilitre of urine.

DISCUSSION AND CONCLUSIONS

Nitrazepam and its metabolites have been analysed by HPLC on a column of strong anion-exchange resin. The mechanism for this separation is poorly understood but has previously been observed with molecules containing substituted nitrogen moieties[11].

The technique is rapid, moderately sensitive and, unlike gas chromatographic analysis, allows separation and quantification of both nitrazepam and its metabolites without the necessity for an hydrolysis stage. It has been demonstrated that the procedure is applicable to the analysis of relatively high levels of nitrazepam in urine, but a more efficient multi-stage clean-up procedure, such as is used for gas chromatographic analysis, may be required for more dilute samples.

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Note

Use of high-performance liquid chromatography to measure plasma concentrations of *p*-chlorophenoxyisobutyric acid after administration of clofibrate to humans

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(Received February 21st, 1977)

Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) is an important hypolipidaemic agent[1–4]. It is hydrolysed during (or after) absorption from the gastrointestinal tract so that only the corresponding acid is detected in the peripheral plasma[5–7]. Plasma concentrations of this acid, *p*-chlorophenoxyisobutyric acid (CPIB), have usually been measured by the spectrophotometric method of Barrett and Thorp[8] or more recently by gas chromatography[6,9,10]. The specificity of the spectrophotometric method is adequate only for controlled studies in human volunteers; otherwise, gas chromatography has been preferred[6]. Furthermore, the variable background encountered using the spectrophotometric method limits the accuracy of measurement of low plasma concentrations of CPIB.

The use of high-performance liquid chromatography (HPLC) would improve the specificity of the method and its accuracy for measurement of lower concentrations of CPIB.

EXPERIMENTAL

Materials

Analytical grade diethyl ether, which was redistilled before use, acetonitrile and methanol (Spectrograde) were obtained from Fisons, Loughborough, Great Britain. 4-Chloro-2-methylphenoxyacetic acid (CMPA) was available from Aldrich, Wembley, Great Britain and *p*-chlorophenoxyisobutyric acid (CPIB) was a gift from Bristol-Myers, New York, U.S.A.

Extraction

The internal standard CMPA (50 μg) in methanol and 3 *M* HCl (0.5 ml) were added to plasma (1 ml) in a 12-ml glass-stoppered centrifuge tube, which was shaken for 15 sec and allowed to stand for 5 min. Diethyl ether (6 ml) was added to the contents of the tube, the mixture shaken for 30 sec and centrifuged for 5 min at 2000 *g*.

The ether layer (now containing CPIB extracted from plasma and the internal standard CMPA) was removed, evaporated to dryness under nitrogen at *ca.* 20°, and the resulting residue dissolved in methanol (100 μl). Portions (10 μl) of this solution were injected into the chromatograph using a stop-flow injection technique.

Apparatus and chromatography

A Pye Unicam LC20 liquid chromatograph (Pye Unicam, Cambridge, Great Britain) fitted with a septum injector and a SP6-400 variable wavelength ultraviolet detector linked to a Philips PM 8220 pen recorder was used. The stainless steel column (25 × 0.46 cm I.D.) was packed with C_{18} Partisil (10 μm, Reeve Angel, London, Great Britain). The mobile phase was 27% (v/v) acetonitrile containing 0.4% (w/v) orthophosphate buffer (to maintain the pH at 4.2) at a flow-rate of 2 ml/min and a typical back pressure of 50 bar.

Under these conditions, the retention times of CMPA and CPIB were 6 and 7 min respectively, thus allowing repeat injections every 10 min.

Standard curves were constructed from chromatograms obtained from plasma to which known amounts of CPIB (0–100 μg/ml) had been added (Fig. 3).

The method was used to measure concentrations of CPIB in the plasma of human subjects who had been dosed orally with clofibrate (1 g) during bioavailability studies[11]. These plasma samples were also analysed by the spectrophotometric method[8].

RESULTS AND DISCUSSION

The ultraviolet absorption spectrum of CPIB shows maxima at *ca.* 230 nm and 280 nm and a minima at *ca.* 254 nm in the solvent used (Fig. 1). The spectrum of CMPA is similar. Thus measurement at 254 nm which is common on fixed wavelength detectors, provided poor sensitivity. At this wavelength there was also an interfering peak in blank plasma extracts equivalent to *ca.* 25 μg/ml of CPIB. Monitor-

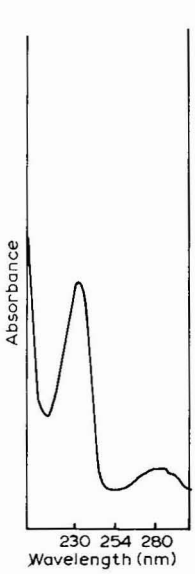

Fig. 1. Ultraviolet spectrum of *p*-chlorophenoxyisobutyric acid in methanol.

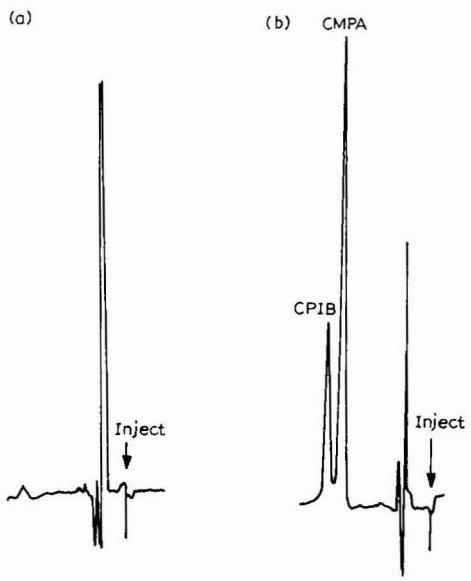

Fig. 2. High-performance liquid chromatogram obtained from (a) control plasma, (b) plasma containing a CPIB concentration of 20 μg/ml (and a CMPA concentration of 50 μg/ml).

ing the column eluate at 230 nm increased the sensitivity to CPIB and decreased the sensitivity to the interfering peak producing an overall limit of detection of about 2 μg/ml (Fig. 2).

Measurement of peak height ratios of CPIB and CMPA with respect to concentrations of CPIB provided a standard curve that was linear ($y = a + bx$, where $a = 0.0385(\pm 0.0384)$, $b = 0.0157(\pm 0.0006)$; the value of the intercept was not significantly different from zero, Fig. 3).

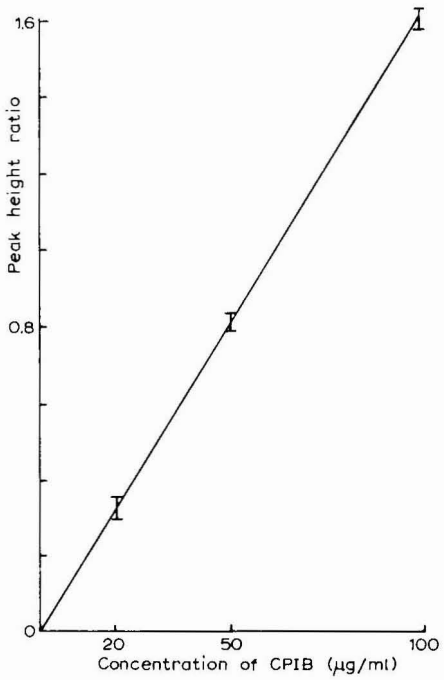

Fig. 3. Standard (calibration) curve for measurement of CPIB in plasma by HPLC. Each point represents the mean + S.D. of three replicates.

The range of three replicate measurements of CPIB added to plasma at concentrations of 20, 50 and 100 μg/ml were 18–22, 49–51 and 99–102 μg/ml, respectively.

The recovery of CPIB added to plasma at concentrations of 20, 50 and 100 μg/ml exceeded 90% and that of the internal standard of CMPA added to plasma at a concentration of 50 μg/ml exceeded 95%.

Concentrations of CPIB in plasma samples withdrawn during bioavailability studies of clofibrate[11] were measured by both HPLC and spectrophotometric methods[8]. There was good agreement of results obtained using either method (Table I), but mean plasma concentrations of CPIB, measured by HPLC, declined with a half-life of 23 h whereas the half-life measured using the spectrophotometric method was 19 h. However, the background (blank) values of CPIB measured were lower and varied less when HPLC was used (< 2 μg/ml in 8 subjects) than when the spectrophotometric method was used [16.7 $\pm$ 5.5 S.D. μg/ml ($n = 8$), range 8.6–24.9 μg/ml].

TABLE I

CONCENTRATIONS OF CPIB IN HUMAN PLASMA

CPIB in the plasma of 8 human subjects was measured using HPLC or spectrophotometry. Results are expressed as μg/ml ± S.D.

Time (h)	*HPLC*	*Spectrophotometric method**
1	28.6 ± 22.3	24.7 ± 24.6
2	48.1 ± 18.6	48.7 ± 26.5
3	55.9 ± 18.5	57.6 ± 23.8
4	60.1 ± 16.4	66.6 ± 21.0
6	64.4 ± 11.7	72.5 ± 17.1
8	64.1 ± 10.3	71.1 ± 15.1
12	60.1 ± 6.1	61.8 ± 14.5
24	43.9 ± 10.6	40.7 ± 15.7
32	33.4 ± 7.8	27.7 ± 12.4
48	19.1 ± 6.7	16.5 ± 9.5

* Values corrected for pre-dose (blank) concentration of 16.7 ± 5.5 μg/ml.

The HPLC method described, appears as accurate and is more convenient than reported gas chromatographic methods[6,9,10,12–14]. Nevertheless, the assay needs to be applied to the plasma of patients receiving multi-drug therapy in order to provide a more rigorous test of its specificity. However, many basic drugs would not be extracted from acidified plasma and the versatility of HPLC, would be expected to allow CPIB to be separated from interfering neutral and acidic drugs.

