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THE ELECTRON-CAPTURE DETECTOR

II. DESIGN AND PERFORMANCE

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(Received November 17th, 1980)

SUMMARY

The model developed in Part I is used to estimate the performance of electron-capture detectors operating in coulometric, constant-current and constant-frequency modes. Coulometric operation is difficult to achieve directly but coulometric responses can be estimated by extrapolation. Detection limits can be substantially less than 1 pg and this mode of operation is preferred where practicable. Where coulometry cannot be attained either directly or by extrapolation, the constant-frequency mode should be utilised with calibration standards for quantitative analysis. The constant-current mode offers a wide dynamic range but responses are non-linear for strongly electron-capturing materials.

INTRODUCTION

The electron-capture detector (ECD) has been widely used for the analysis of trace amounts of strongly electron-capturing materials such as, for example, some explosives or pesticide residues. Detection limits of better than parts per 10^9 (v/v) have been achieved but at these low levels quantitative analysis is difficult, not least because ECD responses under practical working conditions are themselves difficult to predict. In Part I[1] a theoretical model of the operation of the ECD under certain limiting conditions was developed and the extrapolation of results from this model to practical conditions was discussed. The object here is to examine detector design and performance in the light of this model and to make recommendations concerning the optimum operational modes for quantitative analysis with the ECD.

ECD DESIGN

The most important decision facing the ECD designer is the specification of the electron source. For compatibility with gas chromatographic (GC) columns and to ensure that the resolution of chromatographic separations is not affected by dead volumes, detector volume should be small, typically not more than a few millilitres; source size is therefore limited. In addition, since the detector will be required for

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routine work, the source should have the minimum possible requirements for operational controls and for maintenance. The range of choice is thus severely restricted and almost every ECD described to date has relied on a radioactive β particle emitter as the electron source.

Thermal electrons, designated ε, are produced from β particles by interactions with carrier gas molecules

$$\beta + M \rightarrow \beta + M^+ + \varepsilon \quad (1)$$

Typically each β particle has an initial energy ≈ 10 keV and around 30 eV are expended in forming an electron/positive ion pair in normal carrier gases (argon/methane or nitrogen), (see, for example ref. 2, p. 35). Each β particle will thus produce 10^2–10^3 thermal electrons so that a 1-mCi or $4 \cdot 10^7$ β particles per sec source will give $\approx 10^{10}$ electrons per sec and a maximum current of *ca.* 1 nA.

Direct ionisation of the carrier gas as shown in eqn. 1 is the most probable process when nitrogen is the carrier but in argon metastable atoms may be formed.

$$\beta + Ar \rightarrow \beta + Ar^* \ (-11.6 \text{ eV}) \quad (2)$$

These metastable argon atoms have a relatively long lifetime and would be lost from the detector by ventilation unless deactivated. The deactivation process normally involves production of an electron/ion pair by reaction with any polyatomic molecule

$$Ar^* + X \rightarrow Ar + X^+ + \varepsilon \quad (3)$$

Thus with pure argon as carrier the presence of sample increases the electron concentration and thus the detector current, the reverse of the desired electron-capturing effect, leading to anomalous responses. Addition of $\approx 5\%$ methane to the carrier results in all metastables being deactivated by methane and prevents this type of response.

The rate of thermal electron production by eqn. 1 (or by eqns. 2 and 3) is subject to fluctuations due to the stochastic nature of radioactive decay. Noise produced in the ECD system as a result of these fluctuations is a major factor in determining the limits of detection of the system. The noise may be minimised by selecting sources with the maximum possible activity compatible with safety and with the minimum possible β particle energy.

Some relevant properties of currently available β particle sources are given in Table I[3,4]. The maximum current and noise levels are typical values and are taken from Dwight *et al.*[4]; the β particle energy is the value at the maximum of the number/-energy distribution. The rate of electron production is calculated directly from the maximum current assuming that at this maximum all the electrons produced are collected. In a clean system the current is independent of temperature (see, *e.g.*, ref. 5).

Most commercially produced detectors employ ^{63}Ni sources since they are readily available and operate to reasonably high temperatures. Tritium sources seem attractive in that they offer high activity combined with low β particle energy but their performance in practice often falls below expectation, perhaps because of surface contamination effects. The ^{55}Fe source has only recently been described[4]. It has a

TABLE I
SOME PROPERTIES OF ELECTRON SOURCES FOR THE ECD

Properties	*Sources*			
	^{63}Ni foil	*^{3}H titanium foil*	*^{3}H in scandium foil*	*^{55}Fe on nickel alloy foil*
β particle energy (keV)	66	18	18	5.387–5.640
β particle range (mm)	≈ 10	≈ 2.5	≈ 2.5	≈ 0.5
Maximum activity (mCi cm^{-2})	10	170		3
Upper temperature limit (°C)	350	220	325	400
Maximum current (pA)	9 (15-mCi source)	30 (500-mCi source)		0.5 (5-mCi source)
Rate of electron production, Rv (sec^{-1})	$6 \cdot 10^{10}$	$2 \cdot 10^{11}$		$3 \cdot 10^{9}$
Noise level* (pA)	1.5	3		0.1

* Measured at ambient pressure in nitrogen at 21°C.

relatively low activity and rate of electron production but this is compensated for by its low noise level and, as will be seen below, this source appears to have attractive properties for some applications. However, it should be stressed that published information on GC–ECD work with ^{55}Fe sources is extremely limited and the practical value of the source remains to be proven.

A variety of detector geometries have been employed in the past, some important examples of which are shown in Fig. 1. The plane parallel designs tend to have larger volumes than the cylindrical and for this reason the latter are preferred. In the coaxial design the distance from source to anode should be greater than the β particle range to ensure that all β particles are fully deactivated, maximising electron production, and to prevent collisions of energetic β particles with the anode which may cause surface corrosion and increase adsorption rates. The distance should not however be so great as to make efficient electron collection impossible when a narrow (≈ 1 μsec), low-voltage (50 V) pulse is applied to the anode. This condition is easily met for low-diameter tritium or ^{55}Fe detectors, particularly when argon–methane is the carrier gas but it is less readily met with ^{63}Ni sources and nitrogen carrier. Nitrogen will deactivate energetic electrons to thermal levels only inefficiently, methane is very much more effective. Electrons in nitrogen therefore tend to be at somewhat higher energies than in argon–methane and are thus less affected by electric fields and less easily collected. Van de Wiel and Tommassen[5] have clearly demonstrated this effect using a detector with a 10-mCi ^{63}Ni source. They found that a 40-V pulse of <4 μsec duration was sufficient to collect all the electrons in the detector when argon–methane was the carrier gas but a pulse duration >20 μsec was required with nitrogen.

In displaced coaxial cylinder designs, the cell geometry is such that direct collisions of β particles with the anode are unlikely and smaller diameters are possible within the limitation that collisions of β particles with the radioactive source itself should be minimised. Again the distance from the reaction volume to the anode

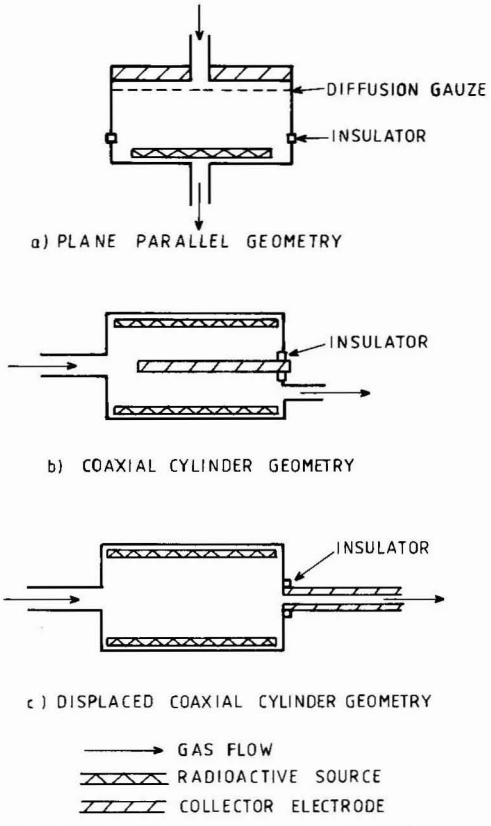

Fig. 1. Schematic diagram of alternative ECD geometries.

should not be great enough to prevent all of the electrons in the cell being collected at each pulse.

Coaxial cylinder cells with ^{63}Ni sources typically have volumes of up to 5 ml and a diameter of 1–2 cm. Displaced coaxial cylinder designs can be rather smaller. Patterson[6] has described such a cell with a total volume of 0.3 ml employing a 7.5-mCi ^{63}Ni source. Even with nitrogen as carrier a 50-V pulse of 0.64 μsec duration was sufficient to collect all electrons present. It is unlikely that other commercially available ECD systems will achieve this performance, but since most manufacturers do not provide a variable pulse width facility it is difficult to check this.

The remaining feature of ECD design which warrants consideration is the need for adequate temperature control. Since the ECD should always be operated at a temperature above that of the GC column in order to minimise contamination by condensation of column effluents, it requires its own temperature control system separate from the GC oven. In addition, since detector response is for many materials a function of temperature, this control system should provide a detector temperature which is well defined throughout the volume and closely controlled. In some commercial designs ECD temperature is controlled only by placing the base of the detector body in contact with a thermostatically controlled metal block. This is likely to produce a temperature gradient within the detector and as such is unsatisfactory.

Devaux and Guiochon[7] argue that in the worst case temperature fluctuations of $\pm 0.01°C$ can give a noise level in the ECD of 1 pA. Their detector current increased strongly with temperature however, suggesting that the system was contaminated, possibly with oxygen in the carrier gas[5]. Probably a more realistic estimate for a reasonably clean system is that by Pellizzari[8] who argues that a fluctuation of $\pm 0.3°C$ will produce an error of 1% in the determined concentration of sample. Likewise, a fluctuation of $\pm 3°C$ will produce an error of 10%. In the absence of impurity, temperature control to $\pm 1°C$ is adequate but oxygen impurity in the carrier gas will severely increase the system noise level unless temperatures are controlled to $\pm 0.1°C$ or better.

DETECTOR PERFORMANCE

Definitions and numerical values

The major concern of this part of the report is to produce numerical estimates of ECD performance for different modes of operation and for materials with different electron capture rate coefficients. The aspects of performance of most interest are the limit of detection (the smallest detectable sample input concentration) and the linear response range (the range of sample input concentrations over which detector response is linear with concentration). Estimates will be based on the assumption that the detector and the carrier gas are clinically clean.

The ECD operating conditions giving optimum detection of a particular material are a function of the ability of that material to capture electrons as measured by the rate constant, k_1, of the electron-capture reaction.

$$\varepsilon + AB \xrightarrow{k_1} AB^- \tag{4}$$

For the present purpose, and quite arbitrarily, materials for which $k_1 \rightarrow 5 \cdot 10^{-7}$ ml sec^{-1}, the collision theory limiting rate constant[9], will be described as being strongly electron capturing and materials for which $k_1 \approx 10^{-10}$ ml sec^{-1} as weakly electron capturing. Materials for which $k_1 < 10^{-10}$ ml sec^{-1} are likely to be more efficiently detected by techniques other than electron capture and are not considered here.

The electron/positive ion recombination rate constant, k_2, and the negative ion/positive ion recombination rate constant, k_3, have comparable values[1], normally in the range 10^{-6} to 10^{-7} ml sec^{-1} (refs. 10 and 11). For these calculations it will be assumed that k_2 and k_3 are equal and a value of $5 \cdot 10^{-7}$ ml sec^{-1} will be adopted.

$$\varepsilon + M^+ \xrightarrow{k_2} \text{neutrals} \tag{5}$$

$$AB^- + M^+ \xrightarrow{k_3} \text{neutrals} \tag{6}$$

The notation adopted is that defined previously[1]; a glossary of symbols employed is given on pp. 208 and 209. The identities

$$\lambda = k_1 c + k_2 \eta_+ + u/v \tag{7}$$

and

$$\alpha = k_2 \eta_+ + u/v \tag{8}$$

will be employed for convenience.

Coulometric operation

It follows[1] that, when the conditions

$$k_1 \bar{\eta}_e \gg u/v \tag{9}$$

and

$$t_p \ll \alpha^{-1} \tag{10}$$

are met the response of an ECD is given by

$$\Delta I_{coul} = I_0 - I = vQB \tag{11}$$

This type of response, where the change in the number of electrons collected per unit time exactly equals the number of sample molecules input in that time, is described as being coulometric. With the possible exception of cases where electrons react with products of the original electron-capturing reaction (eqn. 4), it represents the largest possible response.

In a GC system the gas flow, u, cannot be much less than 0.1 ml sec^{-1}. If it is assumed that eqn. 9 is met at this flow-rate and that positive ion losses by diffusion can be ignored then the pulse periods at which eqn. 10 holds can be evaluated. When $t_p \ll \alpha^{-1}$ and if positive ions are lost only by ventilation then from ref. 1,

$$\eta_+ = Rv/u \tag{12}$$

Using values of Rv from Table I, η_+ and $k_2 \eta_+$ can be estimated and hence the maximum value of t_p estimated as one tenth of the value of $(k_2 \eta_+)^{-1}$; it is assumed that $k_2 \eta_+ \gg u/v$ so that $k_2 \eta_+ = \alpha$, this is true in any practical system. Results are given in Table II.

TABLE II

POSITIVE ION CONCENTRATION AND MAXIMUM PULSE PERIODS FOR COULOMETRIC RESPONSE

Gas flow-rate, $u = 0.1$ ml sec^{-1}. It is assumed that $k_1 \bar{\eta}_e \gg u/v$ and that positive ions are lost only by ventilation. $k_2 = 5 \times 10^{-7}$ ml sec^{-1}, t_p (max) $= 0.1\ (k_2 \eta_+)^{-1}$.

	Rv (sec^{-1})	η_+ (ml^{-1})	$k_2 \eta_+$ (sec^{-1})	$t_{p\,(max)}$ (μsec)
^{55}Fe (5 mCi)	$3 \cdot 10^9$	$3 \cdot 10^{10}$	$1.5 \cdot 10^4$	7.0
^{63}Ni (15 mCi)	$6 \cdot 10^{10}$	$6 \cdot 10^{11}$	$3 \cdot 10^5$	0.3
^{3}H (500 mCi)	$2 \cdot 10^{11}$	$2 \cdot 10^{12}$	$1 \cdot 10^6$	0.1

In real systems positive ion diffusion losses cannot be ignored and positive ion concentrations will be less than and maximum pulse periods greater than those estimated in Table II. Nevertheless Table II does illustrate that very short pulse periods are required for true coulometry and, under these conditions, electron concentrations are very low leading to low dynamic range and a rapid fall-off in sensitivity with increasing sample input. Lovelock and Watson[12] have pointed out that improved performance is achieved by operating at longer pulse periods, typically 100–300 μsec, and making allowance for electron loss by recombination. Provided that the change in detector current ΔI is relatively small, say less than 10% of the detector standing current, then electron recombination is little affected by the presence of sample. The fraction, p, of the electrons produced which are collected by the pulse can be estimated from

$$p = \frac{\eta_e^0 (t_p)}{Rvt_p} = I_0/\varphi \tag{13}$$

where I_0 is the observed current in the absence of sample and φ the maximum possible (zero pulse period) current. The observed sample response ΔI is then converted to the coulometric response ΔI_{coul} by

$$\Delta I_{coul} = \Delta I/p \tag{14}$$

The sample response ΔI should of course be determined under conditions where the condition (eqn. 9), which implies complete sample ionisation, is met. Data from Lovelock *et al.*[13] and calculations by Lovelock and Watson[12] indicate that this is hardly possible at gas flow-rates compatible with chromatographic applications (except perhaps in large-volume detectors) but that complete ionisation is approached as the flow-rate is reduced. Under these conditions the extent of sample ionisation was shown in Part I to be given by

$$x = \frac{k_1 \bar{\eta}_e}{k_1 \bar{\eta}_e + u/v} \tag{15}$$

so that

$$\frac{1}{x} = 1 + \frac{u}{k_1 \bar{\eta}_e v} \tag{16}$$

Hence $x \to 1$ as $u \to 0$ and, provided $\bar{\eta}_e$ is not a function of carrier gas flow-rate, the sample response at complete ionisation can be determined from a plot of the reciprocal response against flow-rate by extrapolating to zero flow[12]. It needs to be stressed that, for this method to be applicable, $\bar{\eta}_e$ must not vary significantly with carrier gas flow-rate so that reactions with impurities such as GC column bleed, which might be expected to vary in concentration with flow, must not be a significant electron loss mechanism. The coulometric response can only be estimated in a clean ECD system.

Limits of detection in the coulometric mode can be calculated from eqn. 11

with ΔI = 2 × system noise and v = 1 ml. This gives the limit in terms of rate c sample input and conversion to the corresponding sample concentration at the lim depends on the shape of the input chromatographic peak. Very crudely, the peak ca be regarded as triangular with widths at half peak height, $t_{\frac{1}{2}}$, of between 5 sec and 3 sec covering the range likely to be encountered in practice. It follows then that th limit of detection in terms of concentration is

$$c_{\min} = B_{\min} t_{\frac{1}{2}} \quad (17$$

Values of $B_{\min}$ and $c_{\min}$ have been calculated for ^{55}Fe-, ^{63}Ni- and ^{3}H-based detector An estimate of the minimum mass which can be detected has also been made on th assumption of a sample molecular weight of 150. The results are given in Table II

TABLE III

LIMITS OF DETECTION IN THE COULOMETRIC MODE

Calculated from $B_{\min} = 2 \times \text{Noise}/vQ$. Limiting concentrations are based on GC peak half-widths of and 30 sec and limiting masses on the basis of a molecular weight of 150.

	Noise (pA)	B_{min} $(ml^{-1}\ sec^{-1})$	$t_{\frac{1}{2}} = 5$ *sec*		$t_{\frac{1}{2}} = 30$ *sec*	
			c_{min} (ml^{-1})	m_{min} *(fg)*	c_{min} (ml^{-1})	m_{min} *(fg)*
^{55}Fe (5 mCi)	0.1	$1.25 \cdot 10^{6}$	$6 \cdot 10^{6}$	1.5	$3.75 \cdot 10^{7}$	9.3
^{63}Ni (15 mCi)	1.5	$1.875 \cdot 10^{7}$	$9 \cdot 10^{7}$	22	$5.6 \cdot 10^{8}$	140
^{3}H (500 mCi)	3	$3.75 \cdot 10^{7}$	$1.9 \cdot 10^{8}$	47	$1.1 \cdot 10^{9}$	275

The limits shown in Table III represent the best possible ECD performance. is unlikely that these limits can be attained directly in a single observation; multip injections to estimate zero flow responses and correction for electron recombinatic are required, complicating the method. Nevertheless this mode of operation is attrac ive for quantitative studies where, for one reason or another, calibration standar are not available[12]. It should be noted that the method employs constant frequen ECD operation and not the constant-current mode now so often supplied in comme cial equipments.

The upper limit to the linear range in coulometric mode is reached wh sample input, B, approaches the rate of electron production, Rv, of the source so th $\bar{\eta_e}$ falls and eqn. 9 cannot be met. The range is therefore certainly less than Rv/B_m and for all three sources is unlikely to exceed $\approx 5 \cdot 10^{2}$.

An alternative approach to coulometry is to employ a d.c. mode detector s that $t_p = 0$ and eqn. 10 is met under all conditions. In the d.c. mode the electro concentration is determined by the rate of electron production and the electron dri velocity between source and collector under the influence of the permanently applie collection voltage. Charge separation effects and the short residence time of electror in the ECD minimise electron/ion recombination. Typically the field in the detect may be 100 V cm^{-1} and under these conditions at atmospheric pressure the electro

drift velocity is $4 \cdot 10^5$ cm sec^{-1} (ref. 10, p. 545). Assuming a cross sectional area in the drift region of 1 cm^2, the electron densities, η_e^0, in ^{55}Fe-, ^{63}Ni- and ^{3}H-based detectors can therefore be estimated as $7.5 \cdot 10^3$ ml^{-1}, $1.5 \cdot 10^5$ ml^{-1} and $5 \cdot 10^5$ ml^{-1} respectively. Taking k_1 to have its largest possible value of $5 \cdot 10^{-7}$ ml sec^{-1}, eqn. 10 can therefore be met in a d.c. ECD for values of u less than about $4 \cdot 10^{-4}$ ml sec^{-1}, $7.5 \cdot 10^{-3}$ ml sec^{-1} and $2.5 \cdot 10^{-2}$ ml sec^{-1} for ^{55}Fe-, ^{63}Ni- and ^{3}H-based detectors, respectively. Such low values are difficult, if not impossible, to reconcile with chromatographic requirements and extrapolation to zero flow of responses determined at a series of flow-rates is still required. In addition d.c. mode ECD operation is problematic because of space charge and contact potential effects[14] which can lead to distortion of peak shapes and, in extreme cases, to spurious peaks. For these reasons the d.c. mode is rarely used.

Operation at constant frequency, long pulse periods

At long pulse periods,

$$t_p \gg \hat{\lambda}^{-1} \tag{18}$$

and with

$$k_1 \bar{\eta}_e \ll u/v \tag{19}$$

the detector response is given by

$$\frac{I_0 - I}{I} = \frac{\bar{\eta}_e^0 k_1 v}{Ru} B \tag{20}$$

and is linear in sample concentration.

For small samples $I \approx I_0$ and, since

$I_0 = \eta_e^0 vQ/t_p$ and $\eta_e^0 = \bar{\eta}_e^0$ at long times,

eqn. 18 can be written as

$$\Delta I = I_0 - I = \frac{\eta_e^{0^2} v^2 Q k_1}{Rut_p} B \tag{21}$$

As was shown previously[1] with $t_p \gg \alpha^{-1}$ then

$$\eta_+ = \bar{\eta}_e^0 = \frac{R}{\alpha} \tag{22}$$

In practice $\alpha = k_2 \eta_+$ since $k_2 \eta_+ \gg u/v$ so that

$$\eta_+ = \bar{\eta}_e^0 = \left(\frac{R}{k_2}\right)^{\frac{1}{2}} \tag{23}$$

and substituting in eqn. 21 gives

$$\Delta I = \frac{v^2 Q k_1}{k_2 u t_p} B \tag{24}$$

The best possible limits of detection in this mode are obtained with k_1 as large and t_p as small as is possible consistent with eqns. 18 and 19 which, for the purposes of these calculations, can be assumed to be when

$$t_p \text{ (min)} = \frac{10}{k_2 \eta_+} \tag{25}$$

and

$$k_1 \text{ (max)} = 0.1 (u/v)\overline{\eta_e^0} \tag{26}$$

Substituting these expressions in eqn. 24 and substituting from eqn. 23 for $\overline{\eta_e^0}$ and η_+ gives

$$\Delta I = \frac{vQB}{100} \tag{27}$$

Thus the greatest possible response in this mode is two orders of magnitude less than the coulometric response.

The upper limit to the range of the detector in this mode is reached when the detector current falls to a level approaching the system noise level I_n. At this limit, eqn. 18 can be written

$$\frac{I_0}{2I_n} = \frac{\overline{\eta_e^0} k_1 v}{Ru} B_{\max} \tag{28}$$

Also from eqn. 18 we have

$$\frac{2I_n}{I_0} = \frac{\overline{\eta_e^0} k_1 v}{Ru} B_{\min} \tag{29}$$

so that the linear range of the system is

$$\frac{B_{\max}}{B_{\min}} = \left(\frac{I_0}{2I_n}\right)^2 \tag{30}$$

The optimum limits of detection for linear responses in the long pulse period mode for three types of detector are listed in Table IV. Also listed are the electron and positive ion concentrations, the linear range and the values of k_1 and t_p calculated from eqns. 25 and 26 with $v = 1$ ml and $u = 1$ ml sec^{-1}. It should be noted that this mode is applicable only to weakly electron capturing materials. For materials with

TABLE IV

LIMITS OF DETECTION IN LONG PULSE PERIOD MODE

Optimum detection limits, B_{min}, in long pulse period constant-frequency mode and the corresponding values of k_1 and t_p (v = 1 ml, u = 1 ml sec^{-1}). For lower values of k_1 or greater values of t_p, B_{min} will rise while for higher values of k_1 or lower values of t_p, B_{min} will decrease but the response will be non-linear.

	R (ml^{-1} sec^{-1})	$\bar{\eta}_e^0 = \bar{\eta}_+$ (ml^{-1})	k_1 (max) (ml sec^{-1})	t_p (min) (ms)	Noise (pA)	B_{min} (ml^{-1} sec^{-1})	Linear range
^{55}Fe (5 mCi)	$3 \cdot 10^9$	$7.7 \cdot 10^7$	$1.2 \cdot 10^{-9}$	260	0.1	$1.25 \cdot 10^8$	$6 \cdot 10^6$
^{63}Ni (15 mCi)	$6 \cdot 10^{10}$	$3.5 \cdot 10^8$	$2.8 \cdot 10^{-10}$	60	1.5	$1.9 \cdot 10^9$	$9 \cdot 10^6$
^{3}H (500 mCi)	$2 \cdot 10^{11}$	$6.3 \cdot 10^8$	$1.6 \cdot 10^{-10}$	30	3	$3.8 \cdot 10^9$	$2.5 \cdot 10^7$

higher electron-capture rate constants, k_1, improved (*i.e.*, lower) limits of detection may be obtained but, for small samples, the response is non-linear in sample concentration. Sensitivity will in fact decrease as sample concentration is increased until $\bar{n}_e$ falls to a value where the condition in eqn. 19 can be met. The best possible response at long pulse periods is obtained with k_1 sufficiently large to ensure that every sample molecule captures an electron. Under this condition the limit is determined from the fraction of the electrons produced in the detector which are actually collected rather than being lost by recombination or ventilation. For small samples and at long pulse periods this fraction is $\bar{n}_e^0/Rt_p$ and for the sources of interest is $\approx 8\%$ or less, at least one order of magnitude less than the coulometric mode.

Materials with lower values of k_1 than those tabulated will give a correspondingly worse (*i.e.*, higher) limit of detection but the linear range will be unaltered.

Operation at constant frequency, intermediate and short pulse periods

The long pulse period mode just described provides excellent linear range for weakly electron capturing materials but it does not provide the optimum lower detection limit. The condition for optimum detection of such materials derived in Part I can be written as

$$\alpha t_p \approx 1.79 \tag{31}$$

The approximation results from the variation in $\bar{\eta}_+$ with t_p. The weakly electron capturing condition is met if $k_1 \bar{\eta}_e \ll u/v$. It was further shown that the sensitivity, S, of the ECD for weakly electron capturing materials is given by

$$S = \frac{dI}{dB} = \frac{k_1 v}{u} \frac{dI}{d\lambda} \tag{32}$$

while

$$I = \frac{\varphi}{\lambda t_p} [1 - \exp(-\lambda t_p)] \tag{33}$$

where $\varphi = RvQ$. Substituting $\lambda t_p = 1.79$ into eqn. 33 gives

$$I = 0.47\varphi \tag{34}$$

φ is the maximum (*i.e.*, d.c.) current available from the detector.

To the extent that η_+ can be regarded as constant, optimum detection of weakly electron-capturing materials is achieved at the pulse period giving a standing current of about half the maximum available (d.c.) current. With $\lambda t_p = 1.79$ it follows that

$$S = -0.3 \frac{k_1 v\varphi}{u\lambda} \tag{35}$$

For small samples, λ can be replaced by α and, under normal operating conditions $\alpha = k_2 \eta_+$ so that both α and φ vary with source strength. At short pulse periods α is linear in source strength R (eqn. 12) and at long pulse periods it is linear in $R^{\frac{1}{2}}$ (eqn. 23). At these intermediate pulse periods an intermediate dependence is expected so that S should increase with source strength but the increase should not be large.

The lower limit of detection can be derived from the expression

$$B_{\min} = \frac{2I_n}{S} \tag{36}$$

provided that S can be estimated. However S is a function of α and hence of η_+ and at these intermediate pulse periods the value of η_+ is a function of pulse period. It is therefore not possible to produce a general expression for $B_{\min}$ in this mode and optimum detection limits need to be determined experimentally. The model can be used only to make the qualitative statements that (a) optimum detection of weakly electron-capturing materials is achieved at a pulse period such that the detector standing current is about half of its maximum (zero pulse period) value, and (b) that for more strongly electron-capturing materials optimum detection is obtained at shorter pulse periods with the limit of detection for strongly electron-capturing materials approaching, but not attaining, the coulometric limit. Since detector responses in this mode cannot be theoretically predicted, quantitative work demands the use of accurately calibrated standard solutions of samples of interest in the relevant concentration ranges. For low-level trace analysis, preparation of such standards may be a major problem in itself[12].

The linear range in this pulse mode is also difficult to estimate from first principles. The condition for linearity of response is that S does not change significantly with sample input which, from eqn. 35, implies that λ is constant. This condition is met for small samples, $k_1 c \ll k_2 \eta_+$, when $\lambda = \alpha$ but this small sample condition requires knowledge of the positive ion concentration if it is to be used quantitatively and this information is not available. In practical ECD systems a linear response (within 5%) is obtained in this mode for samples which remove up to 30% of the available standing current of the detector, corresponding to a range of about 10^2–10^3 in input concentration.

One final point is worth making: at short pulse periods, detection of strongly electron-capturing materials is optimised while more weakly capturing materials are more efficiently detected at longer periods. Short pulse period operation increases the selectivity of the detector for strongly electron-capturing materials. Since these are the materials most likely to be selected for detection by electron capture the short pulse period mode is widely used in practice.

Constant-current operation

The response of an ECD operating in constant current mode was given in Part I as

$$f - f_0 = \frac{f_0 k_1}{\alpha} c \tag{37}$$

and for samples for which $k_1 \bar{\eta}_e \ll u/v$, so that the extent of ionisation is small, this becomes

$$f - f_0 = \frac{f_0 k_1 v}{\alpha u} B \tag{38}$$

At the limit of detection B approaches zero and $f \approx f_0$. The base frequency f_0 giving the optimum limit of detection for a particular sample in this mode is therefore identical with the frequency of operation which gives the optimum limit in the constant-frequency mode. As in the constant-frequency mode, the optimum base frequency f_0 varies from material to material with electron-capturing ability and, for weakly electron-capturing materials, is defined by $\alpha = 1.79 f_0$. The difficulty in calculating values for α from first principles means that the optimum detection condition must be determined experimentally.

Eqn. 38 is the equivalent of eqn. 21 in the constant-frequency mode with the important difference that eqn. 38 is valid for all values of f_0 (or t_p) while eqn. 21 is valid only for small values of f_0. For materials and detector designs for which eqn. 38 holds the linear range is given by

$$\frac{B_{\max}}{B_{\min}} = \frac{f_{\max} - f_0}{2f_n} \tag{39}$$

where $f_{\max}$ is the maximum possible frequency (*i.e.*, the reciprocal of the pulse width and f_n the noise level. Typically $f_{\max}$ is $\approx 10^6$ Hz, $f_0 \approx 10^3$ Hz and $f_n \approx 5$ Hz so that the linear range is 10^5. In practice since α varies with f_0 the linear range is perhaps an order of magnitude less. For weakly electron-capturing materials therefore, constant-current operation combines optimum detection limits with a wide linear range.

For strongly electron-capturing materials the constant-current detector response is given by eqn. 37 and, in the limit where $k_1 \bar{\eta}_e \gg u/v$ so that $B = k_1 \bar{\eta}_e c$,

$$f - f_0 = \frac{f_0}{\alpha \bar{\eta}_e} B \tag{40}$$

If η_+ and therefore α can be regarded as constant, it follows from eqn. 40 that the response to strongly electron-capturing materials in this constant current mode is linear only for small sample inputs where $\bar{\eta}_e = \bar{\eta}_e^0$. As the sample input increases $\bar{\eta}_e$ and, with it, the extent of sample ionisation fall until, for high enough inputs, eqn. 38 holds. Thus for a strongly electron-capturing material in a detector for which $k_1 \eta_e^0 \gg u/v$, the detector response will be large and constant for small sample inputs but will

fall away to a lower level as sample input is increased, subsequently remaining constant at this level up to high inputs. This non-linearity in constant current ECD response is often ignored but it needs to be stressed that optimum linearity is only obtained if the degree of sample ionisation is small and is thus not compatible with optimum detection. As in the constant-frequency mode, optimum detection limits for strongly electron-capturing materials approach but do not attain the coulometric limit. Quantitative work in this mode again demands the use of accurately calibrated samples and is, of course, complicated by the non-linear response.

DISCUSSION

Detector design

The lack of any alternative to β particle emitters as ECD electron sources imposes severe limitations on detector design and application. Such sources are inadequate in two respects which have important practical consequences; they produce a population of electrons at energies which are above thermal energies and at the same time they produce an equal number of positive ions.

The energy distribution of electrons in the ECD is a function of both the carrier gas and time. The initial electron energy is governed by the dynamics of the β particle quenching reaction.

$$\beta + M \rightarrow \beta + M^+ + \varepsilon \qquad (1)$$

Typically each electron will have an excess energy of ≈ 1 eV, this energy is lost by collisions with the carrier gas at a rate which depends on the nature of the carrier gas. At short ECD pulse periods, the average electron energy is likely to be higher than at long pulse periods since in the latter case more time is available for thermalisation. Nitrogen is less efficient than argon–methane mixtures in quenching excited electrons and electron energies will therefore be higher in nitrogen. In general electron-capture reactions are resonance processes, proceeding most rapidly for electrons with a particular energy but at much reduced rates for electrons with only slightly more or less energy (see, *e.g.*, ref. 2, Ch. 6). Variations in electron energy can therefore have profound effects on ECD responses and, since electron-energy distributions in the ECD are not easily estimated, these effects are not readily quantified.

Positive ion effects are also of importance since they directly affect ECD electron concentrations in a way which changes with pulse period and because they may drift to the collector electrode between pulses thus changing the measured detector current[10]. Only under limiting conditions of very short or very long pulse periods can the positive ion concentration and therefore the electron concentration be estimated directly. These conditions are of little practical significance.

Ideally, a source for an ECD should produce electrons at thermal energies with no positive ions. Such a source would provide a detector in which electrons were lost only by ventilation or by collection at the electrode. Higher electron concentrations could be achieved and the time for the electron concentration to reach its maximum value would be increased from milliseconds to seconds. Coulometric detection would then be available in pulsed mode for a wide range of substances and, by varying the pulse period, the selectivity of the detector could be adjusted as desired.

ECD performance

Because of the positive ion concentration effects, quantitative descriptions of ECD performance are not possible in the intermediate pulse period mode which is of most practical interest in GC systems. For a limited range of very strongly electron capturing materials d.c. or short pulse period mode operation will give optimum response, approaching coulometry as the carrier gas flow-rate is reduced. Linear response range will be limited. For other materials optimum responses for small samples are obtained at longer pulse periods with, for weakly electron-capturing materials, the optimum being that period at which the standing current is reduced to half of its d.c. value. At this standing current responses for strongly electron-capturing materials will be about half the coulometric response. For general purpose operation this half maximum current criterion defines the optimum condition for ECD operation in either constant-frequency or constant-current mode. Where interest is primarily in more strongly electron-capturing compounds however, shorter pulse periods will tend to improve responses to these substances while reducing responses to weakly electron-capturing materials, thus increasing the specificity of the detector.

Provided that the optimum pulse frequency for detection of a particular sample is selected, there is no difference in detection limits between constant-current and constant-frequency mode devices. In modern equipments the constant-current mode is often preferred because of its claimed superior linear response range. However, as was shown above, responses in constant-current mode detectors are linear only if $k_1 \bar{\eta}_e \ll u/v$ so that the extent of sample ionisation is negligible, say $< 10\%$. In general this condition is met only for low values of k_1, *i.e.*, for weakly electron-capturing samples, or for very high zero sample pulse frequencies f_0. At such high frequencies $\bar{\eta}_e = R/2f_0$ so that the linearity condition becomes $f_0 \gg k_1 Rv/2u$. For $k_1 \rightarrow 5 \cdot 10^{-7}$ ml sec^{-1} and $u = 1$ ml sec^{-1} this implies that $f_0 > 150$ kHz for a 15-mCi ^{63}Ni source and $f_0 > 7.5$ kHz for a 5-mCi ^{55}Fe source. The required base pulse frequency for the ^{63}Ni source is too high to be practical and neither this source nor ^{3}H can be used in a constant-current mode detector if linearity of response to strongly electron-capturing materials is required. It should be possible to obtain linear responses from a ^{55}Fe source ECD and the low noise level of this source should compensate for the lower sensitivity, giving limits of detection at least comparable to those obtained with ^{63}Ni and ^{3}H sources in the same mode. This limit will be at least one order of magnitude below the coulometric limit, however, and unless a very wide dynamic range is essential, operation at a lower constant frequency where sample ionisation is more nearly complete is preferred. Under such conditions, detection limits may approach the coulometric limit and the linear range is likely to cover at least a factor of 10^2 in sample input concentration.

Although an ECD for GC applications which gives a genuinely coulometric response is difficult, if not impossible, to produce, the coulometric response can be calculated from data obtained under conditions where coulometry is approached. Some uncertainty is introduced by the extrapolation which is required but for strongly electron-capturing materials and in clean ECD systems this uncertainty may be negligible. In view of the great difficulty involved in the preparation of accurate calibration standards at trace levels, say parts per 10^{10} (v/v), this quasi-coulometric approach to the absolute calibration of ECD responses is particularly attractive.

CONCLUSIONS

The constant-current mode ECD now widely used provides a wide dynamic range but responsés to small samples of strongly electron-capturing materials are non-linear in sample concentration. This mode of operation is suitable for qualitative work but at best is highly inconvenient when quantitative results are required. Theoretical considerations and limited experimental evidence[4] suggest that, in constant-current mode, a ^{55}Fe electron source will provide optimum range and limits of detection.

Responses in the constant-frequency mode are linear in sample concentration for

(a) Weakly electron-capturing samples at long pulse periods (typically >5 msec). Such samples are probably better detected using other systems.

(b) Strongly electron-capturing samples at intermediate pulse periods (0.1–1 msec) provided that the sample size is small so that the detector current is not reduced by more than $\approx 20\%$.

(c) Strongly electron-capturing samples under conditions where a coulometric response can be obtained either directly or by extrapolation.

Coulometric detection, whether direct or indirect, requires extremely clean ECD systems and factors such as column bleed present very real problems. On the other hand this mode offers the optimum detection limits, being capable of detecting samples of substantially less than 1 pg of strongly electron-capturing materials in systems where electronic rather than chemical noise predominates. Additionally, responses in this mode are quantitative and calibration using standard solutions is not necessary; this can be a substantial advantage since accurate standard preparation at trace levels is fraught with difficulties.

Exact quantitative descriptions of the ECD operating at intermediate pulse periods are not possible because of lack of information on processes involving positive ions. Quantitative work in this mode therefore requires the use of accurate calibration standards. Limits of detection can approach the coulometric limit but may fall short of this by an order of magnitude or more in systems where impurities such as column bleed provide an important electron loss mechanism.

Future improvements in the performance of the ECD may stem from the development of electron sources which do not at the same time produce positive ions or from techniques which involve the determination of product ions as well as reactant electrons. Some progress in this direction has been made with the plasma chromatograph[17], a drift tube device, but considerable effort is still required to make this type of instrument compatible with GC.

GLOSSARY OF SYMBOLS

v	Detector volume (ml)
B	Rate of sample input to detector (ml^{-1} sec^{-1})
B_{max}	Maximum rate of sample input for which detector response is linear (ml^{-1} sec^{-1})
B_{min}	Minimum detectable rate of sample input (limit of detection) (ml^{-1} sec^{-1})
R	Rate of production of thermal electrons in the detector (ml^{-1} sec^{-1})

u	Carrier gas flow-rate (ml sec^{-1})
Q	Electronic charge (C)
I_0	Detector current with no sample present (A)
I	Detector current, sample present (A)
ΔI	Change in detector current with sample present (A)
ΔI_{coul}	Change in detector current at coulometric limit (A)
I_n	Detector noise level expressed as fluctuation in detector current (A)
φ	Detector current in DC mode ($= RvQ$) (A)
p	Fraction of electrons produced which is collected ($= I_0/\varphi$)
S	Detector sensitivity expressed as the change in detector current with the rate of sample input (A ml sec)
AB	Any sample molecule
AB^-	Product of reaction between sample molecules and electrons
A	Neutral product(s) of AB^- decomposition
M	Any carrier gas molecule
M^+	Positive ion(s) formed by collision of β particles with carrier gas molecules
X	Any impurity molecule
β	Highly energetic electrons
ε	Electrons at (or close to) thermal energies
η_e^0	Number density of thermal electrons in the detector in the absence of sample (ml^{-1})
η_e	Number density of thermal electrons in the detector in the presence of sample (ml^{-1})
$\overline{\eta_e^0}$, $\bar{\eta}_e$	Time averaged values of η_e^0, η_e (ml^{-1})
η_+	Number density of positive ions in the detector (ml^{-1})
$\bar{\eta}_+$	Time averaged value of η_+ (ml^{-1})
η_-	Number density of negative ions in the detector (ml^{-1})
c	Number density of sample molecules in the detector (ml^{-1})
c_{min}	Minimum detectable number density of sample molecules (ml^{-1})
m_{min}	Minimum detectable mass of sample molecules (g)
t_p	Interval between electron collection pulses in pulsed mode ECD (sec)
f	Electron collection pulse frequency (Hz)
f_0	Electron collection pulse frequency in absence of sample (Hz)
f_n	Detector noise level expressed as fluctuation in detector pulse frequency required to maintain constant detector current (Hz)
k_1	Rate constant for attachment of electrons to sample molecules (ml sec^{-1})
k_2	Electron/positive ion recombination rate constant (ml sec^{-1})
k_3	Positive ion/negative ion recombination rate constant (ml sec^{-1})
$t_{\frac{1}{2}}$	Width of gas chromatographic peak at half peak height (sec)

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SIMPLE RELATIONSHIPS CONCERNING MOBILE AND STATIONARY PHASES IN NORMAL- AND REVERSED-PHASE CHROMATOGRAPHY*

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SUMMARY

For normal-phase chromatography on aminopropyl-bonded silicas, the adsorption of the polar modifier of various mixtures of hexane with *n*-alkanols, benzyl alcohol, acetone, dimethylformamide, nitromethane, chloroform and diisopropyl ether were measured. One to three molecules of the polar solvent per bonded aminopropyl chain can be fixed. Simple calculations allow the determination of the relative proportions of amino groups free or bonded with one, two or three modifier molecules for each mobile phase composition. The amount of the polar modifier in the stationary phase increases with its ability to form hydrogen bonds with the amino groups for a given composition of the mobile phase. The amount is larger with any alcohol than with nitromethane, acetone or dimethylformamide.

The capacity factor (k') of various polar solutes (phenols, amines and pyridines) and non-polar solutes (polyaromatic hydrocarbons) decreases when the concentration of polar solvent in the mobile phase increases. When the average number of molecules of the polar modifier in the stationary phase remains less than one per amino group, a linear relationship exists between $1/k'$ and the volume fraction of the polar solvent in the mobile phase. When more than one molecule per amino chain is fixed, the variations of $1/k'$ are not easily linked to the eluent composition.

For reversed-phase chromatography on alkyl-bonded silicas, the ln k' and the selectivity for non-polar and polar solutes vary linearly with NS (N being the total number of chains bonded on the silica surface and S the hydrocarbonaceous surface area of a chain). For instance, the retention time of a solute will be the same using C_8, C_{12} or C_{22} bonded phases with the same value of NS and consequently the same carbon content.

Retentions of polar and non-polar solutes were measured with five different water-miscible organic modifiers (methanol, ethanol, 1-propanol, acetonitrile and tetrahydrofuran). The variations of k' can be related to the volume fraction (x) of

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water by $\ln k' = ax^n + b$ ($0 < x < 0.85$), n depending on the organic solvent nature and a on the solute properties. For each organic modifier, a linear relationship exists between a and the molar volume of the solute. The solubility of solutes was measured in the same mixtures used as mobile phases. The solubility of a given solute in any water–modifier mixture is related to x by $\ln s = -ax^n + b'$, a and n having the same values as those obtained for the retention variation. This equation is verified for x values corresponding to $1 < k' < 200$. Hence a very simple relationship between k' and s ($k's = C$) is valid for all mobile phase compositions.

INTRODUCTION

In partition by normal- and reversed-phase chromatography, the retention of a solute depends on the amount and polarity of the bonded stationary phase, the mobile phase composition, the temperature and the nature of the solute.

In reversed-phase chromatography, Locke[1] has shown that the interactions between *n*-alkyl-bonded chains and solutes are weak and non-selective, and suggested that the relative retention for closely related solutes could be determined solely by the difference in solubility of the two components in the mobile phase. This idea was considered by Karger *et al.*[2], who studied the water solubility theory and predicted retention by using a topological index called molecular connectivity, in which the solute surface area is estimated from the hydrocarbonaceous skeleton of the solute. They obtained good correlations for alkyl and cycloalkyl compounds. Horváth and co-workers[3,4] also showed that the solvophobic effect is mainly responsible for the solute retention. Therefore, even if the hydrocarbonaceous stationary phase plays some role in retention[5], the mobile phase and the solute solubility play a more important role[6–9].

In contrast, in normal-phase or adsorption chromatography, the solute retention is often described as being mainly due to the interactions in the stationary phase. Two approaches to the chromatographic behavior of solutes have been developed. The first describes the stationary phase composition in equilibrium with the eluent. Snyder's[10] and Oscik's[11] theories are two examples of the first approach. According to Snyder[10,12,13], the interaction energy of a solute with any component in the mobile phase is always negligible in comparison with the adsorption energy of the solute or the solvent molecules on the stationary phase surface. This assumption was also used when this theory was extended to non-ideal mobile phases[14] or heterogeneous surfaces[15]. However, the behaviour of aminoalkyl-bonded phases is different and cannot be described accurately by Snyder's theory[15]. Consequently, the activity of this phase should be very low compared with silica or other bonded phases. Moreover, Hammers *et al.*[16] noted that strong interactions occurred between dichloromethane molecules and the bonded amino groups. Strong interactions must occur in the mobile phase between the dichloromethane and amino groups of any solute and this is therefore inconsistent with assumptions about interactions in the mobile phase with polar solutes.

The second approach describes the solute partition between both phases[17,18]. Polar forces are the main forces in the bulk phase, whereas dispersive and polar forces can occur in the eluent. Scott and Kucera[17] observed a linear relationship between the

inverse of the retention volume of a solute and the concentration of the polar modifier in the mobile phase. Another linear relationship is obtained with the eluent density when the polar solvent concentration remains constant. However, this theory only describes dipole–dipole forces in both phases and not hydrogen bond or complex formation, which seem more important with aminoalkyl-bonded phases.

As no theory accurately describes the behaviour of amino-bonded phases, we have tried to define the nature and the composition of the stationary phase in equilibrium with mixtures of *n*-hexane and various polar modifiers and then to explain the variations of the capacity ratio of the solutes with the mobile phase composition.

EXPERIMENTAL

Apparatus

Experiments were performed with a liquid chromatograph assembled from commercially available modules consisting of an Altex 380 pump (Altex, Berkeley, CA, U.S.A.), a Valco six-port sampling valve with a 20-μl loop, a Waters R-401 refractometer (Waters Assoc., Milford, MA, U.S.A.), or a fixed-wavelength UV detector (Altex), and also with a Hewlett-Packard 1084 B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.). The temperature was maintained at 30 $\pm$ 0.1°C with a constant-temperature water-bath. Solute solubilities were measured by the UV technique on a Variscan variable-wavelength spectrophotometer (Varian, Palo Alto, CA, U.S.A.).

Stationary phases

Several chromatographic columns of different lengths were packed with home-made stationary phases described previously[5] and with the commercially available LiChrosorb-NH_2 and RP-8 (10 μm) (Merck, Darmstadt, G.F.R.). The dead times of reversed-phase columns were measured for each mobile phase composition by determining the retention time of sodium nitrate.

Chemicals

All of the alcohols used were of analytical-reagent grade and were obtained from Prolabo (Paris, France). Chloroform and methylene chloride were of LiChrosolv grade and were purchased from Merck. *n*-Hexane, tetrahydrofuran and acetonitrile were of Chromasol grade and were purchased from SDS (Valdone, France). The water was doubly distilled.

Calculations

All calculator-generated data were obtained with a Hewlett-Packard 9825A programmable calculator.

NORMAL-PHASE CHROMATOGRAPHY ON AMINOPROPYL-BONDED SILICAS

Adsorption isotherms

Experimental results. Adsorption of the polar modifier from various mixtures of *n*-hexane with *n*-alkanols, benzyl alcohol, N,N-dimethylformamide, acetone, nitromethane and chloroform was measured on an aminopropyl-bonded stationary phase (LiChrosorb-NH_2) using a frontal analysis technique.

The adsorption isotherms in Fig. 1 show the affinity order of the polar modifier for the amino groups. There is no satisfactory correlation between affinity order and classical measurement of polarity as Snyder's eluent strength ε_0 or Rohrschneider's polarity parameter P' for all of the solvents considered. For instance, acetone has more affinity for amino groups than nitromethane, although acetone is always considered to be less polar than nitromethane.

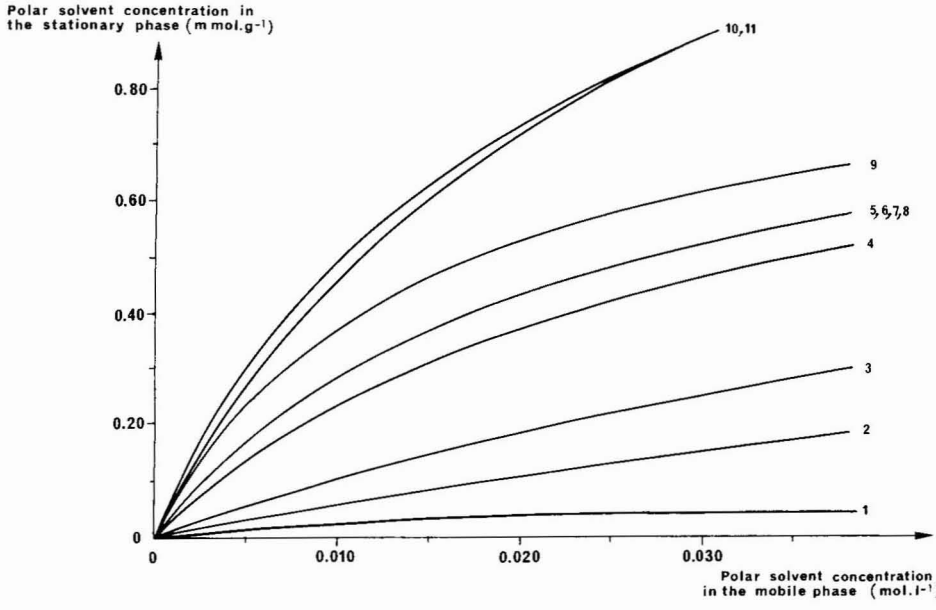

Fig. 1. Adsorption isotherms of polar solvents on LiChrosorb-NH_2 containing 0.9 mmol $\cdot$ g^{-1} of amino chains. Solvents: 1, chloroform; 2, nitromethane; 3, acetone; 4, N,N-dimethylformamide; 5, 1-decanol; 6, 1-octanol; 7, 1-pentanol; 8, 1-propanol; 9, ethanol; 10, methanol; 11, benzylmethanol.

However, as usual, a good correlation is observed for *n*-alkanols:methanol > ethanol > 1-propanol > 1-pentanol > 1-octanol > 1-decanol.

Description of an adsorption model for aminopropyl-bonded silica. We consider that molecules of a polar modifier (P) interact with the amino groups according to the following equilibria:

$$-\overline{NH_2} + P \rightleftharpoons -\overline{NH_2\text{–}P} \qquad K_1 = \frac{|-\overline{NH_2\text{–}P}|}{|-\overline{NH_2}|\,|P|} \tag{1}$$

$$-\overline{NH_2\text{–}P} + P \rightleftharpoons -\overline{NH_2\text{–}P_2} \qquad K_2 = \frac{|-\overline{NH_2\text{–}P_2}|}{|-\overline{NH_2\text{–}P}|\,|P|} \tag{2}$$

$$-\overline{NH_2\text{–}P_2} + P \rightleftharpoons -\overline{NH_2\text{–}P_3} \qquad K_3 = \frac{|-\overline{NH_2\text{–}P_3}|}{|-\overline{NH_2\text{–}P_2}|\,|P|} \tag{3}$$

The number of molecules of P fixed per amino group depends on the polarity of P. For instance, with a slightly polar molecule such as chloroform one obtains only adsorption of one molecule per amino group (Langmuir isotherm). For a strongly polar solvent such as ethanol one obtains adsorption of three alcohol molecules per amino group.

Table I gives equilibrium constants for various *n*-hexane–polar solvent mixtures on aminopropyl-bonded silica (LiChrosorb-NH_2 with 0.9 mmol $\cdot$ g^{-1} of bonded chain). These were determined by regression of the experimental adsorption isotherms.

TABLE I

EQUILIBRIUM CONSTANTS OBTAINED WITH *n*-HEXANE–POLAR MODIFIERS ON LICHROSORB-NH_2 CONTAINING 0.9 mmol $\cdot$ g^{-1} OF BONDED CHAINS

Polar modifier	*Equilibrium constants* ($mol^{-1} \cdot l$)		
	K_1	K_2	K_3
Benzylmethanol	87	12	4
Methanol	71	15	8
Ethanol	50	8,8	1,2
1-Propanol	45.5	–	–
1-Pentanol	45.5	–	–
1-Octanol	46.5	–	–
1-Decanol	46	–	–
N,N-Dimethylformamide	35		
Acetone	13		
Nitromethane	6.7		
Chloroform	0.8		
Dichloromethane	0.7		

We discuss two cases: adsorption of a slightly polar solvent (chloroform) and adsorption of a strongly polar solvent (ethanol).

Adsorption of a slightly polar solvent (chloroform). In this case only one molecule of chloroform is adsorbed per amino group (Fig. 2a) and an adsorption isotherm of the Langmuir type is obtained:

$$|\mathrm{P}|_{\text{total}} = \frac{K_1 N |\mathrm{P}|}{1 + K_1 |\mathrm{P}|}$$

Fig. 2b represents fractions of free amino groups and solvated amino groups (with only one chloroform molecule) plotted against chloroform concentration in the mobile phase (calculated by computer from the K_1 value).

Adsorption of a strongly polar solvent (ethanol). Here, the results indicate that one amino group can adsorb up to three ethanol molecules (Fig. 2c). Fig. 2d shows variations of the number of free and solvated amino groups (with one, two or three ethanol molecules) *versus* ethanol concentration in the mobile phase. The free amino group concentration decreases considerably as the ethanol concentration increases in ethanol–*n*-hexane mixtures.

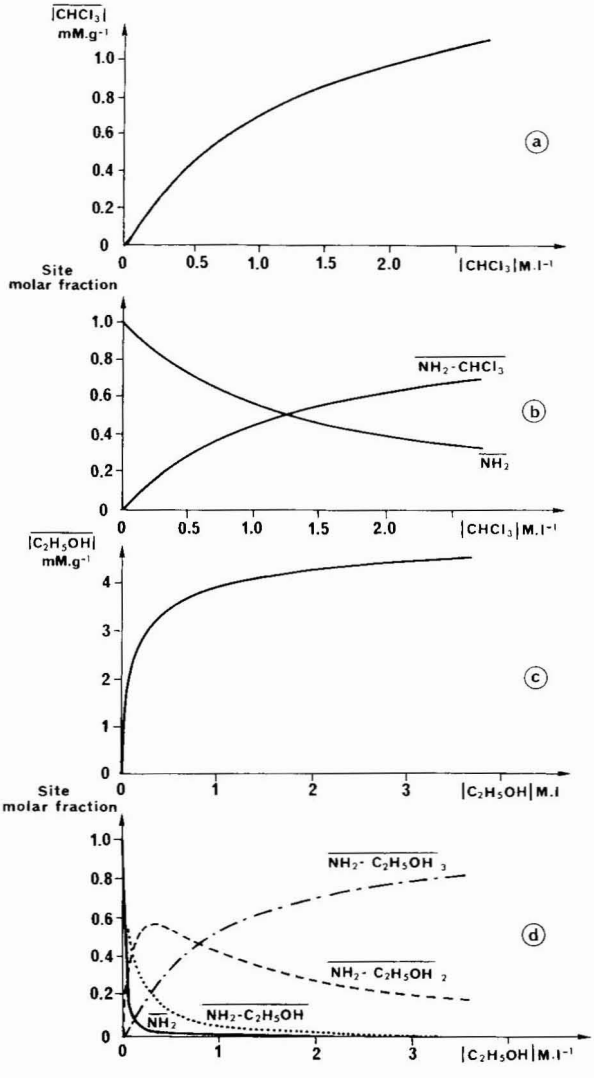

Fig. 2. Adsorption isotherms of chloroform (a) and ethanol (c) on a home-made stationary phase (1.: mmol · g^{-1} of amino bonded chains), and computed molar fraction of free and "solvated" groups wher varying the polar modifier concentration in the mobile phase: chloroform–*n*-hexane (b) and ethanol–*n* hexane (d).

