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Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

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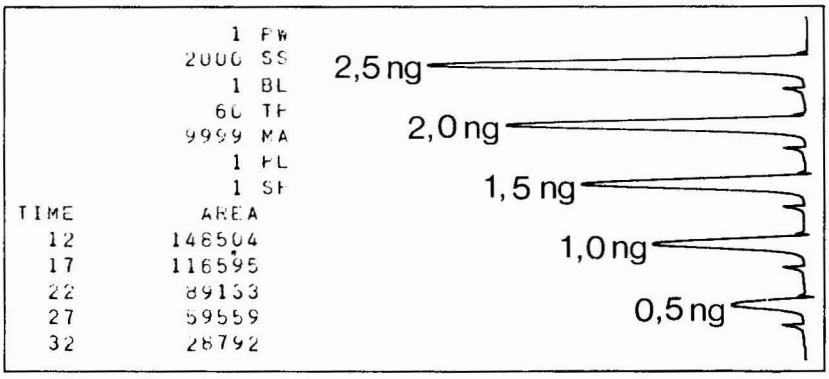

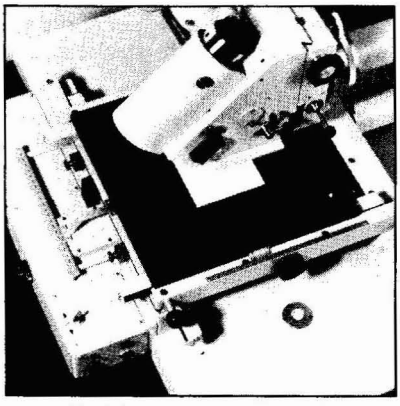

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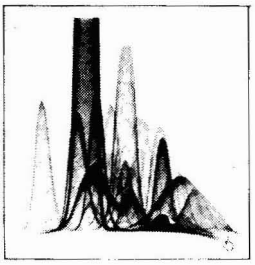

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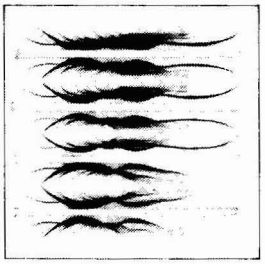

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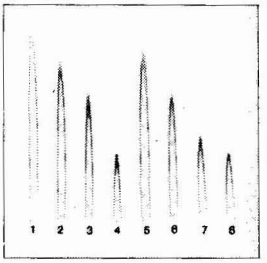

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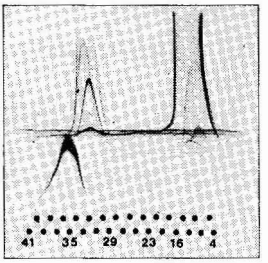

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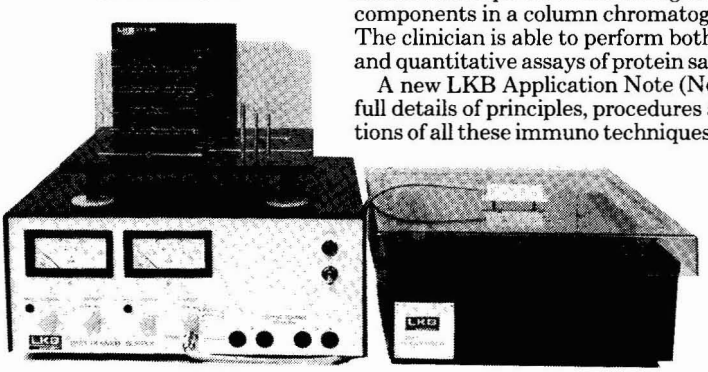

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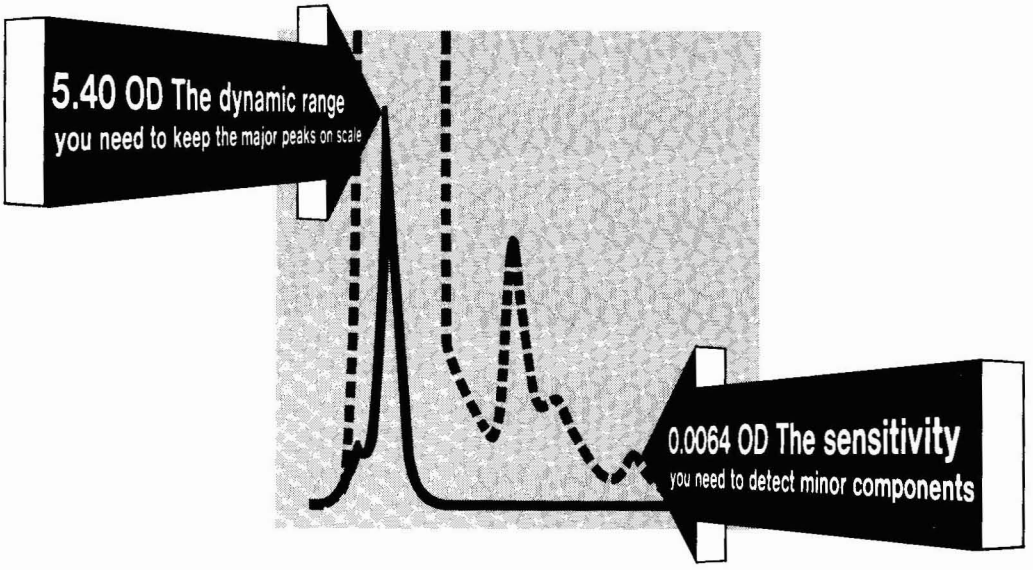

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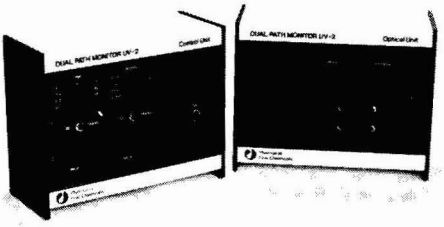

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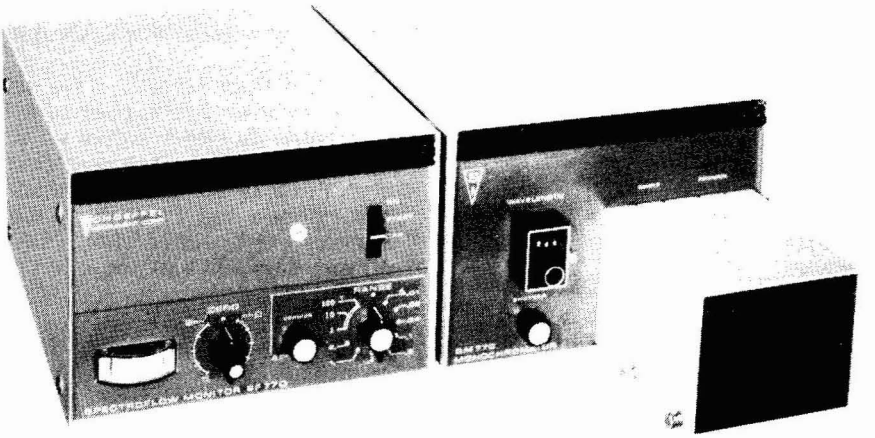

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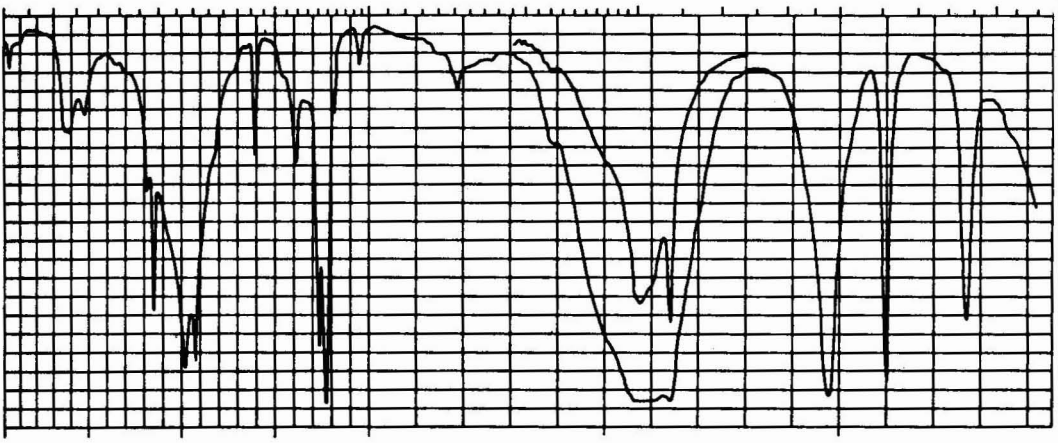

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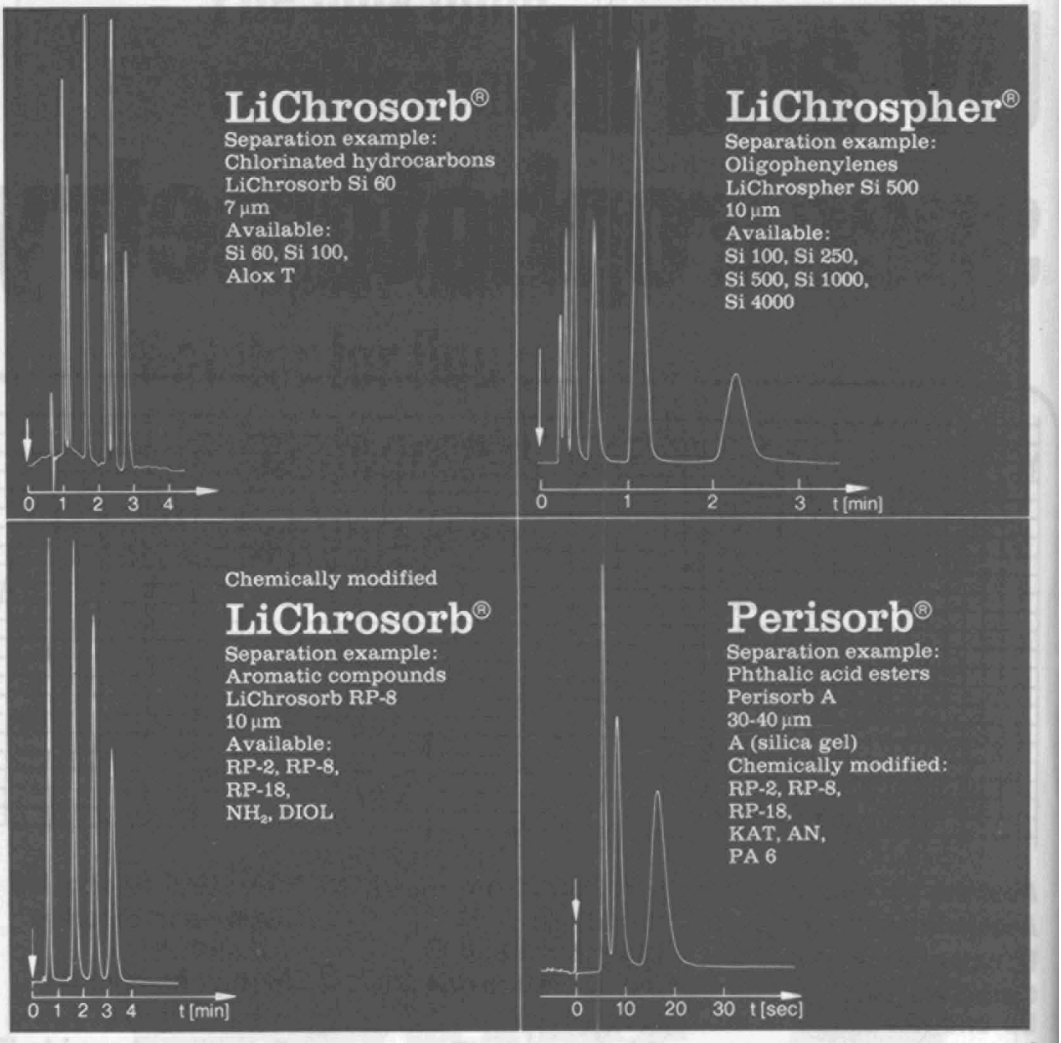

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THEORY OF CHROMATOGRAPHY OF RIGID MOLECULES ON HYDROXYAPATITE COLUMNS WITH SMALL LOADS

IV. ESTIMATION OF THE ADSORPTION ENERGY OF NUCLEOSIDE POLYPHOSPHATES

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(Received June 17th, 1977)

SUMMARY

Taves and Reedy postulated that phosphate ions are adsorbed at hydroxyl positions on the surface of hydroxyapatite, and proposed a model for the adsorption of tripolyphosphate on the crystal surface. On the basis of this model and the hypothesis that a hydroxyl position on the crystal surface corresponds to a chromatographic C site, the experimental chromatogram for a mixture of AMP, ADP, ATP and adenosine tetraphosphate obtained by Bernardi was analysed by using the theory developed in an earlier paper in this series. It was estimated that the energy of adsorption on a C site for a univalent phosphate group on the polyphosphate chain of nucleoside polyphosphate is 0.9–1 kcal/mole and that the adenosine group of the molecule covers at most only one crystal site. Some other experimental parameters were also evaluated. The reasonable results obtained in the calculation strongly support the hypothesis of the one-to-one correspondence between a hydroxyl position and a chromatographic C site and the model of Taves and Reedy.

INTRODUCTION

A model of the surface structure of hydroxyapatite (HA) was proposed by Taves and Reedy[1] on the basis of a comparison of the crystallographic structure of HA with the structure of octacalcium phosphate (OCP). OCP contains alternating sheets of an apatite structure[2–4] and a loose water-rich layer, which are parallel to the $(\vec{b}, \vec{c})$ plane of the crystal structure[5,6]. The apatite structure in OCP consists of a monolayer of apatite cells, each of the four edges (corresponding to four 6_3 axes[2,3]) of each apatite cell being on the interface with the water-rich layer. Two positions that would be occupied by OH^- ions in the HA structure are involved in this edge; with OCP, however, these positions are replaced by a water molecule and an HPO_4^{2-} ion (see Fig. 1 and refs. 5 and 6). Whether the crystal grows as OCP or HA appears to depend on whether one of these positions is occupied by a phosphate ion or a hydroxyl ion[6].

The fact that these positions are on the interface between the HA cell and the water-rich layer in the case of OCP suggests that the crystal surfaces [corresponding to $(\vec{a}, \vec{c})$ and $(\vec{b}, \vec{c})$ planes] of HA must also involve these positions. The fact that OCP grows more rapidly than HA, even though the latter is more stable, is consistent with this hypothesis[6]. Under physiological conditions where the hydroxyl ions in the surrounding solution would be present in much lower concentration than the phosphate ions, these positions on the surfaces of HA must be occupied by many more phosphate ions than hydroxyl ions[6]. Further, it can be estimated that the HPO_4^{2-} ion on the crystal surface (or on the interface in the case of OCP) has oxygen–calcium distances of 2.37, 2.39 and 2.63 Å and a hydrogen bond length of 2.46 Å (see Fig. 1), whereas a hydroxyl in the corresponding position would have only two comparable calcium–oxygen distances and only a weak hydrogen bond, if any[6]. The crystal growth of HA must be blocked when the surface hydroxyl positions are taken up by phosphate ions[1,6]. If unfilled, these positions form grooves running parallel to the $\vec{c}$ axis and these can be considered as adsorption sites for phosphate ions[1,6]. It is probable that the hydroxyl position corresponds to a chromatographic C site, using the nomenclature of earlier

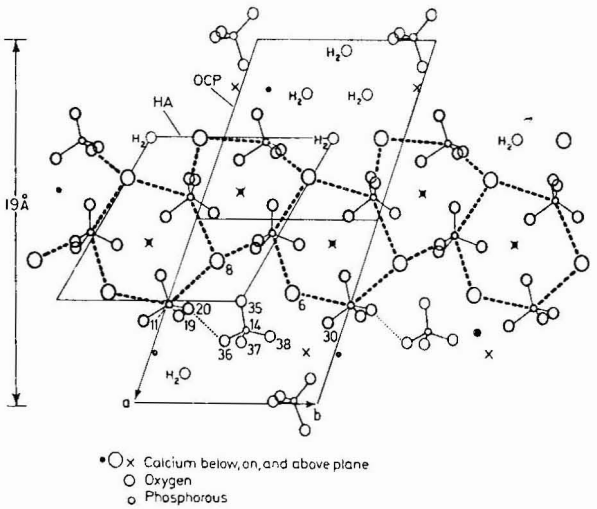

Fig. 1. Atomic positions in OCP on an $(\vec{a}, \vec{b})$ plane at the level of $z \approx 1/4$. The ions connected by dashed lines are those that are common to HA and OCP, and constitute an apatite layer (parallel to $\vec{b}$) inserted between water-rich layers. Two half-unit cells are shown for OCP and one for HA. The key positions are at the corners of the "HA unit cell". To allow the crystal to grow as HA, these must be OH^- ions instead of the H_2O and HPO_4^{2-} shown here (for HPO_4^{2-}, only one of them is shown). This hydrogen phosphate ion (with phosphorus No. 14) has oxygen–calcium distances of 2.37, 2.39 and 2.63 Å and a hydrogen bond length of 2.46 Å (between atoms 20 and 36). A hydroxyl in the position of oxygen No. 35 would have only two comparable calcium–oxygen distances, and only a weak hydrogen bond, if any. In both OCP and HA, two types of the plane alternate along the $\vec{c}$ axis separated by $|\vec{c}|/2$ or 3.44 Å at $z \approx 1/4$ (shown in the figure) and $z \approx 3/4$, respectively, that is, in the second plane of the unit cell the HA cell contents including the water and hydrogen phosphate are located in a reversed or 180° rotated manner. If the corners of the "HA unit cell" are unfilled, they form grooves running in the lateral surfaces parallel to the $\vec{c}$ axis. (This figure is reproduced from ref 1, with the permission of Dr. D. R. Taves).

papers[7–9] in this series (see Fig. 2). It was suggested by Kawasaki (Appendix I in ref. 7) that the C site exists on the side faces of the crystal or the lateral faces parallel to the $\vec{c}$ axis, which is consistent with the hypothesis that a C site corresponds to a hydroxyl position on the ($\vec{a}$, $\vec{c}$) and the ($\vec{b}$, $\vec{c}$) surfaces of HA.

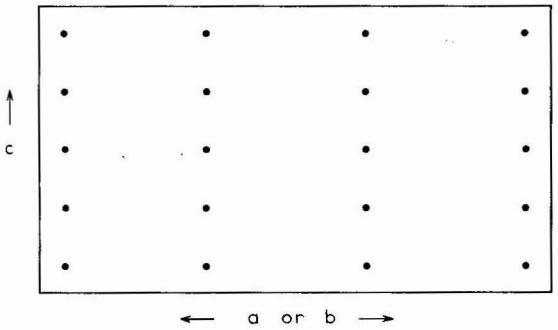

Fig. 2. Schematic representation of hydroxyl positions on the ($\vec{a}$, $\vec{c}$) or the ($\vec{b}$, $\vec{c}$) surface of HA. The minimal interval between the positions in the $\vec{a}$ or $\vec{b}$ direction (*i.e.*, $|\vec{a}|$ or $|\vec{b}|$) and the interval in the $\vec{c}$ direction (*i.e.*, $|\vec{c}|/2$) are 9.42 and 3.44 Å, respectively (*cf.*, Fig. 1 and see refs. 2–4). It is probable that a hydroxyl position constitutes a chromatographic C site (see text). This figure can be compared with Fig. 3 in an earlier paper[7] in which the possible arrangement of P sites is shown.

It was shown by Krane and Glimcher[10] that various nucleoside tri- and diphosphates as well as inorganic pyrophosphate are adsorbed on synthetic apatite crystals at physiological temperature and pH and that the terminal phosphorus atom of the bound nucleotide is transferred, after incubation for several minutes, to a crystal surface phosphate with the formation of pyrophosphate on the crystal and the respective di- and monophosphate (for the transphosphorylation, see Discussion). In connection with this finding, it was proposed by Taves and Reedy[1] that tripolyphosphate is likely to be adsorbed on the surface of HA with its long axis parallel to the $\vec{c}$ axis of the crystal and that the three phosphate groups of the molecule occupy three successive hydroxyl positions [separated by 3.44 Å (see Fig. 2)] of HA, for the following reasons. Firstly, there is a very good fit when the ions on the hydroxyl positions are replaced by a tripolyphosphate, while nothing comparable is found for other orientations or replacements. Secondly, the displacement of HPO_4^{2-} from a hydroxyl position would be much easier than the displacement of a structural phosphate. The former has three favourable electrostatic distances, 2.37–2.63 Å, while the latter has five or more good electrostatic distances.

This model is firmly supported by the HA chromatography of nucleoside mono-, di-, tri- and tetraphosphates and their derivatives carried out by Bernardi[11]. The non-phosphorylated derivatives were not retained by HA equilibrated with 1 m*M* potassium phosphate buffer (pH 6.8); monophosphates were eluted by 1 m*M* potassium phosphate buffer (pH 6.8) but they were retarded; ADP, ATP* and adenosine tetraphosphate were eluted at increasingly higher characteristic phosphate molarities

* Abbreviations: AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate.

with an almost linear relationship between the number of phosphates and the molarity (Fig. 3). This would indicate that the nucleoside phosphates are adsorbed via the phosphate groups (on to C crystal sites) and that the interval between the neighbouring phosphate groups on the polyphosphate chain fits satisfactorily with that between adsorption sites on the crystal surface.

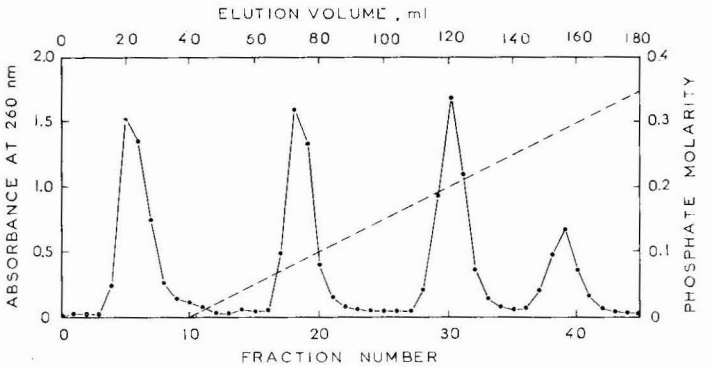

Fig. 3. Chromatography, at room temperature, of a mixture of AMP, ADP, ATP and adenosine tetraphosphate on a 10 × 1 cm HA column. The loading of about 2 mg of the mixture (in a small volume of 0.001 *M* potassium phosphate buffer, pH 6.8) was carried out at zero volume[12]; this amount (for a 10 × 1 cm column) would be small enough for the effect of mutual interactions among molecules adsorbed on HA or the mutual displacement of molecules[13] to be virtually negligible (*cf.*, Figs. 3–5 in ref. 14). Elution was carried out with a linear molarity gradient of potassium phosphate buffer (pH 6.8) (0.001–0.5 *M*; 100 + 100 ml), which was introduced after a volume of 4 ml had been eluted after loading, but which begins at 40 ml owing to the adsorption of the ions of the buffer on to the crystal surface (see Discussion); this was followed by refractometry and is indicated by the broken line (right-hand ordinate). On the abscissa, both the fraction number and the elution volume are shown; the latter was estimated on the basis that the slope of the gradient of phosphate molarity should be 0.5/(100 + 100) = 0.0025 (*M*/ml) (*cf.*, Discussion). The yields were 133, 113, 113 and 50% for AMP, ADP, ATP and adenosine tetraphosphate, respectively, with an overall recovery of 102% (for an explanation of which, see Discussion). For necessary information involved in the figure, see Table II. (This figure is reproduced with slight modifications, from Fig. 1 in ref. 11, with the permission of Prof. G. Bernardi).

In this paper, on the basis of the Taves and Reedy model[1], an attempt is made to apply the theory developed in an earlier paper[7] to the experimental results of the chromatography of a mixture of AMP, ADP, ATP and adenosine tetraphosphate carried out by Bernardi[11], which is shown in Fig. 3, and to evaluate the adsorption energy per univalent phosphate group on the polyphosphate chain of the nucleotides. Some other experimental parameters are also evaluated. The reasonable results of the calculation strongly support the hypothesis that a hydroxyl position on the crystal surface corresponds to a chromatographic C site and the model for the manner of the adsorption of polyphosphate proposed by Taves and Reedy[1].

THEORETICAL

In an earlier paper[7], it was shown that, provided the activity of competing ions is proportional to the molarity, the elution molarity, m_{elu}, at the maximum height*

* Unless s is extremely small, it should be the elution molarity at the maximal height of the chromatographic peak that can be described by eqn. 15 in ref. 7 or eqn. 1 in this paper (see ref. 9).

of each chromatographic peak of a mixture with a small load can be described by eqn. 15 in ref. 7, or by

$$s = \frac{1}{(x' + 1)\cdot\varphi'\cdot\beta_3\,\sigma\,e^{x\varepsilon_3/kT}}\cdot[(\varphi'\cdot m_{elu} + 1)^{x'+1} - (\varphi'\cdot m_{in} + 1)^{x'+1}] \qquad (1)$$

in which

$$s = g\,L \qquad (2)$$

and

$$\varphi' = \frac{\Lambda_2}{m} \qquad (3)$$

In eqn. 2, g is a constant representing the slope (molarity per unit column length) of the linear gradient of competing ions and L is the length of the column; in eqn. 3, Λ_2 is a parameter proportional to the activity of competing ions defined by eqn. 3 in ref. 7 and m is the corresponding molarity. Therefore, φ' is a constant assuming proportionality between the activity and the molarity. In eqn. 1, m_{in} is the molarity of competing ions in the initial buffer before the gradient is applied; x' is the number of sites of HA on which competing ions cannot be adsorbed owing to the presence of an adsorbed molecule; x is the number of adsorption groups of the molecule that can react with sites of HA; $-\varepsilon_3$ ($\varepsilon_3 > 0$) is the adsorption energy of an adsorption group of the molecule on to one of the sites of HA*; and σ and β_3 are constants related to the symmetry of the molecule** and to the property of the column, respectively.

Now, we propose a model for the adsorption of AMP, ADP, ATP and adenosine tetraphosphate on the HA surface as shown in Table I, in which ε'' is the adsorption energy (to one of the crystal sites) of a phosphate group of AMP or the terminal phosphate group of the polyphosphate chain of ADP, ATP and adenosine tetraphosphate; ε' is the adsorption energy for one univalent phosphate group on the polyphosphate chain of ADP, ATP and adenosine tetraphosphate; and x_0' is the number of sites of HA on which competing ions cannot be adsorbed owing to the presence of the terminal phosphate group plus the adenosine part of the nucleotide, assuming that a univalent phosphate on the polyphosphate chain covers only one crystal site. It seems also reasonable to assume that a terminal phosphate group covers only one crystal site, according to which $x_0' - 1$ indicates the number

* It is evident that the term $x\varepsilon_3$ in eqn. 1 can be re-written as $x_1\varepsilon' + x_2\varepsilon''$ if the molecule contains two types of adsorption groups with different adsorption energies, ε' and ε'', which is the case with nucleoside phosphate (*cf.*, Table I).

** In a following paper[15], it will be mentioned that the theory for rigid molecules can be extended to the general case of flexible molecules and that σ represents not only the symmetry but also the flexibility of the molecule. It is reasonable to consider that the molecule of nucleoside phosphate is not completely rigid. It also seems reasonable to assume, however, that the values of σ for AMP, ADP, ATP and adenosine tetraphosphate are almost equal, because except for the part of the molecule fixed on the crystal surface or the phosphate chain (see below), the structures of these molecules are identical.

TABLE I

CHROMATOGRAPHIC MODELS OF AMP, ADP, ATP AND ADENOSINE TETRAPHOSPHATE

In the last column, symbols indicating the phosphate elution molarities at the maximal height of the chromatographic peaks are also shown for ADP, ATP and adenosine tetraphosphate. AMP is not retained on the column, however (see Fig. 3).

Molecule	*Adsorption energy per molecule*	x'	*Phosphate elution molarity*
AMP	ε''	x'_0	Not retained on the column
ADP	$\varepsilon'' + \varepsilon'$	$x'_0 + 1$	$m_{\mathrm{elu(ADP)}}$
ATP	$\varepsilon'' + 2\varepsilon'$	$x'_0 + 2$	$m_{\mathrm{elu(ATP)}}$
Adenosine tetraphosphate	$\varepsilon'' + 3\varepsilon'$	$x'_0 + 3$	$m_{\mathrm{elu(AtetraP)}}$

of crystal sites covered by the adenosine group of the nucleoside phosphate. On the basis of this model, eqn. 1 can be re-written for ADP, ATP and adenosine tetraphosphate as

$$\beta\sigma e^{\varepsilon''/kT} = \frac{a}{(x'_0 + 2)\cdot\varphi'\cdot s_{(\mathrm{ADP})}\cdot e^{\varepsilon'/kT}} \tag{4}$$

$$\beta\sigma e^{\varepsilon''/kT} = \frac{b}{(x'_0 + 3)\cdot\varphi'\cdot s_{(\mathrm{ATP})}\cdot (e^{\varepsilon'/kT})^2} \tag{5}$$

and

$$\beta\sigma e^{\varepsilon''/kT} = \frac{c}{(x'_0 + 4)\cdot\varphi'\cdot s_{(\mathrm{AtetraP})}\cdot (e^{\varepsilon'/kT})^3} \tag{6}$$

respectively, in which

$$a = (\varphi'\cdot m_{\mathrm{elu(ADP)}} + 1)^{x'_0+2} - (\varphi'\cdot m_{\mathrm{in}} + 1)^{x'_0+2} \tag{7}$$

$$b = (\varphi'\cdot m_{\mathrm{elu(ATP)}} + 1)^{x'_0+3} - (\varphi'\cdot m_{\mathrm{in}} + 1)^{x'_0+3} \tag{8}$$

and

$$c = (\varphi'\cdot m_{\mathrm{elu(AtetraP)}} + 1)^{x'_0+4} - (\varphi'\cdot m_{\mathrm{in}} + 1)^{x'_0+4} \tag{9}$$

where the subscript AtetraP represents adenosine tetraphosphate. In eqns. 4–6, β is used instead of β_3; $s_{(\mathrm{ADP})}$, $s_{(\mathrm{ATP})}$ and $s_{(\mathrm{AtetraP})}$ are the effective values of s (eqn. 2) for ADP, ATP and adenosine tetraphosphate, respectively, which are equal to s provided that the R_F values before the gradient of phosphate ions is applied are exactly zero. Actually, however, these values [$R_{F,\mathrm{in(ADP)}}$, $R_{F,\mathrm{in(ATP)}}$ and $R_{F,\mathrm{in(AtetraP)}}$] must be slightly greater than zero and increase in the order $0 \lesssim R_{F,\mathrm{in(AtetraP)}} < R_{F,\mathrm{in(ATP)}} < R_{F,\mathrm{in(ADP)}}$, which means that the effective lengths [$L_{(\mathrm{ADP})}$, $L_{(\mathrm{ATP})}$ and $L_{(\mathrm{AtetraP})}$] of the column for these molecules are smaller than the actual length (L) and decrease in the

order of $L \gtrsim L_{(\mathrm{AtetraP})} > L_{(\mathrm{ATP})} > L_{(\mathrm{ADP})}$. This also means that $s \gtrsim s_{(\mathrm{AtetraP})} > s_{(\mathrm{ATP})} > s_{(\mathrm{ADP})}$ (see eqn. 2). Now, by eliminating $\beta\sigma e^{\varepsilon''/kT}$ between eqns. 4 and 5 and between eqns. 5 and 6, we obtain

$$e^{\varepsilon'/kT} = \frac{s_{(\mathrm{ADP})}}{s_{(\mathrm{ATP})}} \cdot \frac{x_0' + 2}{x_0' + 3} \cdot \frac{b}{a} \tag{10}$$

and

$$e^{\varepsilon'/kT} = \frac{s_{(\mathrm{ATP})}}{s_{(\mathrm{AtetraP})}} \cdot \frac{x_0' + 3}{x_0' + 4} \cdot \frac{c}{b} \tag{11}$$

Further, by eliminating $e^{\varepsilon'/kT}$ between eqns. 10 and 11, and by using eqn. 7, we obtain

$$m_{\mathrm{elu(ADP)}} = \frac{1}{\varphi'} \cdot \left\{ \left[\frac{s_{(\mathrm{ADP})} \cdot s_{(\mathrm{AtetraP})}}{s_{(\mathrm{ATP})^2}} \cdot \frac{(x_0' + 2) \cdot (x_0' + 4)}{(x_0' + 3)^2} \cdot \frac{b^2}{c} + \right. \right.$$

$$\left. \left. + (\varphi' \cdot m_{\mathrm{in}} + 1)^{x_0'+2} \right]^{\frac{1}{x_0'+2}} - 1 \right\} \tag{12}$$

Now, if the values of x_0', $s_{(\mathrm{ADP})}$, $s_{(\mathrm{ATP})}$, $s_{(\mathrm{AtetraP})}$, φ', m_{in}, $m_{\mathrm{elu(ATP)}}$ and $m_{\mathrm{elu(AtetraP)}}$ are known, then the values of $m_{\mathrm{elu(ADP)}}$ and $e^{\varepsilon'/kT}$ can be estimated by using eqn. 12 and eqn. 10 (or eqn. 11), respectively, which enables one to evaluate $\beta\sigma e^{\varepsilon''/kT}$ by using one of eqns. 4–6.

In order to estimate these values by using Fig. 3 or the information involved in it as shown in Table II, we consider the following successive approximation method. Let us introduce several hypothetical values such as 1, 2, 3, ... into x_0' and, as a first step of the successive approximation, let us assume that $s_{(\mathrm{ADP})} = s_{(\mathrm{ATP})} = s_{(\mathrm{AtetraP})} = s$ (see Table II). It should be noted that this assumption would be slightly different from the actual case in Fig. 3 but that it can be realized, if the gradient of phosphate ions can be applied immediately after loading of the sample is completed. We substitute the hypothetical values of x_0', $s_{(\mathrm{ADP})}$, $s_{(\mathrm{ATP})}$ and $s_{(\mathrm{AtetraP})}$ and the experimental values of m_{in}, $m_{\mathrm{elu(ATP)}}$ and $m_{\mathrm{elu(AtetraP)}}$ (see Table II) into eqn. 12, which now expresses $m_{\mathrm{elu(ADP)}}$ as a function of φ' only. Fig. 4 illustrates the dependence of $m_{\mathrm{elu(ADP)}}$ on $\log_{10} \varphi'$ for different values of x_0'. It can be seen that there exists a finite value of φ' which gives $m_{\mathrm{elu(ADP)}}$ a minimal value and that $m_{\mathrm{elu(ADP)}}$ tends to different finite values when φ' tends to zero (*i.e.*, $\log_{10} \varphi'$ tends to $-\infty$) and $+\infty$, respectively, or we can write

$$\lim_{\varphi' \to 0} m_{\mathrm{elu(ADP)}} = \frac{s_{(\mathrm{ADP})} \cdot s_{(\mathrm{AtetraP})}}{s_{(\mathrm{ATP})}^2} \cdot \frac{[m_{\mathrm{elu(ATP)}} - m_{\mathrm{in}}]^2}{m_{\mathrm{elu(AtetraP)}} - m_{\mathrm{in}}} + m_{\mathrm{in}} \tag{13}$$

and

$$\lim_{\varphi' \to +\infty} m_{\mathrm{elu(ADP)}} = \left\{ \frac{s_{(\mathrm{ADP})} \cdot s_{(\mathrm{AtetraP})}}{s_{(\mathrm{ATP})}^2} \cdot \frac{(x_0' + 2) \cdot (x_0' + 4)}{(x_0' + 3)^2} \times \right.$$

$$\left. \times \frac{[m_{\mathrm{elu(ATP)}}^{x_0'+3} - m_{\mathrm{in}}^{x_0'+3}]^2}{m_{\mathrm{elu(AtetraP)}}^{x_0'+4} - m_{\mathrm{in}}^{x_0'+4}} + m_{\mathrm{in}}^{x_0'+2} \right\}^{\frac{1}{x_0'+2}} \tag{14}$$

TABLE II

INFORMATION INVOLVED IN FIG. 3

Parameter	*Symbol*	*Information*
Column length	L	10 (cm)
Column diameter	$\varnothing$	1 (cm)
Slope of phosphate molarity gradient (in molarity per unit elution volume)	grad	0.0025 (M/ml)
Interstitial volume per unit length of the column	v	0.628 (ml/cm)*
Total interstitial volume of the column	V_T	6.28 (ml)
Slope of phosphate molarity gradient (in molarity per unit column length)	g	0.00157 (M/cm)**
Product of g and L	s	0.0157 (M)
Molarity of phosphate ions before the gradient begins	m_{in}	0.001 (M)***
Volume of the solvent eluted from sample loading until the gradient begins	V'	40 (ml)
Temperature	T	Room temperature§
Elution volume at the maximal height of AMP chromatographic peak	$V_{(AMP)}$	20 (ml)
B or R_F value at the maximal height of AMP peak	$B_{in(AMP)}$ or $R_{F,\,in(AMP)}$	0.314§§
Phosphate elution molarity at the maximal height of ADP peak	$m_{elu(ADP)}$	0.08_3 (M)
Phosphate elution molarity at the maximal height of ATP peak	$m_{elu(ATP)}$	0.20_0 (M)
Phosphate elution molarity at the maximal height of adenosine tetraphosphate peak	$m_{elu(AtetraP)}$	0.28_7 (M)

* Calculated by the relationship $v = \varnothing^2/4 \cdot \pi \cdot 0.8 = 0.628\ \varnothing^2$, where the factor 0.8 is the ratio of the interstitial volume to the total packed volume of HA estimated by Bernardi[16].

** Calculated by the relationship $g = v \cdot \text{grad}$.

*** This is not precisely the experimental value, but it must be close to the true value (see Discussion).

§ The value of ε' is estimated by assuming that room temperature is 25° (see Table IV).

§§ Calculated by the relationship $B_{in(AMP)}$ or $R_{F,\,in(AMP)} = V_T/V_{(AMP)}$ (*cf.*, eqn. 20).

It is evident that, in order for φ' to have a physical meaning, $\varphi' > 0$ (see eqn. 3).

Now, in order for eqn. 12 to have a physical meaning, there must be a one-to-one correspondence between $m_{elu(ADP)}$ and φ', which is evident because if the "activity coefficient" φ' (see eqn. 3) is given, $m_{elu(ADP)}$ should be defined uniquely, while, for $m_{elu(ADP)}$ to be defined, φ' must have a unique value. This means that the part of each curve in Fig. 4 which can have a physical meaning is either the point at which m_{elu} is at a minimum or the region in which $\lim_{\varphi' \to +\infty} m_{elu} < m_{elu} < \lim_{\varphi' \to 0} m_{elu}$. The latter possibility can be excluded, because, provided that this is the situation, it is possible that, when φ' varies (for instance, by the addition of an organic solvent to the system), $m_{elu(ADP)}$ varies but that both $m_{elu(ATP)}$ and $m_{elu(AtetraP)}$ continue to have constant values. This is due to the fact that the shape of each curve in Fig. 4 is constant provided that both $m_{elu(ATP)}$ and $m_{elu(AtetraP)}$ are constant, which is not the actual situation. The reason why the mathematical expression, eqn. 12, involves physically meaningless relationships is evident because, if $m_{elu(ADP)}$, $m_{elu(ATP)}$ and $m_{elu(AtetraP)}$ are given, eqn. 12 can be considered as the equation for φ'; this should be soluble (provided that it has solutions) whatever combinations among the values of $m_{elu(ADP)}$, $m_{elu(ATP)}$ and

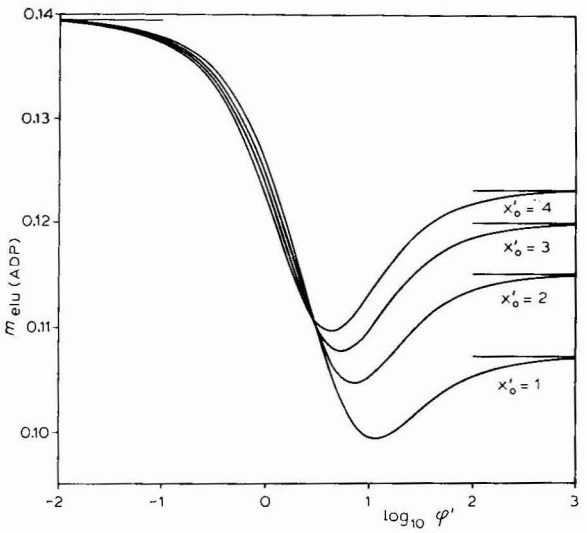

Fig. 4. $m_{elu(ADP)}$ as a function of $\log_{10}\varphi'$ for several values of x'_0 calculated in the first cycle of the successive approximations. Two asymptotes obtained when $\log_{10}\varphi'$ tends to both $-\infty$ and $+\infty$ are also shown for each curve of $m_{elu(ADP)}$ (for details, see text).

$m_{elu(AtetraP)}$ may be assumed; it is only one of these combinations that can have a physical meaning. Hence, the elution molarity of ADP must be written as

$$m_{elu(ADP)} = \min_{\varphi'} \left(\frac{1}{\varphi'} \cdot \left\{ \left[\frac{s_{(ADP)} \cdot s_{(AtetraP)}}{s_{(ATP)}^2} \cdot \frac{(x'_0 + 2)\cdot(x'_0 + 4)}{(x'_0 + 3)^2} \cdot \frac{b^2}{c} + \right. \right. \right.$$

$$\left. \left. \left. + (\varphi' \cdot m_{in} + 1)^{x'_0+2} \right]^{\frac{1}{x'_0+2}} - 1 \right\} \right) \qquad (15)$$

from which the value of φ' can be determined at the same time. In the Appendix, we give a mathematical proof for a more general relationship than eqn. 15:

$$\left(\frac{\partial M_1}{\partial \varphi'} \right)_{M_2, M_3} = 0 \qquad (15')$$

where M_1, M_2 and M_3 represent any one of $m_{elu(ADP)}$, $m_{elu(ATP)}$ and $m_{elu(AtetraP)}$, respectively.

The following step of the calculation is to substitute the values of $m_{elu(ADP)}$ and φ' obtained from eqn. 15 into eqn. 10, or only the latter value into eqn. 11, which gives the value of $e^{\varepsilon'/kT}$. We then substitute $m_{elu(ADP)}$ or the experimental value of either $m_{elu(ATP)}$ or $m_{elu(AtetraP)}$, the experimental value of m_{in} and the hypothetical values of x'_0 and $s_{(ADP)}$, $s_{(ATP)}$ or $s_{(AtetraP)}$ into eqn. 4, 5 or 6, which now gives the value of $\beta\sigma e^{\varepsilon''/kT}$. By using this value of $\beta\sigma e^{\varepsilon''/kT}$, we can calculate the values of B or R_F (see ref. 7), B_{in} or $R_{F,in}$, in the initial state of the chromatography before the gradient is

applied (in which the phosphate molarity is m_{in}) for AMP, ADP, ATP and adenosine tetraphosphate, respectively, by the following relationships (*cf.*, eqn. 1 in ref. 7):

$$B_{in(AMP)} = R_{F,in(AMP)} = \frac{1}{1 + \beta\sigma e^{\varepsilon''/kT}(\varphi' m_{in} + 1)^{-x_0'}} \tag{16}$$

$$B_{in(ADP)} = R_{F,in(ADP)} = \frac{1}{1 + \beta\sigma e^{\varepsilon''/kT} e^{\varepsilon'/kT}(\varphi' m_{in} + 1)^{-x_0'-1}} \tag{17}$$

$$B_{in(ATP)} = R_{F,in(ATP)} = \frac{1}{1 + \beta\sigma e^{\varepsilon''/kT}(e^{\varepsilon'/kT})^2(\varphi' m_{in} + 1)^{-x_0'-2}} \tag{18}$$

and

$$B_{in(AtetraP)} = R_{F,in(AtetraP)} = \frac{1}{1 + \beta\sigma e^{\varepsilon''/kT}(e^{\varepsilon'/kT})^3(\varphi' m_{in} + 1)^{-x_0'-3}} \tag{19}$$

It follows from eqn. 16 that the theoretical value of the elution volume of AMP, $V_{(AMP)}$, is

$$V_{(AMP)} = \frac{V_T}{R_{F,in(AMP)}} \tag{20}$$

where V_T is the total interstitial volume of the column (see Table II). The effective lengths of the column and the effective values of s for ADP, ATP and adenosine tetraphosphate can be calculated, by using eqns. 17–19, respectively, as

$$L_{(ADP)} = L - \frac{V'}{v} \cdot R_{F,in(ADP)} \tag{21}$$

$$L_{(ATP)} = L - \frac{V'}{v} \cdot R_{F,in(ATP)} \tag{22}$$

$$L_{(AtetraP)} = L - \frac{V'}{v} \cdot R_{F,in(AtetraP)} \tag{23}$$

$$s_{(ADP)} = g\, L_{(ADP)} \tag{24}$$

$$s_{(ATP)} = g\, L_{(ATP)} \tag{25}$$

and

$$s_{(AtetraP)} = g\, L_{(AtetraP)} \tag{26}$$

where V' is the volume of the initial buffer eluted from loading the sample until the gradient begins (see Table II) and v is the interstitial volume per unit length of the column (see Table II).

The first cycle of the successive approximation is now finished. The second

cycle begins by substituting the values of $s_{(ADP)}$, $s_{(ATP)}$ and $s_{(AtetraP)}$ obtained by eqns. 24–26 into eqn. 15, and the values of $m_{elu(ADP)}$, φ', $V_{(AMP)}$, $s_{(ADP)}$, $s_{(ATP)}$, $s_{(AtetraP)}$, etc., are again calculated through eqns. 15–26. These values must be slightly different from the values calculated in the preceding cycle. The discrepancy between the values obtained in the nth and the $n-1$th cycles must tend to zero when the value of n increases, however. Hence, the theoretical values of $m_{elu(ADP)}$, φ', $V_{(AMP)}$ [or $R_{F,in(AMP)}$], etc., can finally be estimated for each hypothetical value of x'_0. The values of $m_{elu(ADP)}$ and $V_{(AMP)}$ [or $R_{F,in(AMP)}$] are compared with the experimental values (see Table II) and the value of x'_0 is determined, from which it follows that the values of φ', $e^{\varepsilon'/kT}$ (or ε'), $\beta\sigma e^{\varepsilon''/kT}$, $B_{in(AMP)}$ [or $R_{F,in(AMP)}$], $B_{in(ADP)}$ [or $R_{F,in(ADP)}$], $B_{in(ATP)}$ [or $R_{F,in(ATP)}$], $B_{in(AtetraP)}$ [or $R_{F,in(AtetraP)}$], $L_{(ADP)}$, $L_{(ATP)}$, $L_{(AtetraP)}$, $s_{(ADP)}$, $s_{(ATP)}$ and $s_{(AtetraP)}$ are finally estimated. Fig. 5 summarizes the method for the evaluation of these parameters.

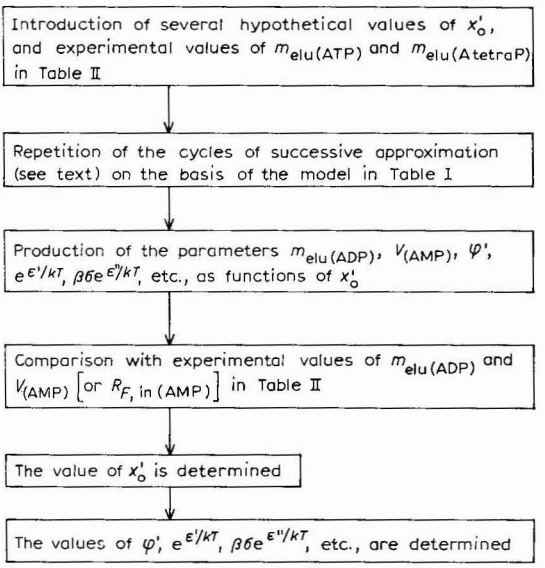

Fig. 5. Method for the estimation of the values of x_0, φ', $e^{\varepsilon'/kT}$, $\beta\sigma e^{\varepsilon''/kT}$, etc.

RESULTS OF CALCULATIONS

Table III shows values of φ', $m_{elu(ADP)}$ and $V_{(AMP)}$ calculated in the first to sixth cycles of the successive approximation for the case when $x'_0 = 2$, in which it can be seen that six cycles are sufficient to give good enough fits of the values with those obtained in the preceding cycle. Similar calculations were carried out assuming $x'_0 =$ 1, 3, 4, 5, 6 and 7. In all instances, six cycles were sufficient to give good fits. Fig. 6 illustrates $m_{elu(ADP)}$ as a function of $\log_{10} \varphi'$ obtained through eqn. 12 in the last of the six cycles for $x'_0 = 1$–4. The value of $m_{elu(ADP)}$ with a physical meaning is at the minimum of each curve (see Theoretical). In Fig. 6, the experimental value of $m_{elu(ADP)}$ [0.083 M (see Table II)] is also shown; this is closest to the minimum of the curve with $x'_0 = 2$ (0.087 M), but also close to the minimum of the curve with $x'_0 = 1$ (0.077 M).