ACKNOWLEDGEMENTS

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Note

Chromatography of histones on hydroxyapatite columns

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It has been shown that hydroxyapatite is a valuable column support for the fractionation of non-histone proteins[1,2]. Bernardi *et al.*[3] found that basic proteins can also be chromatographed on hydroxyapatite by competition for their binding sites (the phosphate groups of hydroxyapatite) with cations.

This paper describes the chromatography of bovine histones on hydroxyapatite with gradients of sodium phosphate or sodium chloride, both in 5 *M* urea. The arginine-rich histones H3 and H4 are, together with histone H2A, eluted first, followed by the slightly lysine-rich histone H2B and then by the lysine-rich histone H1. From the chromatographic behaviour of poly(L-lysine) and poly(L-arginine) it is concluded that on the hydroxyapatite column the histones are mainly separated according to their lysine content, and that the guanidino group of arginine contributes only slightly to the binding of histones to hydroxyapatite.

EXPERIMENTAL

Materials

Poly(L-lysine) (M_r = 16,500) and poly(L-arginine) (M_r = 15,000) were purchased from Miles-Yeda (Rehovoth, Israel).

Preparation of histones

Chromatin was isolated from bovine lymphocytes as described previously[2]. Histones were extracted from chromatin according to Panyim *et al.*[4].

Chromatography on hydroxyapatite columns

Hydroxyapatite was prepared according to the method of Tiselius *et al.*[5]. The urea used throughout this study was prepared as a 10 *M* stock solution and deionized on a column of Ionenaustauscher V mixed-bed resin (Merck, Darmstadt, G.F.R.). The prepared buffers were stored in the cold and used within 2 days of preparation.

Columns containing 20 ml of hydroxyapatite were loaded with about 20 mg of protein in equilibration buffer (see legends of figures), the protein was washed in with the same buffer and the column was eluted with linear gradients as indicated in the figures. All operations were carried out at 4°. Elution molarities were checked by measuring the conductivity and compared with standard graphs for the appropriate buffers.

The protein concentration was determined from the absorbance at 230 nm using the relationship 1 absorbance unit ≡ 0.30 mg/ml, which was established for histones by nitrogen determination by the Kjeldahl method[6].

Polyacrylamide gel electrophoresis

Electrophoresis was performed on gels of 15% acrylamide, 0.9 *N* acetic acid and 2.5 *M* urea as described by Panyim and Chalkley[7].

Amino acid analyses

Total amino acid compositions were determined by using a modified Model 120c Beckman amino acid analyser. Samples containing 200–300 μg of protein were hydrolysed in 6 *N* hydrochloric acid at 110° for 22 h in sealed tubes under a nitrogen atmosphere.

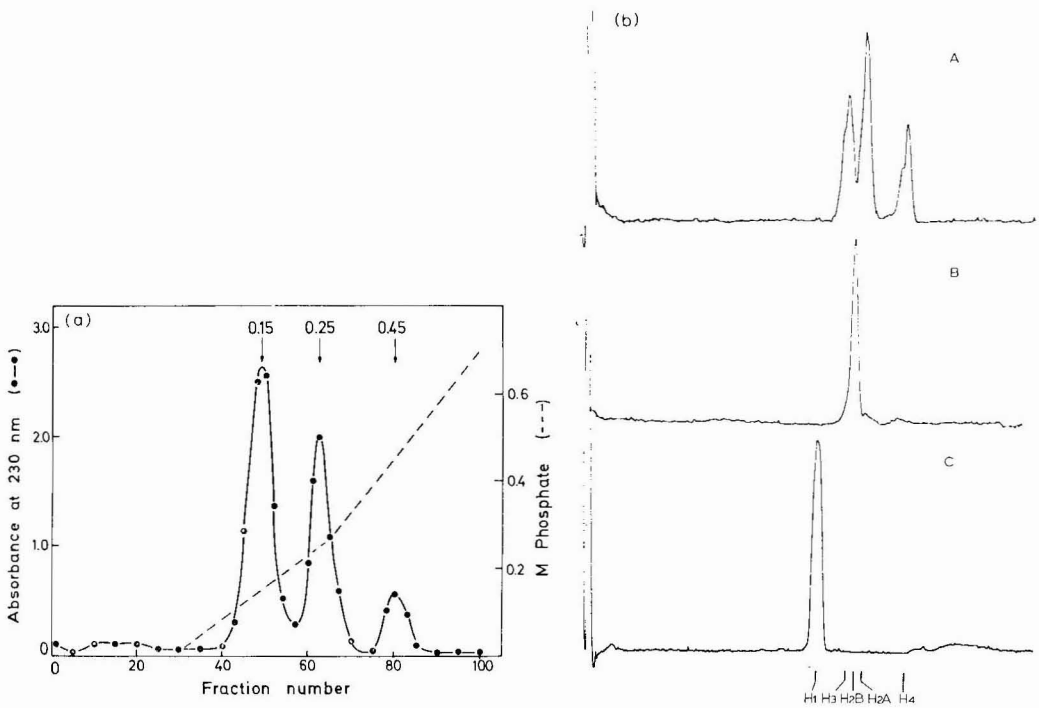

Fig. 1. (a) Chromatography of histones on hydroxyapatite with a gradient of sodium phosphate (pH 6.8) in 5 *M* urea. Histone (25 mg) was dissolved in 1 m*M* sodium phosphate (pH 6.8), 5 *M* urea and loaded on a 20-ml hydroxyapatite column equilibrated with the same buffer. Elution was carried out with two successive sodium phosphate molarity gradients (each 50 + 50 ml), 1 m*M*–0.3 *M* and 0.3–0.8 *M*, both in 5 *M* urea. Fractions of 2.8 ml were collected. (b) Electrophoresis of hydroxyapatite column fractions in acidic urea–polyacrylamide gels. (A) Histones eluted with 0.15 *M*, (B) with 0.25 *M* and (C) with 0.45 *M* sodium phosphate (pH 6.8) in 5 *M* urea.

RESULTS AND DISCUSSION

Fig. 1a shows the pattern of elution of bovine histones from hydroxyapatite with a gradient of sodium phosphate (pH 6.8) in 5 *M* urea. With 0.15 *M* phosphate, histones H3, H2A and H4 elute together from the column, as revealed by acidic urea–polyacrylamide electrophoresis (Fig. 1b). The two following peaks, with 0.25 and 0.45 *M* phosphate, consist of histone H2B and H1, respectively, as shown by polyacrylamide gel electrophoresis and amino acid analysis (Table I). The amounts of protein recovered in the peaks were 50, 30 and 8%, respectively.

TABLE I

AMINO ACID ANALYSES OF FRACTIONS FROM BOVINE HISTONES

No corrections have been made for losses during hydrolysis.

Amino acid	*Amount in fraction (mole per 100 mole)*	
	0.25 M phosphate peak = H2B	*0.45 M phosphate peak = H1*
Lysine	15.7	25.4
Histidine	3.8	0
Arginine	6.4	1.9
Aspartic acid	5.2	2.4
Threonine	6.6	5.6
Serine	9.3	6.3
Glutamic acid	8.9	3.5
Proline	4.4	9.1
Glycine	5.9	6.6
Alanine	10.8	25.1
Cysteine	0	0
Valine	7.6	5.1
Methionine	Trace	0
Isoleucine	5.4	1.3
Leucine	5.7	4.6
Tyrosine	2.4	0.2
Phenylalanine	1.7	0.4

A similar elution pattern was obtained with a gradient of sodium chloride in 5 *M* urea, 1 m*M* sodium phosphate (pH 6.8) (Fig. 2). Again, the arginine-rich histones H3, H4 and the slightly lysine-rich histone H2A appear first in one peak at 0.05 *M* NaCl. Histone H2A is also found in the second peak at 0.38 *M* NaCl, together with the other slightly lysine-rich histone H2B. Histone H1, which has the highest lysine content, elutes with 0.85 *M* NaCl.

A combination of NaCl and phosphate elution is shown in Fig. 3a. The sodium chloride concentration is fixed at 0.6 *M* so that all of the histones, except histone H1, will flow through the column. Histone H1 is then eluted with a phosphate gradient in 5 *M* urea, 0.6 *M* NaCl, and appears in two peaks comprising 2.8% and 7.6% of the material applied, respectively, with similar electrophoretic mobilities in acidic urea gels (Fig. 3b). Whether the chromatographic separation is caused by a microheterogeneity of histone H1 or is due to modifications of the histone by acetylation or phosphorylation has not been investigated. The strong binding of histone H1 to hydroxyapatite has been shown previously by Bernardi *et al.*[3], who found that 0.55 *M*

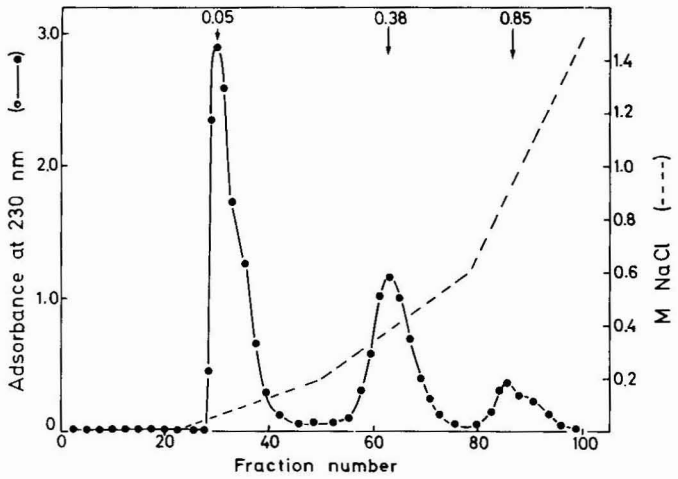

Fig. 2. Chromatography of histones on hydroxyapatite with a gradient of sodium chloride in 5 *M* urea, 1 m*M* sodium phosphate (pH 6.8). Histone (67 mg) was dissolved in 1 m*M* sodium phosphate (pH 6.8), 5 *M* urea and loaded on an 80-ml hydroxyapatite column. Elution was carried out with three successive sodium chloride gradients (each 80 + 80 ml), 0.0–0.2 *M*, 0.2–0.6 *M* and 0.6–1.5 *M*, all in 5 *M* urea, 1 m*M* sodium phosphate (pH 6.8). Fractions of 5.8 ml were collected.