Retention of solutes on amino-bonded phases

Description of the retention model. Although the decrease in the capacity ratio of solutes with increasing concentration of the polar modifier in the eluent is wel known in normal-phase chromatography, only a few data on these variations with amino-bonded phases are available. Linear relationships between the logarithm of the capacity ratio and the polar solvent concentration were mainly observed with various eluent mixtures[19,20].

(a) Mixture of an apolar solvent with a slightly polar solvent. The mobile phase can be considered as ideal and the activity of any species is equal to its concentration. However, the isotherm determination always shows only one molecule of the most polar solvent per amino-bonded group, even with high concentrations. If the solute S can form complexes with the polar solvent in the mobile phase according to

$$S + P \rightleftharpoons SP \qquad K = \frac{|SP|}{|S|\,|P|} \tag{4}$$

and assuming that the solute molecule mainly interacts with free amino groups in the stationary phase according to

$$-\overline{NH_2} + S \rightleftharpoons -\overline{NH_2{-}S} \qquad K_S = \frac{|-\overline{NH_2{-}S}|}{|-\overline{NH_2}|\,|S|} \tag{5}$$

the following relationship is obtained:

$$\frac{1}{k'} = \frac{V}{mr}\,\frac{1 + (K_1 + K)\,|P| + K_1K\,|P|^2}{K_S} \tag{6}$$

where m is the mass of the stationary phase, V the volume of the mobile phase in the chromatographic column and r the total concentration of free and solvated amino groups ($r = (-\overline{NH_2}) + (-\overline{NH_2{-}S}) + (-\overline{NH_2{-}P})$. Hence the inverse of the capacity ratio of a solute is related to the concentration of the most polar solvent by a parabolic equation.

(b) Mixtures of an apolar solvent with a strongly polar solvent. For ethanol–*n*-hexane mixtures, polar solvent molecules solvate each amino group when the polar modifier concentration in the mobile phase is increased. In fact, the interaction energy between amino groups and alcohols is so large that the fixation of one molecule of the polar solvent per amino group occurs only for very low concentrations, generally less than 0.2% (v/v). The activities of the various species are roughly proportional to their concentrations and eqn. 6 can be written as

$$\frac{1}{k'} = \frac{V}{mr}\,\frac{1 + (K_1 + K)\,\gamma(P) + K_1K\gamma^2(P)^2}{K_S} \tag{7}$$

where γ is the mean activity coefficient of the polar eluent in the mixture, roughly equal to its infinite dilution activity coefficient. For very low concentrations of the polar solvent, the term $K_1K\gamma^2(P)^2$ is negligible and eqn. 7 can be written as

$$\frac{1}{k'} = \frac{V}{mr}\,\frac{1 + (K_1 + K)\,\gamma(P)}{K_S} \tag{8}$$

Then, the inverse of the capacity ratio varies linearly with the concentration of the polar solvent in the eluent.

Experimental verification. Two sets of experiments were performed with amino-

bonded phases and various solvent mixtures: the first with *n*-hexane and dichloromethane mixtures for aniline or phenol derivatives and the second with *n*-hexane and *n*-alkanol mixtures for the pyridine derivative separations.

(a) Mixture of an apolar solvent with a slightly polar solvent. Fig. 3. shows the parabolic variations of the capacity ratio of some aniline derivatives eluted with cyclohexane–dichloromethane mixtures (Fig. 3a) and some phenol derivatives eluted with cyclohexane–trichloromethane mixtures (Fig. 3b). According to eqn. 6, we observe that the ratio of the second degree coefficient to the constant term is equal to the product K_1K. Similarly, the ratio of the first degree coefficient of the constant term is equal to the sum $K_1 + K$. Then the calculation of K and K_1 is easy.

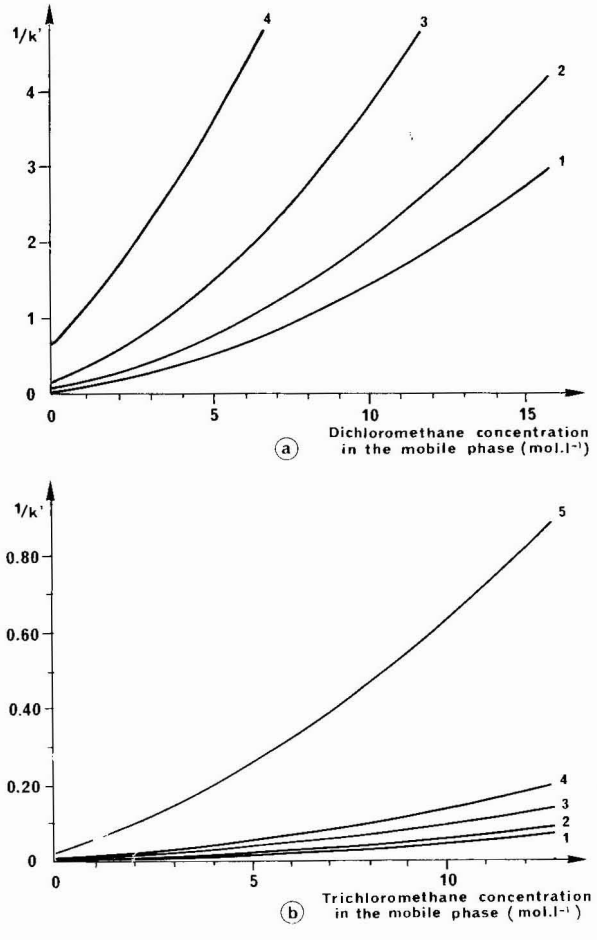

Fig. 3. (a) Variation of $1/k'$ with dichloromethane concentration in the mobile phase (dichloromethane–*n*-hexane mixtures) for aniline derivatives. Column: 15 cm × 4 mm I.D. packed with a stationary phase containing 1.5 mmol · g^{-1} of bonded aminopropyl chains. Flow-rate, 1 ml/min; UV detection at 254 nm. Solutes: 1, aniline; 2, *o*-toluidine; 3, N-methylaniline; 4, N,N-dimethylaniline. (b) Variation of $1/k'$ with trichloromethane concentration in the mobile phase for phenol derivatives. Solutes: 1, *m*-cresol; 2, 3,4-dimethylphenol; 3, *o*-cresol; 4, 2,5-dimethylphenol; 5, 2,6-dimethylphenol.

Table II gives the coefficients of the parabolic curves for the aniline derivatives, as determined by regression analysis, and the two equilibrium constants K and K_1. Table II shows that the equilibrium constant K_1 between the polar solvent and the amino-bonded chains does not depend on the nature of the solute and remains roughly equal to 0.6 $mol^{-1} \cdot l$, which is very close to the value calculated from isotherm determination, *viz.*, 0.7 $mol^{-1} \cdot l$.

TABLE II

COEFFICIENT OF THE FITTED PARABOLIC CURVE FOR VARIATIONS OF $1/k'$ *VERSUS* THE POLAR MODIFIER CONCENTRATION IN THE MOBILE PHASE FOR ANILINE DERIVATIVES

Conditions as in Fig. 3a.

Solute	$\frac{V}{mr} \cdot \frac{1}{K_S}$	$\frac{V}{mr} \cdot \frac{K_1 + K}{K_S}$	$\frac{V}{mr} \cdot \frac{K_1 K}{K_S}$	K_1	K
		$(mol^{-1} \cdot l)$	$(mol^{-2} \cdot l^2)$	$(mol^{-1} \cdot l)$	$(mol^{-1} \cdot l)$
N,N-Dimethylaniline	0.688	0.439	0.025	0.57	0.06
N-methylaniline	0.202	0.148	0.002	0.56	0.18
o-Toluidine	0.087	0.071	0.0116	0.58	0.23
Aniline	0.058	0.051	0.008	0.66	0.22

In contrast, the association constant K between the solute molecule and dichloromethane increases from N,N-dimethylaniline to aniline, that is, with increasing accessibility of the nitrogen atom of the solute molecule. As the constant increases simultaneously with the order of elution of the aniline derivatives, the distribution constant K_S of the solute between the two phases also increases from N,N-dimethylaniline to aniline.

(b) Mixture of an apolar and a strongly polar solvent. As predicted by eqn. 8, the inverse of the capacity ratio of various alkylpyridines eluted with *n*-hexane–methanol mixtures is linearly related to the concentration of the polar solvent in the mobile phase (Fig. 4a). The slopes of the straight lines obtained depend on the structure of the solute and, for instance, the influence of the alcohol concentration is less with 3,4-dimethylpyridine than with 2,6-dimethylpyridine or pyridine.

Fig. 4b shows the influence of the hydrocarbonaceous chain of the alcohol on the elution of 2,6-dimethylpyridine. For each alcohol a linear relationship was observed between the inverse of the capacity ratio of the solute and the mobile phase composition. Further, for a constant composition of the mobile phase (expressed as the volume fraction of the alcohol in the eluent) the solute retention is lower when the number of carbon atoms in the alcohol molecule decreases.

REVERSED-PHASE CHROMATOGRAPHY ON *n*-ALKYL BONDED PHASES

Role of the stationary phase

The influence of the surface concentration and of the length of bonded *n*-alkyl chains was studied previously[5]. C_{18} bonded phases with various surface coverages were synthesized and it was shown that the solute capacity factors and relative reten-

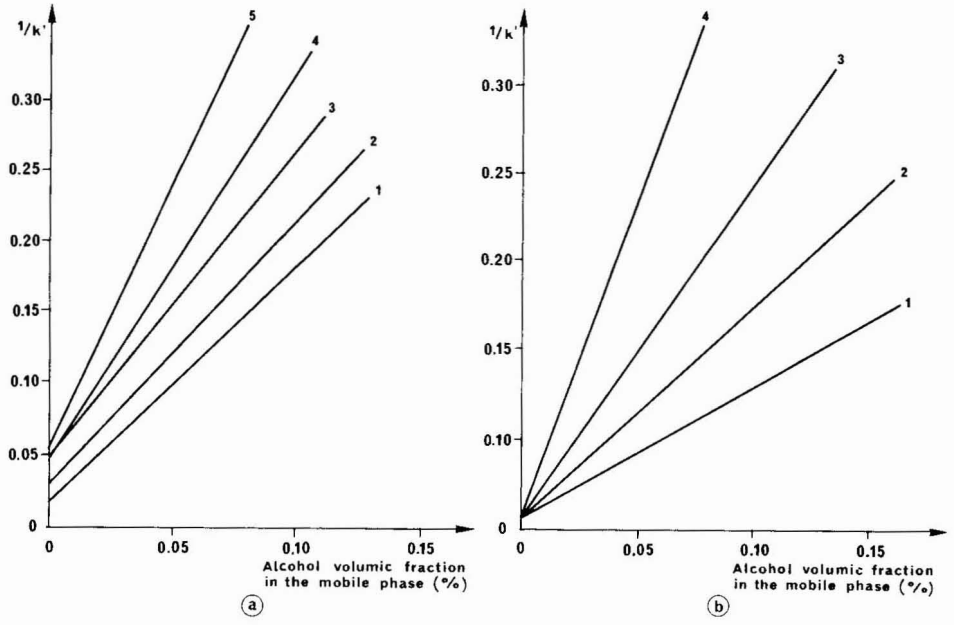

Fig. 4. (a) Variation of $1/k'$ with volume fraction of methanol in methanol–*n*-hexane mixtures for pyridine derivatives. Flow-rate, 1 ml/min; UV detection at 254 nm. Solutes: 1, 3,4-dimethylpyridine; 2, 4-dimethylpyridine; 3, 3-methylpyridine; 4, 2-methylpyridine; 5, 2,6-dimethylpyridine. (b) Variation of $1/k'$ with polar modifier concentration in the mobile phase for 2,6-dimethylpyridine. Solutes: 1, 1-octanol; 2, 1-pentanol; 3, 1-propanol; 4, methanol.

tions increase with increasing surface concentration. For a constant surface concentration of bonded chains, the capacity factor and relative retention increase with increasing chain length. Roumeliotis and Unger[21] correlated k'/S_{BET} (where S_{BET} is the specific surface area of silica) with the hydrocarbonaceous surface area of the bonded chain.

In this study, several stationary phases with various surface concentrations and various lengths of bonded chains (C_4–C_{22}) were synthesized from the same batch of silica and using the same bonding method (from *n*-alkyltrichlorosilane). The hydrocarbonaceous surface area (S) of a chain was calculated from Bondi's relationship[22] and the number of bonded chains per square nanometre of silica (N) from carbon microanalysis[23]. The capacity factors of various polar and non-polar solutes were measured with the above stationary phases, the eluting mixture being the same. The results are shown in Fig. 5. A linear relationship is observed between the capacity factor of pyrene (Fig. 5a) and the bonded hydrocarbonaceous surface per square nanometre of silica. A similar relationship is obtained for the relative retentions of pyrene and phenanthrene (Fig. 5b). So, using a C_{12}, C_{16}, C_{18} or C_{22} bonded phase having about the same hydrocarbonaceous surface area NS and the same eluent, the capacity factors and relative retentions obtained are very similar.

The same results were observed for a solute with a long alkyl chain such as 1-tetradecanol (Fig. 5c). Thus, the chain length has no specific role in the retention mechanism. Berendsen and De Galan[24] have shown from a geometrical model that

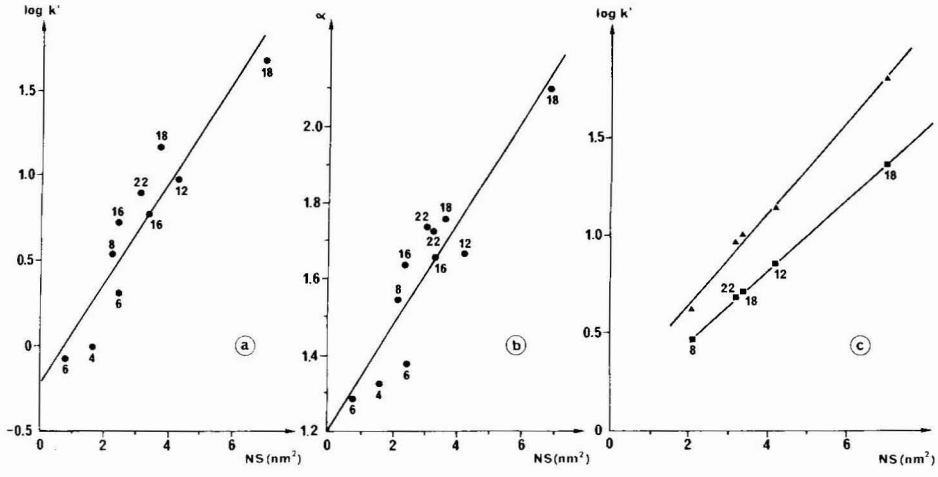

Fig. 5. Variation of logarithm of capacity factor and relative retention with hydrocarbonaceous surface area of the bonded phase (*NS*) per nm^2 of silica. Stationary phase: *n*-alkyl chains bonded on experimental Spherosil (specific surface area, 520 $m^2\ g^{-1}$; average particle diameter, 5 μm). Column: 15 cm × 4 mm I.D. (a) Capacity factor of pyrene eluted with methanol–water (70:30); (b) relative retention of pyrene and anthracene with the same eluent; (c) capacity factor of 1-tetradecanol (▲) and 1-decanol (■) eluted with acetonitrile–water (80:20). The numbers above the experimental points correspond to the bonded chain lengths.

the surface concentration is limited to 3.8 $\mu mole \cdot m^{-2}$ for a trimethyl chain and a lower value for a longer chain. The advantages of bonding a longer chain are to increase the hydrocarbonaceous surface area and to obtain a more selective bonded phase. For instance, Takayama *et al.*[25] synthesized a C_{30} bonded phase and so achieved a very difficult separation of fatty acids.

Role of the mobile phase

Relationship between retention and mobile phase composition. Retentions of non-polar solutes (various polycyclic aromatic compounds) and polar solutes (*n*-alkanols) were measured with various mixtures of water and a water-miscible organic solvent. Five solvents were studied: methanol, ethanol, 1-propanol, acetonitrile and tetrahydrofuran. The relationship between ln k' for the solutes and the volume fraction of water (x) in the eluting mixture is shown in Fig. 6. A linear relationship exists only with methanol as the organic modifier (Fig. 6a). For non-polar solutes the rate of change of ln k' with x increases from acetonitrile to ethanol, to tetrahydrofuran and then to 1-propanol (Fig. 6b–e). Polar solutes eluted with acetonitrile–water mixtures show the same behaviour as non-polar solutes (Fig. 6f). The order of elution of non-polar solutes is the same for each modifier and is related to the solute size; therefore, the heaviest compounds are retained most in the column. The curves obtained with tetrahydrofuran–water mixtures are not well separated; a consequence is that the selectivity between these solutes is not as high as with the other modifier–water mixtures.

Another observation is that for each organic modifier the recorded capacity factor is smaller than that expected from the beginning of the curve when the volume

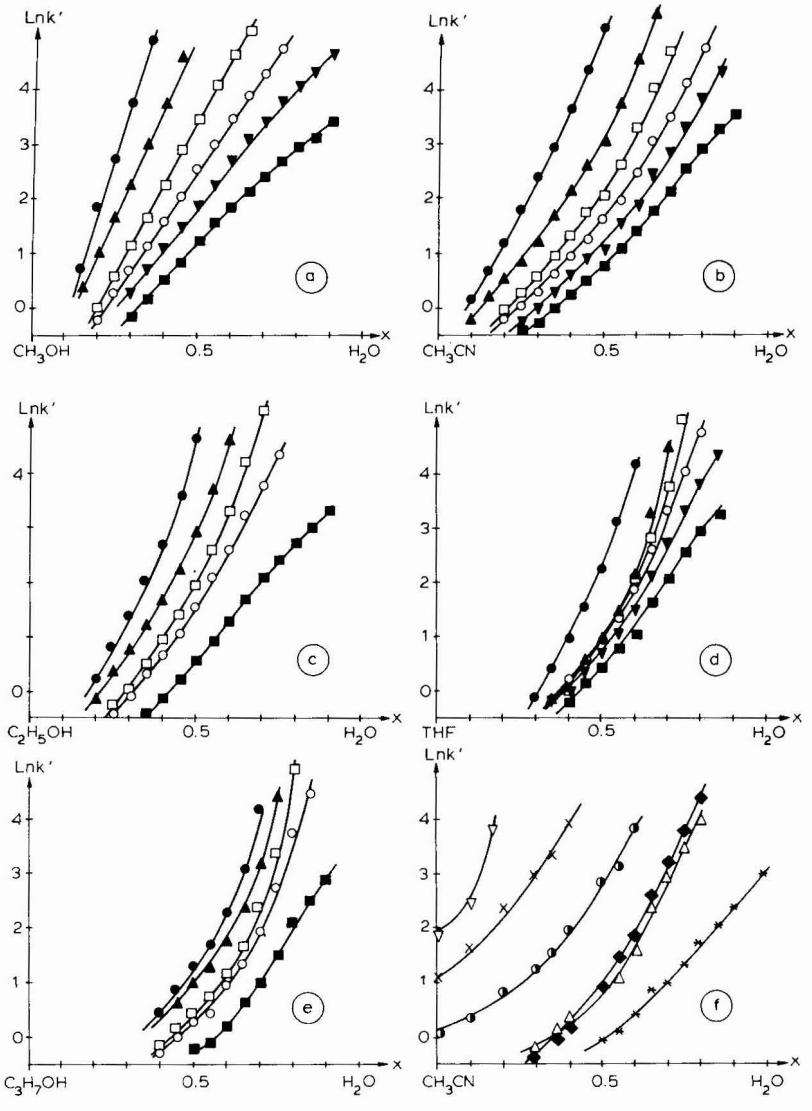

Fig. 6. Variation of logarithm of capacity factor with volume fraction of water in the eluent for various solutes. Column: 5 cm (a–e) and 15 cm for (f); I.D. 4 mm. Packing: *n*-octylsilica (RP-8). Eluent: mixtures of water and (a) methanol, (b) acetonitrile, (c) ethanol, (d) tetrahydrofuran, (e) 1-propanol and (f) acetonitrile. Solute: ■, benzene; ▼, toluene; ○, *p*-xylene; □, phenanthrene; ▲, perylene; ●, tetraphenylnaphthalene; ∗, 1-pentanol; ◑, 1-decanol; ×, 1-tetradecanol; ▽, 1-hexadecanol; ♦, 1-naphthol; △, 2-naphthylamine.

fraction of water is higher than 0.80 or 0.85. This effect is particularly noticeable with the benzene and toluene curves.

In reversed-phase studies, a linear relationship between ln k' and x was frequently observed with methanol–water mixtures and with other modifiers at low water contents. Some workers[2,3,26,27] also noticed that the linear relationship was not always checked, especially when the water content was high. Similar observations

have been reported in thin-layer chromatography on silanized silica gel or on silica gel coated with paraffin oil[29–31]. Recently, Schoenmakers and co-workers[26–28] proposed the parabolic dependence $\ln k' = ax^2 + bx + c$ to represent the experimental curves mathematically.

As we observed that the rate of change in $\ln k'$ with x was dependent on the nature of the modifier, the mathematical model chosen for the study of the experimental curves was $\ln k' = ax^n + b$. For each organic modifier and up to $x = 0.8$, a and n were calculated for each solute (Table III).

(a) Examination of n. Values of n vary between 1.9 and 2.5 for aromatic hydrocarbons with acetonitrile as modifier; with ethanol n varies between 2.5 and 3, with tetrahydrofuran between 3.5 and 4.8 and with 1-propanol between 3.5 and 5. Hence, for this type of solute, n depends mainly on the organic modifier. However, if we look at each column in Table III, we can see that n also depends on the solutes and, except for tetrahydrofuran, for any modifier n decreases as the solute size increases.

If n is about 2 for aromatic hydrocarbons eluted with acetonitrile–water mixtures, n does not have the same value for n-alkanol solutes eluted with the same

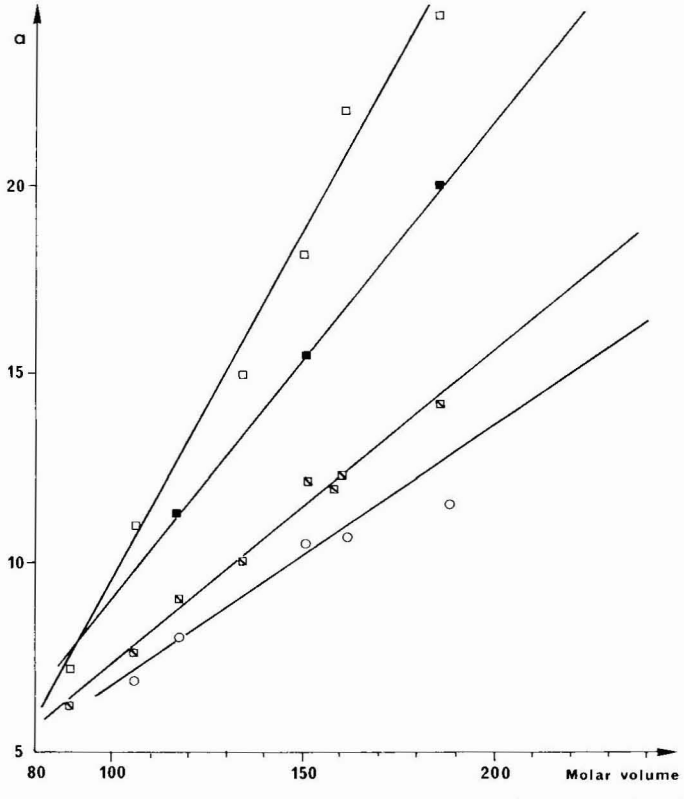

Fig. 7. Variation of the coefficient a obtained from $\ln k' = ax^n + b$ with the molar volume of aromatic compounds for different organic modifiers. Experimental conditions as in Fig. 6. ⧅, Methanol; ○, acetonitrile; ■, ethanol; ⫶ ⌊ tetrahydrofuran.

TABLE III

a AND n VALUES OBTAINED BY THE RELATIONSHIP $\ln k' = ax^n + b$ FOR AROMATIC HYDROCARBONS ELUTED WITH WATER–METHANOL (OR ACETONITRILE, ETHANOL, TETRAHYDROFURAN OR 1-PROPANOL) MIXTURES AND FOR POLAR SOLUTES ELUTED WITH WATER–ACETONITRILE MIXTURES

For experimental conditions, see Fig. 6.

Solute	CH_3OH		CH_3CN		C_2H_5OH		THF		C_3H_7OH		*Solute*	CH_3CN	
	a	*n*	*a*	*n*	*a*	*n*	*a*	*n*	*a*	*n*		*a*	*n*
Benzene	6.2	1		2.6							1-Pentanol	3.2	2.7
Toluene	7.5	1	6.8	2.5			7.2	3.0			1-Decanol	8.7	1.8
p-Xylene	9	1	8.0	2.3	11.2	3.0	11.0	3.5	9.8	4.5	1-Tetradecanol	8.2	1
2-Ethyltoluene	10	1	8.9	2.2			15.1	3.8			1-Naphthol	9.8	3.2
Phenanthrene	12.2	1	10.2	2.1	15.7	3.0	18.1	4.2	15.0	5.0	2-Naphthylamine	9	3.4
Fluoranthene	12.3	1	10.7	2.1			22	4.8					
Perylene	14.2	1	11.5	1.9	20	2.6	24.5	4.7	12.2	3.7			
Tetraphenylnaphthalene	19	1	15.3	1.7	27.5	2.5	25	3.5	14.4	3.5			

mixtures and it varies from 2.5 for 1-pentanol to 1 for 1-tetradecanol. 1-Naphthol and 2-naphthylamine give the same n value.

(b) Examination of a. In Table III for aromatic hydrocarbons, a increases with increasing solute size; Fig. 7 shows a linear relationship between a and the molar volume of solutes. It has been shown[32] that for homologous compounds the logarithm of the water solubility is linearly related to the molar volume.

Thus, for non-polar solutes, as n depends mainly on the organic modifier, it is possible to predict retention using the molar volume of the solute.

Relationship between solubility of the solute and mobile phase composition. The solubility (s) of phenanthrene was measured in the same mixtures as the eluting mixtures previously studied. Fig. 8 shows the logarithms of phenanthrene solubility *versus* the volume fraction of water (x). There is a linear relationship between the solubility and x for methanol–water mixtures, but not for the other organic modifiers

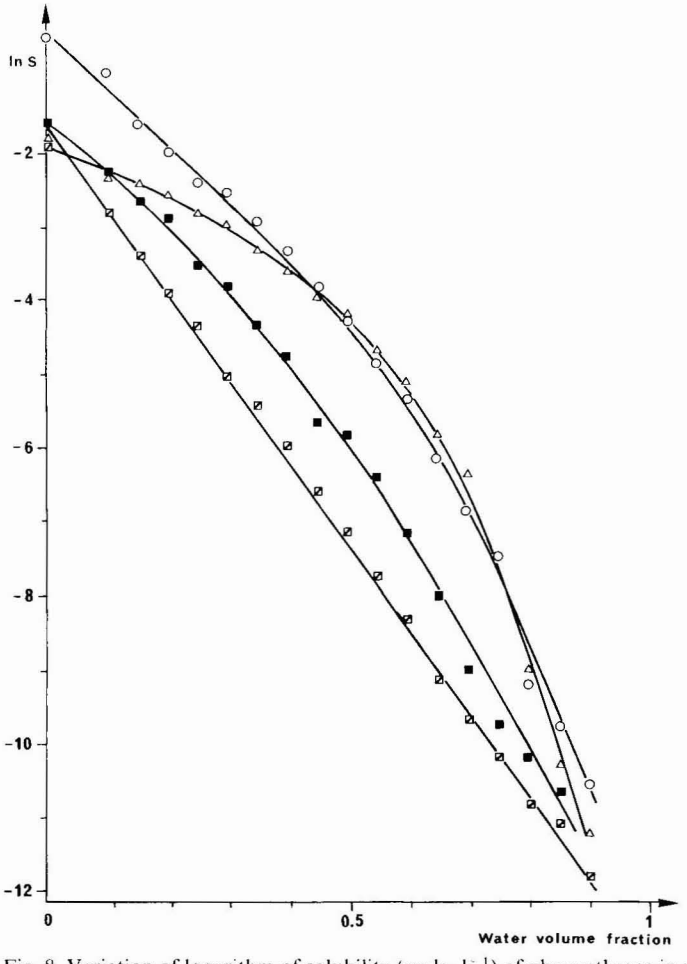

Fig. 8. Variation of logarithm of solubility (mole $\cdot$ l^{-1}) of phenanthrene in water–organic solvent mixtures with the volume fraction of water. ⊠, Methanol; ○, acetonitrile; ■, ethanol; △, 1-propanol.

(the solubility in water–tetrahydrofuran mixtures could not be measured because separation occurred).

Because of this linear relationship with methanol–water mixtures, the same mathematical analysis of experimental curves was carried out, using the equation $\ln s = a'x^{n'} + b'$. Experimental values of a' and n' are given in Table IV.

TABLE IV

a' AND n' VALUES OBTAINED ON REPRESENTING EXPERIMENTAL CURVES OF THE SOLUBILITY OF PHENANTHRENE IN WATER METHANOL (OR ETHANOL, ACETONITRILE OR 1-PROPANOL) MIXTURES BY THE RELATIONSHIP $\ln s = a'x^{n'} + b'$

This relationship is checked in the x range for k' between 1 and 200.

Parameter	*Organic modifier*			
	CH_3OH	CH_3CN	C_2H_5OH	C_3H_7OH
a'	−11.4	−10.5	−15.5	−15.1
n'	1	2.1	3.0	5.0
x range for $1 < k' < 200$	$0.15 < x < 0.65$	$0.20 < x < 0.75$	$0.25 < x < 0.75$	$0.35 < x < 0.85$

Comparison of these values with those obtained by $\ln k' = ax^n + b$ for phenanthrene in Table III shows that $a' \approx -a$ and $n' = n$. Hence for phenanthrene and one given modifier the capacity factor is related to x by $\ln k' = ax^n + b$ and solubility is related to x by $\ln s = -ax^n + b'$. Note that the relationship $\ln s = -ax^n + b$ is checked for x values corresponding to the range of k' between 1 and 200.

It appears that the retention curves look like solubility curves. This analogy has been mentioned in reversed-phase thin-layer chromatography by Markowski *et al.*[33], and indicates the predominating role of the mobile phase.

Relationship between retention and solubility. If we compare the two experimental relationships between s and x and between k' and n, the capacity factor and the solubility are directly related by $k's = e^{b+b'} = C$, where C is a constant. For a given organic modifier this constant is independant of x. This means that when the only variable parameter is the water content in the eluting mixture, the change in retention is governed only by the change in solubility in the mobile phase.

The retention can be defined by[17]

$$k' = \frac{\Sigma \text{ (solute–stationary phase interactions)}}{\Sigma \text{ (solute–mobile phase interactions)}}$$

The solubility can be considered as a measure of the solute–mobile phase interactions. Hence the $k's$ reflects the solute–stationary phase interactions. As this product is independent of the water content of the mobile phase, x, the solute–stationary phase interactions are independent of the water content. Hence the stationary phase has the same composition (in the x range checked for the above relationship, *i.e.*, $x < 0.8$).

Considering the constant $k's$ value of phenanthrene obtained with four organic

modifiers in Table V, this constant is nearly the same for the three alcohols (about 0.02) and is much higher for acetonitrile (0.13). The solubility of phenanthrene in the three pure alcohols (s_0) is also the same and is much higher in pure acetonitrile. If, for each modifier, the water content of the mobile phase is adjusted so as to provide the same solubility (s) of phenanthrene, the phenanthrene capacity factor will be the same for the three alcohols (as $k' = C/s$). It will be about six times higher for acetonitrile as the modifier.

TABLE V

$k's$ VALUES OBTAINED FOR PHENANTHRENE ELUTED WITH WATER–ORGANIC MODIFIER MIXTURES AND PHENANTHRENE SOLUBILITY (s_0) IN THE PURE ORGANIC MODIFIERS (FOR $x = 0$)

Parameter	*Organic modifier*			
	CH_3OH	C_2H_5OH	C_3H_7OH	CH_3CN
$k's$ (mole·l^{-1})	0.019 ± 0.001	0.021 ± 0.001	0.020 ± 0.001	0.13 ± 0.01
s_0 (mole·l^{-1} at 25°C)	0.15	0.19	0.17	0.65

This difference in retention can be easily explained if we consider that the non-polar alkyl-bonded chains are covered with one or several layers of pure organic modifier (or of a mixture very enriched with pure modifier). Table VI gives the $k's$ values of some solutes eluted with ethanol–water mixtures and solute solubilities (s_0) in ethanol.

TABLE VI

VALUES OF $k's$ OBTAINED WITH SOME SOLUTES ELUTED WITH WATER–ETHANOL MIXTURES (15 cm COLUMN PACKED WITH RP-18) AND SOLUBILITIES OF THESE SOLUTES IN PURE ETHANOL AT 25°C

Parameter	*Solute*				
	Acetanilide	*p-Ethoxy-acetanilide*	*p-Methyl-acetanilide*	*Benzamide*	*o-Nitrophenol*
$k's$ (mole·l^{-1})	0.30 ± 0.04	0.08 ± 0.01	0.11 ± 0.01	0.24 ± 0.03	0.36 ± 0.04
s_0 (mole·l^{-1})	1.83	0.37	0.62	1.20	2.25
$k's/s_0$	0.16	0.22	0.18	0.20	0.16

It can be seen that the constant $k's$ is related to s_0, as the ratio $k's/s_0$ is constant. Therefore, $k' = C's_0/s$ and the solute–stationary phase interactions are in fact the interactions between the solute and the organic solvent coated on the stationary phase; the more soluble the solute is in the organic solvent, the stronger these interactions are.

Scott and Kucera[34] showed that for a low water content the bonded chains were wetted by the organic solvent; our results are in complete agreement.

We have seen above that the bonded chains play no specific role in the retention mechanism and that the only important parameter for solute capacity factors and relative retentions is the total hydrocarbonaceous surface area NS. In fact, the hydrophobic hydrocarbonaceous chains are only a support for a layer of organic solvent and, as the solute does not interact directly with the bonded chains, there is no specific interaction between the bonded bristle and the solute.

CONCLUSION

In this study, some simple relationships concerning solute retention have been given. The optimization of chromatographic separations can easily be attained with these relationships, particularly in reversed-phase chromatography. The very simple relationship between the k' and s for a solute in the mobile phase provides a useful means for the rapid choice of operating conditions.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOBASES, NUCLEOSIDES AND NUCLEOTIDES

I. MOBILE PHASE COMPOSITION FOR THE SEPARATION OF CHARGED SOLUTES BY REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

To find optimal conditions for the separation of nucleobases, nucleosides and nucleotides by reversed-phase chromatography, the polarity, pH, ion concentration and polarity of the buffer ions of the mobile phase were varied. A systematic study of the effects of these parameters on retardation led to the formulation of the following simple rules: (1) methanol changes only column capacity ratios, not selectivity; (2) pH influences both column capacity ratios and selectivity; (3) the concentration of buffer ions had hardly any effect on retardation; (4) the hydrophobicity of the buffer ions has strong effects only on column capacity ratios and selectivity of solutes with opposite charge; and (5) a mixture of buffer ions with different hydrophobicities has the chromatographic properties of a buffer ion with intermediate hydrophobicity.

INTRODUCTION

Nucleobases and nucleosides have been separated by standard methods on cation exchangers at acidic pH[1] and nucleotides on anion exchangers at neutral or basic pH[2]. Nucleobases and nucleosides have also been separated on anion exchangers at basic pH[3,4], and under these conditions the simultaneous separation of all compounds on one column was possible[5,6]. The introduction of reversed phases facilitated this simultaneous separation[7,8], especially when hydrophobic buffer ions were used[9]. More recently, affinity chromatography of nucleobases has been reported, using immobilized thymidine as the stationary phase[10]. This class of substances can be separated successfully under many different conditions[1–30] owing to their intrinsic physical properties, combining acidic, basic, polar and lipophilic moieties within one chemical structure.

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To optimize chromatographic conditions, one should use all of these physical properties. It is important to know the effects of different mobile phase parameters such as polarity, pH, ion concentration and polarity of the buffer ions on the retardation of solutes with different physical properties.

The relationship between the resolution of two solutes and selectivity and column capacity ratios can be formulated as[31]

$$R_s = \frac{1}{2} \cdot \frac{\overline{k'}}{\overline{k'} + 1} \cdot \frac{1 - \alpha}{1 + \alpha} \left(\frac{L}{H}\right)^{\frac{1}{2}}$$

where R_s = resolution, $\overline{k'}$ = mean column capacity ratio, α = selectivity, L = length of the column and H = height equivalent to a theoretical plate. Although H is not independent of k'[32], this equation provides a theoretical guide to the optimal composition of mobile phases in chromatography. In general, it is easier to increase k' values than α values. k' values above about 4 are used only if many compounds have to be separated, because the resolution is already at 80% of its maximal value. If the resolution is insufficient, the mobile phase should be changed in such a way that the selectivity (α) is more affected than column capacity ratios (k'). In practice, the optimal separation conditions should combine small column capacity ratios with high selectivity. Therefore, the effects of the polarity of the mobile phase, its pH, ion concentration and the polarity of the cations on column capacity ratio and selectivity were studied systematically on a reversed-phase column.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment consisted of an Altex Model 100A pump, a Rheodyne 7105 injector, a reversed-phase column (5 μm, RP-8, Riedel-de Haën, Hannover, G.F.R.; self-packed, 300 × 3 mm I.D.) and a Perkin-Elmer LC-55 variable wavelength detector set at 260 nm. Retention times were recorded with a stopwatch and were reproducible with a standard deviation of 0.5%. The ion concentrations of the mobile phase are always given for the cations and the total volume. The pH was measured with an E-516 Titriskop Metrohm Herisau pH meter, and is given for the final mobile phase composition. The flow-rate was 1.5 ml/min. The pressure varied with the composition of the mobile phase between 110 and 150 bar. All experiments were carried out at room temperature.

RESULTS AND DISCUSSION

In addition to the solutes listed in Table I*, the following compounds were investigated the same way (data not shown): 8-methoxy-cAMP (B), 8-hydroxy-cAMP (B), 8-bromo-cAMP (B), 5-aminoimidazole-4-carboxamide-1-ribose-3′,5′-monophosphate (AICAR) (B), xanthosine-3′,5′-monophosphate (B), adenosine-N^1-

* Abbreviations used: cAMP = adenosine-3′,5′-monophosphate; 5′-AMP = adenosine-5′-monophosphate; cPMP = purine-riboside-3′,5′-monophosphate; B = Boehringer (Mannheim, G.F.R.); S = Sigma (St. Louis, MO, U.S.A.); P = Pharma-Waldhof (Mannheim, G.F.R.); M = Merck (Darmstadt, G.F.R.).

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF SOLUTES

No.	*Compound*	*Source*	*Hydrophobicity* at pH 3.0*	*Electrophoretic mobility** at pH 3.0*
1	cAMP	P	0.8	−3.1
2	N^6-Monobutyryl-cAMP	B	3.9	−8.4
3	6-Chloro-cPMP	B	2.0	−10.8
4	8-Amino-cAMP	B	0.6	+0.5
5	8-Dimethylamino-cAMP	B	3.5	−0.1
6	8-Benzylamino-cAMP	B	11.2	+0.7
7	5′-AMP	B	0.3	−2.9
8	Adenosine	M	1.1	+11.1
9	Adenine	B	0.8	+19.5

* Hydrophobicity is expressed as the k' value on the reversed-phase column in 10% methanol–10 m*M* ammonium formate (pH 3.0).

** The electrophoretic mobility is expressed as the rate of movement in cm/h of the solutes to the negative pole (positive sign) or positive pole (negative sign) of a Pherograph high-voltage electrophoretic apparatus. The electrophoretic movement is divided by the R_F value of the solute in ascending paper chromatography, to correct for adsorption of the solutes to the paper.

oxide (S), 2′-deoxyadenosine (M), 5′-tosyladenosine (P), 2′,3′-isopropylideneadenosine (S), 2′,3′-O-*p*-methoxybenzylideneadenosine (S), guanosine (P), 2′-deoxyguanosine (M), cytidine (P), 2′-deoxycytidine (P), uridine (P), thymidine (B) and purine riboside (S). The compounds in Table I were chosen for the following reasons:

(1) cAMP, adenosine and adenine have similar polarities but different charges;

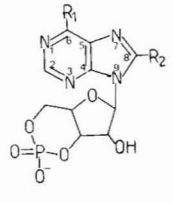
(2) cAMP and 5′-AMP have identical charges at pH 3.0 but differ in polarity;

(3) 8-amino-cAMP, 8-dimethylamino-cAMP and 8-benzylamino-cAMP have similar charges but their polarities differ widely;

(4) N^6-monobutyryl-cAMP and 6-chloro-cPMP were chosen as the least protonated solutes at pH 3.0.

The structures of these cyclic nucleotide derivatives are shown in Fig. 1.

The electrophoretic mobilities of the solutes were determined in 0.04 *M* citrate–hydrochloric acid (pH 3.0) on Whatman 3MM paper using Pherograph high-voltage electrophoresis at 2000 V. Adsorption of the solutes to this paper was determined in this buffer using ascending paper chromatography. As an indication of the charge of the solutes at pH 3.0, the electrophoretic mobility was divided by the R_F value for paper chromatography.



No	R_1	R_2
1	$-NH_2$	$-H$
2	$-NH-CO-CH_2-CH_2-CH_3$	$-H$
3	$-Cl$	$-H$
4	$-NH_2$	$-NH_2$
5	$-NH_2$	$-N(CH_3)_2$
6	$-NH_2$	$-NH-CH_2-C_6H_5$

Fig. 1. Structures of the cyclic nucleotide derivatives.

The silanol groups that are still present after chemical modification of the silica gel matrix often have no influence on retardation[33], and this occurred with this column; retention times of polar compounds did not increase at higher methanol concentrations (data not shown). Therefore, it is assumed that all chromatographic effects are due to interactions between components of the mobile phase, the solutes and the octyl moieties of the stationary phase. In most experiments the pH was kept at 3.0, as at this pH most compounds are partially protonated (Table I). Different substituted ammonium ions were used to give a specific hydrophobicity[34].

Polarity of the mobile phase

An increase in methanol concentration decreases the retention times in reversed-phase chromatography (Fig. 2). The effect of the hydrophobic constituents at the adenine moiety on column capacity ratios is shown in the order of elution of 8-amino-cAMP (4), 8-dimethylamino-cAMP (5) and 8-benzylamino-cAMP (6). The methanol concentration has virtually no effect on selectivity, probably indicating that

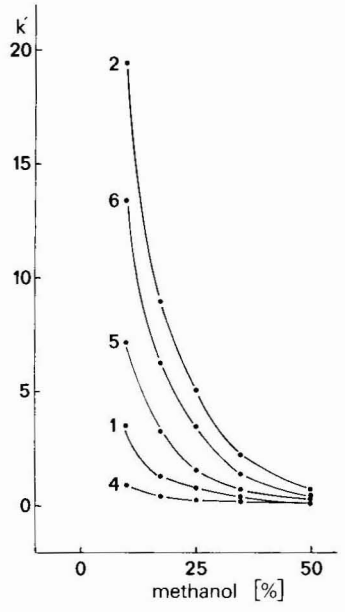


Fig. 2. Influence of methanol concentration on column capacity ratios. Mobile phase: 10 m*M* tributylammonium formate (pH 3.0). Solutes (see Table I): 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 4 = 8-amino-cAMP; 5 = 8-dimethylamino-cAMP; 6 = 8-benzylamino-cAMP.

methanol does not modify the type but rather the intensity of interaction between solutes and the stationary phase; it competes with the solute for occupation of the octyl groups.

As methanol changes only k' values, a decrease in methanol concentration does not result in a much improved resolution of two solutes with small selectivity, but in a longer analysis time.

pH of the mobile phase

The pH of the mobile phase may affect the polarity of the solutes more strongly than the polarity of the mobile and stationary phases. Protonation of basic groups of the solutes results in an increase in polarity, and therefore in a decrease in retention time. The pK value of cyclic AMP (1) is approximately 3.4 (Table I), which agrees well with the significant change in k' values between pH 3 and 4 (Fig. 3). Substitution of the N^6-amino group in N^6-monobutyryl-cAMP (2) decreases the pK value to approximately 2.5 (Table I), and shifts the pH-sensitive part of the curve to values below pH 3.0. By removal of the amino group as in 6-chloro-cPMP (3), no protonation occurs (Table I) and the pH has little effect on retardation. Hence the pH of the mobile phase only influences the k' values of those solutes which have a pK value in the region where the pH was changed. Because most compounds have different pK values, this parameter is particularly useful in modifying selectivity.

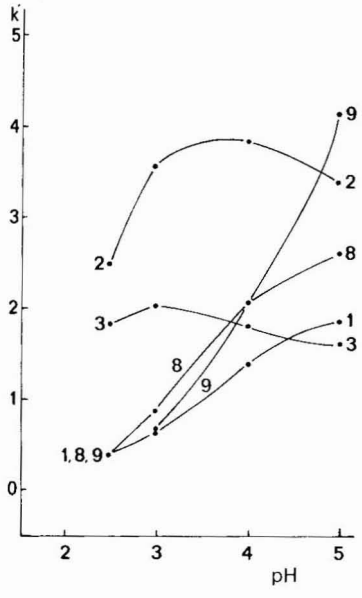


Fig. 3. Influence of pH on column capacity ratios. Mobile phase: 10% methanol–10 mM ammonium formate. Solutes (see Table I): 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 3 = 6-chloro-cPMP; 8 = adenosine; 9 = adenine.

Ion concentration of the mobile phase

The concentration of the buffer ions was varied between 0.001 and 0.1 M. Solutes were injected at a relatively low concentration of 1 μM. Changes in ion concentration may change the equilibrium of the solutes in ion-paired and non-paired forms. The concentration of buffer ions also may change the polarity of the mobile phase[35,36]. Fig. 4 shows that the ion concentration has noticable but small effects on retardation and selectivity on a reversed-phase column. This parameter is therefore of minor importance for optimizing mobile phase compositions for a reversed-phase column.

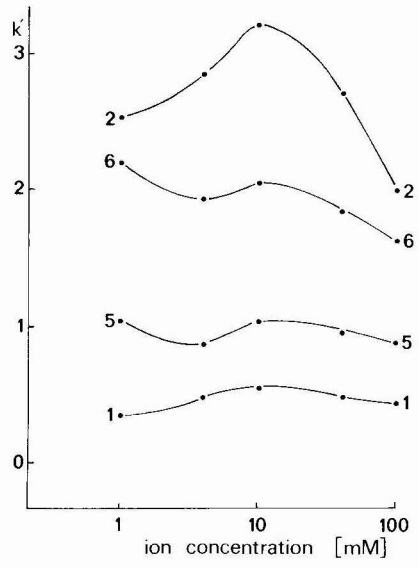


Fig. 4. Influence of concentration of buffer ions on column capacity ratios. Mobile phase: 30% methanol–tributylammonium formate (pH 3.0). Solutes: (see Table I): 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 5 = 8-dimethylamino-cAMP; 6 = 8-benzylamino-cAMP.

Hydrophobicity of the buffer ions

Increasing hydrophobicity of the cations results in an increase in the k' values of negatively charged solutes [Fig. 5A, cyclic AMP (1) and 5′-AMP (7)] and a slight decrease in the k' values of positively charged solutes [adenosine (8) and adenine (9) in Fig. 5A].

Retardation of the cyclic AMP derivatives with a distinct net negative charge at pH 3.0 is strongly influenced by increasing hydrophobicity of the cations [N^6-monobutyryl-cAMP (2) and 6-chloro-cPMP (3) in Fig. 5B]. The retention times of the cyclic AMP derivatives with a close to zero net charge, such as 8-amino-cAMP (4), 8-dimethylamino-cAMP (5) and 8-benzylamino-cAMP (6), are only slightly influenced by the polarity of the buffer ions (Fig. 5B). These results demonstrate the anion-exchange properties of a reversed-phase column in the presence of hydrophobic buffer cations.

Reversed-phase interactions are involved in the retardation mechanism, as is shown by the constant differences in the k' values of 8-amino-cAMP (4), 8-dimethylamino-cAMP (5) and 8-benzylamino-cAMP (6). A lively discussion is going on in the literature on the mechanism by which charged solutes are retarded on reversed-phase matrices in the presence of hydrophobic ions. The result is a variety of terms for this type of chromatography, such as "soap chromatography"[37,38], "ion-pair chromatography"[39], "solvent-generated dynamic ion-exchange chromatography"[40,41], "hetaeric chromatography"[42], "paired-ion chromatography"[43], "detergent-based cation exchange"[40], "solvophobic-ion chromatography"[9], "surfactant chromatography"[44] and "ion interaction"[45]. This variety indicates the uncertainty that exists concerning the retention mechanism of this type of chromatography. For the practice of chromatography it may be sufficient to state that increasing hydrophobicity of

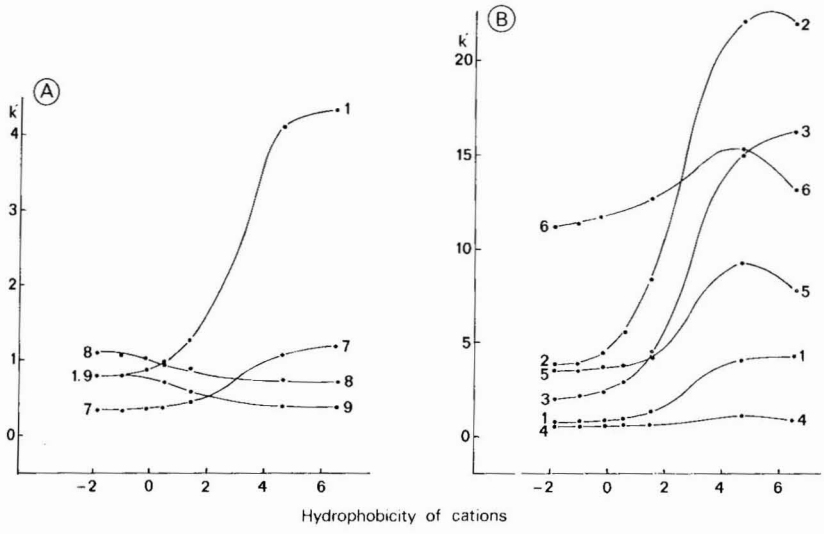


Fig. 5. Influence of the hydrophobicity of the cations on column capacity ratios. Abscissa: hydrophobicity of the cations, calculated with hydrophobic fragmental constants[34]. Cations from left to right on each curve: ethanolammonium, ammonium, monoethylammonium, diethylammonium, triethylammonium, tributylammonium and tetrabutylammonium. Mobile phase: 10% methanol–10 m*M* cations (pH 3.0), adjusted with formic acid. Solutes: see Table I and Fig. 1.

buffer ions results in unchanged retardation of solutes without charge, in unchanged or diminished retardation of solutes with the same charge as the modifying buffer ion, and in stronger retardation of solutes with an opposite charge of the modifying buffer ion.

Mixture of buffer ions with different hydrophobicities

The change of a buffer ion by subsequent substitutions through hydrophobic groups can only result in discrete values of the hydrophobicity of the buffer ions. It will be time consuming to find the specific buffer ion which has the desired hydrophobicity. A mixture of two buffer ions with different hydrophobicities may act as a buffer ion with intermediate hydrophobicity. In Fig. 6 the ratio of concentrations of two cations with different hydrophobicities is varied; the change in selectivity is comparable to the change in Fig. 5, where the hydrophobicity of only one cation was varied. A comparison of Figs. 5 and 6 shows that a mixture such as 10 m*M* tributylammonium formate plus 100 m*M* ammonium formate results in a similar slectivity to 10 m*M* triethylammonium formate.

Mixtures of buffer ions with different hydrophobicities can provide a continuous range of hydrophobicities, and it is easier to find an optimal mixture than a cation with optimal hydrophobicity.

Practical aspects of reversed-phase chromatography

The action of mobile phase components on the retardation of charged solutes by reversed-phase chromatography can be summarized by the following simple rules:

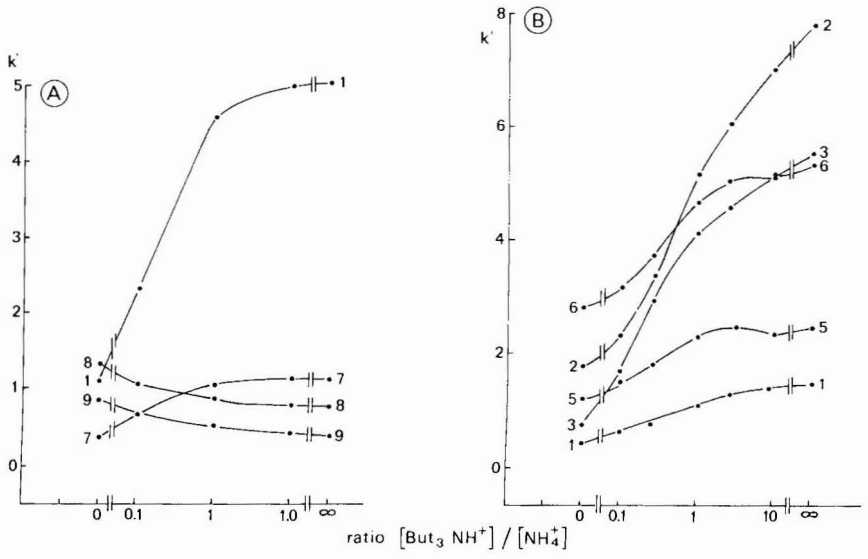


Fig. 6. Influence of a mixture of cations with different hydrophobicities on column capacity ratios. Abscissa: ratio of tributylammonium (But_3NH^+) and ammonium (NH_4^+) concentrations; this ratio was produced by mixing 10 m*M* tributylammonium formate with different concentrations ammonium formate, except where the ratio is 0 or ∞ which were obtained by applying 10 m*M* ammonium formate or 10 m*M* tributylammonium formate, respectively. A, 5% methanol (pH 3.0); B, 25% methanol (pH 3.0). Solutes: see Table I and Fig. 1.

(1) Methanol reduces only the column capacity ratios, without having a strong effect on selectivity.

(2) The pH of the mobile phase influences both column capacity ratios and selectivity. Its action depends mainly on the p*K* values of the solutes.

(3) The concentration of buffer ions has only minor effects on column capacity ratios and selectivity.

(4) The hydrophobicity of a buffer ion has pronounced effects on column capacity ratios and the selectivity of solutes with opposite charge, and small effects on the retardation of solutes without charge or the same charge as the buffer ions.

(5) A mixture of buffer ions with different hydrophobicities acts as a buffer ion with intermediate hydrophobicity. The hydrophobicity of a mixture is determined by the ratio of the concentrations of the components rather than by their absolute concentrations.