TABLE III

VALUES OF φ', $m_{elu(ADP)}$ AND $V_{(AMP)}$ CALCULATED IN THE FIRST TO THE SIXTH CYCLES OF THE SUCCESSIVE APPROXIMATION FOR THE CASE WHEN $x_0' = 2$

Number of cycle	φ'	$m_{elu(ADP)}$ *(M)*	$V_{(AMP)}$ *(ml)*
1	7.3	0.1048	20.5
2	4.6	0.0873	24.6
3	4.3	0.0844	27.6
4	4.6	0.0866	27.2
5	4.6	0.0870	26.7
6	4.6	0.0866	26.7

On the other hand, it can be shown that the experimental value of $V_{(AMP)}$ [20 ml (see Table II)] is closest to the theoretical value obtained assuming $x_0' = 1$ (22 ml) and next to the value corresponding to $x_0' = 2$ (27 ml). Table IV summarizes the values of several parameters finally determined for both $x_0' = 1$ and $x_0' = 2$.

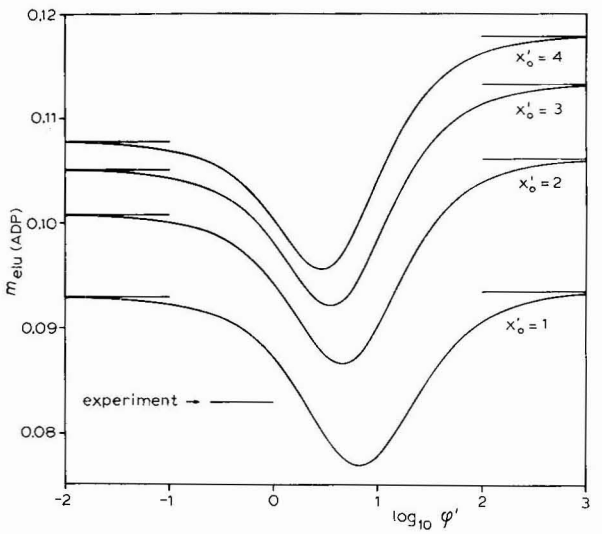

Fig. 6. $m_{elu(ADP)}$ as a function of $\log_{10} \varphi'$ for several values of x_0' calculated in the last of the six cycles of the successive approximation. Two asymptotes obtained when $\log_{10} \varphi'$ tends to both $-\infty$ and $+\infty$ are also shown for each curve of $m_{elu(ADP)}$. The minimum of each curve gives the theoretical value of $m_{elu(ADP)}$. It can be seen that the best fit with the experiment is obtained when $x_0' = 2$ (for details, see text).

DISCUSSION

All calculations were carried out by assuming that the molarity, m_{in}, of phosphate ions before the gradient begins is equal to the molarity of both the solvent of the sample solution and the starting buffer with which the gradient was made, *i.e.*, 0.001 *M* (see legend of Fig. 3). In the experiment in Fig. 3, however, the gradient of phosphate ions was introduced into the column after a volume of only 4 ml had been eluted after loading (which was done at zero volume; see legend of Fig. 3), and it can

TABLE IV

ESTIMATED VALUES OF SEVERAL EXPERIMENTAL PARAMETERS FOR $x'_0 = 1$ AND $x'_0 = 2$

Parameter	x'_0		*Parameter*	x'_0	
	1	*2*		*1*	*2*
φ'	6.7	4.6	$B_{in(ATP)}$ or $R_{F,in(ATP)}$	0.013	0.013
$e^{\varepsilon'/kT}$	5.45	4.80	$B_{in(AtetraP)}$ or $R_{F,in(AtetraP)}$	0.0025	0.0028
ε'/kT	1.70	1.57	$L_{(ADP)}$ (cm)	5.6	6.2
ε' (kcal/mole)*	1.00	0.93	$L_{(ATP)}$ (cm)	9.1	9.2
$\beta\sigma e^{\varepsilon''/kT}$	2.53	3.29	$L_{(AtetraP)}$ (cm)	9.8	9.8
$B_{in(AMP)}$ or $R_{F,in(AMP)}$	0.28	0.23	$s_{(ADP)}$ (M)	0.0089	0.0097
$B_{in(ADP)}$ or $R_{F,in(ADP)}$	0.068	0.060	$s_{(ATP)}$ (M)	0.0144	0.0144
			$s_{(AtetraP)}$ (M)	0.0155	0.0154

* Calculated assuming that $T = 25°$.

be seen that the gradient begins at an elution volume of 40 ml or that the volume of the solvent eluting between the introduction and the beginning of the gradient is 36 ml, which is much larger than the total interstitial volume of the column, *i.e.*, 6.28 ml (see Table II). This would indicate that the gradient is delayed owing to the adsorption of the ions of the buffer on to HA. On the other hand, it can generally be observed that the actual slope of the gradient is virtually equal to the slope that would be realized if there were no adsorption of the ions on the crystal surface*; this means that the delay of the gradient occurs immediately after the gradient is introduced and that, after the initial delay, any part of the gradient migrates with the same rate and $R_F = 1$. This may be due to the fact that the concentration of the ions in the interstices of the column is extremely high and virtually independent of the adsorption on to the crystal surface (except just at the beginning of the gradient). In Fig. 3, it is difficult to know the exact molarity of the phosphate between the introduction and the beginning of the gradient; this must be small because refractometry shows it to be virtually zero[12], but it is probably higher than 0.001 *M*. Eqns. 16–19 show that the values of $R_{F,in}$ vary only very slightly with a variation in the value of m_{in}, provided, however, that $\varphi' \cdot m_{in} \ll 1$.

It is also assumed that the adsorption of a molecule on the surface of HA is unique; this is correct if a particular orientation of the molecule on the crystal surface is energetically much more stable than the other possible orientations, and could be applied approximately to all nucleoside phosphates in Fig. 3. However, the exactness of the approximation must be different for different molecules and it must be better following the order of the values of the adsorption energies in the most stable orientation, *i.e.*, following the order adenosine tetraphosphate, ATP, ADP and AMP. According to this consideration, the theoretical values of $V_{(AMP)}$ and $m_{elu(ADP)}$ estimated on the basis of the approximation of the unique adsorption of the molecule and by using the experimental values of both $m_{elu(ATP)}$ and $m_{elu(AtetraP)}$ (see Table II) might

* In the experiment in Fig. 3, however, it was reported[11] that the volume of each chromatographic fraction is 3 ml. If the slope of the gradient is calculated by using this value, it becomes slightly larger. This must be due to the fact that the measurement of the fraction volume is not exact[12].

be slightly larger than the corresponding experimental values; this suggests that the value 2 estimated for x_0' is more probable than the value 1 because, if $x_0' = 2$, both the theoretical values of $m_{elu(ADP)}$ (0.087 M) and $V_{(AMP)}$ (27 ml) are larger than the corresponding experimental values [$m_{elu(ADP)} = 0.083$ M and $V_{(AMP)} = 20$ ml], whereas if $x_0' = 1$, the theoretical value of $m_{elu(ADP)}$ (0.077 M) is smaller than the experimental value (see Results of calculations). On the other hand, however, a similar experiment for poly-L-lysine suggests that the theoretical value of $m_{elu(ADP)}$ would be slightly smaller than the experimental value owing to a slight deviation from linearity of the relationship between A_2 and m (see ref. 15).

In this step, it is difficult to determine the exact value of x_0'. However, a value of 1–2 estimated for x_0', which would indicate that the adenosine group of adsorbed nucleosides covers at most only one crystal C site (see Theoretical), seems reasonable and consistent with the hypothesis that a C site corresponds to a void of the hydroxyl ion of HA (see Introduction). The value (0.9–1 kcal/mole) estimated for ε' or the adsorption energy for a univalent phosphate group on the polyphosphate chain of the nucleotide (Table IV) is also reasonable and about twice the value (0.5 kcal/mole) estimated for a carboxyl group on the basis of the microheterogeneous model of tropocollagen[17]. As these reasonable conclusions were reached on the basis of the assumption that each phosphate group on the polyphosphate chain should be adsorbed on a C site with a good fit between the intervals of neighbouring phosphate groups and neighbouring C sites, and as the good fit of the intervals would be possible only if a C site corresponds to a hydroxyl position of HA (see Introduction), it can be concluded that these results verify the hypothesis of the one-to-one correspondence between a C site and a hydroxyl position (void of the hydroxyl ion).

Jung *et al.*[18] suggested, however, from adsorption experiments with inorganic pyrophosphate and two diphosphonates (containing a P–C–P structure in contrast to a P–O–P structure in pyrophosphate), namely disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) and disodium dichloromethylene diphosphonate (Cl_2MDP), the existence of not only a common binding site but also a separate site preferential for pyrophosphate. This suggestion is justified[18] by the following arguments: (a) A Scatchard plot (a plot of the ratio of the number of molecules bound to the free concentration in solution as a function of the amount bound) shows that the affinity constant of the first class of sites is highest for EHDP, followed by pyrophosphate, and smallest for Cl_2MDP; but the index for the total binding capacity is largest for pyrophosphate, followed by EHDP and smallest for Cl_2MDP*. When the concentration of pyrophosphate in solution (pH 7.4) is high, a binding of about two molecules per surface unit cell of HA can be estimated (under some assumptions, of course); this is greater than the value predicted by the model of Taves and Reedy[1] (see Introduction). (b) The addition of EHDP displaces pyrophosphate previously bound on HA, but when EHDP is added in amounts up to 50% of the pyrophosphate previously present on the HA, the displaced pyrophosphate and the added EHDP are in molar proportions of 1:3. For larger amounts of EHDP, the relative displacement of

* It is necessary, however, to be careful enough about how the mutual interactions among molecules adsorbed on the surface of HA[13] influence the values obtained through the Scatchard plot, which would indicate, in the ideal case, the affinity of molecule for HA and the binding capacity of HA.

pyrophosphate decreases. These observations are consistent with the existence of two types of adsorption sites*. It can be considered, however, that chromatography reflects mainly the state where the density of molecules on the crystal surface is small enough, because the development of molecules is possible only when the density is small, the state where a considerable proportion of molecules in a section of the column is in solution being realized when the density is small. In this state, the strongest site must be used mainly for the adsorption of molecules, and this must be the C site.

It was shown by Krane and Glimcher[10] that synthetic apatite crystals form complexes with various nucleoside tri- and diphosphates as well as inorganic pyrophosphate at physiological temperature and pH. The terminal phosphorus atom of the bound nucleotide was transferred to a crystal surface phosphate with the formation of pyrophosphate on the crystal and the respective nucleoside di- and monophosphate. It was also shown by Krane and Glimcher[10] that the susceptibility to inorganic pyrophosphatase of the pyrophosphate bound to the crystal was different, depending on whether the pyrophosphate was bound as such from solution (82% hydrolysed) or whether it was formed from the transphosphorylation reaction with ATP (16% hydrolysed). On the other hand, when the crystals from both types of experiment were dissolved and the pyrophosphate was isolated and allowed to react with pyrophosphatase, essentially all of the inorganic pyrophosphate was converted into inorganic orthophosphate. Krane and Glimcher suggested[10] that this difference in enzymatic susceptibility is due to a different orientation of the pyrophosphate on the surface of the HA, depending on the origin of the pyrophosphate. A reasonable atomic explanation was given by Taves and Reedy[1], who considered that the position of the resulting pyrophosphate from the transphosphorylation reaction is roughly perpendicular rather than parallel to the $\vec{c}$ axis of HA. It was reported by Bernardi[11] that the chromatography of a mixture of AMP, ADP, ATP and adenosine tetraphosphate on an HA column (Fig. 3) gave yields of 133, 113, 113 and 50%, respectively. It is probable that this is due to the fact that the terminal phosphorus of the bound nucleoside di-, tri- and tetraphosphates was partially transferred to a crystal surface phosphate with the formation of pyrophosphate on the crystal and the respective nucleoside mono-, di- and triphosphate.

Another example of the molecular reaction that is catalysed by HA on its crystal surface is the degradation of RNA at a high temperature (70°), reported by Martinson and Wagenaar[19]. In this instance, it is considered[19] that the crystal surface of HA is not directly involved in the degradation of RNA but that the degradation is due to a presumably very high concentration of Ca^{2+} ions near the crystal surface, perhaps held there by the requirement of electrostatic charge balance. It was also reported[19] that there is no detectable degradation in the case of DNA, which is consistent with the work of Bernardi[20], who showed that, at room temperature, no significant changes in the physical, chemical and biological properties of native DNA take place upon the adsorption–elution process on HA columns.

Finally, the value of the parameter φ' (4.6–6.7) estimated in this work (Table IV) for competing phosphate ion is comparable to the value of 5 estimated in earlier work[7] for competing potassium ion [see Fig. 2 (e) in ref. 7]. However, this value is

* The possibility that the second type of crystal site corresponds to a P site[7,8] can almost be excluded, because this site reacts with the basic group of the molecule.

much larger than the value of 0.6 estimated for phosphate ion[7], which was based on the experimental data for tropocollagen (Fig. 5 in ref. 7); it was shown that $x'\varphi' = 179$, and the value of 300 estimated for the parameter x' for tropocollagen by assuming that no C sites under the adsorbed molecule can react with phosphate ions*. The estimation of the value of x', which is based on the well known molecular dimensions and shape of tropocollagen, must be correct at least in its order of magnitude, whereas if the value of 4.6–6.7 for the parameter φ' estimated in this work is substituted into the relationship $x'\varphi' = 179$, one obtains $x' = 27$–39; this is much less than 300. Tropocollagen is a rod-like molecule with three polypeptide chains forming a triple helix, which involves mainly glycyl and imino residues occupying 1/3 and 1/5–1/4 of the total residues, respectively; it is considered that the conformation of the triple helix is similar to that of $(\text{Gly–Pro–Pro})_n$ (see ref. 21). As glycyl residues occupying 1/3 of the total residues have no side-chains, it might be possible that phosphate ions of the buffer can enter the spaces among presumably protruding side-chains of aspartic and glutamic residues under the adsorbed molecule, carboxyl groups of which are reacting with C sites; this would reduce considerably the value of x'. In a following paper[15], it will be shown that it is probable that competing ions can enter the spaces among protruding side-chains of poly-L-lysine, ε-amino groups of which are reacting with P sites[7,8] of HA. One cannot, however, exclude the possibility that the adsorption of phosphate ions on to the molecular surface of tropocollagen, if it occurs to a high extent, changes the apparent values of x'.

APPENDIX

A mathematical proof for eqn. 15′ is given below. According to our chromatographic model, it can be considered that, under any experimental condition where φ' has a constant value, M_1, M_2 and M_3 are functions of two parameters (concerning the property of the sample molecule) ε' and $\varepsilon^* \equiv \varepsilon'' + kT \log \sigma$ if x_0' is given, respectively, or it can be written

$$M_1 = \mathrm{f}_1(\varepsilon', \varepsilon^*) \tag{A1}$$

$$M_2 = \mathrm{f}_2(\varepsilon', \varepsilon^*) \tag{A2}$$

$$M_3 = \mathrm{f}_3(\varepsilon', \varepsilon^*) \tag{A3}$$

Now, eliminating ε' and ε^* among eqns. A1–A3, one has

$$M_1 = F(M_2, M_3) \tag{A4}$$

or

$$\mathrm{d}M_1 = \frac{\partial F}{\partial M_2} \cdot \mathrm{d}M_2 + \frac{\partial F}{\partial M_3} \cdot \mathrm{d}M_3 \tag{A5}$$

* It can be considered that tropocollagen is adsorbed on to C sites by using carboxyl groups of aspartic and glutamic residues[7]. These residues occupy about one tenth of the total residues.

As the function F involves φ' as a parameter, it is possible to re-write eqns. A4 and A5, respectively, as

$$M_1 = \Phi(\varphi'; M_2, M_3) \tag{A6}$$

and

$$dM_1 = \left(\frac{\partial \Phi}{\partial M_2}\right)_{\varphi', M_3} dM_2 + \left(\frac{\partial \Phi}{\partial M_3}\right)_{\varphi', M_2} dM_3 \tag{A7}$$

On the other hand, it is possible to consider that eqn. A6 has the form

$$M_1 = \Phi[\varphi', M_2(\varphi'), M_3(\varphi')] \tag{A8}$$

from which we can derive

$$dM_1 = \left(\frac{\partial \Phi}{\partial \varphi'}\right)_{M_2, M_3} d\varphi' + \left(\frac{\partial \Phi}{\partial M_2}\right)_{\varphi', M_3} dM_2 + \left(\frac{\partial \Phi}{\partial M_3}\right)_{\varphi', M_2} dM_3 \tag{A9}$$

Now, by comparing eqn. A9 with eqn. A7, it can be concluded that

$$\left(\frac{\partial \Phi}{\partial \varphi'}\right)_{M_2, M_3} = 0 \tag{A10}$$

which is eqn. 15′. It should be noted that $(\partial \Phi)_{M_2, M_3}$ is a virtual displacement.

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IDENTIFICATION OF ERGOT-PEPTIDE ALKALOIDS, BASED ON GAS–LIQUID CHROMATOGRAPHY OF THE PEPTIDE MOIETY

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SUMMARY

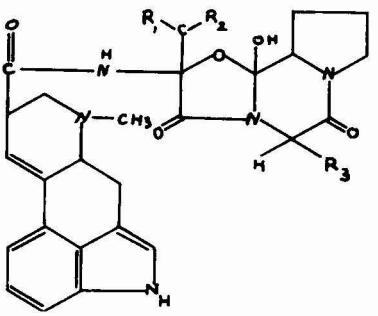
A simple and rapid method for the identification of ergot-peptide alkaloids is described. At temperatures around 300° instantaneous degradation of the free alkaloids occurs in the injection port of the gas chromatograph, each alkaloid yielding a specific set of peptide degradation products, which are subsequently separated on a SE-30 column. Since the lysergic acid moiety cannot be seen in the gas chromatogram, the separation of alkaloids which differ in that part of the molecule is not possible and should be done by thin-layer chromatography or high-performance liquid chromatography. However, combination of these two techniques with the present method provides an excellent identification of all of the possible ergot-peptide alkaloids, including stereoisomers.

INTRODUCTION

Despite numerous attempts, the identification of the ergot-peptide alkaloids has remained cumbersome due to the relatively small differences in the molecular structure of the peptide moiety (Fig. 1). The identification is further complicated by the potential occurrence of isomerization and/or hydrolysis breakdown products[1]. These products result from changes in the lysergic acid moiety, and the hydrolysis breakdown products can be separated relatively easily[1–4].

For the parent alkaloids, ergotamine, ergosine, ergostine, ergocristine, ergokryptine and ergocornine, a large number of thin-layer chromatography (TLC) systems has been described[2,5–7], but, with one exception[7], none has provided an adequate separation of all of these six components. The advantages of high-performance liquid chromatography (HPLC) have not yet been fully investigated[4,8–11] and it remains to be seen whether this technique is able to provide adequate separation efficiency, since the small differences in the peptide moiety hardly seem to affect the chromatographic properties of the total molecule.

Because of the thermal instability and low vapour pressure of the ergot-peptide alkaloids, gas–liquid chromatography (GLC) had been found to be unsuitable for the analysis of this class of compounds[12]. Yet, some observations in a case of death



	R_3			
	$CH_2C_6H_5$	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	
$R_1=R_2=H$	ERGOTAMINE	ERGOSINE	-	ERGOTAMINE-GROUP
$R_1=R_2=CH_3$	ERGOCRISTINE	ERGOKRYPTINE	ERGOCORNINE	ERGOTOXINE-GROUP
$R_1=H;R_2=CH_3$	ERGOSTINE	-	-	-

Fig. 1. Structures of the ergot-peptide alkaloids.

involving ergotamine[13], as well as recent work by Szepesi and Gazdag[14], indicated that GLC may still have some potential. The objective of the present study was to test the applicability of GLC to the identification of the six ergot-peptide alkaloids.

EXPERIMENTAL

Materials

All of the chemicals and solvents were obtained from E. Merck, Darmstadt, G.F.R. and were of analytical grade.

Reference samples of ergotamine, ergosine, ergostine, ergocristine, ergokryptine and ergocornine were kindly supplied as hydrogen maleate or ditartrate salts by Sandoz, Basle, Switzerland. The salts of the respective alkaloids were each dissolved in demineralized water, and the solution was adjusted to pH 10 with 4 *N* ammonia and extracted with chloroform. The chloroform extracts were evaporated to dryness under a gentle stream of nitrogen in a water-bath at 50°, and the residues were taken up in ethanol to give solutions *ca.* 10^{-3} *M* in free base. The free bases were found to be at least 99% pure using the TLC system of Phillips and Gardiner[15]. Volumes of 5 μl of the solutions of the free bases were used in the GLC experiments.

Reference samples of L-phenylalanine-L-proline lactam (L-Phe-L-Pro lactam), L-phenylalanine-D-proline lactam (L-Phe-D-Pro lactam) and pyroergotamine were also gifts from Sandoz. They were used as received and dissolved in ethanol to yield *ca.* 10^{-3} *M* solutions, 5 μl of which were used in the GLC experiments.

Gas chromatography

Analyses were done on a Hewlett-Packard Model 5830 instrument, equipped with a hydrogen flame detector. Glass columns (1.8 m × 2 mm I.D.) coated with

HMDS were packed with 3% SE-30 on Chromosorb G HP (80–100 mesh). The injection port had a glass inlet liner (2 mm I.D.) and was kept at 300°. The oven temperature was 225°, and the detector temperature was 300°. Nitrogen was used as carrier gas at a flow-rate of 30 ml/min. The flow-rates of hydrogen and air in the detector were 30 ml/min and 300 ml/min, respectively.

Thin-layer chromatography

The system of Phillips and Gardiner[15] was used, with chloroform–methanol (9:1) on NaOH-impregnated precoated silica gel plates (silica gel F_{254}; E. Merck). Development was carried out in unsaturated chambers, whereas detection was done in UV light of wavelength 254 nm and 365 nm, followed by spraying with a 5% (w/v) solution of 4-dimethylaminobenzaldehyde in methanol–hydrochloric acid (1:1) (DMBA).

Gas chromatography–mass spectrometry (GC–MS)

Analyses were done on a Finnigan 3300 GC/MS/COM instrument. For chemical ionization (CI) spectra, methane was used as carrier gas and as reactant gas, with the gas chromatograph coupled to the mass spectrometer by a 2 mm I.D. stainless steel tube and a venting valve. The methane flow-rate was 20 ml/min, resulting in an ion-chamber pressure of *ca.* 0.5 Torr. The ion-source temperature was 250°, the electron energy 70 eV and the ion-repeller voltage was 3 V. For electron impact spectra, helium was used as carrier gas, with the gas chromatograph coupled to the mass spectrometer by an all-glass jet separator. The ion-source pressure was kept at *ca.* 10^{-5} Torr, the ion-source temperature was 250° and the electron energy 70 eV.

High-resolution mass spectrometry

Exact mass determinations were obtained on an AEI Model MS-9 instrument via a direct inlet system with a probe temperature of 180°. The electron energy was 70 eV and the acceleration voltage 8 kV. Calculations were carried out on an Atlas–Ferranti computer system.

RESULTS AND DISCUSSION

During a recent investigation into the cause of death of a drug addict we applied hydrolysis in strongly acid medium[16] to the post-mortem blood samples so as to liberate any bound drug from blood cells or blood proteins. An alkaline chloroform extract of the hydrolyzate then revealed the presence of L-Phe-D-Pro lactam, L-Phe-L-Pro lactam and a pyruvoyl precursor of L-Phe-D-Pro lactam[13]. Since these products, in particular L-Phe-D-Pro lactam, are characteristic of the decomposition of ergotamine under acidic conditions[1], we concluded that the latter drug had been present in the blood of the deceased.

Fig. 2 shows the decomposition pathways for ergotamine. However, further investigations into the quantitative aspects of the lactam formation showed that this degradation took place to only a limited extent during the acid hydrolysis, but that reproducible and instantaneous degradation was achieved in the injection port of the gas chromatograph. Fig. 3 represents a gas chromatogram obtained by injecting a

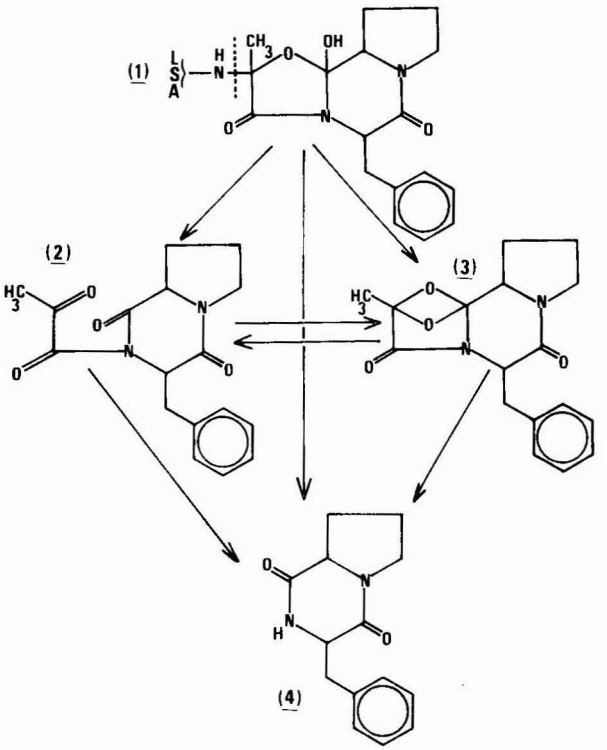


Fig. 2. Decomposition pathways of ergotamine. 1 = Ergotamine. LSA indicates the lysergic acid moiety. The dashed line indicates that the cleavage takes place between the α-nitrogen atom and the α-carbon atom of the amino acid involved, namely α-hydroxyalanine. This results in a pyruvoyl precursor of phenylalanine-proline lactam which can have structure 2 or 3 (ref. 17). Structure 3 is that of pyroergotamine, a reference sample of which showed the same GC and MS behaviour as the above pyruvoyl precursor. However, this does not preclude structure 2 (which is more stable) for this precursor, but for which no reference sample was available. Structure 4 is the phenylalanine-proline lactam, which is obtained in two forms, namely L-Phe-D-Pro lactam and L-Phe-L-Pro lactam.

freshly prepared solution of ergotamine base in ethanol. The identity of the decomposition products was further confirmed by GC–MS and high-resolution MS, and by comparison with authentic reference samples[17].

The optimal injection-port temperature was 300°. Lower temperatures, down to 180°, decreased the amount but not the number of decomposition products. Other solvents, such as benzene, dichloromethane, chloroform, dichloroethane and acetonitrile, gave exactly the same degradation patterns, but diethyl ether, dimethyl sulphoxide and carbon disulphide were less satisfactory, probably due to the poor solubility of ergotamine in these solvents.

The other ergot-peptide alkaloids, ergosine, ergostine, ergocristine, ergokryptine and ergocornine, showed similar decomposition patterns in the injection port, in that they all gave cyclic lactams containing two amino acids and a precursor of these lactams which, besides these two amino acids, also contained a deaminated third hydroxy-amino acid. This was confirmed by GC–MS. Table I summarizes the

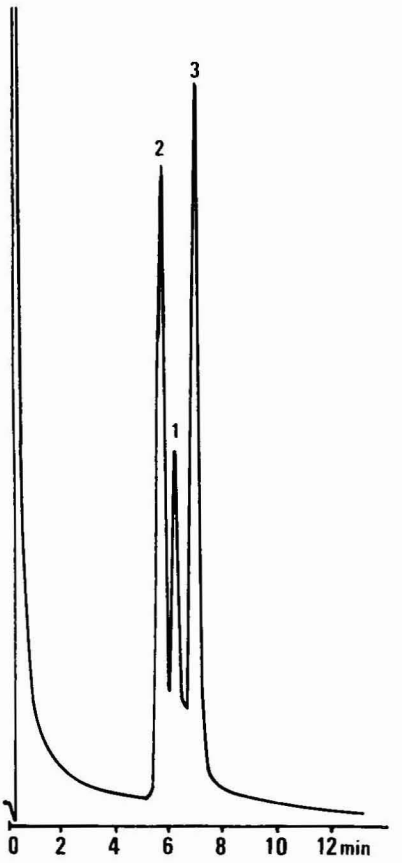


Fig. 3. Gas chromatogram of ergotamine base on SE-30. Peaks: 1 = L-Phe-L-Pro lactam; 2 = L-Phe-D-Pro lactam; 3 = pyruvoyl precursor of L-Phe-D-Pro lactam.

various degradation products, their retention times and retention indices[18] on SE-30, and their quasi-molecular ions in CI–MS. Fig. 4 shows a gas chromatogram of a mixture of the six ergot-peptide alkaloids, injected as bases in ethanol. It can be seen that each individual alkaloid can be identified by the presence of two or three characteristic degradation products.

It is interesting to note that the formation of an L–L dipeptide lactam together with an L–D dipeptide lactam can only be seen with ergotamine, ergostine and ergocristine, the three alkaloids that contain both phenylalanine and proline. The other alkaloids, which do not contain phenylalanine, yield only one dipeptide lactam. Two reasons may account for the latter phenomenon: the degradation occurs in favour of only one configuration, or, if two configurations are formed, the separation of these two is not accomplished under the present GLC conditions. Since the respective L–L and L–D dipeptide lactams derived from ergosine, ergokryptine and ergocornine were not available this question remained unanswered.

Our results do not seem to be in agreement with those of Szepesi and Gazdag[14]. These workers studied the GLC behaviour of dihydroergotoxine alkaloids and

TABLE I

GLC DEGRADATION PRODUCTS, RETENTION TIMES, RETENTION INDICES AND QUASI-MOLECULAR IONS OF THE ERGOT-PEPTIDE ALKALOIDS STUDIED

Alkaloid	*Amino acids in peptide moiety*			*Degradation products detectable by GC*	*Peak number in Fig. 4*	*Retention time (min)*	*Retention index*	*MH^+ (CI–MS)*
Ergotamine	α-hydroxy-Ala	Phe	Pro	L-Phe-L-Pro lactam	1	6.33	2300	245
				L-Phe-D-Pro lactam	2	5.76	2275	245
				pyruvoyl-Phe-Pro lactam*	3	7.00	2340	315
Ergosine	α-hydroxy-Ala	Leu	Pro	Leu-Pro lactam	4	1.93	1900	211
				pyruvoyl-Leu-Pro lactam*	5	2.81	2075	281
Ergostine	α-hydroxy-α--aminobutyric acid	Phe	Pro	L-Phe-L-Pro lactam	1	6.33	2300	245
				L-Phe-D-Pro lactam	2	5.76	2275	245
				α-ketobutyryl-Phe-Pro lactam*	6	9.20	2435	329
Ergocristine	α-hydroxy-Val	Phe	Pro	L-Phe-L-Pro lactam	1	6.33	2300	245
				L-Phe-D-Pro lactam	2	5.76	2275	245
				α-ketoisovaleryl-Phe-Pro-lactam*	7	10.58	2480	343
Ergokryptine	α-hydroxy-Val	Leu	Pro	Leu-Pro lactam	4	1.93	1900	211
				α-ketoisovaleryl-Leu-Pro lactam*	8	4.28	2175	309
Ergocornine	α-hydroxy-Val	Val	Pro	Val-Pro lactam	9	1.51	1810	197
				α-ketoisovaleryl-Val-Pro lactam*	10	3.55	2100	295

* The exact structure of this component is unknown at the present time. As indicated in Fig. 2, pyruvoyl-Phe-Pro lactam may be present as an α,β-diketo structure (2) or as a structure with a dioxane ring (3). This also applies to the other deaminated tripeptide lactam components in this table.

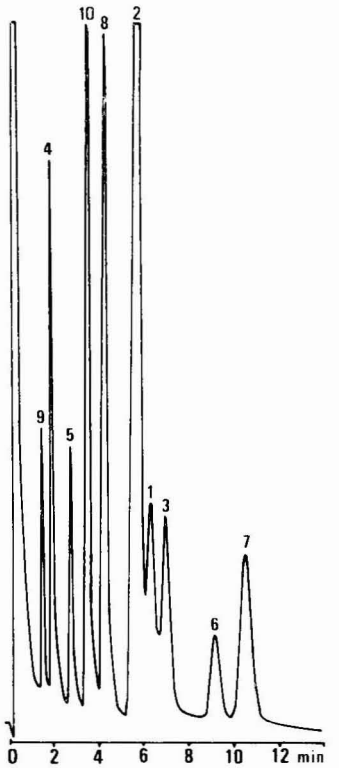


Fig. 4. Gas chromatogram of the six ergot-peptide alkaloids on SE-30. Ergotamine: peaks 1, 2 and 3; ergosine: 4 and 5; ergostine: 1, 2 and 6; ergocristine: 1, 2 and 7; ergokryptine: 4 and 8: ergocornine: 9 and 10.

reported only one GLC peak for the cornine, kryptine and cristine peptide moieties using steel injection ports at temperatures around 235°. Furthermore, they assumed that there was cleavage of the amide bond between the lysergic acid moiety and the peptide moiety, resulting in a tripeptide moiety containing three nitrogen atoms. In our studies, GC–MS and high-resolution MS showed that the initial cleavage takes place between the α-nitrogen atom and the α-carbon atom of the α-hydroxy-amino acid in the peptide moiety, resulting in a deaminated tripeptide structure*. We found no difference between the behaviour of steel liners and glass liners.

Since the present method only identifies the peptide part of the molecule, differentiation between components that differ in their lysergic acid moiety (stereo-isomers such as -ine and -inine components, *aci*-components and derivatives such as lumi- and dihydro-components) is not possible. Yet, as the latter products can be differentiated by TLC[3,13] or HPLC[4], combination of these techniques with the present GLC method allows unequivocal identification of all of the ergot alkaloids, also in mixtures. On the other hand, differences in the structure of the lysergic acid moiety

* Szepesi and Gazdag have recently obtained GC–MS information on the site of cleavage of the peptide moiety which is in agreement with our findings[19].

do not affect the GLC degradation of the peptide moiety. For example, *aci*-ergotamine, ergotamine and dihydroergotamine were found to give the same gas chromatogram as ergotamine (see Fig. 3).

Combination of the present GLC technique with TLC or HPLC systems will also provide a more reliable analysis of materials, suspected of containing lysergide (LSD). For example, when subjected to TLC, various street drug samples show a multitude of blue fluorescent spots giving a blue colour with DMBA and have R_F values close to that of LSD which may be caused by ergot-peptide alkaloids. By collecting the spots from the plate and subjecting them to GLC, it can easily be established whether these products contain a peptide moiety.

It should be noted that neither lysergide, lysergic acid nor the lysergic acid moiety of ergot-peptide alkaloids can be detected under the present GLC conditions.

The quantitative aspects of the method are still under investigation. With ergotamine, it seems that the ratio in which the three degradation components are formed differs slightly, but that the total area of the three peaks remains fairly constant with a variation coefficient of *ca.* 5%.

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QUANTITATIVE ANALYSIS OF DIHYDROERGOTOXINE ALKALOIDS BY GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A rapid, sensitive and specific method for the analysis of the dihydroergotoxine alkaloids (dihydroergocornine, dihydroergocryptine and dihydroergocristine) by gas chromatography and gas chromatography–mass spectrometry is described. The method is based on the quantitative thermal decomposition of the compounds in the injection port of the gas chromatograph, using a 3% SE-30 column with a nitrogen detector. The sensitivity is about 1–10 ng. Gas chromatography–mass spectrometry was used for the structure elucidation of the thermal decomposition products of the dihydroergotoxine alkaloids and for the determination of these compounds at the picogram level.

INTRODUCTION

Dihydroergotoxine (DET) is a mixture of dihydroergocornine (DECO), dihydroergocryptine (DECY) and dihydroergocristine (DECI). The methanesulphonate salt of dihydroergotoxine, Hydergine, is used in the treatment of peripheral and cerebral vascular diseases. It has an α-adrenergic blocking action and produces a generalized peripheral vasodilatation without lowering the blood pressure in normotensive patients, but it may cause a reduction in blood pressure when administered to hypertensive patients. DET is obtained by hydrogenation of the ergotoxine fraction of the Claviceps alkaloids. This fraction is composed of ergocornine, ergocryptine and ergocristine, in various ratios depending on the species of ergot. The isolation and the physical and chemical characteristics of the DET alkaloids were first described by Stoll and Hofmann[1] in 1943. Since then, many attempts have been made to develop sensitive and specific analytical methods for the identification and determination of the ergot and dihydroergot alkaloids.

The DET alkaloids can be separated by paper[2,3], thin-layer (TLC)[4–7], liquid[8,9] and gas chromatography (GC)[10] or by counter-current distribution[11]. Most methods proposed for the determination of the DET alkaloids are based on separation by

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TLC and quantitation of the separated alkaloids either directly by UV or UV–visible spectrophotodensitometry or fluorodensitometry[12–14] or indirectly by eluting the active substance from the layer and measuring the UV absorption or photometrically by Van Urk's colour reaction[15–17].

All of these thin-layers methods are time consuming and, except for the fluorodensitometric detection[14], are insensitive. Recently, a rapid GC method[10] for the separation and determination of the DET alkaloids has been developed, using an all-metal injector and column system with flame ionization detection. The procedure is based on the quantitative decomposition of the compounds, catalysed by a metal surface, and the subsequent separation and detection of the peptide moieties formed from various DET alkaloids. However, 5 μg of each compound is required for adequate quantitation.

In this paper we describe a rapid, sensitive and specific procedure for the analysis of DET alkaloids by GC and combined GC–mass spectrometry (GC–MS). The method is based on a quantitative, non-catalysed, thermal decomposition of the DET alkaloids into a peptide moiety and dihydrolysergic acid amide (DLAA) in the injection port of the gas chromatograph. A normal all-glass system and a selective and sensitive nitrogen detector are used, and a sensitivity of about 1–10 ng for the various DET alkaloids is obtained. With the aid of the combination of GC and chemical ionization (CI) mass fragmentography, a 10-fold increase in sensitivity compared with the GC method used is obtained. Electron impact (EI) and chemical ionization mass spectrometry were used to elucidate the mechanism of the thermal decomposition of the DET alkaloids.

EXPERIMENTAL

Reagents

All solvents were of analytical-reagent grade and were obtained from Merck (Darmstadt, G.F.R.). The methanesulphonates of dihydroergocornine, dihydroergocryptine and dihydroergocristine were obtained from United Pharmaceutical Works (Prague, Czechoslovakia).

For the liberation of the bases of the DET alkaloids from their methanesulphonates, the strongly basic anion exchanger Dowex 1-X2 (100–200 mesh; Fluka, Buchs, Switzerland) in the hydroxide form was used in a methanolic medium. The hydroxide form of the anion exchanger was prepared by eluting a chromatographic column (20 × 2 cm) packed with about 10 g of Dowex 1-X2 with 2.5 ml 1 *N* sodium hydroxide solution until the eluate was alkaline to phenophthalein, followed by rinsing with water until the eluate was neutral; the product was stored under methanol until required.

A stock solution of a 1:1:1 mixture of the DET alkaloids was prepared by dissolving 30.0 mg of each of the methanesulphonates in 5.00 ml of methanol in a 15-ml glass-stoppered tube. After adding 200 mg of air-dried anion exchanger, swirling on a Vortex–Genie mixer for 10 min and allowing the resin to settle down, the solution was used for the preparation of the calibration standards.

An internal standard (I.S.) solution of 5.00 mg/ml of codeine hydrochloride was prepared by dissolving 250.0 mg of codeine hydrochloride (Merck), in 50.0 ml of methanol. Calibration standards containing 0.50 mg of I.S. and 0.10, 0.20, 0.50,

1.00, 2.00, 3.00, 4.00 and 5.00 mg/ml respectively of each of the methanesulphonates of the DET alkaloids were prepared by diluting mixtures of 100 μl of I.S. and aliquots of the DET stock solution to 1.00 ml with methanol in 1-ml Reacti-vials (Pierce, Rockford, Ill., U.S.A., Cat. No. 13221). These solutions were stable for at least 4 weeks when stored in a refrigerator. Volumes of 1 μl were injected into the gas chromatograph.

Samples

A commercial dihydroergotoxine methanesulphonate sample was dissolved in methanol to a final concentration of each component of about 0.2% (w/v). A dihydroergotoxine methanesulphonate formulation (oral tablets of 1.5 mg, sublingual tablets of 0.25 mg, oral solutions of 1.00 mg/ml and injection solutions of 0.30 mg/ml) was extracted either directly (solution) or after powdering and homogenization (tablet) with three portions of 20 ml of chloroform, the combined extracts were filtered, evaporated to dryness under reduced pressure in a Büchi Rotavapor and the residue was dissolved in a suitable volume of methanol to a final concentration of each component of about 0.07% (w/v).

After applying about 100 mg of Dowex 1-X2 (OH^-) anion exchanger, aliquots of these methanolic solutions were treated as described for the preparation of the calibration standards.

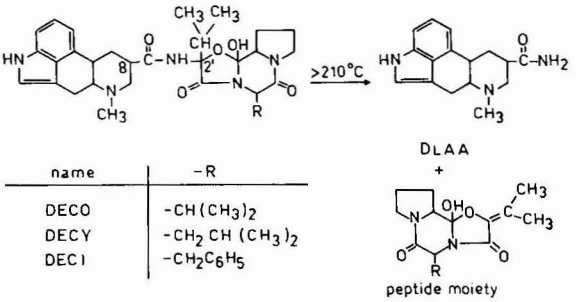
Gas chromatography

A Varian Model 2100 gas chromatograph equipped with a phosphorus/nitrogen detector containing a rubidium sulphate salt tip and a Varian Model A25 1-mV recorder were used. A Varian CDS 101 Chromatography Integrator was employed for the measurement of peak retention times and peak areas.

A U-shaped glass column (125 cm × 3.8 mm I.D.) packed with 3% SE-30 on Supelcoport, 80–100 mesh (Supelco, Bellefonte, Pa., U.S.A.), was found to be the most suitable. The following GC operating parameters were used: column temperature, isothermal at 200° for 21 min, then programmed from 200° to 270° at 10°/min (for routine measurements the chromatograph is programmed after two runs); detector temperature, 250°; injector temperature, 225°; nitrogen flow-rate, 26 ml/min; hydrogen flow-rate, 34 ml/min; air flow-rate, 180 ml/min; amplifier range, 10^{-12} A/mV; electrometer attenuation setting, ×2–128; recorder chart speed, 2 mm/min.

Gas chromatography–mass spectrometry

Mass spectrometry was performed on a Finnigan 1015D GC–MS system equipped with an EI source and on a Finnigan 3200F GC–MS system equipped with a CI source. Both mass spectrometers were coupled to a Finnigan 6000 computer. The GC conditions were as stated under *Gas chromatography* but the carrier gas was helium for the EI instrument and methane for the CI mass spectrometer. The separator oven and transfer line were kept at 260°. The CI source temperature was regulated at 130° while the source pressure was maintained at 900 μmHg by the methane column flow.



name	-R
DECO	$-CH(CH_3)_2$
DECY	$-CH_2CH(CH_3)_2$
DECI	$-CH_2C_6H_5$

Fig. 1. Structures of the dihydroergotoxine alkaloids and their thermal decomposition products.

RESULTS

Gas chromatography

The GC analysis of the DET alkaloids in their base or salt forms cannot be achieved because of their low volatility and thermal breakdown in the gas chromatograph. Hence the determination of these alkaloids by GC is possible only if a complete and reproducible decomposition is observed.

Using a suitable injection temperature, the DET alkaloids bases were each split reproducibly into two components. The structures and the decomposition of these compounds into a peptide moiety and dihydrolysergic acid amide (DLAA) are

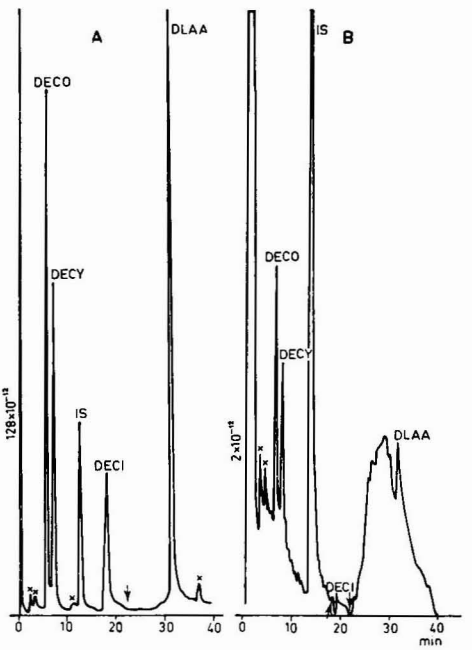


Fig. 2. Gas chromatograms of calibration standards containing (A) 0.5 μg/μl of I.S. and 1 μg/μl of DECO, DECY and DECI, and (B) 0.05 μg/μl of I.S. and 0.01 μg/μl DECO, DECY and DECI. Volume injected: 1 μl. X = unknown thermal decomposition products. Arrows indicate programmed at 10°/min from 200° to 270°.

TABLE I

RELATIVE RETENTION TIMES ON 3% SE-30 OF THE DECOMPOSITION PRODUCTS OF DIHYDROERGOCORNINE, DIHYDROERGOCRYPTINE, DIHYDROERGOCRISTINE AND THE INTERNAL STANDARD

Compound	*Relative retention time*
Internal standard	1.00 (retention time = 12.67 min)
Dihydroergocornine	0.46
Dihydroergocryptine	0.57
Dihydroergocristine	1.42
Dihydrolysergic acid amide	2.42

shown in Fig. 1. These decomposition products were confirmed with the aid of GC–MS (see below). As these peptide moieties are different (Fig. 1), after GC separation they can be used for the analysis of the corresponding DET alkaloids. Figs. 2a and 2b show the GC analysis of calibration standards containing 1 and 0.01 μg/μl, respectively, of each of the DET alkaloids. The relative retention times of these compounds were measured (Table I).

To establish the optimal conditions for the thermal decomposition, the influence of the injection temperature on the separation of the DET alkaloids was investigated. Fig. 3 shows gas chromatograms for the separation of these compounds at injection temperatures of 200° (a), 225° (b), 250° (c) and 275° (d). From Fig. 3 it

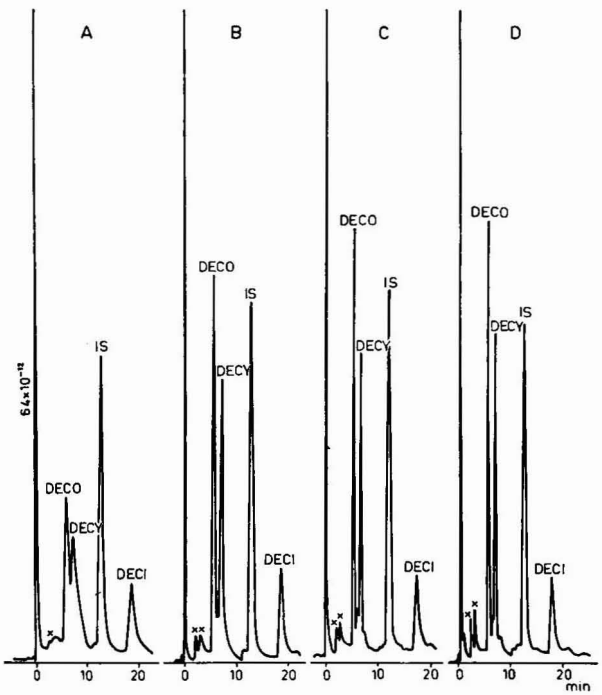


Fig. 3. Gas chromatograms of the separation of the DET alkaloids at injection temperatures of (a) 200°, (b) 225°, (c) 250° and (d) 275°. Injected: 1 μl of the calibration standard containing 0.5 μg/μl of I.S., DECO, DECY and DECI. X = unknown thermal decomposition product.

can be seen that a satisfactory separation and a minor further breakdown of the alkaloids were obtained by using an injection temperature between 225° and 250°. In further experiments a temperature of 225° was employed.

Fig. 4 shows the gas chromatograms of a dihydroergotoxine methane sulphonate solution before (a) and after (b) the addition of the strongly basic anion exchanger Dowex 1-X2. This figure clearly demonstrates that the analysis of these alkaloids in their salt forms cannot be performed without excessive decomposition.

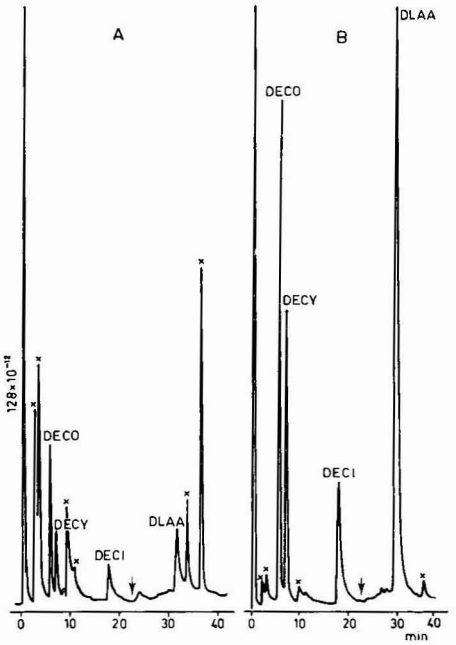


Fig. 4. Gas chromatograms of a dihydroergotoxine methane sulphonate solution containing 1 μg/μl of DECO, DECY and DECI before (a) and after (b) addition of the basic anion exchanger Dowex 1-X2. Injected volume: 1 μl. X = unknown decomposition product. Arrows indicate programmed at 10°/min from 200° to 270°.

After the GC analysis of the calibration standards, calibration graphs for the three DET alkaloids were constructed by plotting the peak-area ratios of dihydroergocornine, dihydroergocryptine, dihydroergocristine and the I.S. against the concentration of these alkaloids in the various calibration standards (Fig. 5). It can be seen that the calibration graphs for the three DET alkaloids show a linear relationship from 0.10 up to 5.00 mg/ml. One of the calibration standards was chromatographed several times daily prior to the determination of the unknown samples; the peak areas of the DET alkaloids and the I.S. were recorded and the ratios were compared with the calibration graphs in order to check the condition of the column and the detector.