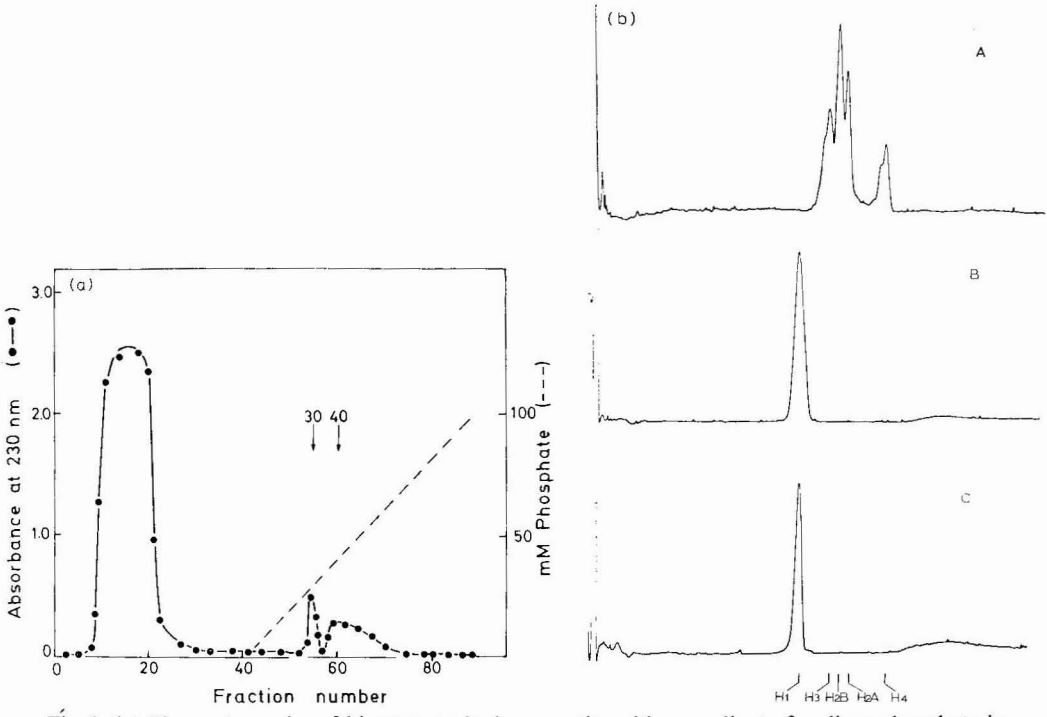

Fig. 3. (a) Chromatography of histones on hydroxyapatite with a gradient of sodium phosphate in 5 *M* urea, 0.6 *M* NaCl. Histone (83.9) was dissolved in 5 *M* urea, 0.6 *M* NaCl, 1 m*M* sodium phosphate (pH 6.8) and loaded on a 100-ml hydroxyapatite column. The unadsorbed material was washed out with the same buffer and the column developed with a 1 m*M*–0.1 *M* sodium phosphate gradient (130 + 130 ml) in 5 *M* urea, 0.6 *M* NaCl. Fractions of 2.9 ml were collected. (b) Electrophoresis of hydroxyapatite column fractions in acidic urea–polyacrylamide gels. (A) Histones, not adsorbed on the column; (B) histone eluted with 30 m*M* and (C) histone eluted with 40 m*M* sodium phosphate (pH 6.8) in 5 *M* urea, 0.6 *M* NaCl.

sodium phosphate is needed for elution. The lower value found in this study (see Fig. 1) may be explained by the addition of 5 *M* urea to all buffers.

The basic nature of the histone molecules is due mainly to the amino acids lysine and arginine, which have isoelectric points of 9.74 and 10.76, respectively. Histidine contributes only slightly.

To investigate the different behaviours of the histones on hydroxyapatite further, the elution molarities of the synthetic polymers poly(L-lysine) and poly(L-arginine) were determined. Table II shows that poly(L-arginine) elutes with 0.12 *M* NaCl in 5 *M* urea, 1 m*M* sodium phosphate, whereas a 10-fold higher NaCl concentration is needed to elute poly(L-lysine). The corresponding sodium phosphate concentrations in 5 *M* urea are 0.4 and 0.7 *M*, respectively. These results suggest that it it is not the basicity of the molecule that determines the chromatographic behaviour on the column, but rather the ε-NH_2 group of lysine binds much more strongly to the phosphate groups of hydroxyapatite than the guanidino group of arginine. This finding fits well with the unexpected observation of Bartley and Chalkley[8] that there is no significant contribution of the hydrogen bonds between arginine and the phosphate groups of DNA towards the strength of DNA–histone binding. The different chromatographic behaviour of lysine and arginine could explain the separation of histones on hydroxyapatite: if the histones are arranged according to the number of lysine residues they contain (Table III), the order obtained is the same as that in which they are eluted from the column.

TABLE II

ELUTION MOLARITIES (mole/l) OF SYNTHETIC POLYPEPTIDES FROM HYDROXYAPATITE COLUMNS

Polypeptide	*Eluting solvent*	
	NaCl in 5 M urea 1 mM sodium phosphate (pH 6.8)	*Sodium phosphate (pH 6.8) in 5 M urea*
Poly(L-arginine)	0.12	0.4
Poly(L-lysine)	1.2	0.7

TABLE III

LYSINE CONTENT OF CALF THYMUS HISTONES

Histone	*Lysine residues per molecule*	*Reference*
H1 (F1)	59	4
H2B (F2b)	20	9
H2A (F2a2)	14	10
H3 (F3)	13	11
H4 (F2a1)	11	12

ACKNOWLEDGEMENTS

The author thanks Heinz Pachowsky and Alfred Schöffski for excellent technical assistance and Walter Vötsch for the amino acid analyses.

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Note

Separation of aromatic sulphinylamines by thin-layer chromatography. II

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The analysis of dichlorosulphinylamine isomers described here is a continuation of earlier thin-layer chromatographic (TLC) studies on arylsulphinylamines[1,2]. Examination by thermal analysis has enabled the stability of the arylsulphinylamines to be determined[3], making it possible to apply gas chromatography for their analysis[4,5].

The arylsulphinylamines show extremely high reactivity, owing to the sulphinyl group, Ar–N=S=O, which makes them suitable for use in certain chemical processes[6,7]. The dipole moments and proton magnetic resonance spectra of substituted N-sulphinylanilines indicate that these compounds adopt preferentially a *cis*- or *syn*-structure (Z-configuration) with respect to the central N=S bond[8–10]. The *trans*-sulphinylation reaction was developed and its kinetics were investigated using gas chromatography[11].

In this paper we describe TLC investigations of isomeric dichlorosulphinylanilines.

EXPERIMENTAL

Reagents and materials

The following pure reagents were used: benzene, chloroform, amyl alcohol, carbon tetrachloride, diethyl ether and ethyl acetate (POCh, Gliwice, Poland); *n*-hexane (VEB Jenapharm-Laborchemie, Apolda, G.D.R.); and acetone (Chem. Inds., Oświęcim, Poland). The solvents were dehydrated and distilled under vacuum, and the amounts of moisture present were tested using the Van der Meulen reagent and by means of gas–liquid chromatography.

Silica gel on aluminium TLC sheets (DC-Alufolien Kieselgel, Art. 5553, Merck, Darmstadt, G.F.R.) 20 × 20 cm, layer thickness 0.25 mm, were used.

Dichlorosulphinylamines. The dichlorosulphinylamines were prepared by the Michaelis and Herz[12,13] method, 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dichlorosulphinylaniline being investigated. The purities of the products were checked by using NMR and mass spectrometry and gas–liquid chromatography.

To protect the dichlorosulphinylamines against moisture, they were stored in sealed glass ampoules.

Procedure

The aromatic sulphinylamines were used as 20% solutions in benzene, and a mixed solution was prepared by mixing 1 cm^3 of each benzene solution. The solutions of the samples and those of standard substances were placed on the plate 2.5 cm from the edge, and the plate was then dried for 5 min at 60–70°. The dried plate was inserted in a chromatographic chamber lined with filter-paper saturated with the solvent system. The migration distance of the solvent front was 15 cm.

A gaseous developing agent, nitrogen dioxide, was used, which was prepared by dropping 1–5 ml of hydrochloric acid (18%) into 10 ml of 20% sodium nitrite solution. The nitrogen dioxide liberated was collected under a 5-l cover-glass for 30 min, then the plate bearing the developed and dried chromatogram was placed under the cover-glass. After 30–60 sec, the plate was removed from under the cover-glass and allowed to stand in air for 24 h until well rounded yellow, orange and brown spots appeared.

RESULTS

To study optimal TLC conditions for the separation of the dichlorosulphinylamines, a variety of polar and non-polar solvent systems were employed (Table I). The R_F values obtained are given in Table II.