During the last 2 years we have applied these rules to several separation problems using different columns. For our studies on the degradation of cyclic nucleotide derivatives by a liver homogenate[46], simultaneous separation of nucleotides, nucleosides and nucleobases was desirable. For the separation of degradation products of 2′-(2,4-dinitrophenoxy)-cAMP, a reversed-phase column was chosen because of the high hydrophobicity of the dinitrophenoxy moiety. A relatively high pH was used in order to discriminate between the charge of phosphate diesters (cAMP) and phosphate monoesters (5′-AMP). Phosphate buffer has a good buffering capacity at pH 6, whereas the ion concentration is not important. First, the methanol concentration was varied to obtain a preliminary separation, then the selectivity between the group

of nucleosides and the group of nucleotides was improved by adding a small amount of tributylammonium formate. Finally, the optimum compromise between resolution and analysis time was found by variation of the methanol concentration (Fig. 7A).

The appearance of 2′-(2,4-dinitrophenoxy)-5′-AMP among the degradation products of this cyclic nucleotide derivative was surprising, as 5′-AMP did not appear as one of the degradation products of cyclic AMP. To prove that the compound which forms peak 3 has a negative charge, more tributylammonium formate was added to the mobile phase. The nucleotides disappeared from the chromatogram, whereas the retention time of the nucleoside remained unchanged (Fig. 7B).

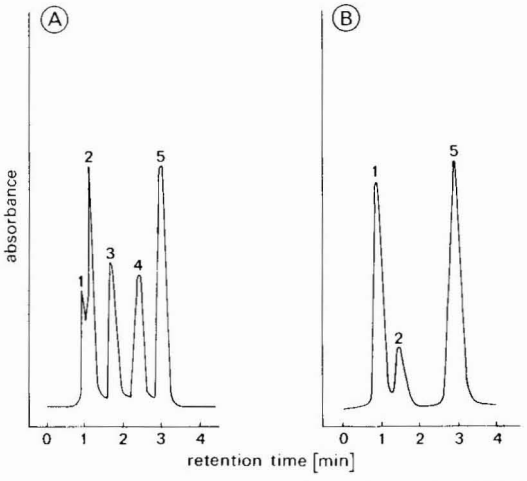


Fig. 7. Separation of 2′-(2,4-dinitrophenoxy)-cAMP incubated with a liver homogenate for 8 h[46]. Stationary phase: RP-18 (Machery, Nagel & Co., Düren, G.F.R.), 300 × 3 mm. Mobile phase: A, 8 m*M* Na_2HPO_4/H_3PO_4–1 m*M* tributylammonium formate–48% methanol (pH 6.6); B, 8 m*M* Na_2HPO_4/H_3PO_4–10 m*M* tributylammonium formate–48% methanol (pH 6.6). Peaks: 1 and 2 = UV-absorbing compound from the liver homogenate; 3 = 2′-(2,4-dinitrophenoxy)-5′-AMP; 4 = 2′-(2,4-dinitrophenoxy)-cAMP; 5 = 2′-(2,4-dinitrophenoxy)inosine.

Another example is kinetic studies of cyclic nucleotide phosphodiesterase under non-equilibrium conditions which require the separation of minute amounts of product from large amounts of substrate. Because peak tailing occurs more often than an extended front of the peak, the high substrate peak should be the last one in the chromatogram. As enzyme preparations are rarely pure, the further degradation products should also be analysed. For cyclic nucleotide phosphodiesterase the optimal separation would take place when all nucleosides and 5′-AMP coincide in one peak just after the injection peak and cyclic AMP appears later in the chromatogram. A reversed phase gives the correct order of elution of 5′-AMP and cyclic AMP. Cyclic AMP becomes the most lipophilic compound on using triethylammonium or tributylammonium formate as the mobile phase buffer (*cf.*, Fig. 5A).

The pH should be below 5, otherwise 5′-AMP acquires two negative charges and therefore two lipophilic counter ions. With triethylammonium formate as the buffer ion adenosine appeared after 5′-AMP, whereas with tributylammonium formate 5′-AMP appeared after adenosine. By mixing different concentrations of trieth-

ylammonium with tributylammonium ions a mixture was obtained that resulted i identical retardation times of adenosine and 5′-AMP. Separation between this pea and cyclic AMP was optimized by variation of the methanol concentration (Fig. 8A

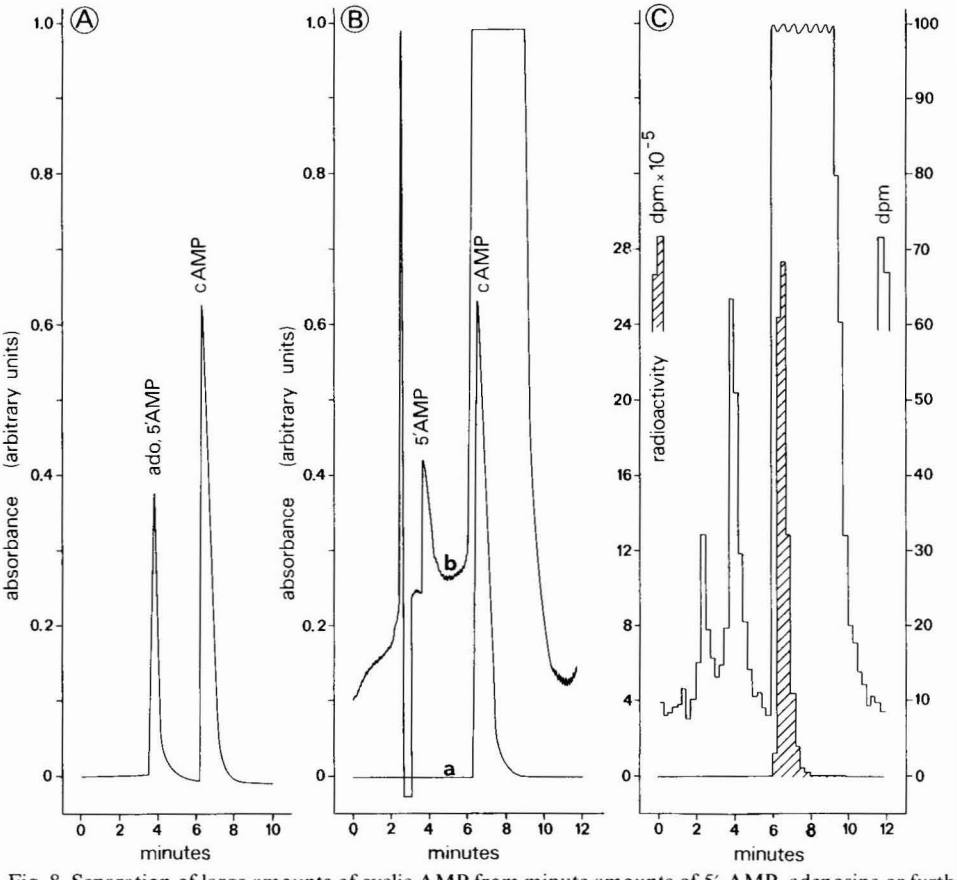


Fig. 8. Separation of large amounts of cyclic AMP from minute amounts of 5′-AMP, adenosine or furthe degradation products. Equipment: Altex Model 100A high-pressure pump, Valco 7000 p.s.i. injecto Laboratory Data Control UVIII (1203) detector (254 nm) and a Kipp BD 40 flat-bed recorder. Stationar phase: reversed-phase LiChrosorb 5RP-18 (250 cm × 4.6 mm) with Vydac 201 SC pre-column (100 cm 2.1 mm). Mobile phase: 5 m*M* triethylammonium formate–0.5 m*M* tributylammonium formate–25 methanol (pH 4.5) (adjusted with formic acid); flow-rate, 1 ml/min; pressure, 306 bar. (A) Separation c $0.5 \cdot 10^{-9}$ moles of adenosine, $0.5 \cdot 10^{-9}$ moles of 5′-AMP and $5 \cdot 10^{-9}$ moles of cyclic AMP. Injectio volume: 10 μl. Sensitivity: detector at 0.128, recorder at 10 mV; thus, one arbitrary unit ≡ 0.128 absorb ance unit. (B) Separation of 10^{-7} moles of cyclic AMP from $2 \cdot 10^{-12}$ moles of 5′-AMP. Injection volume 100 μl. Sensitivity: a = detector at 2.048, recorder at 10 mV (one arbitrary unit ≡ 2.048 absorbance unit) b = detector at 0.002, recorder at 2 mV (one arbitrary unit ≡ 0.0004 absorbance unit). Cyclic AMP wa purified previously under the same chromatographic conditions. (C) Separation of 10^{-10} moles c [^{3}H]cAMP from 10^{-15} moles of [^{3}H]-5′-AMP and possibly further degradation products: 10^{-10} moles c [2,8-^{3}H]cAMP (52 Ci/mmol; Radiochemical Centre, Amersham, Great Britain) were in ubated in 25 m*M* Tris–HCl–2 m*M* $MgCl_2$ (pH 7.5) with $2.5 \cdot 10^{-10}$ g of cyclic nucleotide phosphodiesterase (Boehringer) in total volume of 10 μl. Ten seconds after the initiation the reaction was stopped by injection of th incubation mixture on to the column. The eluent was divided into 0.25-ml fractions, the radioactivity c which was determined. [2,8-^{3}H]cAMP was purified previously under the same chromatographic con ditions.

The background noise of the UV detector is approximately 0.00001 absorbance unit. Fig. 8B shows the separation of 10^{-7} moles of cyclic AMP from $2 \cdot 10^{-12}$ moles of 5′-AMP. By making use of radioactive cyclic AMP the sensitivity is determined by the specific activity of the radioactive label. Fig. 8C shows the separation of 10^{-10} moles of [^{3}H]cAMP from 10^{-15} moles of degradation products (5′-AMP and/or nucleosides). The detection limit can be reduced still further if [^{32}P]cAMP (1000–3000 Ci/mmole, NEG-011; New England Nuclear, Boston, MA, U.S.A.) is used. The detection limit would be then 10^{-17} moles, which is only 6 million molecules.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOBASES, NUCLEOSIDES AND NUCLEOTIDES

II. MOBILE PHASE COMPOSITION FOR THE SEPARATION OF CHARGED SOLUTES BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The polarity, pH, ion concentration and polarity of the buffer ions of the mobile phase were modified systematically in order to find optimal conditions for the separation of nucleobases, nucleosides and nucleotides by ion-exchange chromatography. The effects of these mobile phase parameters on the retardation of 26 nucleobases, nucleosides and nucleotides on the cation exchanger Partisil-10 SCX and the anion exchanger Partisil-10 SAX were examined and resulted in the formulation of the following simple rules:

(1) These ion exchangers also have reversed-phase and normal-phase properties; their occurrence is determined by the polarity of the mobile phase.

(2) The ion concentration has an effect only on ion-exchange properties.

(3) The pH of the mobile phase determines the degree of protonation of the solutes, and therefore the charge and polarity. Many solutes have different pK values, thus providing a tool for the variation of selectivity.

(4) The polarity of the buffer ions has an indirect effect on ion-exchange chromatography through differential distribution of the buffer ions over the two phases. This differential distribution is caused by reversed-phase or normal-phase characteristics.

The integration of these three types of chromatography has been demonstrated by some examples.

INTRODUCTION

Charged solutes have commonly been separated by ion-exchange chromatography[1–9]. Nucleosides, nucleobases and nucleotides have been separated by ion-ex-

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change chromatography and reversed-phase chromatography[5–31]. Reversed-phase chromatography is increasingly replacing ion-exchange chromatography, especially after the introduction of hydrophobic ions as a component of the mobile phase[32–37]. A disadvantage of ion-exchange chromatography is the unpredictability of some separations; the solutes are not always eluted in the sequence of their charges. Often these complexities can be diminished by the addition of non-polar solvents to the mobile phase[38–41].

Samuelson[38] studied the distribution of ethanol over the two phases of an ion exchanger used in low-pressure liquid chromatography. He found that with up to 30% ethanol in the mobile phase the concentration of ethanol in the stationary phase is always higher than that in the mobile phase. If the concentration of ethanol in the mobile phase is above 35%, then its concentration in the stationary phase is always lower than that in the mobile phase[38]. Eksteen *et al.*[21] used these results to explain the retardation of nucleobases and nucleosides on an anion exchanger in the presence of varying ethanol concentrations. These experiments suggest that reversed-phase and normal-phase interactions may take place in ion exchangers. In reversed-phase chromatography the ion concentration has little effect on retardation[42], but in ion-exchange chromatography it has a pronounced effect[1,2].

The simultaneous occurrence of reversed-phase and ion-exchange interactions may result in unpredictable separations. Therefore, the effects of the polarity, pH, buffer ion concentration and polarity of the buffer ions in the mobile phase on the retardation of 26 nucleobases, nucleosides and nucleotides was studied systematically on anion and cation exchangers. The results led to rules for practical ion-exchange chromatography and a few applications are shown.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment consisted of an Altex Model 100A pump, a Rheodyne 7105 injector and a Perkin-Elmer LC-55 variable-wavelength detector set at 260 nm. The columns were packed with the anion exchanger Partisil-10 SAX and the cation exchanger Partisil-10 SCX (Whatman; 250 × 4.6 mm I.D.). The concentrations of the mobile phase components are always given for the total mixture. The ion concentration is given for the cations.

The pH was measured with an E-516 Titriskop Metrohm Herisau pH meter. Retention times were recorded with a stop-watch and were reproducible with a standard deviation of 0.5%. The solutes were dissolved in water. The flow-rate was 2 ml/min with a pressure between 35 and 70 bar, depending on the column and the composition of the mobile phase. All experiments were carried out at room temperature.

RESULTS AND DISCUSSION

In addition to the solutes listed in Table I* and shown in Fig. 1, the following

* Abbreviations used: cAMP = adenosine-3′,5′-monophosphate; 5′-AMP = adenosine-5′-monophosphate; cPMP = purine-riboside-3′,5′-monophosphate; B = Boehringer (Mannheim, G.F.R.); S = Sigma (St. Louis, MO, U.S.A.); P = Pharma-Waldhof (Mannheim, G.F.R.); M = Merck (Darmstadt, G.F.R.); k' = column capacity ratio; α^{cAMP} = selectivity to cAMP.

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF SOLUTES

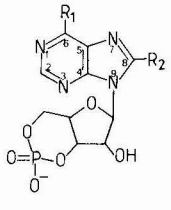
No.	*Compound*	*Source*	*Hydrophobicity* at pH 3.0*	*Electrophoretic mobility** at pH 3.0*
1	cAMP	P	0.8	− 3.1
2	N^6-Monobutyryl-cAMP	B	3.9	− 8.4
3	6-Chloro-cPMP	B	2.0	−10.8
4	8-Amino-cAMP	B	0.6	+ 0.5
5	8-Dimethylamino-cAMP	B	3.5	+ 0.1
6	8-Benzylamino-cAMP	B	11.2	+ 0.7
7	5′-AMP	B	0.3	− 2.9
8	Adenosine	M	1.1	+11.1
9	Adenine	B	0.8	+19.5
10	8-Bromo-cAMP	B	2.0	− 5.6
11	8-Methoxy-cAMP	B	1.0	− 2.9

* Hydrophobicity is expressed as the column capacity ratios (k') of the solutes on a reversed-phase column (RP-8, Riedel-de Haën, Hannover, G.F.R.) in 10% methanol–10 m*M* ammonium formate (pH 3.0)[42].

** The electrophoretic mobility is expressed as the rate of movement in cm/h of the solutes to the negative pole (positive sign) or positive pole (negative sign) of a Pherograph high-voltage electrophoretic apparatus. The electrophoretic movement is divided by the R_F value of the solute in ascending paper chromatography, and thus corrected for adsorption of the solutes to the paper.

compounds were investigated the same way (data not shown, except in Figs. 6 and 7): 8-hydroxy-cAMP (B), xanthosine-3′,5′-monophosphate (B), 5-aminoimidazole-4-carboxamide-1-ribose-3′,5′-monophosphate (AICAR) (B), adenosine-N^1-oxide (S), 2′-deoxyadenosine (M), 5′-tosyladenosine (P), 2′,3′-isopropylideneadenosine (S), 2′,3′-O-*p*-methoxybenzylideneadenosine (S), guanosine (P), 2′-deoxyguanosine (M), cytidine (P), 2′-deoxycytidine (P), uridine (P), thymidine (B) and purine riboside (S).

The electrophoretic mobility of the solutes was determined in 0.04 *M* citrate–hydrochloric acid (pH 3.0) on Whatman 3MM paper using Pherograph high-voltage electrophoresis at 2000 V. Adsorption of the solutes to this paper was determined in this buffer by ascending paper chromatography. As an indication of the charge of the solutes at pH 3.0 the electrophoretic mobility was divided by the R_F value for paper chromatography.



No	R_1	R_2
1	$-NH_2$	-H
2	$-NH-CO-CH_2-CH_2-CH_3$	-H
3	-Cl	-H
4	$-NH_2$	$-NH_2$
5	$-NH_2$	$-N(CH_3)_2$
6	$-NH_2$	$-NH-CH_2-C_6H_5$
10	$-NH_2$	-Br
11	$-NH_2$	$-O-CH_3$

Fig. 1. Structures of the cyclic nucleotide derivatives.

Structure of the ion exchangers

The information on the structure of Partisil-10 SAX and SCX was kindly provided by Dr. F. Rabel, Whatman Inc. (personal communication). Partisil-10 SAX and SCX are chemically modified silica gel resins, which still contain silanol groups. The functional groups are shown in Fig. 2.

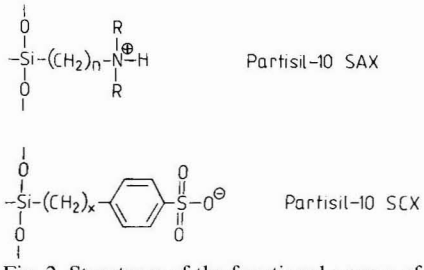


Fig. 2. Structures of the functional groups of Partisil-10 SAX and Partisil-10 SCX.

In the anion exchanger Partisil-10 SAX, the tertiary ammonium moiety for ion-exchange properties is connected to the silica gel matrix via an aliphatic spacer. In Partisil-10 SCX, cation-exchange properties are derived from the sulphonyl moiety, which is connected to the silica gel matrix via a spacer composed of aliphatic and aromatic moieties.

Anion exchanger

In ion-exchange chromatography the buffer ion concentration and the pH are the important mobile phase parameters that influence the retardation of charged

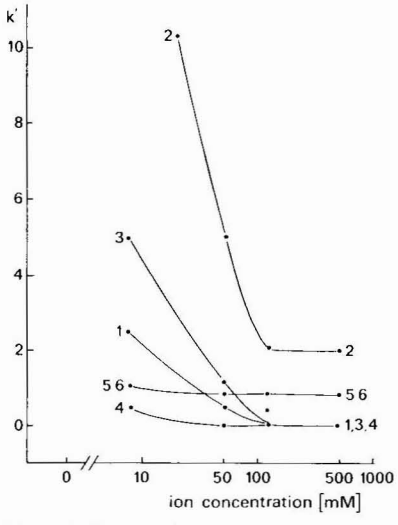


Fig. 3. Influence of ion concentration on column capacity ratios. Stationary phase: Partisil-10 SAX anion exchanger. Mobile phase: sodium phosphate buffer (pH 3.0). Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 3 = 6-chloro-cPMP; 4 = 8-amino-cAMP; 5 = 8-dimethylamino-cAMP; 6 = 8-benzylamino-cAMP.

solutes. The ion concentration mainly affects column capacity ratios and pH affects column capacity ratios and selectivity. In Fig. 3 the buffer ion concentration increased from 6 to 500 m*M*. Above about 100 m*M* the ion concentration no longer influences the retention times. Although ion-exchange chromatography is virtually absent, there is still retention, especially of the nucleotides with hydrophobic substituents (Fig. 3, Table I). Reversed-phase interactions can be diminished by the addition of an apolar solvent to the mobile phase[42]. The addition of small amounts of acetonitrile to the mobile phase (Fig. 4) results in a reduction of the retention times of the nucleotides with hydrophobic substituents (Fig. 4, Table I). This may indicate that acetonitrile removes reversed-phase interactions. At high acetonitrile concentrations, the retention of most nucleotides increases. This suggests the introduction of normal-phase chromatography, probably due to activation of silanol groups at high acetonitrile concentrations.

The effects of the mobile phase components on the retardation of charged solutes by ion-exchange chromatography can be shown more clearly for a cation-exchange column.

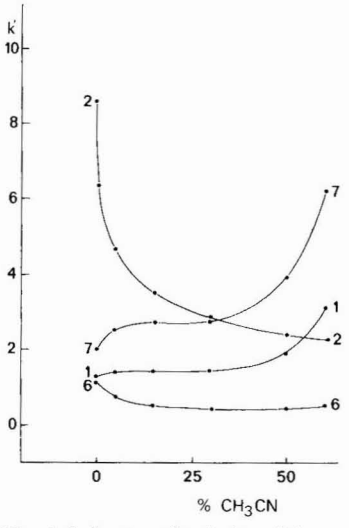


Fig. 4. Influence of polarity of the mobile phase on column capacity ratios. Stationary phase: Partisil-10 SAX anion exchanger. Mobile phase: 20 m*M* triethylammonium formate (pH 3.0). Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 6 = 8-benzylamino-cAMP; 7 = 5′-AMP.

Cation exchanger

Partisil-10 SCX consists of a silica gel matrix to which sulphonyl groups are coupled via a spacer composed of aliphatic and aromatic moieties (Fig. 2); silanol groups are present (Dr. F. Rabel, Whatman Inc., personal communication). Due to these components, the following types of chromatography can take place on this cation exchanger: cation exchange on the sulphonyl groups; reversed-phase chromatography on the aromatic and aliphatic spacer; and normal-phase chromatography on the unprotected silanol groups of the matrix. Each of these types of chromatography may be effective.

Polarity of the mobile phase

If reversed-phase, cation-exchange and normal-phase interactions are involve in the retardation of a solute on a cation exchanger, the polarity of the mobile phas will determine the types of interactions that are predominant. Reversed-phase an cation-exchange properties may be present if the mobile phase does not contain a apolar solvent. Addition of apolar solvent to the mobile phase will remove the re versed-phase properties. Silanol groups may become activated at a high concen tration of an apolar solvent in the mobile phase.

Cation-exchange properties will be present at all polarities. An explanation fo the effects of the polarity of the mobile phase on retardation of the nucleobases an nucleosides will be given later.

In buffer alone several nucleotides are retarded on a cation exchanger (Fig. 5) The retention times of the nucleobases, nucleosides and nucleotides are not very wel correlated with their charges (Fig. 6a). The addition of acetonitrile to the mobil phase results in a reduction of the retention times of the nucleotides, especially of th hydrophobic ones, such as N^6-monobutyryl-cAMP (2), and 8-dimethylamino-cAMI (5) (Fig. 5). Between approximately 15% and 40% acetonitrile, the retention times o the nucleobases, nucleosides and nucleotides change only slightly (Fig. 5).

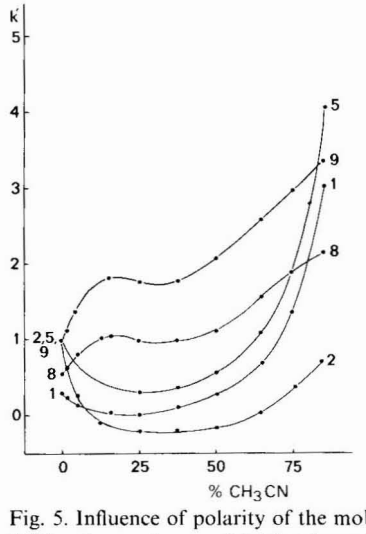


Fig. 5. Influence of polarity of the mobile phase on column capacity ratios. Stationary phase: Partisil-1(SCX cation exchanger. Mobile phase: 5 m*M* triethylammonium formate (pH 3.0). Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 5 = 8-dimethylamino-cAMP; 8 = adenosine; 9 = adenine.

The order of elution from the column follows the order of electrophoretic mobility only with 25% acetonitrile in the mobile phase (Fig. 6b).

The decrease in the retention times of the nucleotides on the cation exchange between 0% and 25% acetonitrile ($\Delta k'$ SCX) is proportional to the hydrophobicity o the solutes (k' RP-8) (Fig. 7). This clearly shows that reversed-phase interaction occur in the absence of acetonitrile, and that acetonitrile removes these reversed phase interactions.

Above 50% acetonitrile in the mobile phase, nucleotides become even more

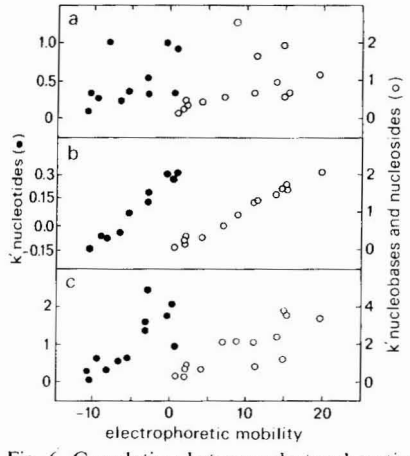


Fig. 6. Correlation between electrophoretic mobility of the solutes and their column capacity ratios at different polarities of the mobile phase. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 m*M* triethylammonium formate (pH 3.0). Solutes: 26 nucleotides, nucleosides and nucleobases (see Experimental). (a) no acetonitrile; (b) 25% acetonitrile; (c) 75% acetonitrile; ●, column capacity ratios of nucleotides; ○, column capacity ratios of nucleobases and nucleosides. For the expression of electrophoretic mobility, see Table I.

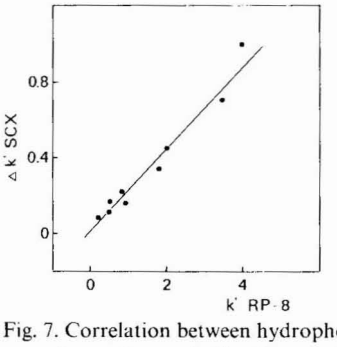


Fig. 7. Correlation between hydrophobicity of the nucleotides and the difference in column capacity ratios on an ion exchanger in the absence of acetonitrile and with 25% acetonitrile. Abscissa: hydrophobicity of the nucleotides, expressed as column capacity ratios on a reversed-phase column (k' RP-8[42]). Ordinate: decrease in column capacity ratios on Partisil-10 SCX cation exchanger if the mobile phase changed from 5 m*M* triethylammonium formate (pH 3.0) to 5 m*M* triethylammonium formate–25% acetonitrile (pH 3.0) ($\Delta k'$ SCX).

retarded (Fig. 5) and, as in the absence of acetonitrile, the order of elution does not correspond closely to the order of the electrophoretic mobilities of the solutes (Fig. 6c). The selectivity of the nucleotides in relation to cAMP changes minimally above 65% acetonitrile (Fig. 8). Only 5′-AMP is retarded more strongly than cAMP at increasing acetonitrile concentrations. This suggests retardation by normal-phase interactions of the phosphate moieties with the silanol groups. This phosphate moiety is identical in all cyclic nucleotides, is more polar in 5′-AMP and is not present in the nucleobases and nucleosides.

The presence of reversed-phase, cation-exchange and normal-phase chromatography on one column can be very confusing when optimizing separations, as the effects of the mobile phase parameters cannot be easily predicted. Retardation on an

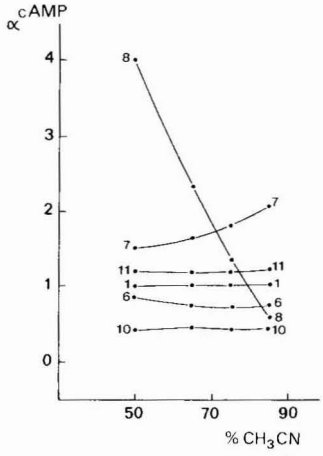


Fig. 8. Influence of polarity of the mobile phase on the selectivity of the solutes to cAMP (α^{cAMP}) at high acetonitrile concentrations in the mobile phase. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 m*M* triethylammonium formate (pH 3.0). Solutes: 1 = cAMP; 6 = 8-benzylamino-cAMP; 7 = 5′-AMP; 8 = adenosine; 10 = 8-bromo-cAMP; 11 = 8-methoxy-cAMP.

ion-exchange column is caused solely by ion-exchange properties, if a moderate amount of apolar solvent is added to the mobile phase.

Concentration of buffer ions

The buffer ion concentration is that mobile phase component which is always used to change column capacity ratios in ion-exchange chromatography[1,2]. An increase in concentration decreases retention times. The ion concentration has minor

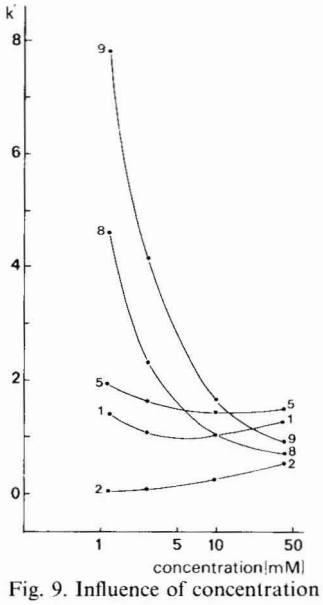


Fig. 9. Influence of concentration of buffer ions on column capacity ratios. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: triethylammonium formate–75% acetonitrile (pH 3.0). Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 5 = 8-dimethylamino-cAMP; 8 = adenosine; 9 = adenine.

effects on reversed-phase chromatography[42]. To show the effect on normal-phase chromatography, this parameter was varied in the presence of 75% acetonitrile. Fig. 9 clearly shows that the ion concentration has minor effects on the nucleotides, which were assumed to be mainly retarded by normal-phase chromatography. The ion concentration strongly influences the retention times of the nucleobases and nucleosides, which is as expected for cation-exchange interactions.

pH of the mobile phase

The pH of the mobile phase influences the charge of the solutes, and consequently their polarity. Protonation of a basic atom group results in stronger cation-exchange interactions, and in an increase in polarity. Both phenomena are shown in Fig. 10A. At 65% acetonitrile, a decrease in pH results in stronger cation-exchange interactions of the nucleobases and nucleosides, and in stronger normal-phase interactions of the nucleotides. Most solutes will have different pK values; therefore, this parameter should also have a strong effect on selectivity. This is shown for some nucleotides in Fig. 10B.

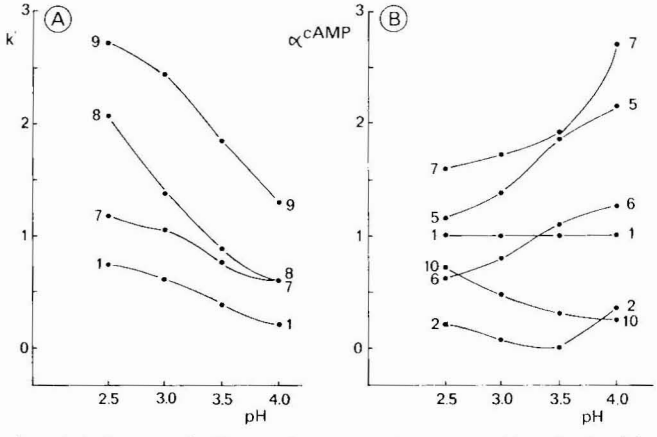


Fig. 10. Influence of pH on column capacity ratios (A) and selectivity to cAMP (B). Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 mM triethylammonium formate–65% acetonitrile. Solutes: 1 = cAMP; 2 = N^6-monobutyryl-cAMP; 5 = 8-dimethylamino-cAMP; 6 = 8-benzylamino-cAMP; 7 = 5′-AMP; 8 = adenosine; 9 = adenine; 10 = 8-bromo-cAMP.

Polarity of the buffer ions

The polarity of the buffer ions has a strong effect on retardation on a reversed-phase column, partly due to differential distribution between the mobile phase and the stationary phase[37,42]. On this cation exchanger not only do cation-exchange properties occur, but normal-phase and reversed-phase interactions can also take place. Owing to these normal-phase and reversed-phase properties, the polarity of the buffer ions may have an effect on the concentrations of these ions in the stationary phase and, consequently, on the retardation of a solute by ion-exchange interactions. To study the effect of this parameter on retardation, the polarity of the cations was varied for all compounds at different acetonitrile concentrations. The concentration of cations in the mobile phase is kept constant. The results for one nucleoside are shown in Fig. 11.

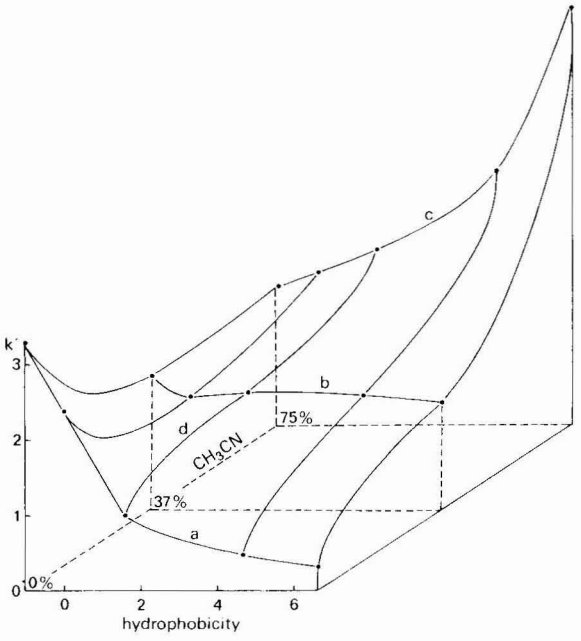


Fig. 11. Influence of hydrophobicity of the buffer ions and polarity of the mobile phase on column capacity ratios of adenosine. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 5 m*M* cations (pH 3.0). Cations used: ammonium, ethylammonium, triethylammonium, tributylammonium and tetrabutyl ammonium. Their hydrophobicities were calculated with the hydrophobic fragmental constant[44]. For (a) (b), (c) and (d), see text.

In the absence of acetonitrile (Fig. 11, curve a), reversed-phase properties ar present in this cation exchanger, which results in attraction of hydrophobic com ponents of the mobile phase by the stationary phase. An increase in the hydropho bicity of the cations results in an increase in their concentration in the stationar phase, and therefore in reduced cation-exchange interactions (Fig. 11, curve a). A 37.5 % acetonitrile (Fig. 11, curve b), the polarities of the two phases are the same, s that the hydrophobicity of the cations has no effect on the distribution of the cation over the two phases, and also has no effect on the retardation of adenosine (Fig. 11 curve b). At 75 % acetonitrile (Fig. 11, curve c) the stationary phase is more pola than the mobile phase. An increase in the hydrophobicity of the cations results in decrease in their concentration in the stationary phase, and therefore in longer reten tion times of solutes retarded by cation-exchange interactions (Fig. 11, curve c).

The influence of different acetonitrile concentrations on the retardation c nucleobases and nucleosides on this cation exchanger in the presence of triethylam monium formate can now be more easily explained (Fig. 5; Fig. 11, curve d). Triethyl ammonium is a hydrophobic cation and is attracted to the stationary phase by re versed-phase interactions that occur in the absence of acetonitrile. Addition of ac etonitrile to the mobile phase removes the reversed-phase characteristics, and th concentration of triethylammonium ions in the stationary phase decreases and reten tion times increase. Between 15 % and 40 % the polarities of the two phases ar identical, and no changes in retention times are observed. A further increase in th

acetonitrile concentration makes the stationary phase more polar than the mobile phase, resulting in a further decrease in the concentration of triethylammonium ions in the stationary phase. The retention times of the nucleosides and the nucleobases will increase still further (Fig. 5; Fig. 11, curve d).

Practical aspects of ion-exchange chromatography

The increasing replacement of ion-exchange columns for the separation of charged solutes is based mainly on the ease of handling mobile phase parameters in reversed-phase chromatography. Although this replacement is justified by excellent separations of almost all classes of compounds, ion-exchange columns can be even more valuable if their properties are understood better. The following rules may help to optimize mobile phase compositions for ion-exchange chromatography:

(1) Ion-exchange columns based on a silica gel matrix also have reversed-phase and normal-phase properties. The polarity of the mobile phase determines the types of interactions that can be involved in retardation.

(2) The pH of the mobile phase is a general parameter affecting column capacity ratios and especially selectivity. This parameter is important if the solutes have different pK values.

(3) The ion concentration has negligible effects on reversed-phase and normal-phase interactions, but has strong effects on ion-exchange interactions. Higher ion concentrations reduce the retention times of solutes retarded by ion-exchange interactions.

(4) The polarity of the buffer ions has an indirect effect on ion-exchange interactions owing to a differential distribution of the buffer ions between the two phases. This partition is caused by the presence of reversed-phase and normal-phase properties.

During the last 2 years these rules have been applied to different separation problems on a variety of ion-exchange columns. Combining them with the rules for the separation of charged solutes by reversed-phase chromatography[42], 2′-(2,4-dinitrophenoxy)-cAMP and 2′-(2,4-dinitrophenoxy)-5′-AMP can be separated in several chromatographic systems (Fig. 12). On a reversed-phase column a high pH is necessary in order to obtain full expression of the charge differences between phosphate diesters and phosphate monoesters. Neither the polarity of the buffer ions nor their concentration is decisive. The methanol concentration was varied in order to achieve sufficient resolution and minimal analysis times (Fig. 12A). On an anion exchanger the reversed-phase properties should be removed by the addition of a moderate amount of acetonitrile, because both compounds have the very lipophilic dinitrophenoxy moiety in common. Neither pH nor the polarity of the buffer ions is decisive. The ion concentration was varied in order to obtain an optimal separation (Fig. 12B). On a cation exchanger a high concentration of acetonitrile is required in order to introduce normal-phase chromatography. Neither pH, the polarity of the buffer ions, nor ion concentration is decisive. The acetonitrile concentration was varied in order to achieve separation (Fig. 11C). On this cation exchanger there is still another range of parameters with which separation of these negative solutes is possible. Both solutes have a very hydrophobic dinitrophenoxy moiety, but they have a different polarity at the phosphorus moiety. Reversed-phase properties in a cation exchanger can produce selectivity for these solutes. Only acetonitrile is an important

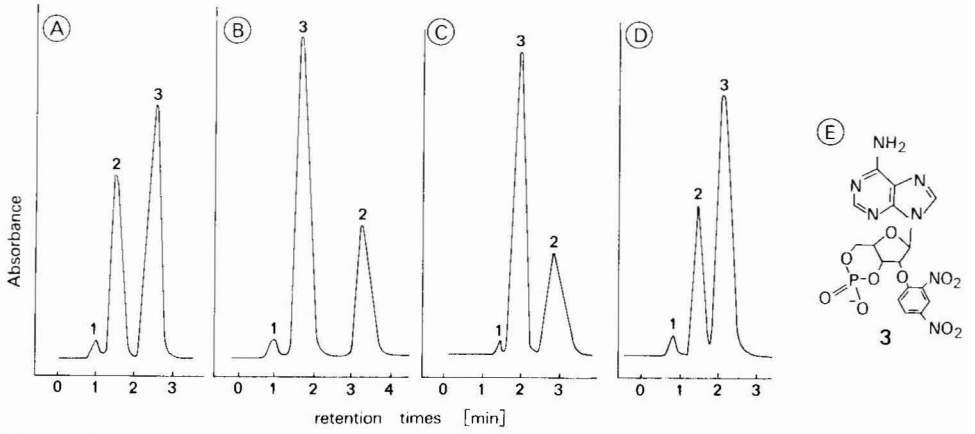


Fig. 12. Separation of 2′-(2,4-dinitrophenoxy)-cAMP and 2′-(2,4-dinitrophenoxy)-5′-AMP in different chromatographic systems. (A) Reversed-phase column (RP-8, Riedel-de Haën, 300 × 3 mm I.D., self-packed) in 15 m*M* Na_2HPO_4/H_3PO_4–46% methanol (pH 6.6); flow-rate, 2 ml/min. (B) Anion exchanger (Partisil-10 SAX) in 25 m*M* triethylammonium formate–20% acetonitrile (pH 3.0); flow-rate, 3 ml/min. (C) Cation exchanger (Partisil-10 SCX) in 2 m*M* triethylammonium formate–73% acetonitrile (pH 3.0); flow-rate, 3 ml/min. (D) Cation exchanger (Partisil-10 SCX) in 2 m*M* triethylammonium formate–1.5% acetonitrile (pH 3.0); flow-rate, 3 ml/min. (E) Structure of 2′-(2,4-dinitrophenoxy)-cAMP. Solutes: 1 = impurity, probably 2,4-dinitrophenol; 2 = 2′-(2,4-dinitrophenoxy)-5′-AMP; 3 = 2′-(2,4-dinitrophenoxy)-cAMP.

parameter for reducing retention times. The other parameters will hardly have any effect (Fig. 11D). Notice the order of elution in these four separations.

For the study of the degradation of cyclic nucleotide derivatives in a liver homogenate[43] a separation system was required for the simultaneous analysis of nucleotides, nucleobases and nucleosides. It would be practical if chromatography is relatively independent of derivatization of the cAMP molecule. The nucleotides can be separated on the basis of the phosphate moiety and the nucleosides and nucleobases according to their charge and polarity. Therefore, a mixture of cation-exchange and normal-phase chromatography was chosen. The pH and polarity of the buffer ions are relatively unimportant. The retention times of the nucleotides were varied with acetonitrile (normal-phase chromatography) and the retention times of the nucleobases and nucleosides were varied with the ion concentration (cation-exchange chromatography). A chromatogram is shown in Fig. 13.

S-Adenosyl-L-methionine (Boehringer; Fig. 14) was chromatographed on another Partisil-10 SCX column that differed from that described above by the necessity for a 100-fold higher ion-concentration in order to obtain the same retention times in cation-exchange interactions. The S-adenosyl-L-methionine peak kept a constant column capacity ratio of 0.8 if the ammonium acetate concentration was above 1.5 *M*. At this high ion concentration the peak could be shifted to the dead volume by adding propanol, again indicating reversed-phase properties. By decreasing the ion concentration in the presence of propanol the S-adenosyl-L-methionine peak was shifted to higher column capacity ratios, indicating cation-exchange interactions.

Only if reversed-phase and cation-exchange properties are present simultaneously do two S-adenosyl-L-methionine peaks appear. The peaks are interconver-

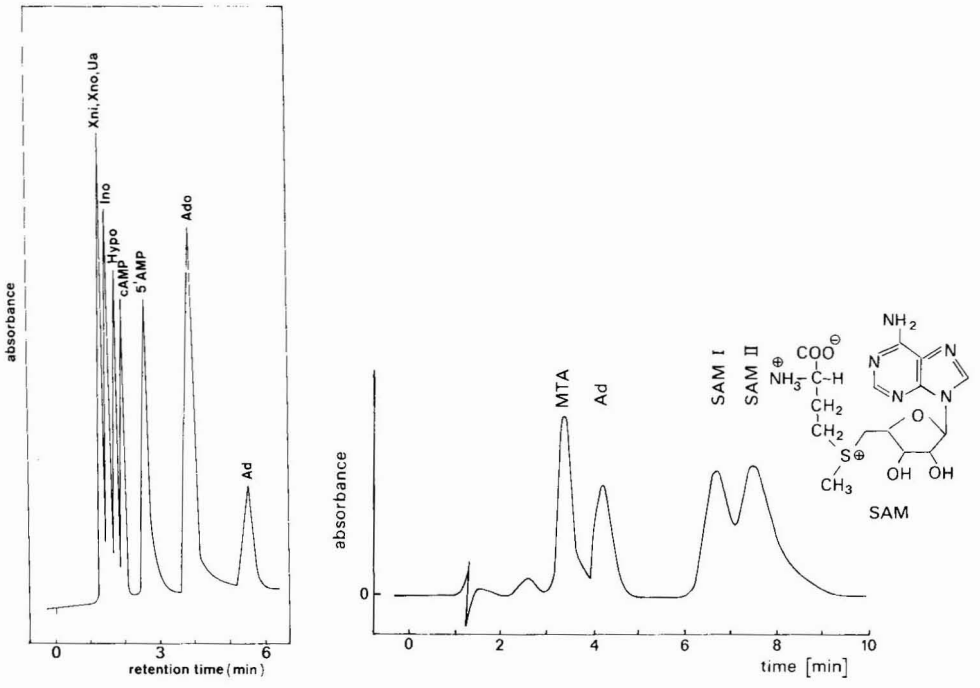


Fig. 13. Separation of cyclic AMP and its enzymatic degradation products by mixed chromatography. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 3 m*M* triethylammonium formate–67% acetonitrile (pH 3.0); flow-rate, 3 ml/min. Solutes: Xni = xanthine; Xno = xanthosine; Ua = uric acid; Ino = inosine; Hypo = hypoxanthine; cAMP = adenosine-3′,5′-monophosphate; Ado = adenosine; Ad = adenine.

Fig. 14. Separation of two stereoisomers of S-adenosyl-L-methionine by mixed chromatography. Stationary phase: Partisil-10 SCX cation exchanger. Mobile phase: 1.1 *M* ammonium acetate (pH 5.6); flow-rate, 2 ml/min. Sample: commercial S-adenosyl-L-methionine was boiled for 5 min at pH 1 in 0.1 *M* HCl, which resulted in racemization and partial degradation. Peaks: MTA = 5′-deoxy-5′-methylthioadenosine; Ad = adenine; SAM I = (−)-S-adenosyl-L-methionine; SAM II = (+)-S-adenosyl-L-methionine.

tible on heating and are equally sensitive to alkaline pH. Most probably these two peaks are the two stereoisomers of S-adenosyl-L-methionine at the sulphonium atom (Fig. 14).

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REACTIVE POLYMERS

XL*. STUDY OF THE INTERNAL STRUCTURE OF THERMALLY TREATED POLYMERIC SORBENTS BASED ON GLYCIDYL METHACRYLATE COPOLYMERS

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SUMMARY

A decrease in the specific pore volume and specific surface area and an overall narrowing of the pore distribution are the main changes in the internal structure of thermally treated copolymers based on glycidyl methacrylate–ethylene dimethacrylate. They are also closely connected with changes in the chromatographic properties of polymeric sorbents thus treated. The decrease in the pore volume may be directly correlated with the decrease in the retention volumes.

INTRODUCTION

The chromatographic properties of macroporous polymeric sorbents are predominantly determined by their internal structure and chemical composition. The specific surface area and the specific pore volume or average pore radius are the characteristics most frequently used in the description of the internal structure of polymeric sorbents. In most instances, these data are obtained by the dynamic nitrogen desorption method[1–3]. Some further valuable findings may be provided by electron microscopy[4–6], which can show the globular order of microparticles and of their sizes. In addition, mercury porosimetry[7,8] provides important information on the pore distribution at pore radii over 4 nm.

In a previous paper[9] we described changes in the chromatographic properties of copolymers based on glycidyl methacrylate–ethylene dimethacrylate (GMA–EDMA) which had been exposed to brief heating, due to which same destruction had already taken place.

In this study we concentrated our attention on the internal structure of such thermally treated copolymers by combining mercury porosimetry and the sorption method based on capillary condensation of nitrogen. The data obtained were correlated with chromatographic results.

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EXPERIMENTAL

The thermal treatment of copolymers and conditions for the gas chromatographic measurement of the retention of sorbates have already been described[9].

The specific pore volume was determined by the method of capillary condensation of nitrogen containing less than 1 % of hydrogen using a Quantasorb apparatus (Quantachrome, Greenvale, NY, U.S.A.).

Porosimetric measurements

Prior to measurements, the copolymer samples were dried at room temperature and 0.6 kPa for 15 h. After being transferred into the measuring dilatometer, they were evacuated at 6 Pa for 1 h, then the dilatometer was filled with mercury at the same pressure. A Model 225 porosimeter (Carlo Erba, Milan, Italy) was used with a pressure range of $0–2 \cdot 10^5$ kPa, and an IBM 1370-135 computer was employed to process the results.

RESULTS AND DISCUSSION

The results of porosimetric measurements on thermally treated macroporous GMA–EDMA copolymers and the pore volumes determined by the method of capillary condensation of nitrogen are given in Table I. Differences in the V_p values obtained by the individual methods are due to the fact that the sorption method is suitable for pore radii between 1.5 and 30 nm, while mercury porosimetry records pores of radius 4 nm and greater. In both instances the cylindrical pore structure model was used. The V_p values obtained by mercury porosimetry are closer to reality, whereas the results of the sorption method detect the participation of micropores in the internal structure.

TABLE I

POROUS STRUCTURES OF INITIAL AND THERMALLY TREATED SAMPLES OF GLYCIDYL METHACRYLATE (GMA)–ETHYLENE DIMETHACRYLATE (EDMA) COPOLYMERS

Copolymer No.	*Modification conditions*		*Mercury porosimetry**			*Capillary condensation of N_2*: V_p (cm^3/g)*
	Temperature (°C)	*Time (h)*	*V_p (cm^3/g)*	*S_{Hg} (m^2/g)*	*$d/2$ (nm)*	
1**	–	–	0.89	144	4–230	0.29
2	250	1	0.80	135	4–190	0.29
3	250	2	0.78	127	4–130	0.23
4	250	4	0.70	113	4–130	0.21
5	260	2	0.74	120	4–110	0.18
6	280	1	0.52	79	4–110	0.19
7***	–	–	1.22	194	4–200	0.31
8	250	1	0.76	99	4–120	0.21
9	250	2	0.63	64	5–120	0.18
10	260	2	0.50	58	5–80	0.09

* V_p = specific pore volume; S_{Hg} = specific surface area; $d/2$ = pore distribution.
** GMA:EDMA ratio in polymerization mixture = 45:55 (w/w).
*** GMA:EDMA ratio in polymerization mixture = 60:40 (w/w).

The results show that in both starting copolymers (samples 1 and 7) the thermal treatment leads to a decrease in the total pore volume. In agreement with earlier findings[9], this decrease is more pronounced for the copolymer with the higher GMA content. If, for example, an identical depolymerization mechanism is assumed for both incorporated monomeric units, then the release of one GMA molecule is at least twice as likely as the release of the cross-linking agent that has reacted on both sides. This is why the copolymer richer in GMA is degraded more quickly. The specific surface area (Table I) were obtained by calculation from porosimetric measurements. Even though their accuracy is lower than that of data obtained by the sorption method[9], it is obvious that the decrease in the specific surface area of thermally treated copolymers is in agreement with the decrease in the specific pore volume.

Morphological changes caused by the thermal treatment have the character of a sintering process and are reflected in the overall pore distribution. As demonstrated by Table I and Figs. 1 and 2, the upper boundary of the pore size decreases. This is due to the decrease in the particle volume caused by heating. Of the original 150–180 μm fraction, 30–50 % of the particles have a diameter smaller than 150 μm, depending on the conditions of treatment[10]. It may be assumed that heating leads to sintering, and hence to the disappearance of micropores. This is suggested by the decrease in the V_p values measured by the sorption method and by the initial shape of the porosimetric curves of some of the thermally treated copolymers (predominantly in samples with a higher GMA content). Hence in the sintering process there is an overall narrowing of the pore distribution, which is a useful effect for the chromatographic sorbent. The results show that this narrowing of the pore distribution is affected by the sintering temperature rather than by the duration of thermal treatment (Figs. 1 and 2).

Table II demonstrates the main advantage of thermally treated GMA copolymers used as chromatographic sorbents, namely a reduction in retention times compared with the original sorbent. This, in turn, makes possible acceleration of the analysis or reduction of the working temperatures during the analysis. It can also be seen that the reduction in the retentions of non-polar hydrocarbons is greater than that of polar sorbates, in accordance with an earlier finding[9] that the polarity of thermally treated GMA copolymers is higher than that of the original copolymers.

Correlation between the porosimetric and chromatographic data (Figs. 3 and 4) shows that there is a linear dependence between the decrease in the total pore volume of thermally treated copolymers and the reduction in the retentions on these samples. It should be pointed out, however, that deviations appear in the slopes of the straight lines obtained for copolymers with various contents of GMA. Under the same conditions of thermal treatment, the decrease in the pore volume of copolymer 1 is smaller (Fig. 3) than that of copolymer 7 (Fig. 4). The relatively larger decrease in the retentions of sorbates on sorbents derived from copolymer 1 may be explained through the fact that the internal structure of this less polar sorbent affects the retentions of compounds much more strongly. Hence, the decrease in the pore volume is also more markedly reflected in the retentions of both polar and non-polar sorbents. This is also indicated by the almost identical slopes of the straight lines for both hydrocarbons and polar sorbates (Fig. 3). In contrast, with the thermally treated and more polar copolymer 7, its chemical composition plays a more important role in the retention, which is also indicated by different slopes of the dependences for hydro-

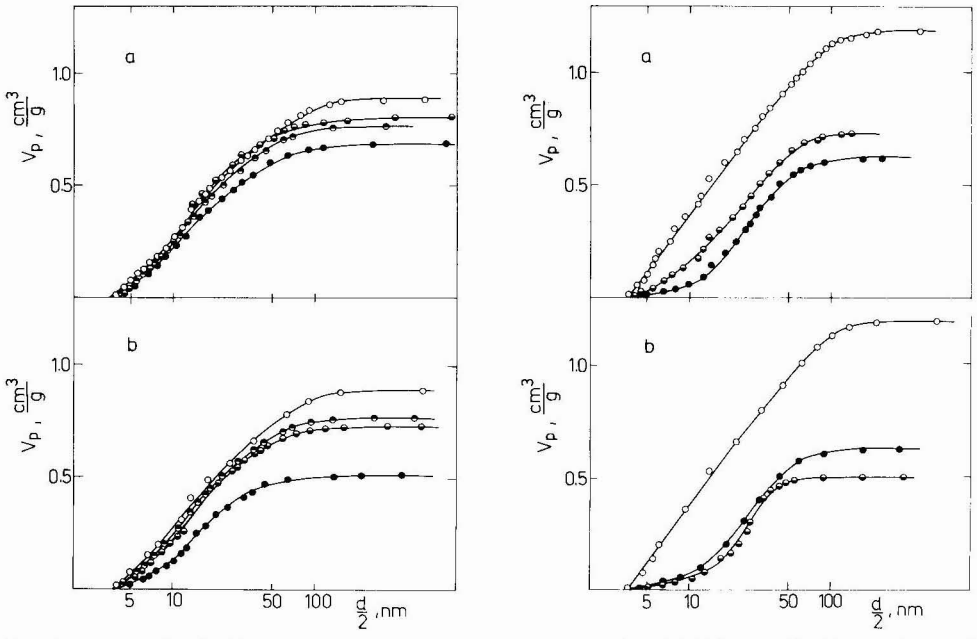


Fig. 1. Integral distribution curves of pore sizes of copolymers 1–6. (a) Effect of the time of thermal treatment: ○, copolymer 1 (original); ◒, copolymer 2 (250°C, 1 h); ◓, copolymer 3 (250°C, 2 h); ●, copolymer 4 (250°C, 4 h). (b) Effect of temperature: ○, copolymer 1 (original); ◓, copolymer 3 (250°C, 2 h); ◒, copolymer 5 (260°C, 2 h); ●, copolymer 6 (280°C, 1 h).

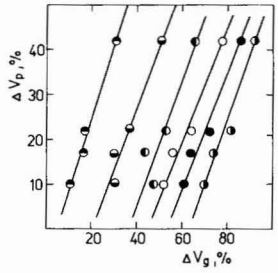
Fig. 2. Integral distribution curves of pore sizes of copolymers 7–10 (a) Effect of the time of thermal treatment: ○, copolymer 7 (original); , copolymer 8 (250°C, 1 h); ●, copolymer 9 (250°C, 2 h). (b) Effect of temperature: ○, copolymer 7 (original); ●, copolymer 9 (250°C, 2 h); ◓, copolymer 10 (260°C, 2 h).

TABLE II

RELATIVE RETENTION VOLUMES OF SORBENTS ON THERMALLY TREATED SAMPLES OF COPOLYMERS RELATED TO THEIR RETENTION VOLUMES ON THE INITIAL COPOLYMER

Values determined at 150°C.