The reproducibility of the method was determined by four replicate analyses on several days of five calibration standards, and the results are summarized in Table II. The limit of sensitivity for the DET alkaloids varied from 1 to 10 ng and was derived from the GC response of a calibration standard containing 0.01 μg/μl of each alkaloid (Fig. 2).

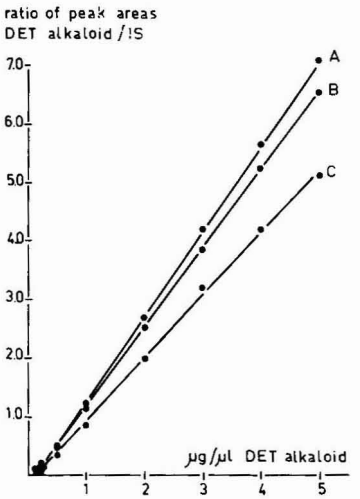


Fig. 5. Calibration graphs for (A) DECO, $y = 1.443\,x - 0.155$, $r = 0.99$; (B) DECY, $y = 1.3367\,x - 0.130$, $r = 0.99$; (C) DECI, $y = 1.0943\,x - 0.153$, $r = 0.99$. Concentration of I.S.: 0.5 µg/µl.

To evaluate the accuracy of the method, synthetic samples were assayed. These samples were prepared by adding 10.0 mg of each of the dihydroergotoxine methanesulphonates to about 2 g of tablet powder, containing all tablet ingredients except the DET alkaloids. The mean recovery in three experiments was 99.0 ± 1.5% for DECO, 98.5 ± 1.8% for DECY and 98.2 ± 2.4% for DECI.

The GC method was applied to the determination of the DET alkaloids in commercial samples and pharmaceutical preparations. The mean dihydroergotoxine methane sulphonate content of five commercial samples was 95.2 ± 2.9%, including a mean DECO content of 30.9 ± 0.8%, a mean DECY content of 32.8 ± 2.1% and a mean DECI content of 31.5 ± 3.6%. In the analysis of three portions of ten tablets of 1.5 mg obtained from two manufacturers, a mean dihydroergotoxine methane sulphonate content of 92.9 ± 3.5% of the declared value, including a mean DECO content of 32.8 ± 1.8%, a mean DECY content of 32.3 ± 2.5% and a mean DECI content of 27.8 ± 3.5%, was found.

Gas chromatography–mass spectrometry

The decomposition products of the DET alkaloids were established by means

TABLE II

RESULTS OF REPRODUCIBILITY STUDIES

Concentration of compound (µg/µl)	*Standard deviation (%)* *		
	Dihydroergocornine	*Dihydroergocryptine*	*Dihydroergocristine*
0.10	6	11	16
0.20	3	6	6
0.50	2	2	4
1.00	1	1	3
3.00	1	2	3

* Relative standard deviation (%) = $(\sigma/\bar{X}) \cdot 100$ of the peak area ratio of DET alkaloid to I.S.

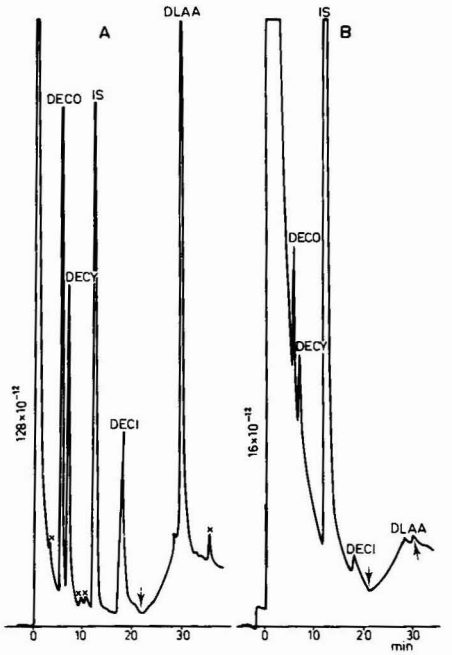


Fig. 6. Gas chromatograms of calibration standards containing (A) 1 μg/μl and (B) 0.1 μg/μl of the DET alkaloids using flame-ionization detection. Injected volume: 1 μl. Arrows indicate programmed at 10°/min from 200° to 270°.

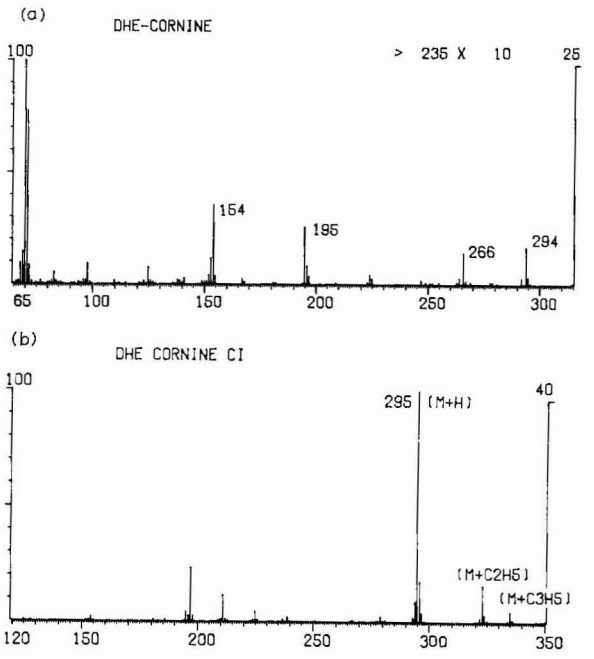


Fig. 7. (a) EI mass spectrum of the DECO peptide moiety. The masses indicated in the spectrum are explained in Fig. 8. (b) The methane CI spectrum of the DECO peptide moiety.

of EI and CI mass spectrometry. The thermal degradation of these substances involves a cleavage of the NH–C_2^1 peptide bond and a hydrogen transfer from the peptide moiety to the DLAA, as indicated in Fig. 1. The peptide moiety is characteristic of the DET alkaloids. In the gas chromatograph these moieties are eluted at their respective retention times before the DLAA molecule (Fig. 2).

Figs. 7a and 7b show the EI and CI spectra of the DECO peptide moiety. The ion at *m/e* 294 (Fig. 7a) is the molecular ion of the peptide and corresponds to the quasi-molecular ion at *m/e* 295 (Fig. 7b). The ions above *m/e* 295 are the (M +

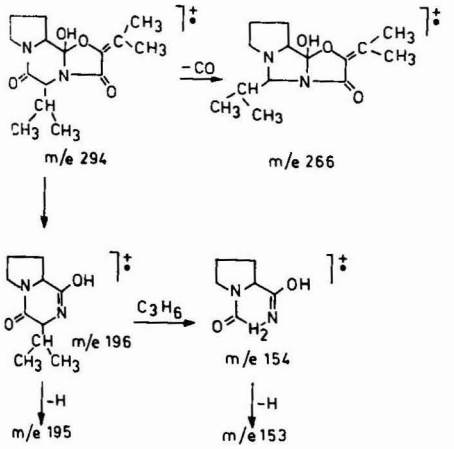


Fig. 8. The fragmentation scheme for the DECO EI mass spectrum.

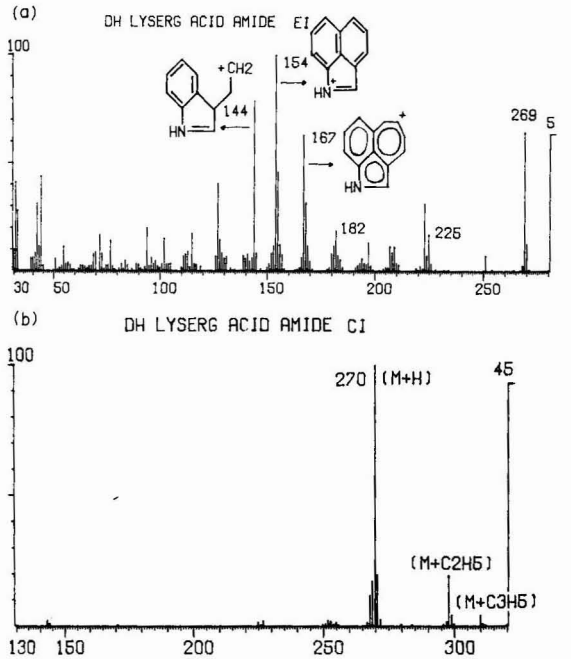


Fig. 9. (a) The EI mass spectrum of DLAA. (b) The methane CI mass spectrum of DLAA.

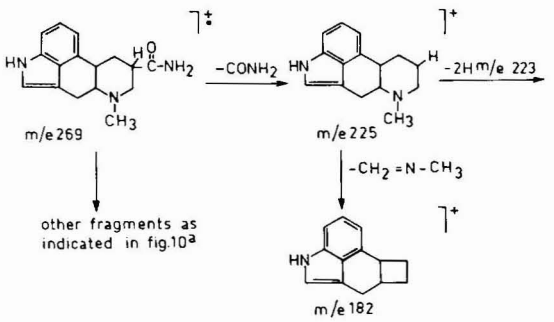


Fig. 10. The fragmentation scheme for the DLAA EI spectrum.

$C_2H_5)^+$ and the $(M+C_3H_5)^+$ ions usually found in CI with methane as reagent gas. The additional structural information as obtained from the EI spectrum is summarized in Fig. 8.

Figs. 9a and 9b show the EI and CI spectra of DLAA. The ion at *m/e* 269 (Fig. 9a) represents the molecular ion; in Fig. 9b the quasi-molecular ion at *m/e* 270 and the C_2H_5 and C_3H_5 addition products are shown. The major fragments of the EI spectrum are indicated in Fig. 10. These fragments agree with those observed by Voigt *et al.*[18] except for the mass difference of 2 a.m.u. due to differences in the ergotoxine and the dihydroergotoxine alkaloids. The fragments indicated in Fig. 9a are identical with those described by Voigt *et al.*

Similar results were obtained for dihydroergocryptine and dihydroergocristine. The major fragments and their intensities are compared with fragments of dihydroergocornine and are listed in Table III for both EI and CI.

In addition to the identification studies, we used the CI instrument for mass fragmentography. In Fig. 11 the computer-added trace of the three *m/e* values 295, 309 and 343 for the quasi-molecular ions of the DET alkaloids and the *m/e* value 300 of the I.S. codeine is shown. The amount per DET alkaloid injected into the gas chromatograph was 500 pg.

TABLE III

MASSES AND RELATIVE INTENSITIES OF THE MAJOR FRAGMENTS OF THE PEPTIDE MOIETIES

Ionization	*Fragment**	*Dihydroergo-cornine***	*Dihydroergo-cryptine***	*Dihydroergo-cristine***
EI	M^+	294 (1.7)	308 (1.9)	342 (3.8)
	$(M-CO)^+$	266 (1.4)	280 (0)	314 (0.6)
	$(M-C_5H_6O_2)^+ (= F_1)$	196 (8)	210 (10)	244 (11)
	$(F_1-H)^+$	195 (26)	209 (20)	243 (10)
	$(F_1-R)^+$	154 (35)	154 (37)	154 (32)
	$(F_1-R-H)^+$	153 (12)	153 (5)	153 (3)
CI	$(M+H)^+$	295 (100)	309 (100)	343 (100)
	$(M+C_2H_5)^+$	323 (16)	337 (19)	371 (17)
	$(M+C_3H_5)^+$	335 (5)	349 (6)	383 (6)

* R is the substituent group indicated in Fig. 1.

** The numbers in parentheses are the relative abundances of the respective *m/e* values.

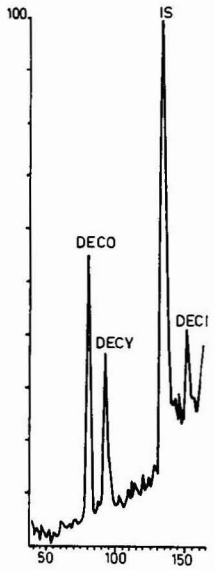


Fig. 11. Computer-added mass fragmentogram traces of a DET sample containing 500 pg of each compound.

DISCUSSION

The GC procedure described here is more specific and sensitive than previously reported chromatographic methods, the lowest measurable concentration of the dihydroergotoxine alkaloids varying from 1 to 10 ng. The method is also less time consuming, as the analysis of these alkaloids can be performed in about 35 min.

The results demonstrate that the most important steps in the procedure for DET are the conversion of the salt forms of the alkaloids into their free base forms with the aid of the strongly basic anion exchanger Dowex 1-X2 (Fig. 4) and the selection of a suitable injection temperature (Fig. 3).

As the "peptide moieties" of the DET alkaloids (Fig. 1) have a nitrogen content varying from 8.2 to 9.5%, it is obvious that a nitrogen detector should be used with these compounds. Using this detector, a minimal solvent response and a selective and sensitive response for the nitrogen-containing compounds is observed in the analysis of methanolic DET solutions (Fig. 2). Our GC method can, of course, also be performed with a flame-ionization detector, as shown in Fig. 6. However, as would be expected with this detector, a much higher solvent response, a decreased sensitivity and a lack of selectivity are obtained in the assay of the DET alkaloids. From the results obtained in the analysis of a number of commercial samples and pharmaceutical preparations, it can be concluded that our method is suitable for detecting and determining the DET alkaloids in these products.

Compared with the GC method previously reported by Szepesi and Gazdag[10], the procedure described here has the advantages of much higher sensitivity and selectivity. The favourable results obtained with the standard glass column system suggest that the thermal decomposition of the DET alkaloids is not catalysed by a

metal surface. By using mass spectrometry we could identify the thermal degradation products.

Although the structure of the peptide moiety could not be established with certainty, the point of cleavage at the NH–C_2^1 bond is certain, which is in contrast to the suggested CO–NH cleavage proposed by Szepesi and Gazdag[10]. The formation of a double bond between C_2^1 and the isopropyl group instead of the dioxocyclobutane ring proposed by Hofmann[19] is speculative but seems possible considering the relative mass spectral stability of the peptide moiety.

Using the CI mass spectrometer in the mass fragmentography mode, we were able to detect even smaller amounts of the DET compounds. Quantification could be performed easily at the 500-pg level.

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GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE ASSAY OF AMINOGLYCOSIDE ANTIBIOTICS IN SERUM

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SUMMARY

A gas–liquid chromatographic (GLC) method is presented for the rapid analysis of gentamicin, tobramycin, netilmicin, and amikacin from human serum. This procedure may have application to all aminoglycoside drugs. The three isomers of gentamicin are resolved as two bands, while tobramycin, netilmicin, and amikacin appear in this system as single bands. Normal serum constituents do not interfere with chromatograms. Thus far, no assay interference has been found in cases where other drugs and antibiotics were administered concurrently with aminoglycoside therapy. Dose-response data demonstrating linear recovery are included for all four aminoglycosides as well as a comparison of the GLC method with the microbiological method for the assay of gentamicin and amikacin.

Quantitation is based upon the relative response of the antibiotics to a fixed amount of the internal standards, either kanamycin A or paromomycin B. These standards are clearly resolved as symmetrical peaks from the antibiotics of assay interest. Isothermal chromatographic analysis time is less than 8 min, while total assay time per single serum specimen is approximately 50 min. Preparation of serum includes: precipitation, evaporative drying of the supernatant, a two-stage derivatization (N-trimethylsilylimidazole, N-heptafluorobutyrylimidazole), and a single hexane extraction with a water wash. The methodology described may be applied to the analysis of other compounds (*e.g.*, saccharides, amino-saccharides, amino acids, etc.) which do not readily partition into an organic phase.

INTRODUCTION

Aminoglycoside antibiotics have become the agents of choice for a variety of serious infections involving Gram-negative bacilli. All compounds in this group have a close toxic–therapeutic ratio. Thus, the dose recommended to ensure biologic activity may prove toxic in a substantial portion of patients.

Current methods to measure serum levels of aminoglycoside include the microbiological assay, enzymatic assay, and radioimmunoassay. All of these techni-

ques have certain limitations which make them less than ideal for routine laboratory use in a general hospital.

The microbiological assay is the most commonly used, but may require 4–24 h of incubation[1–3] and can be quite inaccurate[4]. The enzymatic assay[5] is an excellent alternative, but the instability of enzymes[6] as well as the cost of a scintillation counter may present difficulties. The radioimmunoassay is rapid (3–4 h), very sensitive and accurate, and is not susceptible to interference from concurrent antimicrobials. The disadvantages are the reluctance of clinical laboratories to use radioactive materials, and the requirement for specific antibody for each aminoglycoside.

We have attempted to develop an aminoglycoside assay utilizing gas–liquid chromatography (GLC). This system offers the potential advantages of rapid performance (less than one hour for determination); accuracy, based on internal standards; readily available reagents which do not require radiolabeling, stabilization of enzymes or growth of microorganisms; and use of equipment which is reasonably priced and is versatile enough to be used for other chemical determinations in a laboratory.

METHODS

Serum preparation

Human serum containing an aminoglycoside drug was accurately delivered in 0.1-ml aliquots, into 10 × 75 mm disposable culture tubes (Fig. 1). 0.25 ml of a 2-μg/ml aqueous stock solution of kanamycin A was added as internal standard to sera containing gentamicin, tobramycin, or netilmicin. For standardization of amikacin, 0.25 ml of a 12-μg/ml aqueous stock of paromomycin B was added. The solutions were placed in a sandbath maintained at 81°–83°, allowed to equilibrate (3–5

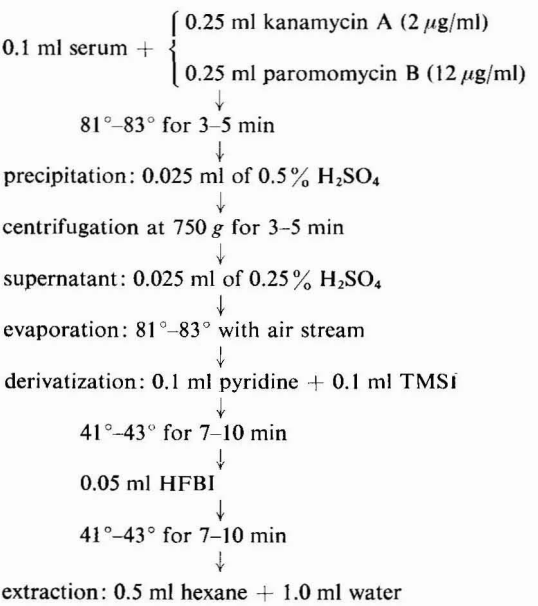

Fig. 1. Preparation technique for GLC analysis of serum aminoglycoside levels.

min), and precipitated by the addition of 0.025 ml of 0.5% H_2SO_4. The milky, finely granular specimens were immediately centrifuged at 750 *g* for 3–5 min. The clear supernatants were transferred to clean 10 × 75 mm tubes and supplemented with 0.025 ml of 0.25% H_2SO_4 (pH 4.5–5.0). Evaporation of the acidified supernatant required approximately 5 min in the 81°–83° sandbath under a vigorous air stream. At apparent dryness the tubes were sealed with cork stoppers (size no. 1).

Derivatization and extraction

To the dry residues, 0.1 ml of silylation grade pyridine and 0.1 ml of TMSI (N-trimethylsilylimidazole; Pierce, Rockford, Ill., U.S.A.) were added. The tubes were resealed and incubated at 41°–43° for 7–10 min. The reaction mixtures were then supplemented with 0.05 ml of HFBI (N-heptafluorobutyrylimidazole; Pierce), and reincubated at 41°–43° for 7–10 min. Reagent-grade hexanes (0.5 ml) and 1.0 ml of distilled water were added, the tubes were recorked, and the phases mixed by repeated inversion. Routinely 2.0 μl of the organic phase were injected into the chromatograph.

Chromatographic conditions and instrumentation

A Shimadzu GC4BMPF gas chromatograph equipped with an electron capture detector (ECD) (^{63}Ni, 10 mC) was used. The detector was operated at a pulse width of 8 μsec, a frequency of 10 kHz, and a pulse voltage of 50 V. The electrometer input sensitivity was 10 MΩ. The output range was 20 mV for gentamicin, tobramycin, and netilmicin, and 40 or 80 mV for amikacin. Gentamicin, tobramycin and netilmicin analyses were performed using silanized pyrex columns (2 m × 3 mm I.D.) packed with 3% OV-101 coated onto 80–100 mesh Chromosorb W AW DMCS (Applied Science Labs., State College, Pa., U.S.A.). Nitrogen (column head pressure 2.8 kg/cm²; flow-rate, 60 ml/min) was the carrier. The detector and injector were kept at 287° with the oven at 272°. Amikacin was chromatographed on 1% OV-17 with nitrogen carrier (column head pressure, 3.45 kg/cm²; flow, 80 ml/min) at detector, injector and oven temperatures of 277°, 277°, and 262°. New columns were flow-conditioned for 2–3 days prior to attachment to the ECD. Derivatized aminoglycoside (equivalent to 60 μg per ml serum) was periodically injected during this time. The columns retained sufficient efficiency and resolution capabilities for one year of continuous operation. A Shimadzu R-201 strip-chart recorder was operated at 2.5 mm/min. Peak areas were measured by a Shimadzu ITG-2A electronic digital integrator (threshold, 200 μV; count delay, 60 sec; minimum detectable slope, 50 μV/min.).

Standard serum antibiotic solutions

Aqueous stock solutions (200 μg/ml) were prepared from standard powders of gentamicin, tobramycin, and netilmicin. For each drug, 0.5 ml of the stock was added to 4.5 ml of normal human serum obtained from the hospital blood bank. The final concentration was 20 μg/ml which was two-fold serially diluted in serum down to 0.625 μg/ml. Serum containing amikacin was prepared in a similar fashion, but was diluted to larger concentrations, reflecting the higher therapeutic serum levels. The serum was stored at −20° (non-frostfree freezer). No change in chromatographic response was noted over 2–3 months of frozen storage.

Internal standard stock solutions

Aqueous stock solutions (200 μg/ml) were prepared from standard powder of kanamycin A and paromycin B. No significant changes in chromatographic response were noted during 2–3 months of 5° storage. Working internal standards (kanamycin A, 2 μg/ml; paromomycin B, 12 μg/ml) were carefully prepared by dilution of these stocks.

Serum aminoglycoside levels by microbiological assay

Serum levels of gentamicin and amikacin were measured by the agar well microbiological method[1,2].

Stability of the gentamicin–kanamycin A ratio

Sera containing known concentrations of gentamicin were supplemented with kanamycin A, derivatized, extracted, and promptly chromatographed. These preparations (0.5 ml hexane over approximately 1.2 ml aqueous) were stored at −20° and reinjected at 24, 48, and 120 h. The areas of the two derivatized drugs were measured to monitor the stability of their ratio.

Blind comparison of serum levels by microbiological and GLC technique

Dilutions of gentamicin in serum were prepared at 1, 5, 10, and 20 μg per ml serum (10 separate aliquots for each concentration). Twenty-one dilutions of amikacin in serum were prepared over the range 0–64 μg per ml serum. These specimens were encoded, frozen at −20°, and distributed for blind analysis by GLC and microbiological technique.

Quantitation

Determinations of aminoglycoside concentrations in human sera were based on the relative response of each drug to a fixed amount of the appropriate internal standard. The calculation of serum gentamicin (Gm) levels with kanamycin A (KmA) as the internal standard is used for illustrative purposes.

$$F_{Gm} = \frac{\text{area Gm}}{\text{area KmA}} \cdot \frac{5\ \mu\text{g KmA per ml serum}}{\mu\text{g Gm per ml serum}} \tag{1}$$

$$\mu\text{g Gm per ml serum} = \frac{\text{area Gm}}{\text{area KmA}} \cdot \frac{5\ \mu\text{g KmA per ml serum}}{F_{Gm}} \tag{2}$$

Eqn. 1 can be used to calculate the concentration response factor of gentamicin relative to kanamycin A, F_{Gm}, while eqn. 2 incorporates this factor into direct calculation of the serum levels. Table I lists the area ratios and F values obtained from increasing concentrations of gentamicin (0.6–20 μg Gm per ml serum). The F_{Gm} values were not constant over this range of known gentamicin concentrations.

To correct for the observed changes in F_{Gm}, a modification of this calculation method was attempted. Experimental area ratios were compared to the area ratios of the known standards (0.6–20 μg/ml). Gentamicin was calculated using the mean of

TABLE I

CHROMATOGRAPHIC RESPONSE TO STANDARD CONCENTRATIONS IN HUMAN SERUM OF GENTAMICIN

Abbreviations: Gm, gentamicin; KmA, kanamycin A; concentration response factor F_{Gm} = $\frac{\text{area Gm}}{\text{area KmA}} \cdot \frac{5\ \mu g\ KmA}{\mu g\ Gm}$

$\left(\frac{\mu g\ Gm}{ml\ serum}\right)$	$\left(\frac{area\ Gm}{area\ KmA^*}\right)^{**}$	F_{Gm}
0.63	0.052 ± 0.004	0.435
1.25	0.129 ± 0.005	0.516
2.50	0.290 ± 0.007	0.580
5.00	0.716 ± 0.040	0.716
10.00	1.359 ± 0.049	0.680
20.00	2.475 ± 0.132	0.619

* 5 μg KmA per ml serum.

** Mean of 11 separate trials ± 1 SEM; area measurement by electronic digital integrator.

the F values obtained from the next lowest and next highest area ratios of the known standards. Thus, using Table I, an experimental area ratio (Gm/KmA) of 0.200 would be calculated using an F_{Gm} value of 0.548 [= (0.516 + 0.580)/2].

RESULTS

Retention times

The relative retention times of derivatized gentamicin (three primary isomers), tobramycin, and netilmicin were measured relative to kanamycin A (Table II). The absolute retention time of kanamycin A was between 6.0 and 6.3 min. Gentamicin isomers C_{1A} and C_2 are not resolved in this system and occur in one chromatographic band. Gentamicin isomer C_1 is cleanly resolved from both kanamycin A and the combined C_{1A}–C_2 band. Since the proportion of each isomer may vary among antibiotic lots, quantitation of gentamicin was based on a sum of the peak areas —the combined C_{1A}–C_2 peak plus the single C_1 peak. Fig. 2 is a chromatogram which illustrates the relationships between the bands derived from sera supplemented with each of the three authentic gentamicin isomers and kanamycin A.

TABLE II

RETENTION TIMES FOR DERIVATIZED GENTAMICIN, TOBRAMYCIN, AND NETILMICIN RELATIVE TO KANAMYCIN A (RRT_{KmA})

Kanamycin A absolute retention time, 6.0–6.3 min. RRT_{KmA} may vary ±0.005.

Aminoglycoside	RRT_{KmA}
Gentamicin C_1	0.601
Gentamicin C_{1A}	0.484
Gentamicin C_2	0.487
Tobramycin	0.613
Netilmicin	0.627

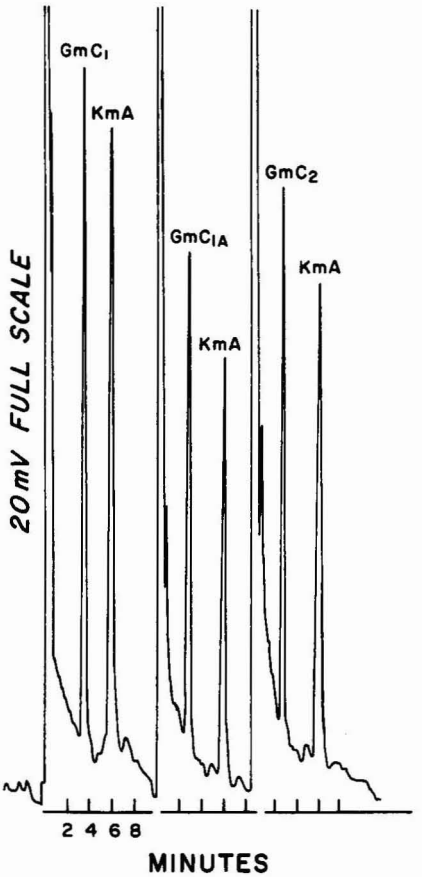

Fig. 2. Gentamicin: chromatographic recovery of isomers C_1, C_{1A}, and C_2 from serum.

The retention time of amikacin relative to paromomycin B was 1.73 ($RRT_{PmB} = 1.73$) on 1% OV-17. The absolute retention time of paromomycin B was usually in the range 2.9–3.1 min. Among other aminoglycosides, the RRT_{KmA} for kanamycin B was 0.714. The antibiotic preparation designated "kanamycin base" responds in this system with the retention characteristics of kanamycin A ($RRT_{KmA} = 1.0$, $RRT_{KmB} = 1.408$).

Chromatographic tracings

Figs. 3, 4 and 5 demonstrate dose-response chromatograms for derivatized gentamicin, tobramycin, and netilmicin, respectively, on 3% OV-101 (0.6–20 μg per ml serum). Fig. 6 illustrates derivatized amikacin on 1% OV-17 (5.0–60 μg per ml serum). A new run can be initiated safely each 8–8.5 min. For gentamicin, tobramycin, and netilmicin, the optimum calculated range of drug quantities presented to the detector is 0.26 ng (0.63 μg per ml serum) to 8.0 ng (20 μg per ml serum).

Quantitation

Tables I, III, IV and V list the area ratios and concentration response factors (F) obtained from dose-response studies of gentamicin, tobramycin, netilmicin and

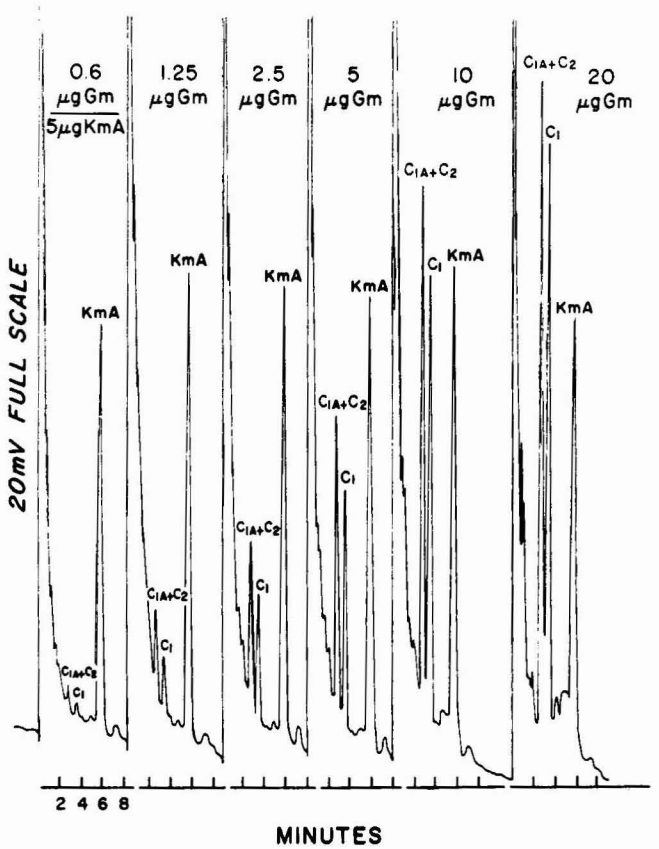

Fig. 3. Gentamicin: chromatographic dose-response from serum.

amikacin in human serum. In general, a two-fold increase in drug concentration is correlated with an approximate doubling of area ratios.

Concentration response factor (F_{Gm}) varied from 0.580 to 0.716 over the range 2.5–20 μg/ml (Table I). The concentration response factors calculated from 0.63 μg/ml (0.435) and 1.25 μg/ml (0.516) may indicate a substantial decrease in unit response to gentamicin at these low serum levels. Since F_{Gm} values were variable, the calculation techniques described in Methods were applied.

Table III indicates that F_{Tm} may be firm over the range from 1.25 to 20 μg/ml. A possible decrease in unit response is again noted at 0.63 μg/ml. At 10 μg/ml, the F_{Tm} of 0.945 is clearly outside the trend of the other values.

Serum recovery data for netilmicin (Table IV) suggests a unit detector response approximately half that found with gentamicin. The F_{Nm} values from 2.5 to 10 μg/ml are very consistent. Curiously, F_{Nm} at 0.63 μg/ml (0.299) is in line with the other three consistent values, while F_{Nm} at 1.25 μg/ml (0.224) suggests a decrease in unit response. An increase in response is observed at 20 μg/ml (0.377).

Amikacin (Table V), tested over its elevated range of expected therapeutic

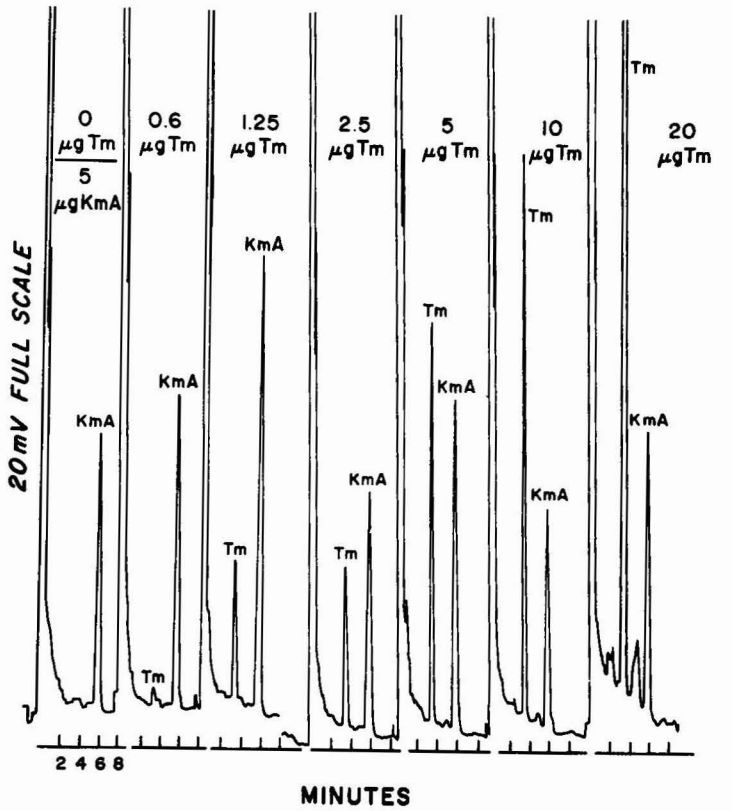

Fig. 4. Tobramycin: chromatographic dose-response from serum.

serum concentrations, appears to have the highest, as well as the most consistent, concentration response factors.

Stability of derivatized drug

The stability of derivatized aminoglycoside ratios was examined (Table IV) to determine if specimens could be prepared at one time and reliably analyzed later. In this experiment, the ratios (Gm/KmA) were reasonably stable up to 48 h, with decomposition seen at 120 h. This 120-h decomposition may indicate a faster hydrolysis of gentamicin ethers and amides relative to those of kanamycin A.

Comparison between assay systems

Blind comparison studies relating the chromatographic and microbiological serum gentamicin assay systems and establishing 95% confidence limits are summarized in Table VII. Both techniques provide good estimates of the actual values. For the levels 5, 10 and 20 μg Gm per ml serum, GLC values tend to underestimate, while the microbiological test slightly overestimates the actual values. The standard errors over this range are somewhat lower with the chromatographic technique.

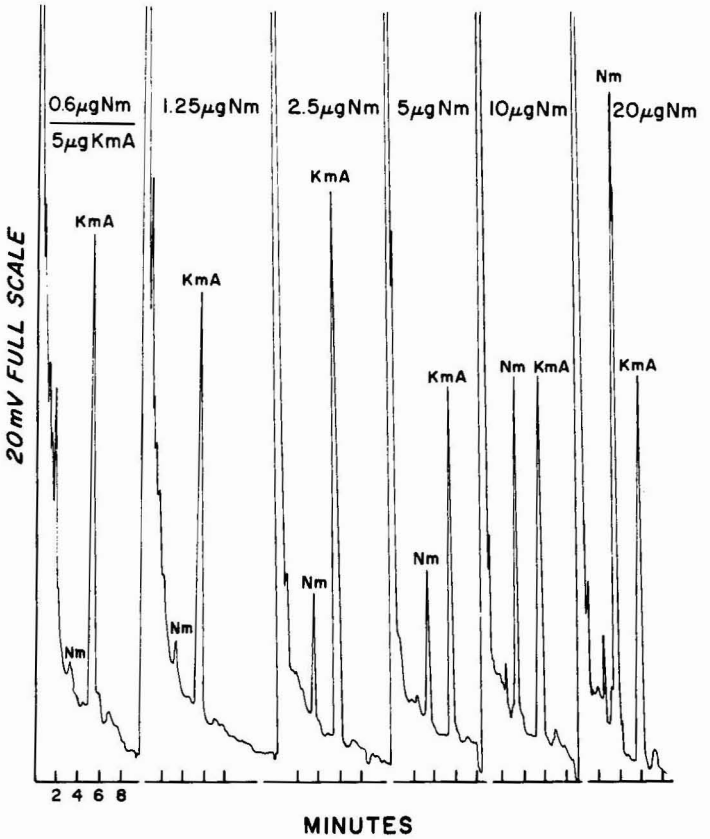

Fig. 5. Netilmicin: chromatographic dose-response from serum.

Statistical analysis of the two assay systems for amikacin showed that the precision of GLC was not significantly different from that of the microbiological technique (Table VIII). The means of percent deviations from actual amikacin concentrations were 11.2% by the GLC estimate and 14.2% by the microbiological estimate.

DISCUSSION

Effective techniques have been developed in isomer separation and potency determinations from standard powders, preparations, and ointments for at least five aminoglycosides[8]. These analyses generally include: silylation, lyophilization, flame ionization detection, and some forms of internal and external standardization. The internal standards (*e.g.*, trilaurin) are not good homologues of aminoglycosides in terms of physical and reactive characteristics. In general, these procedures are excellent for potency studies in milligram ranges, but are inappropriate for quantitation of aminoglycoside antibiotics in serum.

The analytical system for aminoglycosides described in the report appears to combine speed and relative simplicity with acceptable accuracy and precision. A

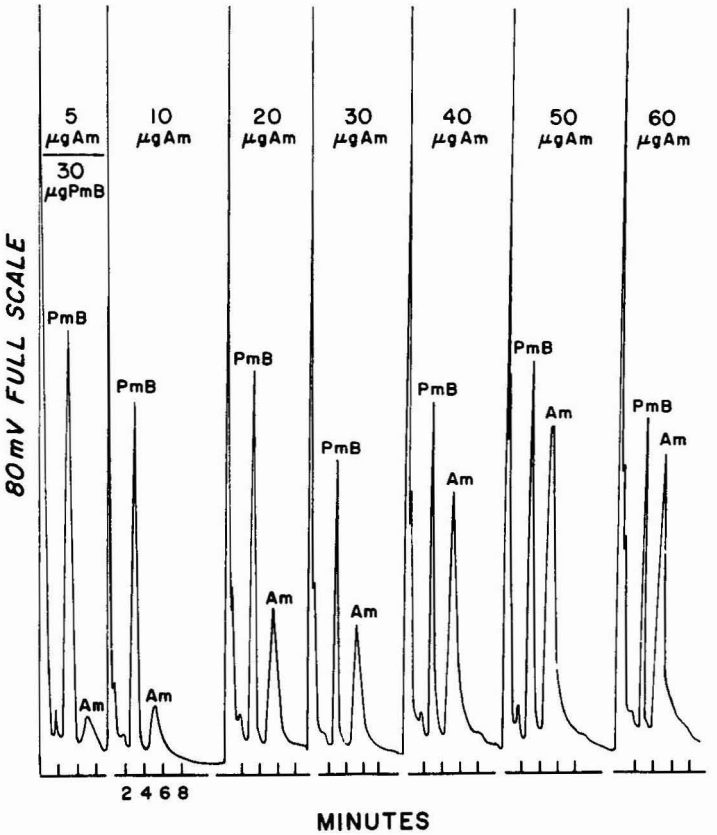

Fig. 6. Amikacin: chromatographic dose-response from serum.

TABLE III

CHROMATOGRAPHIC RESPONSE TO STANDARD CONCENTRATIONS OF TOBRAMICIN IN HUMAN SERUM

Abbreviations: Tm, tobramycin; KmA, kanamycin A; concentration response factor $F_{Tm} = \frac{\text{area Tm}}{\text{area KmA}} \cdot \frac{5\,\mu\text{g KmA}}{\mu\text{g Tm}}$.

$\left(\frac{\mu g\ Tm}{ml\ serum}\right)$	$\left(\frac{area\ Tm}{area\ KmA^{*}}\right)^{**}$	F_{Tm}
0.6	0.064	0.533
1.25	0.203	0.812
2.5	0.432	0.864
5.0	0.812	0.812
10.0	1.890	0.945
20.0	2.996	0.749

* 5 μg KmA per ml serum.

** Mean of three separate trials; area measurement by electronic digital integrator.

TABLE IV

CHROMATOGRAPHIC RESPONSE TO STANDARD CONCENTRATIONS OF NETILMICIN IN HUMAN SERUM

Abbreviations: Nm, netilmicin; KmA, kanamycin A; concentration response factor $F_{Nm} = \frac{\text{area Nm}}{\text{area KmA}} \cdot \frac{5\ \mu\text{g KmA}}{\mu\text{g Nm}}$.

$\left(\frac{\mu g\ Nm}{ml\ serum}\right)$	$\left(\frac{area\ Nm}{area\ KmA^{*}}\right)^{**}$	F_{Nm}
0.063	0.036 ± 0.003	0.299
1.25	0.056 ± 0.005	0.224
2.5	0.154 ± 0.013	0.308
5.0	0.310 ± 0.008	0.310
10.0	0.603 ± 0.031	0.302
20.0	1.507 ± 0.057	0.377

* 5 μg KmA per ml serum.

** Mean of 5 separate trials ±1 SEM; area measurement by electronic digital integrator.

TABLE V

CHROMATOGRAPHIC RESPONSE TO STANDARD CONCENTRATIONS OF AMIKACIN IN HUMAN SERUM

Abbreviations: Am, amikacin; PmB, paromomycin B; concentration response factor $F_{Am} = \frac{\text{area Am}}{\text{area PmB}} \cdot \frac{30\ \mu\text{g PmB}}{\mu\text{g Am}}$.

$\left(\frac{\mu g\ Am}{ml\ serum}\right)$	$\left(\frac{area\ Am}{area\ PmB^{*}}\right)^{**}$	F_{Am}
0.5	0.144	0.864
10	0 307	0.921
20	0.699	1.049
30	0.942	0.942
40	1.532	1.150
50	1.783	1.070
60	2.246	1.123

* 30 μg PmB per ml serum.

** Mean of 3 separate trials; area measurement by electronic digital integrator.

TABLE VI

STABILITY OF GENTAMICIN/KANAMYCIN A AREA RATIOS DURING STORAGE OF DERIVATIZED SPECIMENS

Derivatized preparations stored at −20° (0.5 ml hexane and approximately 1.2 ml aqueous). Ratios are the mean of two separate trials.

μg Gm per ml serum	*Area gentamicin/area kanamycin A*			
	0 h	*24 h*	*48 h*	*120 h*
0.63	0.057	0.064	0.061	0.057
1.25	0.122	0.122	0.144	0.113
2.5	0.260	0.268	0.255	0.154
5.0	0.774	0.768	0.811	0.633
10.0	1.199	1.253	1.315	1.104

TABLE VII

BLIND COMPARISON OF GLC AND MICROBIOLOGICAL ASSAYS FOR SERUM GENTAMICIN LEVELS

μg Gm per ml serum (actual)	*μg Gm per ml serum (estimated)*	
	*GLC assay**	*Microbiological assay**
1	1.11 ± 0.076	0.90 ± 0.049
5	4.72 ± 0.265	5.56 ± 0.356
10	9.55 ± 0.503	11.11 ± 0.964
20	19.19 ± 0.639	20.91 ± 0.716

* Mean of 10 separate trials ± 1 SEM.

TABLE VIII

COMPARISON OF GLC AND MICROBIOLOGICAL ASSAYS FOR SERUM AMIKACIN LEVELS

Actual concn. by supplementing serum with known quantities of drug (μg/ml)	*Concn. estimated*	
	GLC (μg/ml)	*Microbiological (μg/ml)*
0	0	0
0	0	0
4	2.8	5.2
6	5.2	5.8
8	7.5	8.4
8	8.6	8.6
12	10.3	15.0
15	13.8	13.0
16	16.5	18.0
20	16.9	22.0
24	24.6	25.0
24	22.7	20.0
30	23.4	30.0
32	34.3	45.0
40	41.7	30.0
43	40.9	55.0
48	55.8	50.0
51	41.6	45.0
53	40.2	34.0
64	44.8	50.0
64	64.6	65.0
Number of trials, *N*	21	
Degrees of freedom, *df*	20	
Variance	36.996	50.048
Variance ratio, *F*	1.353	

single serum specimen may be quantitated within 50 min. Since the chromatographic bands of the derivatized drugs are directly visualized, the emergence of new or unique peaks relative to the internal standard may indicate modification of the drug by host- or microbe-mediated reactions. The technique should be applicable to all aminoglycoside drugs with variations only in choice of internal standard and chromatographic conditions, *i.e.* liquid phase, temperature, carrier rate. The conditions described here

for amikacin can be applied to butirosin A and B, paromomycin A, streptomycin, and neomycin. These antibiotics are primarily in the 450–600 molecular weight range. In addition, the procedures may be used for the broad range of saccharides, aminosaccharides, and other compounds which do not readily partition into an organic phase. A combination of silylation and acylation for catecholamines has been described[9].

No interfering chromatographic bands have been observed in normal serum when compared to serum containing authentic aminoglycoside. Studies now in progress with serum from patients indicate to date no interference from simultaneously administered cephalosporins, penicillins, chloramphenicol, or clindamycin. A variety of other drugs also were being administered to these patients.

These are several points of the assay procedure worthy of expanded discussion. Optimal results are achieved when preparation temperatures do not exceed 85°. The formation of silyl ethers (TMSI reaction, specific for hydroxyl groups) does occur more rapidly at higher temperatures (*e.g.* 75°) but a compromise has been chosen between time and temperature to generate reproducible chromatograms under the mildest of preparation conditions. The formation of heptafluorobutyrylamides (HFBI reaction) above 70° has resulted in the appearance of multiple chromatographic bands. Our most reliable results with both silylation and acylation have been found at derivatization temperatures from 40° to 55°. Reproducible derivatizations of hydroxyl and amino groups with HFBI alone, as well as acylation attempts with trifluoroacetylimidazole and trifluoroacetic anhydride, have been unsatisfactory.

Derivatized gentamicin is stable for at least 48 h at −20°. Preliminary data indicate derivatized tobramycin and amikacin to be similarly stable. However, derivatized netilmicin appears to hydrolyze rapidly within 24 h. Drying of the hexane phase and resolubilization results in almost complete loss of chromatographic response.

In the protein precipitation step, the fluid should become opaque, white, and finely granular in order to produce a clear supernatant. The addition of 0.25% H_2SO_4 to that supernatant is vital for consistent chromatography. This may suggest that formation of heptafluorobutyrylamides is favored when amino groups of the trimethylsilylated antibiotic are charged. Supernatants which have not been thoroughly dried appear cloudy after the TMSI step and result in a cloudy final hexane phase. Chromatographic results from these specimens are extremely unreliable.

Since kanamycin A and paromomycin B are members of the same class of compounds as the four aminoglycosides discussed, they serve as nearly ideal internal standards. These markers share similar characteristics such as molecular weight and configuration, reactive hydroxyl and amino groups, stability, solubility, and interaction with chromatographic systems. Stock solutions of the standards are easily handled on a routine basis.

Certain lots of kanamycin A, however, may contain a trace band which elutes at $RRT_{KmA} = 0.61$. This band does not conform to authentic kanamycin B ($RRT_{KmA} = 0.71$), but does occur in the region of gentamicin C_1, tobramycin, and netilmicin. The maximum relative area of this band is less than 0.2 μg aminoglycoside per ml serum. Therefore, it is necessary to check internal standard lots by addition to normal serum without other aminoglycosides. An analagous band has not been observed from paromomycin B.

Extremely low absolute integration values for the internal standards can lead

to substantial quantitative error. If the value is less than 50% of the area expected, the specimen should be redone.

In general, the relative sensitivity of ECD operation and preparation techniques are designed to favor linear reproducible response over therapeutic aminoglycoside ranges. The minimum detectable limit may in fact be a hundred-fold lower than the 0.6 μg aminoglycoside per ml serum level.

CONCLUSION

Serum levels of gentamicin, tobramycin, netilmicin, and amikacin can be reliably assayed using gas–liquid chromatography and electron capture detection. The internal standards used are kanamycin A and paromomycin B. Specimen preparation includes: precipitation of the serum–internal standard mixture; evaporation of the resulting supernatant; two-stage derivatization with TMSI and HFBI; and extraction into hexane with water wash. Linear, reproducible dose–response curves of physiological levels are presented. Gentamicin and amikacin serum levels by GLC compare well to those determined by the microbiological method. Interfering peaks were not detected in the sera of patients and normal individuals.

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CHROM. 10,500

STUDIES OF MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. APPLICATION TO GEL PERMEATION CHROMATOGRAPHY OF TECHNIQUES DEVELOPED FOR MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Various micro high-performance liquid chromatographic techniques have been successfully applied to gel permeation chromatography by reducing detector cell volume, injection volume and void volume in ports and joints. Phthalic esters, various oligomers and polymers were separated on appropriate micro columns, as well as on ordinary wide-bore columns. Use of these micro columns means that smaller amounts of expensive packing materials and carrier solvents are required, and that the chromatographic operations and instruments can be simplified.