TABLE I

SOLVENT SYSTEMS EMPLOYED TO SEPARATE DICHLOROSULPHINYLAMINES

No.	*Components*
I	Benzene
II	Chloroform
III	Chloroform–*n*-hexane (80:20)
IV	Chloroform–ethyl acetate–*n*-hexane (40:40:20)
V	Benzene–amyl alcohol–carbon tetrachloride (20:10:70)
VI	Benzene–*n*-hexane–diethyl ether (60:20:20)
VII	Benzene–acetone–chloroform (40:10:50)
VIII	Carbon tetrachloride–*n*-hexane–diethyl ether (50:10:40)
IX	Benzene–chloroform (50:50)
X	Benzene–*n*-hexane–ethyl acetate (70:20:10)
XI	Toluene

The spots were eluted from the plate using benzene and then identified by gas–liquid chromatography. It was found that the spots remained stable and were not subject to hydrolysis on silica gel.

The dichlorosulphinylamines are very alike in their behaviour, especially 2,3- and 2,4-dichlorosulphinylaniline, which led to considerable difficulties in their separation even though the chromatograms were developed twice. The best separation was obtained with solvent systems III, VI, VII and IX, where the differences in the R_F values were sufficient to enable the mixtures to be completely separated in routine work. 2,5-, 2,6-, 3,4- and 3,5-dichlorosulphinylaniline showed considerable differences in their R_F values.

TABLE II

$R_F \times 100$ VALUES OF DICHLOROSULPHINYLAMINES

a, $R_F \times 100$ for single samples; b, $R_F \times 100$ for mixtures of dichlorosulphinylamine isomers.

Dichloro-sulphinyl-amine	*Solvent system**																					
	I		*II*		*III*		*IV*		*V*		*VI*		*VII*		*VIII*		*IX*		*X*		*XI*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
2,3-	54	54	71	68	60	61	70	71	70	71	70	68	71	70	75	75	60	60	63	60	47	48
2,4-	53	54	68	68	55	54	70	71	66	67	69	68	71	70	75	75	58	60	62	60	49	48
2,5-	63	63	72	73	60	61	75	75	74	76	77	77	75	75	80	81	65	66	70	69	60	60
2,6-	73	72	79	78	74	74	78	75	84	84	83	85	81	81	85	85	75	76	80	79	73	72
3,4-	37	33	55	52	34	34	60	60	62	63	47	43	58	55	57	53	38	39	46	46	30	30
3,5-	54	52	63	59	48	42	69	68	67	67	62	57	67	63	68	67	56	57	60	60	47	48

* Solvent systems according to Table I.

TABLE III

COLOURS OF SPOTS OF DICHLOROSULPHINYLAMINES

Dichlorosulphinylamine	*Colour of spot*
2,3-	Orange
2,4-	Brown
2,5-	Bright yellow
2,6-	Bright brown
3,4-	Dark red
3,5-	Red-orange

It was found that with a short developing time (30–60 sec) in the nitrogen dioxide atmosphere, the individual isomeric dichlorosulphinylamines could be identified on the chromatograms by using the specific colours of the spots (Table III).

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Note

Thin-layer chromatographic analysis of phenytoin and its hydroxy metabolites

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(First received October 12th, 1976; revised manuscript received February 14th, 1977)

Phenytoin (5,5-diphenylhydantoin) is a widely used anticonvulsant agent that undergoes extensive metabolic oxidation in the body prior to its excretion in urine[1]. The major metabolites of phenytoin in humans and most experimental animals are 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (Dihydrodiol), and 5-(3,4-dihydroxyphenyl)-5-phenylhydantoin (Catechol)[2–6]. These metabolites are excreted in the urine primarily as glucuronide conjugates. However, glucuronide of 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) is identified as the major urinary metabolite of the drug in dogs[5]. The presence of *m*-HPPH in the urine of humans and other laboratory animals is also reported[1–6] which is believed to be an artifact arising from the labile dihydrodiol metabolite during the isolation process.

While several gas[1,3,6] and liquid[7,8] chromatographic methods are reported for the simultaneous analysis of phenytoin and its hydroxy metabolites, there appears to be no convenient thin-layer chromatographic (TLC) method currently available for the simultaneous analysis of these compounds. The present paper describes a simple and rapid TLC method that utilizes neutral solvent systems for the analysis of phenytoin and its major hydroxy metabolites. Also, application of the TLC procedure in the study of phenytoin metabolism in the rat is reported.

EXPERIMENTAL

Thin-layer chromatographic procedure

Silica gel 60 F_{254} pre-coated TLC plates (E. Merck, Darmstadt, G.F.R.), 20 × 5 or 20 × 20 cm, layer thickness 0.25 mm, were utilized after activation at 105° for 5 min. Appropriate amounts of test compounds in ethyl acetate or methanol were spotted on TLC plates and developed in solvent system A, B or C (Table I) by the ascending technique. The resolved compounds on chromatograms were detected by either viewing under short-wavelength ultraviolet (UV) light (254 nm) or by exposing to iodine vapors.

Administration of [^{14}C]phenytoin to rats

Two male Sprague-Dawley rats, weighing 180–200 g, were housed in individual metabolism cages and were given rat chow (Ralston Purina, St. Louis, Mo., U.S.A.) and tap water *ad libitum*. [4-^{14}C]Phenytoin (spec. act. 50.5 mCi/mmole, New England

TABLE I

TLC OF PHENYTOIN AND ITS HYDROXY METABOLITES

Solvent systems: A, chloroform–ethyl acetate–methanol (5:1:1); B, chloroform–acetone–methanol (6:1:1); C, chloroform–methanol (3:1).

Compound	R_F* *Solvent system*			*Color observed (Detection limit, μg)*	
	A	*B*	*C*	*UV*	*Iodine*
Phenytoin	0.49 ± 0.02	0.51 ± 0.03	0.70 ± 0.03	Blue (2)	Brown (10)
m-HPPH	0.34 ± 0.01	0.30 ± 0.01	0.57 ± 0.02	Blue (1)	Brown (1)
p-HPPH	0.32 ± 0.01	0.29 ± 0.01	0.56 ± 0.02	Blue (1)	Brown (1)
Catechol	0.29 ± 0.01	0.18 ± 0.01	0.37 ± 0.01	Blue (2)	Brown (5)
Dihydrodiol	0.15 ± 0.01	0.09 ± 0.01	0.17 ± 0.01	Blue (5)	Brown (5)

* Mean of six determinations ± standard error.

Nuclear, Boston, Mass., U.S.A.) was diluted with appropriate amount of non-radioactive phenytoin (Eastman-Kodak, Rochester, N.Y., U.S.A.) in distilled water containing a few drops of 0.01 *N* sodium hydroxide and administered to the rats by the intraperitoneal route at a dose of 50 mg/kg, 50 μCi/kg. Urine samples were collected for 24 h in brown glass bottles and stored at −17° until time of analysis.

Isolation of urinary metabolites

The general procedure described by Rao *et al.*[9] was followed. The rat urine samples (15 ml) were chromatographed on Amberlite XAD-2 column (Drug-Skreen adsorbent cartridges, Brinkmann, Westbury, N.Y., U.S.A.). The methanol eluate (40 ml) was evaporated to dryness *in vacuo*, and the residue was taken up in 20 ml of distilled water containing 1 g of sodium acetate and pH adjusted to 7.4 with glacial acetic acid. The enzymatic hydrolysis of conjugated phenytoin metabolites was carried out at room temperature for 24 h by the addition of 2000 units of glucuronidase-sulfatase (Sigma, St. Louis, Mo., U.S.A.). The reaction mixture was then extracted 6 times with 10 ml of ethyl acetate and each extract was dried over anhydrous sodium sulfate and filtered. The pooled ethyl acetate extracts were evaporated to dryness *in vacuo* and the residue analyzed by TLC for phenytoin and its hydroxy metabolites as described above.

Quantitation of phenytoin and its hydroxy metabolites excreted in the rat urine was performed by their isolation and measurement of radioactivity. To isolate the compounds, chromatograms were developed in solvent system A and the spots corresponding to reference compounds (visualized under the UV light) were scraped into scintillation vials. A 15-ml volume of liquid scintillation cocktail (Insta-gel, Packard, Downers Grove, Ill., U.S.A.) was added to each vial and radioactivity measured in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The TLC data on the resolution and detection of phenytoin and its four hydroxy metabolites are given in Table I. When necessary, better resolution of *m*-HPPH and *p*-HPPH can be achieved by separate analysis of these two isomers on TLC plates developed in 4% methanol in diethyl ether (solvent system D, R_F 0.32 and 0.28, respectively).

The earlier TLC systems reported for the separation of phenytoin and its

hydroxy metabolites have used acidic solvent systems for the development of chromatograms[2,10]. Since the instability of the dihydrodiol metabolite of phenytoin under acidic conditions to form phenolic products is well recognized[2,6], the neutral solvent systems described in the present paper are particularly suited for phenytoin metabolism studies. In addition, the TLC procedure reported here is rapid since the chromatographic development time for solvent system A, B or C is about 1 h.

The urinary excretory profile of [4-^{14}C]phenytoin in the rat based on the above TLC procedure is given in Table II. The identity of *p*-HPPH was confirmed by its isolation (1% methanol in ethyl acetate was used for its extraction from TLC plates) and re-chromatography in solvent system D. These results are in general agreement with the earlier phenytoin metabolism studies in the rat[1–6].

Currently, we are utilizing this TLC procedure in the studies involving oral metabolism and gingival tissue adverse reactions induced by prolonged administration of phenytoin[11].