Sorbate	*Copolymer No.*									
	1	2	3	4	5	6	7	8	9	10
Pentane	1	0.48	0.32	0.35	0.44	0.22	1	0.58	0.50	0.38
Hexane	1	0.44	0.28	0.30	0.40	0.17	1	0.59	0.52	0.42
Heptane	1	0.39	0.24	0.27	0.36	0.14	1	0.55	0.47	0.41
Octane	1	0.34	0.19	0.20	0.32	0.10	1	0.50	0.43	0.37
Nonane	1	0.30	0.14	0.16	0.26	0.08	1	0.46	0.38	0.33
Benzene	1	0.52	0.44	0.47	0.56	0.34	1	0.78	0.73	0.70
2-Butanone	1	0.53	0.47	0.50	0.56	0.36	1	0.81	0.73	0.70
Nitromethane	1	0.89	0.75	0.83	0.82	0.69	1	0.99	0.97	0.96
Methanol	1	0.39	0.53	0.73	1.25	0.51	1	1.09	1.08	1.12
Ethanol	1	0.78	0.71	0.76	0.82	0.64	1	0.98	0.95	0.96
Propanol	1	0.69	0.62	0.63	0.70	0.49	1	0.89	0.85	0.83
Butanol	1	0.58	0.50	0.51	0.61	0.37	1	0.80	0.74	0.70



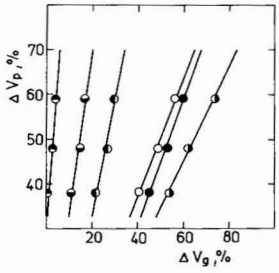


Fig. 3. Effect of decrease in the specific pore volume of samples obtained by the thermal treatment of copolymer 1 on the reduction of the retention volumes of sorbates; ○, pentane; ●, heptane; ◑, nonane; ◐, benzene; ◒, propanol; ◓, nitromethane.

Fig. 4. Effect of decrease in the specific pore volume of samples obtained by the treatment of copolymer 7 on the reduction of the retention volumes of sorbates. Sorbates as in Fig. 3.

carbons and polar sorbates (Fig. 4). Similarly, the sintering effect affects the reduction of the retentions of non-polar compounds compared with the polar compounds.

The correlation between the chromatographic and porosimetric data indicates close relationships between changes in the internal structure and changes in the chromatographic properties of modified sorbents. The conclusions drawn from this study suggest further trends in controlling the structure of polymeric sorbents that possess optimal properties for practical chromatographic analyses.

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CHROM. 13,697

RECOVERY OF VAPOURS FROM SOLID ADSORBENTS*

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SUMMARY

Losses due to polymerization of vapours collected from the atmosphere using a solid adsorbent (activated carbon), that catalyses this polymerization were examined. The losses of styrene vapour sampled on activated carbon that occur when elution is delayed were studied. Tests performed using an adsorbent treated with an inhibitor of the polymerization reaction (catechol), which is effective only when the granules remain in contact with the eluate, are described.

INTRODUCTION

For the efficient utilization of solid adsorbents for the sampling of organic vapours, several factors must be carefully controlled[1], in order to avoid possibilities of error.

When using a fixed adsorbent bed within which air containing the vapours flows, several parameters affecting the collection efficiency must be borne in mind, including the adsorption capacity, the particle size, the packing of the adsorbent, the size of the spaces between the particles with respect to the mean free path of the molecules, which determines whether viscous or molecular flow predominates, and the contact time between gas and solid[2,3].

Other errors may occur in the vapour phase. The tenacity with which the surface of the solid retains the vapour molecules varies greatly, depending on the characteristics of the different molecules (*e.g.*, polarizability) and on the fraction of the adsorbent covered, so that the recovery is never completely quantitative. The error is smaller with near-saturation of the retention capacity of the adsorbent for the vapours being considered, because the fraction retained (not desorbed) is negligible.

* This research was carried out as part of the project "Patologia da ambiente di Lavoro" of the Consiglio Nationale delle Ricerche.

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These are factors of a general nature, although their incidence is different in the various instances and they require a careful preliminary calibration. However, there can also be specific causes, linked to particular properties of a substance, that can have a pronounced effect on the recovery process, such as transformations undergone by the vapours on the adsorbent.

The possibility that some molecules may be polymerized or oxidized, favoured in these processes by the adsorbent, is of practical importance. This may occur particularly with activated carbon, with a consequent loss of sampled vapours.

The tests described in this paper were carried out in order to study this important practical aspect of vapour sampling on a bed of activated granular carbon.

EXPERIMENTAL

Materials

Activated carbon of surface area 1400 m^2/g and particle size 15–30 mesh (some tests were performed using a particle size between 30 and 100 mesh) was used.

Styrene vapour was obtained by heating polystyrene at 600°C.

Procedure

For sampling styrene vapour, the apparatus shown in Fig. 1 was used. It consisted of a cylindrical copper reactor with capillary holes at the base, a copper tube connected with the outside which, after immersion in melting ice, was divided into two parallel lines, each consisting of a small tube containing 1 g of granular carbon, a flow meter and a suction pump.

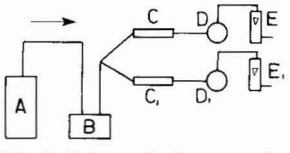

Fig. 1. Schematic diagram of apparatus. A = Reactor; B = refrigerator; C, C_1 = adsorbent beds; D, D_1 = suction pumps; E, E_1 = flow meters.

A 200-mg amount of expanded polystyrene was placed in the reactor and inserted in a muffle furnace at 600°C. The vapour produced was aspirated by the pump at a flow-rate of 0.5 l/min in equal proportions into the two small tubes containing carbon, one of which served as a reference standard (a reference standard had to be used for each test because the composition of the products resulting from the thermal degradation of polystyrene is influenced by the secondary reactions, which are affected by various factors, such as the time the vapour remains in the hottest zone and the thickness and compactness of the sample). The volatile products of pyrolysis of polystyrene in an inert atmosphere are monomer (44%), dimer (22%), trimer (27%), toluene and carbon monoxide[4]. The products vary, depending on the operating conditions, particularly in the presence of oxygen. At 400°C the degradation should be complete; at higher temperatures toluene, styrene, benzene and ethylene are produced. Between 400 and 700°C the highest percentage of styrene is obtained[5].

After the reaction, the standard tube was immediately eluted with carbon

dioxide (5 ml), whereas the other tube was eluted, using the same amount of solvent, after a time t which varied from test to test. The elution was effected by immersing the adsorbent in the solvent for a fixed period (30 min), then the carbon granules were removed by filtration. This method was preferred to percolation because it is more practical and easier to repeat.

The amount of styrene present in the eluate was determined by means of a DANI Model 3400 gas chromatograph using a 2 m × 1/4 in I.D. column packed with 3% SE-30 on Chromosorb and a flame-ionization detector. The number of theoretical plates was 3500 at 60°C, calculated on the styrene, with a retention time of 5.3 min; the carrier gas (nitrogen) flow-rate was 6 cm/sec. The calculations were carried out based on the toluene peak as internal standard, which is always present (t_R = 2.5 min) and which is not subject to variation with time.

RESULTS AND DISCUSSION

The results obtained are presented in Fig. 2.

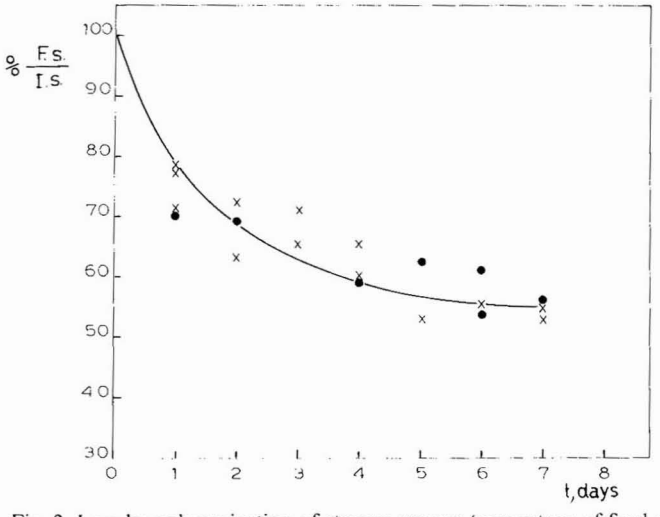

Fig. 2. Loss by polymerization of styrene vapour (percentage of final styrene, F.s., with respect to initial styrene, I.s.) with time. Adsorbent carbon size: ×, 15–30 mesh; ●, 30–100 mesh.

The reduction in the amount of styrene produced on delaying the time when the elution is carried out is fairly regular. The variations in the results, even though the tests were performed in a strictly standardized way, are inevitable because of the many factors that may affect the different tests.

The recovery losses are between 15% and 32% with elution after 24 h, and between 20% and 43% with elution after 48 h. These losses refer to an adsorbent bed of 1 g of carbon with an average total load of 50 μg of styrene, for tubes kept without special precautions with regard to light and at a temperature between 20 and 25°C.

Additional tests consisted of ascertaining the persistence of the vapour loss due to polymerization in the presence of carbon under different conditions, and of attempts to overcome this drawback by means of an inhibitor of the reaction.

Confirmation of the loss of styrene due to the catalysing effect of carbon on its polymerization was obtained by eluting the two tubes simultaneously, but leaving the eluate in one of them in the presence of carbon for various periods. The losses that occur in the eluate that is in the presence of carbon (compared with the control eluate) are of the same order as those which occur when the elution is delayed (Fig. 3).

To prevent the losses due to polymerization of the vapour, catechol was used as an inhibitor of the reaction, both in the liquid phase and in the vapour phase.

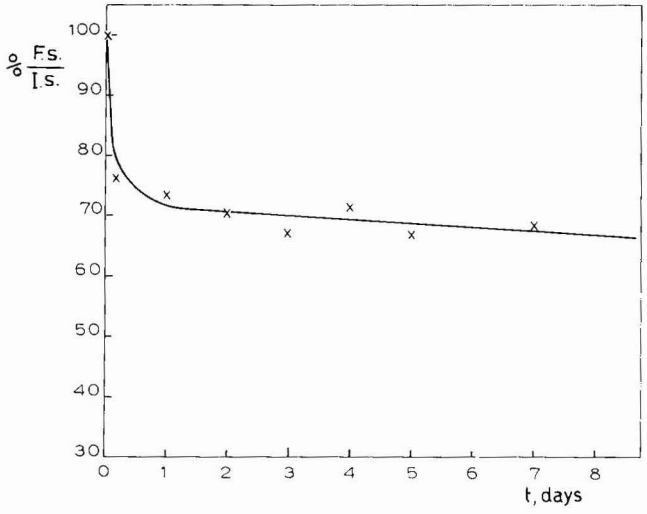

Fig. 3. Loss by polymerization of styrene in solution with time.

Liquid phase: solution of catechol in methanol

The granules of the adsorbent were treated with the solution; after evaporation of the solvent, the tubes were prepared and the tests described were performed. The amounts by weight of catechol, relative to the carbon, were 1:200 and 1:5.

With delayed elution there is in any case a reduction of the styrene produced comparable to that which occurs with the adsorbent untreated with the inhibitor. The behaviour is different if the carbon granules treated with catechol are left in the eluate; in this instance the amount of styrene produced remains constant with time.

Gaseous phase

Passing the catechol vapour over the carbon granules, both before and after adsorption of the styrene, does not lead to a suppression or decrease of the polymerization with time. On the contrary, the catechol vapour inhibits the reaction only when they pass through the adsorbent tube simultaneously with the styrene vapour, *i.e.*, during the sampling. In this instance there is a suppression of the polymerization and the amounts of styrene in the eluate remain constant, even if the elution is carried out after an interval of several days.

The behaviour of catechol can be explained as follows. When the catechol molecules are adsorbed on the surface of the adsorbent, they cannot perform their

inhibiting action because of their lack of mobility. The inhibition occurs when the catechol vapour is mixed with the styrene vapour before entering the tube and also when, on elution, the catechol regains its mobility in the eluate.

CONCLUSIONS

The tests performed with styrene show how the transformations undergone by the sampled vapour as a result of the action of the adsorbent can affect the final results. These results for styrene must be extended to all substances, *e.g.*, vinyl chloride and formaldehyde, that may also under transformation under the influence of carbon.

On the basis of the various tests, it is possible to conclude that prior treatment of the adsorbent with an inhibitor may prevent the loss with the monomer vapour sampled. In the specific case examined it was established that, if carbon granules previously treated with catechol (in solution or in the gaseous state) are left in the eluate, losses due to the catalytic action of carbon are prevented. In this way the analysis can be performed after an interval of time, with the certainty that the results obtained will not be affected by errors due to losses caused by the adsorbent.

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CHROM. 13,677

APPLICATIONS OF IMMOBILISED PHENYLBORONIC ACIDS AS SUPPORTS FOR GROUP-SPECIFIC LIGANDS IN THE AFFINITY CHROMATOGRAPHY OF ENZYMES

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(First received December 8th, 1980; revised manuscript received January 26th, 1981)

SUMMARY

Several different aminophenylboronic acid (APBA)–agarose matrices have been compared.

Nucleotides are able to bind to these matrices in the presence of either NaCl or $MgCl_2$. NAD^+ and FAD are more tightly bound to these columns when compared with other nucleotides.

The properties of NAD^+ and $NADP^+$ complexes with immobilised phenylboronic acids have been compared using pure 6-phosphogluconate and alcohol dehydrogenases. The $NADP^+$-dependent enzyme bound to the $NADP^+$ complex. Conversely the NAD^+-dependent enzyme was only retarded by the NAD^+-immobilised APBA complex.

Glucose-6-phosphate dehydrogenase from yeast has been partially purified on $NADP^+$ complexed to immobilized boronic acid–agarose columns.

The desorption of bound enzymes to nucleotide–phenyl boronate columns can be effected by salt gradients, the addition of diols; monosaccharides, sorbitol, cofactor competition, by lowering the pH of the eluent or by specific retardation.

Purification factors of 14-fold were achieved for yeast glucose-6-phosphate dehydrogenase by choosing the appropriate concentration of presaturating $NADP^+$. The enzyme was selectively retarded and did not require specific elution.

Similar experiments enabled yeast hexokinase to be purified by presaturation of APBA-agarose with ATP. Elution of the enzyme was dependent upon the concentration of ATP used to presaturate the column. The enzyme was simply retarded by the column and required no (specific) eluent.

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INTRODUCTION

The ability of borates to form complexes with alcohols and particularly vicinal diols has been known for some time[1,2]. Biologically useful applications of the latter property using immobilised phenylboronate to selectively interact with diols were developed by Gilham and co-workers[3,4]. Thus, tRNA and tRNA-isoacceptors have been purified from yeast[5]. Other separations of ribonucleotides and ribonucleotide (ADPR)–protein conjugates have also been described[6–8]. Smaller molecules, phenolates[9], catecholamines[10], and sugars[8] have been separated by a variety of phenylboronates immobilised to polyacrylamide, Sephadex and polystyrene.

Immobilised phenylboronates have also found interesting applications in the enzymic assays of adenylate cyclase[11], ribonucleotide triphosphate reductase[12] and a phosphodiesterase[13].

Other applications of immobilised phenylboronic acids utilise a different property of boronic acids, namely their ability to inhibit proteases. Thus, the purification of proteases from *Bacillus subtilis* has been reported[14].

Three variables are particularly important for the design of successful affinity chromatographic separations. These are matrix, spacer arm, and ligand.

(i) Matrix: in the application of immobilised boronates, unlike many affinity systems, the ligands have been mainly based on supports other than agarose. It could be argued that cellulose and other glucose-based polysaccharides would be intrinsically unsuitable because of potential interactions between the ligand (boronate) and the matrix. This feature of the latter polymers is likely to be less pronounced for agarose which has fewer available hydroxyl groups, none of which have the 1,2-*cis*-diol configuration.

(ii) Spacers: we have examined the properties of agarose-immobilised phenylboronates with hydrophobic arms because of the known importance of hydrophobic "spacer arms" to enhance some low affinity interactions[15].

(iii) Ligand: the ligand used in previous work has been phenylboronate (PBA), most frequently as the derivative 3-aminophenylboronate (Fig. 1a). One of the purposes of this investigation was to examine different methods of immobilising aminophenylboronic acid to agarose and to select from these the most suitable adsorbent for nucleotide-based affinity separations. Thus, we hoped to be able to overcome undesirable features of immobilised nucleotides which include (a) the need for complex synthesis of derivatives suitable for immobilisation; (b) the instability of many described covalently attached nucleotides and the resulting limited life of such columns; (c) the cost; (d) the need to carry out separate immobilisation reactions for each nucleotide derivative.

We present data which suggest that phenylboronate–agarose adsorbents can retard nucleotides sufficiently to permit chromatography of dehydrogenases and kinases from yeast extracts.

EXPERIMENTAL

Materials

Enzymes, their substrates and nucleotides were purchased from Boehringer (Mannheim, G.F.R.). 3-Aminophenylboronic acid (APBA), 1,4-butanediol-digly-

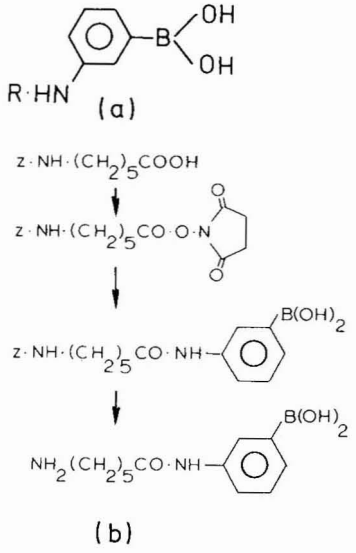

Fig. 1. The structure of phenylboronic acid (a) and synthesis of 6-aminocaproyl-3-aminophenylboronic acid (b).

cidyl ether, and diphenylcarbazone were obtained from Aldrich (Milwaukee, WI, U.S.A.). Cyanogen bromide and yeast enzyme concentrate were obtained from Sigma (St. Louis, MO, U.S.A.). Hydrogen bromide and 6-aminocaproic acid were purchased from BDH (Poole, Great Britain). Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Matrex Gel phenylboronate was obtained from S. Fulton (Amicon, Lexington, MA, U.S.A.). (Ligand concentration was 41 μmoles/ml).

Methods

Thin-layer chromatography (TLC) was performed on Kieselgel GF 254-coated plates (Merck, Darmstadt, G.F.R.). The spots of boron-containing compounds were visualized by spraying with a 0.2% (w/v) solution of diphenylcarbazone in methanol[16]. A 0.2% (w/v) ninhydrin solution in acetone was used for detecting primary amino groups. Nuclear magnetic resonance (NMR) spectra were recorded at 220 MHz on a Perkin-Elmer R-34 instrument.

Measurements of retention volumes. (Least volume before nucleotide could be detected). Each nucleotide (1 mg) was dissolved in 50 m*M* Hepes buffer, pH 8.45 (0.2 ml) containing 0.1 *M* $MgCl_2$ and applied to a column of immobilized APBA (1 ml) attached to CNBr-activated Sepharose 6B (ligand concentration, 8 mg APBA/ml wet weight). The temperature of the column was 4°C throughout. Flow-rates were maintained at 1.0 ml/h using a peristaltic pump (LKB Varioperpex). Fractions (0.5 ml) were collected and monitored for O.D. at 260 nm.

Immobilisation methods. (a) Cyanogen bromide activation: Sepharose 6B (25 g moist weight) was activated with cyanogen bromide (2 g) according to the method of Axen *et al.*[17]. The gel was washed with ice cold 0.1 *M* $NaHCO_3$, pH 9.5 and added to a solution of APBA (500 mg) or aminocaproyl aminophenyl boronic acid (ACA-PBA, 500 mg) in 0.1 *M* $NaHCO_3$, pH 9.3 (20 ml), the suspension was rotated over-

night on a Coulter mixer at 4°C. The matrix was washed with 0.1 *M* $NaHCO_3$. The ligand concentration was estimated spectrophotometrically by subtracting the amount of unbound ligand from the total added initially.

(b) Epoxy activation: The method of Porath[18] was used to couple APBA to Sepharose 6B. The ligand concentration of the matrix was estimated spectrophotometrically by subtracting the amount of unbound APBA from the total amount added.

Synthesis of 6-aminocaproyl-3-aminophenylboronic acid (see Fig. 1b).

(a) Synthesis of benzyloxycarbonyl 6-aminocaproic acid (Z-ACA): 6-Aminocaproic acid (10 mmoles) was dissolved in 4 *M* NaOH (50 ml). Benzyloxycarbonylchloride (20 mmoles) was added with cooling and shaking over a period of 20 min. During the latter period, additional 4 *M* NaOH (50 ml) was added. The solution was extracted with diethyl ether (3 × 100 ml). The aqueous phase was acidified with HCl at 0°C, and extracted with ethyl acetate (3 × 100 ml). The ethyl acetate extracts were combined, dried (Na_2SO_4) and evaporated to dryness. The resulting solid was recrystallised from chloroform m.p. 54–56°C (Lit.[19] m.p. 54°C) yield 85%. TLC showed one spot (R_F, 0.41, ethyl acetate; 0.66, ethanol). NMR [d_6-dimethyl sulphoxide (DMSO)]: δ 1.38 (complex multiplet, 1.2 to 1.6, 3 × CH_2): 2.2 (t, J = 6 Hz, CH_2): 3.0 (q, J = 6 Hz, CH_2): 5.20 (s, $ArCH_2$): 7.23 (complex m, NH): 7.36 (s = 5ArH): 9.8 (s, CO_2H) ^2H_2O exchangeable.

(b) Synthesis of benzyloxycarbonyl 6-aminocaproyl-N-hydroxysuccinimide ester (Z-ACA-NHS): Dicyclohexylcarbodiimide (5.5 mmoles) was added to a solution of Z-ACA (5 mmoles) plus N-hydroxysuccinimide (5.5 mmoles) in dioxan (25 ml) with cooling. The reaction was left for 10 h at 12°C. The white precipitate which formed (dicyclohexylurea) was filtered off and the remaining solution evaporated to give an oil. The oil was washed with water followed by 1 *M* HCl and was used for the next step without further purification.

(c) Synthesis of benzyloxycarbonyl-6-aminocaproyl-3-aminophenylboronic acid (Z-ACA-APBA). To a solution of Z-ACA-NHS (5 mmoles) in ethanol (10 ml) under nitrogen was added APBA (5 mmoles) in water (10 ml) containing $NaHCO_3$ (10 mmoles). The mixture was left for 10 h at room temperature. The precipitate which formed was filtered off, washed with water and recrystallized from ethyl acetate to give Z-ACA-APBA as plates m.p. 172–174°C (yield, 20–40%).

TLC revealed a single UV-absorbing spot (R_F, 0.32, (ethyl acetate); 0.76 (ethanol); 0.84 (ethanol–water, 1:1) (APBA gave R_F, 0.26 (ethyl acetate)). NMR (d_6-DMSO): δ 1.4 (complex multiplet, 1.2 to 1.7, 3 × CH_2): 2.25 (t, J = 6 Hz, CH_2): 2.97 (q, J = 6 Hz, CH_2): 4.97 (s, $ArCH_2$): 7.3 (s, 5ArH): 7.17–7.93 (complex multiplet, H). APBA gave (^2H_2O): 7.07 (complex multiplet): 7.28 (d, J = 2 Hz): 7.34 (d, J = 2 Hz) all aromatic H atoms. λ_{max} (dioxan) = 253, 284 (shoulder).

(d) Synthesis of 6-aminocaproyl-3-aminophenylboronic acid (ACA-APBA): Benzyloxycarbonyl-6-aminocaproyl-3-aminophenylboronic acid (150 mg) was added to 45% (v/v) HBr in acetic acid (4 ml) and acetic acid (4 ml). The mixture was left at room temperature for 40 min. Diethyl ether (100 ml) was added to precipitate the HBr salt of ACA-APBA as a white hygroscopic powder. The precipitate was further washed with diethyl ether (500 ml) and dried *in vacuo*. The product moved as a single spot on TLC (SiO_2) R_F, 0.18 (ethanol); (cellulose) 0.68, (ethanol), m.p. > 250°C (d). Yield, 80%. NMR (d_6-DMSO): significant peaks were δ 1.13 (t, J = 6 Hz, CH_2); 1.4 (complex multiplet, CH_2); 1.65 (complex multiplet, CH_2); 2.38 (broad t, J = 6 Hz,

CH_2); 2.85 (t, J = 6 Hz, CH_2); 7.62 (complex multiplet, 4 ArH); 7.92 (d, J = 6 Hz) and 9.88 (s, $B(OH)_2$) in the presence of ^2H_2O the peaks at δ 7.92 and 9.88 were removed by exchange. $\lambda_{max.}$ (0.1 *M* sodium phosphate buffer, pH 7.0), 242 nm.

RESULTS AND DISCUSSION

Of the eleven nucleotides tested for retardation by CNBr-immobilized APBA, only NAD^+, FAD and NADH seemed to bind tightly (Table I). The effect of varying the pH and salt concentration on the retention of nucleotides was examined (Table II). It can be seen from Table II that Hepes buffer, pH 8.45 improved the binding of FAD when compared with phosphate buffer (pH 7.0) but that either 1 *M* NaCl or 0.1 *M* $MgCl_2$ produced a further 6–10-fold increase in retention volume.

TABLE I

RELATIVE RETARDATION OF NUCLEOTIDES BY PBA-AGAROSE COLUMNS

Nucleotides (1 mg) were separately applied in 50 m*M* Hepes–NaOH buffer, pH 8.45 containing 0.1 *M* $MgCl_2$ to columns (1 ml) of PBA–Sepharose 6B (CNBr-activated); ligand concentration, 58 μmoles/ml (wet weight) at 0°C at a flow-rate of 1.0 ml/h. Retention volumes were measured as in Fig. 2.

Nucleotide	*Retention volume (× V_0)*	*Capacity (μmol/ml)*
NAD^+	>15	5–7
NADH	>15	5–7
$NADP^+$	9–10	5–7
FAD	>15	NT*
Adenosine	8	NT
AMP	6	NT
ADP	6.5	NT
ATP	5.5	4
GMP	5.5	NT
GDP	6	NT
GTP	5	NT

* NT = not tested.

TABLE II

THE EFFECT OF VARYING THE pH AND SALT CONCENTRATION ON THE INTERACTION OF FAD WITH PBA–AGAROSE COLUMNS

Columns containing PBA–Sepharose 6B (CNBr-activated, 1 ml) were equilibrated with the appropriate buffer before application of a pulse of 1 m*M* FAD (0.2 ml) at 4. Flow-rate 0.7 ml/h. Retention volumes were measured as in Fig. 2.

	Retention volume (× V_0)
0.1 *M* potassium phosphate, pH 7.0	2.5
0.1 *M* potassium phosphate + 1 *M* NaCl	4.6
50 m*M* Hepes, pH 8.4	4.0
50 m*M* Hepes + 1 *M* NaCl	26
50 m*M* Hepes + 0.1 *M* $MgCl_2$	25

These results indicate that nucleotides could be used as affinity ligands when complexed to immobilized phenylboronic acid–agarose provided that enzyme and enzyme-nucleotide interactions are stable to either 1 *M* NaCl or 0.1 *M* $MgCl_2$ and to pH values in excess of 8.0 and preferably 8.4.

The increased stability of the PBA–nucleotide interaction in the presence of 1 *M* NaCl or 0.1 *M* $MgCl_2$ has useful chromatographic applications. Thus, enzyme samples can be applied to a nucleotide–PBA–agarose complex at low salt concentrations (the complex being stabilised with $MgCl_2$). The bound enzyme may be desorbed using either 1 *M* NaCl or nucleotide (see below).

The interaction of $NADP^+$ with immobilised PBA was determined to some extent by the chemistry of the linkage with the column. Fig. 2 shows the differing abilities of PBA derivatives to bind $NADP^+$ at various applied $NADP^+$ concentrations. The retention volume was also affected by the amount of nucleotide applied to columns of immobilized phenylboronic acid (Fig. 2), decreasing in response to an increase in the amount of $NADP^+$ applied. This change of retention volume was clearly related to the capacity of the boronate ligand to bind the nucleotides. Thus the retention volumes of both epoxide and CNBr-immobilized PBA fell below ten column volumes when more than 2–3 mg of $NADP^+$ were applied. Columns with retention volumes in excess of ten column volumes were effective as affinity supports. Thus, we conclude from our experiments with CNBr-coupled aminocaproyl-3-aminophenylboronic acid that, at the ligand concentration used, this derivative of phenylboronic acid with $NADP^+$ cannot easily be used for affinity experiments.

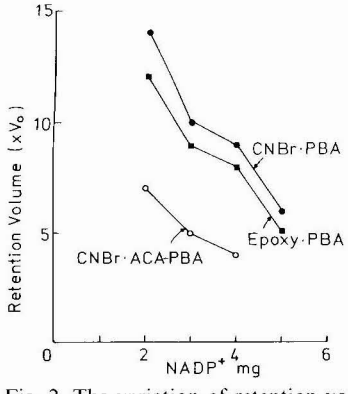

Fig. 2. The variation of retention volume with the concentration of applied $NADP^+$ for three types of immobilised PBA. Buffer was continuously applied until the applied nucleotide emerged. The retention volume is defined as the lowest volume after which the nucleotide was detected and is expressed as a multiple of the column volume. The ligands were prepared as described in Experimental. The ligand concentrations were 58 μmoles/ml (CNBr-PBA); 146 μmoles/ml (epoxy-PBA) and 34 μmoles/ml (CNBr-ACA-PBA). Temperature, buffers and flow-rate used were as quoted in Table II. $NADP^+$ was applied in 0.2 ml to each column (1 ml).

We have chromatographed purified enzymes on both NAD^+ and $NADP^+$-presaturated phenylboronic acid–agarose columns (Fig. 3). Neither yeast alcohol dehydrogenase (ADH) nor 6-phosphogluconate dehydrogenase (6-PGDH) were able to bind tightly to the NAD^+-phenylboronic acid complexes, whereas the $NADP^+$-specific yeast 6-PGDH bound tightly to the $NADP^+$–PBA columns.

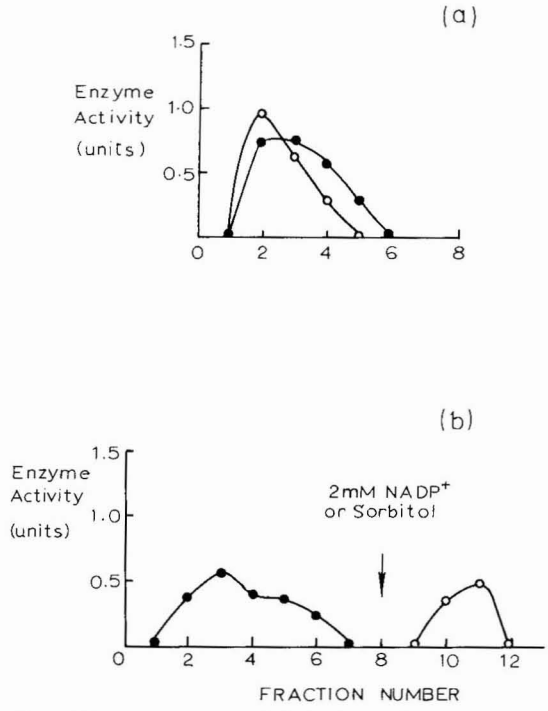

Fig. 3. The chromatographic behaviour of purified yeast ADH and yeast 6-PGDH on NAD(P)$^+$-PBA-Sepharose. NAD$^+$ (a) or NADP$^+$ (b) (2 mg) was applied to APBA–Sepharose 6B (1 ml) (CNBr-activated) and the resulting adsorbent washed with 0.05 *M* Hepes–NaOH buffer, pH 8.45 containing 0.1 *M* $MgCl_2$ (1.0 ml). Columns were then loaded with a mixture of ADH (3 U) and 6-PGDH (2 U) (in 0.2 ml) and washed with the same buffer (7 ml). Elution buffers contained 2 m*M* NAD(P)$^+$ or 20 m*M* sorbitol. ●, ADH; ○, 6-PGDH (×5).

Yeast protein mixtures were applied to NADP$^+$–PBA columns and the results (Fig. 4) confirm those obtained with purified enzymes. Thus, ADH appeared in the early fractions of the column whereas a linear gradient of KCl eluted glucose-6-phosphate dehydrogenase (G-6-PDH), which was separated from the main peaks of protein.

We do not understand why NAD$^+$-presaturated columns did not retard NAD$^+$-dependent dehydrogenases. Similar differences in behaviour have been observed for ribose-immobilised NAD$^+$ and NADP$^+$. Several well documented examples of successful application of such NADP$^+$-affinity columns exist[20,21] but few for NAD$^+$ systems. The reasons may be (i) that a large percentage (50%) of non-productive species are formed by attachment via the adenine ribose of the dinucleotide; or (ii) that NAD$^+$-dependent enzymes use both ribose moieties for successful binding of the cofactor[22]. The apparent inconsistency between free solution studies on periodate-oxidized (ribose) nucleotides (to which dehydrogenases do not bind) and ribose-immobilised NADP$^+$-affinity columns (which do bind NADP$^+$-dependent dehydrogenases) can be explained by the fact that the ribose ring in the latter has been reformed into a morpholine derivative and may well retain the molecular configuration of the original nucleotide.

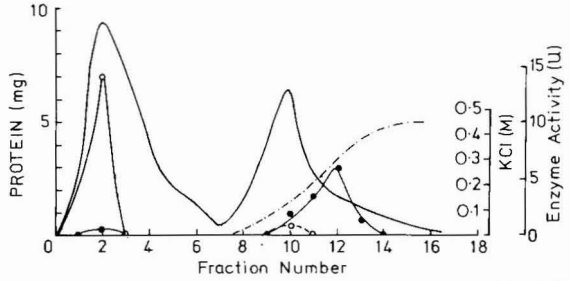

Fig. 4. The chromatography of yeast proteins on NAD(P)$^+$–PBA–Sepharose. Yeast enzyme concentrate (52 mg) in Hepes–NaOH buffer (1 ml), pH 8.45 (plus 0.1 *M* $MgCl_2$) containing G-6-PDH (5.5 U) and ADH (16 U) applied to a column of PBA–Sepharose 6B (CNBr-activated) (3 ml) presaturated with 6 mg $NADP^+$ (prepared as described in Fig. 3). Elution was carried out with a gradient of 0 to 0.5 *M* KCl (– · –) in the same Hepes buffer (20 ml total volume). Fractions of 2 ml were collected. Samples were assayed for G-6-PDH (●), ADH (○) and protein (——).

Application of similar protein mixtures from yeast extracts to $NADP^+$–presaturated aminocaproyl aminophenylboronate columns (Fig. 5) resulted in retardation of the $NADP^+$-dependent G-6-PDH dehydrogenase. A specific elution step was not used and a 14-fold purification factor was achieved with 66% yield. This gel did not perform particularly well in $NADP^+$-saturation studies (Fig. 1) (partly because of its low ligand concentration, 34 μmoles/ml), which might explain why the enzyme did not require a separate elution step to be recovered from the column. Indeed boronate columns appear to be especially useful because of the manner in which enzymes may be selectively retarded[23].

Matrex gel phenylboronate (ligand concentration, 41 μmoles/ml) columns were also able to retard G-6-PDH from yeast protein when presaturated with 2 mg of $NADP^+$ per 1 ml column (Fig. 6). In this experiment, a purification factor of 12-fold and yield of 69% were recorded. Elution in the latter instance was effected by 2 m*M* $NADP^+$.

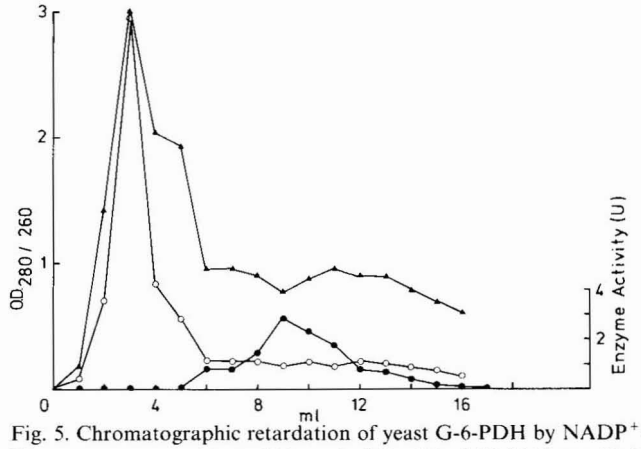

Fig. 5. Chromatographic retardation of yeast G-6-PDH by $NADP^+$-presaturated ACA-PBA–Sepharose. Yeast enzyme concentrate (7.1 mg in 0.5 ml) in 0.05 *M* Hepes–NaOH buffer (1 ml), pH 8.5 (plus 0.1 *M* $MgCl_2$) was applied to a column of ACA-PBA–Sepharose (1 ml) which had been preequilibrated with the above buffer (5 ml) followed by a solution containing $NADP^+$ (2 mg) in the same buffer (0.5 ml) followed by buffer (1 ml). The concentrate contained 18 U of G-6-PDH. Fractions of 1 ml were collected at a flow-rate of 1 ml/h. Fractions were assayed for enzyme activity (●), O.D. 260 (▲) and O.D. 280 (○).

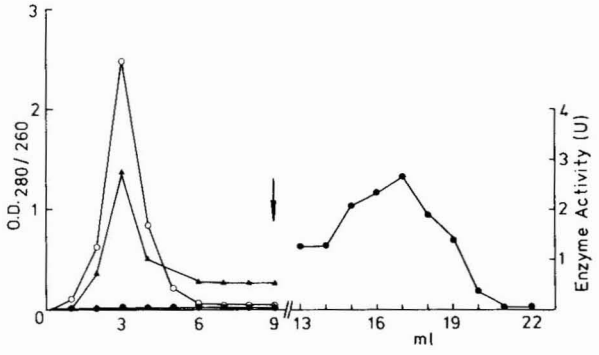

Fig. 6. Chromatography of yeast G-6-PDH by $NADP^+$-presaturated Matrex gel–PBA. Yeast enzyme concentrate (7.5 mg in 0.5 ml) in buffer (ligand concentration, 41 μmoles/ml) (see Fig. 5) was applied to a column of Matrex gel PBA (1 ml) preequilibrated as described in Fig. 5. The concentrate contained 19.4 U of G-6-PDH. Fractions (1 ml) were collected and assayed for enzyme activity (●), O.D. 260 (▲) and O.D. 280 (○). Elution was effected with 2 m*M* $NADP^+$ (arrow).

Thus, various elution procedures can be adopted which depend to some extent on the nature of the boronate ligand, its concentration and the agarose used as a matrix. Cross-linking of the matrix may have some effect and may explain the difference between Matrex gel® and Sepharose® since Matrex gel is cross-linked whereas Sepharose is not.

The specificity of "ligand mediated" separations using boronate columns can be changed by replacing $NADP^+$ with other nucleotides. Thus, we have used Matrex gel phenylboronate–ATP complexes to retard hexokinase. Control columns containing no ATP retard little or no hexokinase. Small amounts of material are bound however, which are released from the column with 100 m*M* sorbitol (Fig. 7).

On the other hand, when ATP was included in the irrigation buffer prior to the application of yeast extract, hexokinase was retarded (Fig. 8). Furthermore, the elu-

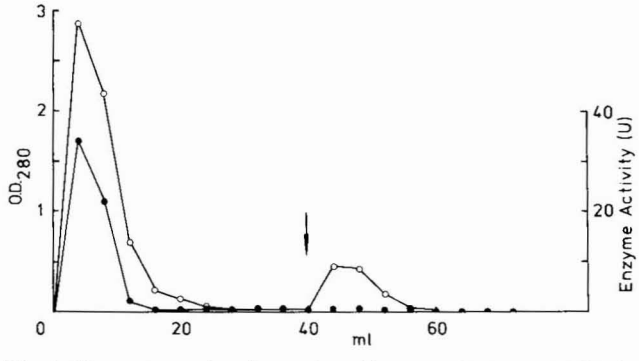

Fig. 7. Chromatography of yeast hexokinase on Matrex gel–PBA in the absence of presaturating ligand. Yeast enzyme concentrate (1 ml) in buffer (see Fig. 5) was applied to a column of Matrex gel–PBA (1 ml) (ligand concentration, 41 μmoles/ml) preequilibrated with 50 m*M* Hepes–NaOH buffer (containing 0.1 *M* $MgCl_2$), pH 8.45. The concentrate contained 62 U of hexokinase. Fractions (4 ml) were collected and assayed for hexokinase activity (●) and O.D. 280 (○). Elution was effected with 100 m*M* sorbitol in buffer (arrow).

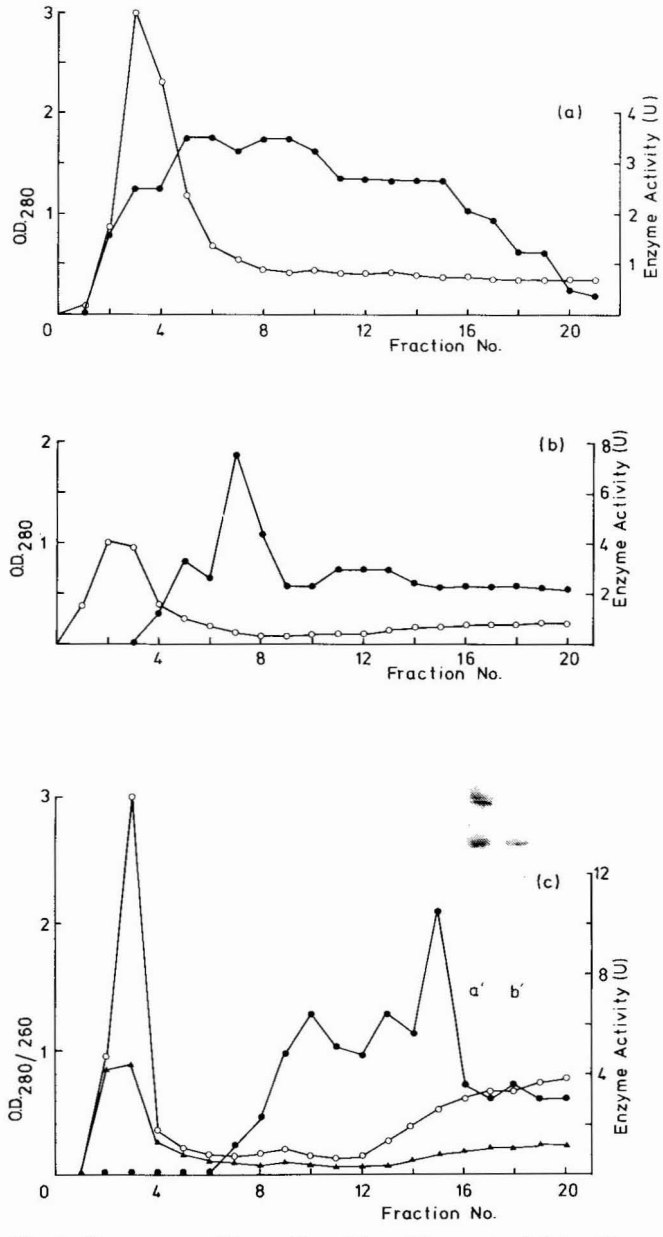

Fig. 8. Chromatographic profiles of hexokinase retarded by Matrex gel PBA presaturated with three different concentrations of ATP. Yeast enzyme concentrate containing hexokinase [60 (c), 66 (b) and 80 (a) U, respectively] in buffer (see Fig. 5) was applied to a column of Matrex gel PBA (4 ml, ligand concentration, 41 μmoles/ml) preequilibrated with ATP [8 (c), 12 (b) and 22 (a) mg in 0.5, 0.5 and 1 ml, respectively] in 50 m*M* Hepes–NaOH buffer (containing 0.1 *M* $MgCl_2$), pH 8.45 followed by the same buffer (4 ml). Fractions (4 ml) were collected and assayed for hexokinase activity (●), O.D. 280 (○) and 260 (▲). Insert Fig. 8c: Fractions were pooled (6 to 26) and analysed by sodium dodecyl sulphate–polyacryl-amide gel electrophoresis (gel a'). A commercial preparation of pure yeast hexokinase (Boehringer) is compared (gel b').

tion profile of hexokinase changed on increasing the amount of presaturating ATP from 8 to 22 mg. The purification factor was least (ten-fold) when the ATP concentration was highest. The precise ATP concentration required for maximizing the emergent specific activity is expected to be different for each batch of yeast protein. From the data presented a value between 8–12 mg ATP per (4-ml) column gave the best results.

Previously reported examples of immobilised nucleotides have been described where the ligand was attached covalently and irreversibly to support matrices. In the latter instances complex chemical syntheses are required prior to coupling the ligand which can incur considerable expense especially when large scale applications are considered.

We believe, despite the problem of preparing suitable immobilised phenylboronic acids that this ligand, when used as a complex with nucleotides, may overcome many of the criticisms of immobilised cofactors as group-specific ligands.

At present, two problems remain to be resolved.

(i) The pK of the immobilised PBA (8.9, see ref. 20 and Fig. 9) used in this study is by no means ideal, since the chromatography requires pH values in excess of 8.0. Work is in progress to synthesise more suitable boronic acids.

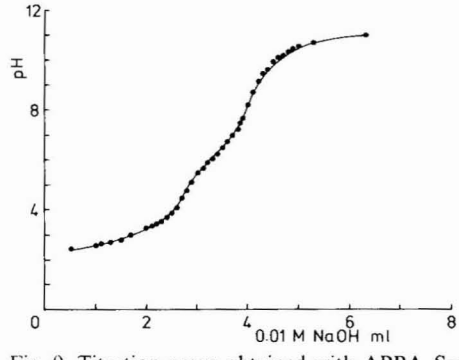

Fig. 9. Titration curve obtained with APBA–Sepharose (CNBr-activated). Samples of gel were washed with distilled water and the pH adjusted to a value of 2.5 with dilute HCl. The suspension was then titrated with 0.01 M NaOH with constant stirring under nitrogen. The titration was carried out at room temperature. Ligand concentration 8 mg APBA per ml gel (wet weight).

(ii) The ligand concentrations used in this work appear to limit the enzyme capacity of these gels. Therefore, a device is needed to augment the ligand concentration[24]. However, the ubiquitous nature of PBA–coenzyme interactions enables a very wide range of immobilised cofactors to be prepared with the same column. The column is not sensitive to enzymic degradation, and the ligand which is complexed to the boronic acid is readily replaced (or exchanged) simply by stripping the PBA matrix with low pH buffers or sorbitol (followed by urea and NaOH to remove any adsorbed protein, for cross-linked gels only).

Other applications of immobilised PBA are under investigation in this laboratory.

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IMPROVED COLUMN EFFICIENCY IN CHROMATOGRAPHIC ANALYSIS OF SUGARS ON CATION-EXCHANGE RESINS BY USE OF WATER–TRIETHYLAMINE ELUENTS

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SUMMARY

An improvement in the chromatographic separation of sugars and sugar alcohols on cation exchangers (Ca^{2+}) with water as eluent is presented. Addition of 0.001 *M* triethylamine to the eluent catalyses the mutarotation of reducing sugars, and results in reduced peak widths. Complete resolution is obtained for glucose, mannose and fructose in $3\frac{1}{2}$ min, for glucose, mannose, fructose, mannitol and glucitol in 9 min and for sucrose, glucose and fructose in $3\frac{1}{2}$ min. A nearly complete resolution of lactose, glucose and galactose is achieved in 3 min. The influence of the triethylamine content, column temperature and eluent flow-rate has been determined. Procedures are given for column preparation, activation and regeneration.

INTRODUCTION

Liquid chromatography of sugars using ion exchangers has become routine. The use of anion exchangers with borate buffers[1] and with ethanol–water[2], and the use of cation exchangers with ethanol–water[3], as eluent have been reported. Many improvements have been achieved, and a comprehensive review is provided by Jandera and Churáček[4]. The methods given by Samuelson and co-workers[2,3] result in a low column efficiency and are not in common use. However, in combination with borate buffers, anion exchangers are frequently employed for the separation of simple mixtures of sugars[5–10]. Although this method also leads to low column efficiencies, it has the advantage of a tailor-made eluent through variation of the buffer concentration and pH[7].

Two other systems are commonly employed: alkylamine-modified silica together with acetonitrile–water, and cation exchangers (Ca^{2+}) with pure water as the eluent. Silica can be modified with alkylamine both chemically[11–13] and physically[14–16]. It has the advantage of being resistant to high pressures, enabling the use of high eluent flow-rates. Separations can also be carried out at room temperature with simple apparatus. In the "Ca-column" system, the main function of the ion exchanger is to immobilize Ca^{2+}, while separation is the result of the different complexing

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abilities of the polyols with Ca^{2+}. However, the degree of cross-linking is also impor tant, polymeric sugars being better separated at low degrees and mono- and dimeri sugars at high degrees. Advantages of this system are cheap eluent, high colum capacity and stability and complete elution of all sugars injected[17].

In contrast with the borate–anion exchanger system, the elution sequenc cannot be changed in the modified silica system nor in that of the cation exchange (Ca^{2+}). There is a remarkable difference between the elution sequences of the last tw systems: on alkylamine-modified silica, sugars are eluted in order of increasing degre of polymerization, first monomeric then oligomeric and polymeric; the opposite i true for the cation exchanger (Ca^{2+}) system. Choice of the appropriate method will therefore, depend on the nature and quantity of the sugars to be separated. Althoug the use of silica columns is increasing, this method will not supersede the two ion exchange methods because of the different elution sequences.

In this paper we describe an extension of the applicability of the cation ex changer (Ca^{2+}). Addition of a small amount of triethylamine (TEA) to the eluen catalyses the mutarotation of reducing sugars, resulting in reduced peak widths with out affecting the elution times. The separations are thus possible at room tempera ture, with no separate peaks for sugar anomers. The overall result is a greatly im proved method of analysis.

EXPERIMENTAL

Apparatus

The column comprised a 316 stainless-steel Lichroma tube (Chrompack, Mid delburg, The Netherlands), 250 mm × 4.6 mm I.D., fitted with filters (pore size 2 μm and thermostated in a water-bath. The eluent was deaerated by heating it to 10°C above the column temperature; it was pumped, after passage through a 2-μm filter, by a solvent delivery system (Model 6000 A, Waters Assoc.). Injection was performed with a Rheodyne Type 7010 sample valve, fitted with a 10-μl loop, and filled by suction using a peristaltic pump. Detection was by a differential refractometer (Type R401, Waters Assoc.). The specifications of the cation-exchange resin Aminex A-5 (Bio-Rad Labs., Richmond, CA, U.S.A.) were as follows: matrix, polystyrene and divinylbenzene with 8% cross-linking; functional group, sulphonic acid; particle diam eter, 13 ± 2 μm; exchange capacity, 1.7 mequiv./ml; maximum allowable tempera ture, 150°C.

Column preparation and quality

Several methods of column preparation are given in literature. Rapp *et al.*[18] pumped a slurry of the resin in ethanol–water (75:25) into the column without any pretreatment. Ladisch *et al.*[19] carried out stepwise washings and extractions in order to purify the resin and to convert it into the appropriate ionic form. Scobell *et al.*[20] used the same procedure, but with the resin inside the column, which is less laborious Ladisch and Scobell also employed sedimentation to obtain a more uniform particle size. We did not use sedimentation as we supposed the resin Aminex A-5 to be within 13 ± 2 μm. Our method, resembling that of Scobell *et al.*[20], is as follows.

(1) A weighed amount of Aminex A-5 (*ca.* 1.5 g per ml column content) was placed into a column and the eluent pump was connected;

(2) The resin was washed with 100 ml distilled water, then with 300 ml of 0.2 *M* $CaCl_2$ in distilled water and finally with 100 ml distilled water;

(3) The resin was pumped out of the column into a beaker together with four times its volume of water;

(4) The analysis column was fitted with a stainless-steel filter at the bottom and a filling column at the top, and then filled with distilled water;

(5) The resin was slurried over 5 min using an ultrasonic bath and poured into the filling column, the residual volume of which was filled with water. The top of this column was connected to the eluent pump. This procedure is carried out quickly in order to avoid selective sedimentation;

(6) During 1 h, water was pumped through the column, maintaining a pressure drop of 50 p.s.i. per cm of bed height;

(7) The filling column was disconnected, the analysis column filled and levelled and fitted with a stainless-steel filter.

The flow direction was marked on the column in order to avoid disturbance of the column packing. (The resin resists applied pressure; Rapp *et al.*[18] even used up to 20% higher pressure drops. The procedure is carried out at room temperature.)

The column, prepared in this way and used at 85°C with an eluent flow-rate of 0.5 ml/min, gave a plate height for the reference substance D-mannitol of 80 μm (6 × particle diameter). It has been used for 3 months under various conditions: temperatures from 15 to 95°C; eluent changes between distilled water, 0.001 *M* TEA, KOH solution at pH 11 and 0.2 *M* $CaCl_2$ in water; and with longer stops of eluent flow and heating. The column performance, with reference to D-mannitol was unaltered over this period, in accordance with the experience of Angyal *et al.*[17].

Standard conditions

Column: 250 × 4.6 mm stainless steel filled with Aminex A-5 (Ca^{2+}); temperature, 45°C. Eluent: 0.001 *M* triethylamine in water; flow-rate, 0.5 ml/min. Injected amount: 0.1 mg of each sugar. Attenuation of the refractometer: × 32.

RESULTS AND DISCUSSION

Separation of sugars with this cation exchanger (Ca^{2+}), with water as eluent, is generally performed at elevated temperatures[19,20]. Owing to a higher rate of mass transfer, the peak widths decrease. At lower temperatures, double peaks of the different sugar anomers occur[21], which fuse at higher temperatures due to the increased mutarotation rate[17]. (The same behaviour is observed in the chromatography of sugars on anion exchangers with ethanol–water as eluent[22]). On the other hand, components are more strongly adsorbed (exothermic adsorption) at lower temperatures and, therefore, the resolution can be improved if the influence of the lower mass-transfer rate is small and the effect of mutarotation is suppressed.

TEA as a catalyst for mutarotation

Since mutarotation is catalysed by hydroxide ions, the use of an eluent having a high pH should improve the chromatographic separation[17]. The use of an amine, especially TEA, is preferred for several reasons: stainless steel is more resistant to corrosion in amines than in alkalis[23]; with TEA, the eluent pump and sample valve

will not be blocked by evaporation residues during shut-down periods; TEA has a boiling point which is relatively high in comparison to the column temperature; and TEA, unlike polyamines, does not result in too high a viscosity. To elucidate the effect of TEA addition, several experiments have been carried out at room temperature.

D-Glucose solutions in water and in 0.001 *M* TEA were analysed at set times after preparation, using our column with pure water as eluent and under circumstances in which the anomers are separated (45°C, flow-rate 0.5 ml/min). Freshly prepared solutions contain mainly the α-form, while at equilibrium the β-form is predominant, as confirmed by polarimetry. Plots of peak areas for the α- and β-forms *versus* time elapsed after preparation of the solutions are presented in Fig. 1. Apparently the mutarotation rate is increased by a factor of *ca.* 20 by the addition of 0.001 *M* TEA. Other results of this experiment are that β-glucose is eluted before α-glucose, and that both forms give the same quantitative response. For quantitative analysis, therefore, it does not matter whether an equilibrium mixture is injected or not.

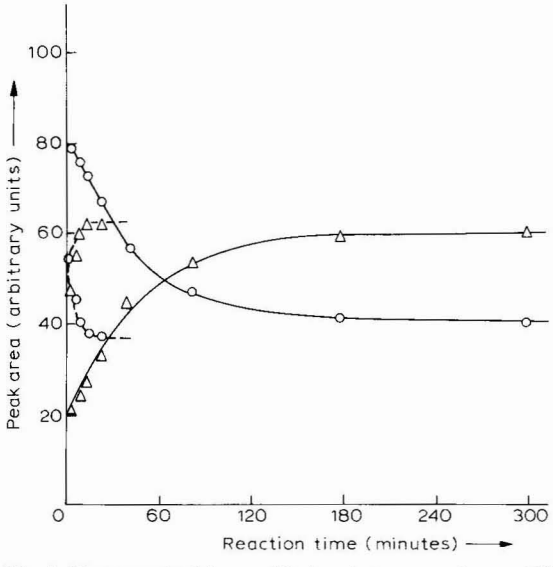

Fig. 1. Time required for equilibrium between α-glucose (○) and β-glucose (△) at 22°C: ———, a freshly prepared solution of glucose in water; – – –, the same solution but after addition of 0.001 *M* triethylamine.