INTRODUCTION

Gel permeation chromatography (GPC) has a unique separation mechanism based on differences in the molecular size of the sample components. It is a powerful method, especially for the separation and identification of high-molecular-weight substances and the determination of molecular-weight distribution[1–6]. Column dimensions of *ca.* 8 mm I.D. and 30–100 cm length have been generally adopted[7–9], except in a few instances[6,10]. The diameter of these columns is appreciably larger than those of the columns used in other high-performance liquid chromatographic (HPLC) methods, for the following reason. The partition coefficient (K_D) in GPC is 0–1 because of the chromatographic mechanism, so that a solute component gives a relatively small dispersion. Therefore, if HPLC columns (2–3 mm I.D.) are used for GPC, the dispersion in the columns becomes so small that the dispersion in void volume in other parts of the apparatus (mainly joints and injection ports) becomes significant.

However, use of wide-bore columns requires large amounts of expensive packing materials and carrier solvents. Furthermore, the solute components may disperse widely, resulting in a decrease in the sensitivity.

We have developed micro high-performance liquid chromatography (MHPLC) for various columns by using the many available techniques[11]. If MHPLC techniques

could be applied to GPC, then the consumption of packing materials, carrier solvents and sample solutions would be decreased and the sensitivity increased. The dispersion of sample components would be smaller, and miniaturization of GPC columns would make it smaller still, perhaps as little as 1–5 μl. We have investigated these possibilities, using a detector cell system and injection system suited to GPC micro columns, and achieved the separation of phthalic esters, oligomers and polymers.

EXPERIMENTAL

Packing materials and preparation of micro columns

The packing materials used are listed in Table I, by their commercial names. Fluororesin was used as the main column material. GPC micro columns were prepared as described for MHPLC[11]. The dispersion of a solute component in GPC micro column is so small that the detector cell volume and the injection volume must be smaller than that for ordinary MHPLC, and "on-column" and "on-cell" systems must be adopted in order to minimize the dispersion in other parts of the apparatus. These problems were solved by the application of MHPLC techniques.

TABLE I

PHYSICAL CHARACTERISTICS OF PACKING MATERIALS

*Packing materials**	*Particle size (μm)*	*Excluded mol.wt.***
TSK G1000H	15	$1 \cdot 10^3$
TSK G2000H	—	$1 \cdot 10^4$
TSK G4000H	5	$4 \cdot 10^5$
Shodex A-801	10	$1 \cdot 10^3$
Shodex A-802	10	$5 \cdot 10^3$
Shodex A-804	10	$5 \cdot 10^5$
HSG-10	8 ~ 10	$3 \cdot 10^2$
HSG-15	7 ~ 9	$3 \cdot 10^3$
HSG-50	8 ~ 10	$1 \cdot 10^6$

* These are the commercial names.
** Based on polystyrene mol.wt.

Chromatographic technique

Methods similar to those used in ordinary MHPLC were employed for the feed of carrier liquid and the injection of sample solution. The method of injection was slightly different from that described previously[11]. The sample solution was drawn into a stainless steel tube, with *ca.* 0.1 μl of air, and the tip tube mopped with gauze before being connected to the micro column. The air disappeared in the micro-column owing to the pressurized passage of the carrier liquid, and it did not influence the chromatographic operation. A UV spectrophotometer equipped with a micro flow-cell was used as the detector system.

RESULT AND DISCUSSION

Relationship between flow-rate and column efficiency

Fig. 1 shows the relationship between flow-rate and column efficiency on the GPC micro columns packed with various stationary phases, in the region of low

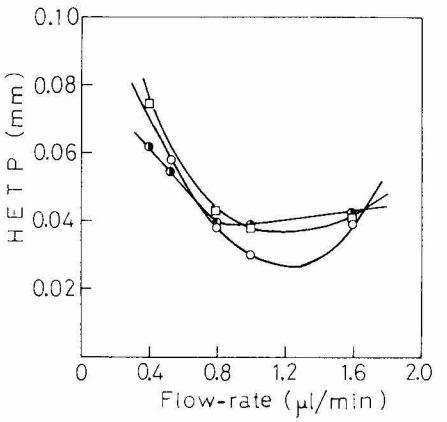

Fig. 1. Relationship between HETP and flow-rate. Columns: ○, 25 cm × 0.5 mm I.D. Shodex A-801; □, 16 cm × 0.5 mm I.D. TSK G2000H; ◑, 16 cm × 0.5 mm I.D. HSG-10. Sample, 1% benzene in tetrahydrofuran; injection volume, 0.01 μl; eluent, THF; detection wavelength, 254 nm.

flow-rate. In the region of high flow-rate, the relationship was similar to that in the ordinary GPC columns. However, column efficiency was reduced at extremely low flow-rates, which does not occur with ordinary GPC columns. The increase of HETP in flow-rates below 1 μl/min may result from the fact that the contribution to column efficiency by longitudinal diffusion of molecules, corresponding to the second term in van Deemter equation, is generally negligible in HPLC, but, at the low flow-rates in GPC micro columns, this diffusion in the mobile phase is no longer negligible and may contribute to the column efficiency. The minimum HETP values in Fig. 1 (0.03–0.04 mm) are not inferior to those obtaining in ordinary GPC.

The influence of sample size on the column efficiency and the separation of sample components

The sample size must be reduced in proportion to the column size and the column capacity. Fig. 2 shows the effects of varying the sample size on the resolution: the smaller the sample, the better the resolution. The injection volume must therefore be as small as possible, preferably 0.02 μl or less. Such very small amounts of sample solution can be injected by using the method developed during the investigation of MHPLC[11].

The influence of flow-cell volume on the resolution

Use of a GPC micro column may make the dispersion of solute components very small (less than a few microlitres), and the volume of the micro flow-cell must be suitably reduced. The influence of flow-cell volume on the separation of sample components was investigated and the results are shown in Fig. 3. With a flow-cell of volume 0.63 μl, the separation of dioctyl phthalate (DOP) and dibutyl phthalate (DBP) was incomplete. However, DOP and DBP were satisfactorily separated using a micro flow-cell of volume 0.13 μl. These results show that the cell volume should preferably be 0.1 μl or less.

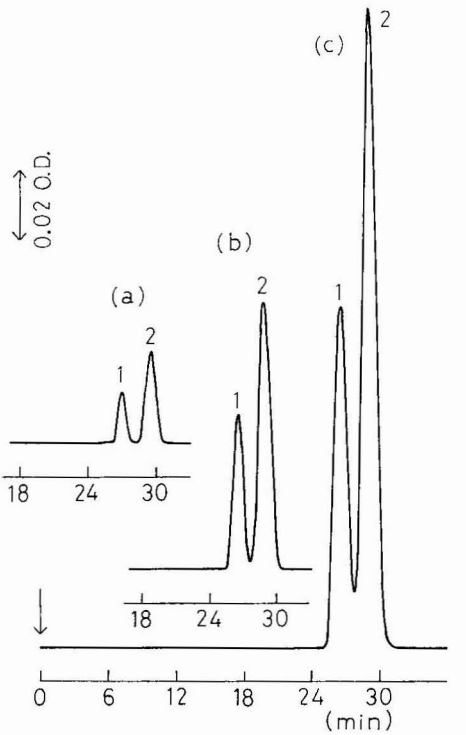

Fig. 2. Effect of sample size on resolution. Column, 16 cm × 0.5 mm I.D. Shodex A-801. Peaks: 1 = dioctyl phthalate; 2 = dibutyl phthalate. Sample, 0.49% DOP and 0.51% DBP in tetrahydrofuran. Sample size: (a) 0.006 μl; (b) 0.02 μl; (c) 0.06 μl. Eluent, tetrahydrofutan; flow-rate, 0.67 μl/min; detection wavelength, 244 nm.

Examples of GPC separation

Separation of phthalic esters. Fig. 4 shows a chromatogram of dilauryl phthalate (DLP), DOP, DBP and diethyl phthalate (DEP) on a 25 cm × 0.5 mm I.D. Teflon column packed with Shodex A-801. They were satisfactorily separated, and the dispersion of each peak was only 1.5 μl. The GPC micro column gave HETP values of 0.04–0.08 mm, which are not inferior to those obtaining in ordinary GPC using relatively wide-bore columns.

The separation of the phthalic esters was carried out on a 50 cm × 0.25 mm I.D. column packed with TSK G1000H, and on a 31 cm × 1.0 mm I.D. column packed with Shodex A-801. As the cross-sectional area of the former column is only one-thousandth that of an ordinary GPC column, chromatographic operating conditions may be reduced by a factor of 10^3. Carrier flow-rate was reduced to 0.4 μl/min and sample amounts to 50 ng, and consequently the dispersion of chromatogram to 0.7 μl. The total amount of carrier liquid required to complete chromatographic run was 16 μl. Under these conditions, good separations were achieved within 40 min. The separation of phthalic esters on the 1.0 mm column was somewhat better than on the 0.5 mm column, and was achieved within 18 min. A typical chromatogram of dinonyl phthalate (DNP), DLP, diheptyl phthalate (DHP), DBP, DEP and dimethyl phthalate (DMP) on the 1.0 mm column is shown in Fig. 5.

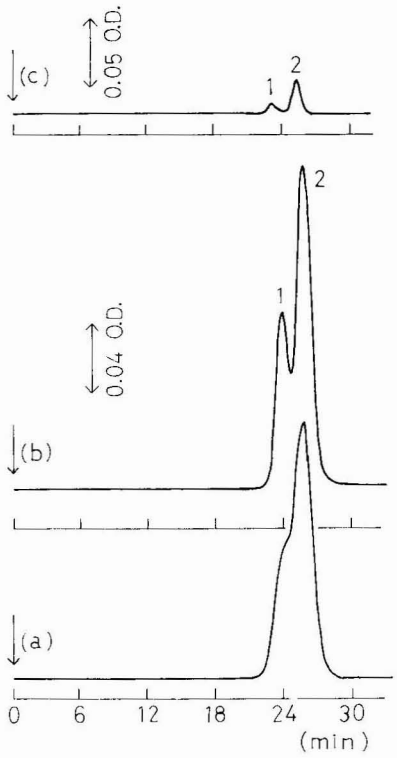

Fig. 3. Effect of cell volume and sample size on resolution. Column, 12 cm × 0.5 mm I.D. Shodex A-801. Sample: 0.47% DOP and 0.53% DBP. Sample size: (a) and (b) 0.06 μl; (c) very small, Cell volume: (a) 0.64 μl; (b) and (c) 0.13 μl. Peaks: 1, DOP; 2, DBP. Eluent, tetrahydrofuran; flow-rate, 0.67 μl/min.

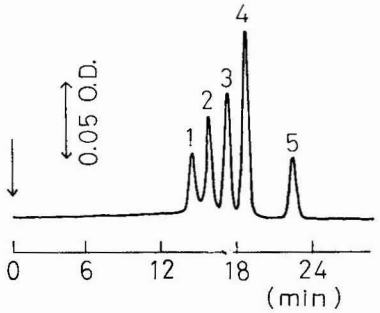

Fig. 4. Separation of a mixture of DLP, DOP, DBP, DEP, and benzene. Peaks: 1 = DLP; 2 = DOP; 3 = DBP; 4 = DEP; 5 = benzene. Column, 25 cm × 0.5 mm I.D. Shodex A-801. Sample: 0.8% DLP, 0.8% DOP, 0.8% DBP, 1.0% DEP and 1.3% benzene in tetrahydrofuran. Sample size, 0.01 μl; eluent, tetrahydrofuran; flow-rate, 1.6 μl/min.

These results show that the micro columns are applicable to GPC, as the column efficiency and separation are as good as the commercially available wide-bore columns can achieve.

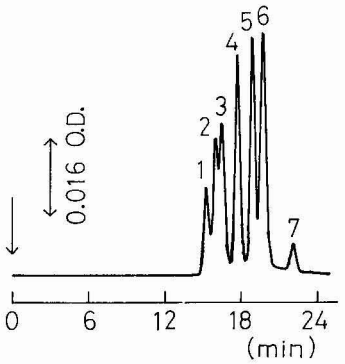

Fig. 5. Separation of a mixture of DLP, DNP, DHP, DBP, DEP, DMP and benzene. Peaks: 1 = DLP; 2 = DNP; 3 = DHP; 4 = DBP; 5 = DEP; 6 = DMP; 7 = benzene. Column, 32 cm × 1 mm I.D. TSK G1000H. Sample: 0.60% DLP, 0.62% DNP, 0.62% DHP, 0.57% DBP, 0.63% DEP, 0.61% DMP and 0.97% benzene in tetrahydrofuran. Sample size, 0.006 μl; eluent, tetrahydrofuran; flow-rate, 8 μl/min.

Separations of oligomers. It was relatively difficult to separate oligomers satisfactorily in a short time when ordinary equipment and column sets were used. Therefore, the separation was achieved with very long columns[12], recycle systems[12,13] or soft gels[14]. However, the analysis time was long in all cases. Recently, it was shown that high resolution is attainable using columns packed with very small gel particles, and the application of high-resolution GPC to oligomers and plasticizers was reported[8].

GPC of oligomers on the 0.5-mm GPC micro column did not give successful results, apparently because of the insufficient column capacity. The problem was solved by using the 1.0-mm GPC micro column. A chromatogram of polystyrene 600 is shown in Fig. 6. The sample components are well separated, but a faster and better separation may be possible using smaller diameter particles as packing material (*ca.* 2–3 μm).

Typical gel permeation chromatograms of oligomers of epoxy resin utilized in adhesives are shown in Fig. 7. In the separation of Epicoat 828, peaks corresponding

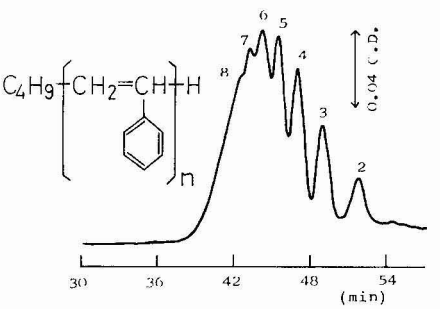

Fig. 6. Separation of polystyrene oligomers. Numbers at peaks are the degree of polymerization. Column, 31 cm × 1 mm I.D. TSK G2000H. Sample, 1.2% polystyrene 600 in tetrahydrofuran; sample size, 0.12 μl; eluent, tetrahydrofuran; flow-rate, 2.67 μl/min.

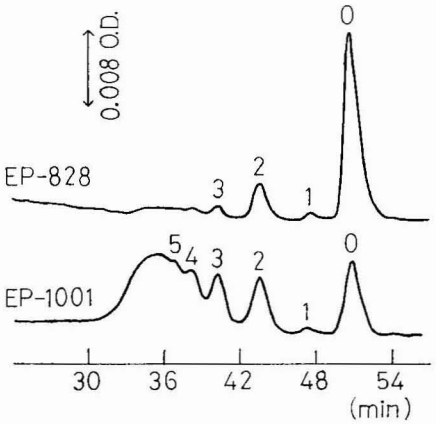

Fig. 7. Separation of polyepoxide oligomers. Column, 31 cm × 1 mm I.D. TSK G2000H. Sample, 1% Epicoat 828 and 1% Epicoat 1001 in tetrahydrofuran; Sample size, 0.12 μl; eluent, tetrahydrofuran; flow-rate, 2.67 μl/min.

to degrees of polymerization 0, 1, 2 and 3 were observed and in the separation of Epicoat 1001, peaks corresponding to degrees of polymerization 0, 1, 2, 3, 4, 5 and 6 were observed. These results show that oligomers can be well separated on appropriate GPC micro columns in a very short time. The separation may be improved by varying the chromatographic conditions.

The separation of standard polystyrene. A mixture of high-molecular-weight polymers could be separated by micro columns packed with polystyrene-gel particles with pore sizes corresponding to 400,000–500,000 excluded-molecular-weight. A typical separation of standard narrow-distribution polystyrenes on a 34 cm × 0.5 mm I.D. Teflon column packed with Shodex A-804 is shown in Fig. 8. The separation by GPC micro column was not inferior to that by the ordinary wide-bore column[7,10]. The separations were also carried out at flow-rates varying in the range 1–8 μl/min. The analysis time for one complete separation run was 14 min at a flow-rate of 4 μl/min, and only 7 min at a flow-rate of 8 μl/min. Thus, the separative analysis of polymers can be achieved in a short time using GPC micro columns.

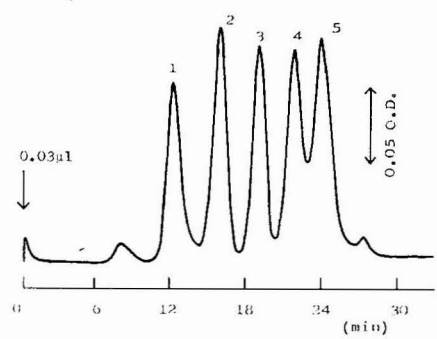

Fig. 8. Separation of standard polystyrenes. Column, 34 cm × 0.5 mm I.D. Shodex A-804. Sample size, 0.03 μl; eluent, tetrahydrofuran; flow-rate, 2 μl/min. Peaks: 1 = 498,000 (0.9%); 2 = 110,000 (0.9%); 3 = 37,000 (0.8%); 4 = 10,000 (1.0%); 5 = 2100 (1.1%).

CONCLUSION

Micro GPC was developed by application of the various techniques of MHPLC to GPC, and the required amounts of sample, packing material and carrier solvent are decreased. Micro GPC can be easily performed by simple instruments and operations, and is useful for the analysis of various polymers and oligomers. It would be applicable to preparatory experiments to select the operating conditions for fractionation by GPC.

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SEPARATION OF POLYCHLOROBIPHENYLS FROM CHLORINATED PESTICIDES IN SEDIMENT AND OYSTER SAMPLES FOR ANALYSIS BY GAS CHROMATOGRAPHY

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SUMMARY

Polychlorobiphenyls (PCBs) have been separated from DDT and its analogs and from the other common chlorinated pesticides by adsorption chromatography on columns of alumina and charcoal. Elution from alumina columns with increasing fractional amounts of hexane first isolates dieldrin and heptachlor epoxide from a mixture of chlorinated pesticides and PCBs. The remaining fraction, when added to a charcoal column, can be separated into two fractions, one containing the chlorinated pesticides, the other containing the PCBs, by eluting with acetone–diethyl ether (25:75) and benzene, respectively. The PCBs and the pesticides are then determined by gas chromatography on the separate column eluates without cross interference. The method is applicable to samples prepared by multi-pesticide residue methodology and should be applicable to sample extracts prepared for gas chromatography. Recoveries of the PCBs (Aroclors) and the chlorinated pesticides are good and the method is applicable to sediment and marine biota samples.

INTRODUCTION

The ubiquitous nature of the polychlorobiphenyls (PCBs) in the environment adds to the problems always present in the analysis of biota and soil samples for chlorinated pesticides. Attempts, more or less successful, have been made by Reynolds[1,2], Holden and Marsden[3], Armour and Burke[4], Feltz[5] and Berg *et al.*[6] to solve this problem by various partitioning procedures utilizing alumina, silica gel, Celite and/or charcoal as adsorbents. Other problems which hamper these procedures include the difficulty in obtaining subsequent supplies of alumina with repetitive adsorbent characteristics and maintaining the required technical expertise of the analyst in preparing the adsorbents for analytical use.

A method of analysis is presented which isolates the PCBs from pesticides, thereby permitting the qualitative and semi-quantitative analyses of each of these chemical groupings separately. The procedure incorporates features of the previous studies with strict modifications necessary to successfully complete the analyses.

Biological samples and soil samples are used to illustrate the applicability of the analytical procedure.

EXPERIMENTAL

Reagents

Woelm Neutral Alumina (or an equivalent) for column chromatography. Fisher No. 5-690 activated charcoal (50–200 mesh) (no substitute) from Fisher Scientific, Fair Lawn, N.J., U.S.A. 6 *N* Nitric acid (reagent grade). Sodium sulfate, anhydrous granular, heated and stored at 200°. QUSO-G30, unreductionized precipitated silica (Philadelphia Quartz Co., Philadelphia, Pa., U.S.A.). Desiccant mix (10% QUSO, 90% anhydrous sodium sulfate). Ottawa sand (acid washed), Cat. No. 3382 (J. T. Baker, Phillipsburg, N.J., U.S.A.). Isooctane (redistilled). Diethyl ether, Mallinckrodt No. 0844. Acetone, benzene, hexane and light petroleum (nanograde; Mallinckrodt St. Louis, Mo., U.S.A.).

Copper powder, as received from the supplier (J. T. Baker, Phillipsburg, N.J., U.S.A.), was treated for *ca.* 30 sec with 6 *N* nitric acid to remove surface oxides. The acid was decanted, the copper was rinsed several times with distilled water, followed by a rinse with acetone, and then air dried under a stream of nitrogen.

The alumina was treated as follows. 95 g of alumina (heated at 120° for 5 h) were weighed into a dry 500 ml ground-glass-stoppered erlenmeyer flask. 3 ml of water were slowly added and the flask was shaken until the heat dissipated. The procedure was repeated with the addition of 2 ml of water, and the treated alumina was mixed for 2 h and then stored in a desiccator. It was necessary to check the relative activity of each new batch (or manufacture's lot number) of alumina.

The charcoal was treated as follows. The charcoal was refluxed with acetone on a steam-bath, cooled and the solvent was removed by suction: the procedure was repeated and the charcoal was filtered. The filter-cake in the funnel was washed with cold acetone, air dried and stored at 135° until needed.

Standard pesticide and PCB solutions

Stock solutions were prepared of technical grade chlordane (1 mg/ml), of a mixture containing 1 μg/ml each of lindane, heptachlor, heptachlor epoxide, aldrin, *p,p'*-DDD, *p,p'*-DDT, *p,p'*-DDE and dieldrin and of 1 mg/ml each of the PCBs (Aroclors) 1242, 1248, 1254 and 1260 (Monsanto, St. Louis, Mo., U.S.A.). Solutions used for fortifying sample materials were as follows: chlordane (0.1 μg/μl); PCBs (1 μg/10 μl); lindane (2.5 ng/5 μl); heptachlor, heptachlor epoxide and aldrin (5 ng/5 μl); DDT, DDE, DDD and dieldrin (each 10 ng/5 μl). Standard solutions for gas chromatography (GC) were as follows: α- and γ-chlordane (each 0.5 ng/5 μl); technical grade chlordane (1 ng/5 μl); lindane (0.2 ng/5 μl); aldrin, heptachlor, heptachlor epoxide, DDD, DDE, DDT and dieldrin (each 0.4 ng/5 μl); PCBs (each 10 ng/5 μl). All of the pesticide and PCB standard solutions were prepared with nanograde hexane.

Apparatus

Aerograph 1200 and 204 gas chromatographs were used. The Aerograph 1200 contained a glass column (6 ft. × 0.125 in.) packed with 4% SE-30–6% SP-4201 on

Chromosorb W (100–120 mesh). The Aerograph 204 contained a glass column (6 ft. × 0.125 in.) with 4% SE-30–6% QF-1 on Chromosorb W (80–100 mesh). The operating conditions of the gas chromatographs were:

	1200	204
Column temperature	180°	185°
Injector temperature	215°	200°
Detector temperature	200°	200°
Nitrogen gas flow-rate	25 ml/min	30 ml/min

Both instruments contained an electron capture detector with a tritium-foil source.

For GC–mass spectrometry (MS), a Varian 1400 gas chromatograph coupled to a Finnegan 3000 mass spectrometer. The 1400 was equipped with a glass column (6 ft. × 2 mm I.D.) packed with 4% SE-30–6% SP-4201 on Supelcoport (100–120 mesh). The operating conditions were: column temperature, 210°; transfer-line temperature, 250°; gas-jet separator temperature, 225°; flow-rate of helium gas, 12 ml/min; sensitivity, 10^{-7} A/V; electron-multiplier voltage, 2.25 kV; electron-ionization current, 6.95 eV.

Biota samples were homogenized in either a Sorvall Omni-Mixer or a Waring Blendor. Sample extracts were concentrated on a rotary evaporator. Prior to use, all of the glassware was soaked in acidic dichromate solution, followed by a thorough rinse with water and acetone. The dried glassware was stored in an oven at 200° until used. Glass wool was rinsed with hexane and acetone and heated overnight at 200°.

Sample preparation

Oyster shells were opened by cracking the hinge; water was removed by shaking the shell after it had opened but before the adductor muscle was cut. The entire animal was removed and mixed in a Waring Blendor. A 30-g amount of the homogenate was weighed into a pint Mason jar and the jars were chilled (but not frozen) in a freezer for 30 min. Desiccant mix (120 g) was added to the chilled sample and thoroughly incorporated with the oyster tissue by means of a spoon after which the mixture was frozen. The frozen mixture was ground in the Sorvall Omni-Mixer until the material was free-flowing. The sample was maintained at freezing temperature during the mixing stage, and stored frozen until needed for analysis.

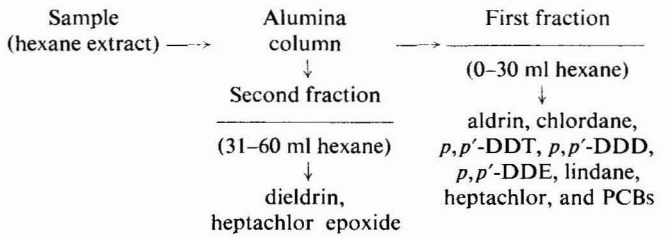
Screened soil samples were air dried at room temperature for at least 72 h, then blended in a Waring Blendor for 1 min. The soil was transferred to Mason jars and stored in a freezer until needed for analysis.

The oyster and soil samples were soxhlet extracted for 8 h with light petroleum for the oysters and acetone–light petroleum (1:9) for the soils.

Sample clean-up

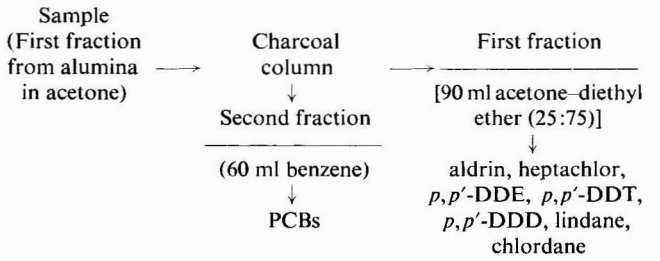
Alumina column. To a chromatographic column (10 × 30 mm, Kontes Chromaflex, Cat. No. K-420320 or an equivalent) containing a glass-wool plug were added 1.0 cm of anhydrous sodium sulfate, 8 cm of alumina adsorbent and finally 1.0 cm of anhydrous sodium sulfate. The column was tapped to ensure proper settling of the granules. Each layer must be flat and not tilted otherwise poor chromatography will result.

The hexane solution of the concentrated extract (less than 1.0 ml) was carefully added to the top of the dry adsorbent in the column. Hexane eluates were collected in 50 ml graduated cylinders and transferred to round bottom flasks for concentration. Eluate volumes will vary from batch to batch of alumina. The following elution scheme details the pesticide separation:



Charcoal column. A Pyrex micro chromatographic tube (140 × 6 mm I.D.) with a 50 ml reservoir was plugged by a small wad of solvent-washed glass wool. *ca.* 25 mm of acid-washed Ottawa sand was added to the column to retain the fines from the charcoal. An acetone slurry of the adsorbent was add until the height of the charcoal was 90 mm. [The acetone drains almost instantaneously through the micro tube, and the analyst should be ready to immediately add the concentrated residue of the first fraction from the alumina column (concentrated in acetone to 1 ml) to the charcoal column.] The flask was rinsed with several 1.0 ml portions of acetone and the rinsings were added to the column.

The DDT group of pesticides was eluted with 90 ml of 25% acetone in diethyl ether. A subsequent elution of the same column with 60 ml of benzene removed the PCBs from the column as noted in the following elution scheme:



Analysis

Analyses were made by GC, and the identification of the PCBs was confirmed by MS.

Elemental sulfur may be present in soils and perhaps in some biological materials; if present it will pass through the described analytical schemes and will be evident in the gas chromatograms[7] thus confusing the interpretation of the chromatograms. In order to avoid this problem, the extract or any fraction of the extract from the clean-up procedures may be treated as follows. The sample extract is evaporated to dryness; 1 ml each of hexane and isooctane, plus 100 mg of treated copper powder,

are added to the residue. The mixture is shaken vigorously and allowed to stand for 1 h; it is then evaporated to dryness and appropriate dilutions are made for GC analysis. Additional amounts of copper may be necessary depending the amount of sulfur present in the sample.

RESULTS AND DISCUSSION

A summation of the elution of the chlorinated organic pesticides and the PCBs from the alumina column is given in Table I. Heptachlor epoxide and dieldrin were removed from the column by extending the elution solvent beyond the 30 ml volume with an additional, but separate, elution volume of 30 ml (total 60 ml). The PCBs remained an integral part of the mixture containing the pesticides in the first 30 ml of eluate. The elution pattern of alumina column Fraction 1 on the charcoal column, Table II, shows that the pesticides were separated from the PCBs by means of the acetone–diethyl ether eluent. The PCBs were subsequently removed from the charcoal

TABLE I

PERCENTAGE RECOVERY OF PESTICIDES ELUTED FROM NEUTRAL ALUMINA

Compound		*First fraction*				*Second fraction*
		0–15 ml	*15–20 ml*	*20–25 ml*	*25–30 ml*	*30–60 ml*
Lindane				10	90	
Heptachlor		100				
Aldrin		100				
Heptachlor epoxide						100
p,p'-DDE		100				
Dieldrin						100
p,p'-DDD				50	50	
p,p'-DDT		100				
PCBs		100				
Chlordane	γ	10	80	10		
	α	80	20			

TABLE II

PERCENTAGE RECOVERY OF PESTICIDES ELUTED FROM CHARCOAL

Compound	*First fraction 90 ml acetone–diethyl ether (25:75)*			*Second fraction 60 ml benzene*	
	0–30 ml	*30–60 ml*	*60–90 ml*	*0–30 ml*	*30–60 ml*
Lindane	30	40	30		
Heptachlor	100				
Aldrin	100				
p,p'-DDE	50	50			
p,p'-DDT	80	20			
PCBs				80	20
Chlordane	100				

column with benzene. Known amounts of pesticides and PCBs (Aroclor 1254) were added to soils and oyster samples; the samples were analyzed as described herein to check the efficiency of the analytical procedure. Recoveries of the added chemicals to the soils and the oysters were consistent and acceptable (Tables III and IV). The limits of detectability of the chemicals examined (Table V) refer to those obtained from pure solutions and they are also applicable to sample extracts; at times, however,

TABLE III

RECOVERY OF PESTICIDES AND PCBs FROM FORTIFIED SOIL SAMPLES

Pesticide	*Fortified (ppm)*	*Recovered (ppm)*	*Recovered (%)*
Lindane	0.0013	0.0011	84.6
Heptachlor	0.0027	0.0022	81.5
Aldrin	0.0027	0.0023	85.2
Heptachlor epoxide	0.0027	0.0025	92.6
p,p'-DDE	0.0027	0.0025	92.6
Dieldrin	0.0027	0.0026	96.3
p,p'-DDD	0.0027	0.0027	100
p,p'-DDT	0.0027	0.0027	100
γ-Chlordane	0.0033	0.0035	106
α-Chlordane	0.0033	0.0037	112
PCB (Aroclor 1254)	0.066	0.066	100

TABLE IV

RECOVERY OF PESTICIDES AND PCBs FROM FORTIFIED OYSTERS

Pesticide	*Fortified (ppm)*	*Recovered (ppm)*	*Recovery (%)*
Heptachlor	0.0027	0.0019	70.4
Aldrin	0.0027	0.0021	77.8
Heptachlor epoxide	0.0027	0.0028	102
α-Chlordane	0.0033	0.0033	100
PCB (Aroclor 1254)	0.066	0.045	68.2

TABLE V

LIMITS OF DETECTABILITY OF PESTICIDES AND PCBs USING THE DESCRIBED PROCEDURE UNDER IDEAL CONDITIONS

Pesticide	*Detectability (ppb*)*
Lindane	0.04
Heptachlor	0.05
Aldrin	0.06
Heptachlor epoxide	0.10
p,p'-DDE	0.14
Dieldrin	0.14
p,p'-DDD	0.25
p,p'-DDT	0.33
γ-Chlordane	0.10
α-Chlordane	0.11
Aroclors 1254, 1260 (PCBs)	6.5

* The American billion (10^9) is meant.

it may be difficult, if not impossible, to minimize background contaminant levels sufficiently to observe this low level of detectability. Many PCBs are present in the environment and their composition is variable, resulting usually in multicomponent peaks on the gas chromatogram. However, when using the recommended analytical procedure, there is always a sufficient number of identifiing peaks to illustrate that they are indeed PCBs and this fact can be confirmed by mass spectrometry. It may be possible to estimate the amount of PCBs in a sample using the procedure described but, in many instances, it is more important to acertain their presence or absence under a given set of circumstances.

ACKNOWLEDGEMENT

The GC–MS confirmation of the PCBs was performed by Karl Yanagihara, whose assistance is greatly appreciated.

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UNE MÉTHODE SIMPLE DE SÉPARATION ET D'ESTÉRIFICATION DES ACIDES AMINÉS EN VUE DE LEUR PASSAGE EN SPECTROMÉTRIE DE MASSE

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SUMMARY

An easy method of separation and esterification of amino acids prior to their mass spectrometric analysis

An easy method for the identification of amino acids in a mixture by mass spectrometry (MS) without using a gas chromatography–MS apparatus is described. After separation on a classical amino acid analyzer without prior purification, amino acids are esterified using dimethoxypropane at ambient temperature and analyzed by MS.

INTRODUCTION

Si l'on veut trouver ou confirmer la structure des acides aminés d'un mélange par spectrométrie de masse, il faut deux étapes préliminaires: l'obtention de dérivés volatils et la séparation des constituants du mélange. Dans le couplage chromatographie en phase gazeuse–spectrométrie de masse (GC–MS), les deux étapes sont réalisées dans cet ordre.

Si l'on ne dispose pas de l'appareillage nécessaire à cette technique, on peut opérer en sens inverse en réalisant d'abord une séparation sur résine échangeuse d'ions en tampons volatils puis l'estérification de chaque acide aminé avant passage en MS.

Mais, après chromatographie, on obtient des acides aminés contaminés par des produits relargués par la résine et par des sels non volatils formés entre la base contenue dans le tampon et les anions présents dans l'échantillon.

D'autre part, les techniques d'estérification sont souvent délicates à mettre en oeuvre. Aussi, nous avons étudié une méthode simple qui permet de résoudre ces différents problèmes.

MATÉRIEL ET MÉTHODES

Les produits chimiques utilisés sont des produits purs pour analyse sauf le

diméthoxypropane (Merck, pour synthèse). Seule la pyridine est purifiée par distillation. Les esters méthyliques témoins sont des produits Fluka et Cyclochemical.

Estérification

Dans des tubes de verre bouchés émeri de 150 × 17 mm, on introduit l'acide aminé, le méthanol, la solution d'acide chlorhydrique (d = 1.19) et le diméthoxypropane. Après réaction à température ordinaire, le mélange est évaporé par un courant d'azote et repris par 3 ml d'eau. Cette solution est extraite successivement par 10 ml d'éther, 5 ml de dichloréthane et deux fois 2 ml d'éther.

Chaque extraction est faite directement dans le tube par agitation modérée pendant 1 min et aspiration de la phase organique avec une pipette reliée au vide. La phase aqueuse qui contient l'ester méthylique est ensuite évaporée ou lyophylisée.

Purification complémentaire

Dans le cas où l'acide aminé est contaminé par une importante quantité de sels non volatils, on ajoute dans le tube où se trouve le résidu d'évaporation 2 ml de dichloréthane et l'on fait barboter de l'ammoniac pendant 5 min; on filtre; on chasse l'excès d'ammoniac par un courant d'azote; on ajoute 0.2 ml d'acide acétique et on évapore la solution pour obtenir l'ester méthylique sous forme d'acétate.

Dosage des esters méthyliques

Au cours de l'étude de la réaction d'estérification, le dosage des esters est réalisé par colorimétrie en les transformant en acides hydroxamiques. Ceux-ci donnent des complexes ferriques dont le maximum d'absorbance est situé vers 520 nm[1,2]. La concentration de chaque ester est proportionnelle à la densité optique lue.

Chromatographie en phase gazeuse des esters méthyliques

Les esters méthyliques des amino-acides ont été analysés en GC dans les conditions expérimentales suivantes: appareil Carlo Erba Fractovap 2.200 muni d'un détecteur à ionisation de flamme; colonne de verre (0.3 × 200 cm) remplie de Chromosorb W AW DMCS (80–100 mesh) contenant 2.4% de OV-225; la colonne a été conditionnée à 210°; la température de l'injecteur et du détecteur est de 310°; la température de la colonne est programmée pour un palier de 8 min à 90° puis une augmentation de 5°/min jusqu'à 160°.

Spectrométrie de masse des esters méthyliques

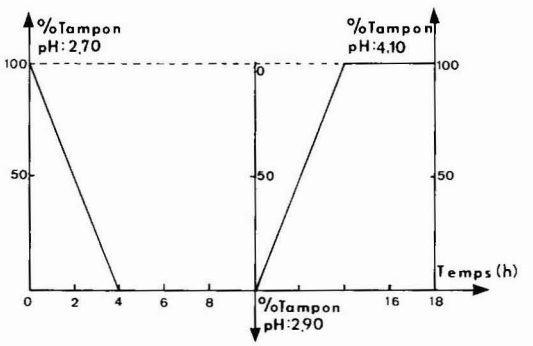
Les spectres de masse ont été réalisés dans les conditions suivantes: appareil type AEI MS-902; énergie des électrons 70 eV; courant d'émission du filament 100 μA; température du bloc source 110°; température de la sonde d'introduction entre 30° et 80°.

Séparation des acides aminés sur résines échangeuses d'ions

Elle est effectuée au moyen d'un appareil Carlo Erba modèle 3A27. Les acides aminés acides et neutres sont séparés sur une colonne Carlo Erba AN-55 de 550 × 9 mm I.D. remplie avec une résine Carlo Erba 3AR4A25. Les acides aminés basiques sont séparés sur une colonne Carlo Erba AN-15 de 150 × 9 mm I.D. remplie avec une résine Carlo Erba 3AR2A25. Les colonnes sont thermostatées à 50°.

Avant d'être mises dans les colonnes, les résines sont transformées en sels de pyridinium. Les tampons servant à l'élution sont exprimés en concentration de pyridine. Ils sont préparés en milieu aqueux en ajustant le pH par de l'acide acétique[3]. On utilise: tampon pH 2.70, 0.1 *M*; tampon pH 2.90, 0.1 *M*; tampon pH 4.10, 0.8 *M*; tampon pH 5.00, 1 *M*. Avant d'être posé, l'échantillon est acidifié par un tampon 0.1 *M* (pH 2.20) préparé en ajoutant une quantité convenable d'acide formique au tampon pH 2.70.

Un appareil LKB Ultrograd est utilisé pour la préparation des gradients. Pour la séparation des acides aminés acides et neutres, on équilibre la colonne avec le tampon pH 2.70. Après la pose de l'échantillon, on élue avec le gradient donné Fig. 1. Pour la séparation des acides aminés basiques, on équilibre la colonne avec le tampon pH 2.90 et après pose de l'échantillon, on élue avec le gradient donné Fig. 2. Après chaque séparation, les colonnes sont régénérées par du tampon pH 5.00.



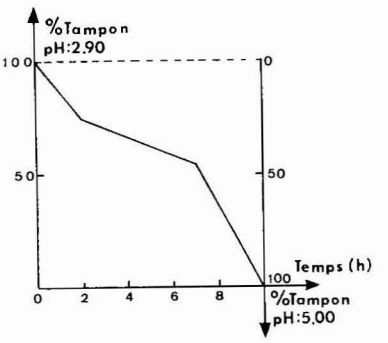


Fig. 1. Gradient d'élution des acides aminés acides et neutres: pourcentage en volume du tampon pH 2.7 par rapport au tampon pH 2.9 puis du tampon pH 2.9 par rapport au tampon pH 4.1 en fonction du temps (h).

Fig. 2. Gradient d'élution des acides aminés basiques: pourcentage en volume du tampon pH 2.9 par rapport au tampon pH 5 en fonction du temps (h).

Tous les tampons sont pompés à une vitesse de 60 ml/h. En sortie de colonne, l'éluat est divisé en deux parties: l'une est collectée au moyen d'un collecteur LKB Minirac en fraction d'une durée de 5 min, l'autre est diluée dans de l'eau et envoyée par une pompe Technicon P1 dans le réacteur du chromatographe pour mettre en évidence la présence des acides aminés par réaction avec la ninhydrine (Fig. 3).

Au moment où on collecte dans le premier tube, on pompe une solution de nitrate d'ammonium 10^{-4} *M* à la place de l'eau avec la pompe P1. Le pic obtenu sur le tracé permet, en tenant compte de la vitesse de déroulement du papier de l'enregistreur et de la durée de collection dans chaque tube, de savoir à quels tubes correspond chaque partie du tracé.

Après séparation, on réunit les tubes correspondant à chaque fraction. On amène le pH de ces fractions à la neutralité en ajoutant une solution concentrée d'ammoniaque de façon à déplacer la pyridine, puis on lyophilise.

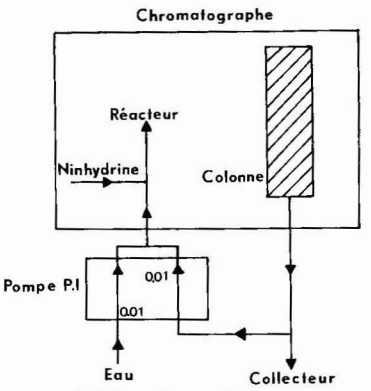


Fig. 3. Schéma du système utilisé pour collecter et détecter les acides aminés (diamètre interne des tuyaux de pompe, 0.01 pouce).

RÉSULTATS ET DISCUSSIONS

Choix de la méthode d'estérification

Comme il est particulièrement intéressant de pouvoir utiliser une méthode d'estérification ayant lieu à température ordinaire et ne nécessitant pas la manipulation de réactifs très toxiques ou difficiles à préparer et à conserver, nous avons repris le principe proposé par Rachele[4] pour l'estérification à l'échelle préparative de quelques acides aminés par le mélange diméthoxypropane, solution aqueuse d'acide chlorhydrique. Nous avons cherché quelles étaient les meilleures conditions de réaction et de purification de l'ester méthylique formé.

Nous avons observé que l'adjonction de méthanol diminue la polymérisation du diméthoxypropane, ce qui facilite la purification.

En faisant varier la quantité de diméthoxypropane et en gardant constantes les quantités d'acides aminés (10 μM), de solution d'acide chlorhydrique (0.1 ml; soit environ 24 mM HCl) et de méthanol (0.5 ml) dans le mélange réactionnel, on détermine quelle doit être la proportion entre les quantités de diméthoxypropane et de solution d'acide chlorhydrique ajoutées. Pour des temps de réaction de 3, 6 et 24 h, la quantité de diméthoxypropane doit être comprise entre 0.7 et 2.8 ml (5.5 et 22 mM environ) (Fig. 4). Une quantité trop faible de diméthoxypropane ne permet

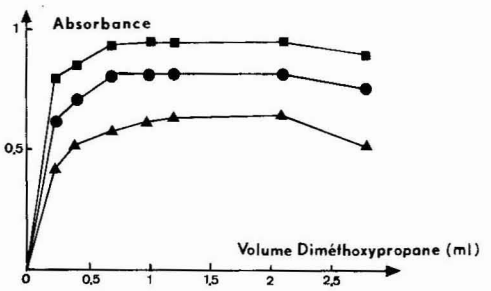


Fig. 4. Quantité d'ester formé, exprimée en unité d'absorbance, en fonction du volume (ml) de diméthoxypropane ajouté pour un temps de réaction de 3 h (▲), 6 h (●) et 24 h (■). Dosage par formation d'hydroxamate ferrique.

pas de transformer toute l'eau ajoutée avec la solution d'acide chlorhydrique, une quantité trop forte a un effet de dilution préjudiciable à la cinétique de la réaction. On a choisi un rapport entre les quantités de diméthoxypropane et de solution d'acide chlorhydrique de 10:1.

Si l'on ajoute des quantités croissantes du mélange diméthoxypropane–solution d'acide chlorhydrique 10:1 à une quantité fixe d'acide aminé (10 μM) et de méthanol (0.5 ml), le meilleur rendement correspond à un volume de 2.2 ml du mélange (Fig. 5). Les courbes tracées Figs. 4 et 5 correspondent à la formation de l'ester méthylique de l'alanine. Des résultats analogues ont été obtenus pour d'autres acides aminés.

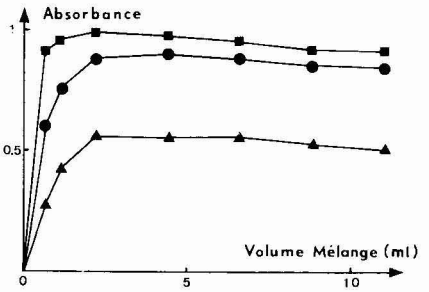
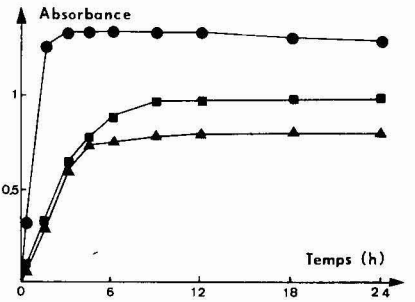


Fig. 5. Quantité d'ester formé, exprimée en unité d'absorbance, en fonction du volume du mélange (ml) diméthoxypropane–solution d'acide chlorhydrique (10:1), pour un temps de réaction de 3 h (▲), 6 h (●) et 24 h (■). Dosage par formation d'hydroxamate ferrique.

Si l'on ajoute à 10 μM d'un acide aminé, 0.5 ml de méthanol, 0.2 ml de solution d'acide chlorhydrique et 2 ml de diméthoxypropane et si l'on étudie la cinétique de la réaction pour différents types d'acides aminés, on voit que l'équilibre est pratiquement atteint après 12 h (Figs. 6 et 7).



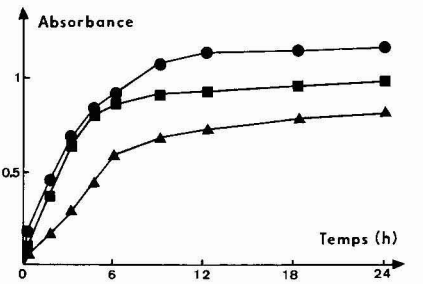


Fig. 6. Quantité d'ester formé, exprimée en unité d'absorbance, en fonction du temps (h) pour la lysine (▲), l'alanine (■) et l'acide aspartique (●). Dosage par formation d'hydroxamate ferrique.

Fig. 7. Quantité d'ester formé, exprimée en unité d'absorbance, en fonction du temps (h) pour la sérine (▲), la proline (■) et la phénylalanine (●). Dosage par formation d'hydroxamate ferrique.

En se basant sur ces résultats, nous utilisons pour estérifier les acides aminés, les conditions expérimentales suivantes: méthanol 0.5 ml, solution d'acide chlorhydrique 0.2 ml, diméthoxypropane 2 ml, temps de réaction 24 h.

Les solvants d'extraction ont été choisis pour permettre l'élimination des produits secondaires de la réaction et de certains des contaminants dus au passage sur résine. Le procédé retenu est simple et rapide.

Dans le cas où l'échantillon posé en chromatographie sur résine échangeuse d'ion est très chargé en sels non volatils, une quantité importante de ces sels peut être éluée avec certains des acides aminés. Pour pouvoir estérifier, ceux-ci, il faut ajouter un volume de solution d'acide chlorhydrique et de diméthoxypropane suffisant pour que l'acide chlorhydrique soit en excès et puisse jouer de rôle de catalyseur. Il faut également opérer une étape de purification complémentaire pour éliminer tous les sels.

Étude des esters en chromatographie en phase gazeuse et en spectrométrie de masse

Pour les identifier et déterminer leur pureté, les esters méthyliques préparés et purifiés en une étape et en deux étapes à partir des acides aminés suivants: acide aspartique, acide glutamique, thréonine, sérine, proline, glycine, alanine, valine, leucine, isoleucine, méthionine, cystéine, phénylalanine, tyrosine, lysine et histidine, sont analysés en GC et en MS.

En GC chaque ester donne un seul pic, identique à celui obtenu avec l'ester méthylique témoin, sauf pour l'ester de l'histidine qui est décomposé sur la colonne[5].

En MS, ces esters donnent un spectre proche de celui décrit par Biemann *et al.*[6] pour les esters éthyliques compte tenu des différences de masse des ions ayant le groupement ester. Pour identifier rapidement un acide aminé, nous utilisons le Tableau I où sont indiquées la masse du pic de base ainsi que la masse de deux ions caractéristiques avec leur pourcentage par rapport au pic de base.