TABLE II

METABOLISM OF [4-^{14}C]PHENYTOIN IN THE RAT

Percentage urinary excretion of ^{14}C-labeled phenytoin and its hydroxy metabolites 24 h after a single intraperitoneal dose of [4-^{14}C]phenytoin (50 mg/kg; 50 μCi/kg). The metabolite conjugates were subjected to enzyme hydrolysis prior to the analysis. The recovery efficiency for the various compounds from urine were: Phenytoin, 92%; *p*-HPPH, 90%; Catechol, 85%; Dihydrodiol, 88%.

Compound	*Percent of dose*		
	Rat No. 1	*Rat No. 2*	*Average*
Phenytoin	1.9	1.7	1.8
p-HPPH	55.8	59.6	57.7
Catechol	2.1	1.9	2.0
Dihydrodiol	26.2	24.4	25.3

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Note

Quantitative thin-layer chromatographic assay for the β-neoagarotetraose hydrolase of *Pseudomonas atlantica**

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(Received July 19th, 1976)

Quantitative densitometry of thin-layer chromatograms has been used to assay sugars[1,2], lipids[3–5] and several other substances[6–10]. Thin-layer chromatography (TLC) also has been used to evaluate the neoagarobiose series of oligosaccharides obtained on enzymic hydrolysis of agarose[11], and as a quantitative assay for intestinal disaccharidases[12] and for the β-neoagarotetrase of *Cytophaga flevensis*[13]. The latter method involved scanning photographs of the chromatograms but the experimental details have not been published.

Agarose is degraded to monomeric sugars, in *Pseudomonas atlantica*, by an extracellular β-agarase and cell-bound oligosaccharidases, β-neoagarotetraose hydrolase and α-neoagarobiose hydrolase. The β-neoagarotetraose hydrolase hydrolyzes neoagarotetraose (O-3,6-anhydro-α-L-galactopyranosyl (1→3)-O-β-D-galactopyranosyl (1→4)-O-3,6-anhydro-α-L-galactopyranosyl (1→3)-D-galactose) by cleaving the central β(1→4) linkage to yield two molecules of neoagarobiose[14] (Fig. 1). Neoagarobiose (O-3,6-anhydro-α-L-galactopyranosyl (1→3)-D-galactose) is further hydrolyzed to the monomers 3,6-anhydro-L-galactose and D-galactose by an α-neoagarobiose hydrolase[15].

Purification and characterization of β-neoagarotetraose hydrolase required development of a sensitive and specific assay method. In crude enzyme preparations, where both β-neoagarotetraose hydrolase and α-neoagarobiose hydrolase are present, the quantitative assay should measure specifically activity of the β-neoagarotetraose hydrolase. Thus a method involving measurement of reducing sugars could not be used since both enzymes increase the reducing power. Activity of β-neoagarotetraose hydrolase was determined by measuring the concentration of neoagarotetraose on thin-layer chromatograms, after visualization of the sugar by charring. In this paper, a technical description of the assay is presented and possible applications discussed.

EXPERIMENTAL

Materials

Neoagarotetraose was prepared as previously described[16].

* This work is based on a thesis submitted by D. Groleau to the faculty of graduate studies, McGill University, in partial fulfillment of the M.Sc. degree requirements.

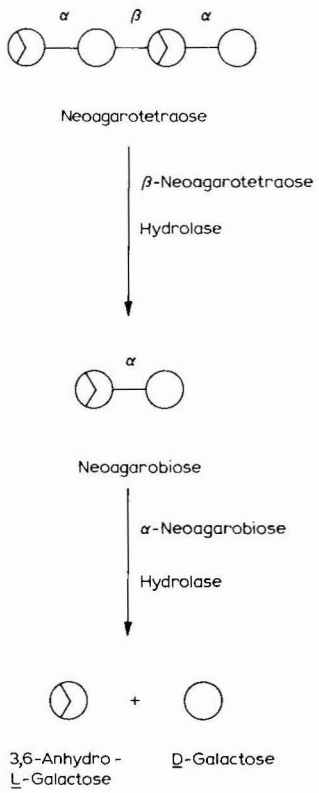

Fig. 1. Hydrolysis of neoagarotetraose by a mixture of β-neoagarotetraose hydrolase plus α-neoagarobiose hydrolase from *Pseudomonas atlantica*.

Silica gel thin-layers. MN-Silica gel N-MR (no binder, Macherey, Nagel & Co., Düren, G.F.R.), 30 g in 80 ml of a 1.0% (w/v) aqueous ammonium bisulphate solution, was layered to a thickness of 0.3 mm on 20 × 20 cm glass plates using a TLC-plate coater (Camag, Muttenz, Switzerland) and then air dried.

Chromatography. The freshly prepared solvent system was: butan-1-ol–ethanol–5% (w/v) aqueous ammonium bisulphate (3:1:1).

Methods

Application of samples. Samples were applied from a 25-μl microsyringe (Hamilton) equipped with a repeating dispenser delivering 0.5 μl. The spots were dried with a current of cold air.

Charring. Following chromatography, the plates were air-dried for about 1 h, heated at 175° for 30 min in a large oven provided with a fan and then cooled at room temperature for 20–30 min. Absorbance was measured within 1–2 h, with no significant change in absorbance after 6 h.

Densitometry. Quantitative densitometry was performed using a Vitatron TLD 100 densitometer (Vitatron, Dieren, The Netherlands). Absorbance of each peak was recorded as a number of pulses. The instrument settings were: transmission

(—log) mode; interference filter no 445; diaphragm, 0.25 mm; integrator, position 7; scanning speed, 0.5 cm/min; recorder speed, 1 cm/min.

Measurement of enzymic activity. Enzyme assays were carried out at room temperature in a serological plate covered with parafilm to prevent evaporation. For a 100 μl assay, the mixture consisted of 50 μl of 1.58×10^{-2} *M* neoagarotetraose (mol.wt. 630); 10 μl of 1 *M* NaCl, 0.01 *M* Tris buffer pH 7.2; and 40 μl of the enzyme preparation. At regular intervals, 2.5 μl of the assay mixture was applied in duplicate to the plate. Enzyme solution, equivalent to the amount present in 2.5 μl of the assay mixture, was applied as control.

RESULTS AND DISCUSSION

The procedure for charring neoagarotetraose was a modification of the technique of Touchstone *et al.*[4] for lipids and steroids. On heating, the oxidizing agent ammonium bisulphate is decomposed to acid that chars the sugars to yield dark-brown spots. Charring of sugars was reported to give quite stable spots[2].

Ammonium bisulphate was not incorporated into the silica gel by dipping the plates into a saturated ethanolic solution of this salt, as originally described, but rather added to the silica gel immediately before spreading the slurry on glass plates. The concentration of ammonium bisulphate used (1.0% w/v) was found to be optimal; lower concentrations yielded pale spots whereas higher concentrations had a deleterious effect on separation of sugars and on shape of the spots. Integration values for charred spots were higher and more reliable when the oxidizing agent was also added to the solvent system. Chromatography was started within 30 min as neoagarotetraose was slightly degraded by the ammonium bisulphate present in the layer. Under the conditions described, a linear relationship between absorbance (expressed as a number of pulses) and concentration of neoagarotetraose was obtained within the 1–22 μg range (Fig. 2).

Products of hydrolysis of neoagarotetraose (Fig. 1) with an enzyme preparation containing β-neoagarotetraose hydrolase and α-neoagarobiose hydrolase are shown

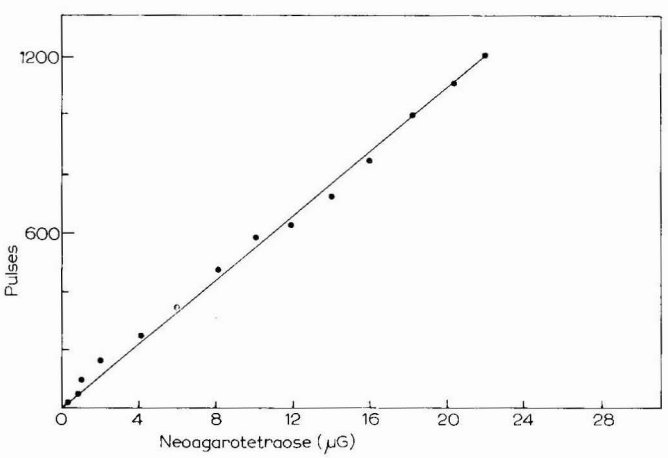

Fig. 2. Calibration curve for neoagarotetraose after charring and densitometry.