Influence of TEA concentration

To determine the optimum TEA concentration for analysis, a reference sample was injected using eluents comprising various amounts of TEA in water. First, sufficient eluent had to be pumped through in order to reach a "steady state". The results are presented in Table I. Retention times are almost independent of the TEA concentration; however, the peak width of glucose decreases with increasing TEA content. Concentrations of TEA higher than 1 m*M* do not further improve the analysis.

TEA from the eluent will gradually displace Ca^{2+} from the ion exchanger, resulting in decreased retention times and in low separation efficiency. This effect is illustrated in Table II. Concentrations higher than 0.001 *M* TEA should not be used,

TABLE I

ELUTION TIME AND PEAK WIDTH AS A FUNCTION OF TRIETHYLAMINE CONCENTRATION IN THE WATER ELUENT

t_R = Elution time in minutes; $w^1_{\frac{1}{2}h}$ = peak width at half height in minutes.

*Sugar***	*Triethylamine concentration (mM)**							
	0.2 (9)		*0.4 (5)*		*0.6 (3)*		*1.0 (3)*	
	t_R	$w^1_{\frac{1}{2}h}$	t_R	$w^1_{\frac{1}{2}h}$	t_R	$w^1_{\frac{1}{2}h}$	t_R	$w^1_{\frac{1}{2}h}$
Glucose	3.63	0.37	3.63	0.31	3.61	0.27	3.59	0.26
Fructose	5.07	0.34	5.07	0.34	5.07	0.33	5.05	0.32
Mannitol	6.89	0.44	6.93	0.44	6.91	0.43	6.85	0.42
Glucitol	9.23	0.57	9.23	0.56	9.15	0.54	9.13	0.54

* The column conditioning time is given in parentheses.

** All sugar products mentioned in this paper belong to the D-series.

TABLE II

COLUMN EFFICIENCY BEFORE AND AFTER 2000 ml OF 0.001 *M* TRIETHYLAMINE ELUENT HAS BEEN PUMPED THROUGH

N = Number of theoretical plates. t_R and $w^1_{\frac{1}{2}h}$ as in Table I.

	Ca^{2+}-column (freshly regenerated)–water	*Ca^{2+}-column (after 2000 ml 0.001 M TEA eluent)–0.001 M TEA*	*TEA-column*–water*
Mannitol			
t_R	7.09	6.71	4.03
$w^1_{\frac{1}{2}h}$	0.41	0.38	0.31
N	1656	1728	936
Glucitol			
t_R	9.59	8.85	4.95
$w^1_{\frac{1}{2}h}$	0.54	0.50	0.36
N	1747	1736	1047
Resolution (mannitol–glucitol)	2.63	2.43	1.37

* Realized by pumping of 150 ml of 0.1 *M* TEA · HCl.

and, depending on the resolution needed, the column should be regenerated after $\geqslant 2$ l of eluent have been pumped through it. Column regeneration can be performed as described below.

Influence of column temperature

The results of experiments carried out to determine the influence of column temperature on the resolution are given in Table III. The eluents pure water and 0.001

M TEA are compared. While at low column temperatures, using water, double peaks occur for the reducing sugars, single peaks are obtained under all circumstances using TEA. At higher temperatures the use of TEA results in a substantial decrease of peak width for these sugars. The peak widths for the sugar alcohols, which do not mutarotate, are only slightly affected. The mutarotation rate decreases in the sequence: fructose > mannose > glucose, as can be seen from the occurrence of double peaks. The most significant improvement of the analytical result by adding TEA is obtained for the slowly mutarotating sugars. Aitzetmüller[14] reported improved resolution, apart from increased elution times, upon adding polyamine to an acetonitrile–water eluent using silica columns. This improved resolution can also be explained by increased mutarotation.

TABLE III

ELUTION DATA FOR SUGARS AT VARIOUS COLUMN TEMPERATURES WITH (+) AND WITHOUT (−) 0.001 *M* TEA

t_R and $w_{\frac{1}{2}h}$ as in Table I.

Sugar	*Column temperature (°C)*						
	25 (+/−)	*35 (+/−)*	*45 (+/−)*	*55 (+/−)*	*65 (+)*	*75 (+)*	*85 (+/−)*
Glucose							
t_R	3.67/ 3.65*	3.59/ 3.65*	3.61/3.63*	3.66/3.67*	3.63	3.57	3.68/3.69
$w_{\frac{1}{2}h}$	0.42/ −**	0.30/ −	0.23/ −	0.20/ −	0.19	0.17	0.17/0.24
Mannose							
t_R	4.35/ 4.37*	4.35/ 4.32*	4.25/4.34*	4.23/4.29	4.25	4.14	−/4.21
$w_{\frac{1}{2}h}$	0.31/ −	0.27/ −	0.25/ −	0.22/0.51	0.20	0.19	−/0.23
Fructose							
t_R	5.69/ 6.03*	5.31/ 5.61*	5.07/5.29	4.93/4.99	4.75	4.51	4.51/4.53
$w_{\frac{1}{2}h}$	0.43/ −	0.35/ −	0.30/0.94	0.25/0.60	0.22	0.22	0.20/0.24
Mannitol							
t_R	7.93/ 8.21	7.31/ 7.61	6.85/7.09	6.53/6.69	6.21	5.85	5.79/5.87
$w_{\frac{1}{2}h}$	0.58/ 0.62	0.47/ 0.49	0.40/0.41	0.32/0.35	0.28	0.26	0.24/0.25
Glucitol							
t_R	11.4 /12.1	10.1 /10.7	9.09/9.59	8.41/8.75	7.79	7.19	6.99/7.11
$w_{\frac{1}{2}h}$	0.78/ 0.87	0.60/ 0.66	0.51/0.54	0.41/0.44	0.34	0.30	0.28/0.28

* Elution time is calculated according to $t_R = t_0 (1 + k_\alpha' f_\alpha + k_\beta' f_\beta)$, where t_0 is the elution time in minutes of an unretained compound, k_α' and k_β' are the capacities of anomers α and β, respectively, and f_α and f_β are the corresponding mol fractions.

** Double peak.

Influence of eluent flow-rate

Results of experiments at different eluent flow-rates are presented in Table IV. Peak widths decrease with decreasing eluent flow-rate, while elution volumes hardly change. The improvement in column efficiency, however, is not sufficient to justify the longer elution times.

TABLE IV

ELUTION DATA FOR SUGARS AT VARIOUS ELUENT FLOW-RATES

V_R = Elution volume in ml; $w_{\frac{1}{2}h}$ = peak width at half height in μl.

Sugar	*Eluent flow-rate (ml/min)*						
	0.1	*0.2*	*0.3*	*0.4*	*0.5*	*0.6*	*0.7*
Sucrose							
V_R		1.53	1.52	1.52	1.49	1.47	1.41
$w_{\frac{1}{2}h}$		93	100	104	112	110	118
Lactose							
V_R	1.68	1.62	1.59	1.57	1.58	1.56	1.57
$w_{\frac{1}{2}h}$	83	98	106	112	120	120	126
Glucose							
V_R	1.92	1.86	1.83	1.82	1.81	1.80	1.81
$w_{\frac{1}{2}h}$	77	90	98	102	106	110	112
Galactose							
V_R	2.18	2.10	2.07	2.05	2.06	2.03	2.03
$w_{\frac{1}{2}h}$	79	93	100	109	112	113	118
Mannose							
V_R		2.15	2.13	2.12	2.10	2.08	2.08
$w_{\frac{1}{2}h}$		91	106	107	112	113	120
Fructose							
V_R	2.56	2.47	2.43	2.42	2.41	2.40	2.41
$w_{\frac{1}{2}h}$	88	102	116	120	128	130	134
Mannitol							
V_R	3.39	3.27	3.23	3.21	3.20	3.18	3.18
$w_{\frac{1}{2}h}$	108	124	143	156	165	168	179
Glucitol							
V_R	4.33	4.19	4.14	4.11	4.09	4.07	4.08
$w_{\frac{1}{2}h}$	135	150	175	192	200	211	227

Addition of 0.001 *M* TEA to the eluent causes the pH to rise to 10.8, and during the elution isomerization and/or degradation of the sugars may occur. We studied this possibility by determining the quantitative response, peak form and elution volume of the different sugars as a function of the eluent flow-rate. No influence could be found and, therefore, isomerization and degradation of the sugars at high pH can be neglected.

Column regeneration and activation

Column regeneration and activation can be performed by pumping through the column successively: 100 ml distilled water, 100 ml 0.2 *M* $CaCl_2$ in water, 100 ml

distilled water and 50 ml of 0.001 *M* TEA eluent. Regeneration can be carried ou overnight, and, therefore, the procedure has not been optimized. The frequency c regeneration will depend on the resolution needed. On the basis of an average elutio volume of 2 ml and a regeneration step after 2000 ml eluent, 1000 analyses can b carried out between two steps.

For reactivation and deactivation of the column, some time is needed. This i illustrated in Table V. Six column volumes of TEA eluent must be pumped through i order to attain a "steady state". This amount of amine corresponds to only 1/300 c the total column capacity. The increased rate of mutarotation, is therefore not due t the column being in the amine form, but merely to the higher pH throughout th column. For deactivation (not regeneration) of the column, which means lowering th pH inside the resin, even more time is needed. A hundred column volumes of wate eluent have to be pumped through to convert an activated column into a "normal column (Ca^{2+}).

TABLE V

COLUMN ACTIVATION

t_R and $w_{\frac{1}{2}h}$ as in Table I.

Sugar	*Column volumes of eluent*				
	0	*2.4*	*4.8*	*6.0*	*7.5*
Glucose					
t_R	3.51*	3.51*	3.67	3.71	3.69
	3.95*	3.95*			
$w_{\frac{1}{2}h}$	–	–	0.29	0.24	0.24
Fructose					
t_R	5.19	5.19	5.19	5.19	5.19
$w_{\frac{1}{2}h}$	0.70	0.66	0.38	0.32	0.31
Mannitol					
t_R	7.01	7.01	6.99	7.01	6.99
$w_{\frac{1}{2}h}$	0.41	0.40	0.40	0.40	0.40
Glucitol					
t_R	9.35	9.29	9.29	9.33	9.27
$w_{\frac{1}{2}h}$	0.52	0.52	0.53	0.53	0.52

* Double peak.

Application of the method to some common sugar mixtures

The addition of TEA to the eluent results in a large reduction in peak widths of the mutarotating sugars. Therefore, better separations within shorter analysis times are obtained, as compared with previous methods. The separations of some common sugar mixtures are now presented to illustrate the method.

Glucose, mannose, fructose. This product mixture is obtained from the isomerization of glucose to fructose, where mannose is a side-product. The "isomerose" mixture is used as a sweetener in the food industry, as an alternative to sucrose and "invert-sugar". Analysis can be performed using an anion exchanger with borate as

eluent[6], a cation exchanger (Ca^{2+}) with water as eluent[19] or unmodified silica with acetonitrile containing a trace of water[24]. In these three systems the analysis time needed is *ca.* 30 min and only the first system yields complete separation. No literature data are available for the analysis of this mixture using propylamine-modified silica with acetonitrile–water, and interpretation of other data suggests that separation will be difficult.

From the elution data in Table III, it is seen that the resolution is dependent on the column temperature. With increasing temperature, the glucose–mannose resolution improves, while that of mannose–fructose worsens. The optimum temperature is 45°C, and, using our standard analytical conditions, complete separation is obtained within 6 min (see Fig. 2). In serial analysis the time required can be reduced to $3\frac{1}{2}$ min.

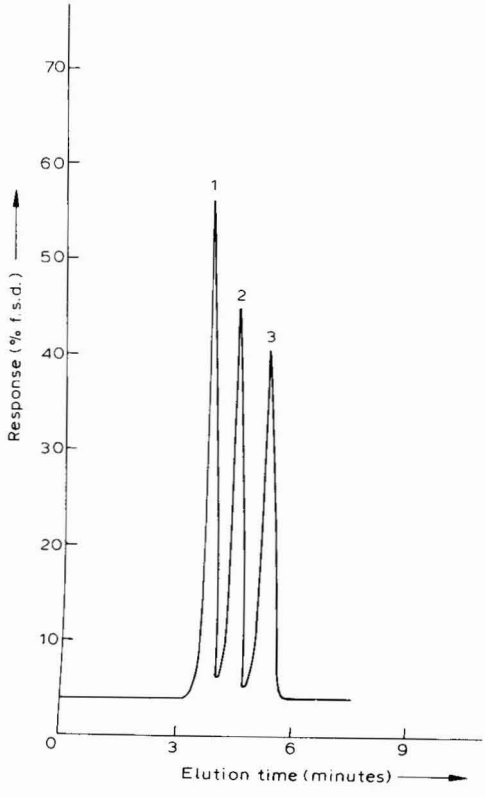

Fig. 2. Chromatogram of glucose (1), mannose (2) and fructose (3) under standard conditions. f.s.d. = Full scale deflection.

Peak heights remain constant within 2%. From the injection of different amounts of sugar, a linear calibration curve could be constructed. If the signal-to-noise ratio is higher than 10, the detection limit for our system is 1 μg sugar, corresponding to 0.1 g/l with an injection volume of 10 μl.

Glucose, mannose, fructose, mannitol, glucitol. This product mixture is obtained from the simultaneous isomerization and hydrogenation of glucose. The sugar al-

cohols obtained by hydrogenation are eluted after the sugars, and a complete separation is possible using the conditions described above. A typical chromatogram is shown in Fig. 3. The time needed to separate the five components is 12 min; in serial analysis, 9 min. The detection limit for the sugar alcohols is a factor of 2 higher than for the sugars owing to the longer elution times.

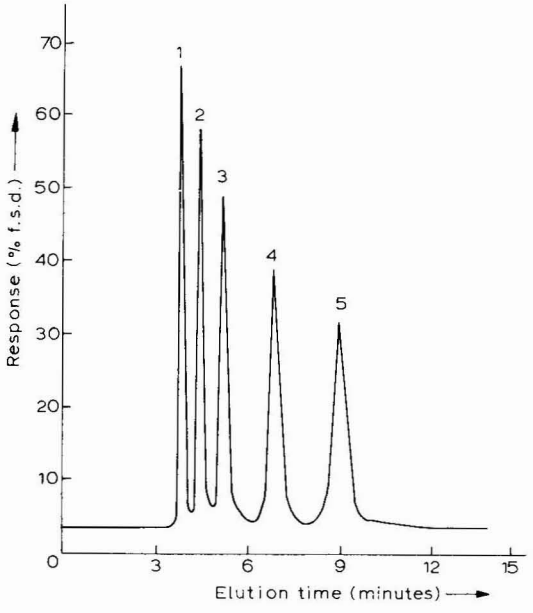

Fig. 3. Chromatogram of glucose (1), mannose (2), fructose (3), mannitol (4) and glucitol (5) under standard conditions.

Lactose, glucose, galactose. People suffering from lactose intolerance cannot digest lactose or "milk-sugar". Hydrolysis of lactose to the digestible sugars glucose and galactose is a useful method of overcoming this problem. In this reaction, some oligomeric sugars are formed as side-products. Chromatographic separation of the main components lactose, glucose and galactose can be achieved using an anion exchanger with borate as eluent[25] and a cation exchanger with water as eluent[19]. The time needed for the analysis is 30 min, which can be reduced in serial analysis to 20 and 10 min respectively for the two systems. No data are available on the use of modified silica with acetonitrile–water as eluent for the analysis of this sugar mixture. Such analysis will probably be difficult.

With our system, separation at 55°C is achieved within 6 min as is shown in Fig. 4. The quantitative results are similar to those already mentioned for the glucose–mannose–fructose mixture. An advantage of our system over the modified silica system is that oligomeric sugars are eluted before the monomeric sugars, which enables accurate estimation.

Sucrose, glucose, fructose. Sucrose, glucose and fructose are the most commonly used sweeteners. A glucose–fructose (1:1) mixture obtained by hydrolysis of sucrose is called "invert-sugar". Many methods for chromatographic separation of this mixture are given in the literature: anion exchangers with borate as eluent[9,10,25],

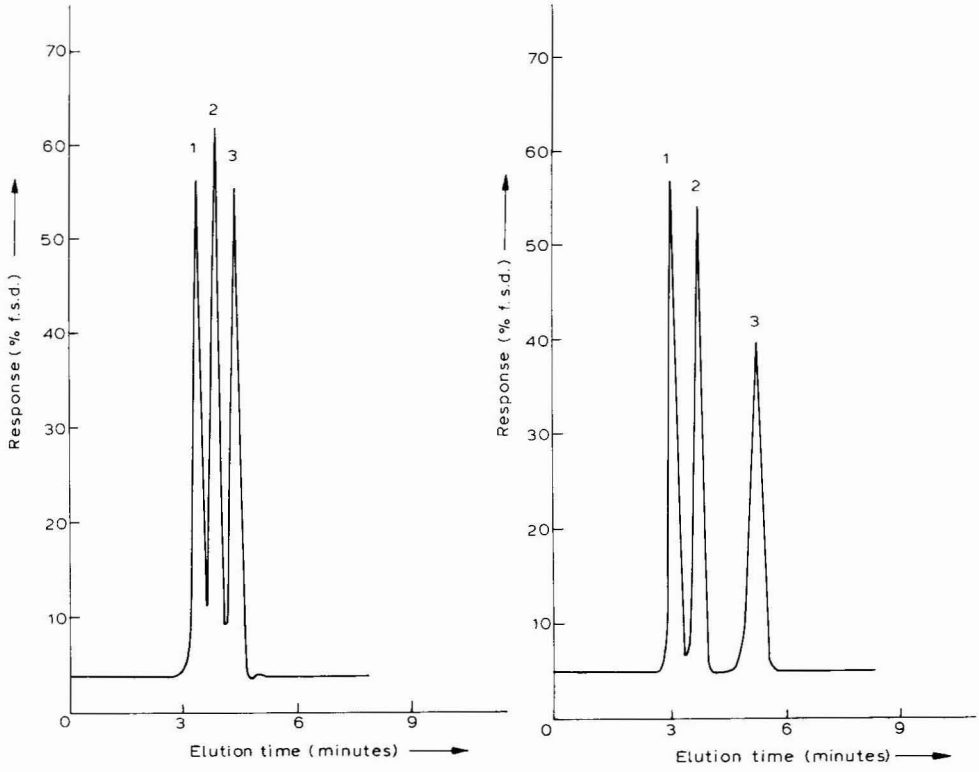

Fig. 4. Chromatogram of lactose (1), glucose (2) and galactose (3) under standard conditions, but with a column temperature of 55°C.

Fig. 5. Chromatogram of sucrose (1), glucose (2) and fructose (3) under standard conditions.

cation exchangers with water[18–20,26] and alkylamine-modified silica gel with acetonitrile–water[10,11]. For the anion- and cation-exchanger systems, the time required for a single analysis is 30 min, and in the latter case, as little as 15 min for serial analysis. With the silica gel system, 10 min are usually necessary, and the time cannot be less in serial analysis because of baseline disturbances caused by injection.

Sucrose, glucose and fructose can be separated under identical conditions to those used for glucose–mannose–fructose, as shown in Fig. 5. The analysis requires $3\frac{1}{2}$ min if carried out serially. The quantitative results are similar to those obtained for glucose–mannose–fructose. The three components could also be separated using only water as eluent at 85°C. The time needed for elution is the same, but addition of TEA to the eluent enables the use of a much lower column temperature.

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GASCHROMATOGRAPHISCHE ERFASSUNG VON 6-DESOXYHEXOSEN, PENTOSEN UND HEXOSEN AUS HERZWIRKSAMEN GLYKOSIDEN

I. GAS–FLÜSSIGKEITS-CHROMATOGRAPHIE AN GEPACKTEN SÄULEN

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SUMMARY

Gas chromatographic determination of 6-deoxyhexoses, pentoses and hexoses of cardiac glycosides. I. Gas–liquid chromatography on packed columns

A new, relatively simple and accurate method allows the identification and quantification of the monosaccharide components of cardiac glycosides.

After hydrolysis under standard conditions the trimethylsilyl ethers of digitoxose, cymarose, fucose, rhamnose, 6-deoxyallose, 6-deoxygulose, 6-deoxyglucose, 6-deoxyidose, 6-deoxytalose, 3-O-methylglucose, allose, altrose, gulose, idose, glucose, mannose, galactose, talose, arabinose, lyxose, ribose and xylose can be identified by gas chromatography on OV-101 or OV-17 as stationary phases. The retention times of the main peaks of each of the analysed monosaccharides, and the ratios of the peak areas of the different anomers were found to be characteristic and of good reproducibility.

With phenyl-β-D-glucopyranoside as internal standard the quantification of the absolute amount of sugar present in cardiac glycosides of known constitution is possible, the recovery after hydrolysis being 71–92%. The method allows determination of the composition of the sugar chain in new cardiac glycosides and gives better information in a shorter time of analysis than other methods.

EINLEITUNG

Die meisten Herzglykoside enthalten ungewöhnliche, sonst in der Natur nur selten vorkommende Zucker, die hinsichtlich ihrer Struktur[1], Biogenese[2–4] sowie ihres Einflusses auf die Löslichkeit bzw. pharmakodynamischen Eigenschaften einzelner Substanzen[5,6] besonderes Interesse beanspruchen. Da diese Naturstoffe oft nur in geringen Mengen isoliert werden können, ist man für die Identifizierung der Zuckerkomponente(n) vorwiegend auf chromatographische Vergleiche angewiesen. Dafür hatte man früher die Papierchromatographie herangezogen, wobei Verwendung vier verschiedener Fliessmittelsysteme eine eindeutige Sicherung der nach Säurehydrolyse des Herzglykosides erhaltenen Monosaccharid-Bausteine ermöglichte[7].

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Eine Verbesserung der Trennung konnte durch Komplexbildung erzielt werden, indem das Papier mit Boratpuffer[7] oder Natriummolybdat[8] imprägniert wurde; auch diese Methode war jedoch mit grossem Zeitaufwand verbunden.

Als bisher einfachstes Verfahren zur eindeutigen Unterscheidung der Zuckerkomponenten erwies sich eine Kombination von Dünnschichtchromatographie, Papierchromatographie und Hochspannungselektrophorese[9].

Da unserer Arbeitsgruppe in den letzten Jahren die Isolierung einer grossen Anzahl neuer Herzglykoside aus *Convallaria majalis*[10,11], *Erysimum cheiri*[12] und *Urginea maritima*[13] gelang und die Auffindung weiterer herzaktiver Verbindungen zu erwarten ist, schien es angebracht, eine zeitsparende und weniger umständliche Methode auszuarbeiten. Wegen des geringen Substanzbedarfes bot sich für diesen Zweck im besonderen die Gaschromatographie an[14].

Zur Vervollständigung der Resultate sollten neben den in diesen Verbindungen häufig vorkommenden 6-Desoxyhexosen auch sämtliche Hexosen und Pentosen in die Untersuchungen miteinbezogen werden, zumal gerade in letzter Zeit solche Zukker in Cardenoliden aufgefunden wurden[10,11].

EXPERIMENTELLES

Vergleichssubstanzen

D-Arabinose, D-Lyxôse, D-Ribose, D-Xylose, D-Allose, D-Altrose, D-Galaktose, D-Gulose, D-Glucose, D-Idose, D-Mannose, D-Talose, D-Digitoxose, D-Fucose (6-Desoxy-D-galaktose) und L-Rhamnose (6-Desoxy-L-mannose), Sigma (St. Louis, MO, U.S.A.); 6-Desoxy-D-allose, 6-Desoxy-D-gulose; 6-Desoxy-D-glucose, 6-Desoxy-L-idose, 6-Desoxy-L-talose, 3-O-Methyl-D-glucose: Professor Dr. T. Reichstein, Basel, Schweiz).

Die Glykoside waren isolierte Reinsubstanzen aus *Convallaria majalis*[10,11,15–17], *Erysimum cheiri*[12], *Adonis vernalis*[18] und *Ornithogalum boucheanum*[19], die Glykoside Sarmentosid A und Strogosid verdanken wir Herrn Professor Dr. T. Reichstein.

Zum Vergleich wurden 6-Desoxy-allose aus Strophallosid und Strophanollosid[15], 6-Desoxy-gulose aus Desglucocheirotoxol und Perigulosid[16], 6-Desoxy-glucose aus Glucoallisid[12], Fucose aus Cheirosid A[12], Rhamnose aus Thollosid, Lokundjosid und Rhodexin A[10,17], 6-Desoxy-talose aus Sarmentosid A und Strogosid, Digitoxose aus Helveticosid[12], Cymarose aus Cymarin[18], Allose aus Bipindogenin-β-D-allosid[10], Glucose aus Cheirosid A und Glucoallisid[12], Arabinose aus Strophanthidin-β-D-allomethylosido-α-L-arabinosid[11] und Xylose aus Glykosid A_1[19] durch Hydrolyse im Mikromassstab hergestellt.

Als innerer Standard wurde Phenyl-β-D-glucopyranosid, Fluka (Buchs, Schweiz) verwendet. Die Substanzen wurden vor der Einwaage im Hochvakuum getrocknet. Alle verwendeten Lösungsmittel waren p.A. Qualität, Merck (Darmstadt, B.R.D.).

Gaschromatographie

(1) Varian Aerograph 2740 Flammenionisations detektor (FID); Säule; Pyrex 10 ft. × 1/4 in. O.D. × 2 mm I.D.; Trennmaterial: 1.6% OV-101 auf Chromosorb G AW DMCS (100–120 mesh); Trägergas: Stickstoff 20 ml/min, Wasserstoff 30

ml/min., Synthetische Luft 300 ml/min; Temperatur: Injektor 230°C, Detektor 240°C; Temperaturprogramm 100–240°C (2°/min); Empfindlichkeit 32 × 10^{-11}; Integrator: Perkin-Elmer SIP-1.

(2) Perkin-Elmer F 33/3 (FID); Säule, Pyrex 6 ft. × 1/4 in. O.D. × 2 mm I.D.; Trennmaterial: 6% OV-17 auf Chromosorb W AW DMCS (100–120 mesh); Trägergas: Stickstoff 13.5 ml/min, Wasserstoff 30 ml/min, Synthetische Luft 300 ml/min.; Temperatur: Injektor/Detektor 225°C; Temperaturprogramm 100–200°C (2°/min); Empfindlichkeit 8 · 10; Integrator: Perkin-Elmer M-2.

Hydrolyse (Methode 1)

0.50–1.00 mg Glykosid wurden in einem Kochglas in 0.3 ml Kiliani-Mischung (3.5 ml Eisessig, 5.5 ml destilliertes Wasser und 1.0 ml konz. Salzsäure[20]) gelöst, gut verschlossen und 1 h auf 100°C (Trockenschrank) erhitzt. Nach dem Abkühlen verdünnten wir mit dem doppelten Volumen Wasser und extrahierten die wässrige Lösung zweimal mit je 0.5 ml Chloroform, um die Aglykon-Artefakte abzutrennen[1]. Die Neutralisation der sauren Lösung erfolgte mit Dowex 44 (Serva, Heidelberg, B.R.D.); OH^--Form; Säule, 10 × 1 cm I.D. Dem Eluat setzten wir 0.20 mg Standard (Phenyl-β-D-glucopyranosid) in Form einer methanolischen Lösung zu und brachten unter wiederholtem Zusatz von Methyläthylketon–*n*-Propanol (1:1) bei max. 40°C und unter vermindertem Druck zur Trockene.

Hydrolyse (Methode 2)

Bei dieser Methode versetzten wir 0.50–1.00 mg Glykosid mit 2 ml Aceton und 5 ml 0.05 *N* Schwefelsäure und erhitzten 2 h auf 95°C. Nach dem Abkühlen wurde die Lösung mit Dowex 44 (OH^--Form; Säule: 10 × 1 cm I.D.) neutralisiert und nach Zusatz von 0.20 mg Standard unter wiederholter Zugabe von Methyläthylketon–*n*-Propanol (1:1) bei max. 40°C und vermindertem Druck in einem 3-ml Reacti-Vial (Pierce, Rotterdam, Niederlande) zur Trockene gebracht.

Äquilibrierung der freien Zucker in saurer Lösung

Wir lösten 0.20–0.50 mg Zucker in 0.3 ml Kliani-Mischung und erhitzten 30 min auf 100°C. Die anschliessende Aufarbeitung erfolgte wie unter *Hydrolyse (Methode 1)* angegeben.

Äquilibrierung der freien Zucker in wässriger Lösung

Die Äquilibrierung wurde nach Wulff[21] ausgeführt. Das Abdampfen der wässrigen Phase erfolgte wie oben.

Derivatisierung

Das nach Hydrolyse oder Äquilibrierung erhaltene Zuckergemisch lösten wir in wasserfreiem Pyridin, wobei für 0.20 mg Zucker 100 µl Pyridin verwendet wurden, und setzten pro 100 µl Pyridin 10 µl Hexamethyldisilazan und 10 µl Trimethylchlorsilan (Pierce, Rotterdam, Niederlande) zu[22]. Nach gutem Durchschütteln wurde 1 µl dieser Suspension direkt in den Gaschromatographen eingespritzt. Die Lösungen waren bei Aufbewahrung im Exsiccator und Kühlschrank mindestens drei Tage verwendbar.

Quantitative Bestimmung von Zuckern in Glykosiden

Die verschiedenen, in wässriger Lösung äquilibrierten Zucker wurden mit Standard (204.8 mg Phenyl-β-D-glucopyranosid in 100.0 ml Methanol) versetzt und wie oben weiterbehandelt. Die zur Derivatisierung verwendeten Lösungsmittel- und Reagentienmengen wählten wir so, dass die Substanzkonzentrationen denjenigen der Probeneinspritzlösungen entsprachen. Für die Berechnung[23] wurden folgende gemittelte Standard-Korrekturfaktoren verwendet, da die für die einzelnen Zucker gefundenen Werte jeweils nur um $\pm 10\%$ (rel.) differierten: 2,6-Bisdesoxyhexosen 0.90; 6-Desoxyhexosen 0.85; Hexosen und 3-O-Methylhexosen 0.95; Pentosen 1.0.

ERGEBNISSE

Die Gaschromatographie wurde in letzter Zeit häufig zur Identifizierung und quantitativen Bestimmung von Zuckern herangezogen. Zwar müssen die Monosaccharide zu diesem Zweck in flüchtige Derivate (z.B. Trimethylsilyl-(TMS-)Äther[24–34], Acetalditole[35], Methyläther[35–37] oder Trifluoroacetate[34,38]) überführt werden, doch wird dieser Nachteil bei weitem durch die erreichbare Trennkapazität und den Zeitgewinn wettgemacht[14,39]. Für die in der vorliegenden Arbeit in erster Linie angestrebte Identifizierung der Monosaccharid-Bausteine verschiedener herzwirksamer Glykoside empfahl sich besonders die Verwendung der TMS-Derivate, welche zum einen durch einfache, in kurzer Zeit quantitativ verlaufende Umsetzung hergestellt werden können und zum anderen durch Stabilisierung der anomeren furanoiden, pyranoiden bzw. auch heptanoiden Formen im Gaschromatogramm charakteristische "fingerprints" der einzelnen Zucker ergeben[40–42], woraus eine zusätzliche Absicherung der Analysenergebnisse resultiert.

Bei der Wahl des gaschromatographischen Trennsystems gingen wir davon aus, dass die Trennung einer grossen Anzahl von Hexosen, Desoxyhexosen und Pentosen möglich sein sollte. Zu diesem Zweck musste ein Programm über einen grossen Temperaturbereich gewählt werden, da einerseits eine gute Abtrennung der relativ niedrig siedenden TMS-Derivate der Bisdesoxyhexosen vom Lösungsmittel und anderseits eine gute Auftrennung der höher siedenden TMS-Hexosen bei möglichst kurzer Analysenzeit zu gewährleisten war. Wegen der hohen Temperaturstabilität setzten wir dazu die Siliconphasen OV-101 bzw. OV-17 ein.

Da die Erfassung der Monosaccharid-Bausteine von Glykosiden eine saure Hydrolyse voraussetzt, wurden auch die Vergleichssubstanzen den für die Glykosidspaltung notwendigen Hydrolysebedingungen unterworfen. Die Behandlungszeit musste in Übereinstimmung mit Wulff[21] allerdings für die freien Zucker herabgesetzt werden, um der Kiliani-Spaltung von Herzglykosiden vergleichbare Bedingungen zu schaffen, da ja die freien Zucker sofort den bekannten Zersetzungsreaktionen ausgesetzt waren. Vergleichende Untersuchungen von freier und durch saure Hydrolyse aus Monoglykosiden gewonnener Glucose, Rhamnose und Fucose ergaben, dass die freien Monosaccharide nach halbstündiger Behandlung mit Kiliani-Mischung die gleichen "fingerprints" wie die aus den Glykosiden nach einstündiger Hydrolyse gewonnenen Zucker aufwiesen. Wir konnten daher einzelne Zucker, die nur in geringen Mengen als Reinsubstanzen zur Verfügung standen, durch Hydrolyse von Monoglykosiden gewinnen, ohne eine Verfälschung der Analysenergebnisse befürchten zu müssen. Für den Nachweis der 2,6-Bisdesoxyzucker verwendeten wir ein schonendes

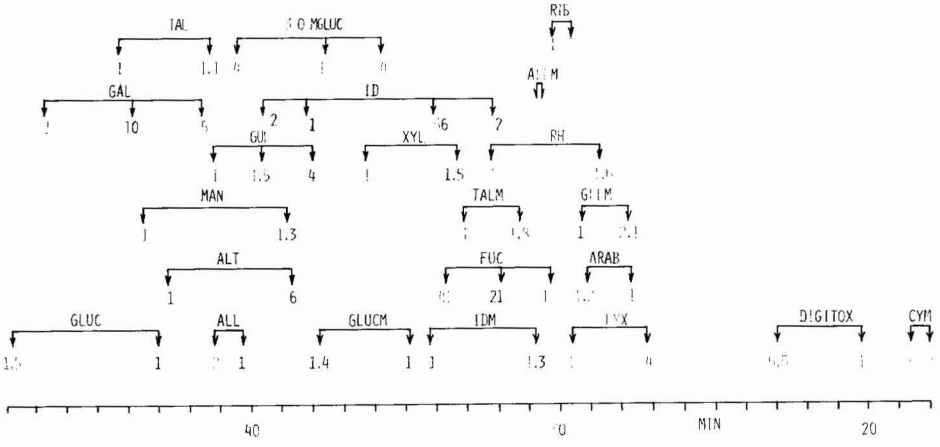

Fig. 1. Retentionszeiten der TMS-Monosaccharide nach saurer Hydrolyse sowie Peakflächenverhältnisse der anomeren Formen an OV-101; Säule: Pyrex 10 ft. × 1/4 in. O.D. × 2 mm I.D., 1.6% OV-101 auf Chromosorb G (100–120 mesh); Trägergas: N_2, 20 ml/min; Temperatur: Injektor: 230°C, Detektor: 240°C; Ofen, Programm 100–240°C (2°/min). CYM = Cymarose, DIGITOX = Digitoxose, LYX = Lyxose, ARAB = Arabinose, GULM = 6-Desoxygulose, RH = Rhamnose, ALLM = 6-Desoxyallose, RIB = Ribose, IDM = 6-Desoxyidose, FUC = Fucose, TALM = 6-Desoxytalose, XYL = Xylose, ID = Idose, 3-O-MGLUC = 3-O-Methylglucose, GLUCM = 6-Desoxyglucose, ALL = Allose, ALT = Altrose, MAN = Mannose, GUL = Gulose, GLUC = Glucose, GAL = Galaktose, TAL = Talose.

Hydrolyseverfahren, da sich diese unter den oben angegebenen Bedingungen vollständig zersetzten[1,12].

Die gaschromatographische Trennung der auf diese Weise aufbereiteten, persilylierten Zucker an OV-101 (Fig. 1) zeigte, dass die verwendeten Bedingungen in den meisten Fällen eine sichere Identifizierung der Zucker zulassen, wenn man davon ausgeht, dass in Herzglykosiden maximal drei verschiedene Zuckerkomponenten zu erwarten sind. Nach Behandlung mit Säure traten jeweils zwei bis drei Hauptpeaks auf, die mindestens 90% der Gesamtpeakfläche aufwiesen, weshalb wir nur diese für die Auswertung heranzogen. Bei Retentionszeiten unter 35 min wurde noch bei Differenzen von 0.6 min und bei Retentionszeiten über 35 min bei Unterschieden von 0.9 min (4σ-Trennung) eine für die sichere Identifizierung der Peaks ausreichende Auflösung gefunden. Die wenigen verbleibenden kritischen Paare führten wir einer Trennung an der mittelpolaren Silikonphase OV-17 zu (Fig. 2) und konnten feststellen, dass diese Vorgangsweise eine gute Ergänzung zu den an OV-101 erzielbaren Ergebnissen darstellt. Die aus Figs. 1 und 2 ersichtlichen Peakflächenverhältnisse der anomeren Zucker erwiesen sich bei strikter Einhaltung der standardisierten Hydrolysebedingungen als gut reproduzierbar (max. ± 15% rel.). In Wasser äquilibrierte Monosaccharide ergaben normalerweise gleiche Hauptpeaks mit allerdings unterschiedlicher Flächenverteilung. Eine Ausnahme bildeten Gulose und Altrose, welche nach Behandlung mit Säure jeweils einen zusätzlichen Hauptpeak kürzerer Retentionszeit zeigten. Dies kann als Hinweis dafür gewertet werden, dass in diesen Fällen bei der Säurehydrolyse leicht Anhydroprodukte entstehen[4,3].

Um die Leistungsfähigkeit des neuen Verfahrens zu prüfen, wurde eine grosse Anzahl von herzwirksamen Glykosiden aus *Convallaria majalis*, *Erysimum cheiri*,

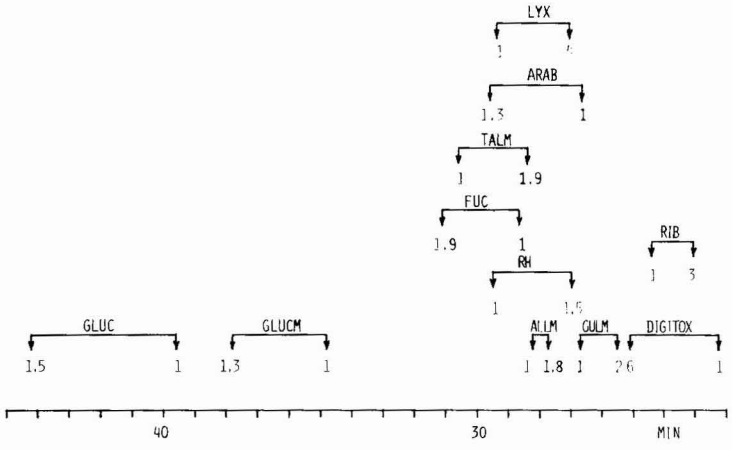

Fig. 2. Retentionszeiten der TMS-Monosaccharide nach saurer Hydrolyse sowie Peakflächenverhältniss der anomeren Formen an OV-17; Säule: Pyrex 6 ft. × 1/4 in. O.D. × 2 mm I.D., 6% OV-17 au Chromosorb W (100–120 mesh); Trägergas: N_2, 13.5 ml/min; Temperatur: Injektor/Detektor 225°C Ofen, Programm 100–200°C (2°/min). Abkürzungen vergleiche Fig. 1.

Adonis vernalis und *Ornithogalum Boucheanum* hydrolysiert und die Zuckerbausteine mit Hilfe von Phenyl-β-D-glucopyranosid als innerem Standard quantifiziert. Die fü die Berechnung notwendigen Standard-Korrekturfaktoren bestimmten wir unte Verwendung von in wässriger Lösung äquilibrierten Vergleichssubstanzen unter Be rücksichtigung der in Fig. 1 bzw. Fig. 2 ersichtlichen Hauptpeaks, sodass die bei de

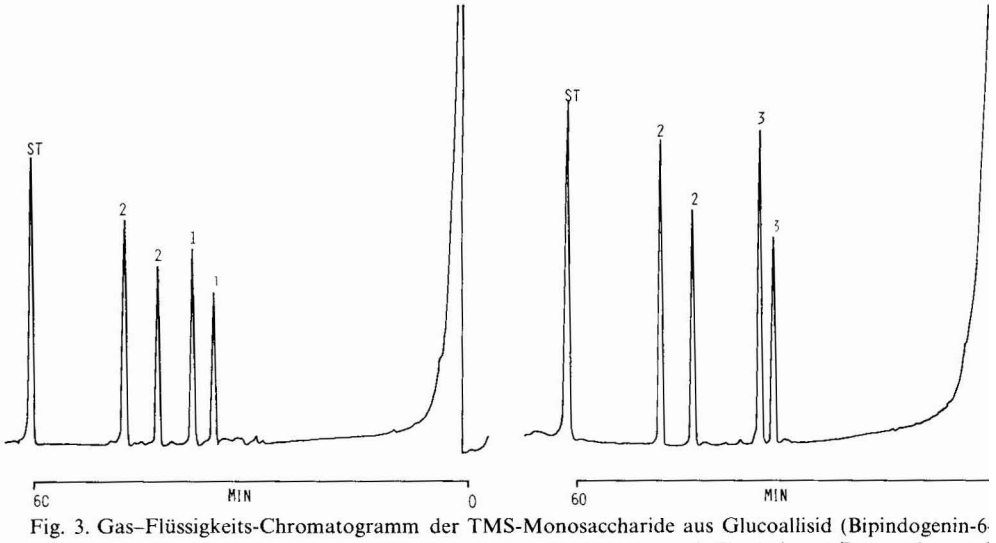

Fig. 3. Gas–Flüssigkeits-Chromatogramm der TMS-Monosaccharide aus Glucoallisid (Bipindogenin-6 desoxyglucosido-glucosid) nach saurer Hydrolyse, Trennbedingungen vgl. Fig. 1. 1 = 6-Desoxyglucose, 2 = Glucose, ST = Phenyl-β-D-glucopyranosid.

Fig. 4. Gas–Flüssigkeits-Chromatogramm der TMS-Monosaccharide aus Cheirosid A (Uzarigenin-fucosi do-glucosid) nach saurer Hydrolyse, Trennbedingungen vgl. Fig. 1. 2 = Glucose, 3 = Fucose, ST = Phenyl-β-D-glucopyranosid.

Hydrolyse und Probenaufbereitung nicht zu vermeidenden Substanzverluste erfasst werden konnten. Bei Einsatz von 0.50–1.00 mg Glykosid erhielten wir gut auswertbare Gaschromatogramme (Figs. 3–6) und stellten fest, dass unter den angegebenen Bedingungen 71–92% der in den Glykosiden enthaltenen absoluten Zuckermengen wiederzufinden waren (Tabelle I). Bei Di- bzw. Triglykosiden waren die mit den Faktoren korrigierten Peakflächenverhältnisse mit dem molaren Verhältnis der Zucker gut korrelierbar.

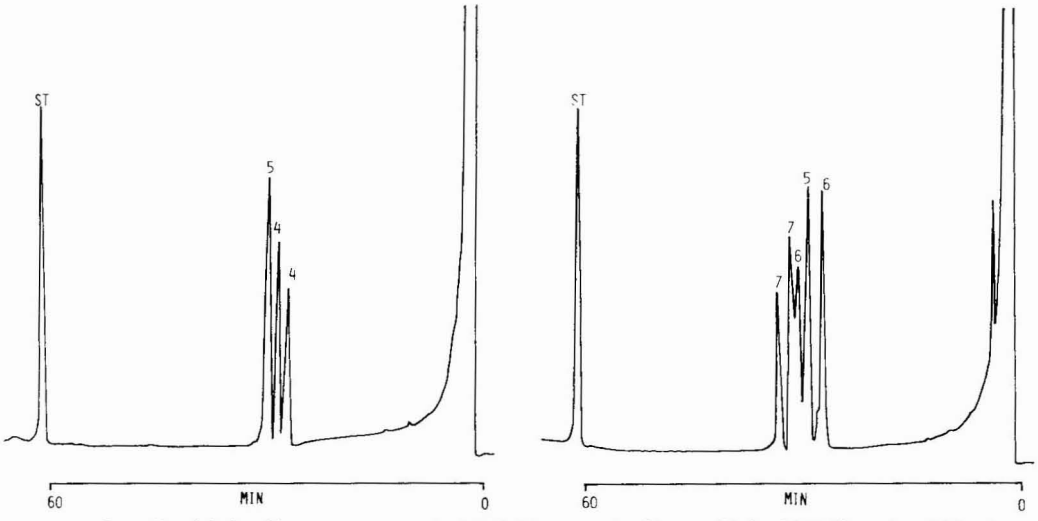

Fig. 5. Gas–Flüssigkeits-Chromatogramm der TMS-Monosaccharide aus Glykosid U (Strophanthidin-6-desoxyallosido-arabinosid) nach saurer Hydrolyse, Trennbedingungen vgl. Fig. 1. 4 = Arabinose, 5 = 6-Dexoxyallose, ST = Phenyl-β-D-glucopyranosid.

Fig. 6. Gas–Flüssigkeits-Chromatogramm der TMS-Monosaccharide aus Glykosid A_1 (Lit. 19) nach saurer Hydrolyse, Trennbedingungen vgl. Fig. 1. 5 = 6-Desoxyallose, 6 = Rhamnose, 7 = Xylose, ST = Phenyl-β-D-glucopyranosid.

Das beschriebene Verfahren eignet sich somit nicht nur für die Identifizierung der in herzwirksamen Glykosiden auftretenden Monosaccharid-Bausteine, sondern lässt unter genauer Einhaltung der standardisierten Hydrolysebedingungen auch sichere Aussagen über die Zusammensetzung der Zuckerkette zu. Der Zeit- und Informationsgewinn gegenüber den früher verwendeten Verfahren ist erheblich.

ZUSAMMENFASSUNG

Ein neues, im Vergleich zu bisher bekannten Methoden einfaches und genaues Verfahren ermöglicht die Identifizierung und quantitative Bestimmung von in herzwirksamen Glykosiden vorkommenden Monosaccharid-Bausteinen. Nach hydrolytischer Spaltung bei standardisierten Bedingungen gelingt unter Verwendung von OV-101 bzw. OV-17 als stationäre Phase die gaschromatographische Identifizierung der Trimethylsilyläther von Digitoxose, Cymarose, Fucose, Rhamnose, 6-Desoxyallose, 6-Desoxygulose, 6-Desoxyglucose, 6-Desoxyidose, 6-Desoxytalose, 3-O-Methylglucose, Allose, Altrose, Gulose, Idose, Glucose, Mannose, Galaktose, Talose,

TABELLE I

WIEDERFINDUNGSRATEN DER ZUCKER NACH SÄUREHYDROLYTISCHER SPALTUNG VON HERZWIRKSAMEN GLYKOSIDEN BEKANNTER KONSTITUTION

Glykosid	*Zucker-komponente(n)*	*Einwaage (mg)*	*Rückgewinnung (%)*
Convallatoxol	Rhamnose	0.98	88
Periplorhamnosid	Rhamnose	0.78	71
Desglucocheirotoxin	6-Desoxygulose	1.00	91
Perigulosid	6-Desoxygulose	0.67	88
Strophallosid	6-Desoxyallose	0.70	85
Peripallosid	6-Desoxyallose	0.83	85
Cheirosid A	Fucose	0.97	83
	Glucose		92
Glucoallisid	6-Desoxyglucose	0.83	88
	Glucose		89
Glykosid U[11]	6-Desoxyallose	0.90	83
	Arabinose		81
Glykosid A_1[19]	6-Desoxyallose	1.07	84
	Xylose		87
	Rhamnose		82
Sarmentosid A	6-Desoxytalose	0.56	78
Cymarin	Cymarose	0.65	73
Helveticosid	Digitoxose	0.73	75

Arabinose, Lyxose, Ribose und Xylose, wobei als Identifizierungsmerkmale neben den Retentionszeiten der Hauptpeaks auch die gut reproduzierbare Peakflächenverteilung ("fingerprints") der mit diesem System trennbaren anomeren Formen der einzelnen TMS-Monosaccharide herangezogen wurden. Mit Hilfe von Phenyl-β-D-glucopyranosid als innerem Standard konnten bei Analyse der Zuckerkomponenten von herzwirksamen Glykosiden bekannter Struktur 71–92 % der absoluten Monosaccharidmenge wiedergefunden werden. Das Verfahren erlaubt somit auch die rasche Feststellung der quantitativen Zusammensetzung der Zuckerketten unbekannter Glykoside.

DANK

Für die Überlassung von Vergleichssubstanzen danken wir Herrn Professor Dr. T. Reichstein, Basel, Schweiz auf das herzlichste. Die verwendeten gaschromatographischen Einheiten wurden durch Mittel der Österreichischen Apothekerkammer bzw. aus Sondermitteln des Österreichischen Bundesministeriums für Wissenschaft und Forschung finanziert.

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AFFINITY TECHNIQUE FOR THE ISOLATION OF POLYPEPTIDES CONTAINING ARGININE MODIFIED WITH CYCLOHEXANE-1,2-DIONE, AND THEIR ANALYSIS BY COMBINED GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A technique is described whereby a polypeptide containing an arginine residue that has been modified with cyclohexane-1,2-dione may be digested with a protease and any arginine-containing peptides specifically adsorbed to an affinity column consisting of immobilized borate. After desorption, the peptides may be converted into a derivative compatible with N-trifluoroacetylation and permethylation and then subjected to analysis by combined gas–liquid chromatography–mass spectrometry. Alternatively, after isolation the cyclohexanedione group may be removed and the peptide analysed by conventional procedures. Improved reaction conditions, involving use of urea, for modification with cyclohexanedione are described that were used successfully to modify insulin and a 65-residue haem-containing fragment from cytochrome-*c*. The sequence Arg–Gly–Phe was identified by mass spectrometry in a peptide isolated by affinity chromatography of a digest of cyclohexanedione-modified insulin. The methods described in this paper are appropriate both to primary structure determination and in structure–function studies via chemical modification of arginine residues.

INTRODUCTION

The analysis of partial hydrolysates of proteins and polypeptides by combined gas–liquid chromatography–mass spectrometry (GLC–MS)[1–6] offers several advantages over techniques of amino acid sequence determination that rely on the Edman degradation. To be able to exploit these advantages, the oligopeptide derivatives which it is necessary to prepare must both be volatile and possess good mass spectral characteristics. N-Trifluoroacetylation followed by permethylation is suitable and is directly applicable to peptides containing residues of any of the commonly occurring amino acids, except arginine[7]. This amino acid does not give rise to volatile products

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owing to the high basicity of its guanido group, which must therefore first be modified in a separate reaction.

The fact that arginine residues are chemically distinct from residues of the other amino acids permits their selective modification. Of the various methods available, the products of hydrazinolysis, condensation with pentane-2,4-dione and treatment with cyclohexane-1,2-dione (CHD) in sodium hydroxide solution have proved chemically compatible with the permethylation reaction involving dimethylsulphinyl sodium and methyl iodide[3,7,8]. CHD in sodium hydroxide solution has been used in conjunction with an earlier type of permethylation reaction[9]. When CHD is used in borate buffers, its selectivity for arginine residues becomes complete[10–13]. In summary, the adduct that is formed possesses a 1,2-diol function which is stabilized by borate. At low pH, the borate–diol complex dissociates and the free adduct is stable. At alkaline pH in the absence of borate, the adduct spontaneously regenerates CHD and the guanido group, a process which may be accelerated by the addition of nucleophiles such as hydroxylamine. At pH > 12, a rearrangement takes place to yield a compound containing a spiro carbon atom. Trypsin does not cleave on the C-terminal side of arginine residues modified with CHD, a feature that is useful for amino acid sequence determination, especially in view of the reversible nature of the modification (compare the utility of reversible acylation of lysine residues[14]).

Peptides modified with CHD may be isolated by the usual techniques of gel filtration, paper chromatography and electrophoresis, if necessary using a radioactively labelled reagent[15]. The ability of the 1,2-diol function to bind borate can be exploited for affinity chromatography on a column of immobilized borate. Any peptides so isolated and therefore containing such a modified arginine residue could be identified mass spectrometrically either after alkaline rearrangement to the spiro form[7] or after regeneration of the guanido group followed by one of the procedures for arginine-containing peptides, usually hydrazinolysis or condensation with pentane-1,4-dione. An advantage of direct alkaline rearrangement of the diol is that this would retain the distinction between an arginine residue that had been modified with CHD and one that had not. Alternatively, after isolation on the borate column and regeneration of the guanido group, the peptides would, of course, be amenable to sequence determination by a non-mass spectrometric method such as the Edman degradation. This paper describes some investigations concerning the formation, isolation and analysis of the adducts of CHD with various arginine-containing polypeptides.

EXPERIMENTAL

Unless otherwise specified, reagents were of analytical-reagent grade and were used without further purification. High-voltage paper electrophoresis and staining of separated peptides were as previously described[16]. Cellulose acetate electrophoresis was performed in a Shandon MMB tank on strips of cellulose acetate (Schleicher & Schüll, Dassel, G.F.R.) and using a buffer prepared by dissolving 3.1 g of boric acid in 200 ml of deionized 8 *M* urea whilst diluting with water and 1 *M* sodium hydroxide solution; the final volume was 250 ml (pH 8.0). The 3-cm wide strips drew approximately 0.05 mA at 200 V over a length of approximately 15 cm. After air drying

at 80–100°C, staining was effected with Ponceau S (0.2% in 3% acetic acid) and destaining with 3% acetic acid. Prior to acid hydrolysis for amino acid analysis, 20 μl of mercaptoacetic acid (reagent grade, Hopkin & Williams, Chadwick Heath, Great Britain) was added when required.

Preparation of the CHD adduct of porcine insulin

Boric acid (1.55 g) was dissolved in water as the pH was adjusted to 9.0 with 5 *M* sodium hydroxide solution; the final volume was 50 ml (0.5 *M*). A 1.00-g amount of CHD (Aldrich, Milwaukee, WI, U.S.A.) was dissolved in 28.6 ml of buffer and the pH was readjusted with 5 *M* sodium hydroxide solution to pH 9.0 (0.3 *M* CHD). Boric acid (1.55 g) was dissolved in deionized 8 *M* urea and the pH was adjusted to 9.0 with 5 *M* sodium hydroxide solution; the final volume was 50 ml (0.5 *M*). CHD (175 mg) was dissolved in 5.0 ml of this buffer and the pH was readjusted with 5 *M* sodium hydroxide solution to pH 9.0 (0.3 *M* CHD). Four 100-mg batches of porcine insulin (Monocomponent, Novo) were taken. To lot A were added 5 ml of water and then 5 ml of buffered CHD solution. To lot B were added 5 ml of deionized 8 *M* urea and then 5 ml of 0.5 *M* borate buffer (pH 9.0). To lot C were added 5 ml of deionized 8 *M* urea and then 5 ml of buffered CHD solution. To lot D were added 5 ml of deionized 8 *M* urea and then 5 ml of buffered CHD–urea solution. All samples were soluble and were incubated for 2 h at 37°C. Each was then rinsed with 10 ml of 30% acetic acid into a length of boiled (1% w/v sodium hydrogen carbonate solution, 15 min) 18/32 Visking dialysis tubing and dialysed at 4°C against 5 l of cold 1% acetic acid with stirring. The diffusate was changed after 3 h, a further 17.5 h and a further 5 h. The bag contents were then freeze-dried. Yields were 99–100 mg. Samples were hydrolysed for amino acid analysis. In each instance, a duplicate hydrolysis was performed in the presence of 20 μl of mercaptoacetic acid. Samples were also examined by cellulose acetate electrophoresis.

Affinity chromatography of modified insulins and control

Samples (approximately 2 mg) of the modified insulins and the unmodified control (B) were dissolved in 0.5 ml of 1% N-ethylmorpholine (Koch-Light, Colnbrook, Great Britain; redistilled before use) buffer (pH 8.0 with acetic acid) and applied to a column (bed dimensions 80 × 5.5 mm diameter) containing *m*-aminophenylboronic acid coupled to epoxy-activated Sepharose 6B[7] that had been equilibrated and was then eluted with the same buffer. Fractions of 2.5 ml were collected. After the fifth fraction had eluted, the eluent was replaced with 1% acetic acid. The UV spectra of fractions were determined.