TABLEAU I

PIC DE BASE ET PICS CARACTÉRISTIQUES DES ESTERS MÉTHYLIQUES DES ACIDES AMINÉS

Acide aminé	*Pic de base*	*Autres pics*
Glycine	30	89 (8%)
Alanine	44	88 (3%), 103 (2%)
Sérine	60	88 (45%), 42 (30%)
Méthionine	61	56 (75%), 104 (37%)
Proline	70	129 (1%)
Valine	72	88 (35%), 55 (28%)
Cystéine	76	88 (70%), 59 (51%)
Histidine	82	110 (26%)
Glutamique	84	116 (61%), 56 (41%)
Lysine	84	56 (25%), 101 (17%)
Leucine	86	88 (27%), 30 (14%)
Isoleucine	86	88 (44%), 30 (22%)
Phénylalanine	88	120 (71%), 91 (21%)
Thréonine	89	74 (84%), 57 (75%)
Aspartique	102	70 (30%), 88 (20%)
Tyrosine	107	88 (36%), 195 (11%)

Application

Nous utilisons couramment cette méthode dans l'étude des acides aminés libres ou résultant de l'hydrolyse de peptides séparés à partir de culture de mycelium de champignons supérieurs (*Lacaria Lacata* Scop. ex Fr). A titre d'exemple, l'identification des acides aminés de deux hydrolysats est donnée Fig. 8. Ces hydrolysats correspondant à des fractions séparées sur DEAE-Sephadex en tampon phosphate, ont des concentrations très importantes en ions phosphates et en ions chlorures. Néanmoins, ils sont posés directement en chromatographie sur résine échangeuse d'ions. Après adjonction d'ammoniaque, les fractions sont lyophilisées, estérifiées et purifiées en deux temps. Les spectres de masse obtenus sont analogues à ceux des composés purs et permettent une identification immédiate.

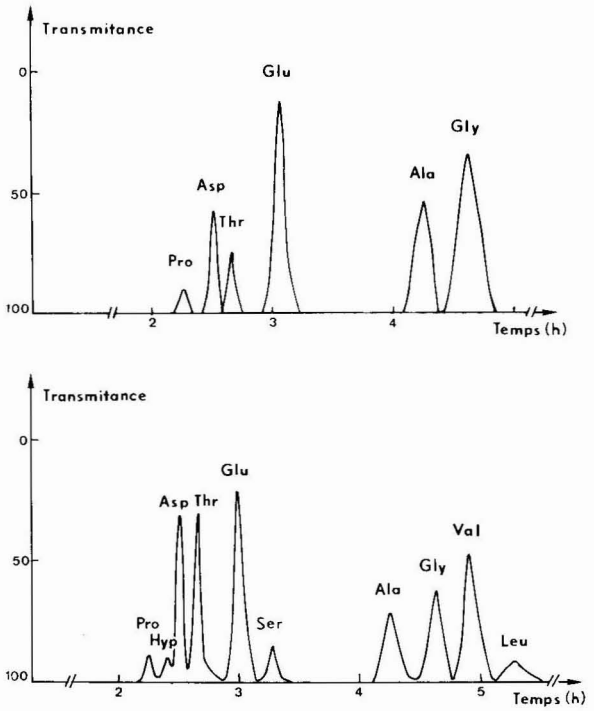


Fig. 8. Chromatographies des acides aminés acides et neutres résultant de l'hydrolyse de deux fractions séparées sur DEAE-Sephadex en tampon phosphate. L'identification a été faite par spectrométrie de masse. Hyp = Hydroxyproline

CONCLUSION

Le procédé proposé est plus long que celui basé sur le couplage GC–MS; mais il nécessite seulement l'emploi d'un appareillage classique de chromatographie d'acides aminés sur résine échangeuse d'ions sans purification préalable de l'échantillon. De plus, les techniques à mettre en oeuvre sont simples. Seuls des réactifs faciles à manipuler sont utilisés.

REMERCIEMENT

Nous remercions Mademoiselle Salandre pour sa collaboration technique.

RÉSUMÉ

Les auteurs décrivent un procédé simple à mettre en oeuvre permettant de séparer les acides aminés sur un appareillage classique de chromatographie sur résine échangeuse d'ions puis de les estérifier par une nouvelle méthode utilisant le diméthoxypropane à température ordinaire. Après purification, les esters méthyliques des acides aminés ainsi obtenus sont étudiés par spectrométrie de masse.

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ZUR KENNTNIS DES ELUTIONSVERHALTENS EINIGER PFLANZENSCHUTZMITTELWIRKSTOFFKLASSEN BEI DER GELCHROMATOGRAPHIE

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(Eingegangen am 8. August 1977)

SUMMARY

Evaluation of the elution behaviour of some classes of pesticides in gel chromatography

The elution behaviour of insecticides and herbicides of the organophosphorus, carbamate, phenylurea, triazine, chlorophenoxy acid and ester types has been studied on gel such as Sephadex, Bio-beads, and Merckogel. By comparing the elution volumes of the compounds within each class it was found that in most cases the order of elution does not follow the pure gel permeation principle, but is modified by several influences of substituents, π-systems and the overall polarities of the compounds chromatographed. In triazine herbicides some linear relationships within several subgroups have been evaluated.

The effects described may be used for differentiation and identification of pesticides.

EINLEITUNG

Die Gelchromatographie findet in letzter Zeit zunehmend auch zur Trennung von Verbindungen mit niedrigen Molekulargewichten ($100 < \text{M.G.} < 1000$) Anwendung. Dabei spielen mehrere physikalische Vorgänge in unterschiedlichem Masse eine Rolle. Nach dem Molekularsiebeffekt werden die Substanzen lediglich aufgrund der Unterschiede in ihren Molvolumina, in der Regel in der Reihenfolge ihrer Molgewichte, differenziert. Häufig kommt es aber zwischen funktionellen Gruppen oder den energetischen Feldern ganzer Bindungssysteme der Probenkomponenten und der Gelmatrix zu Nahewechselwirkungen, so dass die Elutionsvolumina der Komponenten in solchen Fällen nicht mehr von der Molekülgrösse allein bestimmt werden. Eine Reihe von Autoren untersuchte die Wechselwirkungen zwischen verschiedenen Gelen einerseits und mehreren Verbindungsklassen andererseits. So wurde u.a. von Wilk *et al.*[1], Joustra *et al.*[2], Oelert[3], Brook und Munday[4], Streuli[5], Determann und Lampert[6], Berek und Bakoš[7], Čoupek *et al.*[8], Klimisch und Ambrosius[9] und Shopova *et al.*[10] das Elutionsverhalten verschiedener aliphatischer und aromatischer Kohlenwasserstoffe an Sephadex LH-20 mit unterschiedlichen Elutionsmitteln untersucht;

Klimisch und Reese[11], Čoupek *et al.*[8] sowie Asche und Oelert[12] ermittelten das gelchromatographische Verhalten ein- und mehrkerniger Aromaten sowie das von Aminen und Phenolen an Polystyrolgelen; ferner untersuchten Oelert[13] das Elutionsverhalten von Mineralölen und Kohlenwasserstoffen, Klimisch und Reese[11] das der Polyaromaten an einem Vinylacetatgel.

Über das Elutionsverhalten von Verbindungsklassen, die als Pflanzenschutzmittelwirkstoffe eingesetzt werden, wurde bisher nur für insektizide Phosphorsäureester von Ruzicka *et al.*[14] und von den Autoren[15] dieser Arbeit an Sephadex LH-20 berichtet. In einer Reihe weiterer Publikationen[16–22] wird der Einsatz der Gelchromatographie zur Abtrennung von Pflanzenschutzmittelrückständen aus pflanzlichen und tierischen Rohextrakten beschrieben.

Infolge des ihr hauptsächlich zugrundeliegenden Trennprinzips kann die Gelchromatographie grundsätzlich auf alle Stoffe zerstörungsfrei angewendet werden. Voraussetzung ist das Vorhandensein eines Spektrums von Gelsystemen mit einer Palette weitstreuender Porendurchmesser. Die sehr lange Wiederverwendbarkeit der gelchromatographischen Trennsäulen ermöglicht eine Automatisierung ihres Betriebes, die gelegentlich schon versucht wurde[22,23]. Damit eröffnen sich besonders viele Einsatzmöglichkeiten für solche Verfahren. Deshalb erschien es uns sinnvoll, das Elutionsverhalten von insektiziden Phosphorsäureestern, insektiziden und herbiziden Carbaminsäureestern, herbiziden Phenylharnstoff- und Triazinderivaten sowie von herbiziden Phenoxyalkancarbonsäuren und -estern an verschiedenen Gelsystemen zu untersuchen.

EXPERIMENTELLES

Geräte

Rotationsverdampfer: Vapsilator KRV 65/30 (Chemophor, Zürich, Schweiz); gelchromatographische Ausrüstung, bestehend aus Pumpen: Modell CMP 3 (Chromatronix, Berkeley, Calif., U.S.A.) bzw. Modell T4P 1001 SC (Chemie und Filter, Heidelberg, B.R.D.), Probenaufgabeventil: Modell SV-8031 (Chromatronix); Säulen: Glas, 1 m × 20 mm i.D. Modell SR 25/1000 (Pharmacia, Uppsala, Schweden) bzw. Glas, 1 m × 25 mm i.D. oder 0.5 m × 15 mm i.D. (Quickfit, Wiesbaden, B.R.D.); Detektoren: Modell Fractoscan UV-Monitor (Buchler, Fort Lee, N.J., U.S.A.) bzw. Modell 1205 H (vormals Hupe und Busch, jetzt Hewlett-Packard, Frankfurt, B.R.D.); und Kompensationsschreiber: Modell Servogor RE 511 (Metrawatt, Nürnberg, B.R.D.).

Materialien

Äthanol, abs., unvergällt (Monopolverwaltung Berlin); Hexan, p.a. (Merck, Darmstadt, B.R.D.); Isopropanol, p.a. (Merck); Methanol, p.a. (Merck); Tetrahydrofuran, rein (Merck), HCl-behandelt und über Na destilliert.

Gele: Sephadex LH-20 (Pharmacia), Bio-Beads SX-4 (Bio-Rad Labs., München, B.R.D.), Merckogel OR-500 (Merck).

Die in den Tabellen aufgeführten Pflanzenschutzmittelwirkstoffe besassen den höchstmöglichen, von den Herstellern erhältlichen Reinheitsgrad.

Säulenherstellung

Die zur Füllung der Säulen verwendeten Gele wurden jeweils 24 h in dem betreffenden Elutionsmittel gequollen. Vor dem Füllen der Säulen wurden die Gelsuspensionen im Ultraschallbad gerührt, um evtl. Teilchenagglomerate aufzulösen und Luftblasen zu entfernen. Nach beendeter Füllung wurde solange Lösungsmittel mit einer Durchflussrate von 50 ml/h durch die Säule gepumpt, bis sich eine konstante Gelbetthöhe ergab. Die Fixierung des Gelbettes erfolgte durch zwei Adapter.

Ermittlung der Elutionsvolumina

Zur Ermittlung der Elutionsvolumina der einzelnen Wirkstoffe wurden jeweils 0.5 ml einer Reinlösung in Konzentrationen zwischen 1 und 10 μg/ml mit Hilfe des Probenaufgabeventils auf die Säule gegeben. Das aus der Säule ausfliessende Eluat durchströmte die Küvette des UV-Monitors. Die Messung erfolgte in der Regel bei 254 nm. In Fällen zu schwacher Absorption bei dieser Wellenlänge wurde im Absorptionsmaximum des Stoffes gemessen. Die erhaltenen Chromatogramme des UV-Detektors wurden graphisch ausgewertet. Der Schnittpunkt der Wendetangenten der Peakflanken ergibt den Wert des absoluten Elutionsvolumens. Für diejenigen Verbindungen, die im UV-Bereich nicht absorbieren, wurden die Punkte für das Gelchromatogramm dadurch gefunden, dass das Säuleneluat in 4-ml Fraktionen aufgefangen, diese anschliessend gaschromatographisch untersucht und deren Konzentrationen gegen die eluierten Milliliter aufgetragen wurden. In den Tabellen I–IV, und VI und VII finden sich die auf das Elutionsvolumen von Benzol bezogenen, relativen Elutionsvolumina der einzelnen Wirkstoffe. Die Werte konnten mit einem relativen Fehler von 1–2% Genauigkeit bestimmt werden.

ERGEBNISSE UND DISKUSSION

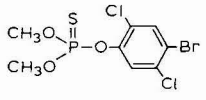
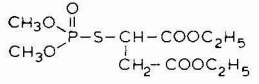
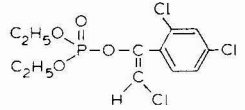
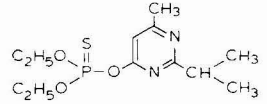
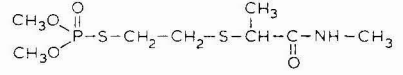
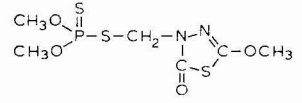
Insektizide Phosphorsäureester

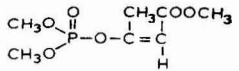
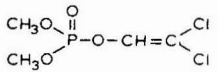
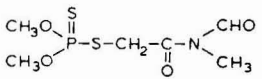
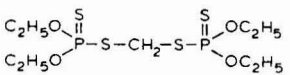
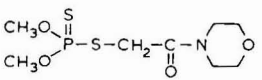
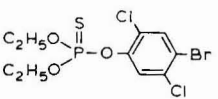
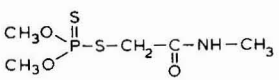
In Tabelle I sind die relativen Elutionsvolumina von 37 Phosphorsäureesterinsektiziden an drei Gelsystemen (in der Reihenfolge steigender Elutionswerte an Sephadex) aufgelistet, mit deren Hilfe sie sich voneinander eindeutig unterscheiden und identifizieren lassen. Dabei gilt das Kriterium, dass die Elutionskennwerte samt ihrer Streubereiche von jeweils $\pm$ 0.02 Einheiten sich nicht überlappen. Von 666 paarweisen Kombinationsmöglichkeiten können mit diesen drei Gelsystemen lediglich 10 nicht sicher differenziert werden. Die Tabelle I macht ferner deutlich, dass Molgewichtsabnahme und Elutionsvolumenzunahme an Sephadex LH-20/Äthanol keineswegs exakt parallel verlaufen, wie es ein streng gelchromatographisches Trennprinzip bewirken würde. Vielmehr treten andere, strukturspezifische Nahewechselwirkungskräfte in Konkurrenz zu den gelchromatographischen Vorgängen und tragen so zu der Möglichkeit bei, zwischen den Individuen einer Stoffklasse zu differenzieren. Doppelbindungen und aromatische Systeme in den Molekülen der Wirkstoffe dürften dabei erheblichen Anteil haben, wie schon Wilk *et al.*[1] hervorhoben.

Es zeigt sich, dass Verbindungen, die ein mehrkerniges Ringsystem aufweisen, wie Azinphos-äthyl, Azinphos-methyl, Coumaphos und Coumithoat, stärker zurückgehalten werden als manche kleineren Moleküle, die nur einen aromatischen Kern besitzen. Ferner lassen sich Regeln über dem Molekülsortierungsprinzip entgegen-

TABELLE I

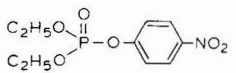
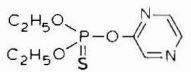
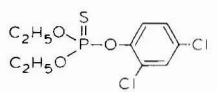
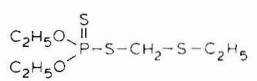
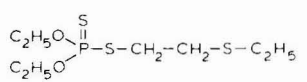
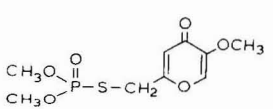
DIE RELATIVEN ELUTIONSVOLUMINA INSEKTIZIDER PHOSPHORSÄUREESTER AN VERSCHIEDENEN GELSYSTEMEN (BENZOL = 1.0)

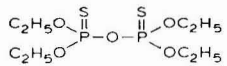
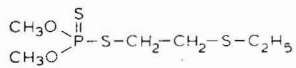
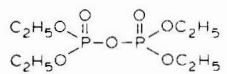
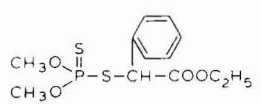
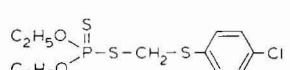
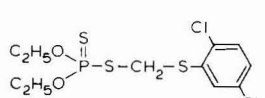
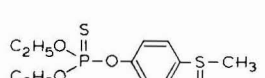
Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/Äthanol*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
Bromophos		366.00	0.88	0.88	0.82
Malaoxon		314.29	0.88	0.98	0.80
Chlorfenvinphos		359.50	0.89	0.98	0.76
Diazinon		304.35	0.89	0.81	0.78
Vamidothion		287.35	0.89	1.20	0.79
Methidathion		302.33	0.92	1.03	0.84

Mevinphos		224.15	0.92	1.29	0.85
Dichlorvos		220.98	0.94	1.13	0.86
Formothion		257.27	0.95	0.81	0.80
Ethion		384.48	0.95	0.77	0.82
Morphothion		269.33	0.96	1.17	0.79
Bromophos-äthyl		394.00	0.96	0.85	0.78
Dimethoat		229.28	0.97	1.15	0.80

(Fortsetzung S. 176)

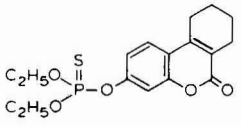
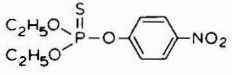
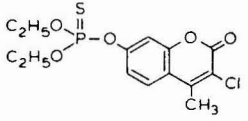
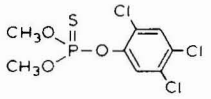
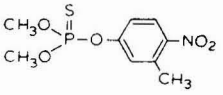
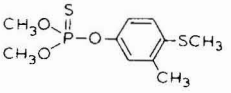
TABELLE I (*Fortsetzung*)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/Äthanol*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
Paraoxon		276.20	0.97	1.05	0.78
Zinophos		284.24	0.98	1.05	0.82
Dichlofenthion		315.17	1.00	0.97	0.82
Phorate		260.38	1.02	1.11	0.83
Disulfoton		274.40	1.02	0.94	0.80
Endothion		280.44	1.04	1.27	0.82

Sulfotepp		322.33	1.06	0.91	0.76
Thiometon		246.35	1.06	0.94	0.86
Tepp		290.26	1.06	0.94	0.96
Phenthoat		320.37	1.07	0.96	0.80
Carbophenothion		342.96	1.09	0.88	0.77
Phenkapton		377.33	1.10	0.83	0.77
Fensulfothion		308 35	1.11	0.95	0.77

(Fortsetzung S. 178)

TABELLE I (*Fortsetzung*)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/Äthanol*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
Coumithoate		364.36	1.12	0.89	0.78
Parathion		291.27	1.16	0.96	0.76
Coumaphos		362.78	1.17	0.95	0.75
Fenchlorphos		321.56	1.17	0.98	0.79
Fenitrothion		277.24	1.22	1.00	0.80
Fenthion		278.33	1.23	1.01	0.85

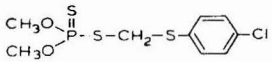
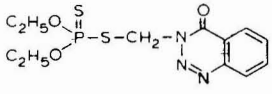
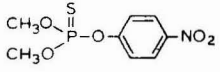
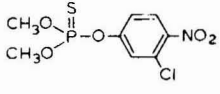
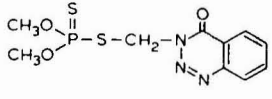
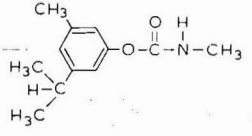
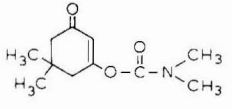
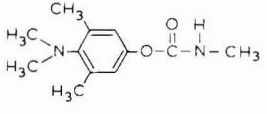
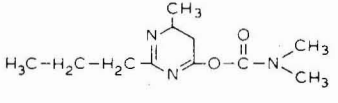
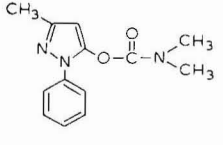
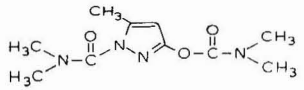
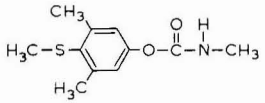
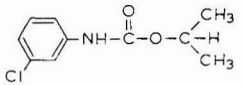
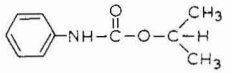
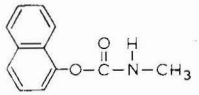
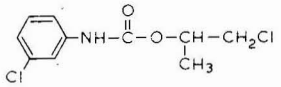
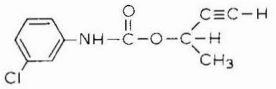
Carbophenothion-methyl		**314.91**	**1.24**	**0.98**	**0.81**
Azinphos-äthyl		**345.36**	**1.27**	**0.93**	**0.82**
Parathion-methyl		**263.21**	**1.32**	**1.05**	**0.80**
Chlorthion		**297.66**	**1.37**	**0.89**	**0.77**
Azinphos-methyl		**317.33**	**1.56**	**1.02**	**0.77**

TABELLE II

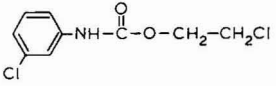
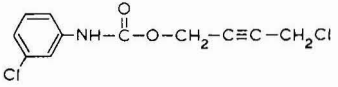
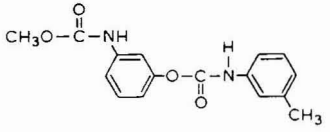
DIE RELATIVEN ELUTIONSVOLUMINA INSEKTIZIDER UND HERBIZIDER CARBAMINSÄUREESTER AN VERSCHIEDENEN GEL-SYSTEMEN (BENZOL = 1.0)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/ Isopropanol*	*Sephadex LH-20/ Tetrahydrofuran*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
Promecarb		207.26	0.70	0.92	0.79	0.78
Dimetan		211.26	0.83	0.94	0.88	0.83
Zectran		222.29	0.86	0.94	0.80	0.78
Pyramat		212 27	0.92	0.92	0.82	0.86
Pyrolan		231.26	0.96	0.94	0.88	0.84

Dimetilan		240.26	0.96	1.01	0.81	0.87
Methiocarb		212.29	0.99	0.98	0.85	0.80
Chlorpropham		213.67	1.06	0.87	0.78	0.74
Propham		179.22	1.10	0.90	0.82	0.80
Carbaryl		201.23	1.30	1.08	0.92	0.82
CPPC		249.11	1.38	0.87	0.79	0.74
Chlorbufam		233.66	1.52	0.90	0.81	0.75

(Fortsetzung S. 182)

TABELLE II (*Fortsetzung*)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/ Isopropanol*	*Sephadex LH-20/ Tetrahydrofuran*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
CEPC		235.09	1.71	0.93	0.82	0.74
Barban		258.19	1.86	0.92	0.82	0.73
Phenmedipham		300.19	1.99	0.93	0.79	0.69

wirkende Substituenteneinflüsse auf die Elutionsvolumina ableiten. Ersatz eines H-Atoms (oder einer CH_3-Gruppe) durch ein Cl-Atom erhöht das Elutionsvolumen V_{El}, wie die Paare Parathion-methyl (M.G. $\approx$ 263; $V_{El} = 1.32$) und Chlorthion (M.G. $\approx$ 298; $V_{El} = 1.37$) bzw. Carbophenothion (M.G. $\approx$ 343; $V_{El} = 1.09$) und Phenkapton (M.G. $\approx$ 377; $V_{El} = 1.10$) bzw. Dichlofenthion (M.G. $\approx$ 315; $V_{El} = 1.00$) und Fenchlorphos (M.G. $\approx$ 322; $V_{El} = 1.17$) bzw. Fenitrothion (M.G. $\approx$ 277; $V_{El} = 1.22$) und Chlorthion (M.G. $\approx$ 298; $V_{El} = 1.37$) zeigen. Innerhalb der zur Verfügung stehenden Stoffe dieser Verbindungsklasse sind die Strukturvarietäten zu erheblich, als dass man weitere Gesetzmässigkeiten mit einiger Sicherheit erkennen könnte.

Im System Merckogel OR-500/Tetrahydrofuran (THF) sind die Abweichungen vom Molekülsortierverhalten bereits zahlenmässig geringer und weitaus kleiner. Vor allem scheinen keine störenden Wechselwirkungen zwischen dem Gel und den aromatischen Phosphorsäureestern mehr einzutreten. Im System Bio-Beads SX 4/THF schliesslich weichen die Eluierungsfolgen der Wirkstoffe nur noch selten von den Erwartungen aus der reinen Gelchromatographie ab.

Insektizide und herbizide Carbaminsäureester

Für die insektizid- bzw. herbizidwirksamen Carbaminsäureester stellen schon die drei ersten Systeme der Tabelle II, Sephadex LH-20/Isopropanol, Sephadex LH-20/THF und Merckogel OR-500/THF eine leistungsfähige Identifizierungskombination dar. Alle 15 untersuchten Verbindungen können differenziert werden.

Auch hier ist, besonders beim System Sephadex LH-20/Äthanol, der Elutionsvolumen erhöhende Effekt der Cl-Substitution erkennbar (Chlorpropham, CPPC). Ansonsten überlagern sich offensichtlich diverse Einflüsse. Bei den insektiziden N,N-Dimethylcarbamaten scheinen übliche adsorptionschromatographische Vorgänge zu überwiegen: Die Elutionsfolge liegt in der Reihenfolge steigenden Molekulargewichts.

Bei den übrigen Gelsystemen werden die Effekte geringfügiger und damit weniger deutlich. Umkehrungen einzelner Elutionsfolgen treten auf, was der Differenzierung zu Identifizierungszwecken entgegenkommt. Die deutlich niedrigeren Elutionsvolumina der meisten Verbindungen im Vergleich zum Benzol (= 1.0) ist das Ergebnis verminderter Wechselwirkungsfähigkeit dieser Gele mit aromatischen Systemen.

Phenylharnstoffherbizide

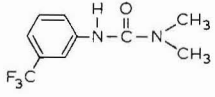
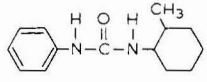
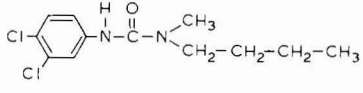
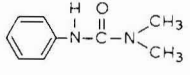
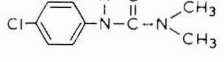
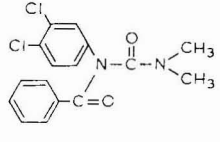
Von den 16 Wirkstoffen wird an den ausgewählten Gelsystemen der Tabelle III nur das Substanzenpaar Monolinuron–Metobromuron noch nicht befriedigend getrennt.

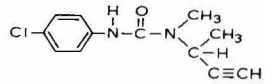
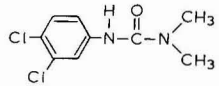
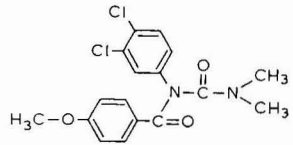
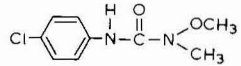
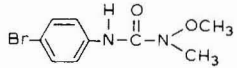
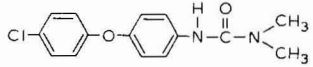
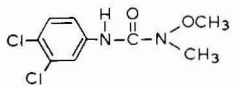
Auch hier kann der Cl-Substitutionseffekt, wenn auch nicht besonders ausgeprägt, beobachtet werden (vgl. z.B. Fenuron–Monuron–Diuron). Darüberhinaus wird durch Ersatz von Cl durch Br wiederum eine retentionserhöhende Wirkung beobachtet (vgl. Monolinuron–Metobromuron; Linuron–Chlorbromuron), worüber bei Klassen einfacherer Verbindungen bereits Brook und Munday[4] berichteten. Sehr deutlich ist ferner das retentionserhöhende Ergebnis nach dem Austausch der N-Methyl- gegen die N-Methoxy-Gruppe (vgl. Monuron $\rightarrow$ Monolinuron bzw. Diuron $\rightarrow$ Linuron).

Der Übergang vom Äthanol zu Isopropanol als Quell- und Elutionsmittel bewirkt ein Schrumpfen des Sephadexvolumens um *ca.* 20% und zugleich die Tren-

TABELLE III

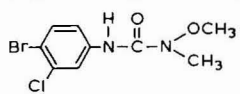
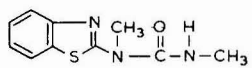
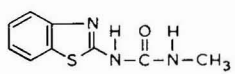
DIE RELATIVEN ELUTIONSVOLUMINA HERBIZIDER PHENYLHARNSTOFFDERIVATE AN VERSCHIEDENEN GELSYSTEMEN (BENZOL = 1.0)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/ Äthanol*	*Sephadex LH-20/ Isopropanol*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
Fluometuron		232.21	0.76	0.89	0.85	0.76
Siduron		232.33	0.85	0.97	0.83	0.77
Neburon		275.18	0.88	0.96	0.74	0.75
Fenuron		164.21	0.95	1.07	0.96	0.85
Monuron		198.66	0.96	1.07	0.92	0.80
Benzomarc		337.25	1.01	1.08	0.81	0.80

Buturon		236.70	1.02	1.18	0.85	0.94
Diuron		233.10	1.02	1.10	0.89	0.83
Metoxymarc		367.28	1.04	1.24	0.82	0.76
Monolinuron		214.65	1.05	1.16	0.88	0.98
Metobromuron		259.11	1.06	1.16	0.84	0.96
Chloroxuron		290.75	1.07	1.24	0.81	0.76
Linuron		249.10	1.11	1.23	0.81	0.80

(Fortsetzung S. 186)

TABELLE III (*Fortsetzung*)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/ Äthanol*	*Sephadex LH-20/ Isopropanol*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
Chlorbromuron		293.56	1.13	1.28	0.85	0.79
Methabenzthiazuron		221.29	1.29	1.37	0.93	0.89
Benzthiazuron		207.26	1.54	1.76	0.98	0.85

nung der meisten, mit Äthanol noch nicht aufgelösten Stoffpaare. Dies legt die Vermutung nahe, dass abnehmende Porengrösse der Gelmatrix mit einer Zunahme der Fähigkeit zu adsorptionschromatographischen Wechselwirkungen einhergeht.

Die obengenannten Einflüsse schwinden auch für die Phenylharnstoffe in den letzten beiden Gelsystemen merklich, und es gelten auch hier die gleichen Aussagen wie bei den Carbaminsäureestern.

Herbizide 1,3,5-Triazinderivate

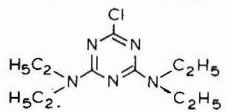
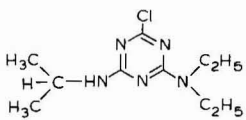
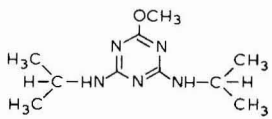
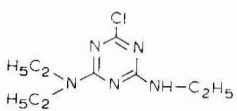
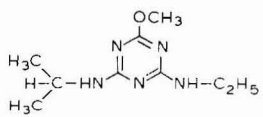
Wie die Tabelle IV zeigt, gelingt die Auftrennung der —meist herbizidwirksamen— 17 Triazinderivate an vier Gelsystemen deutlich unvollkommener (7 Paar-Kombinationen werden nicht ausreichend differenziert). Das liegt in der teilweise sehr engen Strukturverwandtschaft der Individuen dieser Gruppe begründet. Da uns hier einige Verbindungen mit nur sehr geringen Strukturabwandlungen zur Verfügung standen (vgl. Übersicht in Tabelle V), die ihrerseits teilweise wieder in Untergruppen zusammengefasst werden konnten, wurden einige Verhaltensvergleichsstudien unternommen. Das Ergebnis lässt sich folgendermassen zusammenfassen: Das rein gelchromatographische Verhalten dieser Triazinderivate wird stark überlagert vom Einfluss anderer Wechselwirkungskräfte. Der Substituentenaustausch am ($C_{(2)}$-Atom des heterozyklischen Ringes (–OCH_3, –Cl, –SCH_3) bei sonst gleichbleibendem Molekülbau bewirkt keine Elutionsverschiebung, wie sie nach rein gelchromatographischen Verhalten zu erwarten wäre, vielmehr ist sie zuweilen gleichsinnig mit dem Molgewichtsanstieg und vor allem sind die Unterschiede in den Elutionsvolumina bei –Cl ↔ –OCH_3 viel grösser, dagegen beim Vergleich –Cl mit –SCH_3 sehr gering oder gleich Null. Ferner wird das Elutionsverhalten sehr empfindlich von den NH-Gruppen in 4- und 6-Stellung beeinflusst. So lassen sich die Chlor- bzw. die Methoxy- bzw. die Methylmercaptotriazine, die noch 2 NH-Gruppen besitzen und dieselben ihrseits nicht zu unterschiedlich substituiert sind, als eine quasi homologe Reihe auffassen. Regressionsrechnungen ergaben, dass die relativen Elutionsvolumina V_{El} der Methylmercapto-Triazine Simetryn, Desmetryn, Ametryn, Prometryn und Terbutryn eine lineare Funktion des dekadischen Logarithmus ihrer Molekulargewichte sind. Und zwar gilt im System Sephadex LH-20/Äthanol $V_{El} = -4.280 \cdot \log$ M.G. $+ 11.169$ (Bestimmtheitsmass 94.6%), im System Sephadex LH-20/THF $V_{El} = -3.245 \cdot \log$ M.G. $+ 8.579$ (Bestimmtheitsmass 96.6%), im System Merckogel OR-500/THF $V_{El} = -1.205 \cdot \log$ M.G. $+ 3.616$ (Bestimmtheitsmass 96.7%). Im System Bio-Beads SX 4/THF liess sich die lineare Abhängigkeit statistisch nicht sicher stellen, desgleichen nicht für die Chlor-Triazinderivate Simazin, Norazin, Atrazin, Propazin. Die Anzahl der für die Untersuchung zur Verfügung stehenden Verbindungen reicht offenbar nicht aus, um gesicherte Schlussfolgerungen über die Zusammenhänge ziehen zu können. Ähnlich muss über die Methoxy-Triazinderivate geurteilt werden, obwohl die drei untersuchten Glieder dieser Reihe in allen Systemen recht gut einer Geraden $V_{El} = a \log$ M.G. $+ b$ zugeordnet werden können.

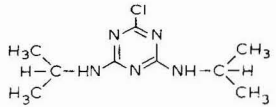
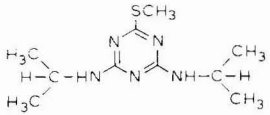
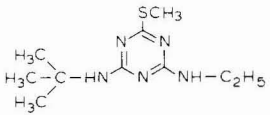
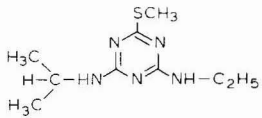
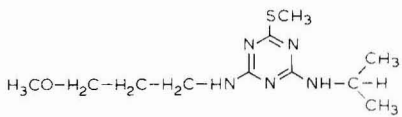
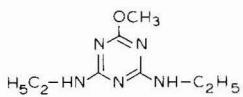
Herbizide Phenoxyalkancarbonsäuren

Alle 9 Säuren dieser in der Praxis besonders wichtigen Herbizidwirkstoffklasse lassen sich an den Systemen Sephadex LH-20/Äthanol und Merckogel OR-500/THF differenzieren (vgl. Tabelle VI). Auch bei den Phenoxyalkancarbonsäuren macht sich der Chlorsubstitutionseffekt im System LH-20/Äthanol deutlich, im System Bio-

TABELLE IV

DIE RELATIVEN ELUTIONSVOLUMINA HERBIZIDER 1,3,5-TRIAZINDERIVATE AN VERSCHIEDENEN GELSYSTEMEN (BENZOL = 1.0)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/ Äthanol*	*Sephadex LH-20/ Tetrahydrofuran*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
Chlorazin		257.77	0.82	0.74	0.73	0.81
Ipazin		243.74	0.87	0.81	0.74	0.77
Prometon		225.92	0.88	0.98	0.77	0.75
Trietazin		229.72	0.91	0.84	0.78	0.79
Atraton		211.27	0.91	1.08	0.82	0.78

Propazin		230.09	0.95	0.83	0.75	0.74
Prometryn		241.37	0.96	0.82	0.74	0 75
Terbutryn		241.36	1.01	0.88	0.75	0.77
Ametryn		227.33	1.02	0.92	0.77	0.75
Methoprotryn		271.39	1.03	0.85	0.74	0.75
Simeton		198.15	1.07	1.26	0.86	0.80

(Fortsetzung S. 190)

TABELLE IV (*Fortsetzung*)

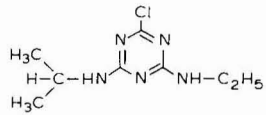
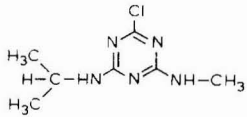
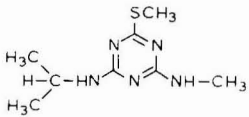
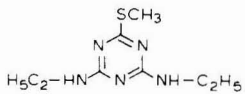
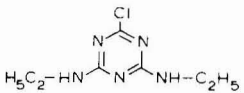
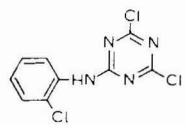
Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/ Äthanol*	*Sephadex LH-20/ Tetrahydrofuran*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
Atrazin		215.69	1.08	0.89	0.80	0.75
Norazin		201.66	1.18	0.99	0.82	0.72
Desmetryn		213.31	1.21	1.04	0.82	0.78
Simetryn		213.31	1.22	1.01	0.80	0.74
Simazin		201.66	1.22	1.01	0.83	0.76
Anilazin		275.54	1.26	0.85	0.81	0.72

TABELLE V

SYSTEMATIK DER UNTERSUCHTEN 1,3,5-TRIAZINDERIVATE

R-C(4)	*C(6)-R'*	*-Cl*	*-OCH3*	*-SCH3*
H_5C_2–NH	NH–C_2H_5	Simazin	Simeton	Simetryn
$(H_3C)_2$CH–NH	NH–CH_3	Norazin		Desmetryn
$(H_3C)_2$CH–NH	NH–C_2H_5	Atrazin	Atraton	Ametryn
$(H_3C)_2$CH–NH	NH–CH(CH_3)$_2$	Propazin	Prometon	Prometryn
$(H_3C)_3$C–NH	NH–C_2H_5			Terbutryn
$H_3CO{\cdot}H_6C_3{\cdot}$NH	NH–CH(CH_3)$_2$			Methoprotryn
$(H_5C_2)_2$N	NH–C_2H_5	Trietazin		
$(H_5C_2)_2$N	NH–CH(CH_3)$_2$	Ipazin		
$(H_5C_2)_2$N	N(C_2H_5)$_2$	Chlorazin		
Cl	NH–C$_6$H$_4$–Cl	Anilazin		

Beads SX-4/THF geringfügig bemerkbar. Dagegen scheint sich beim System Merckogel OR-500/THF die Gesamtpolarität der Moleküle mehr auf das Elutionsverhalten auszuwirken.

Herbizide Phenoxyalkancarbonsäureester

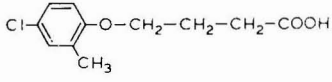
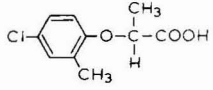
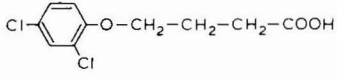
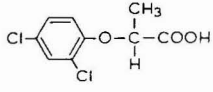
Bei den 11 untersuchten Estern der Tabelle VII können mit Hilfe der verwendeten drei Gelsysteme von den denkbaren Kombinationen 2 Paare nicht voneinander unterschieden werden.

Äus Gründen des höheren Molgewichts, aber besonders wegen der verminderten Polarität, eluieren die Ester in allen Systemen deutlich früher als die zugehörigen Säuren. Jedoch nehmen strukturspezifische Kräfte Einfluss auf das gelchromatographische Verhalten der Ester. Innerhalb der Mecoprop-Ester-Reihe ist das Molekülsortierprinzip nahezu gewahrt.

Zusammenfassend kann abgeleitet werden, dass bei der Gelschromatographie

TABELLE VI

DIE RELATIVEN ELUTIONSVOLUMINA HERBIZIDER PHENOXYALKANCARBONSÄUREN AN VERSCHIEDENEN GELSYSTEMEN (BENZOL = 1.0)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/Äthanol*	*Merckogel OR-500/Tetrahydrofuran*	*Bio-Beads SX-4/Tetrahydrofuran*
γ-MCPB		228.67	1.00	0.76	0.76
Mecoprop		214.65	1.01	0.89	0.76
γ-(2,4-D)-B		249.09	1.07	0.76	0.76
Dichlorprop		235.07	1.14	1.33	0.76

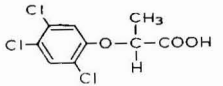
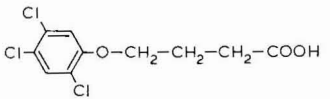
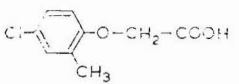
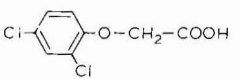
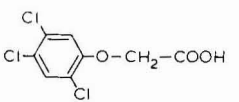
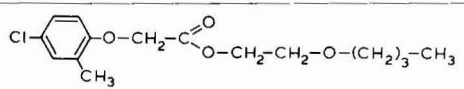
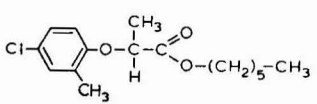
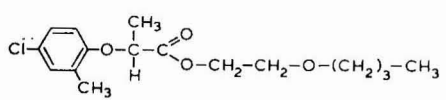
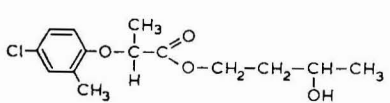
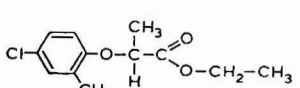
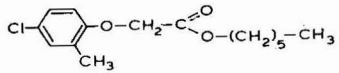
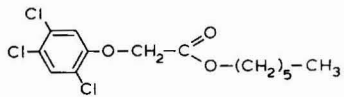
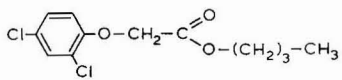
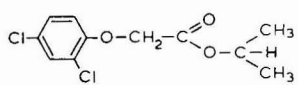
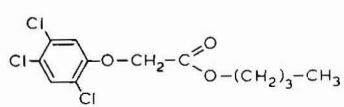
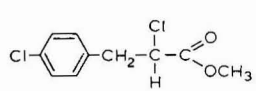
Fenoprop		269.51	1.17	1.52	0.77
γ-(2,4,5-T)-B		283.54	1.18	0.75	0.75
MCPA		200.62	1.21	1.22	0.78
2,4-D		221.04	1.34	1.07	0.85
2,4,5-T		255.49	1.48	0.73	0.78

TABELLE VII

DIE RELATIVEN ELUTIONSVOLUMINA HERBIZIDER PHENOXYALKANCARBONSÄUREESTER AN VERSCHIEDENEN GELSYSTEMEN (BENZOL = 1.0)

Wirkstoff	*Formel*	*M.G.*	*Sephadex LH-20/ Äthanol*	*Merckogel OR-500/ Tetrahydrofuran*	*Bio-Beads SX-4/ Tetrahydrofuran*
MCPA-butoxyäthylester		300.78	0.77	0.67	0.72
Mecoprop-hexylester		298.82	0.84	0.65	0.72
Mecoprop-butoxyäthylester		314.81	0.87	0.67	0.71
Mecoprop-hydroxybutylester		286.76	0.89	0.70	0.70
Mecoprop-äthylester		242.71	0.94	0.74	0.71

MCPA-hexylester		284.79	0.99	0.68	0.71
2,4,5-T-hexylester		339.66	1.05	0.66	0.70
2,4-D-butylester		277.16	1.08	0.74	0.74
2,4-D-isopropylester		263.12	1.08	0.75	0.84
2,4,5-T-butylester		311.61	1.11	0.70	0.73
Chlorfenprop-methylester		233.10	1.14	0.84	0.81

der behandelten biozidwirksamen Stoffe (bei gleichbleibendem Elutionsmittel) die chemische Struktur des Gels eine nicht unwesentliche Rolle spielt; es gerät in Nahewechselwirkungen mit den Strukturen der chromatographierenden Komponenten. Daraus können Gemeinsamkeiten für sehr nahe verwandte Substanzen abgeleitet werden. Als Nutzen für die praktische Anwendung ergibt sich aber besonders die Möglichkeit zur Differenzierung und Identifizierung verwandter Substanzen im Bereich kleinerer Moleküle. Dies zu zeigen ist der Zweck der vorgelegten Arbeit. Die Verfasser bedienen sich dieser Methodik bei der Isolierung und Identifizierung von Spurenrückständen in komplexen Vielfachgemischen.

DANK

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ZUSAMMENFASSUNG

Das Elutionsverhalten von insektizid- bzw. herbizidwirksamen Phosphorsäureestern, Carbamaten, Phenylharnstoffen, Triazinen, Chlorphenoxysäuren und -estern wurde an Sephadex LH-20, Bio-Beads SX-4, SX-8 und Merckogel OR-500 mit verschiedenen Elutionsmitteln untersucht. Die Einflüsse der Substituenten, des Bindungssystems der Polarität und des Eluens auf das Elutionsverhalten der einzelnen Verbindungen wurden diskutiert. Dabei zeigte sich, dass bei den verwendeten Elutionsmitteln die oben genannten Einflüsse sich stark bei den polaren Gelen Sephadex LH-20 und Merckogel OR-500 bemerkbar machen, während sie bei den schwach polaren Bio-Beads-Gelen weniger merkbar sind. Ferner werden die Möglichkeiten der Trennung und Identifizierung der einzelnen Verbindungen innerhalb der verschiedenen Wirkstoffklassen anhand der unterschiedlichen Elutionsvolumina diskutiert.

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CHROM. 10,545

ANALYSIS OF RIFAMPICIN AND OF ITS HYDROGENATED DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and complete separation of rifampicin from its quinone was accomplished in 7 min using a filled column of the MicroPak NH_2 type and a chemically bonded phase on silica gel (10 μm). The eluent was chloroform–methanol (97:3) under isocratic conditions. The same columns and conditions were used for a complete separation of all of the reaction products of the hydrogenation of rifampicin, including the dihydro and tetrahydro derivatives. The plate heights were optimally 0.5–1.9 mm. The method is particularly useful for the rapid control of the reaction products of hydrogenation.

INTRODUCTION

Rifamycins were isolated from cultures of *Streptomyces mediterranei* n.sp. as metabolic products and many of the derivatives were shown to possess important therapeutical activities, such as the commonly used rifamycin SV. Similarly, rifampicin or 3-(4-methylpiperazinyl)iminomethylrifamycin SV has been applied successfully in the treatment of various diseases. Some of its physico-chemical properties were described in 1966 by Maggi *et al.*[1], who compared some of the analytical methods available and examined the stability of rifampicin in different media. However, the analysis of rifampicin by liquid chromatography has not been described so far, although Schmit *et al.*[2] analyzed rifamycin SV by high-performance liquid chromatography on a column with ODS Permaphase, employing gradient elution. The mobile phase used was water–methanol. Schmit *et al.*[3] also mentioned the separation of 3-formylrifampicin SV from some contaminants on a Zipax–polyamide column with *n*-hexane–ethanol as eluent.

When following the course of hydrogenation of rifampicin[4], we used high-performance liquid chromatography and found that the product is not a unique dihydro derivative, as one might have expected, but that the reaction course is much more complex.

EXPERIMENTAL

Apparatus

A Varian Model 4100 high-pressure liquid chromatograph (Varian Aerograph, Palo Alto, Calif., U.S.A.) was used. The detector was a Variscan UV spectrophotometer (Varian) with a variable wavelength (190–900 nm). The 10×1 mm cell had a volume of 8 μl. Samples were injected with a Hamilton Type 701 SN (10 μl) micro-syringe using the stop-flow technique. UV spectra were recorded with the Variscan spectrophotometer and with a Specord UV–Vis spectrophotometer (Zeiss, Jena, G.D.R.).

Columns

Stainless-steel columns (25 cm $\times$ 2 mm I.D.) were filled with MicroPak Si-10 and Micro-Pak CN with chemically bonded alkylnitrile groups on silica gel, and with MicroPak NH_2 with chemically bonded alkylamine groups on silica gel. The silica gel was always 10-μm LiChrosorb.

Chemicals

The samples of rifampicin were obtained by purification of its drug-form Rifadin, produced by UMB Drugs (Bucharest, Rumania) under licence from Lepetit (Milan, Italy). The purification was described in detail by Hanuš *et al.*[5]. The hydrogenated derivatives were prepared in this laboratory[5]. The quinone form of rifampicin was obtained by preparative isolation on the silica gel column and its identity was checked by NMR spectroscopy.

The solvents used were methanol for UV spectroscopy (Lachema, Brno, Czechoslovakia) and analytically pure chloroform (Lachema), which were further purified on an activated silica gel column in the usual way.

Chromatographic analysis

Samples of rifampicin or its derivatives were dissolved in chloroform to a concentration of 200–400 μg/ml. The column was injected with 2–10-μl samples.

The mobile phase used was chloroform–methanol in various proportions and it was de-gassed before use. The elution flow-rates were 0.2–0.7 ml/min at pressures of 1.36–3.40 MPa.

Rifampicin and its derivatives were detected in the Variscan spectrophotometer at 334 nm.

Capacity factors (k') were calculated from $k' = (t_R - t_0)t_0$ where t_R is the retention time of the sample and t_0 the retention time of a non-retained compound (*n*-hexane in this work). The theoretical plate height (H) was calculated from the elution chromatogram according to $H = (L/16)\,(w_t/t_R)^2$ where L is the column length and w_t the peak width at the baseline.

RESULTS AND DISCUSSION

Analysis of rifampicin

Before analysis of the samples we removed the various additives, such as starch and magnesium stearate, which adversely affect the analytical results, by

extraction with diethyl ether and passage through a preparative column containing activated silica gel.

An adsorption column containing MicroPak Si-10 and columns filled with silica gel with a chemically bonded phase of the type MicroPak NH_2 and MicroPak CN were used for the analysis, MicroPak NH_2 being the most suitable. When using chloroform–methanol (97:3) as the mobile phase under isocratic conditions, the substance proper was preceded by a contaminant which was identified as a quinone form of rifampicin. The separation of rifampicin from its quinone is illustrated in Fig. 1.