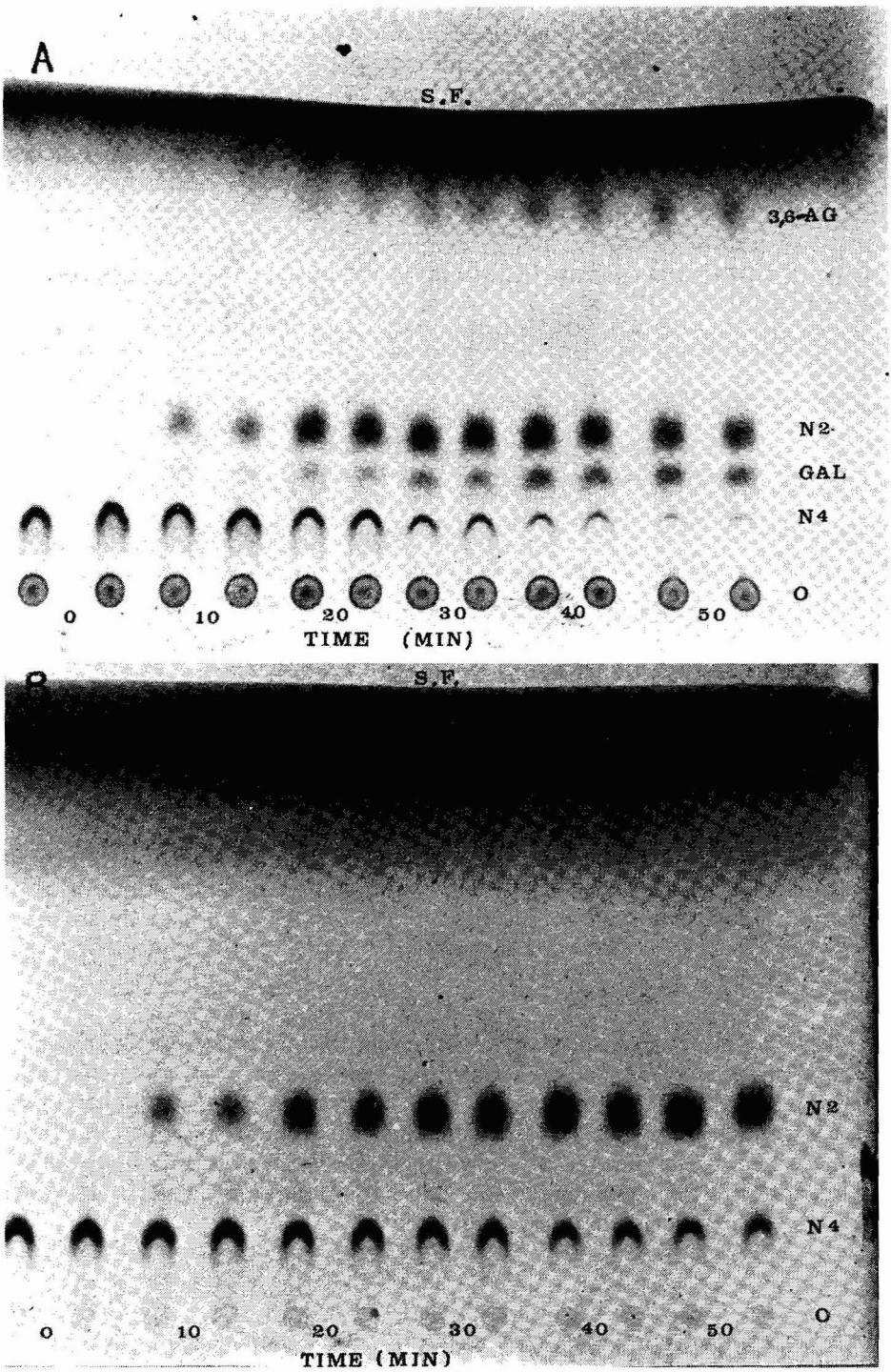

Fig. 3. Products of hydrolysis on TLC obtained on enzymic hydrolysis of neoagarotetraose by a preparation containing β-neoagarotetraose hydrolase and α-neoagarobiose hydrolase (A); and by a purified β-neoagarotetraose hydrolase preparation (B). N2, N4 = Neoagarobiose and -tetraose; GAL = D-galactose; 3,6-AG = 3,6-anhydro-L-galactose; S.F. = solvent front; O = origin.

in Fig. 3A and with a purified β-neoagarotetraose hydrolase preparation in Fig. 3B.

Results of a representative assay are presented in Fig. 4. Aliquots at time 0 contained 12.5 μg of neoagarotetraose and were considered as internal standards. Integration values were corrected on the basis of the value obtained for the internal standard and the concentration of neoagarotetraose determined from the standard curve (Fig. 2). A plot of disappearance of neoagarotetraose *versus* time was used to determine initial velocity of the enzyme (Fig. 4). One unit of β-neoagarotetraose hydrolase was defined as that amount of enzyme hydrolyzing 1 nmole of neoagarotetraose per min at room temperature. The molecular weight of neoagarotetraose was calculated as 630.

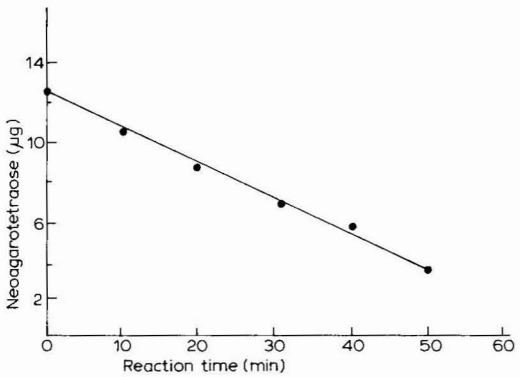

Fig. 4. Hydrolysis of neoagarotetraose by β-neoagarotetraose hydrolase: curve showing the disappearance of neoagarotetraose *versus* time.

Validity of the quantitative assay

The plate to plate variation (22 plates) of the internal standard (12.5 μg of neoagarotetraose) was less than 10% for 17 plates, with a variation within 10–15% for 5 plates. The variation for two identical aliquots on the same plate ranged from 1–7%.

CONCLUSION

The thin-layer chromatographic assay for β-neoagarotetraose hydrolase is accurate and reproducible. The duration of the enzyme assay is of about six hours and three separate assays can be carried out at the same time. The method was used to assay enzyme activity and properties of β-neoagarotetraose hydrolase. It should also be possible to determine the ratio of neoagarobiose to methylated neoagarobiose in different agars by this method, following hydrolysis of the agar samples by a mixture of β-agarase and β-neoagarotetraose hydrolase[17].

ACKNOWLEDGEMENTS

This research was generously supported by a grant from the National Research Council of Canada. We are indebted to Miss Donna Seto for the preparation of the neoagaro-oligosaccharides.

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CHROM. 9995

Note

Thin-layer electrophoretic separation of the degradation products of cyclic adenosine-3′,5′-monophosphate

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(First received October 18th, 1976; revised manuscript received February 7th, 1977)

A method of thin-layer electrophoresis (TLE) developed by Pastuska and Trinks[1] and by Honegger[2] has been successfully applied to the separation of several classes of compounds, including amino acids[2], alkaloids[3], indoles[4] and carbohydrates[5]. However, the TLE of cyclic adenosine-3′,5′-monophosphate (cAMP) and its degradation products has not hitherto been investigated.

The separation of cAMP from its degradation products is important because it is involved in many assays for of cAMP phosphodiesterases, where it is accomplished by using tedious chromatographic methods[6–9]. These methods are mostly concerned with the separation of cAMP from other compounds and not with the separation of the degradation products from each other. This latter separation, however, is important because it can be of use in characterizing a particular biopharmacological situation. It was therefore of interest to develop a simple and rapid method for separating the degradation products of cAMP, and we describe here the use of TLE for this purpose.

MATERIALS AND METHODS

Chemicals and equipment

[8-^{3}H]cAMP (ammonium salt; 21 Ci per mmole) was obtained from the Radiochemical Centre (Amersham, Great Britain). Inosine, hypoxanthine, buffer components, silica gel DC60-F254 TLC plates (200 × 200 mm; 0.1 mm thick) and scintillation chemicals were purchased from E. Merck (Darmstadt, G.F.R.). cAMP, adenosine 5′-monophosphate (AMP) and adenosine were obtained from Boehringer und Soehne (Mannheim, G.F.R.).

A power supply with a 0–600-V facility and a chamber from Desaga (Heidelberg, G.F.R.) were used for TLE.

Thin-layer electrophoresis

Borate buffer solution was made up from solutions of 7.22 g/l of boric acid and 17.6 g/l of sodium carbonate monohydrate, and phosphate buffer from 9.08 g/l of potassium dihydrogen phosphate and 11.8 g/l of disodium hydrogen phosphate dihydrate.

The compounds being investigated were dissolved in twice-distilled water to

give solutions of the following concentration: cAMP and adenosine, 1 mg/ml; inosine and AMP, 1.5 mg/ml, and hypoxanthine 2 mg/ml; these solutions were applied to the thin layers in amounts of 2 μl.

A potential difference of 400 V was applied across the plate; a typical current for 90% phosphate–10% borate combined buffer of pH 6.5 was 40 mA.

After electrophoresis for 60 min, the dried plate was viewed under UV radiation (254 nm), and migration distances towards the cathode were measured.

Thin-layer electrophoresis of [^{3}H]cAMP and its metabolites

A 200-g female Sprague-Dawley rat was decapitated, and the brain was removed immediately into ice-cold 0.16 *M* Tris buffer of pH 7.5 containing, per 100 ml, 100 mg of magnesium chloride trihydrate. The tissue (1 part by weight) was homogenised with the buffer solution (10 parts by weight) for 60 sec in an all-glass system, then the homogenate was centrifuged at 20,000 *g* for 30 min at 5° and stored at 2° until required. This preparation was characterised by biuret protein determination (Lowry *et al.*[10]), and the cAMP phosphodiesterase activity was determined by the method of Poech[11] as follows. A 200-μl portion of the supernatant was incubated with 200 μl of Tris–magnesium chloride buffer solution (pH 7.5) containing 70 nmoles of [^{3}H]cAMP for 10 min at 37°, then the mixtures were stabilised by adding 5 μl of 25% acetic acid and heated in boiling water for 3 min.

A blank comprising 200 μl of the buffer and 200 μl of the [^{3}H]cAMP buffer was used. After centrifugation at 2000 *g* at room temperature for 10 min, 10-μl portions of the supernatant were applied to the silica gel TLE plates, to which marker substances had been added as mentioned above. Electrophoresis was performed for 1 h in 90% phosphate–10% borate buffer medium at pH 6.8. After drying and identification of the substances under UV radiation, the gel from each zone was scraped into a scintillation counting phial. Then 400 μl of twice-distilled water were added to each phial, followed by 20 ml of scintillation fluid, and the radioactivity was counted in a Packard liquid scintillation counter (Tri-Carb 2420), quenching being measured with use of an internal standard. The scintillation fluid used consisted of 150 mg of 2,2′-*p*-phenylenebis-(4-methyl-5-phenyloxazole), 6 g of 2,5-diphenyloxazole, 1000 ml of toluene, 100 ml of dioxan and 400 ml of ethanol.