Performic acid oxidation of insulin

A 50-mg amount of insulin (or derivative) was dissolved in 2 ml of 98% formic acid–100-volume hydrogen peroxide (9:1). After 1 h at room temperature the solution was diluted with 100 ml of water and freeze-dried to a fluffy white powder.

Peptic digestion and application to the affinity column

A 12-mg amount of performic acid-oxidized insulin (or derivative) was dispersed in 0.3 ml of pH 1.9 buffer (formic acid–acetic acid–water, 1:4:45), then 1.5 mg of pepsin (Sigma, St. Louis, MD, U.S.A.) was dissolved in 100 μl of pH 1.9 buffer and

50 μl were added to each sample. After incubating overnight at 37°C, 10-μl aliquots were subjected to high-voltage paper electrophoresis at pH 1.9 and 6.5 and the sheets cut and the separate tracks stained with cadmium–ninhydrin, Pauly, Sakaguchi and α-nitroso-β-naphthol stain. The digests were adjusted to pH 8 with pure N-ethylmorpholine and applied to the borate column as described above for undigested material. Fraction 2 (unbound material) and fractions 7 and 8 (pooled material that bound to the column and was then eluted at low pH) were freeze-dried, taken up in 200 μl of water and 2-μl aliquots were electrophoresed at pH 1.9 and 6.5. Aliquots of 2 μl were hydrolysed in the presence of mercaptoacetic acid for amino acid analysis. Aliquots of 2 μl were treated with 100 μl of 0.2 *M* hydroxylammonium chloride which had been adjusted to pH 7.0 with sodium hydroxide solution, at 37°Cvfor 23 h and were then hydrolysed in the presence of mercaptoacetic acid.

Isolation and analysis of the arginine-containing peptide from CHD-modified insulin

A mixture of 20 mg of CHD-modified insulin (C) and 1.3 mg of V8 protease (Miles Labs., Slough, Great Britain) was dissolved in 1 ml of 1 % N-ethylmorpholine, which had been adjusted to pH 8.0 with acetic acid, and applied to the borate column equilibrated with the N-ethylmorpholine buffer. A 0.2-ml volume of V8 protease solution (2 mg/ml in the same buffer) wass then applied and the flow stopped. After overnight incubation at room temperature (28°C), the column was eluted as described above. Fraction 7 (opalescent) was clarified by addition of 1 drop of glacial acetic acid. The UV spectra of fractions were determined. Unbound and bound material (fractions 2 and 7, respectively) were dried down, taken up in 300 μl of water plus 50 μl of pH 1.9 buffer and 10-μl aliquots were subjected to high-voltage paper electrophoresis at pH 1.9. Fraction 7 was then dried down before treatment with pentane-2,4-dione (reagent grade; BDH, Poole, Great Britain)[8]. Analytical electrophoresis at pH 6.5 showed evidence of approximately 50 % conversion. This was thought to be due to traces of the pH 1.9 buffer neutralizing some of the triethylamine, so the pentane-2,4-dione treatment was repeated. Analytical electrophoresis at pH 6.5 then showed that conversion was essentially complete. The peptide derivative was taken up in 500 μl of 1 % (w/v) ammonium hydrogen carbonate solution, pH 8, and incubated at 37°C for 5 h with α-chymotrypsin (Sigma), added as 50 μl of a 2 mg/ml solution in water. After removal of solvent in a vacuum desiccator, the digest was dissolved in dimethyl sulphoxide, trifluoroacetylated and permethylated; the product was taken up in chloroform and 7 % of the solution analysed by GLC–MS as previously described[6].

RESULTS AND DISCUSSION

Formation of the adduct

When porcine insulin was subjected to reaction conditions recommended for proteins[10], amino acid analysis (A, Table I) showed extensive but nevertheless incomplete derivatization of the single arginine residue at position B22. This was thought to be due to steric hindrance, so denaturing conditions were considered. Guanidinium chloride, a commonly used protein solubilizing and denaturing agent, would be expected to complete effectively with arginyl side-chains for the CHD reagent, so no experiments were performed with it. Urea, the other commonly used denaturant, is also known to react with 1,2-diketones and this was thought to preclude its use during

TABLE I

RESULTS OF AMINO ACID ANALYSIS OF PORCINE INSULIN TREATED IN VARIOUS WAYS (A–D, SEE TEXT)

MA = mercaptoacetic acid; PI = porcine insuline. The results are expressed as ratios, normalised to Leu = 6.00. "Bound" refers to material which bound to the borate column.

Amino acid	*A*		*B*		*C*		*D*		*Bound*		*PI***
	HCl	*HCl/MA**	*HCl*	*HCl/MA**	*HCl*	*HCl/MA**	*HCl*	*HCl/MA**	*HCl*	*HCl/MA**	
Asp	3.19	3.08	2.99	3.26	2.99	3.14	3.01	3.17	3.48	3.21	(3)
Thr	2.25	2.54	2.00	2.90	1.98	2.69	1.98	2.65	1.89	1.85	(2)
Ser	3.06	2.67	2.89	2.76	2.81	2.68	2.82	2.72	2.69	2.52	(3)
Glu	7.13	7.13	7.09	7.18	7.15	7.08	7.12	7.26	6.98	6.74	(7)
Gly	3.85	3.80	3.60	4.00	3.81	4.03	3.64	3.67	3.88	3.50	(4)
Ala	2.09	2.02	1.88	2.02	1.94	1.92	2.00	2.02	1.95	1.93	(2)
Val	3.06	3.02	3.09	3.12	3.09	3.14	3.14	3.21	3.11	3.19	(4)
Cys_2	2.35		2.50		2.38		2.45				(3)
Ile	1.20	1.09	1.25	1.11	1.21	1.15	1.22	1.17	1.30	1.15	(2)
Leu	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	(6)
Tyr	3.29	4.01	3.87	4.03	3.22	3.95	3.16	4.08	3.01	3.92	(4)
Phe	2.88	2.87	3.02	2.87	2.89	2.81	2.87	2.87	2.85	2.89	(3)
	2.02	2.61	2.06	2.01	2.04	2.62	2.01	2.62	2.01	2.82	(2)
Lys	0.76	0.82	0.70	0.75	0.84	0.85	0.84	0.85	0.88	0.87	(1)
Arg	0.42	0.28	0.98	0.94	0.28	0.14	0.31	0.17	0.22	<0.05	(1)

* Values obtained after hydrolysis in the presence of mercaptoacetic acid, which prevents regeneration of arginine from its CHD adduct which is otherwise about 20%[10].

** Composition of porcine insulin[23]. Proline (1 residue) was not quantitated.

*** Hydrolysis of the CHD adduct of arginine in the presence of mercaptoacetic acid gives rise to a neutral product[10] which elutes on our analyser at the histidine position[7].

modifications with CHD[17]. However, guanidines are more reactive than urea[18], so one might expect to achieve a degree of arginine modification in urea solutions. This was found to be the case: a higher degree of derivatization of porcine insulin may be achieved in 4 or 8 *M* urea (Table I). The slight difference between the 4 and 8 *M* results may not be significant. Analysis of the products by cellulose acetate electrophoresis confirmed the results of amino acid analysis. In a phosphate–urea buffer (pH 7.2), CHD-modified insulin is not separated from native insulin. In a borate–urea buffer, CHD-modified insulin runs towards the anode about 1.4 times faster than unmodified insulin, consistent with the introduction of an extra negative charge on complexation with borate. The N-terminal cyanogen bromide fragment of horse-heart cytochrome-*c*, consisting of residues 1–65 and the haem prosthetic group, was also successfully modified at the single arginine residue with CHD in 4 *M* urea (Table II). The use of at least 4 *M* urea is recommended whenever quantitative reaction of CHD with the arginine residues of polypeptides or proteins is being attempted.

Isolation of CHD-modified peptides by affinity chromatography

Substances possessing 1,2- or 1,3-diol functions that can attain a suitable orientation are able to form covalent complexes with borate. Complex formation, which is reversible, is strongly favoured at alkaline pH. Borate attached to a solid support may thus be used to bind specifically such substances[19,20], which may then be released at a

TABLE II

RESULTS OF AMINO ACID ANALYSIS OF THE CHD-ADDUCT OF THE N-TERMINAL CNBr FRAGMENT OF HORSE HEART CYTOCHROME-*c*

All values normalized to Leu = 3.00.

Amino acid	*HCl/MA*	*HCl/ MA**	*PP***
Asp	5.09	5.18	(5)
Thr	6.19	5.90	(7)
Glu	6.68	6.59	(7)
Gly	10.21	9.95	(10)
Ala	3.15	2.99	(3)
Val	2.22	2.10	(3)
Ile	1.56	1.42	(2)
Leu	3.00	3.00	(3)
Tyr	0.99	0.88	(1)
Phe	2.49	2.31	(3)
His***	4.09	4.30	(3)
Lys	10.82	11.54	(11)
Arg	0.28	0	(1)

* See footnote to Table I.

** Composition of the polypeptide[27].

*** See footnote to Table I. Proline (2 residues), cysteine (2 residues) and homoserine (1 residue) not determined.

later stage. This specific recognition of a target species by an immobile ligand has become known as affinity chromatography. *m*-Aminophenylboronic acid is a suitable ligand in that it possesses a nucleophilic group for attachment to an activated support, it is a stable compound, it may be quantitated in solution by UV spectrophotometry and it is readily available. This ligand was coupled to epoxy-activated Sepharose 6B, a support found to be preferable to cyanogen bromide-activated Sepharose 4B[7]. In preliminary experiments, the column produced demonstrated an ability to bind selectively the CHD–guanido adducts of arginine and of Ser–Pro–Phe–agmatine (Agm). As borate–diol complexes are unstable at low pH, it proved possible to release the adducts with dilute acetic acid. The adduct is thus released by a volatile solvent at a pH at which it is stable even in the absence of borate.

In addition to binding small peptides carrying the diol group, the column is equally satisfactory when modified polypeptides are used. Unmodified insulin (B, see Table I) was found not to bind to the borate column (100% of the absorbance at 280 nm was found in fractions 1 and 2), whereas modified insulin (D, see Table I) does bind (15% of the absorbance at 280 nm eluted in fractions 1 and 2 and 85% in fractions 7 and 8; the latter figure agrees well with the value of 83% for the diol content of sample D deduced from the amino acid analysis result in Table I). Bound material, released by 1% acetic acid, was found to be completely derivatized (Table I).

Application to the borate column of a peptic digest of performic acid-oxidized, CHD-modified insulin led to the isolation of a set of peptides containing the modified arginine residue. The digest before application contained a set of peptides similar in

electrophoretic mobility to those which stained for arginine in the case of the control; electrophoresis showed that these were selectively bound on application to the borate column and were released, in fraction 7, on decreasing the pH of the eluent. UV spectrophotometry, electrophoresis and amino acid analysis showed that no peptides were present in fraction 7 of the control. Amino acid analysis of the isolated modified peptides was consistent with the expected cleavage pattern of pepsin around the modified arginine residue:

–Glu–Ala–Leu–Tyr–Leu–Val–Cys–Gly–Glu–Arg–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala
↑ ↑ ↑ ↑ ↑ ↑

The yield based on the Gly, Glu, Leu and Arg values of this analysis was 1 μmol (50 %).

The CHD–diol group is stable even in the absence of borate at the low pH required for peptic digestion. Pepsin is a very convenient protease to use on this account. Digestion with trypsin may be performed in a phosphate buffer at pH 6.8 without decomposition of the CHD adduct[15]. If alkaline conditions are required for the digestion, as is sometimes the case, borate should be present in order to avoid substantial loss of the diol group[10,11]. If a high concentration of borate is used, say 0.1 *M*, then this complexes with the diol group of the adduct at the pH used for application to the borate column and effectively prevents binding to the column. For example, less than 10 % of CHD-modified insulin binds when applied in 0.1 *M* sodium borate at pH 8 to a column equilibrated and then eluted with 1 % N-ethylmorpholine acetate (pH 8). Although it should be possible, by using a longer or more highly substituted column, a lower borate concentration and perhaps a lower pH, to find conditions suitable for the application of alkaline digests, another possibility is to carry out the digestion with the sample bound to this borate column itself. This has been achieved. Digestion with V8 protease[21] of modified insulin bound to the borate column led to the isolation of a single peptide of electrophoretic mobility[22] at pH 6.5 of $M_{Asp} = -0.49$, and at pH 1.9 $M_{Ser} = 1.16$. The expected mobilities of Arg(DHCH)–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala are $M_{Asp} = -0.43$ at pH 6.5 and $M_{Ser} = 1.18$ at pH 1.9. The peptide stained red with the cadmium–ninhydrin reagent and was Sakaguchi negative.

Analysis of the modified peptide by GLC–MS

The peptide isolated by affinity chromatography from the V8 digest of modified insulin was treated with pentane-2,4-dione, digested with chymotrypsin, N-trifluoroacetylated and permethylated and an aliquot of the products analysed by GLC–MS. The selected ion record of *m/e* 150, diagnostic of the modified arginine side-chain, showed one maximum at a retention time of 28 min 22 sec. A full spectrum of the modified arginine-containing tripeptide Arg–Gly–Phe was obtained (Fig. 1), corresponding to residues 22–24 of the B-chain of porcine insulin[23].

CONCLUSION

It is possible to use a column of immobilized borate to isolate arginine-containing peptides modified with cyclohexane-1,2-dione from a proteolytic digest of a modified polypeptide carried out either at acidic or alkaline pH. The modified arginine

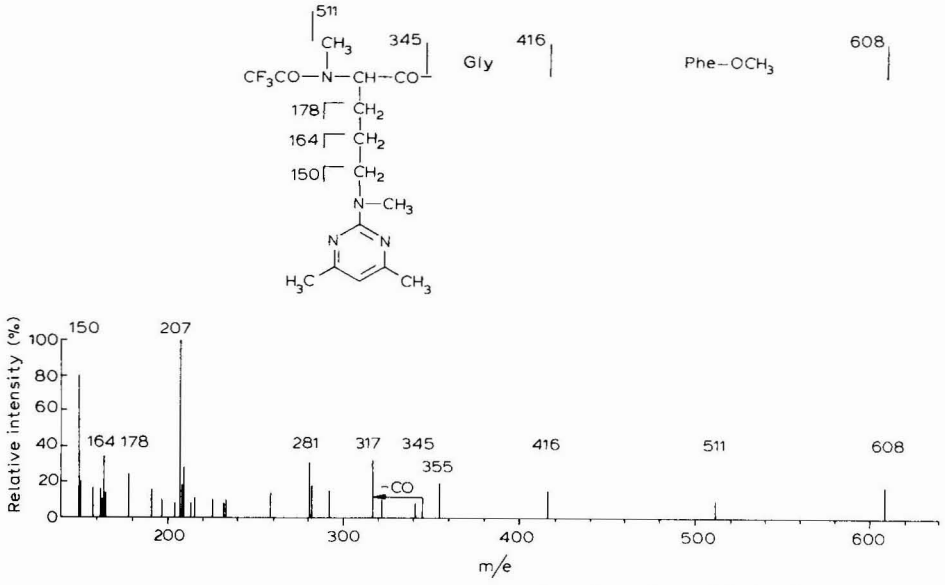

Fig. 1. Mass spectrum showing the sequence "Arg"–Gly–Phe. The arginyl residue has been converted into a residue of 4,6-dimethylpyrimidyl-2-ornithine. In this figure, the usual three-letter code is used to denote the N,O-permethylated amino acid residues. This is the raw mass spectrum ($m/e > 150$) stored as scan 185 of the GLC–MS run described in the text. Ions appearing at m/e 207, 281, 341 and 355 are due to column bleed. The sequence of the tripeptide is easily deduced via the strong sequence ions.

residue may be identified in sequence by GLC–MS. Since the completion of this work, a paper has appeared[24] describing the isolation, on a commercially available borate gel, of an arginine-containing peptide after reaction with CHD. It is of interest that the products of modification of arginine residues with butanedione in borate buffer[25] and with phenylglyoxal in borate buffer[26] are also believed to contain the 1,2-diol moiety and so could in principle be isolated by affinity chromatography on a column of immobilized borate.

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DETERMINATION OF PLASMA AND BRAIN CONCENTRATIONS OF TRAZODONE AND ITS METABOLITE, 1-*m*-CHLOROPHENYLPIPERAZINE, BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific gas chromatographic procedure is described for the quantitation of trazodone and its active metabolite, 1-*m*-chlorophenylpiperazine (mCPP), in plasma and brain. After addition of internal standards, the samples were extracted with benzene and the extracts divided into two portions. One portion was evaporated to dryness, the residue dissolved in methanol and the solution injected into a gas chromatograph equipped with a nitrogen-selective detector, for trazodone quantitation. To the remaining half of the extracts, 100 μl of heptafluorobutyric anhydride solution were added and the metabolite was measured as the heptafluorobutyryl derivative by electron-capture detection. Gas chromatography–mass spectrometry was used to confirm the specificity of the analyses.

The kinetic profile of trazodone and its metabolite was investigated after oral administration of trazodone (25 mg/kg). The parent drug and its metabolite both accumulated in brain, reaching concentrations several times those in plasma. More mCPP than the parent compound entered the brain; the ratio of the area under the curve for trazodone to mCPP in plasma was about 4, whereas in brain it was only about 0.8.

INTRODUCTION

Trazodone,2-{3-[4-(*m*-chlorophenyl)-1-piperazinyl]propyl}-*s*-triazolo[4,3*a*]pyridin-3-(2*H*)-one hydrochloride, a non-tricyclic antidepressant, is extensively metabolized in man and animals, only a small percentage being excreted unchanged in the 24-h urine and faeces[1]. The main products of elimination are hydroxylated derivatives[2,3] and oxotriazolopyridinepropionic acid originating from oxidative cleavage of the parent compound[2,4,5]. There is no evidence that these metabolites are biologically active.

One metabolic pathway includes oxidation of the phenylpiperazine system of the trazodone molecule, with the formation of 1-*m*-chlorophenylpiperazine (mCPP), which has recently been isolated as the glucuronide from rat urine[6]. Pharmacological studies indicated that mCPP was more active than trazodone as an inhibitor of the

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membrane uptake mechanism for serotonin (5HT) in the brain[7], a property shared by some tricyclic antidepressants[8]. Subsequent studies showed that mCPP behaved like a 5HT agonist in the central nervous system[9–11]. Some workers[12] reported that trazodone acted as a central 5HT agonist at high doses but at lower doses it exerted anti-5HTergic activity, suggesting that the former effect might be induced by its metabolite. It is therefore of interest to follow the time courses of the production and elimination of mCPP, because in the studies mentioned[12] the metabolite was not directly identified in trazodone-treated animals. Moreover, in seeking a relationship between the plasma and tissue concentrations and pharmacological effects it is necessary to quantitate both the metabolite and trazodone.

Methods available for the quantitation of trazodone use spectrofluorimetry[1], ^{14}C-labelled compound[2,4], gas chromatography (GC) with flame-ionization detector or mass fragmentographic techniques[13], and are aimed only at the analysis of the parent compound. This paper reports a GC method for the quantitation of trazodone in biological samples, using a simpler and more sensitive procedure, *viz.*, rapid extraction and the use of a nitrogen-selective detector. The procedure includes quantitation of the metabolite by GC with electron-capture detection. GC–mass spectrometry (MS) was used to confirm the presence of the drug and its metabolite. Plasma and brain concentration curves were plotted for both compounds after oral administration of trazodone to rats.

EXPERIMENTAL

Trazodone hyydrochloride was supplied by Angelini (Rome, Italy), mCPP hydrochloride by Aldrich-Europe (Beerse, Belgium), benperidol by Janssen (Beerse, Belgium) and 4-amino-1-(6-chloro-2-pyridyl)piperidine hydrochloride by Clin-Midy (Montpellier, France). Heptafluorobutyric anhydride (HFBA), as a 25% (v/v) solution in ethyl acetate, was obtained from Pierce (Rockford, IL, U.S.A.). Formic acid, *n*-heptane, chloroform and benzene were obtained from Carlo Erba (Milan, Italy).

Apparatus

GC determinations of trazodone were carried out on a Dani 3400 gas chromatograph equipped with a nitrogen–phosphorus detector (NPD). A glass column (1 m × 3 mm I.D.) packed with 80–100-mesh Gas-Chrom Q with 3% OV-1 as the stationary phase (Supelco, Bellefonte, PA, U.S.A.) was used. The temperatures of the column, detector and injector port were maintained at 270°C. The carrier gas was nitrogen at a flow-rate of 30 ml/min.

mCPP heptafluorobutyrate was analysed on a Carlo Erba Fractovap 2150 instrument equipped with a ^{63}Ni electron-capture detector. The chromatographic column was a glass tube (2 m × 3 mm I.D.) packed with 80–100-mesh Supelcoport with 3% OV-17 as the stationary phase (Supelco). The oven, injector port and detector temperatures were 205, 250 and 250°C, respectively. The carrier gas was nitrogen at a flow-rate of 35 ml/min.

GC–MS analysis was performed on an LKB 2091-051 instrument coupled with an LKB-2130 computer system for data acquisition and calculation. The gas chromatograph was operated under the conditions described above and mass spectra were collected in the electron-impact mode at 70 eV.

Animals

Male CD-COBS rats (Charles River, Como, Italy), average weight 250 g, were used.

Extraction from plasma

To 0.5–2 ml of heparin-treated plasma were added 50 μl of a methanolic solution of 4-amino-1-(6-chloro-2-pyridyl)piperidine (2 μg/ml) and water to a final volume of 2 ml, followed by 0.5 ml of 1 *N* sodium hydroxide solution and 10 ml of benzene. The samples were mechanically shaken and centrifuged. An 8.5-ml volume of the benzene phase was evaporated to dryness, the residue dissolved in 0.05 ml of a methanolic solution of benperidol (10 μg/ml)[13] and 1–2 μl were injected into the GC column described for trazodone analysis.

To the remaining benzene phase (1 ml), 100 μl of an ethyl acetate solution of HFBA (25%, v/v) were added and the samples were heated at 60°C for 30 min.

After the reaction the samples were washed with water (1 ml) and 5% aqueous ammonia solution (0.5 ml) and 1–2 μl of the benzene phase were injected into the GC column for mCPP analysis.

Extraction from brain

Brains were homogenized (9 ml/g) in cold acetone–1 *N* formic acid (85:15) and centrifuged. The supernatant was shaken twice with *n*-heptane–chloroform (4:1), the organic phase was discarded and the aqueous phase (0.5 ml) was used for drug extraction as described for plasma.

Internal standard calibration graphs

Drug-free plasma and brain samples containing known amounts of trazodone (0.05–0.5 μg) and mCPP (0.01–0.5 μg) were analysed concurrently with each set of unknown samples.

Calibration graphs were constructed by plotting the ratios of the peak areas of trazodone and mCPP to those of the respective internal standards and comparing the amounts of trazodone and mCPP added. Concentrations of trazodone and mCPP in the unknown samples were subsequently determined from the calibration graphs.

Recovery studies

Percentage recoveries were calculated by comparing the peak-area ratios of trazodone and mCPP heptafluorobutyrate after plasma and brain extraction with the peak-area ratios obtained by direct injection of standard solutions of trazodone or mCPP heptafluorobutyrate.

RESULTS AND DISCUSSION

Flame-ionization detection is not sensitive enough for kinetic studies of trazodone and the biological extracts have to be cleaned up in order to eliminate interfering constituents[13]. The selective detector significantly improves the sensitivity and reduces interference from endogenous constituents. However, electron-capture detection is not very sensitive to high-molecular-weight compounds such as trazodone, which contain only one chlorine atom. We chose the alternative of using an NPD,

achieving high sensitivity and specificity with the nitrogen-containing compound involved. Under the experimental conditions used the ratio of the peak area of trazodone to that of the internal standard was linear in the range 1–10 ng per injection. An additional sample dilution was necessary at concentrations higher than 10 ng per injection; 1 ng per injection (2 μl) was the detection limit, corresponding to 0.05 μg per millilitre of plasma or per gram of brain. The greater response of mCPP heptafluorobutyrate to the ECD provided high sensitivity and specificity in the analysis of the metabolite. Concentrations down to 10 ng per millilitre or per gram of tissue could still be measured. Thus, evaporation of the biological extracts, which resulted in unpredictable losses of mCPP, was avoided. The ratio of the peak area of mCPP heptafluorobutyrate to that of the internal standard was linear in the range from 0.01 ng (2 μl) to 0.5 ng per injection. Extraction from plasma and brain homogenates using *n*-hexane, diethyl ether or benzene was investigated. Benzene was found to be the most suitable as it gave consistent recoveries of both trazodone and mCPP after a single extraction. A summary of the recoveries during the kinetic studies in rats is reported in Table I. Trazodone was extracted reproducibly with a mean recovery of 81 ± 1.3% and a coefficient of variation (C.V.) of 3.6–10%. The recoveries of mCPP from plasma samples were 82–94% with a C.V. of 3.3–9.7%; recoveries from brain homogenates were 83–86% with a C.V. of 4.6–7.2%.

Figs. 1 and 2 are typical chromatograms of extracts from (A) a spiked brain sample, (B) the brain of a rat treated with trazodone (25 mg/kg, p.o.) and (C) a drug-free homogenized brain. The extracts from drug-free plasma or brain show no peaks that could interfere with the analysis of trazodone or its metabolite. Retention times were 4.2 min for trazodone and 5.3 min for the internal standard. The retention times of heptafluorobutyrate derivatives of mCPP and 4-amino-1-(6-chloro-2-pyridyl)-1-piperidine were 4.2 min and 3.3 min, respectively. Specificity of the analysis was confirmed when unknown plasma and brain samples of rats given trazodone were analysed by GC–MS. The mass spectra were identical with those obtained after

TABLE I

RECOVERY OF TRAZODONE AND 1-*m*-CHLOROPHENYLPIPERAZINE FROM PLASMA AND BRAIN

Each value is the mean of five determinations.

Sample	*Amount added (μg)*	*Trazodone*		*1-m-Chlorophenylpiperazine*	
		Amount found (μg ± S.D.)	*Recovery (% ± S.D.)*	*Amount found (μg ± S.D.)*	*Recovery (% ± S.D.)*
Plasma	0.01	–	–	0.008 ± 0.001	82 ± 8
	0.05	0.040 ± 0.003	80 ± 6	0.045 ± 0.004	91 ± 8
	0.10	0.080 ± 0.004	80 ± 4	0.090 ± 0.003	90 ± 3
	0.25	0.205 ± 0.080	82 ± 3	0.230 ± 0.007	92 ± 3
	0.50	0.404 ± 0.038	81 ± 8	0.469 ± 0.029	94 ± 6
Brain	0.05	0.040 ± 0.003	81 ± 5	0.041 ± 0.003	83 ± 6
	0.10	0.080 ± 0.008	80 ± 8	0.085 ± 0.006	85 ± 6
	0.25	0.198 ± 0.010	79 ± 4	0.215 ± 0.009	86 ± 4
	0.50	0.417 ± 0.037	83 ± 7	0.430 ± 0.020	85 ± 4

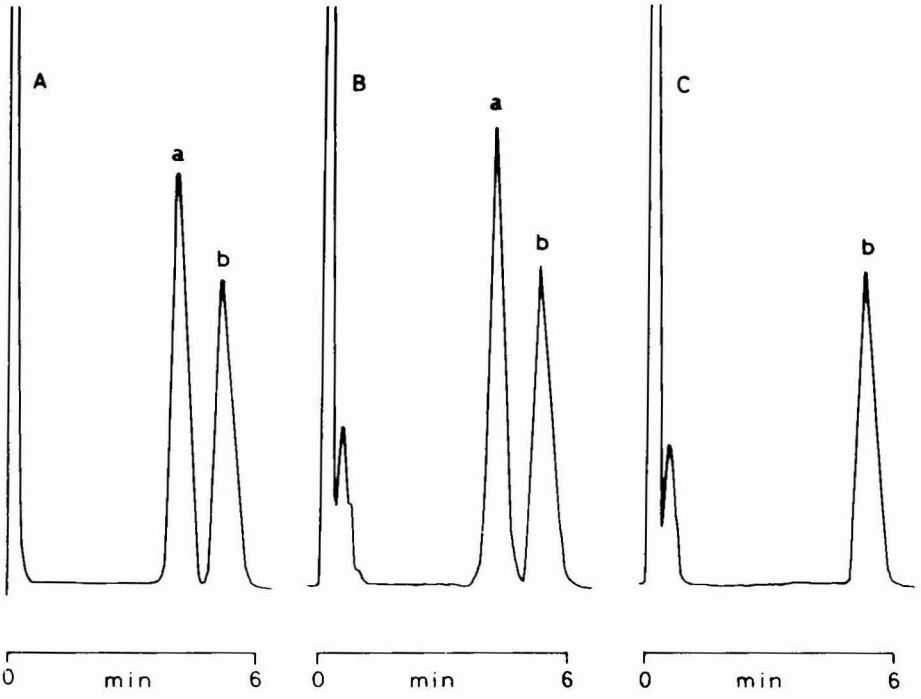

Fig. 1. Gas chromatograms of extracts from spiked brain sample (A), from brain of rats treated with trazodone (B) and from drug-free brain (C). Peaks: (a) trazodone; (b) internal standard.

injection of trazodone or mCPP heptafluorobutyrate. Fig. 3 shows the mass spectrum of mCPP heptafluorobutyrate. There is a base peak at m/e = 195 corresponding to the chlorophenylpiperazine moiety, which is further fragmented to give intense ions at m/e = 166, 139 and 111. In the high mass range the mass spectrum shows characteristic ions at m/e 373 and 357, which correspond to the loss of fluorine and chlorine, respectively, from the molecular ion. The mass spectrum of trazodone has been reported by other workers[13,14].

In order to collect information on the kinetic profile of trazodone and its active metabolite, male rats were treated orally with the parent compound (25 mg/kg) and the plasma and brain were analysed as described. Fig. 4 shows graphs of plasma and brain concentrations *versus* time. Trazodone was rapidly adsorbed, rising to peak plasma concentrations of 1.40 ± 0.40 μg/ml after 5 min. The plasma concentrations showed a biphasic decline thereafter, with an initial phase lasting about 30 min, followed by a second, slower phase with a half-life ($T_{\frac{1}{2}}$) of 50 min. Brain peak concentrations were reached after 15–30 min, declining thereafter in a monoexponential manner with $T_{\frac{1}{2}}$ = 77 min. Similar results have been obtained for the rat by other workers[1,2,13].

mCPP was detected in both plasma and brain 5 min after oral administration of the parent compound, reaching peak concentrations after 1–2 h. The metabolite disappeared from plasma ($T_{\frac{1}{2}}$ 97 min) more slowly than the parent compound, but its brain $T_{\frac{1}{2}}$ (83 min) was comparable to the brain $T_{\frac{1}{2}}$ of trazodone. The metabolite

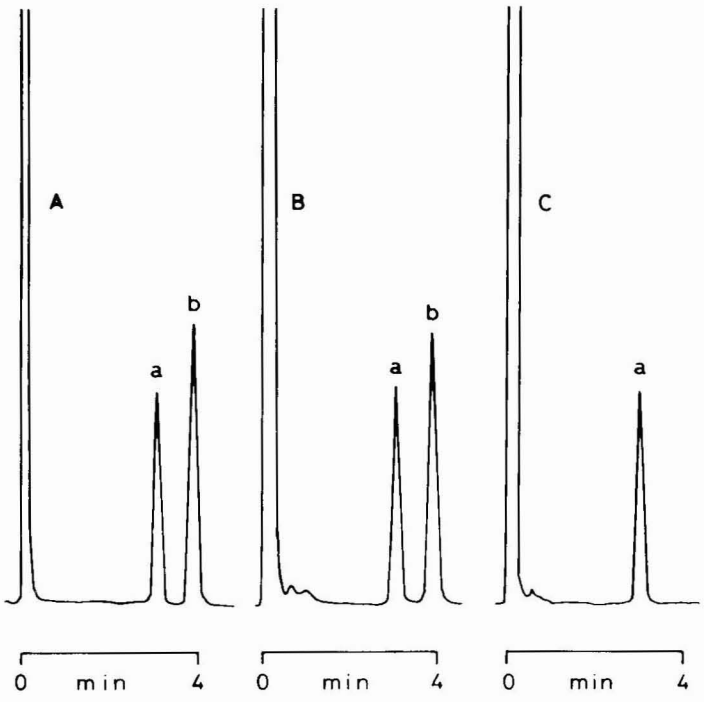

Fig. 2. Gas chromatograms of 1-*m*-chlorophenylpiperazine heptafluorobutyrate (b) and internal standard (a) from spiked brain sample (A), from brain of rats treated with trazodone (B) and from drug-free brain (C).

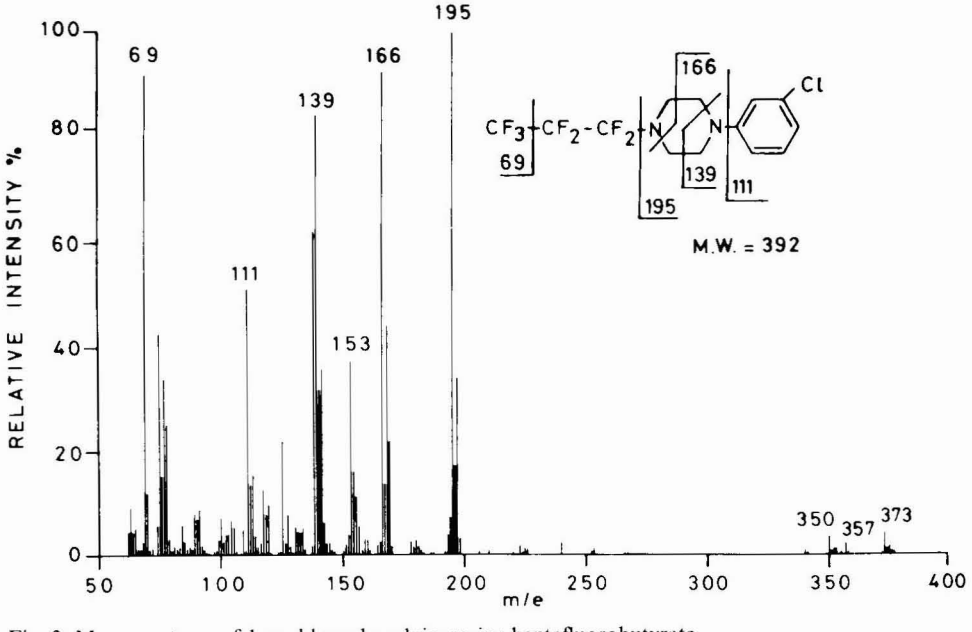

Fig. 3. Mass spectrum of 1-*m*-chlorophenylpiperazine heptafluorobutyrate.

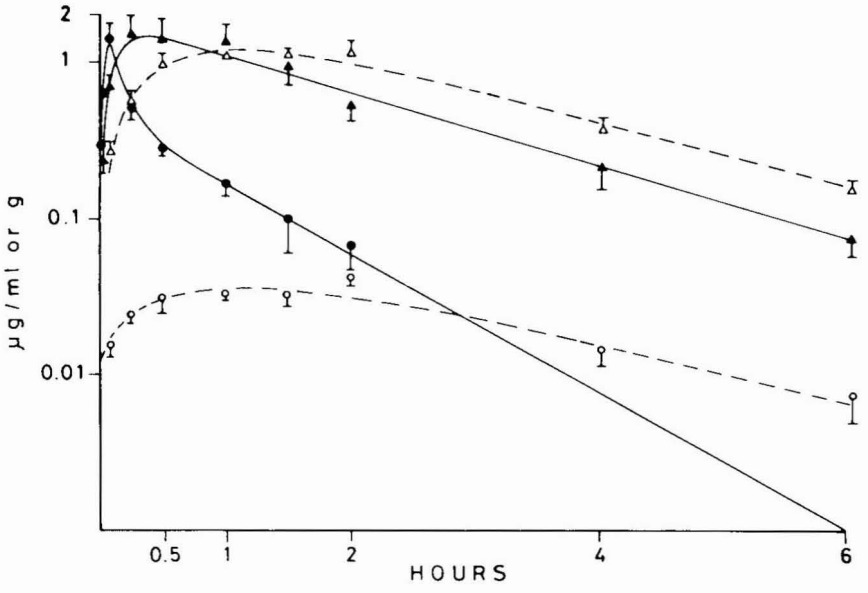

Fig. 4. Plasma (circles) and brain (triangles) concentration–time curves for trazodone (●, ▲) and 1-*m*-chlorophenylpiperazine (○, △) after oral administration of trazodone (25 mg/kg) to rats. The values are means ± S.D. for 4–6 rats.

accumulated more specifically than the parent compound in brain. These findings were reflected in the area under the curves (AUC) (Table II). At the oral dose of trazodone tested, the brain AUC was about 5 times greater than the plasma AUC, whereas the brain AUC of the metabolite was about 26 times greater than the plasma AUC. The AUC ratio of trazodone to mCPP was approximately 4 in plasma and 0.8 in brain. These results suggest that mCPP is a quantitatively significant metabolite of trazodone in the rat brain, confirming the suggestion[12] that it may play an important role as a 5HT agonist after relatively high doses of trazodone in the rat. Data not reported in detail indicate that the brain concentrations of mCPP after oral administration of trazodone to rat are comparable to those reached after pharmacologically and biochemically effective doses of mCPP[15]. Further studies are now in progress to elucidate the role of the metabolite in the pharmacological effects of the parent compound.

TABLE II

PLASMA AND BRAIN AREAS UNDER THE CURVES (AUC) OF TRAZODONE AND 1-*m*-CHLOROPHENYLPIPERAZINE

AUC values were calculated by the trapezoidal rule and extrapolated to infinity.

Compound	*Plasma AUC* (*μg/ml · min*)	*Brain AUC* (*μg/g · min*)
Trazodone	39.81	202.87
1-*m*-Chlorophenylpiperazine	10.01	262.12

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PREPARATIVE CAPILLARY ISOTACHOPHORESIS: A MICRO METHOD FOR THE PURIFICATION OF ERYTHROPOIETIN

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SUMMARY

The micropreparative separation of an *in vivo* active glycoprotein, erythropoietin, using isotachophoresis on an LKB Tachophor equipped with a Tachofrac, a micropreparative fraction collector is described. The preparative technique as described by earlier workers was varied by application of a new time–distance delay determination which produces highly accurate component separations. Using this technique a purification factor of 228 could be obtained for erythropoietin with a recovery of 59%.

INTRODUCTION

Hitherto the main problem in the purification of erythropoietin was the realization of a rapid, efficient fractionation procedure to obtain a product of high yield and purity. Up to now the best method is that due to Myake *et al.*[1], leading to a preparation of 70,400 IU/mg in a six-step procedure. The activity yield was 21%, demonstrating a significant loss of material during fractionation, mainly caused by loss of N-acetylneuraminic acid from erythropoietin. As the LKB Tachophor and Tachofrac had been shown to be powerful tools in protein analysis[2,3] and preparation[4,5], we tested the efficiency of preparative capillary isotachophoresis in overcoming the difficulties in the purification of erythropoietin (Epo).

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

*Materials**

HPMC was obtained from Dow Chem. (Midland, MI, U.S.A.), Tris from Serva (Heidelberg, G.F.R.), 1 *N* hydrochloric acid and barium oxide from Merck (Darmstadt, G.F.R.), glutamic acid, cystine and glycine from Sigma (St. Louis, MO, U.S.A.), Ampholine pH 3.5–9.5 from LKB (Stockholm, Sweden), Epo-HAI from JCL Clinical Research Corp. (Knoxville, TN, U.S.A.), indigo tetrasulphonate from LKB and ammediol from Serva.

All reagents except HPMC were of analytical-reagent grade. HPMC was purified according to the method of Delmotte[2].

Instrumentation and conditions

All investigations were carried out with the LKB Tachophor equipped with a Tachofrac, a micropreparative fraction collector. The microtitration equipment of Cooke Laboratory Products (Alexandria, VA, U.S.A.) was used for the Epo-HAI.

The Tachophor was operated with a detection wavelength of 280 nm and UV gain 175; the capillary length was 43 cm and the capillary temperature 285°K. The strip velocity of the Tachofrac was 0.43 mm/sec. The elution current was 67 μA, chart speed 0.5 mm/sec and counter flow 0.03 μl/sec.

Buffer system

The leading electrolyte was 10 m*M* hydrochloric acid in 0.3% HPMC adjusted to pH 8.00 with Tris. The terminator was 15.38 m*M* Gly adjusted to pH 10.00 with freshly prepared barium hydroxide. Buffer preparation was carried out under nitrogen to avoid carbon dioxide adsorption[2]. Before use the buffers were degassed for 10 min.

Buffer runs

The first four runs of every sequence were carried out for buffer control and system cleaning. A constant current of 150 μA was applied, causing a voltage rise from 2.6 to 23.7 kV. The last buffer run was used to control Ampholine (Fig. 1).

Determination of time–distance delay (f value)

As there is a distance between the UV window and the T-tube outlet of the capillary, every eluted component has a delay against its UV signal. This delay, called the *f* value, is specific for every buffer system and has to be measured by a dyestuff run under preparative conditions with indigo tetrasulphonate when anionic systems are used.

A 1-μl volume of the dye (0.5 mg/ml) was applied to the system, which was kept open at the terminator side. When the dye had covered half the distance between the injection port and the UV window, a counterflow was started. The current was reduced until the zone migration stopped. As the counterflow decreased the zone sharp-

* Abbreviations: HPMC = hydroxypropylmethylcellulose; Tris = trishydroxymethylaminomethane; Glu = glutamic acid; $(Cys)_2$ = cystine; Gly = glycine; Epo-HAI = erythropoietin haemagglutination inhibition assay; ammediol = 2-amino-2-methylpropane-1,3-diol; IU = International Unit.

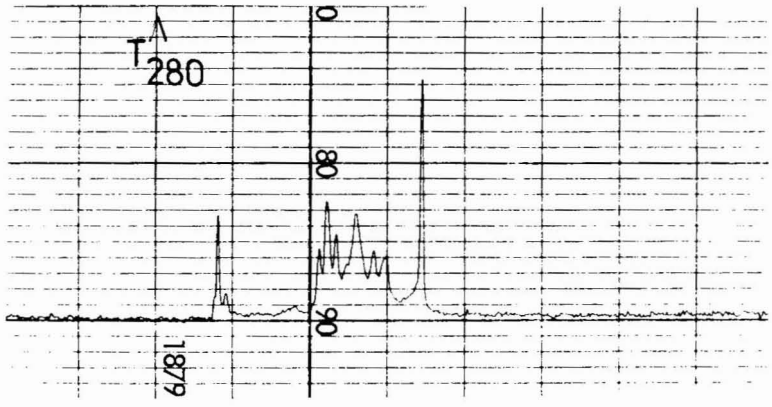

Fig. 1. Buffer run using 1.5 μl of 1 % Ampholine, pH 3.5–9.5. No effect on detection is observed.

ness, the dye zone was observed for several minutes to be sure that no further migration occurred. The corresponding current reduced by 5 % was the elution current. After the counterflow was stopped the system was closed with the terminator valve. After reconstitution of the dye zones, the counterflow was started again. No reduction in resolution could be observed when the system was absolutely closed.

At 0.5 kV before dye detection, the current was decreased to the elution value and the Tachofrac was switched on, followed by the counterflow pump. Each 5-cm distance was marked as a punch hole on the acetate strip, corresponding to a spike on the UV diagram. The following distances had to be estimated:

(*a*) distance of two successive spikes on the UV trace;
(*b*) distance of the first spike to the beginning of the UV signal;
(*c*) punch–punch distance on the strip (= 5 cm = constant);
(*d*) (*b*/*a*) *c*;
(*e*) punch–peak distance on the acetate strip;
(*f*) *e* − *d*.

f must be added to *d* in order to find the eluted component on the strip. The scheme for determination of the *f* value is shown in Fig. 2.

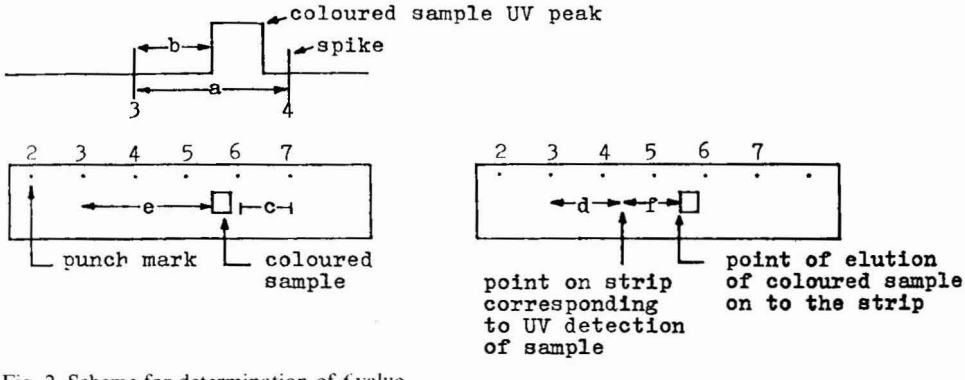

Fig. 2. Scheme for determination of *f* value.

Normally, fractionation of achromatic compounds such as proteins and peptides was carried out according to the parameters estimated in a pre-run with indigo tetrasulphonate. This produces errors when alkaline buffer systems are used because carbon dioxide adsorption changes the f value from run to run; hence the exact positions of components cannot be estimated. The f value remains constant when the buffers in the capillary and in the reservoirs are changed for every run. The f value increases when only the capillary buffers are changed. This problem may be solved by injecting 0.2 μl of dye together with the sample. The f value may then be calculated *in situ* and high accuracy is achieved for component determinations in every run. The dye does not affect the resolution (Fig. 3).

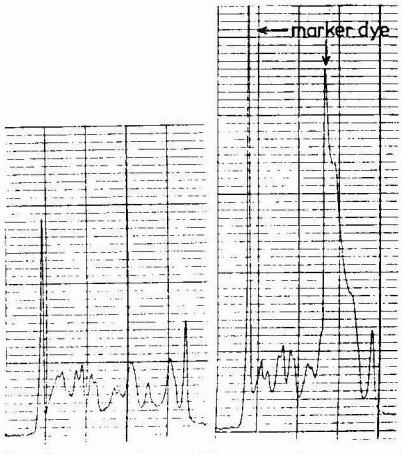

Fig. 3. Comparison of the same diagram region of two runs carried out with and without dye. Although the dye produces mixed zones with sample components, the other components are not effected.

Erythropoietin sample

A urinary protein mixture isolated from the urine of a chronic myeloid leukaemia patient by benzoic acid–acetone precipitation[6] and additional gel filtration on Sephadex G-50 (Pharmacia, Uppsala, Sweden) with an activity of 2 IU/mg standardized against the International Reference Standard (IRS) was used. Biological activity was measured by the exhypoxic polycythaemic mouse assay[7].

Determination of erythropoietin activity

Erythropoietin activity was measured with the Epo-HAI method according to Lange *et al.*[8]. The sample with a bioassayed activity of 2 IU/mg had an HAI activity of 1800 miu (milli-immunological units) calculated with the HAI quantitative chart. First, the erythropoietin activity of the regions formed by Glu and $(Cys)_2$ as discrete spacers[9] were assayed in a screening test. The acetate strip was cut into pieces corresponding to protein regions. The protruding edges of the strips were cut off so that only the small track with the components on it remained. These pieces were prewetted with phosphate-buffered saline (pH 7.4) and then incubated with 50 μl of PBS at 313°K for 20 min to resolubilize the components. The strip was then removed and the solution was assayed for HAI. For peak identification the strip region with erythropoietin activity was cut into pieces according to the calculated zone lengths.

Protein determination

Assuming Myake *et al.*'s erythropoietin preparation[1] to be absolutely pure, we could calculate the amount of erythropoietin present in our sample to be 25.5 ng/mg protein. As a 5-μl sample volume was found to be the optimum for the separation capacity, only 1.9 ng of erythropoietin could be assumed to be present in every preparative run. We tried to calculate erythropoietin concentrations using the Bouguer–Lambert–Beer law and the absorbance coefficient ($A^{1\%}_{1\,cm}$) for erythropoietin, which is 9.26 according to Espada *et al.*[10]. The other parameters necessary for calculation were obtained from the capillary geometry and the UV spectrum: I_0 = 17.5 cm (total absorbance); I = 15.85 cm (total absorbance − signal height); d = 0.05 cm (inner diameter of the capillary); ε = 0.926 (special extinction coefficient = $A^{1\%}_{1\,cm}/10$).

From these parameters, the erythropoietin concentration was calculated to be 929 ng/μl, representing the maximal concentration of the erythropoietin zone. For calculation of the amount of erythropoietin present in this zone, the zone length also had to be determined. The physical constants needed for a calculation depending on the Kohlrausch equation[11] are not known. The factor between the real zone length and UV signal length was therefore estimated by running indigo tetrasulphonate under preparative conditions. The real zone length was measured with a millimetre strip pasted parallel to the capillary. The UV signal length was measured on the UV spectrum. A linear relationship was found between the real zone length and the UV signal length and the gradient factor was 0.3. Using this value, the erythropoietin zone volume was calculated to be 0.2 μl, leading to a total amount of erythropoietin of 193 ng.

Erythropoietin fractionation

In every run, 5 μl of sample (135 miu of erythropoietin) were applied together with 1.5 μl of 1 % Ampholine, 0.3 μl of Glu (1 mg/ml), 0.3 μl of $(Cys)_2$ (1 mg/ml) and 0.2 μl of dye. During a run without fractionation the voltage increased from 2.0 to 17.6 kV at a constant current of 100 μA. During fractionation a final voltage of 14.3 kV was reached. Fig. 4 shows a typical UV spectrum of a fractionation run.

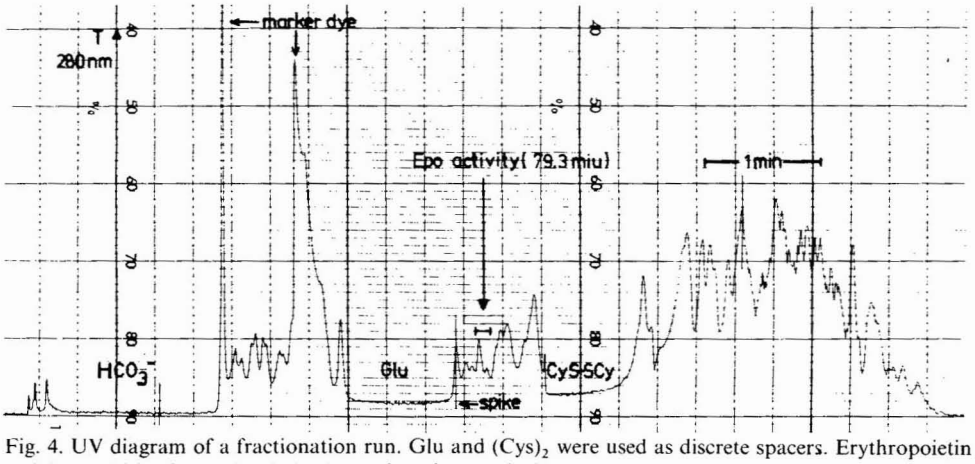

Fig. 4. UV diagram of a fractionation run. Glu and $(Cys)_2$ were used as discrete spacers. Erythropoietin activity could be detected only in the peak region marked.

RESULTS AND DISCUSSION

Preparative capillary isotachophoresis was used to purify erythropoietin with both a high yield and a high purification factor. A suitable buffer system for erythropoietin preparation was found to be 10 m*M* chloride–Tris (pH 8.00)–15.38 m*M* Gly–$Ba(OH)_2$. Chloride–ammediol (pH 9.00)–Gly–$Ba(OH)_2$, which gave a better resolution, was not useful as no erythropoietin activity could be recovered.

A modification of the method for the determination of the *f* value was tested, which gave a highly accurate isolation of components. The importance of this modification was demonstrated by measuring the influence of carbon dioxide on the *f*-value, which leads to continous changes in time–distance delay from run to run.

The mean activity yield of four determinations was 59 ± 3%. A purification factor of 228 ± 11 was calculated for the isotachophoretic fractionation. When the purification factor of benzoic acid–acetone precipitation and Sephadex chromatography is taken into account (= 25), a total factor of 5700 ± 275 for a two-step procedure was calculated from a mean activity recovery of 79.3 miu.

Compared with serum protein, uroprotein mixtures are more complex, containing large numbers of denatured proteins, peptides and fragments of these compounds. This was demonstrated by analytical isotachophoresis of chromatographic uroprotein fractions investigated with Cl^-–Tris (pH 5.8) and Gly–Tris (pH 7.2)[3] according to the method applied for macropreparation with preparative isotachophoresis on the LKB Uniphor[12]. Although the system was not optimized, up to 150 different components could be discriminated. As erythropoietin was only a minor component in our sample, a number of developments were necessary for erythropoietin fractionation. First a buffer system had to be found where the activity recovery was optimal. This proved not to be the system that gave the best resolution of components. The more alkaline pH of Cl^-–ammediol compared to Cl^-–Tris may have caused loss of sialic acid from erythropoietin, thus influencing the p*I* value and the net mobility and leading to activity spread over a wide range of mobilities with concentrations below the detection limit. This suggestion would be in agreement with Lukowsky and Painter[13], whose results of electrofocusing studies showed a significant change of erythropoietin p*I* corresponding to loss of sialic acid.

The second problem was the quantification of the protein present in the zone with erythropoietin activity. Some methods exist for component determination[14–16]. For minor components with zones thinner than the diameter of the UV slit an approximation was made by measuring the peak height in millimetres. This method is useful only when a calibration line obtained from pure standards can be calculated. This was not the case for erythropoietin; hence we were forced to estimate the real zone length of the erythropoietin zone. As calculations depending on the Kohlrausch equation were not possible because of lack of data for erythropoietin, the method described under *Protein determination* was used as an approximation. The estimation of the erythropoietin zone volume could not be carried out with high accuracy in this manner. As the zone length of any component at isotachophoretic equilibrium is directly proportional to the amount of current-transporting charges on the ions in the leading electrolyte, the Kohlrausch equation under constant conditions is reduced to

$$C_L = \text{constant} \cdot C_x$$

where C_L is the concentration of the leading ion and C_x the concentration of a dissociated component[17]. Knowing the relationship between the charge concentrations of erythropoietin and indigo tetrasulphonate, this method could be applied more accurately when physical data for a calculation are not available.

Although the method applied to the protein determination is coarse and more exact methods may lead to a lower yield and purification factor, preparative capillary isotachophoresis seems to be a very successful method. The application of a protein mixture with a higher original erythropoietin activity may open up new possibilities in the field of erythropoietin characterization, purification and quantitation.

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Note

Bestimmung der Totzeit in der "Reversed-phase" Hochleistungsflüssigkeitschromatographie

O. FINI, F. BRUSA und L. CHIESA*
Abteilung Qualitätskontrolle, Wander Chimica S.A., Postfach, 6600 Locarno (Schweiz)
(Eingegangen am 27. Oktober 1980; geänderte Fassung eingegangen am 3. Februar 1981)

Über die Totzeitbestimmung in der Gaschromatographie existieren viele Literaturangaben[1–3]. Bei der Hochleistungsflüssigkeitschromatographie (HPLC) sind die Literaturangaben bisher rar, obwohl die Kenntnis der Totzeit aus folgenden Gründen sehr wichtig ist:

(1) Aus der Totzeit und den Bruttoretentionszeiten können die Nettoretentionszeiten berechnet werden; daraus lassen sich die weiteren wichtigen Parameter errechnen (k-Werte, Bodenzahl usw.).

(2) Aus der Totzeit wird die Güte der Einrichtung geprüft, z.B. Dosiergüte (nach Kaiser[4]).

(3) Aus der Totzeit lassen sich ungünstige HPLC-Bedingungen erkennen, wie Fig. 1 zeigt.

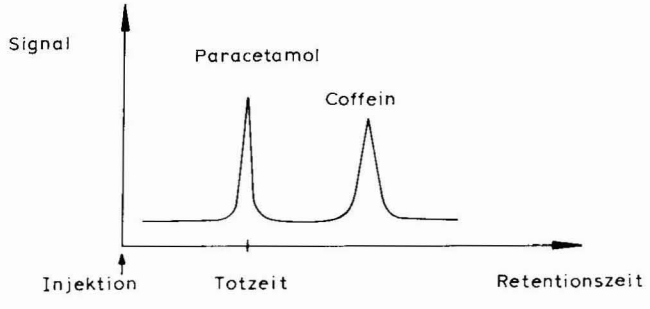

Fig. 1. Chromatogramm-Schema.