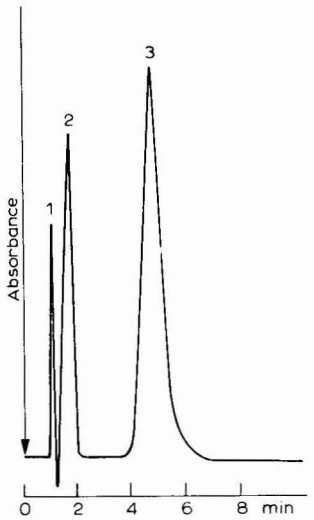

Fig. 1. Chromatogram of a mixture of rifampicin and its quinone form. MicroPak NH_2 (10 μm) 25 cm × 0.2 cm column, elution with chloroform–methanol (97:3), flow-rate 0.7 ml/min. Peaks: 1, *n*-hexane; 2, rifampicin quinone; 3, rifampicin.

In a silica gel adsorption column, rifampicin was separated from the quinone only on using a strongly polar eluent with a high methanol content. The chromatograms obtained were poorly reproducible and the efficiency of the column decreased rapidly. The MicroPak CN column possessed more suitable properties but the separation of the two compounds was not complete.

The contaminant was determined using a standard of rifampicin quinone and UV spectra. These were recorded in the Variscan spectrophotometer in an aqueous medium (pH 7.38) and the results were compared with published values. The spectra of the two substances are shown in Fig. 2.

Table I shows the retention times, capacity factors and the values of measured and reported absorption peaks.

The quinone form of rifampicin found as an impurity accompanying the starting preparation is formed readily by oxidation with atmospheric oxygen and is detected by a purple colour of the solution. The amount of quinone in rifampicin is hence dependent on the conditions and length of storage. In addition to these factors,

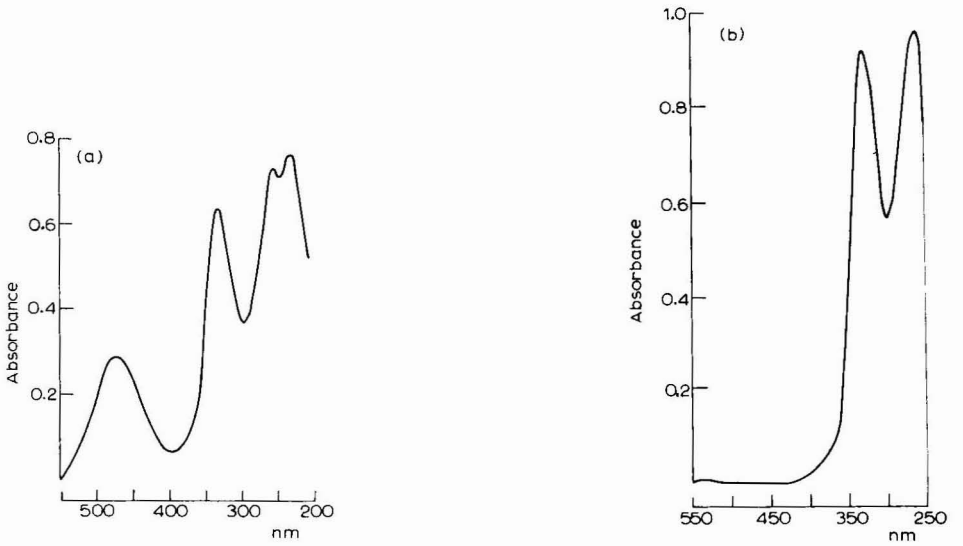

Fig. 2. UV spectrum of rifampicin (a) and its quinone (b) measured in a phosphate buffer of pH 7.38 in a Variscan spectrophotometer.

TABLE I

RETENTION TIMES (t_R), CAPACITY FACTORS (k') AND WAVELENGTHS OF MAXIMUM ABSORPTION ($\lambda_{max.}$) OF RIFAMPICIN AND ITS QUINONE

Compound	t_R *(min)* *	k' **	*Wavelength of maximum absorption (nm)* **	
			Measured values	*Published values*[1]
Rifampicin	4.7	3.7	235, 255, 333, 474	237, 255, 334, 475
Quinone	1.6	0.6	259, 332, 539	260, 332, 540

* Analyzed in a 25 × 0.2 cm column, MicroPak NH_2; elution with chloroform–methanol (97:3) at a flow-rate of 0.7 ml/min.

** Measured in a medium of pH 7.38.

the presence of the quinone form is affected by the preliminary purification procedures, whereupon its content decreases. The formulae of rifampicin and its quinone are shown in Fig. 3.

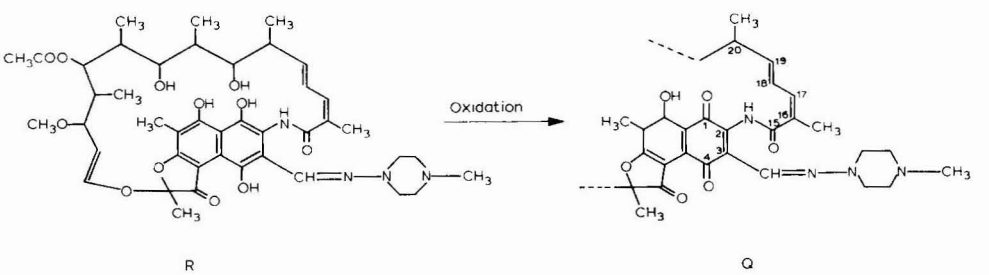

Fig. 3. Structural formulae of rifampicin (R) and its quinone (Q).

Analysis of hydrogenated derivatives of rifampicin

Rifampicin was hydrogenated at low and medium pressure on palladium(II) oxide. In following the course of hydrogenation and checking the purity of the reaction products, the best results were obtained with the MicroPak NH_2 column and chloroform–methanol (97:3) as the mobile phase. Depending on the reaction conditions, the products were hydrogenated at one or two double bonds.

Liquid chromatography showed that in all instances a mixture of four hydrogenated derivatives was formed, eluted from the column as peaks C, D, E en F. The proportions of these compounds differed, depending on the conditions and period of hydrogenation. The overall course of hydrogenation is illustrated in Fig. 4.

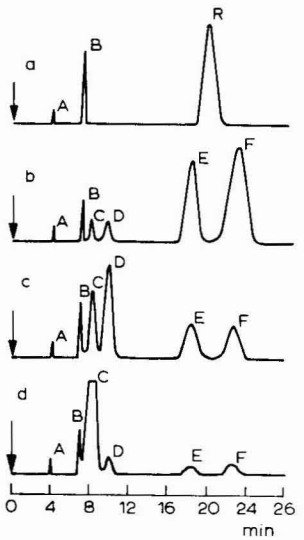

Fig. 4. Course of hydrogenation of rifampicin shown by liquid chromatography. (a) Rifampicin (R) and its quinone form (B). (b) Reaction products after first-degree hydrogenation. A, Solvent; B, quinone form; C, D, E and F, hydrogenated derivatives. (c) Reaction products after second-degree hydrogenation. Peaks as in (b). (d) Reaction products after total hydrogenation. Peaks as in (b).

After hydrogenation to the first degree the reaction products were mainly peaks E and F, fully separated, plus the minor peaks C and D and peaks of solvent A and the quinone form B (Fig. 4b). After hydrogenation to the second degree a product was obtained with major peaks C and D, minor peaks E and F and again peaks of the solvent A and the quinone B (Fig. 4c). After a prolonged total hydrogenation the final product was still a mixture of the substances with predominating peak C (Fig. 4d).

The individual eluates C, D, E and F were isolated and collected by the stop-flow technique and their individual UV spectra were recorded. Comparison of the spectra showed full agreement in the positions of all absorption peaks with the four derivatives. The spectra coincided with the spectrum of pure rifampicin (Fig. 5).

The analogies in the spectral analysis indicate that we are dealing with derivatives in which none of the principal conjugations were disturbed, *i.e.*, the naphthalene

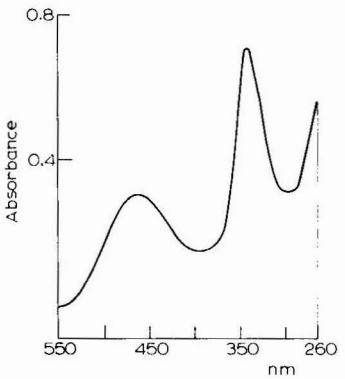

Fig. 5. UV spectrum of the four elution peaks C, D, E and F formed on hydrogenation of rifampicin, measured in ethanol in a Specord UV–VIS spectrophotometer. a, Starting rifampicin; b, hydrogenated derivatives C, D, E and F; c, quinone form of the hydrogenated derivatives.

skeleton was preserved, together with the conjugated C=N bond (see structural formulae in Fig. 3).

The degree of separation of the hydrogenated derivatives of rifampicin was found to depend on the proportion of methanol in the chloroform–methanol mobile phase. With less than 2% (v/v) of methanol, the compounds were not completely eluted from the column and the peaks were broad and tailed. At concentrations higher than 4.5% (v/v), peaks E and F were not separated, while at concentrations above 6% (v/v), even peaks C and D could not be resolved. The optimum concentration of methanol was 2–3% (v/v). Fig. 6 shows that the separation of eluates E and F is much more affected by the methanol concentration than is the separation of derivatives C and D.

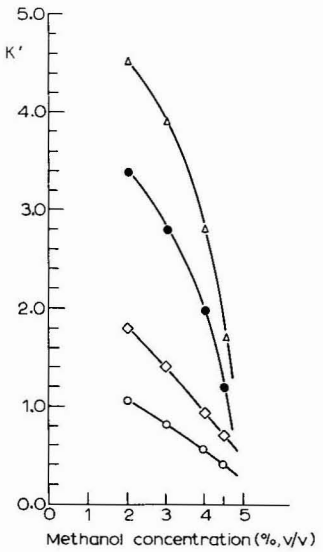

Fig. 6. Effect of methanol concentration in the mobile phase on the separation of hydrogenated derivatives of rifampicin.

TABLE II

DEPENDENCE OF RETENTION TIMES (t_R) AND CAPACITY FACTORS (k') ON CONCENTRATION OF METHANOL IN MOBILE PHASE

Analysis in a 25 × 0.2 cm column; MicroPak NH_2 (10 μm); elution with chloroform–methanol, flow-rate of 0.7 ml/min.

Compound	*Methanol concentration (%, v/v)*							
	2		*3*		*4*		*4.5*	
	t_R *(min)*	k'	t_R *(min)*	k'	t_R *(min)*	k'	t_R *(min)*	k'
B	—	—	1.4	0.4	—	—	1.1	0.1
C	2.05	1.05	1.8	0.8	1.55	0.55	1.4	0.4
D	2.8	1.8	2.4	1.4	1.9	0.9	1.7	0.7
E	4.4	3.4	3.8	2.8	3.0	2.0	2.3	1.2
F	5.5	4.5	4.9	3.9	3.8	2.8	2.8	1.7

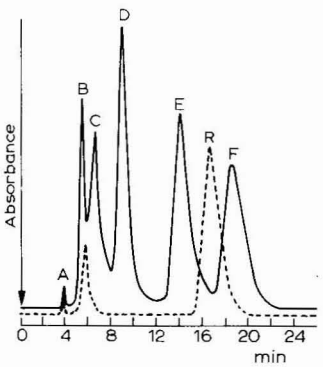

Fig. 7. Chromatograms of the hydrogenation products of rifampicin (solid line) and of rifampicin and its quinone (broken line). Peaks as in Fig. 4(b).

The retention times and the capacity factors of all of the hydrogenated products are summarized in Table II.

The starting preparation of rifampicin could not be separated in any of the systems tested from the hydrogenated forms E and F. The chromatogram in Fig. 7 shows the position of the two peaks E and F and that of rifampicin, lying between them.

The optimal efficiency of the MicroPak NH_2 column expressed as the theoretical plate height was 0.5–1.9 mm. After about 20 days, the efficiency of the column decreased.

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CHROM. 10,552

SEPARATION OF TWO TYPES OF DIPHENYLINDENONE DERIVATIVES OF AMINO ACIDS (ITH- AND DIS-AMINO ACIDS) APPLICABLE TO SEQUENCING OF PROTEINS ON POLYAMIDE SHEETS

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SUMMARY

Several chromatographic systems are proposed that provide the possibility of determining all amino acids commonly found in proteins as diphenylindenone thiohydantoins and diphenylindenone sulphonamides at the picomole level using two-dimensional thin-layer chromatography on polyamide sheets.

INTRODUCTION

2-*p*-Isothiocyanophenyl-3-phenylindenone or diphenylindenonyl isothiocyanate (DIITC) was proposed as a coloured reagent for sequencing proteins and peptides[1]. With the corresponding sulphochloride 2-*p*-chlorsulphophenyl-3-phenylindenone[2] or diphenylindenonesulphonyl chloride (DIS-Cl), a highly sensitive fluorescent method for the detection of N-terminal groups of peptides and proteins was developed[3].

Using DIITC, the N-terminal amino acid was identified on a thin layer of silica gel G as a coloured (diphenyl)indenonylthiohydantoin (ITH) derivative[4] or as a fluorescent isobenzofuran derivative[5]. With DIS-Cl, the N-terminal amino acid was identified on a thin layer of silica gel G as a coloured diphenylindenonesulphonyl (disyl or DIS) amino acid or as a fluorescent isobenzofuran derivative[3] using solvent systems described in an earlier paper[6].

These two types of diphenylindenone derivatives, ITH-amino acids and DIS-amino acids, can be used in a double checking technique in the sequence analysis of proteins. The purpose of this work was to find suitable solvent systems for the separation on polyamide sheets of ITH and DIS derivatives of the amino acids found in native proteins.

EXPERIMENTAL

Polyamide sheets

Cheng-Chin polyamide sheets (15 × 15 cm) from BDH Chemicals, Poole, Great Britain, were cut into 5 × 5 cm squares.

Preparation of ITH derivatives

ITH-amino acids were synthesized, purified and characterized as described earlier[7,8]. These compounds were obtained in micromole amounts in the following way[1]. To a solution of 3 μmole of amino acid in 0.3 ml of 0.4 *M* dimethylallylamine buffer (pH 9.6) was added 0.3 ml of a pyridine solution of 3.39 mg (10 μmole) of DIITC. The reaction was carried out at 40° under a nitrogen atmosphere. After 2 h, the solution was evaporated to dryness *in vacuo* over phosphorus pentoxide and potassium hydroxide. The residue was dissolved in 0.2 ml of a mixture of acetic acid and 6 *M* hydrochloric acid (5:1) and the solution was warmed for 10 min at 80° under nitrogen. The solvents were evaporated over potassium hydroxide. On thin-layer chromatograms the spot of ITH derivative was easily distinguished from the other spots, due mainly to the unreacted reagent and bisdiphenylindenonylthiourea, by its colour and R_F value.

Preparation of DIS derivatives

The preparation, purification and IR characterization of the DIS-amino acids were described earlier[9,10]. For chromatographic purposes, as described earlier[6], DIS-amino acids can be prepared by adding DIS-Cl dissolved in acetone (1 mg/ml) to an equal volume of a 0.1 *M* sodium hydrogen carbonate solution of 5 nmole of amino acid or of an amino acid mixture (the concentration of each amino acid being 5 nmole/ml). After leaving the solution for 3 h in a closed tube at room temperature, it was evaporated to dryness at low pressure. The residue was dissolved in methanol and aliquot volumes were applied on the chromatogram.

The solvents *n*-pentane, benzene, toluene, *n*-butanol, acetic acid, propionic acid and ethylene chlorhydrin were re-distilled.

Chromatography

ITH-amino acids in acetone or methanol solutions and DIS-amino acids in methanol solutions were applied on polyamide sheets (5 × 5 cm) using a micro-capillary tube. The samples were spotted at a distance of 0.5 cm from the edge of the sheet. For optimal separation, the diameter of the spots should not exceed 2 mm. The chromatograms were developed in a 250-ml closed glass chamber, containing 10 ml of solvent system and filter-paper for saturation of the atmosphere. A pre-equilibration of about 10–15 min was used before immersion of the chromatogram. When the solvent front reached the edge of the sheet the latter was removed and dried in a stream of hot air. For the development of a two-dimensional chromatogram the sheet was chromatographed in two solvent systems in perpendicular directions with intermediate drying. The same solvent system was used for about five chromatograms, then replaced with fresh solvent.

ITH-amino acids and DIS-amino acids are coloured compounds and, when spotted in amounts greater than 0.1 nmole, are detected on polyamide sheets as yellow or yellow-orange spots. To detect smaller amounts, the polyamide chromatogram was observed under UV light (365 nm). Diphenylindenone derivatives were detected as dark violet spots on light blue background as a result of the increased absorption of UV light due to the aromatic system (diphenylindenone residue). On treatment of the polyamide sheet with sodium ethoxide solution (5 g of sodium per 100 ml of 96% ethanol) diphenylindenone derivatives were detected as yellow-green fluorescent spots under UV light[3,5].

For repeated use of the polyamide sheets, the layers were washed with the solvents suggested by Wang and Wu[11]: acetone–85% formic acid (9:1) and acetone–29% ammonia (9:1). The chromatogram must be washed immediately after chromatography and location of the spots in order to avoid irreversible adsorption on the polyamide. If the sheet has been treated with sodium ethoxide for UV detection, preliminary washing with water is necessary before dipping it in the above rinse solution. Under these conditions of washing, the sodium ethoxide detection does not decrease the possibility of repeated use of the polyamide sheet.

RESULTS AND DISCUSSION

Separation of ITH-amino acids

Using published data for the separation of phenylthiohydantoin amino acids[12,13] and on the basis of our own investigations, the following solvent systems can be proposed as suitable for the separation of ITH-amino acids: I, toluene–*n*-pentane–glacial acetic acid (60:30:15); II, 60% aqueous acetic acid. The running time in solvent I is 15 min and in solvent II 60 min.

The two solvent systems can be used separately for one-dimensional and in combination for two-dimensional chromatography of ITH-amino acids. A two-dimensional chromatogram for the separation of ITH derivatives of 22 amino acids, DIITC and its by-products in sequencing [mono(diphenyl)indenonylthiourea (MITU) and bis(diphenyl)indenonylthiourea (BITU)] is presented in Fig. 1. On the starting point, 0.5 cm from both edges of the sheet, a mixture containing 0.5 nmole of each shown compound, dissolved in acetone-methanol (1:1), was applied. After consec-

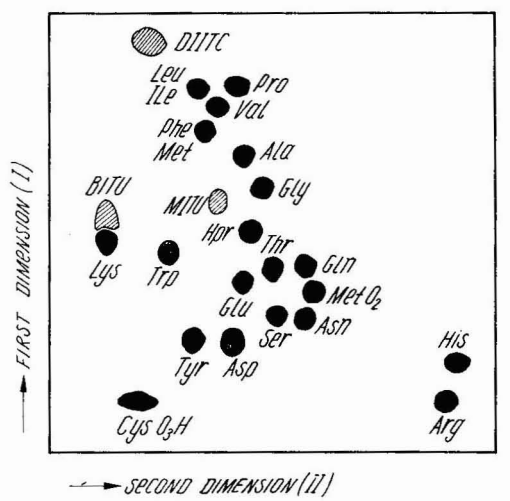

Fig. 1. Two-dimensional separation of 22 (diphenyl)indenonylthiohydantoin (ITH) amino acids, 2-*p*-isothiocyanophenyl-3-phenylindenone (DIITC), mono(diphenyl)indenonylthiourea (MITU) and bis(diphenyl)indenonylthiourea (BITU) on Cheng-Chin polyamide sheet. Solvents: first dimension, solvent I [toluene–*n*-pentane–glacial acetic acid (60:30:15)], 4.5 cm, 15 min; second dimension, solvent II (60% aqueous acetic acid), 4.5 cm, 60 min. ITH derivatives are indicated by abbreviations for the corresponding amino acids; Cys O_3H = cysteic acid; Met O_2 = methionine sulphone.

utive development in two perpendicular directions in systems I and II, 23 coloured spots were detected on the chromatogram. Only the ITH-derivatives of leucine and isoleucine and those of phenylalanine and methionine remained unseparated. ITH-methionine and ITH-phenylalanine can readily be separated in solvent III: *n*-butanol–glacial acetic acid (9:1) (R_F values 0.63 ± 0.01 and 0.71 ± 0.01, respectively). Also in solvent III ITH-leucine and ITH-isoleucine have similar R_F values of 0.52 ± 0.01 and 0.55 ± 0.01, respectively, but their relative positions are very reproducible and their differentiation is reliable.

The spots of ITH-lysine and BITU are close together, but they differ in colour and shape. On the other hand, BITU is usually extracted completely in sequencing. The difference between the colour of the ITH-amino acids (yellow and yellow-orange) and the colour of the reagent and its thiourea products (pink-red) facilitate the identification of the N-terminal amino acid as a coloured spot.

R_F values of the ITH-amino acids, DIITC and its by-products in the proposed solvent systems are presented in Table I (average values from 21 determinations).

The sensitivity of the colour and UV detection of the ITH-amino acids is shown in Fig. 2. On starting points 1–6 of a one-dimensional chromatogram were

TABLE I

R_F VALUES OF ITH-AMINO ACIDS, 2-*p*-ISOTHIOCYANOPHENYL-3-PHENYLINDENONE (DIITC) AND MONO- AND BIS(DIPHENYL)INDENONYLTHIOUREAS (MITU AND BITU) ON POLYAMIDE SHEETS

Distance: 4.5 cm.

ITH-amino acid	*Solvent system*		
	I	*II*	*III*
Alanine	0.61 ± 0.02	0.37 ± 0.02	–
Arginine	0.03 ± 0.01	0.88 ± 0.02	
Asparagine	0.18 ± 0.02	0.48 ± 0.02	
Aspartic acid	0.12 ± 0.01	0.32 ± 0.02	
Cysteic acid	0.00	0.06 ± 0.02	
Glutamine	0.29 ± 0.03	0.50 ± 0.02	
Glutamic acid	0.25 ± 0.03	0.35 ± 0.02	–
Glycine	0.52 ± 0.02	0.41 ± 0.02	
Histidine	0.12 ± 0.02	0.90 ± 0.02	
Hydroxyproline	0.35 ± 0.03	0.39 ± 0.02	–
Isoleucine	0.75 ± 0.02	0.26 ± 0.02	0.55 ± 0.01
Leucine	0.75 ± 0.02	0.24 ± 0.02	0.52 ± 0.01
Lysine	0.31 ± 0.01	0.01 ± 0.00	–
Methionine	0.65 ± 0.02	0.27 ± 0.03	0.63 ± 0.01
Methionine sulphone	0.23 ± 0.02	0.50 ± 0.02	
Phenylalanine	0.67 ± 0.03	0.27 ± 0.02	0.71 ± 0.01
Proline	0.84 ± 0.03	0.32 ± 0.03	
Serine	0.18 ± 0.03	0.44 ± 0.02	
Threonine	0.28 ± 0.02	0.44 ± 0.02	
Tryptophan	0.29 ± 0.03	0.17 ± 0.02	
Tyrosine	0.11 ± 0.02	0.23 ± 0.02	
Valine	0.72 ± 0.02	0.30 ± 0.02	
DIITC	0.93 ± 0.03	0.14 ± 0.01	
MITU	0.44 ± 0.03	0.14 ± 0.01	
BITU	0.39 ± 0.01	0.00	–

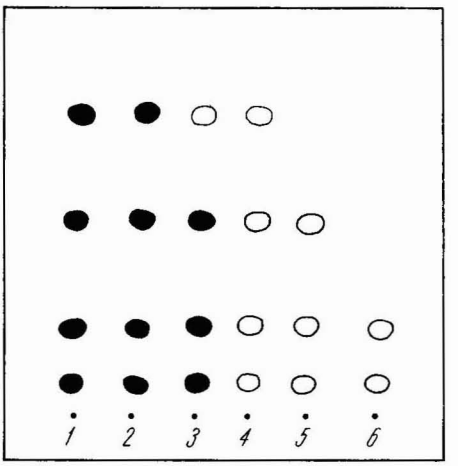

Fig. 2. Sensitivity of detection of a mixture of ITH-amino acids applied in the following amounts: (1) 0.4 nmole of each derivative; (2) 0.2 nmole; (3) 0.1 nmole; (4) 0.05 nmole; (5) 0.02 nmole; (6) 0.01 nmole. The identities of the spots, in order of increasing R_F, are: ITH-tyrosine, ITH-glutamic acid, ITH-glycine, ITH-proline. Chromatography was carried out in a single dimension with solvent I [toluene–*n*-pentane–glacial acetic acid (60:30:15)]. Distance, 4.5 cm. ●, Coloured spot; ○, spot visible only under UV light (directly as a dark violet spot or as a fluorescent spot after treatment with sodium ethoxide).

applied decreasing amounts of the following ITH-amino acids with different R_F values in solvent system I: ITH-tyrosine, ITH-glutamic acid, ITH-glycine and ITH-proline. The amounts spotted were as follows: on starting point 1, 0.4 nmole from each derivative; on starting point 2, 0.2 nmole; on starting point 3, 0.1 nmole; on starting point 4, 0.05 nmole; on starting point 5, 0.02 nmole; and on starting point 6, 0.01 nmole. As can be seen in Fig. 2, the sensitivity of the colour detection is 0.1–0.2 nmole. When the same chromatogram was inspected under UV light the spots originally indicated only by a contour were detected additionally as dark violet spots and the sensitivity increases to 0.01–0.05 nmole. The application of fluorescence detection with sodium ethoxide under UV light does not increase this sensitivity further. This fact can be explained by the slight fluorescence of the polyamide layer, which decreases the contrast of the fluorescent spots and the detection limit is 0.01–0.05 nmole. Therefore, the visual detection of coloured spots and of quenched spots under UV light is recommended.

In this way, the sensitivity of detection of the ITH-amino acids on a polyamide layer as coloured spots and under UV light (0.1–0.01 nmole) is 10–100 times higher than the corresponding sensitivity when the same derivatives are chromatographed on silica gel G (1 nmole)[4]. By inspection of the polyamide sheet under UV light the high sensitivity of the sodium ethoxide detection of ITH-amino acids on silica gel G (0.01–0.04 nmole)[5] was achieved. On the other hand, the sensitivity of detection of ITH-amino acids on a polyamide sheet (0.01–0.05 nmole) under UV light is therefore higher than that for the corresponding phenylthiohydantoin derivatives by quenching the fluorescence of the polyamide layer (0.05–0.2 nmole)[12].

Coloured thiohydantoins for sequencing work and amino acid identification have also been used by Chang *et al.*[14]. The azo group permits the detection of 4-N,N-

dimethylaminoazobenzene-4'-thiohydantoins of amino acids as red spots directly on the polyamide sheet with high sensitivity (1 pmole).

Separation of DIS-amino acids

The solvent systems for the separation of dansyl derivatives according to Woods and Wang[15] on polyamide sheets were unsuitable for the disyl amino acids. We suggest the following solvent systems for their separation: (1) 60% aqueous acetic acid; (2) benzene–propionic acid (1:1); (2a) benzene–propionic acid (8.5:1.5); (3) toluene–ethylene chlorohydrin–25% ammonia (3:5:2)[16]. Solvents 1 and 3 were used without a filter in the chamber for saturation of the atmosphere. The running time in solvent systems 1 and 3 is 60 min, while in systems 2 and 2a it is 30 min.

The R_F values of the disyl amino acids for solvent systems 1 and 2 are given in Table II (averages from ten determinations). Solvent system 2a is suggested for the separation of disylleucine and disylisoleucine. To distinguish disylcysteic acid and disylsulphonic acid, solvent system 3 is suitable.

The reliable detection of most of the disyl derivatives can be achieved ty two-dimensional chromatography (5 × 5 cm) in solvent systems 1 (first dimension) and 2 (second dimension) (Fig. 3). It can be seen that the disyl derivatives of the pairs serine–methionine sulphone and threonine–asparagine are not separated. The sepa-

TABLE II

R_F VALUES OF DISYL AMINO ACIDS, DISYL CHLORIDE, DISYLAMIDE AND DISYL-SULPHONIC ACID ON POLYAMIDE SHEETS

Distance: 4.5 cm.

Disyl amino acid	*Solvent system*			
	1	*2*	*2a*	*3*
Alanine	0.41 ± 0.03	0.62 ± 0.02	–	–
Arginine	0.84 ± 0.02	0.72 ± 0.03	–	–
Asparagine	0.53 ± 0.02	0.37 ± 0.02	–	–
Aspartic acid	0.41 ± 0.01	0.20 ± 0.02	–	–
Cysteic acid	0.00	0.00	0.00	0.79 ± 0.01
Glutamine	0.42 ± 0.01	0.47 ± 0.01	–	–
Glutamic acid	0.42 ± 0.01	0.31 ± 0.01	–	–
Glycine	0.04 ± 0.02	0.41 ± 0.02	–	–
Histidine	0.84 ± 0.02	0.37 ± 0.02	–	–
Isoleucine	0.33 ± 0.02	0.81 ± 0.03	0.66 ± 0.01	–
Leucine	0.32 ± 0.02	0.78 ± 0.01	0.57 ± 0.01	–
ε-Lysine	0.84 ± 0.01	0.27 ± 0.02	–	–
Bis-lysine	0.08 ± 0.01	0.52 ± 0.02	–	–
Methionine sulphone	0.53 ± 0.01	0.28 ± 0.01	–	–
Phenylalanine	0.30 ± 0.02	0.66 ± 0.01	–	–
Proline	0.52 ± 0.02	0.81 ± 0.03	–	–
Serine	0.52 ± 0.02	0.25 ± 0.02	–	–
Threonine	0.53 ± 0.02	0.38 ± 0.02	–	–
Bis-tyrosine	0.00	0.69 ± 0.02	–	–
Valine	0.39 ± 0.02	0.79 ± 0.02	0.58 ± 0.01	–
DIS-amide	0.43 ± 0.02	0.59 ± 0.02	–	–
DIS-chloride	0.30 ± 0.02	0.95 ± 0.01	–	–
DIS-sulphonic acid	0.00	0.00	0.00	0.09 ± 0.02

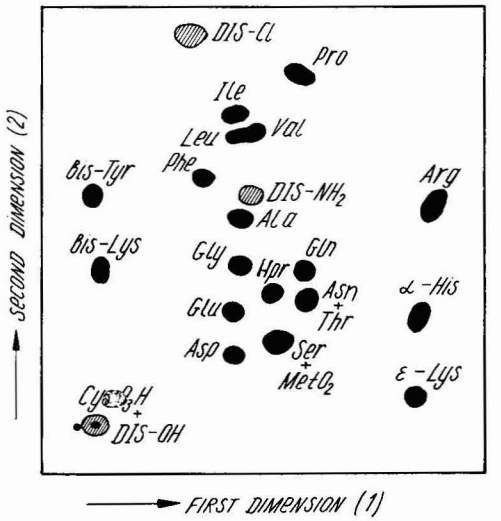

Fig. 3. Two-dimensional chromatogram of disyl amino acids on polyamide sheet (5 × 5 cm). First dimension, 60% aqueous acetic acid; second dimension, benzene–propionic acid (1:1). The starting point is 5 mm from both edges of the polyamide sheet. Disyl derivatives are indicated by abbreviations for the corresponding amino acids.

ration of threonine from asparagine is not necessary, as after hydrolysis one would expect the appearance of a more intense spot, corresponding to disylaspartic acid, the R_F value of which differs considerably from that of disylasparagine. When a single spot appears, it should be due to disylthreonine. Disylcysteic acid and disylsulphonic acid remain at the start. They can be separated with solvent system 3, even on 3 × 3 cm sheets. All disyl amino acids, as well as the artefacts disylamide and disyl chloride, move with the front in solvent 3, whereas disylsulphonic acid remains at the start.

The sensitivity of the technique is shown in Fig. 4. On starting points 1–8 increasing amounts of a mixture of three disyl amino acids (aspartic acid, glutamine and valine) with different R_F values were spotted. The chromatogram was developed with solvent system 2. The amounts spotted were as follows: on starting point 1, 0.0075 nmole (of all disyl amino acids); on starting point 2, 0.01 nmole; on starting point 3, 0.025 nmole; on starting point 4, 0.05 nmole; on starting point 5, 0.075 nmole; on starting point 6, 0.1 nmole; on starting point 7, 0.25 nmole; and on starting point 8, 0.5 nmole (Fig. 4). The results indicate that amounts from 0.25 to 0.1 nmole of the disyl derivatives can be detected as coloured spots. Amounts from 0.1 to 0.05 nmole can be detected by ultraviolet irradiation (365 nm) due to the absorption of the diphenylindenonesulphonyl residue. Amounts from 0.01 to 0.0075 nmole are detected after treatment with sodium ethoxide, following the transformation of the disyl derivatives into fluorescent isobenzofuran derivatives[3]. These results indicate that the sensitivity of detection of the disyl derivatives on polyamide sheets (0.1 nmole) is considerably higher than the corresponding colour detection of the same derivatives on silica gel G (1 nmole). The sensitivity of detection of the disyl amino acids as fluorescent spots is lower than the corresponding fluorescent detection on silica gel G (1 pmole)[6], owing to the fluorescence of the polyester foil of the polyamide sheet,

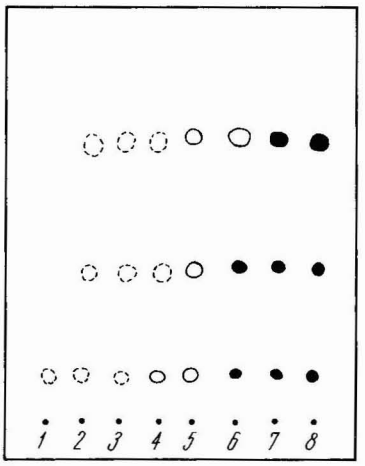

Fig. 4. One-dimensional chromatogram of a mixture of disyl derivatives of aspartic acid, glutamine and valine in increasing amounts from 0.01 to 0.5 nmoles. Solvent system 2: benzene–propionic acid (1:1). ●, Coloured spot; ○, spot visible under UV light; ◌, fluorescent spot visible under UV light after treatment with sodium ethoxide.

which decreases the contrast between the spots and the background. This effect was observed by Zimmer *et al.*[17] in the detection of dansyl amino acids. They established that when the polyester foil is replaced with an aluminium one, which does not fluoresce, the detection limit increases. Under analogous conditions (polyamide layer on the aluminium foil) the detection limit increases to 3 pmole (as a result of the absorption under UV light) and to 1 pmole after treatment with sodium ethoxide.

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DETECTION AND DETERMINATION OF N-NITROSAMINO ACIDS BY THIN-LAYER CHROMATOGRAPHY USING FLUORESCAMINE*

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SUMMARY

A novel procedure is described for the detection and determination of N-nitrosamino acids (NAAs) on activated silica gel thin-layer chromatographic plates. N-Nitrososarcosine, N-nitrosoproline, and N-nitroso-4-hydroxyproline could be detected as fluorophors at the 200-pmole level (20–30 ng) after being irradiated with ultraviolet light and sprayed with fluorescamine reagent. Spectrophotometric determination of the relative fluorescence of 0.4–40 nmoles of NAAs gave rise to similar calibration curves when plotted on a log–log scale. An application of this method to the detection of NAAs in uncooked bacon is described.

INTRODUCTION

N-Nitrosamino acids (NAAs) are considered to be potential precursors to the carcinogenic N-nitrosamines (NAs)[1–11]. N-Nitrosoproline (N-Pro) has been converted to N-nitrosopyrrolidine (N-Pyr) on heating in model food systems[4–6,8,9] and by microbial action[12], and N-nitroso-4-hydroxyproline (N-HO-Pro) has been converted to N-nitroso-3-hydroxypyrrolidine (N-HO-Pyr)[11]. N-Pro has been detected in raw but not in cooked bacon, whereas N-Pyr is found in cooked but not in raw bacon[13], and N-HO-Pyr has been detected in cooked bacon[14]. Production of NAs from various amino acids and sodium nitrite has been demonstrated[7,15].

Volatile NAs are generally determined directly by gas chromatography (GC) combined with high-resolution mass spectrometry (MS). Methodology for analysis of NAs has been recently reviewed[16,17]. NAAs have been detected or determined by thin-layer chromatography (TLC) on paper, cellulose powder or silica gel[18–31]; liquid chromatography (LC)[18,32,33]; polarography[34–39]; thermal energy analysis[32,40,41]; by conversion to their corresponding alkyl[6,9,42–46] or tetramethylsilyl[47] esters followed by GC or GC–MS; cleavage in solution by ultraviolet (UV) irradiation[48,49], hydrobromic acid in glacial acetic acid[50], or thionyl chloride[51] to give nitrite, which is then complexed and determined colorimetrically, or to give the free amino acid, which is converted to a fluorescent derivative[46] and then quantitated by TLC or LC; and

* Contribution Number 980.

cleavage by hydrobromic acid to give nitrosylbromide, which is quantitated by chemiluminescence[52].

Detection of NAAs on TLC plates has been accomplished with a wide variety of reagents including: bromocresol green[19], bromophenol blue[20], diazotized *o*-dianisidine[20], 4-dimethylaminobenzaldehyde[20,21], 4-dimethylaminocinnamaldehyde[20], Dragendorff reagent[20,22], ferric chloride[20], fluorescamine[23], Griess reagent (UV irradiation and 1-naphthylamine–sulfanilic acid)[24–26], isatin[21], iodine[19,27,28], NEDSA reagent (UV irradiation and N-1-naphthylenediamine–sulfanilic acid)[29], ninhydrin[20–22,26,30], 4-nitrobenzenediazonium fluoborate[20], phosphomolybdic acid[20], Preussmann reagent (UV irradiation and palladium chloride–diphenylamine)[24,29], and sulfuric acid and heat[28]. The majority of these reagents have been used only for diagnostic purposes; detection limits of 2, 20, and 500 μg have been reported for NEDSA[29], iodine and sulfuric acid[28], and Preussmann reagents[29], respectively.

In earlier studies it was observed that most NAs[53] and the NAA N-nitroso-N-(phosphonomethyl) glycine[23] could be detected and determined by TLC at the picomole level with fluorescamine. This paper reports an extension of the use of fluorescamine to the detection and determination of other NAAs on TLC plates and its application to some samples of bacon.

EXPERIMENTAL

Materials

L-Proline, 4-hydroxy-L-proline, and sarcosine were purchased from Aldrich (Milwaukee, Wis., U.S.A.). The corresponding NAAs were synthesized by the method of Hansen *et al.*[27] and gave infrared (IR) and nuclear magnetic resonance (NMR) spectra consistent with those reported by Lijinsky *et al.*[54]. Methyl esters were prepared by treatment of the parent NAA with ethereal diazomethane. Standard solutions of NAAs in methylene chloride were freshly prepared before each determination and stored in a refrigerator. Solutions of 0.1 mg/ml fluorescamine (Fisher Scientific, Pittsburgh, Pa., U.S.A.) in acetone were prepared and stored at room temperature in a stoppered flask. Eastman Chromagram sheets (No. 13179; 0.1 mm silica gel) without fluorescent indicator (Fisher) were stored in a dry atmosphere and activated by heating at 105° for 1 h prior to use. Acidic aluminum oxide (W200 acid Woelm from ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.) (10 g) was washed first with 20% hydrochloric acid (100 ml) and then sufficient water until the eluate was neutral, heated at 400° for 4 h, cooled, mixed with water (0.3 ml), placed in a stoppered flask, and kept overnight in a dessicator prior to use. Bacon was purchased from local grocery stores. All other reagents and solvents were of reagent grade and used as received from commercial sources.

Spectral determinations

IR spectra were determined in chloroform or nujol mull on a Beckman IR-20A spectrometer. NMR spectra were determined in pyridine-d_5 on a Varian T-60 spectrometer. Mass spectra were determined on a Finnigan Model 9500 gas chromatograph (1.8 m × 6.5 mm O.D. glass column containing 3% SE-30 ultraphase on high-performance Chromosorb W, 80–100 mesh) coupled to a Model 3100D quadropole mass spectrometer and a Model 6000 computer controlled data aquisition system.

Thin-layer chromatography

Standard solutions of NAAs were spotted, developed in 95% ethanol–benzene–water (4:1:1)[25], dried for 15 min in a vacuum desiccator, and irradiated with UV light using the apparatus previously described[53]. The plates were then sprayed sequentially with fluorescamine solution and triethanolamine (10% in methylene chloride) and viewed under long-wave UV light. Fluorescence intensities were determined[53] and the relative values normalized by assigning to 1.0 nmole of each NAA a value of 10. Detection limits were determined by spotting decreasing volumes of standard solutions of each NAA, developing, irradiating, spraying with fluorescamine reagent, and then viewing under UV light. Mixtures of methanol–chloroform (4:1) and acetonitrile–chloroform–95% ethanol–acetic acid (100:100:97:3) were also used as developers.

Gas chromatography

The methyl esters of NAAs were examined on a Pye Model 104 gas chromatograph (1.5 m × 6.5 mm O.D. glass column containing 5% Carbowax 20M-TPA on high-performance Chromosorb W, 80–100 mesh) fitted with both a flame ionization detector and an alkali flame ionization detector.

Column chromatography

Mixtures to be analyzed were dissolved in methylene chloride (25 ml) and passed through a column of acidic aluminum oxide (2 g). The column was then eluted successively with ethyl acetate, acetone–methanol (3:1), and water (25 ml each), and 20% aqueous acetic acid (50 ml). The final fraction was evaporated to dryness under reduced pressure, dissolved in acetonitrile, filtered if necessary, and analyzed by TLC using methanol–chloroform (4:1) as developer.

Extraction and analysis of bacon

The clean-up procedure is based on one developed by Sen *et al.*[14]. A 50-g sample of ground bacon was extracted in a blender for 10 min with acetonitrile (125 ml), allowed to settle for several minutes and the supernatant filtered through glass wool. The residue was treated in a similar manner and the combined filtrates washed with heptane (2 × 250 ml) and evaporated to dryness under reduced pressure. A methylene chloride solution (25 ml) of this residue was further purified by column chromatography and then analyzed by TLC. Samples spiked by addition of NAAs to the ground bacon were also analyzed.

To confirm the identity of NAAs, samples were spotted, developed in methanol–chloroform (4:1), developed in the other dimension with acetonitrile–chloroform–95% ethanol–acetic acid (100:100:97:3), irradiated, sprayed with fluorescamine reagent and viewed under UV light. In addition, samples were spotted, developed in methanol–chloroform (4:1), irradiated, developed in the other dimension first with a 0.01% solution of fluorescamine in hexane–acetone (4:1)[55] and then with methanol–chloroform (4:1), sprayed with triethanolamine solution and viewed under UV light. Alternatively, after development, the adsorbent at the appropriate R_F was scraped off, collected using the apparatus of Clemett[56], and eluted with 300 μl of ethereal diazomethane. The eluate was concentrated under a stream of dry nitrogen and then examined by GC or GC–MS.

Safety precautions

Many NAs and some NAAs are known to be potent carcinogens. Thus, safety precautions to prevent skin contact and inhalation must be exercised at all times.

RESULTS AND DISCUSSION

Alkyl and some heterocyclic NAs can be cleaved by UV light on activated silica gel TLC plates to give products that yield fluorophors after being sprayed with fluorescamine reagent[53]. Under similar conditions, NAAs also yield fluorophors. This technique is very sensitive and can detect as little as 200 pmoles. Detection limits and R_F values for the three NAAs studied are given in Table I. In this context, detection limit is the minimum amount of an NAA that ultimately gives a detectable visual fluorescence. The NAA N-nitroso-N-(phosphonomethyl) glycine can be detected at the 50-pmole level[23].

TABLE I

THIN-LAYER CHROMATOGRAPHIC R_F VALUES AND VISUAL FLUORESCENCE DETECTION LIMITS OF N-NITROSAMINO ACIDS

N-Nitrosamino acids are determined on activated silica gel by spotting, developing, irradiating with UV light, spraying with fluorescamine reagent, and viewing under long-wave UV light. Amino acids are detected by spraying with ninhydrin reagent and heating. Solvent systems: A, 95% ethanol–benzene–water (4:1:1); B, methanol–chloroform (4:1); C, acetonitrile–chloroform–95% ethanol–acetic acid (100:100:97:3).

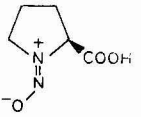
Compound	R_F			*Detection limit**	
	A	*B*	*C*	*Nanograms*	*Picomoles*
N-Nitrososarcosine	0.64, 0.70	0.71	0.75	20	180
Sarcosine	0.23	0.10**	0.02		
N-Nitroso-L-proline	0.63, 0.69	0.72	0.21	30	200
L-Proline	0.33	0.12**	0.03		
N-Nitroso-4-hydroxy-L-proline	0.60, 0.66	0.67	0.13	33	210
4-Hydroxy-L-proline	0.28	0.09**	0.01		

* Determined in solvent system A.

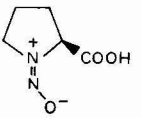
** Streak.

Fig. 1 shows that the calibration curves for the NAAs are not significantly different.

There is a 23-kcal/mole barrier to rotation[57] about the nitrogen–nitrogen bond in the N–N=O system due to contribution from the zwitterionic form. Since NAAs are unsymmetrical, (*E*)- and (*Z*)-conformers (*e.g.* I and II, respectively, for L-proline) are possible and can be resolved by LC[18,33] or TLC[18,19,26,31,58]. In this study it was



(I)



(II)

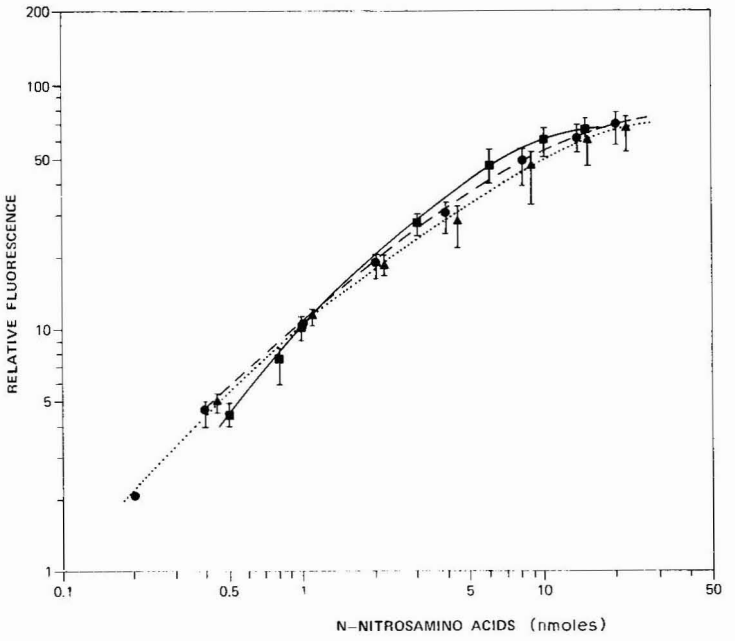


Fig. 1. Calibration curves for UV-irradiated and fluorescamine-treated N-nitrososarcosine (--▲--), N-nitroso-L-proline (—■—), and N-nitroso-4-hydroxy-L-proline (· · ·●· · ·) on silica gel TLC plates. Fluorescence values normalized so that 1.0 nmole gave a relative fluorescence of 10. Error bars are standard deviations calculated from measurements made in triplicate.

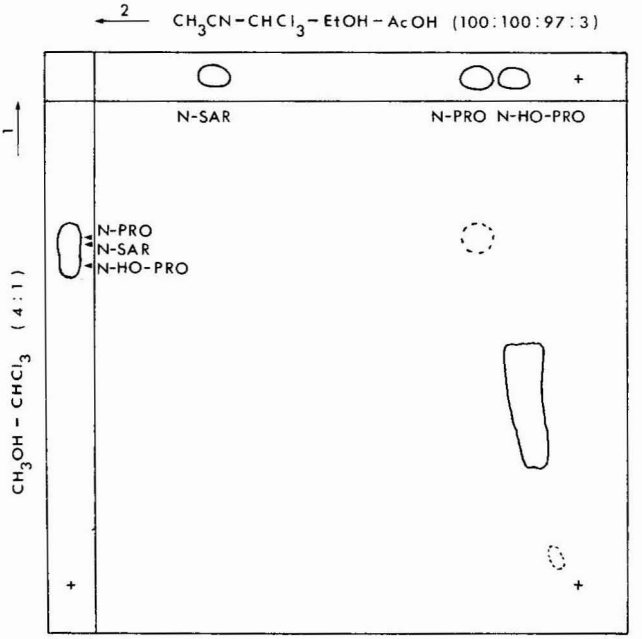


Fig. 2. Two-dimensional thin-layer chromatogram of purified uncooked bacon extract. Silica gel plate was developed, irradiated with UV light, sprayed with fluorescamine reagent, and viewed under long-wave UV light. Mixture of N-nitrososarcosine (N-SAR), N-nitrosoproline (N-PRO), and N-nitrosohydroxyproline (N-HO-PRO) standards spotted alongside.

observed that the conformers could be separated with the ethanol–benzene–water (4:1:1) developer and their ratios determined directly from the TLC plate, thus complementing LC[18,19] or NMR[54] methods of ratio determination. Agreement with the NMR method was good.

Recovery of NAAs in bacon samples spiked at the 1-ppm level from columns of aluminum oxide was variable, ranging from 25–80% (median, 30%) and depended not only upon the brand of adsorbent but also upon the individual lot. The highest recoveries were obtained from W200 acid Woelm (aminotropic) that had been acid washed, heated to 400°, and made to 3% water. This brand was used for the analysis.