RESULTS

The effects of phosphate buffer solutions ranging in pH from 5.0 to 7.8 are shown in Fig. 1, which shows that adenosine and hypoxanthine, and cAMP and AMP, were not resolved; hypoxanthine migrated the furthest. A range of borate buffer solutions (pH 5.5 to 10.5) was also investigated (results not shown); migration distances were small, and cAMP, AMP and hypoxanthine were not separated. In this instance, at each pH value, inosine migrated the furthest.

The effects of phosphate and borate buffer mixtures at pH 6.8 are shown in Fig. 2. Separation of the individual substances improved as the content of phosphate increased up to a maximum of 90%. Complete separation of cAMP, AMP, adenosine, hypoxanthine and inosine was achieved with a combined phosphate–borate buffer of pH 6.8 containing 85 to 95% of phosphate. Optimum separation was with 90% phosphate–10% borate buffer at pH 6.8 in 1 h at 400 V; under these conditions, the

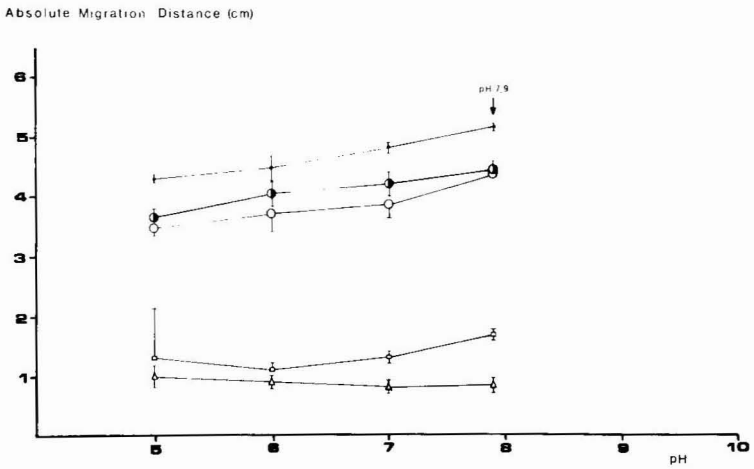

Fig. 1. Migration values of cAMP and degradation products in phosphate buffer solution of pH 5.0 to 7.8 on silica gel layers. Each result is the average of five experiments ± the standard deviation. □ = cAMP; △ = AMP; ○ = adenosine; ● = inosine; ◐ = hypoxanthine.

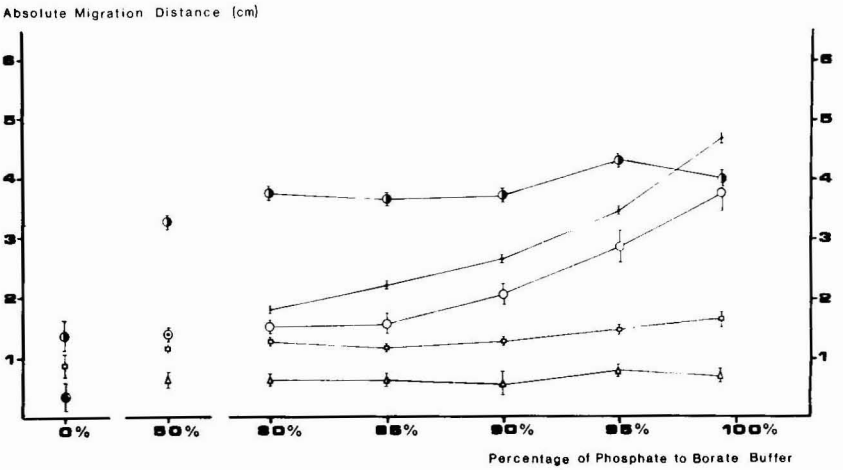

Fig. 2. Migration values of cAMP and degradation products in various combinations of phosphate and borate buffer solutions at pH 6.8 on silica gel layers. Each result is the average of five experiments ± the standard deviation. Symbols as in Fig. 1.

absolute migration distances (± the standard deviation) were 0.8 ± 0.01 for AMP, 1.5 ± 0.05 for cAMP, 2.9 ± 0.025 for adenosine, 3.5 ± 0.025 for inosine, and 4.35 ± 0.025 for hypoxanthine (each value being the mean of five determinations). The developed plate from a typical experiment is shown in Fig. 3.

The final pH values of the buffer solutions remaining in the anode and cathode compartments were 7.27 and 5.77, respectively; the final zone diameter was consistently in the region of 3.5 mm.

The results of the separation of the degradation products of cAMP after hydrolysis with the supernatant fluid from a rat-brain homogenate are presented in Table I.

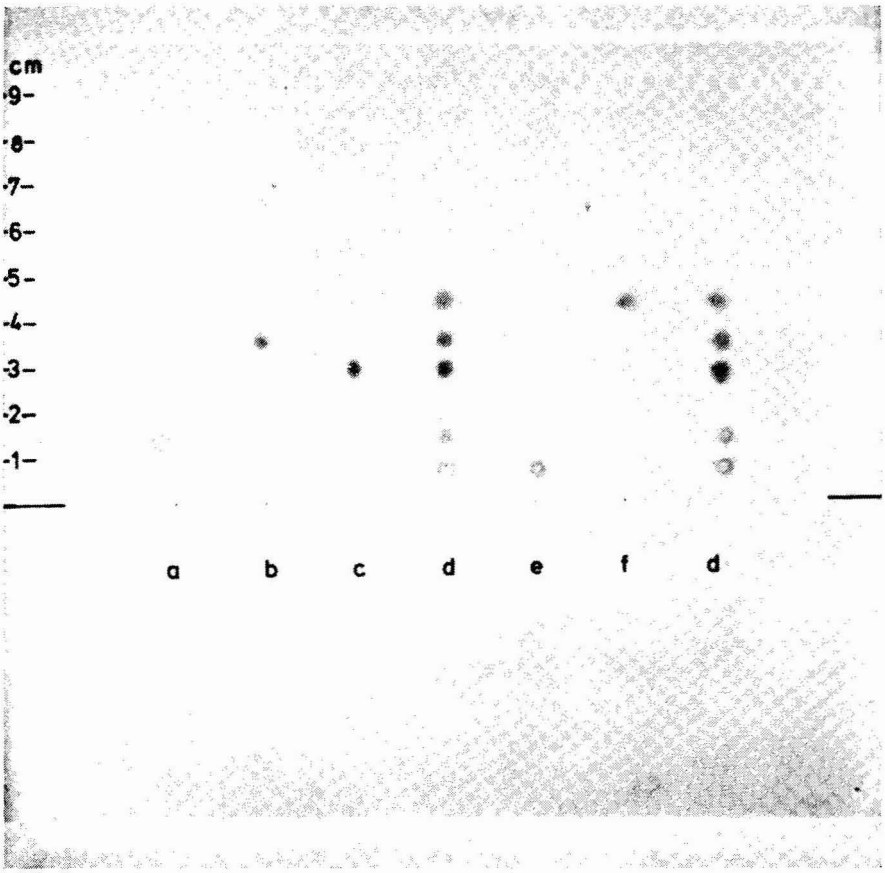

Fig. 3. Photograph (Polaroid Land film) of a developed plate under 254-nm radiation after TLE in 90% phosphate–10% borate buffer solution of pH 6.8; TLE for 1 h at 400 V. a = cAMP; b = inosine; c = adenosine; d = cAMP, inosine, adenosine, AMP and hypoxanthine; e = AMP; f = hypoxanthine.

TABLE I

ANALYSIS OF INCUBATION MIXTURE OF [^{3}H]cAMP WITH RAT-BRAIN HOMOGENATE SUPERNATANT AFTER SEPARATION BY TLE AND SCINTILLATION COUNTING

Results are expressed as the percentage of radioactivity per sample, and each is the average of eight experiments (± the standard deviation). The cAMP phosphodiesterase activity of the supernatant was 1.05×10^{-7} mg of cAMP per mg of protein per min at a cAMP substrate concentration of 0.125 μM.

Substance	*Incubation with supernatant*	*Incubation blank*
AMP	1.1 ± 0.3	2.3 ± 0.8
cAMP	1.83 ± 0.3	94.7 ± 2.1
Hypoxanthine	15.1 ± 0.5	0.1 ± 0.05
Adenosine	25.9 ± 1.8	1.0 ± 0.8
Inosine	54.1 ± 2.0	0.5 ± 0.3
Origin	1.8 ± 0.2	1.3 ± 0.2

Our results demonstrate that this method can be used for the separation and subsequent determination of radioactive labelled cAMP and its degradation products. We suggest that this method could be usefully applied to the study of the degradation pattern of cAMP in various physiological and pharmacological situations.