Im abgebildeten Chromatogramm-Schema ist das Paracetamol zwar von Coffein getrennt, aber die Spezifität der Paracetamol-Bestimmung ist sehr gering, und die Störungsmöglichkeiten sind hoch. Paracetamol wird in diesem Falle von der stationären Phase überhaupt nicht zurückgehalten. Von der Verwendung dieses chromatographischen Systems zur Paracetamol-Bestimmung ist deshalb (dank der Kenntnis der Totzeit) abzuraten.

Es gibt viele pharmazeutische Wirksubstanzen, die mit bestimmten Fliessmitteln bei der Totzeit, oder sogar vorher (z.B. Acetylsalicylsäure), eluiert werden (vgl. Tabelle II).

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DEFINITION DER TOTZEIT T_M

Unter Totzeit T_M versteht man die Verweilzeit der zu bestimmenden Substanzen in der mobilen Phase. Die gemessene Retentionszeit einer Substanz (T_R) ist die Summe aus der Totzeit (T_M) und der Verweilzeit der Substanz in der stationären Phase (T_R').

$$T_R = T_R' + T_M$$

Theoretisch sollte die Totzeit T_M, bei gegebener Säule und Fluss, für alle Substanzen gleich sein[5] und unabhängig von der Fliessmittelzusammensetzung.

EXPERIMENTELLER TEIL

Bestimmung des prozentualen Anteils von flüssiger und fester Phase in der Säule mittels Gravimetrie

Um das exakte Volumen der in der Säule enthaltenen Flüssigkeit zu bestimmen (= theoretisches Totvolumen), wurde folgende Methode angewendet:

(a) Wägen der Säule (einschliesslich stationärer und mobiler Phase) = G_{tot};

(b) Quantitative Transferierung des Säuleninhaltes (fest + flüssig) in einen tarierten Rundkolben. Verdampfung der flüssigen Phase. Bestimmung des Gewichtes der festen Phase = G_{fest};

(c) Wägen der leeren Säule = G_{leer};

(d) Bestimmung des Totalvolumens der leeren Säule aus dem Gewicht der leeren Säule und dem Gewicht der mit Wasser gefüllten Säule = V_{tot}.

Aus diesen Messungen ergibt sich das Volumen der flüssigen Phase = V_{F1}

$$V_{F1} = \frac{G_{tot} - (G_{leer} + G_{fest})}{S_{F1}}$$

wobei S_{F1} das spezifische Gewicht der mobilen Phase darstellt. Das Volumen der stationären Phase (V_{st}) ist gegeben durch:

$$V_{st} = V_{tot} - V_{F1}$$

Messungen. Bei Knauer-Fertigsäulen (Knauer, Oberursel, B.R.D.), LiChrosorb RP-8, 7 μm, 25 × 0.46 cm, ergeben sich folgende Werte (25°C): Volumen der leeren Säule: 4.11 ml; Volumen der flüssigen Phase: 3.03 ml; Volumen der festen Phase: 1.08 ml. Die Erfahrung zeigt, dass sich das Volumen der festen Phase bei Änderung der mobilen Phase nicht ändert (keine "Schrumpfung" oder "Quellung"); daraus folgt, dass V_{st} eine konstante Grösse sein muss. Wenn man annimmt, dass bei der Chromatographie die gesamte, in der Säule weilende Flüssigkeit laufend erneuert wird, und wenn die Volumenkontraktion vernachlässigt wird, so stellt das oben gemessene Volumen der flüssigen Phase V_{F1} das theoretische Totvolumen der Säule dar.

Das Volumen der vor und nach der Säule angeschlossenen Kapillaren ist derart klein, dass es vernachlässigt werden kann; für den untersuchten Säulentyp ergeben sich somit:

bei einem Fluss von 1.00 ml/min eine theoretische Totzeit von 3.03 min, bei 2.00 ml/min eine theoretische Totzeit von 1.52 min, u.s.w.

Bestimmung der Totzeit mittels Inertsubstanzen

Eine Reihe möglicher Inertsubstanzen wurde unter gleichen HPLC-Bedingungen eingesetzt, und die Aufenthaltszeit bis zum Detektor wurde gemessen. Die Experimente wurden mit verschiedenen mobilen Pasen durchgeführt (vgl. Tabelle I).

Diskussion. Die theoretische Totzeit für die gewählten Bedingungen (vgl. Tabellen I und II) ist 3.03 min (s. oben).

Aus der Tabelle I sieht man, dass diese Zeit mit den gefundenen Zeiten von Formamid, Harnstoff und Thioharnstoff gut übereinstimmt. Aus praktischen Gründen (sehr gute UV-Absorption) empfehlen wir, Thioharnstoff zur Bestimmung der Totzeit in der "Reversed-phase" Hochleistungsflüssigkeitschromatographie zu verwenden. Salze, Natriumnitrat usw. kommen mit bestimmten Fliessmitteln zu früh und können nicht zur Bestimmung der Totzeit verwendet werden. Sauerstoff wird hingegen von der stationären Phase zurückgehalten und ist demzufolge zur Bestimmung der Totzeit nicht anwendbar. (Sauerstoff ist in organischen Lösungsmitteln besser löslich als in Wasser.)

Wie aus der Tabelle II ersichtlich ist, gibt es einige pharmazeutische Wirkstoffe, die bei der Anwendung bestimmter mobilen Phasen eine Retentionszeit aufweisen, die kleiner als die Totzeit ist. Dass gewisse Substanzen vor der Totzeit eluiert

TABELLE I

RETENTIONSZEITEN (min) EINIGER INERTSUBSTANZEN, DIE ZUR BESTIMMUNG DER TOTZEIT IN FRAGE KOMMEN KÖNNTEN

Konstant gehaltene Bedingungen: Fluss: 1,0 ml/min; Säule: Knauer-Fertigsäule RP-8, 7 μm, 25 × 0.46 cm, Volume der leeren Säule = 4.11 ml; Einrichtung: Hewlett-Packard Flüssigchromatograph Modell 1084; Temperatur: 25 C Bemerkung: Die Leitung vor und nach der Säule bestand aus kurzen Kapillar-Stahlröhren. Das Volumen dieser Röhren (= 0.05 ml) gegenüber dem Säule-Volumen war derart klein, dass es vernachlässigt wurde.

*Substanz**	*Mobile Phase*						
	Methanol–Wasser				*Methanol–0.1 M $NH_4H_2PO_4$ in Wasser (pH 5.4), 50:50*	*Methanol–0.1 M $(NH_4)_2HPO_4$ in Wasser (pH 8.3), 50:50*	*Methanol–NaCl-Lösung 0.1 M in Wasse (pH 6.2), 50:50*
	100:0	*75:25*	*50:50*	*25:75*			
Natriumnitrat	1.94	2.00	1.93	1.95	2.94	2.96	2.96
Pikrinsäure	1.78	1.65	1.77	2.92	6.70	6.40	6.05
Chromotropsäure-dinatriumsalz	1.61	1.59	1.59	1.63	2.58	2.49	2.39
Formamid	3.00	2.94	2.93	3.02	2.91	2.91	2.94
Wasser	–	–	2.98	3.17	–	–	2.98
Harnstoff	–	2.93	2.93	3.09	2.92	2.91	2.93
Thioharnstoff	2.90	2.88	2.94	3.15	2.94	2.93	2.93
Uracil	3.23	3.23	3.25	3.48	3.24	3.21	3.27
Sauerstoff	3.68	4.50	5.81	7.24	5.95	6.01	6.10

* In möglichst kleiner Menge eingespritzt (50 bis 200 ng). Einspritzvolumen: 10 μl.

TABELLE II

RETENTIONSZEITEN (min) EINIGER PHARMAZEUTISCHER WIRKSTOFFE MIT BEKANNT KLEINEM KAPAZITÄTSFAKTOR

Konstant gehaltene Bedingungen: Fluss: 1.0 ml/min; Säule: Knauer-Fertigsäule RP-8, 7 μm, 25 × 0.46 cm, Volumen der leeren Säule = 4.11 ml.; Einrichtung: Hewlett-Packard Flüssigchromatograph Modell 1084; Temperatur: 25°C; Bemerkung: Die Leitung vor und nach der Säule bestand aus kurzen Kapillar-Stahlröhren. Das Volumen dieser Röhren (= 0.05 ml) gegenüber dem Säule-Volumen war derart klein, dass es vernachlässigt wurde.

*Substanz**	*Mobile Phase*					
	Methanol–Wasser			*Methanol–0.1% Ammoniumcarbonatlösung*		*Methanol–0.1% Na-dihydrogenphosphat, 90:10*
	75:25	*90:10*	*100:0*	*75:25*	*90:10*	
Clopamid	3.60	3.15	3.03	3.55	3.18	3.20
Coffein	3.51	3.35	3.30	3.53	3.34	3.50
Phenobarbital	3.57	3.12	3.02	3.24	3.02	3.20
Acetylsalicylsäure	3.12	3.02	3.07	2.82	2.57	2.64
Paracetamol	3.12	3.04	2.99	3.09	3.07	3.18
Aesculin	2.90	2.80	2.80	2.70	2.58	2.83

* In möglichst kleiner Menge eingespritzt (50 bis 200 ng). Einspritzvolumen: 10 μl.

werden, kann durch die Donnan's Gleichgewichtsverteilungen erklärt werden oder/und dadurch, dass die erwähnten Substanzen unter bestimmten Bedingungen nicht in die Poren der stationären Phase eindringen können (Solvathülle).

Als Richtlinie zur Erstellung von Analysenmethoden schlagen wir vor, die HPLC-Bedingungen so zu wählen, dass der Peak der zu bestimmenden Substanz nach dem Thioharnstoff-Peak kommt und von diesem vollständig getrennt ist.

Homologe Reihe

Analog zur Gaschromatographie besteht auch bei der "Reversed-phase" HPLC die Möglichkeit, die Totzeit durch das Chromatographieren einer homologen Reihe zu berechnen[4,6]. Grundlage dazu ist das lineare Verhältnis zwischen Logarithmus der Netto-Retentionszeit ($T_R' = T_R - T_M$) und der homologen Zahl (n) der entsprechenden Substanz[6].

$$\log (T_R - T_M) = an + b$$

Wir haben Versuche mit nur einer homologen Reihe (*p*-Hydroxy-benzoesäureester) durchgeführt (Tabelle III). Die so errechneten Totzeiten weichen ein wenig von den direkt gemessenen ab. Es scheint, dass es einen weiteren Einflussparameter, wenigstens für die eingesetzte homologe Reihe, gibt, weil die berechnete Totzeit mit abnehmendem Methanol-Anteil in der mobilen Phase, d.h. mit zunehmendem Kapazitätsfaktor, zunimmt (Tabelle IV).

TABELLE III

RETENTIONSZEITEN (min) EINER HOMOLOGEN REIHE

Konstant gehaltene experimentelle Bedingungen: Fluss: 2.0 ml/min. Säule: Knauer-Fertigsäule RP-8, μm, 25 × 0.46 cm; Einrichtung: Hewlett-Packard Flüssigchromatograph Modell 1084; Temperatur 25°C.

Homologe Substanz	*Homologe Zahl*	*Mobile Phase Methanol–Wasser*		
		65:35	*60:40*	*55:45*
p-Hydroxybenzoesäure-				
-methylester	1	2.36	2.73	3.29
-aethylester	2	2.76	3.40	4.44
-propylester	3	3.46	4.61	6.64
-butylester	4	4.58	6.71	10.67

TABELLE IV

VERGLEICH ERRECHNETER UND GEMESSENER TOTZEITEN

Konstant gehaltene experimentelle Bedingungen: Fluss: 2.0 ml/min; Säule: Knauer-Fertigsäule RP-8, μm, 25 × 0.46 cm; Einrichtung: Hewlett-Packard Flüssigchromatograph Modell 1084; Temperatur 25°C.

Mobile phase Methanol–Wasser	*Totzeit, errechnet aus den Retentionszeiten einer homologen Reihe (Tabelle III) (Iterationsmethode) (min)*	*Totzeit durch Thioharnstoff gemessen (min)*
65:35	1.75	1.62
60:40	1.85	1.63
55:45	1.96	1.65

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Note

Determination of polychlorinated biphenyl vapor pressures by capillary gas chromatography

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Transport of organic pollutants through the environment is, to a large extent, governed by two physical properties: water solubility and vapor pressure. For an important class of organic pollulant, polychlorinated biphenyls (PCBs), water solubilities have been determined for individual isomers. Vapor pressures, however, have been determined only for commercial Aroclor® fluids[2]. These mixtures contain fifteen or more isomers. For several reasons vapor pressure data for these mixtures are uncertain; there is no evidence that solutions of PCB isomers exhibit ideal behavior (obey Raoult's Law); vapor pressures for these mixtures at 25°C are extrapolated from data obtained at 100–250°C higher than room temperature; and finally, commercial PCB mixtures are liquids while most of the pure isomers are solids.

Gas chromatography has several advantages as a technique for determining vapor pressures: speed, tolerance to relatively impure compounds, the ability to determine vapor pressures of several compounds simultaneously, and small sample size requirements. This paper reports the use of glass capillary gas chromatography for determining the vapor pressures of individual PCB isomers and chlorinated pesticides.

THEORY

Vapor pressures for two substances at the same temperature are related through the equation:

$$\ln p_1^0 = L_1/L_2 \ln p_2^0 + C \quad (1)$$

where 1 and 2 refer to the test and reference compounds respectively, p^0 is the vapor pressure, L is the heat of vaporization, and C is a constant. The ratio L_1/L_2 and the constant C may be calculated from the relative retention volume (V_R) (or time) of the test to the reference compound and the vapor pressure of the reference compound:

$$\ln (V_R)_1/(V_R)_2 = (1 - L_1/L_2) \ln p_2^0 - C \quad (2)$$

A plot of $\ln (V_R)_1/(V_R)_2$ *vs.* $\ln p_2^0$ should give a straight line with slope $(1 - L_1/L_2)$

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and intercept $-C$. It is assumed that the ratio of the heats of vaporization of the two compounds is independent of temperature over the range of measurements. The derivation of eqns. 1 and 2 is presented in detail by Hamilton[3].

EXPERIMENTAL

Nanogram quantities of individual PCB isomers in hexane were injected directly onto a 1 m × 0.25 mm I.D. wall-coated open tubular (WCOT) glass capillary column installed in a Varian 3700 gas chromatograph. Use of pentane as a solvent instead of hexane produced no difference in retention times. The stationary phase was Apolane-87 (Applied Science Labs., State College, PA, U.S.A.), chosen because of its low polarity and non-chiral structure. Hydrogen at a flow-rate of 2–4 ml/min was used as a carrier gas, and nitrogen at 30 ml/min was used as a make-up gas for the ^{63}Ni electron-capture detector. The column was operated isothermally at 60–130°C. Temperatures indicated by the oven pyrometer agreed well with those measured with a mercury thermometer inserted into the oven. The detector was maintained at 325°C, and the injector at 180°C. Retention times were measured in chart units from the injection point and were expressed relative to the retention time of 2,4,5-trichlorophenoxyacetic acid, isobutyl ester (2,4,5-TIB) which has a vapor pressure of $2.2 \cdot 10^{-5}$ Torr at 25°C[3]. PCB isomers were purchased from Analabs (North Haven, CT, U.S.A.). Pesticides were obtained from the U.S. Environmental Protection Agency Pesticide Repository (Research Triangle Park, NC, U.S.A.). Solvents were pesticide residue quality.

RESULTS

Vapor pressures for the reference compound, 2,4,5-TIB, in the temperature range of our measurements were obtained from Hamilton's relative retention data[3] for 2,4,5-TIB and dibutyl phthalate (DBP). Using his values for L_1/L_2 and C for the 2,4,5-TIB/DBP pair together with DBP vapor pressures at different temperatures (from the equation of Small *et al.*[4], quoted by Hamilton[3]), p^0 was calculated for 2,4,5-TIB in our temperature range using eqn. 1. Regression lines were fitted to $\ln (V_R)_1/(V_R)_2$ *vs.* $\ln p_2{}^0$ plots (Fig. 1) according to eqn. 2 and the parameters L_1/L_2 and C were obtained for each of the test compound/2,4,5-TIB pairs. Two to seven of these plots were constructed for each test compound. Table I gives, for each test compound, the means of the individual regression results for L_1/L_2 and C, and the means of the vapor pressure values calculated from the individual regression results and the vapor pressure for 2,4,5-TIB ($2.2 \cdot 10^{-5}$ Torr at 25°C; $4.1 \cdot 10^{-5}$ Torr at 30°C) using eqn. 1.

DISCUSSION

Selectivity in gas–liquid chromatography is influenced by the volatility of the solute and by chemical interactions between the solute and the stationary phase. If chemical effects can be eliminated or at least minimized, partitioning between the stationary and mobile phases is controlled by solute volatility. Non-polar columns have been employed in several applications involving determinations of boiling points

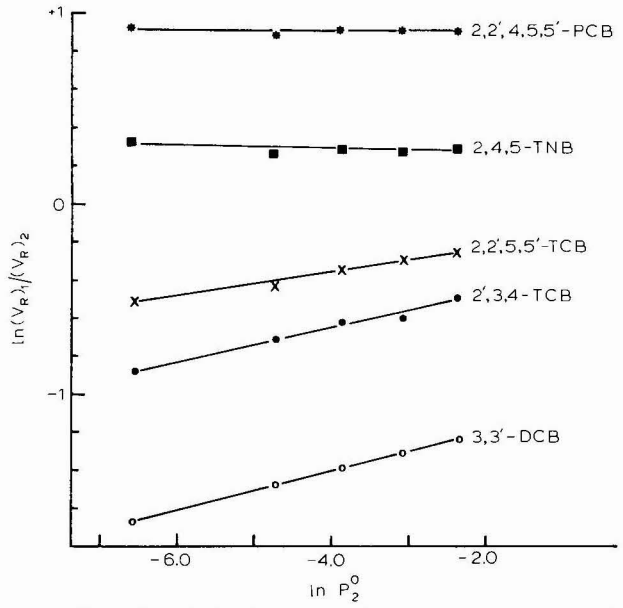


Fig. 1. Plots of $\ln (V_R)_1/(V_R)_2$ *vs.* $\ln p_2^0$ (vapor pressure of 2,4,5-TIB at different temperatures) for PCB isomers and 2,4,5-trichlorophenoxyacetic acid, *n*-butyl ester (2,4,5-TNB).

TABLE I

MEAN VALUES OF L_1/L_2, C, AND VAPOR PRESSURE

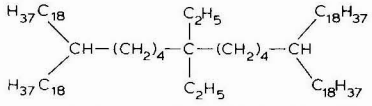
Compound	*Experiments*	L_1/L_2	C	p^0 *(Torr) (25°C)*
Pesticides				
p,p'-DDE	2	1.008 ± 0.063	−1.046 ± 0.270	$(7.4 \pm 2.7) \cdot 10^{-6}$
o,p'-DDT	2	1.030 ± 0.027	−1.312 ± 0.047	$(4.5 \pm 1.3) \cdot 10^{-6}$
2,4,5-T, *n*-butyl ester	7	1.016 ± 0.020	−0.222 ± 0.059	$(1.6 \pm 0.2) \cdot 10^{-5}$
PCB isomers				
3,3'-DCB	5	0.884 ± 0.029	0.900 ± 0.100	$(2.0 \pm 0.5) \cdot 10^{-4}$
2',3,4-TCB	4	0.913 ± 0.016	0.324 ± 0.056	$(8.0 \pm 1.0) \cdot 10^{-5}$
2,2',5,5'-TCB	4	0.925 ± 0.009	0.076 ± 0.039	$(5.5 \pm 0.4) \cdot 10^{-5}$
2,2',4,5,5'-PCB	4	1.000 ± 0.036	−0.897 ± 0.128	$(9.2 \pm 2.3) \cdot 10^{-6}$
2,2',4,4',6,6'-HCB	3	0.975 ± 0.029	−0.828 ± 0.113	$(1.2 \pm 0.2) \cdot 10^{-5}$

or vapor pressures. Packed columns with SE-30 as the stationary phase have been used by Green *et al.*[5] to simulate distillation of hydrocarbons, by Martin *et al.*[6] to calculate boiling points of phenothiazines, and by Hamilton[3] to determine vapor pressures of herbicides. Castello and D'Amato[7,8] found some variation in activity coefficient due to solute–solvent interactions when using SF-96 as a stationary phase to determine the vapor pressures of a series of alkanes.

Use of an open tubular column eliminates adsorption effects due to the presence of a stationary phase support. Stationary phase selectivity can be minimized by choosing a stationary phase of as low polarity as possible. Apolane-87 is an 87-unit

TABLE II

APOLANE-87: STRUCTURE AND McREYNOLDS CONSTANT



Phase	*McReynolds constants**
Apolane-87	71
SF-96	205
SE-30	217

* Sum of five ΔI values (Applied Science Labs.).

hydrocarbon which is non-polar and non-chiral, with McReynolds constants lower than those for either SE-30 or SF-96 (Table II). The 1-m long WCOT column used for these experiments was obviously of lower efficiency than the 30–50-m columns normally used for analytical separations, but the ability to resolve large numbers of compounds of similar volatility is not a major requirement for this technique. Fig. 2

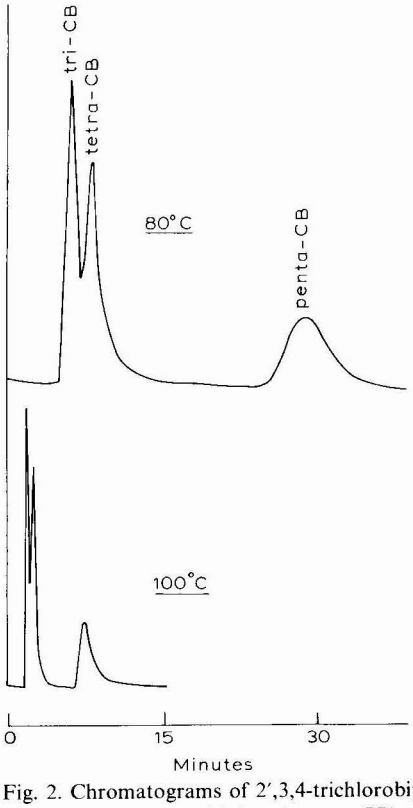


Fig. 2. Chromatograms of 2′,3,4-trichlorobiphenyl (tri-CB), 2,2′,5,5′-tetrachlorobiphenyl (tetra-CB) and 2,2′,4,5,5′-pentachlorobiphenyl (penta-CB) on a 1-m Apolane-87 capillary column at 80 and 100°C.

shows that the resolution of the column was sufficient to allow several compounds to be chromatographed at once. The use of a longer column would have been inconvenient because of the extremely long retention times for compounds of low vapor pressure at low column temperatures. On the 1-m column, 40 min were required to elute 2,4,5-TIB at 70°C and 3 ml/min carrier gas flow-rate, while 1100 min were required to elute the same compound from Hamilton's 0.6-m packed column at 72°C and a carrier gas flow-rate of 66 ml/min[4]. On the 1-m WCOT column, 175 min were required to elute 2,2′,4,5,5′-pentachlorobiphenyl at 60°C.

Table III shows a comparison between vapor pressures determined by this method and those measured by us using gas saturation[9], along with literature values where available[3,10].

TABLE III

COMPARISON OF VAPOR PRESSURES DETERMINED BY GAS CHROMATOGRAPHY AND GAS SATURATION TECHNIQUES

Compound	*Temperature (°C)*	p^0 *(Torr)*		*Literature*
		Gas chromatography	*Gas saturation*[9]	
Pesticides				
p,p′-DDT	30	$14.0 \cdot 10^{-6}$	$13.0 \cdot 10^{-6}$	$6.5 \cdot 10^{-6}$ (ref. 10)
o,p′-DDT	30	$8.4 \cdot 10^{-6}$	$8.8 \cdot 10^{-6}$	$5.5 \cdot 10^{-6}$ (ref. 10)
2,4,5-T, *n*-butyl ester	25	$1.6 \cdot 10^{-5}$	–	$1.5 \cdot 10^{-5}$ (ref. 3)
PCB isomers				
2′,3,4-TCB	25	$8.0 \cdot 10^{-5}$	$10.0 \cdot 10^{-5}$	–
2,2′,5,5′-TCB	25	$5.5 \cdot 10^{-5}$	$1.9 \cdot 10^{-5}$	–
2,2′,4,5,5′-PCB	25	$9.2 \cdot 10^{-6}$	$7.2 \cdot 10^{-6}$	–

CONCLUSIONS

Capillary gas chromatography is a rapid and convenient means of determining vapor pressures. The solid PCB isomers 2′,3,4-trichlorobiphenyl and 2,2′,5,5′-tetrachlorobiphenyl are major components of the commercial fluid Aroclor® 1242 which has a reported vapor pressure of $4.1 \cdot 10^{-4}$ Torr, and 2,2′,4,5,5′-pentachlorobiphenyl is a major component of Aroclor® 1254 which has a reported vapor pressure of $7.7 \cdot 10^{-5}$ Torr[2]. The vapor pressures determined for the solid individual PCB isomers are approximately 5–10 times lower than those reported for the liquid commercial mixtures. Vapor pressures determined by gas chromatography agreed well with those determined by gas saturation.

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Note

Gaschromatographische Erfassung von 6-Desoxyhexosen, Pentosen und Hexosen aus herzwirksamen Glykosiden

II*. Gas–Flüssigkeits-Chromatographie an Glaskapillarsäulen**

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Im Rahmen von Arbeiten zur Konstitutionsbestimmung verschiedener herzwirksamer Glykoside[2–5] hatte sich die Notwendigkeit ergeben, ein einfaches Verfahren zur Identifizierung und Quantifizierung von 6-Desoxyhexosen, Hexosen und Pentosen auszuarbeiten. Die Gaschromatographie der pertrimethylsilylierten Zucker an gepackten Säulen erwies sich zu diesem Zweck gut geeignet[1], wobei allerdings bei einigen Zuckerpaaren eine sichere Zuordnung nur durch Verwendung zweier verschiedener Trennsäulen (OV-101 und OV-17) erreicht werden konnte. Im Hinblick auf eine weitere Vereinfachung stellte sich nun die Frage, ob die bei der Gaschromatographie an Glaskapillarsäulen erzielbare Trennkapazität[6–9] einen Einsatz zweier Trennsysteme erübrigen würde. Um einen Vergleich mit den an gepackten Säulen erreichbaren Ergebnissen zu ermöglichen, untersuchten wir daher das Trennverhalten der 6-Desoxyhexosen, Hexosen und Pentosen sowohl an mit OV-101 als auch an mit OV-17 belegten Dünnfilmglaskapillarsäulen. Daneben versuchten wir durch Einsatz verschiedener Silylierungsreagentien eine Vereinfachung der zur Derivatisierung nötigen Manipulationen zu erzielen.

EXPERIMENTELLES

Vergleichssubstanzen: Wie in Lit. 1 angegeben.

Lösungsmittel: Alle verwendeten Lösungsmittel waren p.A. Qualität (Merck, Darmstadt, B.R.D.).

Gaschromatographie: Gerät Perkin-Elmer F 33 ausgerüstet mit einem Flammenionisationsdetektor (FID). Säule 1: Glaskapillare: 24 m × 0.23 mm I.D., Trennfilm: OV-101; Trägergas (N_2): 0.6 ml/min, Split 1:50; Temperatur: Injektor/Detektor 225°C; Ofen, Programm: 150–240°C, 1°/min; Initialzeit: 20 min; Brenngase: Wasserstoff–Synthetische Luft. Säule 2: Glaskapillare: 24 m × 0.28 mm I.D., Trennfilm: OV-17; Trägergas (N_2): 0.6 ml/min, Split 1:30; Temperatur: Injektor/Detektor 225°C; Ofen, Programm: 150–200°C, 1°/min, Initialzeit: 20 min; Brenngase: Wasser-

* I. Mitt.: Lit. 1.
** Teil der Diplomarbeit I.G.-K., Universität Wien, Wien, 1979.

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stoff–Synthetische Luft. Integrator Perkin-Elmer M-2; Schreiber Perkin-Elmer 056.

Hydrolyse der Glykoside und Äquilibrierung der freien Zucker: wie früher beschrieben[1].

Derivatisierung: Reagens 1: Nach Sweeley *et al.*[10] mit Hexamethyldisilazan und Trimethylchlorsilan in wasserfreiem Pyridin[1]. Reagens 2: 0.2 mg Zucker werden mit 100 µl Tri-Sil Z (Pierce, Rotterdam, Niederlande) versetzt[11]. Reagens 3: 0.2 mg Zucker werden mit 100 µl N,O-Bis-(trimethylsilyl)-trifluoracetamid reinst (Serva, Heidelberg, B.R.D.) versetzt[12]. Reagens 4: 0.2 mg Zucker werden mit 100 µl N-Trimethylsilylimidazol reinst (Serva) versetzt. Von diesen Lösungen wurden 0.5–1.5 µl injiziert.

ERGEBNISSE UND DISKUSSION

Zur Optimierung der gaschromatographischen Bedingungen zogen wir ein Gemisch von Arabinose, Rhamnose und Fucose heran, deren Hauptpeaks bei der Verwendung gepackter Säulen teilweise stark überlappten (Fig. 1). Nach Variation von Gasfluss und Temperaturprogramm konnten wir mit einer OV-101 Glaskapillarsäule bei vergleichbaren Retentionszeiten eine wesentlich bessere Auftrennung erzielen (Fig. 2).

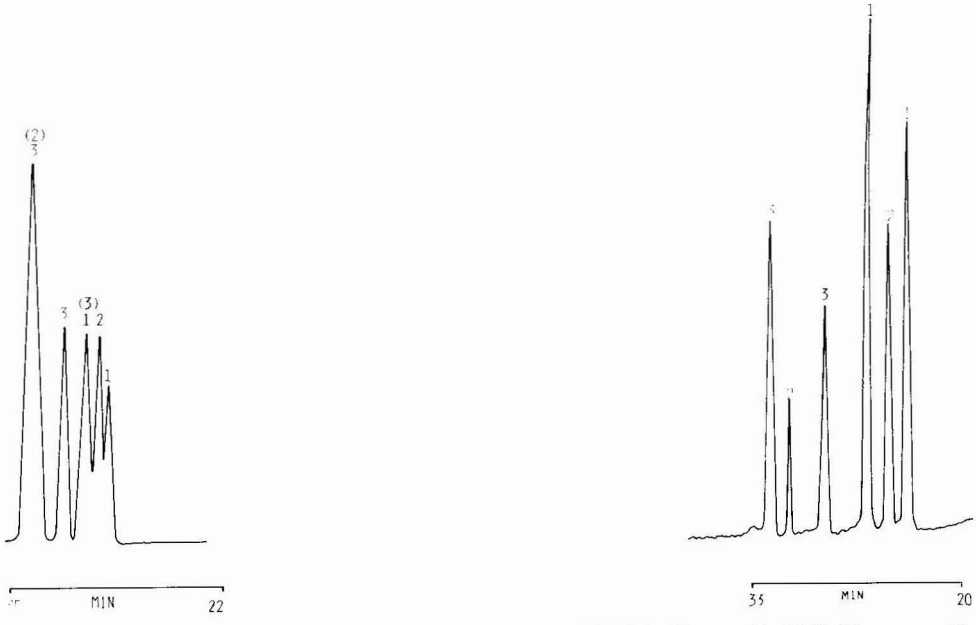

Fig. 1. Gas–Flüssigkeits-Chromatographische Trennung von TMS-Arabinose (1), TMS-Rhamnose (2) und TMS-Fucose (3) an gepackter Säule; Säule: Pyrex 10 ft. × $\frac{1}{4}$ in O.D. × 2 mm I.D.; Trennfilm 1.6% OV-101 auf Chromosorb G (100–120 mesh); Trägergas (N_2): 20 ml/min; Temperatur Injektor 230 C, Detektor 240°C; Programm 100–240°C (2°/min).

Fig. 2. Gas–Flüssigkeits-Chromatographische Trennung von TMS-Arabinose (1), TMS-Rhamnose (2) und TMS-Fucose (3) an Glaskapillarsäule; Säule: Glaskapillare 24 m × 0.23 mm I.D.; Trennfilm: OV-101; Trägergas (N_2): 0.6 ml/min. Split 1:50; Temperatur Injektor/Detektor 225°C; Ofen, Programm 150–240°C, 1°/min; Initialzeit 20 min.

Da sich bei dem bisher verwendeten Derivatisierungsverfahren[1,10] durch die bei der Reaktion auftretende Trübung gewisse Nachteile ergeben hatten, überprüften wir nun andere im Handel erhältliche Reagentien auf ihre Eignung: Während "Tri-Sil Z" hinsichtlich der Haltbarkeit der Lösungen nicht zufriedenstellte, verlief die Reaktion mit N,O-Bis-(trimethylsilyl)-trifluoracetamid (BSTFA) auch nach 30 min Erwärmen auf 60°C nicht vollständig. Erst die Verwendung von Trimethylsilylimidazol-(TSIM) Konzentrat brachte dem früheren Verfahren vergleichbare Ergebnisse, besass jedoch den Vorteil, dass klare Einspritzlösungen erhalten wurden, weshalb wir dieses Reagens bei allen weiteren Untersuchungen verwendeten.

Um einen Überblick über die an OV-101 Glaskapillarsäulen auftretenden Retentionszeiten, die Anzahl der Anomerenpeaks und deren Flächenverhältnisse zu erhalten, dosierten wir zunächst Lösungen der einzelnen pertrimethylsilylierten Reinsubstanzen, die wir zuvor wie früher beschrieben[1] mit Säure vorbehandelt hatten. Ein Vergleich der Retentionszeiten zeigte, dass sich verschiedene Zucker in nur einem, einige aber in zwei Hauptpeaks überlagern mussten.

Zur Gewinnung präziser Daten spritzten wir anschliessend Gemische von Trimethylsilyl (TMS)-Zuckern ähnlicher Retentionszeit ein und fanden bei den Paaren Ribose–6-Desoxygulose, Ribose–6-Desoxyidose, 6-Desoxyidose–6-Desoxytalose, Ribose–Fucose, 6-Desoxyallose–Fucose, 6-Desoxyallose–6-Desoxytalose, Xylose–Fucose, 6-Desoxyglucose–Altrose, Allose–Talose, Talose–Galaktose, Gulose–Mannose und 6-Desoxyidose–Idose eine Überlappung jeweils eines Hauptpeaks. Nahezu gleiche Retentionszeiten wiesen die Hauptpeaks der Paare Lyxose-6-Desoxygulose und Fucose–6-Desoxytalose auf. Von diesen kritischen Paaren konnten allerdings z.B. 6-Desoxyallose–6-Desoxytalose und Allose–Talose trotz unvollständiger Trennung nebeneinander erfasst werden. Die gefundenen Retentionszeiten und Flächenverhältnisse sind in Fig. 3 ersichtlich. Beispiele für die Trennung von aus jeweils fünf Monosacchariden bestehenden Gemischen zeigen Fig. 4 und 5.

Entsprechende Versuche mit einer OV-17 Glaskapillarsäule ergaben die in Fig. 6 ersichtlichen Daten. Wie bei Verwendung von OV-101 stellten wir auch bei OV-17 Überlagerungen von Hauptpeaks einzelner Zuckerpaare fest, doch lagen die auf OV-

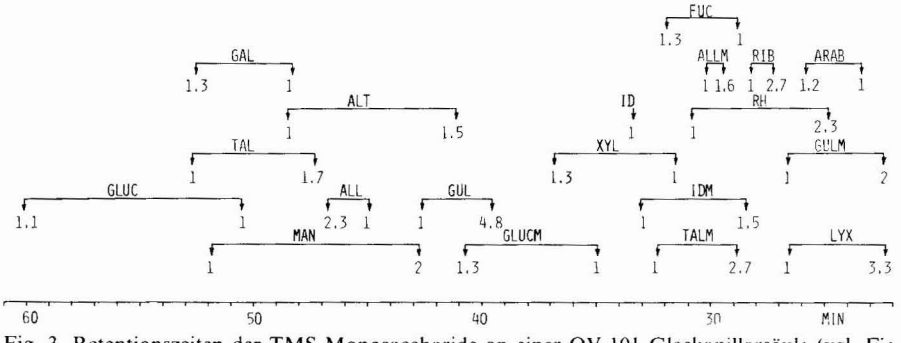

Fig. 3. Retentionszeiten der TMS-Monosaccharide an einer OV-101 Glaskapillarsäule (vgl. Fig. 2) und Peakflächenverhältnisse der anomeren Formen nach Behandlung mit Säure. GULM = 6-Desoxygulose, RIB = Ribose, RH = Rhamnose, ARAB = Arabinose, LYX = Lyxose, ALLM = 6-Desoxyallose, IDM = 6-Desoxyidose, TALM = 6-Desoxytalose, FUC = Fucose, GUL = Gulose, XYL = Xylose, ALT = Altrose, GLUCM = 6-Desoxyglucose, ID = Idose, ALL = Allose, TAL = Talose, GAL = Galaktose, MAN = Mannose, GLUC = Glucose.

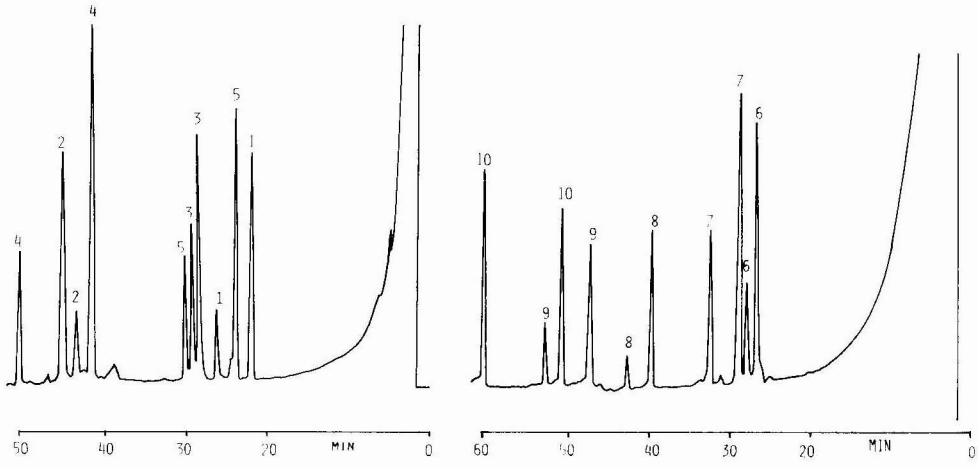

Fig. 4. Gas–Flüssigkeits-Chromatographische Trennung von TMS-Lyxose (1), TMS-Allose (2), TMS-6-Desoxyallose (3), TMS-Mannose (4) und TMS-Rhamnose (5) an einer OV-101 Glaskapillarsäule; Trennbedingungen wie in Fig. 2.

Fig. 5. Gas–Flüssigkeits-Chromatographische Trennung von TMS-Ribose (6), TMS-6-Desoxytalose (7), TMS-Gulose (8), TMS-Talose (9) und TMS-Glucose (10) an einer OV-101 Glaskapillarsäule; Trennbedingungen wie in Fig. 2.

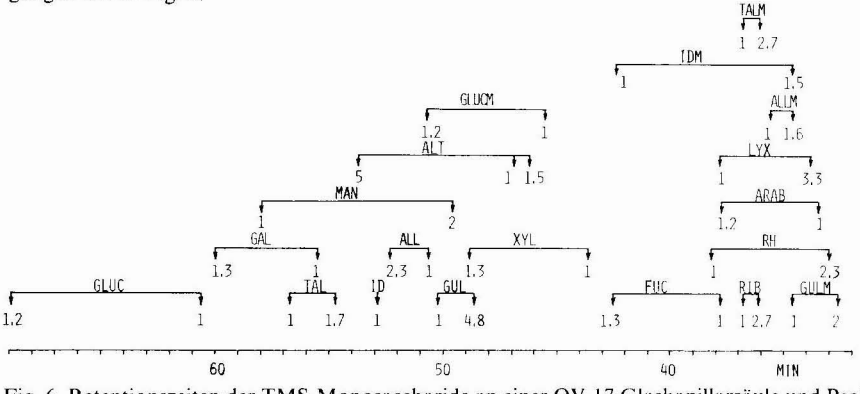

Fig. 6. Retentionszeiten der TMS-Monosaccharide an einer OV-17 Glaskapillarsäule und Peakflächenverhältnisse der anomeren Formen nach Behandlung mit Säure. Glaskapillare 24 m × 0.28 mm I.D., Trennfilm OV-17; Trägergas (N_2) 0.6 ml/min, Split 1:30; Temperatur Injektor/Detektor 225°C; Ofen, Programm 150–200°C, 1°/min; Initialzeit 20 min. Abkürzungen vgl. Fig. 3.

101 gefundenen kritischen Paare nunmehr soweit getrennt vor, dass eine sichere Identifizierung möglich wurde. Für Analysen unbekannter Proben empfiehlt es sich, zunächst an OV-101 zu trennen und in Zweifelsfällen, das heisst bei Vorliegen der aufgezeigten kritischen Paare, eine zusätzliche Bestimmung an OV-17 durchzuführen. Bei der Untersuchung herzwirksamer Glykoside führt normalerweise allein die Verwendung von OV-101 zu raschen und sicheren Ergebnissen, da dieser Glykosidtyp erfahrungsgemäss maximal drei verschiedene Monosaccharid-Bausteine enthält.

Wie bereits bei der Gaschromatographie an gepackten Säulen[1] festgestellt, liessen sich erwartungsgemäss auch die Peakflächenverhältnisse als Zuordnungskriterien heranziehen. Es ist jedoch hervorzuheben, dass wir bei den Analysen an Glaskapillarsäulen teilweise abweichende Verhältniswerte fanden, was zum grossen Teil auf das neue Silylierungsverfahren zurückgeführt werden muss. Da vergleichende Analy-

sen an gepackten und Kapillarsäulen ebenfalls Schwankungen der Flächenverteilung aufzeigten, kann auch ein Einfluss des Probenteilers nicht ausgeschlossen werden. Die in Fig. 3 und 6 angegebenen Flächenverhältnisse müssen daher als Richtwerte betrachtet werden, welche sich allerdings bei strikter Einhaltung der Hydrolyse- bzw. Äquilibrierbedingungen gut reproduzieren lassen.

Auch in quantitativer Hinsicht gab die Analyse unter Verwendung von Glaskapillarsäulen zufriedenstellende Ergebnisse. Eichanalysen mit Monosaccharidgemischen bekannter Zusammensetzung führten zu Korrekturfaktoren, die mit den an gepackten Säulen ermittelten[1] gut korrelierten. Nachdem die Faktoren der Hexosen, Pentosen und 6-Desoxyhexosen nur um $\pm 10\%$ (relativ) differierten, liessen sich näherungsweise auch die Flächenverhältnisse für die Bestimmung der Mol-Verhältnisse heranziehen.

Sowohl bei Verwendung von gepackten als auch von Glaskapillarsäulen ist somit eine sichere Identifizierung der einzelnen Zucker möglich; dies bringt gegenüber den bisher zur Verfügung stehenden Verfahren[14] einen deutlichen Zeitgewinn. Hinsichtlich der Trennkapazität zeigten sich die Kapillarsäulen den gepackten überlegen, wodurch bei komplexen Gemischen leichter interpretierbare Chromatogramme erhalten wurden. Es ist jedoch anzumerken, dass auch bei Anwendung von Kapillarsäulen bei manchen Analysen zwei unterschiedliche stationäre Phasen (OV-101 und OV-17) eingesetzt werden müssen, um die geforderte Analysensicherheit zu gewährleisten. Dennoch können beide Verfahren (Chromatographie an gepackten Säulen oder an Glaskapillarsäulen) im Rahmen der Strukturaufklärung herzwirksamer Glykoside mit Erfolg verwendet werden; im Einzelfall wird die Wahl in Abhängigkeit vom speziellen Trennproblem und vorhandenen technischen Möglichkeiten zu treffen sein.

DANK

Für die Überlassung von Vergleichssubstanzen danken wir Herrn Professor Dr. T. Reichstein, Basel, Schweiz auf das herzlichste. Die verwendeten gaschromatographischen Einheiten wurden durch Mittel der Österreichischen Apothekerkammer bzw. aus Sondermitteln des Österreichischen Bundesministeriums für Wissenschaft und Forschung finanziert.

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Note

Gas chromatographic enzymic determination of amygdalin

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(Received January 21st, 1981)

The evaluation of amygdalin (D-mandelonitrile-β-D-gentiobioside) as a cancer remedy has been the subject of considerable controversy[1,2]. Recently, Stobaugh *et al.*[3] developed a method for the determination of amygdalin in human plasma, based on the gas chromatographic (GC) determination of benzaldehyde formed by enzymatic (β-glucosidase) hydrolysis of the parent glycoside. In this paper, we describe a more sensitive method for the determination of amygdalin in aqueous solution. Enzymatic hydrolysis of amygdalin by β-glucosidase was performed according to the procedure described by Stobaugh *et al.*[3], then the benzaldehyde liberated was converted by pentafluorobenzyloxylamine into its O-pentafluorobenzyl oxime and the derivative was determined by GC with the use of a flame-ionization detector (FID) or an electron-capture detector (ECD).

EXPERIMENTAL

Reagents

Pentafluorobenzyloxylammonium [O-(2,3,4,5,6-pentafluorobenzyl)hydroxylammonium] chloride (PFBOA) was synthesized[4] from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan). α,α-Dichlorodiphenylmethane was used as an internal standard (IS). Amygdalin (Merck, Darmstadt, G.F.R.) was used without further purification (m.p. 221–222°C). β-Glucosidase (powder, prepared from almonds) was obtained from Sigma (St. Louis, MO, U.S.A.), with an activity of 4.0 units/mg (1 unit will liberate 1.0 μmole/min of glucose from Salicin at pH 5.0 at 37°C). Benzaldehyde was distilled and kept under nitrogen.

Apparatus and conditions

A Shimadzu GC-4APF gas chromatograph equipped with a FID and GC-4APE gas chromatograph equipped with a 10-mCi ^{63}Ni ECD were used. A 2-m glass column packed with 3% XE-60 on 80–100-mesh Celite 545 (AW DMCS) was used, with a column temperature of 160°C, a detector temperature of 180°C and a chart speed of 0.25 cm/min.

0021-9673/81/0000–0000/$02.50

Standard procedure

Method A. When the FID was used, amygdalin was dissolved in 50 m*M* acetate buffer (pH 4.8) to give solutions of concentration 2.5–10 μg/ml. To 0.2 ml of the sample solution in a 5 ml centrifuge tube were added 50 μl of β-glucosidase (2.0 units/ml) dissolved in acetate buffer (50 m*M*, pH 4.80). After shaking for 1 h in an incubator of 30°C, 50 μl of an aqueous solution of PFBOA (2.2 mg/ml) were added and the mixture was allowed to stand for 1 h at room temperature. After being saturated with sodium chloride and acidified with 1 drop of 18 *N* sulphuric acid, the O-PFBO derivative of benzaldehyde was extracted with 500 μl of ethyl acetate containing 1 μg of α,α-dichlorodiphenylmethane as an IS, followed by centrifuging for a few minutes to separate two phases. The lower, aqueous layer was removed with a syringe with a long needle, a few grains of anhydrous sodium sulphate were added to dry the ethyl acetate extract, followed by concentration of the solvent into a small volume under a stream of air, and an aliquot of the final solution was injected on to the GC column.

Method B. When the ECD was used, amygdalin was dissolved in 50 m*M* acetate buffer (pH 4.8) to give solutions of concentration 0.5–2.5 μg/ml. Then 0.2 ml of the sample solution was treated by a technique similar to method A, except that this time 2.0 ml of ethyl acetate containing 5 μg of α,α-dichlorodiphenylmethane was used for extraction of the O-PFBO derivative.

RESULTS AND DISCUSSION

Stobaugh *et al.*[3] hydrolysed 3-ml plasma samples spiked with amygdalin at concentrations of 2–20 μg/ml. If a molar equivalent of benzaldehyde was produced by the hydrolytic action of β-glucosidase (in their work, the benzaldehyde recovered was found to be 95% of the amygdalin added), about 1.4 μg of benzaldehyde was produced from 6 μg of amygdalin. They extracted it with 250 μl of chloroform containing an IS and injected directly 1 μl of it on to a gas chromatograph equipped with an FID. However, when GC with an FID was used, the sensitivity limit is about 2 μg in 250 μl of benzaldehyde in ethyl acetate, partly because benzaldehyde is so volatile that it is difficult to concentrate a solution without loss, and partly because the peak of benzaldehyde on the chromatogram occurs on the slope of that of solvent. Hence, Stobaugh *et al.* were forced to take 3-ml plasma samples and to extract with as small a volume as 250 μl of organic solvent.

We have found PFBOA to be an excellent derivatizing agent in the GC determination of low-molecular-weight carbonyl compounds in aqueous solution. The reaction of carbonyl compounds with PFBOA proceeded readily in weakly acidic media (pH 2–5) at room temperature to yield derivatives extractable from the aqueous solution with organic solvents, and the complete removal of the unreacted reagent was easily achieved. The resulting derivatives were stable in organic solvents and very volatile, and therefore the GC separation could be carried out at low temperatures. Also, the O-PFBO were extremely sensitive to the ECD. In this work, PFBOA was applied to the micro-determination of amygdalin. The reagent concentration was made 100–1000-fold greater than that of benzaldehyde to ensure completion of the condensation reaction with a small amount of the aldehyde in the aqueous solution. The conditions for enzymatic hydrolysis of amygdalin were fixed according to the procedure of Stobaugh *et al.*

Experiments were also conducted to determine the effect of pH on β-glucosidase activity. The enzyme rapidly hydrolysed amygdalin to liberate benzaldehyde between pH 4.0 and 7.0. Therefore, incubation solutions were buffered at pH 4.8, taking into consideration conditions for the enzyme activity of β-glucosidase and the condensation reaction with PFBOA. Ethyl acetate was a suitable solvent for the extraction of the oxime. Salting-out improved the extent of extraction. In order to prevent an excess of PFBOA from being extracted, extraction was carried out in the acidic media by adding 1 drop of 18 *N* sulphuric acid.

Typical GC separations are illustrated in Fig. 1. α,α-Dichlorodiphenylmethane was used as an internal standard. Quantitative assay was carried out by comparison of the peak-height ratio with a calibration graph prepared from solutions containing known amounts of amygdalin.

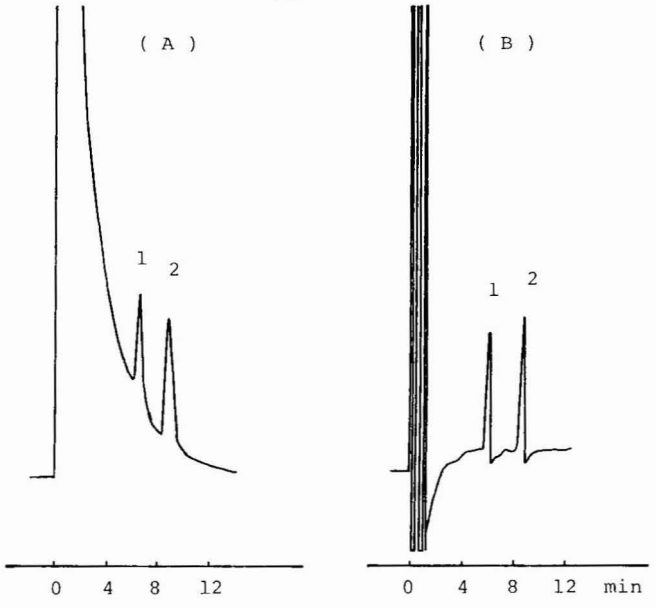

Fig. 1. Gas chromatogram of benzaldehyde produced from hydrolysis of amygdalin by β-glucosidase on a 2.0-m 3% GE-XE column at 160°C. 1 = Benzaldehyde O-pentafluorobenzyloxime; 2 = α,α-dichlorodiphenylmethane. (A) Analysis on a gas chromatograph equipped with a hydrogen FID, using 2.0 μg of amygdalin. (B) Analysis on a gas chromatograph equipped with a ^{63}Ni ECD, using 200 ng of amygdalin.

In the present method, the reaction of benzaldehyde with PFBOA provided a larger molecule, which led to an increase in FID sensitivity and to the possibility of concentration of the extract without perceptible loss of the derivative. Even when an FID was used, the sensitivity limit was improved 10-fold compared with the procedure performed without derivatization by Stobaugh *et al.*, which means that one tenth of the amount of plasma samples is sufficient for analysis. The O-PFBO derivative was very sensitive towards the ECD and was about 40 times more sensitive than α,α-dichlorodiphenylmethane used as an IS. Fig. 1B is a chromatogram obtained on treating 200 ng of amygdalin according to method B. A stable response was observed, even for the injection of 10 pg of benzaldehyde as its O-PFBO.

The overall precision of measurements by method B was a coefficient of variation of 5.03% ($n = 6$) and a correlation coefficient of 0.9989 over the range 0.1–2.0 μg of amygdalin. The advantage of the proposed method is the potential for microdetermination with fairly good precision. The procedure will be useful for monitoring the concentration and behaviour of amygdalin in biological materials.

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Note

Measurement of exposure to xylenes by separate determination of *m* and *p*-methylhippuric acids in urine

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(First received October 27th, 1980; revised manuscript received February 4th, 1981)

Hippuric acid occurs as a metabolite of toluene after inhalation of large doses and *m*- and *p*-methylhippuric acids (*m*- and *p*-MHA) are urinary metabolites of *m* and *p*-xylene. These acids are rapidly excreted in urine over a period of time tha increases with increasing level of exposure[1]. The determination of these acids is there fore a valuable index of exposure to toluene and also to xylenes, which often occur a contaminants of toluene.

Many methods have been described for the determination of these metabolites the first based on colorimetry. These have now been supplanted by various chroma tographic techniques: thin-layer chromatography[2], gas chromatography (GC)[3–5] high-performance liquid chromatography[6–8] and isotachophoresis[9]. GC procedure have not yet produced a simple separation of the various urinary metabolites o xylene, especially *m*- and *p*-MHA.

The aim of this work was to develop a simpler routine technique for hippuric acids; this was achieved with high sensitivity by GC using a thermionic detector anc an original internal standard.

EXPERIMENTAL

Reagents

The following analytical-reagent grade chemicals were used: concentratec hydrochloric acid, sodium chloride, ethyl acetate, methanol, diazomethane–diethy ether solution, benzoylproline (Fluka, Buchs, Switzerland) (internal standard), hip puric acid (Fluka), *m*-MHA* and *p*-MHA*.

Gas chromatographic system

A Hewlett-Packard 5710A gas chromatograph with a 2 m × 2 mm I.D. glass column packed with 2% OV-225 was used, equipped with a nitrogen–phosphorus flame-ionization detector. The carrier gas was nitrogen at a flow-rate of 20 ml/min.

* Kindly supplied by Mr. Rainer Sjöholm, Department of Organic Chemistry, Abo Akademi, Turku, Finland.

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The flame was air (50 ml/min)–hydrogen (3 ml/min). The following temperatures were maintained: column, 210°C; injector, 250°C; and detector, 300°C.

Method

A mixture of 0.5 ml urine, 0.5 ml of concentrated hydrochloric acid, 0.3 g of sodium chloride, 1 ml of 150 mg/l aqueous benzoylproline solution (internal standard) and 3 ml of ethyl acetate was shaken vigorously in a glass screw-capped test-tube for 10 min, and 2 ml of the supernatant ethyl acetate were transferred into another test-tube with 1 ml of methanol. Methylation was effected with diazomethane–diethyl ether solution. A 1-μl volume of the solution obtained was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Table I shows the extraction recovery of the internal standard and Tables II–IV show the results obtained with known amounts of the various hippuric acids as the methyl esters. Fig. 1 shows a chromatogram obtained for a mixture of compounds.

The extraction technique used here is that of Ogata *et al.*[10], which provides at least 99% recoveries of the various hippuric acids. However, with high levels of hippuric acids, the detector becomes saturated and the results are not reproducible. A ten-fold dilution of the sample will prevent this from occurring.