Two samples each of two brands of uncooked bacon were examined for the presence of NAAs. In only one sample of one brand was N-Pro detected at the ppm level. The presence of N-Pro was confirmed by two-dimensional TLC (Fig. 2), by comparison of R_F values of the fluorescamine derivative[55] of the UV photolysis product, and by GC and GC–MS of the methyl ester. N-Pro has been reported at the 1-ppm level in some uncooked bacon samples[5] and not observed in others[9]. N-Nitrososarcosine and N-HO-Pro were not detected in any samples at this level.

ACKNOWLEDGEMENTS

The author wishes to thank Mr. J. Lapointe for technical assistance and Mr. D. Dobson of the Analytical Chemistry Services for obtaining mass spectra.

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CHROM. 10,680

Note

Simple method for improving the efficiency of liquid chromatographic columns filled with soft gels

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Unexpected changes in separation efficiency are sometimes observed in liquid chromatography with columns filled with soft or semi-rigid organic gels. A detailed study of this phenomenon showed that it is connected with changes in size of the swollen gel particles of the column packing. These changes are caused by the injection of solutes that reduce the swelling of the gel considerably or by accidental penetration of air into the gel bed. The practical aspects of these effects are discussed here.

EXPERIMENTAL

The experiments were performed on a simple home-made low-pressure apparatus[1]. Glass columns (15–28 mm I.D.) were filled by the classical low-pressure slurry technique[2]. The volumes of the gel beds (V_t) were 200–400 cm^3. Air and liquid samples were injected into the columns from a 2-cm^3 loop of the injector. The columns were washed with a large volume (*ca.* V_t) of the de-gassed eluent after the injection of air or a liquid that reduced the swelling of the gel considerably. In all experiments, descending elution was applied. A decrease in the gel bed volume was usually observed after injection of air or deswelling liquid and subsequent washing of the column. Therefore, the adjustable column end-pieces were re-set before efficiency testing with diluted solutions of benzene or *n*-heptane. A Model R-4 differential refractometer (Waters Assoc., Milford, Mass., U.S.A.) was used as the detector. The pressure applied was 0.05–0.5 MPa at an elution rate of 0.5–1.0 cm^3/min.

RESULTS AND DISCUSSION

Table I gives several typical examples that show the column efficiencies obtained after injection of air or deswelling liquid into gel bed. The results can be summarized as follows.

(1) Injection of small amounts of air or a liquid that reduces the swelling of the gel particles considerably into the column under controlled conditions may substantially increase the column efficiency. This effect is most pronounced in less efficient columns.

(2) The total filling of the gel bed with air does not necessarily destroy the column. This observation contradicts the statements that are often found in the literature.

TABLE I

COLUMN EFFICIENCIES AFTER INJECTION OF AIR OR LIQUID INTO THE GEL BED

Gel	*Eluent*	*Conditioning*	*Number of theoretical plates per metre*
Sephadex LH-20*	Methanol	—	1400
		2 cm^3 of air	1650
		2 × 2 cm^3 of air	1900
		20 × 2 cm^3 of air	3700
		Complete filling of the gel bed with air	3400
Sephadex LH-20*	Methanol	—	1600
		2 × 2 cm^3 of benzene–methanol (9:1, v/v)	1850
Sephadex LH-20*	Tetrahydrofuran	—	1700
		2 × 2 cm^3 of air	2000
Sephadex LH-20**	Benzene–methanol (77.8:22.2, v/v)	—	4900
		5 × 2 cm^3 of air	5200
Sephadex LH-20**	Methanol	—	5500
		20 × 2 cm^3 of air	6800
Hydroxypropylated Sephadex G-50***	Methanol	—	1500
		2 × 2 cm^3 of air	1750
Bio-Beads SX-3§	Tetrahydrofuran	—	2500
		5 × 2 cm^3 of air	3200
Bio-Beads SX-3§	Benzene–methanol (77.8:22.2, v/v)	—	4300
		2 × 2 cm^3 of air	1500

* Hydroxypropylated crosslinked dextran gel (Pharmacia Fine Chemicals, Uppsala, Sweden). Particle size, 25–125 μm (dry).

** Smallest and largest gel particles removed by sedimentation.

*** Irregular particles. Prepared by hydroxypropylation of Sephadex G-50.

§ Styrene–divinylbenzene gel (Bio-Rad Labs., Richmond, Calif., U.S.A.). Particle size, 37–74 μm (dry).

(3) Injection of air considerably reduces the column efficiency of styrene–divinylbenzene gel if benzene–methanol (77.8:22.2, v/v) is used as the eluent. This mixture is an extremely poor solvent for linear polystyrene. It swells the styrene–divinylbenzene gel only to a small extent and the gel particles tend to form clusters in the slurry. On the other hand, Sephadex LH-20 gel swells in the above mixture to virtually the same extent as in pure methanol and no cluster formation in the slurry was observed.

(4) No changes in the separation ranges according to the size of molecules of oligomers during gel chromatographic experiments after re-swelling of gels were observed. Hence the decreases in gel bed volumes and in the corresponding elution volumes are caused by decreases in the inter-particulate (dead) volumes only.

A possible explanation of these results involves local deswelling of the gel particles in the zone of eluent containing air or deswelling liquid and their subsequent tighter re-packing, which results in the decrease in the gel bed volume and an increase in the column efficiency. If the deswollen gel particles cannot move in the gel

bed sufficiently as the extent of deswelling is too small and/or their mutual adhesion is too great, channels appear in the gel bed and the column efficiency decreases. The gel particles may also be deswollen osmotically by relatively low concentrations of larger solute molecules injected into the column[3–6]. This could explain the frequently observed changes in the column separation efficiencies during gel chromatographic separations with soft gels.

The effects described have been routinely applied for several years in this laboratory for improving the separation efficiencies of low-pressure liquid chromatographic columns packed with soft organic gels. Small amounts of air are injected until no changes in the gel bed volume are observed. The use of air as a deswelling agent is generally most convenient as some adjustable column end-pieces are attacked by organic solvents that can be applied with hydrophilic gels.

In addition to the increase in column efficiency, an advantage of this column "conditioning" is that in the event of accidental penetration of air into the conditioned column its gel bed volume will not change further and the elution volumes of separated substances remain constant. Hence the column need not be re-calibrated.

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CHROM. 10,570

Note

Marihuana metabolites in urine of man

IX. Identification of Δ^9-tetrahydrocannabinol-11-oic acid by thin-layer chromatography

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In 1974, we described a major human metabolite of Δ^9-tetrahydrocannabinol (THC) which we could not positively identify[1]. Its mobility when chromatographed in a thin-layer solvent system of petroleum ether–diethyl ether–glacial acetic acid (50:50:1) (solvent system A), was about the same as that of 8β-hydroxy-THC. It was not the latter, however, as in a solvent system of chloroform–acetone–glacial acetic acid (16:4:1) (solvent system B), the mobilities were different. Then gas–liquid chromatography–mass spectrometry (GLC–MS) specific-ion detection techniques eliminated other metabolites: 11-hydroxy-, 8α-hydroxy-, 8,11-dihydroxy, as well as 8β-hydroxy-THC. It also eliminated the possibility of other compounds of molecular weight 328 (keto-derivatives of THC) and 330 (monohydroxy- or epoxy-derivatives of THC).

At that time Δ^9-THC-11-oic acid (THC-11-oic acid) was not available as a reference standard. Acidic metabolites of THC that were described in the literature had been obtained from rabbits[2,3]. Based upon newer developments in our thin-layer chromatography (TLC) procedures, refinement of our multistep extraction procedure, and the availability of THC-11-oic acid as a reference standard, we can identify this major metabolite as THC-11-oic acid[4,5].

The new development in our TLC procedure, sequential TLC, permits separation of THC compounds into natural neutrals, alcoholic neutrals and acidics. The refinement of our multistep extraction procedure was to extract with hexane at pH 8 instead of 5.5, because it was found that THC-11-oic acid was partially extracted by hexane from an aqueous solution of the latter pH[5]. Using these techniques, we were subsequently able to show THC-11-oic acid as a major THC metabolite. The question arose as to whether or not it was the same major metabolite we had previously found but had not been able to identify.

We used two urine specimens known to contain THC-11-oic acid as determined by our later techniques. These urine specimens were obtained from two separate subjects, one taken 6–12 h after drug ingestion, the other 24–48 h after. These urines were hydrolyzed, concentrated and extracted with hexane at pH 5.5 and the residues of the extracts were separately chromatographed, one in solvent system A, the other in solvent system B[1]. Chromatograms of reference standards of THC-11-oic acid, 8β-

hydroxy-, 11β-hydroxy-, and 8β,11-dihydroxy-THC were also run on each plate. Zones corresponding to the THC-11-oic acid reference standard were eluted with ethanol from untreated chromatograms (Figs. 1A and 1B). These ethanol eluates were then chromatographed in chloroform–acetone–triethylamine (80:20:1) (solvent system C) in which neutral compounds move away from the origin, but in which acidic compounds, such as THC-11-oic acid, remain. Ethanol eluates of the material at the origin of this plate were chromatographed in petroleum ether–ether–glacial acetic acid (50:50:1) (solvent system D) in which acidic compounds move[4]. This procedure is shown in Figs. 2A and 2B.

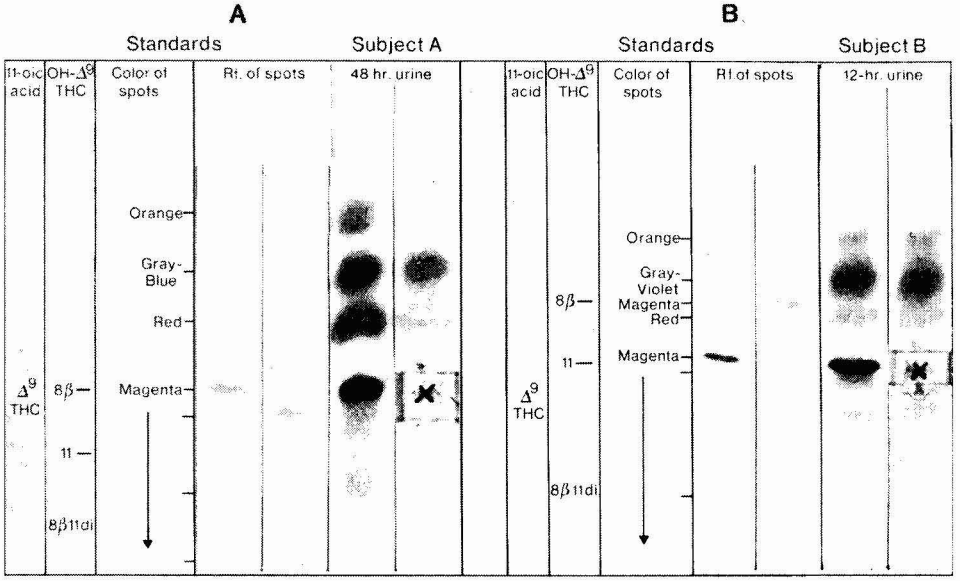

Fig. 1. Chromatograms of hexane extracts prepared at pH 5.5. (A) 48-h post-THC urine of subject A chromatographed in petroleum ether–ether–glacial acetic acid (50:50:1). (B) 12-h post-THC urine of subject B chromatographed in chloroform–acetone–glacial acetic acid (16:4:1). ⁄, Zones of silica gel, suspected to contain THC-11-oic acid, eluted with ethanol and chromatographed sequentially, Fig. 2. Color of spots due to reaction with Fast Blue Salt B. Spots colored orange, gray-blue, gray-violet and yellow probably not due to cannabinoids. *Correction to Fig. 1B:* The R_F value of $\triangle^9$-THC-11-oic acid should be the same as that of 11-hydroxy-$\triangle^9$-THC.

The silica gel zones corresponding to THC-11-oic acid that were eluted from the chromatograms prepared in solvent systems A and B respectively, had the same mobility as THC-11-oic acid when chromatographed sequentially in solvent systems C and D. This confirmed that the previously unidentified metabolite that was extracted in hexane at pH 5.5 was THC-11-oic acid[1].

Since the publication in 1974, others have demonstrated the presence of THC-11-oic acid in the urine of humans after ingestion of THC and we have also confirmed the presence of this metabolite in extracts of equivalent urines prepared by our multi-step extraction procedure and analyzed by high-performance liquid chromatography and GLC–MS[6–10].

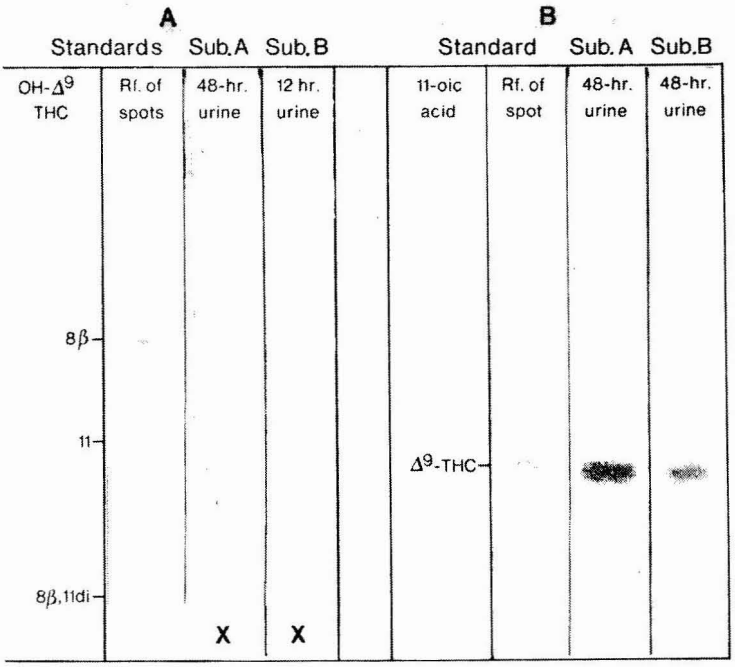

Fig. 2. Sequential chromatography of ethanol eluates of silica gel zones eluted from chromatograms described by Fig. 1. (A) Chromatographed in chloroform–acetone–triethylamine (80:20:1), in which acidic compounds remain at the origin. (B) Chromatograms of the ethanol eluates of the zones described by ×, Fig. 2A, in petroleum ether–ether–glacial acetic acid (50:50:1), in which acids are mobilized[4]. Color of spots due to reaction with Fast Blue Salt B. All spots are magenta colored.

ACKNOWLEDGEMENTS

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Note

Chromatographic determination of 4-nitro-L-histidine

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The synthetic amino acid 4-nitro-L-histidine was first prepared by Tautz *et al.*[1] by direct nitration of histidine. It has been recently used in our laboratory in the solid-phase synthesis of two modified hypothalamic peptide hormones, namely [4-nitro-L-histidine]^2-thyrotropin releasing factor (nitro-TRF) and [4-nitro-L-histidine]^2-luteinizing hormone releasing factor[2,3]; substitution of 4-nitro-L-histidine for L-histidine enhances the acidity of the imidazole side chain, thus providing a tool for studying the role played by histidine in the biological activity of natural peptides.

Acid hydrolysis followed by automatic ion-exchange chromatographic analysis is the usual way to control the synthesis and check the purity of the synthetic peptides[4]. Fig. 1 shows the chromatogram corresponding to a nitro-TRF hydrolysate obtained from a Beckman 120-C autoanalyzer equipped with a M 82 resin and working at standard conditions. Peaks corresponding to 4-nitro-L-histidine and proline are eluted simultaneously and therefore not resolved. Similar results were obtained when using a Beckman-Unichrom autoanalyzer equipped with a M 72 resin. In view of these difficulties, we have studied in detail the problem of chromatographic determination of 4-nitro-L-histidine.

EXPERIMENTAL

4-Nitro-L-histidine was synthesized by treating L-histidine with a sulphuric acid–fuming nitric acid mixture as described by Tautz *et al.*[1]. The product was precipitated at its isoelectric point and recrystallized twice from water: m.p. 197–198°; $[\alpha]_{25}^{D} = -23.5°$ (*c* 1, 5 *N* HCl). NMR spectrum (^2H_2O, NaO^2H): 8.56 ppm (s, 1 H, imidazole proton); $\delta_X = 3.72$ ppm, $\delta_A = 3.15$ ppm, $\delta_B = 3.34$ ppm, $J_{AX} = 11$ Hz, $J_{BX} = 3.5$ Hz, $J_{AB} = 13.8$ Hz) (3H, ABX system of protons from C_α and C_β).

Gas chromatography

A Perkin-Elmer F-11 apparatus equipped with a flame ionization detector was used. The carrier gas (nitrogen) flow-rate was 34 ml/min. Chromatograms were recorded between 60 and 210° employing a temperature gradient of 5°/min. The col-

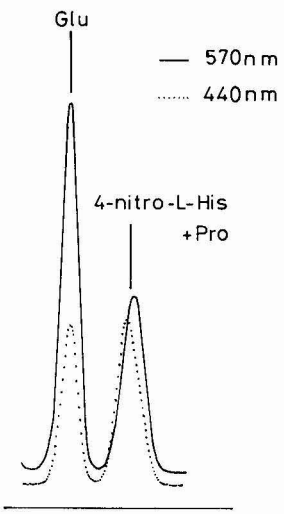

Fig. 1. Ion-exchange chromatogram corresponding to an hydrolysate of nitro-TRF. The analysis was performed on a Beckman 120-C autoanalyzer equipped with a M 82 resin. Temperature, 55°; sodium citrate buffers, pH 3.25 (0.2 *N* Na^+, 0.2 *N* citrate), pH 4.12 (0.4 *N* Na^+, 0.2 *N* citrate), pH 6.40 (1.0 *N* Na^+, 0.2 *N* citrate).

umns contained either Chromosorb–4% poiy(phenyl ether) or Chromosorb–0.65% ethyleneglycol adipate. N-Trifluoroacetyl-*n*-butyl derivatives of amino acids were prepared by treatment of the amino acid with 3 *N* hydrogen chloride–butanol and 25% trifluoroacetic anhydride–methylene chloride following standard procedures[5].

Spectrophotometric determination

Spectra were recorded on a Perkin-Elmer 124 spectrophotometer. The molar extinction coefficient of 4-nitro-L-histidine was determined from the absorptions at 295 nm of 14 solutions havnig concentrations ranging from $8.35 \cdot 10^{-6}$ to $1.67 \cdot 10^{-4}$ *M* (linear regression coefficient, 0.9982).

Ion-exchange chromatography

Acid hydrolyses of peptides were performed with 6 *N* hydrochloric acid in vacuum-degassed sealed tubes at 110° for 24 h, norleucine being added as the internal standard. The resolution of proline and 4-nitro-L-histidine was achieved by using a Beckman 119-C autoanalyzer equipped with an AA 20 resin and operating under the following conditions: column, 46 × 0.6 cm; flow-rates, 35 ml/h (buffer), 17.5 ml/h (ninhydrin); sodium citrate buffers, pH 3.25 (0.2 *N* Na^+, 0.2 *N* citrate), pH 4.12 (0.4 *N* Na^+, 0.2 *N* citrate), pH 6.40 (1.0 *N* Na^+, 0.2 *N* citrate); temperature, 50°. 4-Nitro-L-histidine was quantitated at 570 nm, using a colorimetric constant of 0.530 relative to leucine[6].

The spectra shown in Fig. 3 were recorded after heating at 100° for 20 min a mixture of 1 ml of the ninhydrin solution used by the analyzer and 1 ml of a solution of each amino acid in the initial citrate buffer.

RESULTS AND DISCUSSION

Gas chromatographic analysis

Gas chromatography of N-trifluoroacetyl-4-nitro-L-histidine *n*-butyl ester using columns of either Chromosorb–ethyleneglycol adipate or Chromosorb–poly-(phenyl ether) under the conditions described by Kaiser *et al.*[5] led to no definite peaks. This behaviour is probably due to decomposition of the derivatized 4-nitro-L-histidine

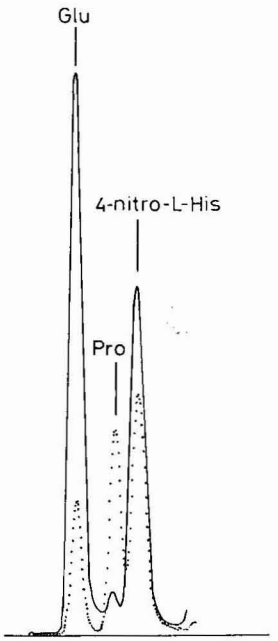

Fig. 2. Ion-exchange chromatogram of a hydrolysate of nitro-TRF obtained using a Beckman 119-C autoanalyzer equipped with a AA 20 resin. For conditions see Experimental section.

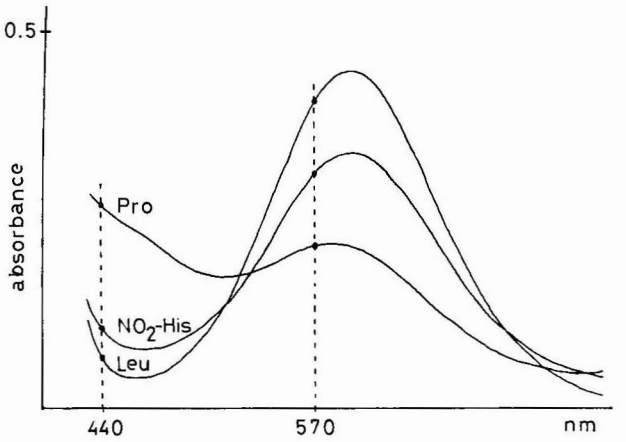

Fig. 3. Visible spectra of the products of reaction of ninhydrin with proline, leucine and 4-nitro-L-histidine.

on the column. An analogous decomposition of derivatized L-histidine has been previously described[7]. However, the application of this method to the analysis of hydrolysates of nitrated analogues of hypothalamic hormones allows the determination of amino acids other than 4-nitro-L-histidine. Proline can also be determined by gas chromatography, thus avoiding the difficulties encountered in its ion-exchange chromatographic separation from 4-nitro-L-histidine (Fig. 1).

Spectrophotometric determination

The combination of gas chromatography and UV spectroscopy allows analysis of most nitrated hypothalamic hormone hydrolysates. The UV spectrum of 4-nitro-L-histidine in acid solution shows an absorption maximum at 295 nm ($\varepsilon = 5661$, 1 *N* HCl) corresponding to the nitroimidazole side chain. Beer's law is obeyed over the whole range of concentrations examined (up to $1.67 \cdot 10^{-4}$ *M*). Thus, 4-nitro-L-histidine can be determined spectrophotometrically from peptide hydrolysates using 1 *N* hydrochloric acid as solvent.

Ion-exchange chromatography

As mentioned in the introduction, proline and 4-nitro-L-histidine are eluted simultaneously when M 82 and M 72 resins are used in Beckman analyzers under standard conditions. We have thoroughly varied the experimental parameters (temperature, buffer pH, buffer ionic strength) but could not resolve the two amino acids. However, the use of a Beckman AA 20 resin in a Beckman 119-C autoanalyzer under standard conditions (see Experimental section) provides a fairly good separation (Fig. 2).

The 570 nm:440 nm absorption ratio at the peak corresponding to 4-nitro-L-histidine in Fig. 2 is lower than the values usually found for other amino acids containing primary amine functions. The visible spectrum of a previously heated mixture of ninhydrin and 4-nitro-L-histidine is shown in Fig. 3 and compared with those of leucine and proline. Although the spectrum is very similar to that of leucine, the 570 nm:440 nm absorption ratio is lower for 4-nitro-L-histidine than for leucine. This suggests that the anomalous shape of the 4-nitro-L-histidine peak in Fig. 2 is not due to an inefficient separation from proline but rather to the chromophoric properties of the reaction product of ninhydrin and 4-nitro-L-histidine.

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CHROM. 10,597

Note

Determination of saccharin in urine by electron-capture gas chromatography after extractive methylation

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The use of the sweetening agent saccharin (2,3-dihydro-3-oxobenzisosulphonazole) in food and cosmetics has increased during the last decade. Although only small amounts are added, questions have recently been raised about its safety[1,2].

The determination of trace amounts of saccharin in urine or blood samples requires selective and sensitive methods. Saccharin has been determined by gas chromatography with flame-ionization detection after methylation with diazomethane[3–7], methyl iodide[7,8], dimethyl sulphate[9] or dimethylformamide dimethylacetal[10] or after silylation[7,11]. Saccharin can also be detected with an electron-capture detector[8,10] after methylation.

Extractive alkylation is a flexible and convenient means of derivatizing organic acids before gas chromatographic analysis[12–15]. Recently, the direct determination of clioquinol in plasma and urine samples after extractive methylation was demonstrated[16].

This paper presents a sensitive and selective method for the determination of saccharin in urine by electron-capture gas chromatography after extractive methylation. The method consists in extraction of saccharin as an ion pair with tetrabutylammonium with methylene chloride containing methyl iodide. Excess of methyl iodide and methylene chloride are removed by evaporation and tetrabutylammonium iodide by extraction with silver sulphate solution.

EXPERIMENTAL

Apparatus

Gas chromatography. A Varian 1400 gas chromatograph with a tritium electron-capture detector operated in the d.c. mode was used. The glass column (150 × 0.2 cm I.D.) was filled with 3% OV-17 on Gas-Chrom Q (80–100 mesh) and operated at 180°. The injector and detector temperatures were 250° and 210°, respectively. The flow-rate of the carrier gas (nitrogen) was 30 ml/min.

Mass spectrometry. N-Methyl- and N-propylsaccharin were identified with an LKB 9000 mass spectrometer. The ionization energy was 70 eV.

Spectrophotometry. The photometric measurements were performed with a Zeiss PMQ II Spectralphotometer.

Reagents and chemicals

N-Methyl and N-propylsaccharin (internal standard) were synthesized from sodium saccharin and methyl or propyl iodide (Merck, Darmstadt, G.F.R.) in dimethyl sulphoxide[8]. Tetrabutylammonium hydrogen sulphate (AB Hässle, Mölndal, Sweden) was neutralized with sodium hydroxide and the solution was diluted to 0.5 *M* with phosphate buffer of pH 7.4 ($\mu = 0.5$). Ethyl acetate (Kebo AB, Solna, Sweden) was distilled before use.

Methods

Determination of extraction constant. The partition experiments were performed with equal phase volumes of methylene chloride and aqueous phosphate buffer of pH 11 ($\mu = 0.1$) using an equilibration time of 30 min in a thermostated water-bath ($25 \pm 0.1°$). The concentration of tetrabutylammonium in the aqueous phase was $5\text{–}50 \cdot 10^{-4}$ *M* and that of saccharin $5.5\text{–}55 \cdot 10^{-4}$ *M*. After separation of the phases, the concentration of saccharin as the anion was determined photometrically in the aqueous phase. The extraction constant was calculated as described elsewhere[17].

Determination of saccharin in urine. The urine sample (4.0 ml) is mixed with 1.0 ml of tetrabutylammonium (0.5 *M*) in buffer of pH 7.4. The solution is shaken for 1 h with a mixture of 0.2 ml of methyl iodide and 0.3 ml of methylene chloride containing N-propylsaccharin (10 μg/ml). An aliquot (10 μl) of the organic phase is transferred into a tube containing 100 μl of ethyl acetate. After evaporation to dryness by a stream of nitrogen, 1.0 ml of ethyl acetate is added and the solution is shaken with saturated silver sulphate solution for 10 min. A 1–4-μl portion of the organic phase is injected into the gas chromatograph.

RESULTS AND DISCUSSION

Extraction of saccharin

Saccharin can be extracted from acidic aqueous solutions into diethyl ether[8] or ethyl acetate[4,6]. The extracts were shown to contain many of the acids normally present in urine[4] and a purification step has to be included before quantitation by gas chromatography with flame-ionization detection.

Saccharin can also be extracted in anionic form as an ion pair with quaternary ammonium ions[18]. The extraction constant of saccharin with tetrabutylammonium as the counter ion was found to be $10^{3.1}$. Saccharin can thus be quantitatively extracted into methylene chloride from a 0.1 *M* solution of tetrabutylammonium using equal phase volumes[19].

Reaction conditions

The high electron-capture response of saccharin after methylation has been demonstrated previously[8,10]. In the extractive alkylation procedure, methyl iodide was used as alkylating reagent, as it has shown to react rapidly with sulphonamides[20]. The time course of the reaction with two different concentrations of methyl iodide is shown in Fig. 1. As the rate of reaction of saccharin is slow compared with most other sulphonamides[20] a high concentration of methyl iodide was used.

A low pH in extractive alkylation procedures helps to prevent hydrolysis of the alkylating reagent[21] and the derivative formed. The acid dissociation constant of

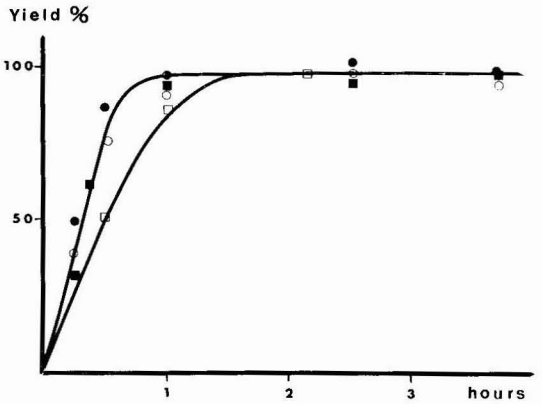

Fig. 1. Time course of the methylation of saccharin. Organic phase (1.0 ml): methylene chloride. Aqueous phase (5.0 ml): 0.1 *M* tetrabutylammonium in buffer (μ = 0.1). ●, pH 7.4, 6 *M* methyl iodide; ○, pH 7.4, 6 *M* methyl iodide, urine present; ■, pH 12, 6 *M* methyl iodide, urine present; □, pH 12, 3 *M* methyl iodide present. Urine constituted 80% of the organic phase.

saccharin ($pK_a = 2.5$) created possibilities for mild conditions in the derivatization step. The rate of methylation of saccharin was the same at pH 7 and 12, as can be seen from Fig. 1. The influence of urine was insignificant. The methyl derivative of saccharin was stable for at least 6 h under the reaction conditions used.

Identity of formed derivatives

Methylation of saccharin with diazomethane has been shown to give a mixture of N- and O-methylsaccharin[6,8,22]. A small amount of 2-methoxycarbonylbenzenesulphonamide, formed through ring fission, has also been found in the reaction mixture[22]. On the other hand, methylation with methyl iodide in dimethyl sulphoxide gave only the N-methyl derivative[8].

Extractive methylation of saccharin gave the N-methyl derivative. The mass spectrum was in good accordance with that obtained by Couch *et al.*[4]. Fragments from an O-methyl derivative could not be detected in the mass spectrum.

Electron-capture response

In a separate study, the sulphonamide moiety was shown to have inherent electrophoretic properties[23]. The minimum detectable concentrations[24] of N-methyl- and N-propylsaccharin were 2.5×10^{-16} and 2.8×10^{-16} mole/sec, respectively. These levels correspond to about 2 pg of derivative injected on a column with 1600 theoretical plates and with a retention of 5 min.

Purification of the reaction mixture

Injection of the organic phase from the methylation reaction directly into the gas chromatograph was not possible as methyl iodide and methylene chloride seriously affected the electron-capture detector. Evaporation was used to remove methyl iodide and methylene chloride.

Tetrabutylammonium iodide is formed as a side-product in the reaction and its degradation products formed in the injector caused long tailing fronts in the chro-

matogram. It was easily removed by extraction with aqueous silver sulphate solution[25].

N-methylsaccharin is quantitatively retained in the organic phase.

Determination of saccharin in urine

The present method was used in the determination of saccharin in urine. Investigations of the fate of saccharin in different animals have been undertaken[6,26,27]. Only small fractions are metabolized, the main fraction being the hydrolysis product 2-sulphamoylbenzoic acid[27]. Its methylation product does not have the same retention as N-methylsaccharin and its electron-capture response is low.

N-Propylsaccharin added directly to methylene chloride was found to be satisfactory as an internal standard as no structurally related compounds could be found that reacted similarily (*e.g.*, phthalimide).

The absolute recovery and precision of the method was $97 \pm 6\%$ ($n = 6$) at the 0.5 μg/ml level as compared to a dilution of crystalline N-methylsaccharin.

A chromatogram from an analysis of 1.2 μg/ml of saccharin in urine is shown in Fig. 2. Saccharin has been detected at concentrations down to 10 ng/ml of urine with this method.

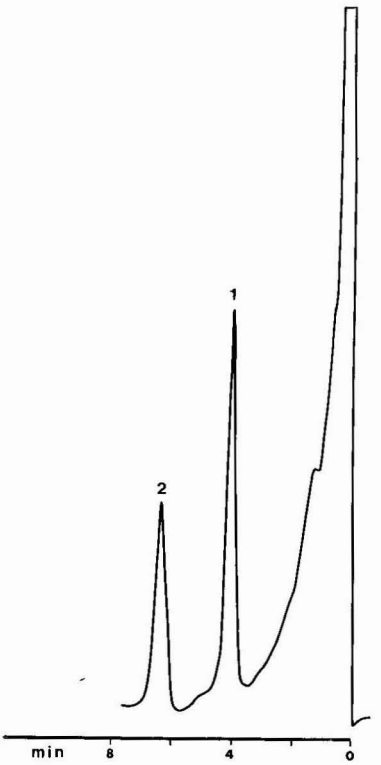

Fig. 2. Gas chromatogram from an analysis of 1.2 μg/ml of saccharin in urine. 1, Saccharin as N-methyl derivative (95 pg injected); 2, internal standard (N-propylsaccharin, 60 pg injecteed).

ACKNOWLEDGEMENTS

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CHROM. 10,616

Note

High-performance liquid chromatographic analysis of 5-fluorouracil in plasma

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5-Fluorouracil (FU) remains an important antineoplastic agent, more than 20 years after its introduction into clinical medicine[1–3]. It is used primarily for the treatment of solid tumours of the breast, colon and rectum, and for disseminated disease, mainly in combination with other chemotherepeutic agents designed to be synergistic in cytotoxicity while minimizing serious side-effects and toxicities[4]. Although FU exerts its anticancer activity following metabolic activation to 5-fluorodeoxyuridine monophosphate (FdUMP) intracellularly, the measurement of free FU plasma concentrations remains the most reasonable clinical pharmacological approach for studying individual variation in metabolism and response.

Several analytical methods have been reported for FU in plasma. We have reported[5] a gas chromatographic procedure, following silylation of FU, that is sensitive to 0.3 μg/ml in plasma and that is applicable to disposition studies following typical bolus dose administration. Others workers[6–8] haved described various gas chromatography–mass spectrometry methods with lower limits of sensitivity (1–25 ng/ml). These have been used mainly to detect FU at considerable time after bolus doses or prolonged intravenous infusion. Retention time data for ftorafur, FU and several metabolites have been reported for a reversed-phase high-performance liquid chromatographic (HPLC) system, but no analytical details were given[9]. In the present paper we report a rapid HPLC assay for FU using a strong anion-exchange column sensitive to 100 ng/ml in plasma.

MATERIALS AND METHODS

A Waters ALC Model 202 liquid chromatograph (Milford, Mass., U.S.A.) equipped with a UK-6 injector and a Model 440 UV detector was used for the analyses. Chromatography was performed on a 30 cm $\times$ 2.0 mm I.D. Aminex A-25 strong anion-exchange column (Bio-Rad, Richmond, Calif., U.S.A.), with a mean particle diameter of 17.5 $\pm$ 2 μm packed at 3000 p.s.i., at a flow-rate of 1.0 ml/min. The mobile phase was 0.3 *M* acetate buffer (pH 4.5) at a flow-rate of 0.7 ml/min, and the column was heated to 30° by a regulated water jacket. The mobile phase was degassed by sonicating for 30 min, and the detector wavelength was 254 nm.

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FU was obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, Md., U.S.A.) and 5-chlorouracil (CU), used as the internal standard, was obtained from K & K Laboratories (Irvine, Calif., U.S.A.). Distilled water was purified by passing it through a reverse osmosis four-filter system (Millipore, Bedford, Mass., U.S.A.). All other reagents and chemicals were of analytical reagent grade.

Plasma samples were either from patients receiving 15 mg/kg bolus doses weekly or from normal human pools. The analytical procedure involved the addition of 10 μg of CU and 1.0 ml of saturated ammonium sulphate to a 1.0-ml plasma sample, followed by rapid shaking for 30 min with 6 ml of ether–*n*-propanol (80:20). The organic layer was removed and evaporated under a stream of filtered air at 60°. The residue was reconstituted in 100 μl of a 0.005 *M* K_2HPO_4 solution (pH 9.8) and 5–25 μl were injected. Quantitative determination in the 100–300 ng/ml range required purification on a 3-cm Sephadex LH-20 (Pharmacia, Piscataway, N.J., U.S.A.) column. FU and CU were eluted with 0.005 *M* K_2HPO_4 (pH 9.8), which was then evaporated and reconstituted as above. Peak height ratios (FU/CU) were used to produce standard curves from spiked pooled plasma and aqueous samples.

RESULTS AND DISCUSSION

Typical chromatograms for a patient sample and a plasma blank are shown in Fig. 1. Retention times for FU and CU were 8.0 and 17.0 min, respectively, under the conditions described. Standard curves from spiked pooled plasma were found to be linear from 0.5 to 40.0 μg/ml (Fig. 2), and the lower limit of sensitivity employing the Sephadex LH-20 column clean-up and a 20-μl injection volume was 100 ng/ml. Typical variation in the analysis of duplicate specimens was found to be of the order of 5–10%, and a 1.0 μg/ml plasma standard had a relative standard deviation of 7.5% when run six times on separate occasions.

Because the high polarity of FU requires a highly polar organic solvent that remains immiscible with the aqueous phase for extraction, the clean-up of samples by

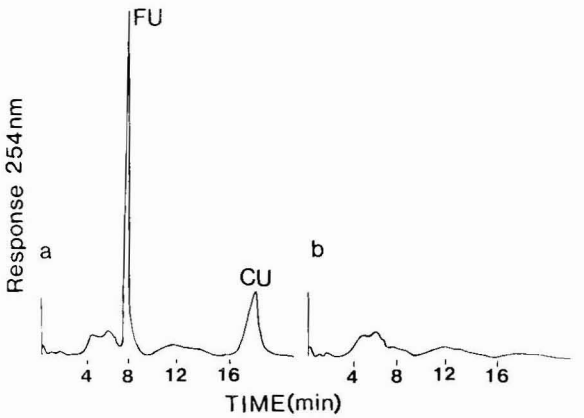

Fig. 1. High-performance liquid chromatograms from (a) a patient receiving a 15 mg/kg bolus intravenous dose of FU and (b) from an extracted patient blank.

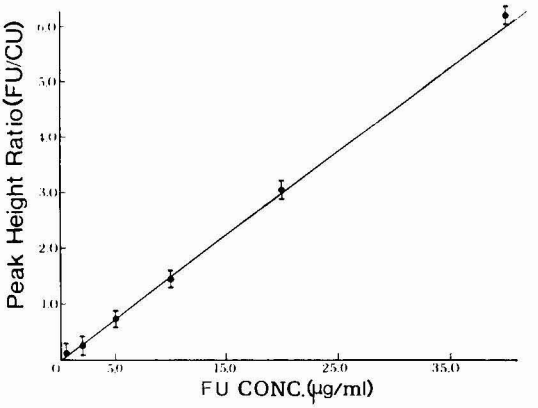

Fig. 2. Standard curve obtained from pooled plasma samples spiked with FU.

extraction is difficult and tedious. Although the chromatogram is generally free from interference at the point of FU elution, the expanded scale required for the detection of concentrations of less than 1 μg/ml causes the FU peak to appear as a shoulder on the interference peak, eluting at *ca.* 6.8 min. The Sephadex column clean-up procedure, although somewhat more time-consuming, eliminates this interference and allows quantitation of levels down to 100 ng/ml. Relative to unextracted standard curves the extraction procedure is *ca.* 80% and 94% efficient for FU and CU, respectively, whereas Sephadex chromatography results in the loss of an additional 10% of each component. pH control was critical for the separation, with lower pH markedly decreasing the retention time and higher pH causing peak broadening. The mildly elevated temperature dramatically sharpened the FU peak to allow increased sensitivity.

The method is somewhat more sensitive than the gas chromatographic procedure previously reported[5]. it is suitable for the routine determination of FU following intravenous or oral bolus dose therapy, and in the support of clinical pharmacology studies of FU up to its sensitivity limitations. Sensitivity could perhaps be

TABLE I

COMPARISON OF GAS AND LIQUID CHROMATOGRAPHIC ANALYSIS OF FLUOROURACIL PLASMA TIME COURSE

The gas chromatographic procedure was a slight modification of that used by Cohen and Brennan[5].

*Time (min)**	*FU concentration (μg/ml)*	
	*GLC***	*HPLC***
0	—	—
5	46.8	52.5
10	30.7	32.3
15	28.9	26.5
60	3.3	4.4
90	0.2	0.5

* Time following 1.0 g (15 mg/kg) bolus intravenous dose.

** Each concentration represents an average of two determinations.

improved two-fold if a continuously variable UV detector were available to allow monitoring at 266 nm, the λ_{max} of FU[10]. Analysis by both methods of samples from patients following 15 mg/mg doses produced comparable results (Table I).

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CHROM. 10,594

Note

Die fluoreszenzspektroskopische Bestimmung des Phytoalexins 3-Methyl-6-methoxy-8-hydroxy-3,4-dihydroisocumarin nach Abtrennung durch Gelfiltration

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Die Bildung von 3-Methyl-6-methoxy-8-hydroxy-3,4-dihydroisocumarin (MMHD) in der Möhrenspeicherwurzel[1] wird durch Pilzbefall[2], Verletzung, niedrige Lagertemperatur[3] und Einwirkung von Äthylen[4] u.a. Wuchsstoffen[5] ausgelöst.

MMHD wird für den Bittergeschmack bei gelagerten Möhren verantwortlich gemacht, obwohl es scheint, dass noch andere Verbindungen beteiligt sind.Gegenüber dem Säugetierorganismus weist es nur schwach toxische Eigenschaften auf[6]. Seine Einstufung als Phytoalexin beruht auf seiner stark fungistatischen Wirkung[2,7].

Lagerungsversuche mit Möhren gebräuchlicher deutscher Sorten sollten über die MMHD-Bildung bei Kaltlagerung, Verletzung und nach Pilzbefall Auskunft geben (darüber wird demnächst an anderer Stelle publiziert). Die dafür ausgearbeitete fluoreszenzspektroskopische Bestimmungsmethode nach Abtrennung des MMHD vom störenden β-Carotin durch Gelfiltration soll hier vorgestellt und die Ergebnisse mit den nach den üblichen UV-spektroskopischen Methoden[3,6–8] gewonnenen verglichen werden.

MATERIAL UND METHODEN

Extraktion

Eine wägbare Menge an MMHD für die Eichlösungen wurde aus Möhren gewonnen, die im Boden überwintert hatten. Wie auch bei den quantitativen MMHD-Bestimmungen (mit 50 g Frischsubstanz) wurde die Frischsubstanz in Gegenwart von 200 ml *n*-Hexan mit dem Ultra-Turrax homogenisiert und mehrere Stunden geschüttelt. Nach dem Absaugen über eine Filternutsche wurde die Prozedur noch einmal wiederholt. Von aliquoten Teilen (meistens 1/10) wurde im Vakuum-Rotationsverdampfer das *n*-Hexan abdestilliert und die Rückstände zweimal mit 2 ml Äthanol behandelt. Nach dem Abzentrifugieren des unlöslichen Anteils gelangten 2 ml Überstand zur chromatographischen Reinigung.

Gelfiltration

Die Trennung erfolgte an Sephadex LH 20 mit Äthanol als Elutionsmittel. Der Beladbarkeit der Säule waren durch die Anwesenheit des grossen β-Carotin-Überschusses Grenzen gesetzt ($\triangleq$ 5 g Möhrenfrischsubstanz).

Kieselgel-Säulenchromatographie

Zur Gewinnung einer befriedigend reinen MMHD-Menge für die Eichlösungen und zur Überprüfung der Ergebnisse von den verschiedenen Bestimmungsmethoden wurde der Sephadex LH 20 eine Kieselgel-Säulenchromatographie[9] [mit Kieselgel Merck (< 0.08 mm) und 1.5% Aceton in Chloroform] nachgeschaltet. Dabei konnten alle Begleitkomponenten, ausser einem Restgehalt an Eugenin (5-Hydroxy-7-methoxy-2-methylchromon), abgetrennt werden.

Dünnschichtchromatographie

Die Dünnschichtchromatographie erfolgte auf Kieselgel G-Schichten mit dem Fliessmittel Toluol–Äthylformiat–Ameisensäure (5:4:1)[7]. Hierdurch war der Nachweis des Eugenin möglich, das oft das MMHD begleitet. Da das Eugenin im Gegensatz zum MMHD nicht zur Fluoreszenz angeregt werden kann, wurde es durch Anfärbung mit diazotierter Sulfanilsäure nachgewiesen.

Fluoreszenzmessung

Die Fluoreszenzmessungen erfolgten in äthanolischer Lösung mit dem Perkin-Elmer Fluorescence Spectrophotometer 204 bei 455 nm. Als Anregungswellenlänge waren sowohl 273 als auch 305 nm brauchbar.

UV-Absorptionsmessung

Sie wurden mit dem Spektralphotometer PMQ II (Carl Zeiss, Oberkochen, B.R.D.) durchgeführt. Bei der Auswahl von Lösungsmittel und Wellenlänge richteten wir uns nach den zum Vergleich dienenden Nachweismethoden.

ERGEBNISSE UND DISKUSSION

Die Eichlösung wies das für das MMHD charakteristische UV-Absorptionsspektrum auf ($\lambda_{max.}$ 216, 267, 300 nm), obwohl das Dünnschichtchromatogramm noch Eugenin-Beimengungen anzeigte. Durch präparative dünnschichtchromatographische Trennung, Isolierung und Messung der Extinktionen konnten über die molaren Extinktionskoeffizienten[6] die Anteile an beiden Verbindungen in der Eichlösung ermittelt werden.

Die Verunreinigung an Eugenin ($\lambda_{max.}$ 211, 229, 249, 256, 290 nm) betrug 10.3%. Die Fluoreszenzmessung wird durch das Eugenin nicht gestört, bei den üblichen UV-Absorptionsmessungen bei 265–267 nm trägt aber der Eugenin-Anteil zur gemessenen Extinktion bei, obwohl sein Spektrum in diesem Bereich ein Minimum aufweist. Da bei den bisher angewandten Bestimmungsmethoden für das MMHD seine Anreicherung lediglich durch die teilweise Abtrennung der Störsubstanzen erfolgte, haben noch andere Komponenten zur Vortäuschung von MMHD beigetragen.

Fig. 1 zeigt die Abtrennung des β-Carotins durch Gelfiltration, das ein starker Quencher ist und deshalb vor der Fluoreszenzmessung quantitativ abgetrennt werden muss. In Fig. 2 ist das Anregungsspektrum von MMHD mit seinen Maxima bei 273 und 305 nm wiedergegeben, das Emissionsmaximum liegt bei 455 nm.

Die Ergebnisse von den verschiedenen Bestimmungsmethoden nach jeweils

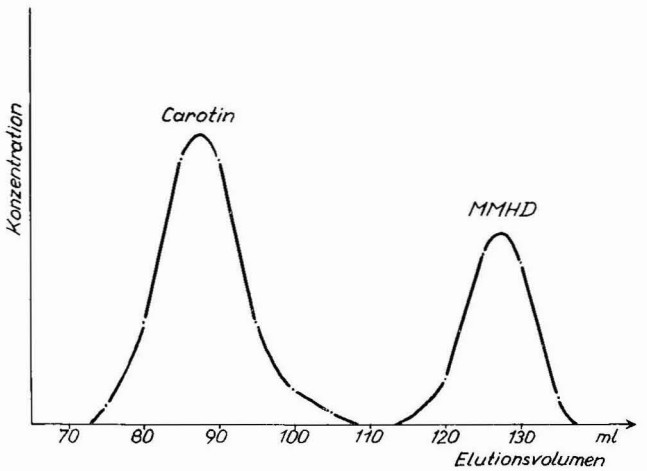

Fig. 1. Trennung von MMHD und β-Carotin durch Gelfiltration an Sephadex LH 20. Säule, 30 cm × 2.1 cm I.D.; Eluant, Äthanol; Durchfluss, 40 ml/h.

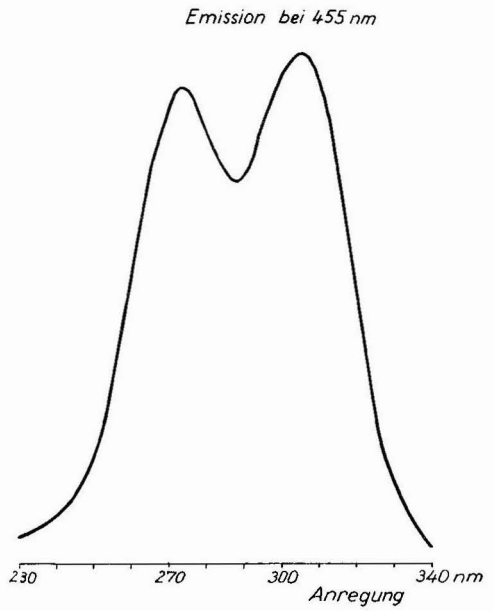

Fig. 2. Fluoreszenz-Anregungsspektrum von MMHD in äthanolischer Lösung.

gleichem Reinigungsschritt sind in Tabelle I gegenübergestellt. Drei Möhrenproben (Nr. 1–3) mit stark unterschiedlichen Gehalten waren dabei zur Analyse gelangt.