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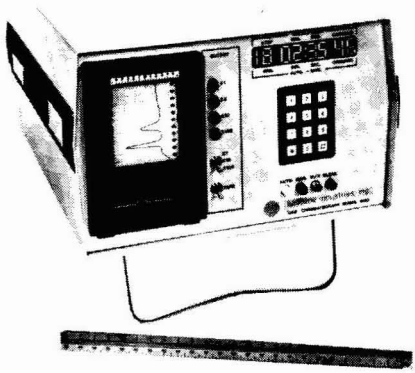
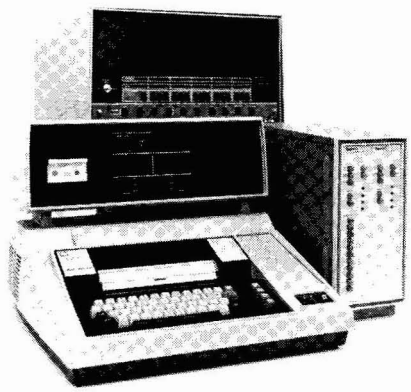
APPARATUS

N-1013

MICROPROCESSOR-CONTROLLED GAS CHROMATOGRAPH

Baseline Industries announce their microprocessor-controlled Series 1030 gas chromatograph. Features include: automatic zero, sample injection, stream selection, column backflushing, calibration, peak gating and integration, temperature programming, automatic recycle, etc. The programme step, time and command are displayed while the peak and/or peak area are recorded.





N-1007

ANALYTICAL PROCESSOR

The Tracor 812 Analytical Processor can be used with any liquid or gas chromatograph. Its minicomputer CPU (Data General Nova 2/10) allows operation with up to 16 GC/LC channels, sophisticated data acquisition and processing for gas and liquid chromatography. The software features use of both first and second derivative of the chromatographic signal.

N-1021

CAPILLARY COLUMN SUPPLIES

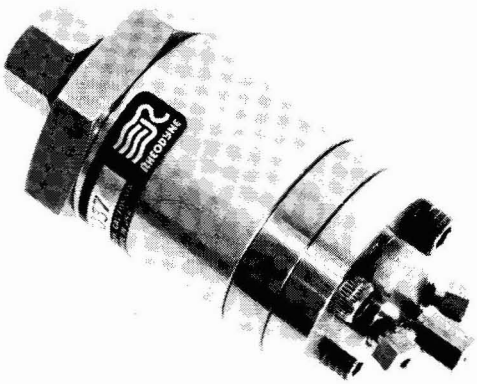
Available from Chrompack is a new brochure/application book on capillary columns, including information on the types of columns available, requirements for injection systems and detectors, quality control and installation, etc., as well as a number of applications.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1014

PRESSURE RELIEF VALVE FOR HPLC

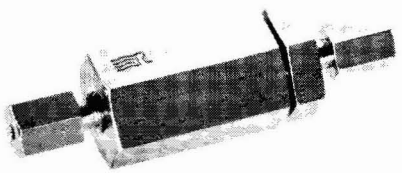
Rheodyne announce a new pressure relief valve (Model 7037) for HPLC equipment, which covers the pressure range 140–500 bar. In and Out ports provide a continuous in-line connection for full-time flushing of the 6-μl chamber. The relief setting can be set anywhere within range; when the system exceeds the setting a vent port opens. Stream contact is limited to type 316 stainless steel.



N-1010

INLET FILTER FOR HPLC

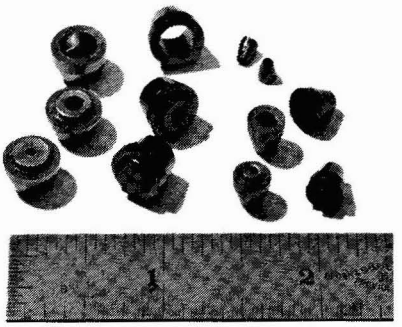
Rheodyne's inlet filter for use with HPLC columns is connected between the sample injection valve and the column. It uses a replaceable 2-μm filter element 3 mm in diameter and 3 mm thick to protect the inlet frit from plugging by particles of the sample or injection valve wear particles. Pressure rating of the filter assembly is 500 bar. Contact with the stream is type 316 stainless steel and PTFE.



N-1016

NEW GRAPHITE FERRULES

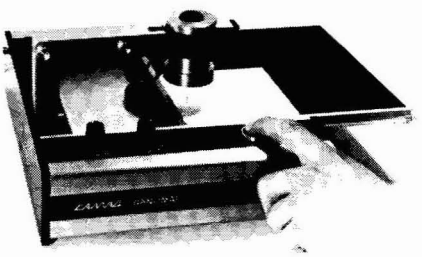
Now available from Analabs a complete line of graphite ferrules for maintaining gas-tight seals on glass and metal columns. The ferrules are malleable and will form to the tubing at temperatures as high as 440°. Sizes range from 0.8 mm capillary through 1/4 inch standard, in ten different size combinations.



N-1023

CAMAG NANOMAT

Recently announced is the new Camag Nanomat, which enables spot application of quantitative and qualitative samples in TLC (especially HPTLC) suitable for both linear plates and circular chromatograms developed in the Camag U-chamber. Transfer of the sample is made by a capillary pipette: glass capillary for normal TLC layers, application capillary with Pt–Ir tip or fixed-volume dosage pipette for HPTLC plates.



PROCEDURES

N-1012

CAMAG TLC BIBLIOGRAPHY

Camag's TLC Bibliography nr. 38 is now available. The editor announces that in future more information will be given on fewer publications. Papers containing interesting methodological information will be annotated in more detail and particular emphasis will be placed on HPTLC.

CHEMICALS

N-1015

SYRINGE CLEANER

Analabs' cleaner (Anakleen) can be used for all types of syringe, including microliter. Diluted 1:49 it is suitable for general laboratory use.

N-1017

GC MATERIALS AND ACCESSORIES CATALOGUE

Perkin-Elmer have available a catalogue listing their complete range of gas chromatography consumable supplies. Full details are given of stationary phases, columns, support materials, porous polymer supports and adsorbents, as well as syringes, tube couplings, etc.

N-1019

FATTY ALCOHOL KITS

Analabs announce the availability of two kits containing even-carbon fatty alcohol standards. One kit contains 25 mg each of 10 common even-carbon saturated alcohols, the second 10 mg each of 10 unsaturated alcohols. All standards have a purity greater than 99% as determined by GC or TLC.

N-1022

PESTICIDES KIT

Analabs, polychlorinated biphenyl (PCB) isomers kit has been developed for the environmental testing of the most commonly encountered PCBs. Included are 5 mg each of 14 PCB isomers and 6 interference pesticides to be used as reference standards. All PCBs and 5 of the pesticides have a chemical purity of greater than 99%.

N-1026

SORBENTS FOR REVERSED-PHASE CHROMATOGRAPHY

Available from EM Labs (Merck) is a 5-page colour brochure describing the mechanism of reversed-phase chromatography as well as its applications in TLC and HPLC. An explanation is included of the use of reversed-phase sorbents to optimize speed, resolution and capacity in HPLC.

MEETING

EUROANALYSIS III

Euroanalysis III will take place in the Science Block of University College Dublin, Ireland, from August 20–25, 1978.

The Conference will emphasize both pure and applied aspects of analytical chemistry. The programme has been designed to appeal to scientists engaged in the use of analytical techniques in problem solving as well as to those involved in research and development of analytical techniques.

Those who intend to participate in the Conference are welcome to offer papers for the scientific sessions. The Scientific Committee will consider papers of special interest and novelty on any relevant topics. The final date for receipt of abstracts is February 28, 1978.

For further information please contact Secretariat Euroanalysis III, Institute for Industrial Research and Standards, Ballymun Road, Dublin 9, Ireland.

NEW BOOKS

Pesticides in the environment, Vol. 3, edited by R.H. White-Stevens, Marcel Dekker, New York, 1977, 384 pp., price US $ 38.50, ISBN 0-8247-6192-8.

Experimental approach to electrochemistry, by N.J. Selley, Edward Arnold, London, 1977, ca. 220 pp., price ca. £ 8.00 (cloth), ca. £ 4.00 (paper), ISBN 0-7131-2540-3 (cloth), 0-7131-2541-1 (paper).

Electroanalytical chemistry. A series of advances, Vol. 10, edited by A.J. Bard, Marcel Dekker, New York, 1977, ca. 280 pp., price US $ 29.75, ISBN 0-8247-6461-7.

Fundamentals of electrochemical analysis, by Z. Galus, Ellis Horwood, Chichester/PWN, Warsaw/Halsted (Wiley), New York, 1976, XVIII + 520 pp., price £ 23.50, US $ 44.65, ISBN 0-85312-036-6.

Essentials of medicinal chemistry, by A. Korolkovos and J.H. Burckhalter, Wiley-Interscience, New York, London, 1976, VII + 697 pp., price US $ 29.95, £ 17.40, ISBN 0-471-12325-0.

Advances in chromatography, Vol. 15, edited by J.C. Giddings, E. Grushka, J. Cazes and P.R. Brown, Marcel Dekker, New York, Basel, 1977, XVII + 331 pp., price Sfr. 98.00, ISBN 0-8247-6500-1.

Analytical pyrolysis, edited by C.E.R. Jones and C.A. Cramers, Elsevier, Amsterdam, Oxford, New York, 1977, X + 424 pp., price Dfl. 96.00, US $ 39.25, ISBN 0-444-41558-0.

Carboniogenic activity of zeolites, by P.A. Jacobs, Elsevier, Amsterdam, Oxford, New York, 1977, XVI + 253 pp., price Dfl. 72.00, US $ 29.50, ISBN 0-444-41556-4.

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4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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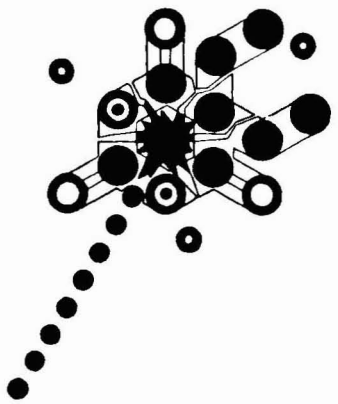

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