The GC separation of *m*- and *p*-MHA is very satisfactory. The specificity of OV-225 is much better than that of SE-30 as used by Kira[5] for the same order of sensitivity; the latter type of packing, even when used at 10% concentration, does not separate the two isomers. Moreover, salicyluric acid and its monomethyl ester inter-

TABLE I

RECOVERY OF BENZOYLPROLINE FROM WATER

	Amount added to water (mg/l)	*Amount found (mg/l) (n = 5)*	*Recovery (%)*	*S.D.*	*Coefficient of variation (%)*
Benzoylproline (mg/l)	100	100.28	100.28	2.91	2.9

TABLE II

RECOVERY OF HIPPURIC ACID FROM URINE

	Amount added to urine (mg/l)	*Amount found (mg/l) (n = 5)*	*Recovery (%)*	*S.D.*	*Coefficient of variation (%)*
Urine (U)	0	120	–	4.8	4
U + hippuric acid (mg/l)	250	303.6	73.1	8.0	2.6
	500	522.1	80.2	9.8	1.9
	1000	885.1	77.4	20.1	2.3

TABLE III

RECOVERY OF *m*-METHYLHIPPURIC ACID FROM URINE

	Amount added to urine (mg/l)	*Amount found (mg/l) (n = 5)*	*Recovery (%)*	*S.D.*	*Coefficient of variation (%)*
Urine (U)	0	0	–	–	–
U + *m*-MHA (mg/l)	28.37	26.98	95	1.30	4.8
	56.75	50.37	88.7	3.09	6.1
	113.5	102.86	90.6	2.04	2

TABLE IV

RECOVERY OF *p*-METHYLHIPPURIC ACID FROM URINE

	Amount added to urine (mg/l)	*Amount found (mg/l) (n = 5)*	*Recovery (%)*	*S.D.*	*Coefficient of variation (%)*
Urine (U)	0	0	–	–	–
U + *p*-MHA (mg/l)	34.25	27.88	81.4	1.43	5.1
	68.5	54.35	79.3	2.90	5.3
	137	112.3	82	2.56	2.3

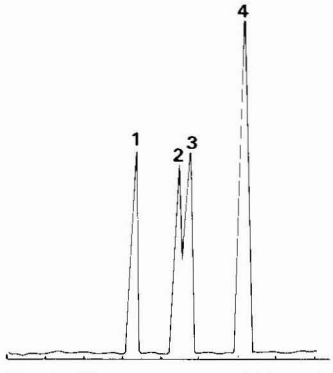

Fig. 1. Chromatogram of hippuric acid (1), *m*-methylhippuric acid (2), *p*-methylhippuric acid (3) and benzoylproline (internal standard) (4).

fere with this peak, whereas on the OV-225 column there is no interference, the retention times being hippuric acid 3.39 min, *m*-MHA 4.51 min, *p*-MHA 4.76 min, internal standard 6.16 min and methyl salicylurate 8.73 min.

We have compared the GC method with that of Engström *et al.*[4], by which hippuric acids were determined as their alkaline hydrolytic products. The correlation between exposure to toluene and urinary concentrations of benzoic and toluic acids

was better with the latter method for high levels corresponding to a heavy inhalation, but this was not our concern as we were more interested in long-term, low-level, exposure. It would seem more useful to evaluate the increase in the levels of the metabolites to achieve a better sensitivity together with a good resolution. This is what we propose here with the OV-225 separation and the thermionic detector, the sensitivity of which averages 1 mg/l with a signal-to-noise ratio of 5.

The relative response of the detector to hippuric acid and benzoylproline (internal standard) was about 1.01, *i.e.* the sensitivity of the detector is almost identical for the two compounds.

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Note

Identification of two *in vitro* metabolic products after liver microsomal incubation of antazoline

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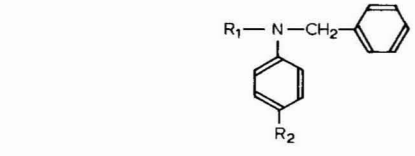
Antazole (Fig. 1) is an antihistaminic drug, with local anaesthetic and some anticholinergic properties. It is claimed to be less irritating to the tissues than most other antihistamines, and only two cases of lethal antazoline intoxication have been reported[1]. Blomquist *et al.*[1] reported two hydrolysis products of antazoline after autopsy in a case of lethal intoxication; (phenylbenzylaminoacetyl)ethylenediamine was the major hydrolysis product, together with N-benzylaniline in trace amounts.



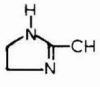
R_1	R_2	Compound
	H	Antazoline [2-(N-benzyl-anilinomenyl)imidazoline]
H	H	N-benzylaniline
H	OH	*p*-benzylaminophenol

Fig. 1. Structures of antazoline, N-benzylaniline and *p*-benzylaminophenol.

No reports on antazoline metabolism, to our knowledge, have been published previously. Therefore, we decided to study the metabolism of this drug to establish whether the two products reported[1] are metabolites or chemical decomposition products.

This paper describes investigations into the *in vitro* metabolism of antazoline using rabbit liver microsomal fraction.

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EXPERIMENTAL

Compounds and reagents

Antazoline hydrochloride was kindly supplied by Ciba-Geigy (Basle, Switzerland). N-Benzylaniline was obtained from BDH (Poole, Great Britain) and *p*-benzylaminophenol from Eastman (Rochester, NY, U.S.A.). $NADPNa_2$, glucose-6-phosphate Na_2 and glucose-6-phosphate dehydrogenase were purchased from Boehringer (Mannheim, G.F.R.), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.), acetic anhydride from BDH, acetonitrile from Fisons (Loughborough, Great Britain; redistilled and kept over calcium chloride at room temperature) AnalaR diethyl ether from BDH (freshly distilled), 2,3,5-triphenyl-2*H*-tetrazolium chloride monohydrate (98%) from Aldrich (Milwaukee, WI, U.S.A.), *n*-pentane from Fisons, iron(III) chloride from May & Baker (Dagenham, Great Britain) and sodium metabisulphite from BDH.

Thin-layer chromatography (TLC)

Glass plates (20 × 20 cm) were spread to a thickness of 0.25 mm with a mixture of silica gel G (Merck, Darmstadt, G.F.R.) and water (1:2). The plates were first allowed to dry at room temperature and were heated for 1 h at 110°C before use. The solvent system was chloroform–acetone (90:20, v/v). The various spots were revealed by spraying with (a) ammoniacal silver nitrate solution, (b) triphenyltetrazolium chloride (TTC), (c) iron(III) chloride (5% in 0.5 *N* hydrochloric acid) and (d) Dragendorff's reagent. These spray reagents were prepared and used according to the methods described in ref. 2.

Gas–liquid chromatography (GLC)

A Perkin-Elmer Model F33 gas chromatograph equipped with a flame-ionization detector and a 1.0-mV Perkin-Elmer 56 recorder were used. All columns were made of glass, 0.64 cm O.D., and the solid supports were acid washed and treated with dimethyldichlorosilane.

System A was a glass column, 2 m long, packed with Chromosorb Q (100–120 mesh) coated with OV-17 (3%, w/w) and operated under the following conditions: hydrogen, 1.12 kg/cm^2; air, 1.68 kg/cm^2; nitrogen, 2.1 kg/cm^2.

System B was a glass column, 2 m long, packed with Chromosorb G (80–100 mesh) coated with OV-17 (5%, w/w) and operated under the same conditions as for system A.

System C was a glass column, 1 m long, packed with Chromosorb W (80–100 mesh) coated with UCW-98 (10%, w/w) and operated under the following conditions: hydrogen, 2.1 kg/cm^2; air, 0.7 kg/cm^2; nitrogen, 1.4 kg/cm^2.

All the columns were conditioned at 250°C for 24 h before use and the injection port temperature was 250°C. Each column was silanized with two 5-μl portions of hexamethyldisilizane (HMDS) before use.

Combined gas–liquid chromatography–mass spectrometry

All mass spectra were obtained using a Perkin-Elmer Model 270 gas chromatograph–mass spectrometer at an electron energy of 70 eV. A 1.0 m × 0.64 cm O.D. glass column packed with UCW-98 (10%, w/w) on Chromosorb W (80–100 mesh)

was used at 190°C (oven temperature); helium (1.4 kg/cm^2) was used as the carrier gas.

Incubation procedure

Antazoline hydrochloride (10 μmol/ml as the base; 1 ml) was incubated at 37°C for 40 min with the microsomal fraction from a liver homogenate of a New Zealand white rabbit[3]. Each incubation mixture contained 1 ml of substrate; 1 ml of cofactor solution consisted of glucose-6-phosphate Na_2 (6 mg, 20 μmol), nicotinamide (0.6 *M*) (0.1 ml, 60 μmol), $NADPNa_2$ (3.4 mg, 4 μmol), distilled water (0.7 ml); phosphate buffer (pH 7.4) (3 ml) and liver microsomal fraction (1 ml) were also used. Glucose-6-phosphate dehydrogenase (2 units) was added to the microsomal fraction preparation. Control experiments were carried out at the same time.

In all instances the incubation mixtures were incubated for 5 min at 37°C with shaking before the addition of the substrate. The incubation reactions were stopped by putting the flasks in ice, and extracted as described.

Extraction procedure

(a) Antazoline and N-benzylaniline. To the incubation mixture (6 ml) was added sodium chloride (2 g); the pH was adjusted to 9–10 with ammonia solution (10%) and the mixture was extracted with freshly distilled diethyl ether. The concentrated ethereal extracts were analysed by GLC systems A and B and by TLC. Also, acetic anhydride was added to ethereal solutions of authentic N-benzylaniline and to the concentrated ethereal extracts of antazoline incubation mixture prior to GLC.

(b) p-Benzylaminophenol. To the incubation mixture (6 ml) was added $Na_2S_2O_5$ (10%, 1 ml) and sodium chloride (2 g), the pH was adjusted to 8.0–8.2 with ammonia solution (10%) and the mixture was extracted with freshly distilled diethyl ether. The concentrated ethereal extracts were examined using GLC with systems A, B and C and by TLC. A separate portion of the concentrated ethereal extracts was allowed to dry under nitrogen; dry acetonitrile (10–15 μl) and BSTFA (10 μl) were added and the mixture allowed to stand at room temperature for 5 min. The trimethylsilyl derivative of *p*-benzylaminophenol was then examined by GLC using system A.

Stability of antazoline during incubation and extraction

Antazoline hydrochloride (10 μmol as the base) was incubated at 37°C for 40 min as described earlier, but omitting the cofactor solution. The incubation mixture was extracted as described above. The concentrated ethereal extracts were examined by GLC using system A and by TLC.

RESULTS AND DISCUSSION

Stability of antazoline during incubation and extraction

When antazoline was incubated under conditions similar to those used in metabolic studies but without the cofactor solutions and the incubation mixture was extracted at pH 9.0 with diethyl ether, the ethereal extract gave only one peak on GLC (system A) and one spot on TLC corresponding to antazoline itself. It is therefore concluded that antazoline is stable under the above conditions.

Identification of metabolic products

N-Benzylaniline. When antazoline was incubated with rabbit liver microsomal fraction plus the necessary cofactor requirements and the incubation mixture was extracted at pH 9.0 with diethyl ether, N-benzylaniline was identified as a metabolic product. TLC of the ethereal extract gave a spot with an R_F value of 0.96 (Table I). GLC of the ethereal extract gave a peak at a retention time of 1.5, 5.0 and 4.0 min on columns A, B and C, respectively (Table I).

TABLE I

GLC AND TLC CHARACTERISTICS OF ANTAZOLINE, N-BENZYLANILINE, *p*-BENZYLAMINOPHENOL AND THEIR TRIMETHYLSILYL AND ACETYL DERIVATIVES

GLC oven temperatures are given in parentheses.

Compound	*GLC retention time (min)*			*TLC R_F values*
	Column A	*Column B*	*Column C*	
Antazoline	11.0 (240°C)	–	–	0.0
N-Benzylaniline	1.5 (220°C)	5.0 (230°C)	4.0 (190°C)	0.96
Acetyl derivative of N-benzylaniline	–	–	8.0 (190°C)	
p-Benzylaminophenol	6.0 (220°C)	17.0 (230°C)	15.0 (190°C)	0.39
Trimethylsilyl derivative *p*-benzylaminophenol	7.0 (215°C)	–	–	

Acetylation gave mainly N-acetylbenzylaniline together with traces of unreacted N-benzylaniline (Table I). GC–MS of the ethereal extract gave fragment ions at *m/e* 91 (100%) and 183 (40%), with the latter fragment ion corresponding to the molecular ion, in agreement with published data[1] (Fig. 2a and b). The above characteristics of the metabolically produced N-benzylaniline are identical with those of the authentic compound.

p-Benzylaminophenol. p-Benzylaminophenol was identified as a metabolic product of antazoline. TLC of the ethereal extract of the incubation mixture (pH 8.0) gave a spot with an R_F value of 0.39 (Table I). Black, violet and yellow spots were obtained upon spraying with ammoniacal silver nitrate, iron(III) chloride and TTC reagents, respectively. GLC of the ethereal extract (pH 8.0) gave a peak with a retention time of 6.0, 17.0 and 15.0 min on columns A, B and C, respectively (Table I). Derivatization with BSTFA gave the trimethylsilyl derivative of *p*-benzylaminophenol (retention time 7.0 min on column A, Table I). GC–MS of the ethereal extract (pH 8.0) showed the presence of the fragment ions *m/e* 91 (100%) and 199 (50%), corresponding to the base peak and the molecular ion, respectively (Fig. 3a and b). GC–MS of the trimethylsilyl derivative gave fragment ions at *m/e* 73 (base peak), 271 (molecular ion), 180, 91 and 75 (Fig. 4a and b). The fragment ions at *m/e* 73 and 75 correspond to the trimethylsilyl derivatives[4,5]. The characteristics of the metabolically produced *p*-benzylaminophenol, described above, are identical with those of the authentic reference compound.

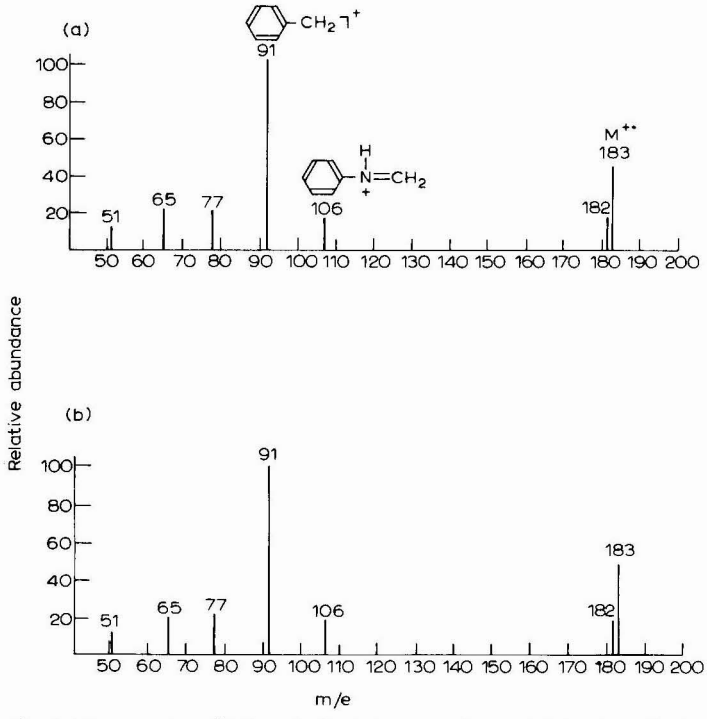

Fig. 2. Mass spectra of (a) synthetic N-benzylaniline and (b) metabolically produced N-benzylaniline.

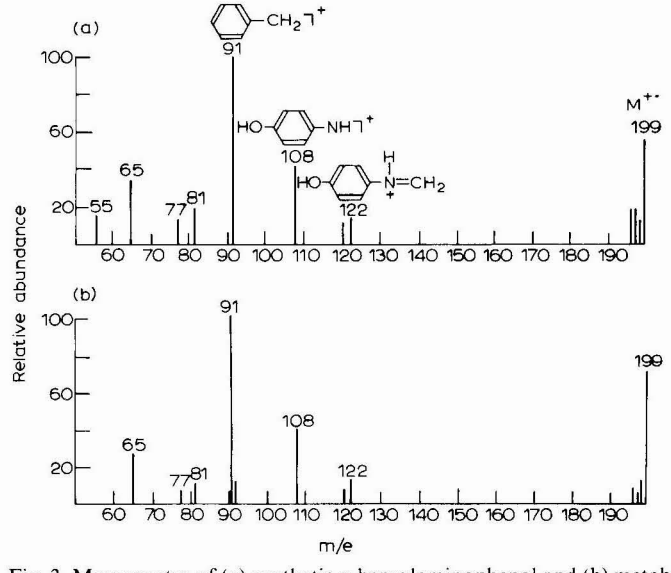

Fig. 3. Mass spectra of (a) synthetic *p*-benzylaminophenol and (b) metabolically produced *p*-benzylaminophenol.

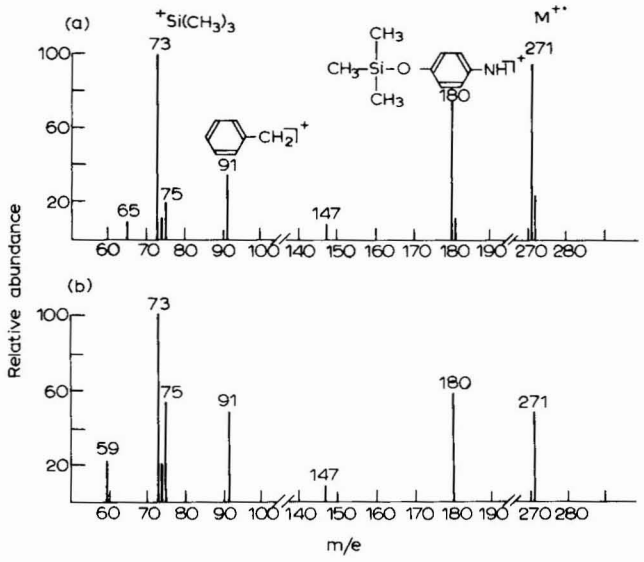

Fig. 4. Mass spectra of (a) synthetic *p*-benzylaminophenol and (b) metabolically produced *p*-benzylaminophenol as their trimethylsilyl derivatives.

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Note

Reversed-phase high-performance liquid chromatographic separation of carbofuran and its five metabolites

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Use of high-performance liquid chromatography (HPLC) for the determination of carbofuran residues has been described. Most reports include methods for the determination of 3-hydroxycarbofuran metabolites in plants[1–5]. Frei *et al.*[6] separated Dns derivatives of N-methylcarbamate pesticides, including carbofuran, in water and soil using a β,β'-oxydipropionitrile column. Moye and St. John[7] reported the use of a C_{18} column in the determination of carbofuran and other N-methylcarbamates. Sparacino and Hines[8] reported the separation of 25 carbamate pesticides, including 3-hydroxycarbofuran and carbofuran, using HPLC with silica, CN and NH_2 columns. Lawrence[9] reported the determination of carbamate pesticide residues, including carbofuran, in crops after separation on a silica column. Lawrence and Leduc[10] developed an analytical procedure for residues of carbofuran, 3-hydroxycarbofuran and 3-ketocarbofuran that used a silica column. We needed a method to separate carbofuran and its metabolites 3-hydroxycarbofuran, 3-hydroxy-7-phenol, 3-ketocarbofuran, 3-keto-7-phenol and 7-phenol, using HPLC. No report could be found on the use of HPLC for the separation of carbofuran and these five carbofuran metabolites.

Because of the variations in retention times and irreversible adsorption often observed with silica HPLC columns, the use of a reversed-phase column was explored. The HPLC separation of carbofuran and its five non-conjugated metabolites using a C_{18} reversed-phase column is described here.

EXPERIMENTAL

Apparatus

A liquid chromatograph (Model 1084B; Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a variable-wavelength UV detector (operated at 220 nm) and a 24 cm × 4.6 mm I.D. Zorbax ODS analytical column with a 5 cm × 4.6 mm I.D. Permaphase ODS guard column (DuPont, Wilmington, DE, U.S.A.) was used.

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Reagents

Methanol and acetonitrile were liquid chromatographic quality (Burdick & Jackson, Muskegon, MI, U.S.A.). Water was filtered through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). The following compounds (see Fig. 1 for structures), used as reference standards, were obtained from FMC (Middleport, NY, U.S.A.): carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate, I); 7-phenol (2,3-dihydro-2,2-dimethyl-7-hydroxybenzofuran, II); 3-OH-carbofuran (2,3-dihydro-2,2-dimethyl-3-hydroxybenzofuranyl-7-methylcarbamate, III); 3-OH-7-phenol (2,3-dihydro-2,2-dimethyl-3,7-dihydroxybenzofuran, IV); 3-CO-carbofuran (2,3-dihydro-2,2-dimethyl-3-ketobenzofuranyl-7-methylcarbamate, V) and 3-CO-7-phenol (2,3-dihydro-2,2-dimethyl-3-keto-7-hydroxybenzofuran, VI). Standards were prepared in methanol or acetonitrile and were stored at −20°C when not in use.

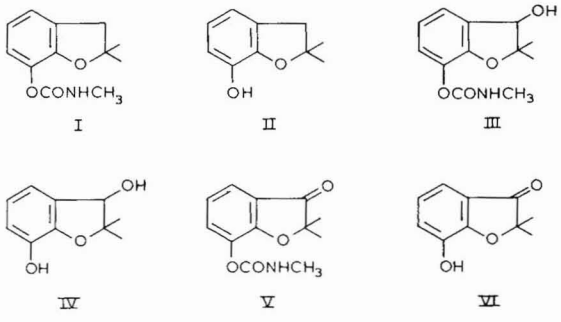

Fig. 1. Carbofuran and its five metabolites: I = carbofuran; II = 7-phenol; III = 3-OH-carbofuran; IV = 3-OH-7-phenol; V = 3-CO-carbofuran; VI = 3-CO-7-phenol.

High-performance liquid chromatography

Chromatographic parameters: column oven temperature 40°C; UV detector: sample beam wavelength 220 nm, reference beam wavelength 430 nm; mobile phase: System A (isocratic) acetonitrile–water (25:75), 2 ml/min; System B (gradient) methanol in water 40, 44, 62, 100 and 100% for retention times of 0, 8, 15, 17 and 30 min, 1.5 ml/min. Following gradient elution, the mobile phase was returned to starting conditions (methanol–water, 40:60) and the system was equilibrated for 10 min before any subsequent injections.

RESULTS AND DISCUSSION

Two solvent systems were investigated. The first was an acetonitrile–water isocratic elution (System A). Fig. 2 shows the resolution of carbofuran and its five metabolites using this system. The corresponding hydrolyzed compound eluted before its parent compound (II before I, IV before III and VI before V). Resolution of carbofuran and its metabolites was not completely satisfactory for our needs using this system because the resolution of I and II varied, especially when comparing resolution before and after flushing the column with 100% acetonitrile. Long equilibration periods were often required to return the column to optimum resolution following the acetonitrile flush. Reduction of the time required for the separation was

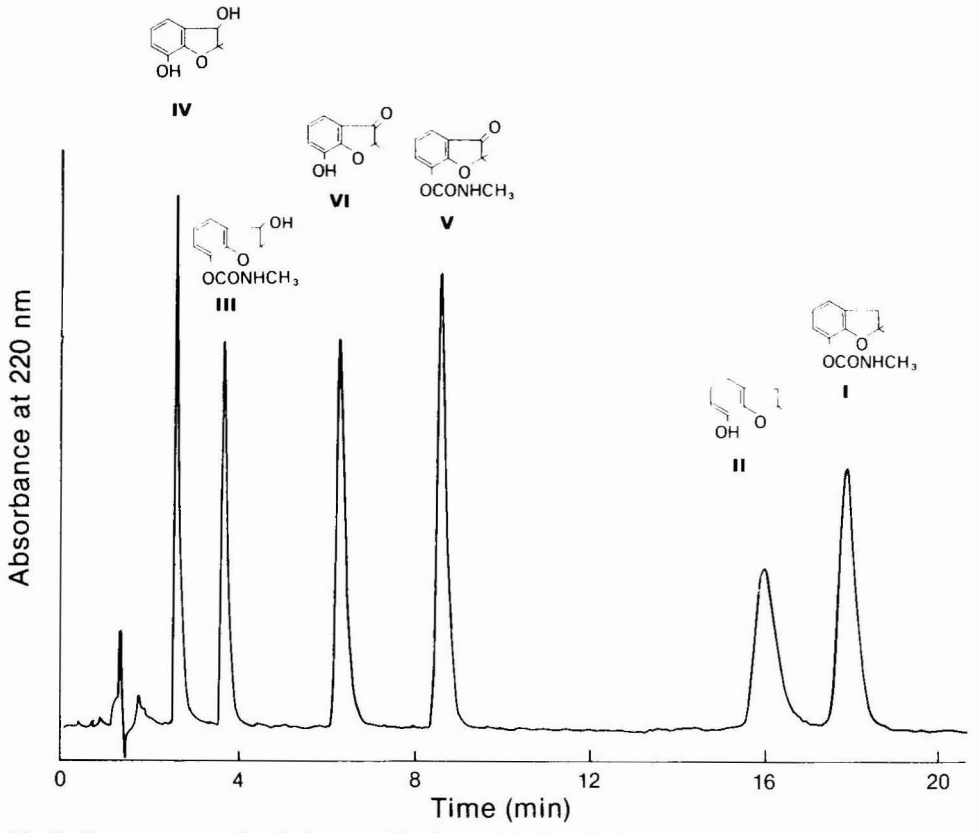

Fig. 2. Chromatogram of carbofuran and its five metabolites. Packing: Zorbax ODS. Column: 25 cm × 4.6 mm I.D. Mobile phase: acetonitrile–water (25:75), 2 ml/min (System A); recorder sensitivity, 0.058 absorbance units full scale; injection volume, 10 μl. Concentration of standards (μg/ml): I, 44.4; II, 48.0; III, 20.0; IV, 40.0; V, 31.6; VI, 12.6.

also desirable. For these reasons, a separation involving gradient elution was also investigated.

Fig. 3 shows a typical chromatogram of carbofuran and its metabolites using a methanol–water gradient elution (System B). The resolution of carbofuran and its five metabolites was satisfactory for our purposes. The order of elution of I and II is reversed compared with that of the isocratic elution using acetonitrile–water. This is not the case for the order of elution of the remaining four metabolites (III, IV, V and VI). Adequate resolution was maintained for consecutive runs using this elution system.

Some problems with the stability of V were encountered. Metcalf *et al.*[1] reported that V is easily hydrolyzed to its phenol (VI) upon standing at 37.5°C in methanol–phosphate buffer solution at pH 9.5. No significant hydrolysis of V to VI was observed when the mixed standards in methanol or acetonitrile were kept at 4°C overnight. Neither was hydrolysis of V observed when these solutions were stored for 1 week at −20°C. No degradation of the other carbofuran metabolites was observed when methanol and acetonitrile solutions were stored at 4°C for a month.

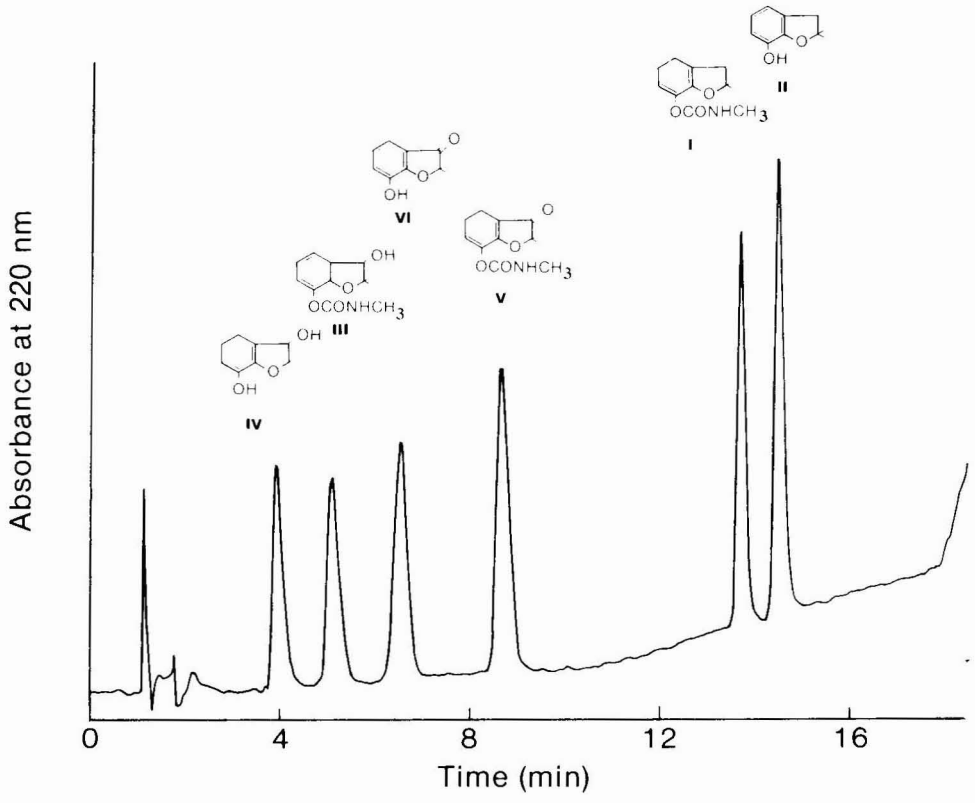

Fig. 3. Chromatogram of carbofuran and its five metabolites. Packing: Zorbax ODS. Column: 25 cm × 4.6 mm I.D. Mobile phase: as described in Experimental, 1.5 ml/min (System B); recorder sensitivity, 0.116 absorbance units full scale; injection volume, 10 μl. Concentration of standards (μg/ml). I, 57.8; II, 61.3; III, 41.8; IV, 36.4; V, 37.3; VI, 28.9.

In our application, these separations were made before collection of fractions and subsequent quantitation of [^{14}C]carbofuran residues in extracts of root crops. We believe that this separation, with UV determination at 220 nm, may be applicable for the quantitative determination of carbofuran residues in some samples without cleanup of the extracts. However, in most cases cleanup of the sample extracts before HPLC determination may be necessary. Data on the extraction study for which these separations were used will be presented in a future publication.

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Note

High-performance liquid chromatography of cyclohexanone oxime in urine and plasma

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Cyclohexanone oxime (Fig. 1) is a chemical intermediate used in the production of nylon 6. In the synthesis of nylon 6, cyclohexanone oxime undergoes the Beckmann rearrangement to form the seven-membered heterocyclic caprolactam.

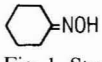

Fig. 1. Structure of cyclohexanone oxime, $C_6H_{11}ON$.

Caprolactam is then hydrolyzed to the straight-chain ε-aminocaproic acid which polymerizes to form the linear polymer. Toxicology data on cyclohexanone oxime is scarce, but the approximate seven-day LD_{50} in the male mouse, by intraperitoneal administration, has been reported[1] to be 250 mg/kg. Upon administration to the rat, cyclohexanone oxime appears to affect primarily the central nervous system. A synergistic central nervous system effect (as evaluated by loss of conditioned reflex) has been reported when cyclohexane, cyclohexanone, cyclohexanol and cyclohexanone oxime (four intermediates in the production of caprolactam) are administered simultaneously to the rat[2]. A decrease in the erythrocyte count and increase in blood methemoglobin levels in the rat after 6–10 week inhalation exposure to cyclohexanone oxime (0.1 or 1.0 mg/m^3) has also been reported[3]. Desquamation of the bronchial epithelium was also observed in the animals exposed to 1 mg/m^3, but no toxic effects were observed after exposure to 0.03 mg/m^3 (ref. 3).

Cyclohexanone oxime, along with cyclohexane, cyclohexanol and cyclohexanone, are also thought to be metabolites of the artificial sweetening agent sodium cyclamate[4–6]. In Drosophila tests, cyclohexanone oxime failed to cause sex-linked lethal mutations[7].

Several photometric[8], polarographic[9], thin-layer chromatographic[10,11], gas chromatographic[6,12] and liquid chromatographic[13] procedures for the determination of cyclohexanone oxime appear in the literature. However, most are indirect, relatively insensitive or unsuitable for the routine determination of trace quantities of the compound. Therefore, a sensitive, highly reproducible high-performance liquid chromatographic (HPLC) procedure for the determination of cyclohexanone oxime, suitable for use in water sample analysis, bioconcentration and metabolism studies, was developed.

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EXPERIMENTAL

Standards

Cyclohexanone oxime (purity 94.7%) was obtained from the Fibers and Plastics Company of Allied Chemical Corporation (Hopewell, VA, U.S.A.). Impurities in the material were water (*ca.* 5%) and cyclohexanone (*ca.* 0.2%). Cyclohexanone oxime was dissolved in the appropriate volume of acetonitrile to yield standards containing 0.005, 0.01, 0.05, 0.1 and 0.5 mg/ml. Standards were analyzed immediately after preparation. Samples (10 μl) were injected onto the column using microliter syringes (Glenco Scientific, Houston, TX, U.S.A.).

Equipment

Reversed-phase chromatography was performed using an LDC (Laboratory Data Control, Riveria Beach, FL, U.S.A.) constametric II-G HPLC system including an LDC Spectromonitor II variable-wavelength absorbance detector. Samples were injected onto the column using a Valco N60 fixed-volume loop injector. Separations were achieved with a Whatman (Clifton, NJ, U.S.A.) Partisil PXS 10/25 ODS-2 column (particle size, 10 μm; column dimensions, 25 cm × 4.5 mm I.D.). A guard column (Whatman) packed with pellicular ODS (particle size, 25–37 μm) was attached preceding the analytical column. The elution rate was 1.0 ml/min, and cyclohexanone oxime was detected at a wavelength of 205 nm, with the absorbance detector at sensitivities of 0.01–1.28 absorbance units full scale (a.u.f.s.). Solvent programming (Gradient Master, LDC) was used to establish the optimum solvent ratio.

Elution solvent

The elution solvent consisted of glass-distilled acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and micro-filtered distilled water. Acetonitrile–water (1:3, v/v) was found to give adequate resolution of cyclohexanone oxime from interfering peaks in urine and plasma samples. The elution solvent was degassed under vacuum before use, and kept under nitrogen during chromatography.

RESULTS AND DISCUSSION

Retention time

At a flow-rate of 1 ml/min, using acetonitrile–water (1:3) as the elution solvent, cyclohexanone oxime eluted as a sharp, symmetrical peak (Fig. 2). The retention times were highly reproducible. Fifteen injections of cyclohexanone oxime over a period of 6 days gave a mean retention time of 574 sec, with a coefficient of variation of 0.54% (Table I).

Precision and sensitivity

Precision was evaluated by injecting, over a two-day period, ten 10-μl aliquots of standard solution containing 0.5 μg cyclohexanone oxime. Reproducibility of peak height was good, with a coefficient of variation of 1.1%, representing the combined errors of injection, detection and flow-rate fluctuation (Table I). Mean sensitivity of detection, mm peak height per ng of cyclohexanone oxime injected (Table I) and the chromatograms shown in Fig. 2 indicate that as little as 10 ng of cyclohexanone oxime can be detected and quantitated.

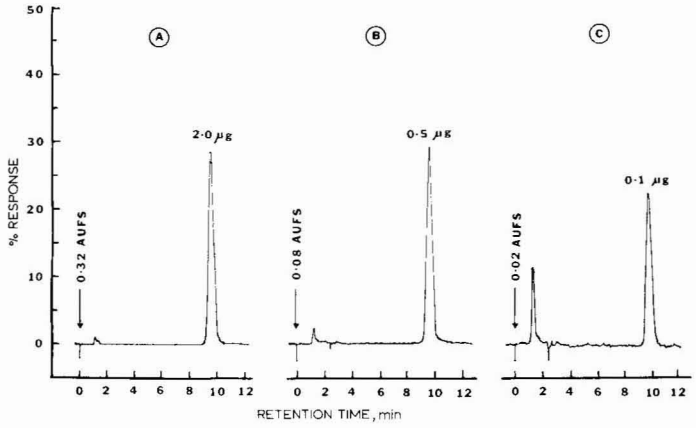

Fig. 2. HPLC chromatogram tracings of cyclohexanone oxime standards; (a) 2.0 μg, (b) 0.5 μg, (c) 0.1 μg. Elution solvent, acetonitrile–water (1:3); flow-rate 1 ml/min.

TABLE I

REPRODUCIBILITY OF RETENTION TIME AND PEAK HEIGHT FOR CYCLOHEXANONE OXIME BY HPLC

Retention time	
Injections, *n**	15
Range (sec)	565–579
Mean	574
Standard deviation (sec)	3.08
Coefficient of variation (%)	0.536
Peak height	
Injections, *n***	10
Range (mm)	85–88
Mean	86.95
Standard deviation (mm)	0.956
Coefficient of variation (%)	1.099
Sensitivity (mm peak height/ng)***	3.478

* Successive injections of cyclohexanone oxime standard (50 ng–5 μg) over a six-day period, using several separately prepared batches of the elution solvent.

** Successive injections of cyclohexanone oxime standard (0.5 μg, sensitivity 0.08 AUFS).

*** Calculated to maximum sensitivity, 0.005 AUFS.

Linearity

The relationship between peak height and quantity of cyclohexanone oxime injected was linear over a range of 50 ng–2.5 μg. In Fig. 3, the peak heights (converted to a common sensitivity) are plotted *vs.* the quantity of cyclohexanone oxime injected.

Recovery of cyclohexanone oxime from rat urine and plasma

For the determination of cyclohexanone oxime in urine, cyclohexanone oxime (dissolved in acetonitrile) was added to aliquots of urine (from untreated rats) to yield concentrations of 0.01, 0.05, 0.1 and 0.25 mg/ml. The spiked samples then were

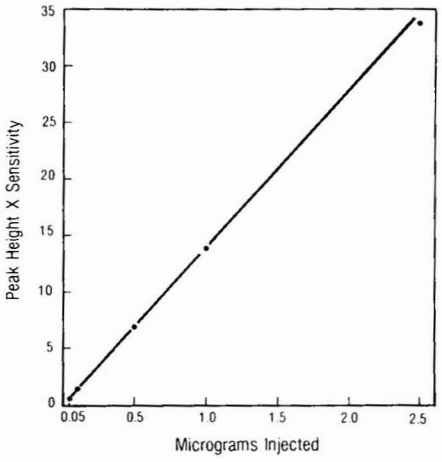

Fig. 3. Linearity, peak height *vs.* amount of cyclohexanone oxime injected (50 ng–2.5 μg).

centrifuged and the supernatants injected directly onto the high-performance liquid chromatograph for determination of cyclohexanone oxime.

For the determination of cyclohexanone oxime in plasma, cyclohexanone oxime (dissolved in acetonitrile) was added to aliquots of plasma (from untreated rats) to yield concentrations of 0.01, 0.05, 0.1 and 0.25 mg/ml. An equal volume of acetonitrile was added to each spiked sample to precipitate the plasma proteins. The

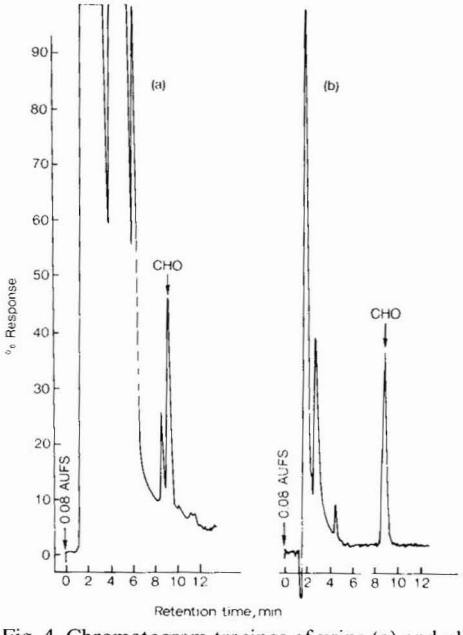

Fig. 4. Chromatogram tracings of urine (a) and plasma (b) spiked to 50 μg/ml with cyclohexanone oxime (CHO). Spiked plasma samples were diluted 1:1 with acetonitrile to precipitate the proteins. The peak (retention time 8.5 min) immediately preceding CHO was not observed in all urine samples.

TABLE II

RECOVERY OF CYCLOHEXANONE OXIME FROM SPIKED URINE AND PLASMA SAMPLES

Recovery given as mean ± standard error.

Cyclohexanone oxime added (mg/ml)	*Recovery (%)*	
	Urine	*Plasma*
0	–	–
0.01	96.9 ± 1.76	104.5 ± 2.56
0.05	102.5 ± 2.87	102.6 ± 1.86
0.1	103.3 ± 2.84	99.8 ± 0.947
0.25	98.7 ± 0.998	97.8 ± 2.10

samples were centrifuged (5 min at 1000 *g*), and the supernatants were removed. The supernatants were then injected onto the high-performance liquid chromatograph for determination of cyclohexanone oxime.

As indicated in Table II, determination of cyclohexanone oxime in both urine and plasma was quantitative over the concentration range investigated. Chromatogram tracings of spiked urine and plasma samples are shown in Figs. 4a and 4b, respectively.

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Note

High-performance liquid chromatographic determination of stress-induced sesquiterpenes of the potato (*Solanum tuberosum*)

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Under the stress of bacterial and fungal infection, potato tuber tissue produces a number of sesquiterpenes that are not native to uninfected tissue[1–8]. These compounds are of interest because of the possibility that such compounds are involved in the resistance of the tuber and the potato plant to infection. Qualitative and quantitative analytical methods are needed for determining the conditions under which sesquiterpenes are formed in the potato tuber. Gas–liquid chromatography (GLC) has been used to separate and quantitate some of these compounds[9–11].

The purpose of this investigation was to establish high-performance liquid chromatography (HPLC) parameters suitable for the separation and quantitation of the four main sesquiterpenes produced in infected potato tissue.

EXPERIMENTAL*

Phytuberin[12–14], katahdinone[15,16], rishitin[17], and lubimin[18–20] (Fig. 1) have been reported as the predominant sesquiterpenes produced in infected potato tissue.

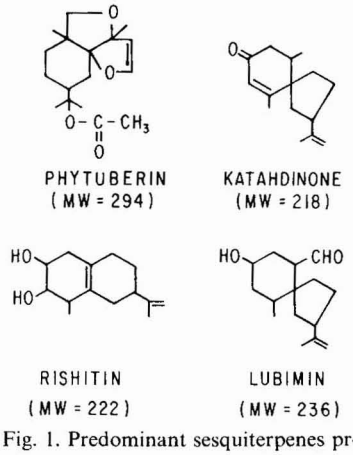

Fig. 1. Predominant sesquiterpenes produced in infected potato tubers. MW = Molecular weight.

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

For the purposes of this study these compounds were isolated from potato tubers infected with *Phytophthora infestans* and were purified by combinations of vacuum steam distillation and column and thin-layer chromatographic (TLC) techniques[6]. The identities of these four compounds were confirmed by mass spectrometry at this laboratory.

Standard solutions containing 0.1 μg/μl of lubimin, rishitin, phytuberin, and katahdinone in methanol were prepared and used to evaluate HPLC separation parameters. The HPLC system was assembled from Waters Assoc. (Milford, MA, U.S.A.) components (solvent delivery system, Model 6000 A; injection system, Model U6K; and variable-wavelength UV detector, Model 450) and a column that was 30 cm $\times$ 3.9 mm I.D. and packed with 10-μm, reversed-phase, μBondapak C_{18} (monomolecular layer of organosilane bonded to porous silica particles). The system was operated isocratically with a binary solvent mixture consisting of methanol–water. The proportions of organic solvent and water were varied over a range of 9:1 to 6:4, in conjunction with a varying flow-rate of 0.4 to 2.0 ml/min, respectively, to determine optimum conditions for separation of the four sesquiterpenes. A wavelength scan was run with the Model 450 variable-wavlength UV detector, and a wavelength of 200 nm was found to be satisfactory for all four compounds. Katahdinone also can be detected at 255 nm.

RESULTS AND DISCUSSION

With a methanol–water (7:3) solvent system, at a flow-rate of 1.0 ml/min and a wavelength of 200 nm, good separation of authentic lubimin, rishitin, phytuberin, and katahdinone was obtained, the resolutions being 1.1, 1.5, and 3.4 for lubimin–rishitin, rishitin–phytuberin and phytuberin–katahdinone, respectively (Fig. 2). Other conditions were investigated, namely, a μBondapak phenyl column (30 cm $\times$ 3.9 mm I.D.) and an acetonitrile–water solvent system. In no case was the resolution as good as that obtained with the methanol–water system and the μBondapak C_{18} column as described above. The early peaks in the chromatogram do not represent impurities in the standards; they were present in the control (solvent only) injection. As expected for the four compounds, the capacity ratio (k') (based on unretained solvent peak) for each compound increased as the amount of water, the more polar solvent in the binary system, was increased (Fig. 3). In addition to tests on extracts spiked with authentic compounds, the identities of lubimin, rishitin, phytuberin, and katahdinone separated from extracts were substantiated further on the HPLC eluate collected in fractions corresponding to the retention volumes of the authentic compounds, then concentrated and spotted on TLC plates; TLC R_F values agreed with those of authentic compounds, and no extraneous compounds were found. Standard curves were developed with known amounts of authentic lubimin, rishitin, phytuberin, and katahdinone (Fig. 4); recorder response was linear throughout the range of weights used to establish the curves with the stated operating conditions. Calculated as peak height (mm) divided by weight of compound (μg) response factors per μg of compound for lubimin, rishitin, phytuberin, and katahdinone were 364, 1674, 698, and 926, respectively.

The precision of the HPLC method was determined with a crude extract of potato tissue that had been infected with *P. infestans* and incubated for 72 h at 20°C

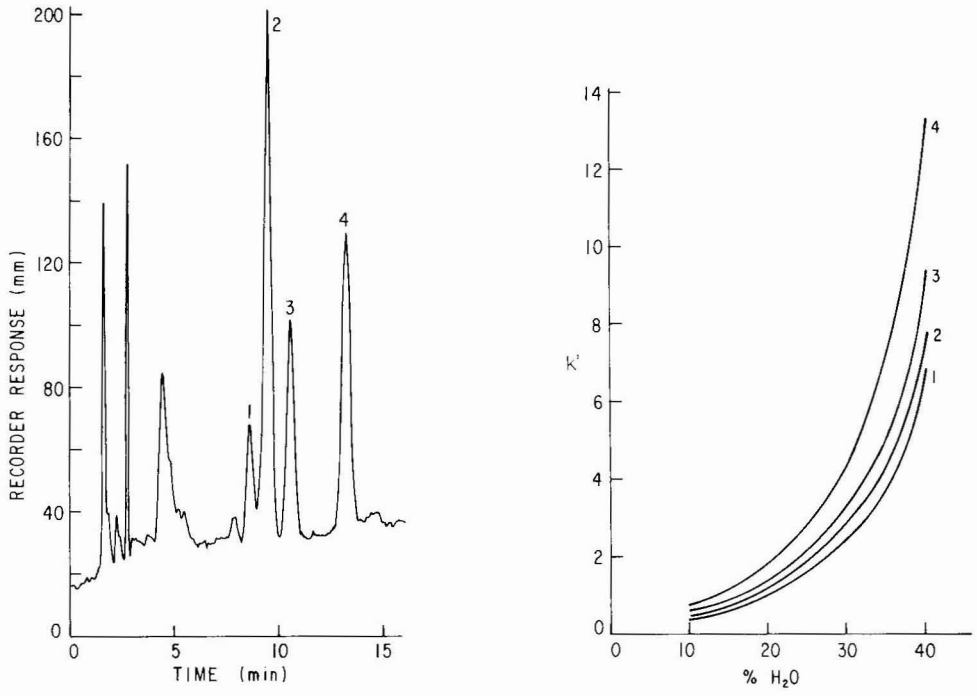

Fig. 2. High-performance liquid chromatogram of authentic sesquiterpenes: lubimin (1), rishitin (2), phytuberin (3), and katahdinone (4) (0.2 μg of each compound). Methanol–water (70:30), 1.0 ml/min, wavelength 200 nm, 0.02 a.u.f.s., reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D.).

Fig. 3. k' values obtained for lubimin (1), rishitin (2), phytuberin (3), and katahdinone (4). Methanol–water system, reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D.) $k' = (t_R - t_{R_0})/t_{R_0}$, where t_{R_0} is the retention time of the solvent front, and t_{R_1} is the retention time of the compound.

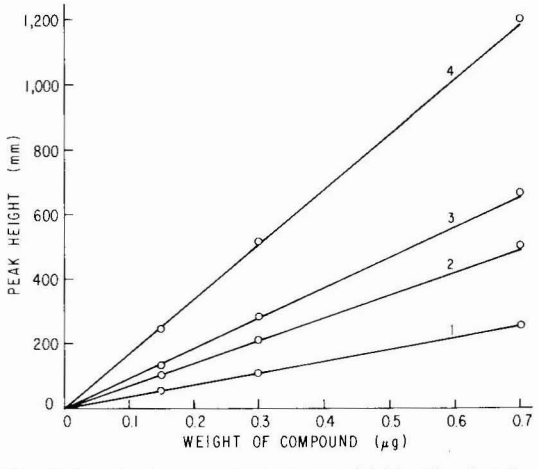

Fig. 4. Standard curves, lubimin (1), rishitin (2), phytuberin (3), and katahdinone (4). HPLC, methanol–water (70:30), 1.0 ml/min, reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D.).

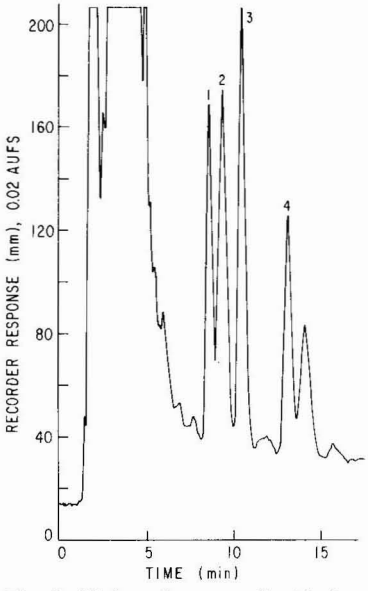

Fig. 5. High-performance liquid chromatogram of sesquiterpenes in a crude extract of potato tissue infected with *Phytophthora infestans:* lubimin (1), rishitin (2), phytuberin (3), and katahdinone (4); 3.0-μ introduction of diluted (30 ×) crude extract. Column and operating conditions as in Fig. 2.

in an ethylene–oxygen atmosphere[7]. The initial extract was too concentrated and had to be diluted 30-fold. A 3.0-μl injection of the diluted extract was used for the precision study. An example of the chromatogram obtained is presented in Fig. 5. A series of 11 determinations for lubimin, rishitin, phytuberin, and katahdinone were made. the standard deviation of the values obtained for the concentration of each compound was calculated. The concentration values shown were calculated back to the original (undiluted) crude extract (Table I).

TABLE I

STANDARD DEVIATIONS OBTAINED FROM REPLICATE (n = 11) HPLC ANALYSES OF A CRUDE EXTRACT OF POTATO TISSUE INFECTED WITH *PHYTOPHTHORA INFESTANS*

Compound	*Average concentration found (μg/μl)**	*S.D.*	*Rel. S.D. (%)*
Lubimin	7.48	0.321	4.29
Rishitin	1.40	0.218	15.57
Phytuberin	4.50	0.308	6.84
Katahdione	2.07	0.056	2.69

* Orig. (undiluted) crude extract.

The percent recoveries of the four compounds were determined by spiking the crude extract with known amounts of each compound (0.15 μg). Five determinations were made with the spiked and unspiked extracts. The average recoveries for lubimin

rishitin, phytuberin, and katahdinone were, 99.8, 92.0, 116.2, and 98.8%, respectively.

As described herein, a reversed-phase, μBondapak C_{18} HPLC column eluted with methanol–water (70:30), at a flow-rate of 1.0 ml/min, can be used routinely to separate and determine the quantities of lubimin, rishitin, phytoberin, and katahdinone in crude extracts of potato tissue.

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Note

Dehydrogenation of terpenoids on chromatoplates

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(First received June 19th, 1980; revised manuscript received January 28th, 1981)

A number of organic reactions in a solid matrix are known to occur[1–4]. For example, El-Maghraby[5] carried out chemical reactions on chromatoplates in order to solve several problems, particularly involving natural products. Materials obtainable from natural sources are often too small for preparing derivatives for comparison with known products or for carrying out reactions in a flask. We describe here the dehydrogenation of terpenoids with *p*-chloranil and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) on chromatoplates.

EXPERIMENTAL AND RESULTS

Glass plates (5 × 20 cm) were coated with a silica gel G slurry, which was prepared by mixing thoroughly one part by mass of silica gel G with two parts by volume of water. After drying in air the plates were stored and heated in an oven for 30 min at 110°C before use.

TABLE I

R_F VALUES OF REACTION PRODUCTS OF TERPENOIDS WITH *p*-CHLORANIL ON SILICA GEL G PLATES

Solvent: benzene–ethyl acetate (9:1 or 8:2).

Compound	*Original compound*		*Reaction product*	
	9:1	*8:2*	*9:1*	*8:2*
Carvone	0.23	0.24	0.23	0.24
Linalool	0.22	0.23	0.27	0.30
Eugenol	0.64	0.65	0.57	0.60
Khusinodiol	0.20	0.24	0.13	0.14
Santonin	0.34	0.35	0.34	0.35
Abietic acid	0.37	0.39	0.46	0.50
Camphene	0.36	0.44	0.81	0.83
Menthol	0.50	0.53	0.72	0.79
Khusol	0.59	0.60	0.59	0.60
Khusilic acid	0.45	0.49	0.32	0.33
Khusilol	0.56	0.58	0.73	0.76
Daucol tosylate	0.78	0.79	0.83	0.87
Podocarpic acid	0.27	0.52	0.66	0.71
Δ^3-Carene	0.55	0.56	0.77	0.84
Khusinol	0.52	0.63	0.78	0.87

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TABLE II

R_F VALUES OF REACTION PRODUCTS OF TERPENOIDS WITH DDQ ON SILICA GEL G PLATES

Solvent: benzene–ethyl acetate (8:2 or 7:3)

Compound	*Original compound*		*Reaction product*	
	8:2	*7:3*	*8:2*	*7:3*
Equol	0.28	0.51	0.09	0.12
Khusinol	0.37	0.62	0.08	0.08
Khusol	0.35	0.56	0.07	0.10
Citral	0.44	0.68	0.06	0.08
Camphene	0.61	0.75	0.07	0.07
Khusinodiol	0.14	0.27	0.05	0.06
Santonin	0.21	0.36	0.03	0.06
Khusilic acid	0.06	0.10	0.03	0.05
Khusilol	0.38	0.53	0.06	0.06
Daucol tosylate	0.56	0.59	0.06	0.08
Podocarpic acid	0.30	0.37	0.10	0.12
Δ^3-Carene	0.50	0.51	0.08	0.09
Carotol	0.37	0.62	0.11	0.13
Daucol	0.51	0.54	0.06	0.07

General method for carrying out the reaction on chromatoplates

Terpenoids in the appropriate solvent were applied to a plate at the starting line together with *p*-chloranil or DDQ dissolved in benzene. The plates were heated in an electric oven at 100°C for 12 h in order to simulate, as far as possible, the normal reaction conditions in a flask. The adsorbed layer was then treated in the normal manner as a chromatographic medium for the resolution of the components of the reaction mixture. Thus, after development with a suitable solvent system, the plates were sprayed with concentrated sulphuric acid–methanol (1:1) and heated in an electric oven at 100°C in order to reveal the spots. The results are given in Tables I and II.

It is interesting that *p*-chloranil or DDQ dehydrogenated khusinol and khusol on the plate. The product was isolated and identified as 1,6-dimethyl-4-isopropylnaphthalene by comparison with an authentic sample identified using thin-layer chromatography and IR spectroscopy.

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PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
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Chromatographic Reviews							220/1							
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