Nachweismethode A (Tabelle I) liefert bei Messung des Rohextraktes völlig falsche Ergebnisse. Die empirisch ermittelte Berechnungsformel nach Extinktionsmessung bei drei verschiedenen Wellenlängen liefert nur bei sehr hohen MMHD-Gehalten und Carotinabtrennung befriedigende Ergebnisse. Selbst nach der Kieselgel-Säulenchromatographie sind die Ergebnisse von Proben mit niedrigen Gehalten un-

TABELLE I

VERGLEICH DER ERGEBNISSE, DIE NACH DREI VERSCHIEDENEN METHODEN ZUR MMHD-BESTIMMUNG GEWONNEN WURDEN: (A)[3,8] UV-ABSORPTIONSMESSUNG BEI 240, 265 und 290 nm, (B)[6,7] UV-ABSORPTIONSMESSUNG BEI 267 nm UND (C) FLUORESZENZMESSUNG BEI 455 nm NACH ANREGUNG BEI 273 ODER 305 nm

Methode	*Proben-Nr.*	*MMHD-Gehalt (mg) pro 100 g Frischsubstanz*		
		Messung des n-Hexan-Extrakts	*Messung nach Sephadex LH 20-Chromatographie*	*Messung nach Sephadex LH 20- und Kieselgel-Chromatographie*
A	1	12.23	0.07	0.04
	2	10.52	2.85	1.18
	3	21.51	9.77	9.42
B	1		0.54	0.24
	2		3.96	1.58
	3		10.70	9.50
C	1		0.14	0.15
	2		1.41	1.33
	3		9.39	9.08

befriedigend. Nachweismethode B liefert in jedem Falle zu hohe Werte, die Abweichungen sind aber bei hohen Gehalten noch akzeptabel. Die fluoreszenzspektroskopische Methode (Nachweismethode C) ist dagegen über einen weiten Konzentrationsbereich anwendbar, wenn zuvor das β-Carotin durch Sephadex LH 20-Chromatographie quantitativ abgetrennt wird. Auf die zeitaufwendige Kieselgel-Säulenchromatographie kann in diesem Falle verzichtet werden.

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Note

Electrophoretic, chromatographic and mass spectrometric procedures for the identification and isotopic assay of amino acid constituents in etamycin

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Etamycin is a peptidolactone antibiotic produced by *Streptomyces griseoviridus*, *S. griseoroseus* and other Streptomyces species[1,2]. The complete structure has been elucidated[3] and the antimicrobial activity described[1,4]. In the earlier studies, the amino acid constituents were identified by classical techniques involving isolation of crystalline hydrolysis products[3,5]. These procedures are unsuitable for biosynthetic investigations and, therefore, appropriate methodology was sought. The present paper describes the use of electrophoretic, paper, thin-layer, ion-exchange and gas chromatographic–mass spectrometric (GC–MS) procedures which provide rapid and effective techniques for the separation of the amino acid constituents in etamycin hydrolysates.

EXPERIMENTAL

Apparatus

High-voltage paper electrophoresis was effected with an apparatus from Gilson Medical Electronics, Middleton, Wisc., U.S.A., using 4% formate buffer, pH 1.9, at 200 mA and 3600 V for 3 h. The amino acid analyzer was a Beckman-Spinco Model 120C, with 0.2 *M* sodium citrate buffer, pH 3.05 and 4.25, flow-rate 34 ml/h. For gas–liquid chromatography (GLC), a Shimadzu Model 4BM gas chromatograph equipped with flame ionization detectors was employed with argon (40 ml/min) as carrier gas. The column was glass (2 m × 3 mm I.D.), packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh). Combined GC–MS was performed on a LKB 9000 instrument equipped with a 6 ft. column of 1% OV-17. A refrigerated liquid scintillation spectrometer (Mark I; Nuclear-Chicago, Des Plaines, Ill., U.S.A.) was employed for radioactivity measurements.

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Materials and methods

Etamycin was obtained by fermentation with *S. griseoviridus* in a chemically defined medium[6] and it was also supplied by Dr. H. Dion (Parke, Davis Co., Detroit, Mich., U.S.A.). L-Alanine, L-threonine, sarcosine, D-leucine and *allo*-hydroxy-D-proline were obtained from commercial sources. Phenylsarcosine, N,β-dimethyl-leucine and 3-hydroxypicolinic acid were kindly supplied by Drs. J. Sheehan and D. Ponzi (Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.) and Dr. L. C. Vining (Dalhousie University, Halifax, Nova Scotia, Canada).

Etamycin samples were hydrolyzed in 6 *N* HCl for 3 h at 15 p.s.i., 121° in a PTFE-lined, screw-capped test-tube. After evaporation *in vacuo*, the residual amino acids were treated as described below. For two-dimensional paper electrophoresis–paper chromatography, amino acids were first separated by high-voltage electrophoresis, as noted above, followed by ascending chromatography with the solvent systems: (a) 1-butanol–acetic acid–water (4:1:5) and (b) methanol–pyridine–water (20:1:5). The thin-layer (Sil Gel 1B-Baker flex, J. T. Baker, Phillipsburgh, N.J., U.S.A.; or silica gel 60 F-254, Merck, Darmstadt, G.F.R.) chromatography (TLC) systems were (1) for *allo*-hydroxyproline, 1-propanol–water–methanol (7:3:2) and (2) for 3-hydroxypicolinic acid, ethanol–water–ammonia (90:5:5). Amino acids were rendered visible with ninhydrin and isatin reagents. 3-Hydroxypicolinic acid was detected under UV light.

For gas–liquid chromatography the authentic amino acids and the dried etamycin hydrolysate were derivatized by conversion to their trifluoroacetylated (TFA) methyl esters[7] and N-formyl methyl esters[8] as described previously. The column temperature was initially 80° with a 4°/min temperature program (for TFA methyl esters) or isothermally at 150° (for N-formyl methyl esters). In the GC–MS apparatus similar methods were employed except for an initial temperature of 60° (for TFA methyl esters) and 115° (for N-formyl methyl esters).

In the investigation of the distribution of radiolabel from [$^{14}CH_3$]L-methionine during biosynthesis of etamycin, *S. griseoviridus* was cultivated first in glucose–yeast extract–malt extract liquid medium and then in a chemically defined medium for 24 h (each) prior to addition of the ^{14}C-labeled precursor[9]. Incubation was continued for 60 min and the etamycin was recovered by extraction of the culture filtrate with benzene. Details of this procedure will be published elsewhere. The etamycin was hydrolyzed as described above, and after paper electrophoresis in parallel with standard amino acids (located with ninhydrin reagent), the individual components were cut out, placed in counting vials, moistened with water (0.1 ml) and counted in Bray's scintillation fluid[10] (10 ml).

RESULTS AND DISCUSSION

The results of two-dimensional paper electrophoresis (PE)–paper chromatography (PC 1 and PC 2) are shown in Table I, together with data from the amino acid analyzer (AAA) and thin-layer chromatography (TLC). Difficulties were encountered in the visualization of 3-hydroxypicolinic acid, which is weakly fluorescent, after PC (second dimension). However, it could be identified on PE (1 or 3 h) or TLC. The latter technique also served to distinguish *allo*-hydroxyproline (R_F = 0.26) from its diastereoisomer (hydroxyproline, R_F = 0.34). The two-dimensional

TABLE I

ELECTROPHORETIC AND CHROMATOGRAPHIC PROPERTIES OF HYDROLYSATE AMINO ACIDS OBTAINED FROM ETAMYCIN

Amino acid	*PE (ref. to sarcosine = 1.00)*	*PC1* (R_F)*	*PC2** (R_F)*	*AAA (min)*	*TLC*** (R_F)*	*GC (min)*	*GC–MS (min)*
L-Alanine	1.08	0.30	0.60	278	—	2.5	1.1
Sarcosine	1.00	0.25	0.60	177	—	5.1	2.3
D-Leucine	0.84	0.57	0.81	362	—	6.2	3.3
L-Threonine	0.82	0.25	0.59	150	—	3.4	2.0
allo-Hydroxy-D-proline	0.76	0.19	0.55	150	0.26	15.7	11.3
N,β-Dimethyl-L-leucine	0.60	0.67	0.91	296	—	6.4[§]	3.0[§]
Phenylsarcosine	0.45	0.55	0.80	320	—	20.2	13.9
3-Hydroxypicolinic acid	0.19[§§]	—	—	—	0.64	—	—

* Solvent = butanol–acetic acid–water (4:1:5), upper phase.

** Solvent = methanol–pyridine–water (20:1:5), 6 h.

*** For *allo*-hydroxyproline, solvent = 1-propanol–water–methanol (7:3:2); for 3-hydroxypicolinic acid, solvent = ethanol–water–ammonia (90:5:5).

[§] Retention times for N,β-dimethylleucine refer to the N-formyl methyl ester; all others refer to trifluoroacetylated methyl esters.

[§§] Electrophoresis for 1 h.

paper electrophoresis–chromatography procedure was necessitated by the overlap of leucine and threonine (PE) and of threonine and *allo*-hydroxyproline (AAA).

GC retention times (min) for the TFA amino acid methyl esters are also presented in Table I. N,β-Dimethylleucine was not detected by this procedure due to steric hindrance during esterification, and was identified instead as the N-formyl methyl ester (prepared with diazomethane). GC–MS data (not shown) of the deriva-

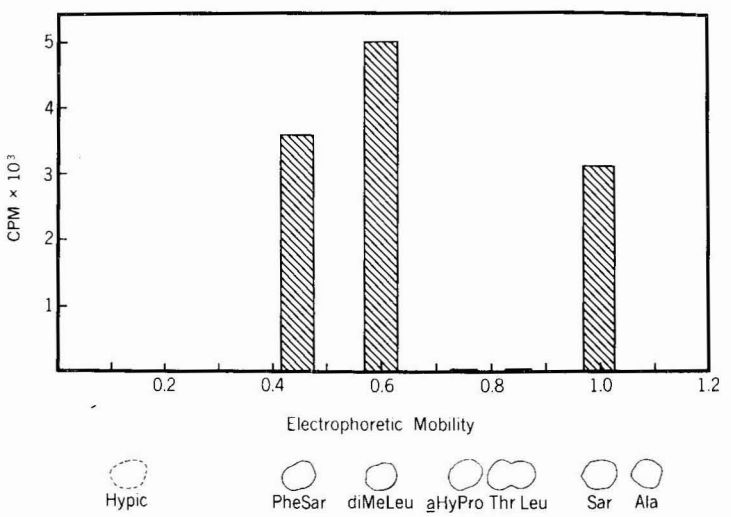

Fig. 1. Paper electrophoretic demonstration of distribution of radiolabel from [$^{14}CH_3$]L-methionine in etamycin hydrolysate.

tized amino acids from an etamycin hydrolysate are virtually identical with those obtained with standard amino acids. In the GC studies, the *allo*-hydroxyproline derivative is the N,O-bis(trifluoroacetyl) methyl ester.

As an example of the application of one of the above separation techniques to biosynthetic studies on etamycin, the distribution of radiolabel from [$^{14}CH_3$]L-methionine into the antibiotic components[6,9] was demonstrated after hydrolysis by means of PE (Fig. 1). The data reveal that the methyl group of methionine is incorporated selectively into sarcosine, N,β-dimethylleucine and phenylsarcosine.

In addition to the use of radioisotopes in conjunction with PE and PC, the GC–MS procedures outlined above could be applied with stable isotopes to biosynthetic labeling studies. The latter approach has been utilized in an investigation of the biogenesis of sarcosine in several actinomycins[8]. Due to the separations achieved, these procedures would also be suitable for studies of directed biosynthesis[11] using analogs of the amino acids present in etamycin.

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Note

Detection of aminophenols, aromatic amines and related compounds on thin-layer plates

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Aminophenols are frequently formed when compounds of industrial or pharmacological importance, such as aniline[1] or prilocaine[2], are metabolised in mammals by the liver microsomal mixed function oxidase systems. Although these metabolites are important, and may exert physiological effects such as methaemoglobin formation[2], their identification is often difficult, since *o*-, *m*- and *p*-isomers often have similar R_F values. We have therefore developed colour reactions to distinguish between them and also to give characteristic colours with related compounds.

EXPERIMENTAL

Thin layer chromatography (TLC) of compounds was carried out on plates coated with silica gel G (0.3 mm thick; Merck, Darmstadt, G.F.R.)[2], 0.5–25 μl of 0.1% solutions being applied.

(a) A freshly prepared 3% aqueous solution (w/v) of selenium dioxide (I) is sprayed onto a dried TLC plate which is then heated at 120° for 15–20 min.

(b) A freshly prepared 1% solution of phenol in 20% Na_2CO_3 (II) is sprayed and the TLC plate heated for 20–25 min at 120°. Overspraying with concentrated HCl may give a colour change immediately or up to 48 h later.

Both systems give coloured spots on a white ground.

RESULTS

The colour reactions found are listed in Table I. Amino acids gave negative results with both sprays. The limits of detection after development were 1–2 μg for all compounds tested. The chromatospots from spray I turned brown-black after a few days, possibly due to decomposition with release of elemental selenium.

DISCUSSION

Selenious acid in sulphuric acid was introduced by Mecke[3] for the detection of opium alkaloids. Since then, it has found little use as a detecting agent, apart from some steroids[4] and phenothiazines[5]. It is known that phenyl selenoxides can be formed[6,7] and that selenium dioxide can react with certain amino group derivatives

TABLE I

COMPOUNDS INVESTIGATED AND OBSERVED COLOUR REACTIONS WITH THE SPRAYS TESTED

Compound	*Spray I*		*Spray II*	
	Initial colour	*After heat*	*After heat*	*HCl overspray*
o-Aminophenol	pale yellow	yellow	dark yellow	peach
m-Aminophenol	—	dark brown	rust-brown	brown
p-Aminophenol	—	purple	blue	red
o-Anisidine	—	purple		lilac
m-Anisidine	—	purple-brown		peach
p-Anisidine	—	light brown		red
o-Aminobenzoic acid	—	rust-brown		
m-Aminobenzoic acid	—	light brown		
p-Aminobenzoic acid	—	lime-green	—	—
3,5-Diaminobenzoic acid	buff	dark brown-purple	light pink	buff
2,4-Dimethoxyaniline	—	maroon	—	purple
2,5-Dimethoxyaniline	—	black-brown	—	green-brown
3,5-Dimethoxyaniline	—	dark brown, yellow halo	—	yellow
2,4-Dichloroaniline	—	blue-purple	—	buff
2,5-Dichloroaniline	—	cream-yellow	—	mustard
3,4-Dichloroaniline	—	mauve	light blue	buff
2-Amino-5-hydroxybenzoic acid	—	light brown-purple	light blue	dark brown
3-Amino-2-hydroxybenzoic acid	—	gold	—	cream
3-Amino-4-hydroxybenzoic acid	yellow	yellow-black	green	brown
2-Amino-1,3-dimethylbenzene	—	purple		light red
2-Amino-1,4-dimethylbenzene	—	green, purple halo	—	light green
3-Amino-1,2-dimethylbenzene	—	rust-brown	—	light green
4-Amino-1,2-dimethylbenzene	—	mustard green	—	yellow
4-Amino-1,3-dimethylbenzene	light brown	mustard, purple halo	—	mauve
2-Amino-5-nitrotoluene	bright yellow	bright yellow	bright yellow	bright yellow
4-Amino-2-nitrotoluene	yellow	lime-green	yellow	cream-yellow
4-Amino-3-nitrotoluene	dark yellow	dark yellow	orange-yellow	deep yellow
Aniline	—	brown-green	—	—
Phenol	—	—	—	—
Eugenol	—	orange-peach	buff	buff
Orcinol	—	rust-brown	pink	cream
Resorcinol	—	brown	khaki	grey-buff
Metol	light brown	rust	blue-grey	brown
Pyrocatechol	buff	dark brown	dark grey	dark brown
Pyrogallol	light brown	brown	brown-yellow	brown-yellow
α-Naphthol	—	green-grey	grey	brown
β-Naphthol	beige	yellow-grey	brown	brown
Cinchonine	—	orange	—	pink
Brucine	—	orange-yellow	—	—
Hydrazine	—	tangerine	—	—
Phenylhydrazine	light yellow	mustard	light yellow	beige

such as hydrazines and phenylhydrazines[8]. Under these conditions, hydrazine[9] and phenylhydrazine[10] are oxidised, as is ascorbic acid, which easily reduces selenious acid[11,12]; the colours found using our spray presumably indicate formation of red elemental selenium, since these compounds gave characteristic tangerine or orange spots. Reducing sugars, such as glucose, were not found to react, in agreement with earlier work[13]. Phenol similarly gave no visible reaction, which was not unexpected,

since early work[14] showed that a colourless product was obtained when phenol was treated with acidic selenium dioxide. Nitrophenols also show no colour change[6], although polyphenols, such as resorcinol, do give coloured products.

The range of colours found by reacting aqueous selenium dioxide with aminophenols, some aromatic amines and related compounds has not previously been reported. A similarly extensive range of colours was found using spray II, which is based on the colorimetric test for *p*-aminophenol described by Brodie and Axelrod[15], and later modified by other workers[2,16]. In this reaction, *p*-aminophenol gives a blue coloration in the presence of phenol and carbonate ions, due to the formation of indophenol. The *o*- and *m*-aminophenols give yellow and buff coloured solutions respectively. It is interesting to note that few compounds react with the alkaline phenol reagent even after heating, although on overspraying with concentrated HCl many aromatic amines, phenols and derivatives give a variety of colours. Both sprays clearly differentiate between *o*-, *m*- and *p*-isomers, and also allow distinction between such similar compounds as 2-amino-1,3-dimethylbenzene and 2-amino-1,4-dimethylbenzene. It is hoped that this will prove useful in studies on the metabolism of aromatic amines, aminophenols and related compounds.

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CHROM. 10,583

Note

Dansylation of amines, phenolic and catecholic amines and amino acids in aprotic solvents

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Reagents which produce fluorescent derivatives have found widespread use in the qualitative and quantitative detection of amines, phenolic and catecholic amines and amino acids[1]. One of the most common of these reagents is 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride). Derivatizations using dansyl chloride are usually carried out in aqueous acetone saturated with sodium carbonate; the derivative is isolated by concentration of the reaction solution, extraction with ethyl acetate followed by thin-layer chromatography (TLC). In this short note a procedure is described for the dansylation of some amines, phenolic amines, catecholic amines and amino acids in aprotic organic solvents (*e.g.*, dimethylformamide (DMF), acetonitrile, acetone and ethyl acetate), in which "naked" fluoride anion, solubilized by means of 18-crown-6, activates amino and hydroxyl hydrogen atoms to displacement by the dansyl group. Derivatization of as little as 25 ng (200 pmole) in 10 μl of solvent is possible; the entire reaction mixture may be applied to TLC plates without preliminary concentration or extraction.

EXPERIMENTAL

Reagents and equipment

Dansyl chloride (Calbiochem, Los Angeles, Calif., U.S.A.), 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) (PCR Inc., Gainesville, Fla., U.S.A.), potassium fluoride and all solvents were used as obtained commercially without purification or drying. Pre-coated silica gel 60 thin-layer plates, 250 μm thick (E. Merck, Darmstadt, G.F.R.; distributed in Canada by BDH, Toronto) were used without prior treatment. Visualization of the dansyl derivatives on TLC plates was achieved by irradiation with long-wave UV light (565 nm) in a light box (Ultraviolet Products Inc., San Gabriel, Calif., U.S.A.). Mass spectra were recorded at 70 eV on an AEI-MS 902S mass spectrometer.

Dansylation procedure

Dansylating solutions were prepared by heating 18-crown-6 (40 mg/ml) and potassium fluoride (8 mg/ml) in DMF, acetonitrile, acetone, ethyl acetate and benzene followed by the addition of dansyl chloride (100 mg/ml) to the cold solution just before use.

p-Tyramine, 3-methoxytyramine, histamine, β-phenylethylamine, dopamine, dibenzylamine, N-methylbenzylamine, *n*-butylamine, aniline, *p*-octopamine, phenylethanolamine, normetanephrine, tryptamine, β-phenylalanine and γ-amino butyric acid were dansylated by the procedures described below.

Method 1. The amine, dissolved in water or ethanol, was introduced into a 0.3-ml Reacti-vial (Pierce, Rockford, Ill., U.S.A.) and evaporated to dryness in a stream of nitrogen. The dansylating solution (50–100 μl) was then added and the mixture heated at 65° in a heating block for 3 h. An aliquot (5–10 μl) of this reaction mixture was then applied to the origin zone of a TLC plate (alternatively the entire reaction mixture may be applied after being concentrated in a stream of nitrogen to *ca.* 25 μl) and developed in benzene–triethylamine (8:1). If the reaction solvent (*e.g.*, DMF) was unsuitable for easy transfer to the chromatograms it may be evaporated to dryness and dissolved in a more suitable solvent (*e.g.*, benzene).

Method 2. The amine, dissolved in acetonitrile, DMF, acetone or ethyl acetate, was introduced into a capillary tube (1.6–1.8 × 100 mm) sealed at one end, concentrated to *ca.* 5 μl and the dansylating solution (10 μl) added. The other end of the capillary was then sealed and the entire tube heated at 65° by submersion in a solvent bath. After 3 h the tube was removed, cooled and the end broken off. The contents after being removed by syringe were applied either in part or *in toto* to the origin zone of the TLC plate.

When the entire reaction mixture was applied to the TLC plates the excess dansyl chloride caused tailing which obscured the separated dansylamine zones. It was necessary in these cases therefore to separate in parallel authentic dansylamines in order to locate accurately the reaction product. The appropriate zones are then extracted with ethyl acetate and re-chromatographed. When only a small fraction of the reaction mixture was chromatographed overloading and tailing did not occur.

The yield of the derivatization reaction was determined for *p*-tyramine. Five different concentrations (6500, 2500, 650, 65 and 25 ng respectively) dissolved in alcohol were transferred to Reacti-vials, dried and derivatized in acetonitrile as described above in Method 1. Each reaction solution was then applied to the origin of a TLC plate. Superimposed on this zone was a known and similar quantity of [α,α-$^2H_2$]bis(dansyl)tyramine[2]. The plate was then developed in benzene–triethylamine (8:1) and the bis(dansyl)tyramine zones, outlined with a metal stylus, removed and placed in special capillary micro-columns and eluted with ethyl acetate as described by Philips *et al.*[2]. Quantitation was achieved by the mass spectrometric integrated ion current technique[2,3]. Yields in all cases were 50–60%.

The identities of the dansyl derivatives of all the compounds investigated were confirmed by comparison of their mass spectra with published spectra (see Table I) and by comparison of their R_F values [in benzene–triethylamine (8:1)] (see Table I) with those of authentic dansyl derivatives[3].

RESULTS AND DISCUSSION

"Naked" fluoride anion, generated from potassium fluoride and crown ethers in aprotic solvents, has been employed in a number of reactions[4–7]. In the dansylation reaction described here, the "electron-rich" fluoride anion probably acts by forming a very strong hydrogen bond with the amino and hydroxyl hydrogen atoms, thereby

TABLE I

R_F VALUES AND DETAILS CONCERNING THE MASS SPECTRA OF SOME DANSYL DERIVATIVES

Dansyl derivative	*R_F value**	*Reference to published mass spectra*
p-Tyramine (bis-dansyl)	0.51	3, 10
3-Methoxytyramine (bis-dansyl)	0.42	10
p-Octopamine (bis-dansyl)	0.17	3, 10
Tryptamine	0.19	3, 10
Histamine (bis-dansyl)	0.33	10
β-Phenylethylamine	0.62	3, 10
Dibenzylamine	0.65	—
N-Methylbenzylamine	0.77	3
n-Butylamine	0.56	10
Phenylethanolamine	0.26	3, 10
Normetanephrine (bis-dansyl)	0.11	3, 10
Dopamine (tris-dansyl)	0.47	10
Aniline	0.25	3
Phenylalanine	0.03	11
γ-Aminobutyric acid**	0.60	10, 11

* Solvent system: benzene–triethylamine (8:1).

** Cyclization to the lactam occurs during dansylation.

increasing the susceptibility of the amino nitrogen or hydroxyl oxygen to attack by the sulfur atom of dansyl chloride.

Dansylation occurred in DMF, acetonitrile, acetone and ethyl acetate, but not in benzene. Although reaction occurred most rapidly in DMF, the low volatility of this solvent rendered it unsuitable for convenient transfer to the TLC plates. Of the other three solvents studied, acetonitrile appeared to produce somewhat higher yields (based on visual comparison of the fluorescence of the zones on TLC plates).

Of the amines studied, four reacted poorly in the conditions described above. For example, about ten times more tryptamine than tyramine was required to give a spot of equal fluorescence, an even greater amount of the β-hydroxylated amines (phenylethanolamine, octopamine and normetanephrine), was required. This anomalous behaviour has not yet been explained.

Two papers by Dünges and co-workers[8,9] have described the dansylation in aprotic solvents (acetone or ethyl acetate) of some barbituric acids in the presence of potassium carbonate. Their method was not successful when applied to the amines listed in Table I. The explanation for this is probably that barbituric acids are very acidic and therefore much more susceptible to dansylation than the weakly acidic phenolic and amino groups.

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Note

Thin-layer chromatographic assay of tetracyclines

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In the last few years, several methods have been evolved for the thin-layer chromatography of tetracyclines[1–5]. In our laboratory, further attempts have been made to develop high-performance procedures for the separation, detection and assay of tetracyclines. A method using cellulose pre-treated with a stationary phase consisting of a buffer and an organic solvent proved to be advantageous. Of the impregnating agents examined, ethylene glycol exhibited the highest efficiency.

For the detection of tetracyclines, the possibility of fluorogen formation was studied. Detection in a strong ammonia atmosphere gives poor results in a quantitative assay, owing to the short duration of the colour obtained. Significantly better results can be achieved with the amines and metal salts proposed by Ragazzi and Veronese[4], but both the delayed formation of the fluorogen and the poor sensitivity of the compounds investigated are disadvantages. During studies with similar fluorogen-forming agents, it was found that by carrying out detection first with metal salts and then with organic solvents, fluorogens with relatively high intensities are formed. The procedure is described in this paper.

EXPERIMENTAL

Cellulose (mikrokristallin, E. Merck, Darmstadt, G.F.R.) (30 g) was suspended in a mixture of 0.2 *M* disodium hydrogen orthophosphate (70 ml) and 0.1 *M* citric acid (84 ml) in a mixer, coated as 0.3-mm layers on glass plates (20 × 20 cm) with the help of a Camag automatic TLC coater and left to dry at room temperature for 24 h. Prior to use, the plates were submerged in a methanolic ethylene glycol solution (20%, v/v) and the superfluous solvent was sucked off by placing the plates on filter-paper. Samples (0.1 μg) of tetracycline dissolved in 1 *N* hydrochloric acid–methanol (1:99) (200 μg/ml) were applied on to the plate, which was divided into three bands (in our experiments, the samples were working standards purified in the laboratory). Development was carried out twice in glass chambers containing a saturated atmosphere of ethyl acetate saturated with water. After development, the first band (A) of the chromatogram was sprayed with a 0.2 *M* magnesium chloride solution–95% ethanol (1:1), the second (B) with methanolic triethanolamine (10%, v/v), and the third (C) first with the reagent used for band A and then after 5 min with the reagent used for band B. Evaluation was carried out with an Opton PMQ-2 spectrophotometer coupled to a Camag-Z-Scanner (excitation, mercury-lamp, 366-nm filter; absorption, 540 nm).

RESULTS

The R_F values obtained (chlorotetracycline, 0.72; α-doxycycline, 0.66; oxytetracycline, 0.47; tetracycline, 0.35; methacycline, 0.20; β-doxycycline, 0.11; and apoterramycin, 0.07) show a satisfactory degree of separation.

Of the above substances, oxytetracycline (OTC), its degradation product apoterramycin (AT) and methacycline (MC) or α-doxycycline (DC) obtainable from it, were studied in detail. A chromatogram obtained by applying equal amounts (0.2 μg) of the compounds and developing them according to this method (Fig. 1) shows compact spots that are easy to evaluate. The increase in intensity achieved by the combined procedure (C) in comparison with those obtained with method A or B is striking.

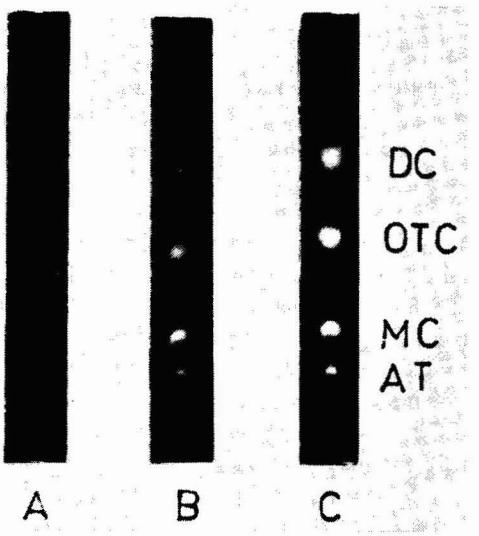

Fig. 1. Chromatograms of tetracyclines. Detection: (A) 0.1 *M* magnesium chloride solution–95% ethanol (1:1); (B) methanolic solution of triethanolamine (10%, v/v); (C) reagent A followed by reagent B.

The fluorograms prepared from the above chromatograms (Fig. 2) permit a quantitative evaluation of these intensity differences with the help of surface integrals (*1*).

In Fig. 3, the fluorescence intensity values procedure C obtained with are plotted as a function of time. With DC and OTC there is virtually no change in intensity between 30 min and 20 h. However, with MC, there is a gradual increase in intensity, but the rate of increase is so low after 2 h that it still permits a satisfactory determination. Under these conditions, for amounts in the range 0.05–1.00 μg, there is a linear correlation between the concentration of tetracyclines and the fluorescence intensities measured.

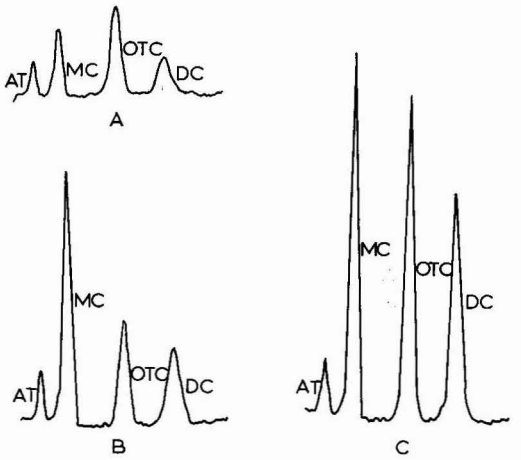

Fig. 2 Fluorograms of the chromatograms shown in Fig. 1.

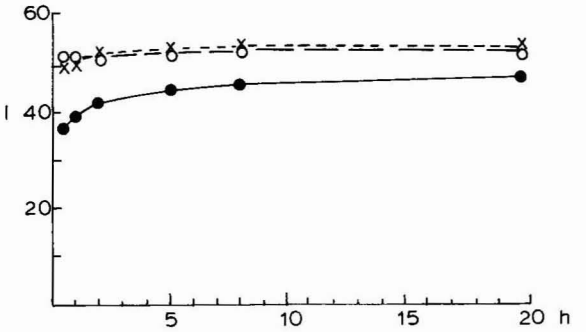

Fig. 3. Intensity (I) changes plotted as a function of time for chromatograms made visible according to method C. ×, DC; ○, OTC; ●, MC.

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CHROM. 10,742

Note

Gas–liquid chromatographic determination of zinc, copper and nickel in marine bottom sediments

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The quantitative determination of metals in marine bottom sediments and minerals by means of atomic-absorption spectrophotometry (AAS) has been subject to some problems associated with the necessity for elimination of interferences due to components of the bed and accompanying elements and to the reagents employed. The application of gas–liquid chromatography (GLC) allows complications inherent in the AAS technique to be eliminated and permits the simultaneous assay of a few elements in one sample.

In order for organic metal complexes to be suitable for GLC assay, they should have a high volatility and a high thermal stability, and a variety of compounds that meet these requirements have been described[1,2]. However, only a few of them have been used in the GLC assay of metals. D'Ascenzo and Wendlandt[3] found that certain metal cations form volatile complexes with diethyldithiocarbamate (DEDTC). Masaryk *et al.*[4] separated zinc from nickel by GLC using this reagent and Krupčík *et al.*[5] used it for assaying nickel(II). Cardwell and Desarro[6] separated chromatographically by DEDTC complexes of nickel, palladium, platinum, zinc, cadmium, copper, lead and mercury. Tavlaridis and Neeb[7] carried out a comparative study on the GLC separation of the DEDTC and bis(trifluoroethyl)dithiocarbamates of zinc, nickel, cadmium, antimony and bismuth.

It should be emphasized that all of the studies mentioned were carried out on artificial mixtures of pure reagents and there are no reports of the application of DEDTC to the GLC assay of several elements in real samples except for the determination of arsenic in urine and water by Daughtrey *et al.*[8].

The object of this work was to determine zinc, copper and nickel in marine bottom sediments in the form of their DEDTC complexes.

EXPERIMENTAL

Analysis of standard solutions of the metals

The concentration range of the standard zinc, copper(II) and nickel chloride solutions was 1–1000 $\mu g/cm^3$ based on the cations. To remove organic contaminants from the standard solutions, an aliquot was extracted for 1 min with chloroform–acetone (5:2, v/v) in a 250-ml separating funnel and the organic phase was discarded.

To the remaining aqueous phase, 5 ml of 2% sodium diethyldithiocarbamate solution, previously freed from metal impurities by extraction with the chloroform–acetone mixture, were added. The DEDTC complexes of the metals were then extracted for 1 min once with 5 ml and three times with 2 ml of the chloroform–acetone mixture. The combined extracts were evaporated to dryness on a water-bath in a stream of oxygen-free nitrogen. To the dry residue, 100 μl of chloroform were added by means of a microsyringe and 2-μl aliquots were then withdrawn for the GLC assay.

Analysis of bottom sediments

A sample of bottom sediment (1.5 g) was dried for 2 h at 110°, powdered in a vibrational agate mortar, and a 1-g amount (weighed exactly) was treated in a microautoclave (Perkin-Elmer Model 3) with a few drops of redistilled water, 2 ml of concentrated nitric acid and 5 ml of 40% hydrofluoric acid for 30 min at 150°. The solution was cooled, treated with 5 ml of 60% perchloric acid and 5 ml of 40% hydrofluoric acid and then evaporated to white fumes (to remove silicon compounds). The residue was again treated with 40% hydrofluoric acid, evaporated and dried. The dry residue was dissolved in 10 cm^3 of 6 *N* hydrochloric acid. The solution was then transferred into a 250-ml beaker, treated with 2 ml of 30% hydrogen peroxide, heated to 50°, 25 ml of lanthanum(III) chloride solution were added [to co-precipitate iron and manganese; lanthanum(III) concentration 1000 μg/ml], the solution was heated to 60° and an excess of a 25% aqueous ammonia solution was added under stirring. The temperature was then increased to 60–70° for 2–3 min and the precipitate was filtered through filter-paper and washed three times with hot ammonia solution. The pH of the filtrate was adjusted to 8 with 6 *N* hydrochloric acid and the solution was transferred into a 500-ml separating funnel. To the aqueous phase, 5 ml of 2% sodium

TABLE I

COLUMNS AND OPERATING CONDITIONS

Column No.	*Dimensions (m × mm I.D.)*	*Stationary phase*	*Column temperature (°C)*	*Total plate number (N*)*	*Resolution (R_s*)*
1	1.5 × 4	3% SE-30	Programmed, 240–290° at 10°/min	584.2	0.39
2	1.5 × 2	3% OV-7	280	403.1	0.44
3	1.5 × 2	5% OV-101	Programmed, 200–290° at 10°/min	293.0	0.5
4	3.0 × 2	Tenax (60–80 mesh)	Programmed, 200–290° at 5°/min	238.5	No resolution
5	1.5 × 2	3% SE-30	Programmed, 240–290° at 10°/min	470.4	0.37
6	1.8 × 2	2.5% BBBT	250	234.3	No resolution
7	1.5 × 2	5% QF-1	235	558.5	0.80

* The values of *N* and R_s were calculated according to Grushka[9].

diethyldithiocarbonate solution, previously freed from metal impurities by extraction with the chloroform–acetone mixture, was added and the solution was analysed as described in the previous section.

Gas–liquid chromatography

A Pye Unicam Series 104 gas chromatograph with a flame-ionization detector was used. In Table I, the column packings and operating conditions for the GLC of the DEDTC complexes of the three metals are given. Nitrogen was used as the carrier gas throughout.

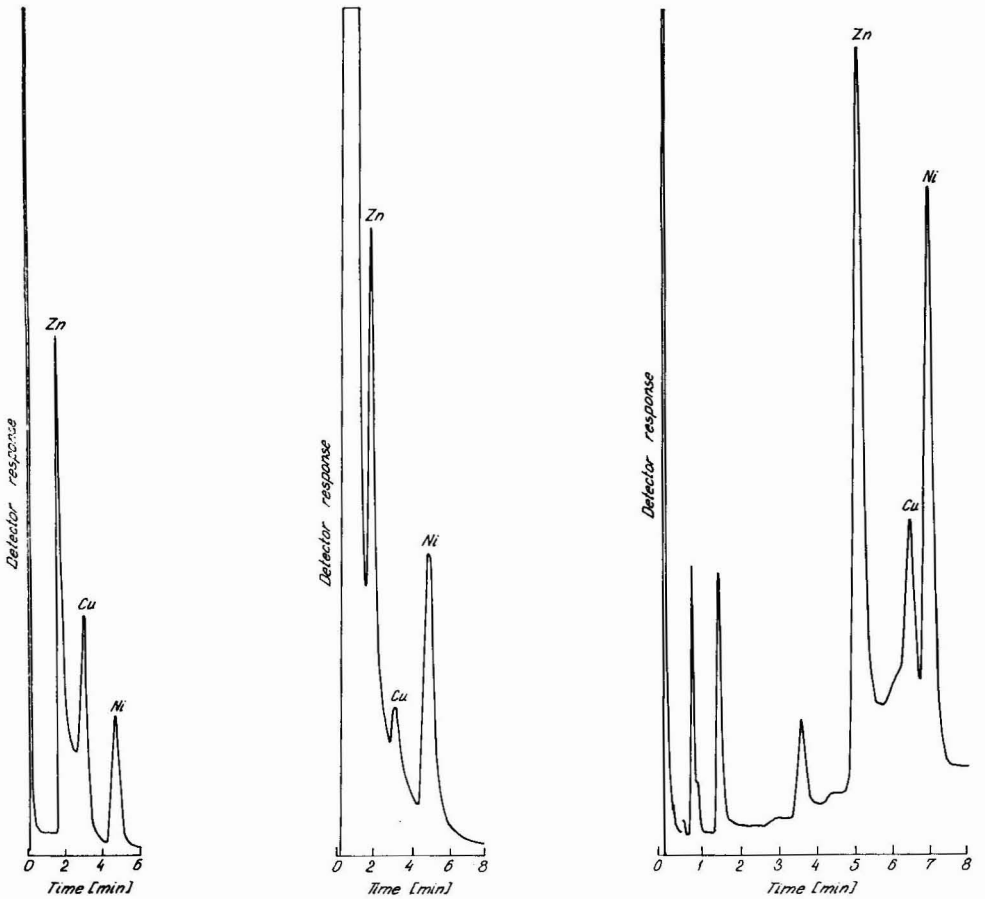

Fig. 1. GLC of bis(N,N-diethyldithiocarbonates) of Zn, Cu and Ni. Glass column No. 7 (Table I), on Chromosorb W-HP (80–100 mesh). Carrier gas, nitrogen, flow-rate 20 ml/min. Column temperature, 235°; detector (FID) temperature, 280°.

Fig. 2. Separation of a marine bottom sediment sample. Concentrations of $Zn(DEDTC)_2$, $Cu(DEDTC)_2$ and $Ni(DEDTC)_2$: 17.1, 1.6 and 4.9 ppm, respectively. Column and other chromatographic conditions as in Fig. 1.

Fig. 3. Chromatogram of a sample of the bottom sediment. Glass column No. 3 (Table I) on Chromosorb W-HP (80–100 mesh). Carrier gas, nitrogen, flow-rate 20 ml/min. Column temperature, programmed from 200° to 290° at 10°/min; detector temperature, 320°.

RESULTS AND DISCUSSION

A chromatogram of a mixture of standard solutions is shown in Fig. 1. The components of the mixture were separated on column No. 7 (Table I). Similar results were obtained with column No. 3, but the temperature had to be programmed over the range 200–290° at a rate of 10°/min. The remaining columns listed in Table I failed to give satisfactory separations of the metal peaks ($R_s < 0.5$).

The chromatogram shown in Fig. 2 was obtained by using column No. 7. It can be seen that the procedure ensures the removal of extraneous metals and allows the separation of the three complexes of zinc, copper and nickel. By comparison of these chromatograms with those of the standard solutions, the concentrations of zinc, copper and nickel in the sample were determined to be 17.1, 1.6 and 4.9 ppm, respectively.

The results of a similar analysis, obtained on column No. 3 packed with OV-101 temperature with programming, are shown in Fig. 3. There are three additional peaks that could not be identified.

Our current efforts are aimed at finding an appropriate internal standard that would permit a more accurate determination of the concentrations of the three metals. Of the compounds tested so far, coronene, anthanthrene and dotriacontane have given encouraging results, which will be published later.

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CHROM. 10,800

Letter to the Editor

Catecholamine determination by gas–liquid chromatography

Sir,

Whilst there is a need for development of relatively simple and rapid techniques for analysis of small concentrations of catecholamines that occur in biological fluids and tissues, it is essential that new methods reported claiming to fulfil these criteria should be reproducible and adaptable in different laboratories. Therefore it is surprising that the report by Lovelady and Foster[1] describing the measurement of adrenaline and noradrenaline in plasma, red blood cells and urine with detection sensitivity in the sub-picogram range has to date produced no comment in this journal with respect to several factors relating to the viability of the assay procedure.

Firstly, apart from the well established radioenzymatic method, the analysis of picogram ($10^{-12} - 10^{-14}$ moles) quantities of catechol- and related biogenic amines by gas chromatographic techniques has been accomplished by means of the highly sensitive mass spectrometry and electron capture detection[2]. In contrast the report by Lovelady and Foster[1] states that quantitative detection of similar amounts of catecholamines can be accomplished by gas–liquid chromatography using a dual hydrogen flame ionization detector with relatively little clean up of the biological extracts. In practice the detection limits of gas chromatography using a flame ionization detector for the determination of compounds extracted from biological fluids does not generally extend below the low nanogram range and it is of interest to note that Lovelady and Foster[1] present data showing linear calibration for adrenaline and noradrenaline from less than 0.2 pg to approximately 2 pg with a minimum detection of "approximately 0.1 pg". This stated detection limit is even numerically below the minimum detectability that is specified for flame ionization detectors on most commercially available gas chromatographs (in the order of 10^{-11} g/sec for alkanes[3]).

Secondly, we have shown elsewhere[4] that the extraction technique used by Lovelady and Foster[1] and also that described in a separate report by Lovelady[5] for extraction of catecholamines from red blood cells is unlikely to give significant recovery of these compounds. That is, the extraction of a protein free extract of red blood cells with water–4-methyl-2-pentanone–*n*-hexane (1:1:1) mixture as described by the authors[1,5] will not yield significant partition of the free catecholamines into the top organic phase, the bulk of catecholamines will remain in the aqueous phase. This is in contrast with the results of Lovelady and Foster[1] and Lovelady[5] that greater than 90% recovery of free catecholamines from red blood cells is achieved following extraction with this solvent system.

Thirdly, Lovelady and Foster[1] show that tripalmitin, used as an internal standard, has a retention time of approximately 11 min on a 6 ft. × $\frac{1}{8}$ in. O.D. steel column packed with 7% DC-11 on Gas-Chrom P operating isothermally at 115° with carrier flow-rate of 20 ml/min. Yet it is well established that gas chromatography of glycerol esters of long chain fatty acids require considerably higher temperatures

higher carrier flow-rates, shorter column length and less percentage liquid phase for their elution from the column. For example triglycerides with carbon number 30–40 (the carbon number of tripalmitin is 48) are consecutively eluted at 250° on a 40-cm column of 2.25% SE-30 on Chromosorb W with carrier flow-rate of 75 ml/min[6]. Furthermore, if tripalmitin is used as an internal standard as stated by the authors[1] in nanogram amounts then it is unlikely to be able to differentiate the added tripalmitin from the endogenous tripalmitin together with the other closely related triglycerides which occur in human plasma at considerably higher concentration[7].

Fourthly, the catecholamine derivatives were prepared by simultaneous reaction with a mixture of bis-(trimethylsilyl)acetamide (BSA)–trifluoroacetic anhydride (TFA) (1:1) in tetrahydrofuran[1,5] and were simply referred to as the "TFA–BSA derivatives"[5]. No further information as to the nature of these compounds have to date been given. Presumably the derivatisation was an attempt to prepare the N-acyl, O-trimethylsilyl ether derivatives originally described by Horning *et al.*[8] where catechol- and related amines were reacted in a two-step sequential reaction with a trimethylsilyl donor then the acyl anhydride in anhydrous conditions. Similar derivatives, again using a two-step procedure have been prepared by other workers[9,10]. The method of Lovelady and Foster[1] also introduces the presence of water following extraction of the "TFA–BSA products" with water–organic solvent pair and we consider this likely to lead to hydrolysis of the formed derivatives. We have attempted to prepare the same derivatives of adrenaline, noradrenaline and dopamine by the method described by Lovelady and Foster[1] and found no formed products which eluted under the chromatographic conditions described in their report.

We are therefore led to conclude that the method of Lovelady and Foster[1] is of doubtful value for the measurement of catecholamines in blood or plasma, especially in the concentrations in which these compounds normally occur.

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Book Review

Methods of surface analysis, edited by A. W. Czanderna, Elsevier, Amsterdam, Oxford, New York, 1975, XIV + 481 pp., price Dfl. 150.00, US$ 62.50, ISBN 0-444-41344-8.

Methods of surface analysis is the major sourcebook and instruction manual for anyone working with newer analytical techniques dealing with solid surfaces. This volume initiates a new series, "Methods and Phenomena: Their Applications in Science and Technology", and includes chapters covering various new methods for the investigation of the chemistry of surfaces written by well known experts.

In the short Introduction, A. W. Czanderna gives a good review of problems that are difficult to solve by the usual methods of surface chemistry, including one of the most important questions, namely "what is the elemental composition of the surface?" He emphasizes that the answer to this question could not be obtained until a few years ago.

Chapter 1 provides an overview of sputtering in surface analysis methods. Table 1 in this chapter allows one to understand clearly the connection between the manner of excitation and the emission obtained. Using this table, a reader can easily assimilate in the nomenclature and possibilities of different new sputtering methods in surface analysis. Chapter 2 gives a comparison of methods of surface analysis involving the use of incident particles to produce an output of detectable particles. The last eight chapters include descriptions of low-energy ion-scattering spectroscopy (LEIS or ISS, Chapter 3), X-ray photoelectron spectroscopy (XPS or ESCA, Chapter 4), Auger electron spectroscopy (AES, Chapter 5), secondary ion mass spectrometry (SIMS, Chapter 6), a combined study of AES and SIMS (Chapter 7), field-ionization mass spectrometry (FIMS, Chapter 8) and infrared reflection–absorption spectroscopy (Chapter 9).

Particularly useful is Chapter 4, which describes the determination of the chemical composition of surfaces, gas–metal reactions, "gas-surface" impurities, reactions and ion exchange on glass and fluoropolymer surfaces, the migration of components in glass, and the determination of the chemical differences between charcoal-supported catalysts of high and low activity. In addition, the investigation of chemical state of sulphur in atmospheric samples and the identification of SO_3, SO_4^{2-}, SO_2 and SO_3^{2-} are considered.

FIMS is a method for gas analysis. The reaction of oxygen with sulphur on metallic elements on surfaces, emitters such as zeolites on platinum, the kinetics of adsorption and chemisorption and the formation of complex ions of copper, silver and gold metals with gases can also be studied by FIMS.

It is unfortunate that Chapter 9 does not consider the possibilities of the new instruments that use Fourier transform in infrared spectrometry, because infrared spectrometry is one of the most useful adjuncts to the chromatography of surfaces.

It would have been better to include in this book the details of low-energy electron diffraction (LEED).

This book must be described as definitive for this branch of chemistry, and the several hundred references up to 1974 contained therein show its comprehensive nature. It is undoubtedly essential to those working in the field of detectors for chromatography.

Modern GLC, LLC and especially GSC and LSC require a knowledge of the chemical composition and the structure of different supports and adsorbents. In this connection, the book is also very useful for specialists trying to improve the quality of these supports and adsorbents. Hence it will undoubtedly not only appeal to research workers studying modern methods of surface analysis but will also be useful to those who are interested in the application of solids of different chemical compositions in chromatography. However, the amorphous and disperse nature of the supports and adsorbents used in chromatography gives rise to difficulties, which is why it is difficult to recommend this book as initial reading for newcomers to the chromatographic applications of these supports and adsorbents. However, in the near future many of these methods will probably find important applications even in investigations of the chemistry of the surfaces of amorphous and disperse solids.

The price of the book seems high and, in view of the indicated limited interest to readers of the Journal of Chromatography, it must be a personal decision whether they consider this interest is sufficient to tempt them to spend this amount of money on it.

Moscow (U.S.S.R.) A. V. KISELEV

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
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4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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