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JOURNAL OF CHROMATOGRAPHY

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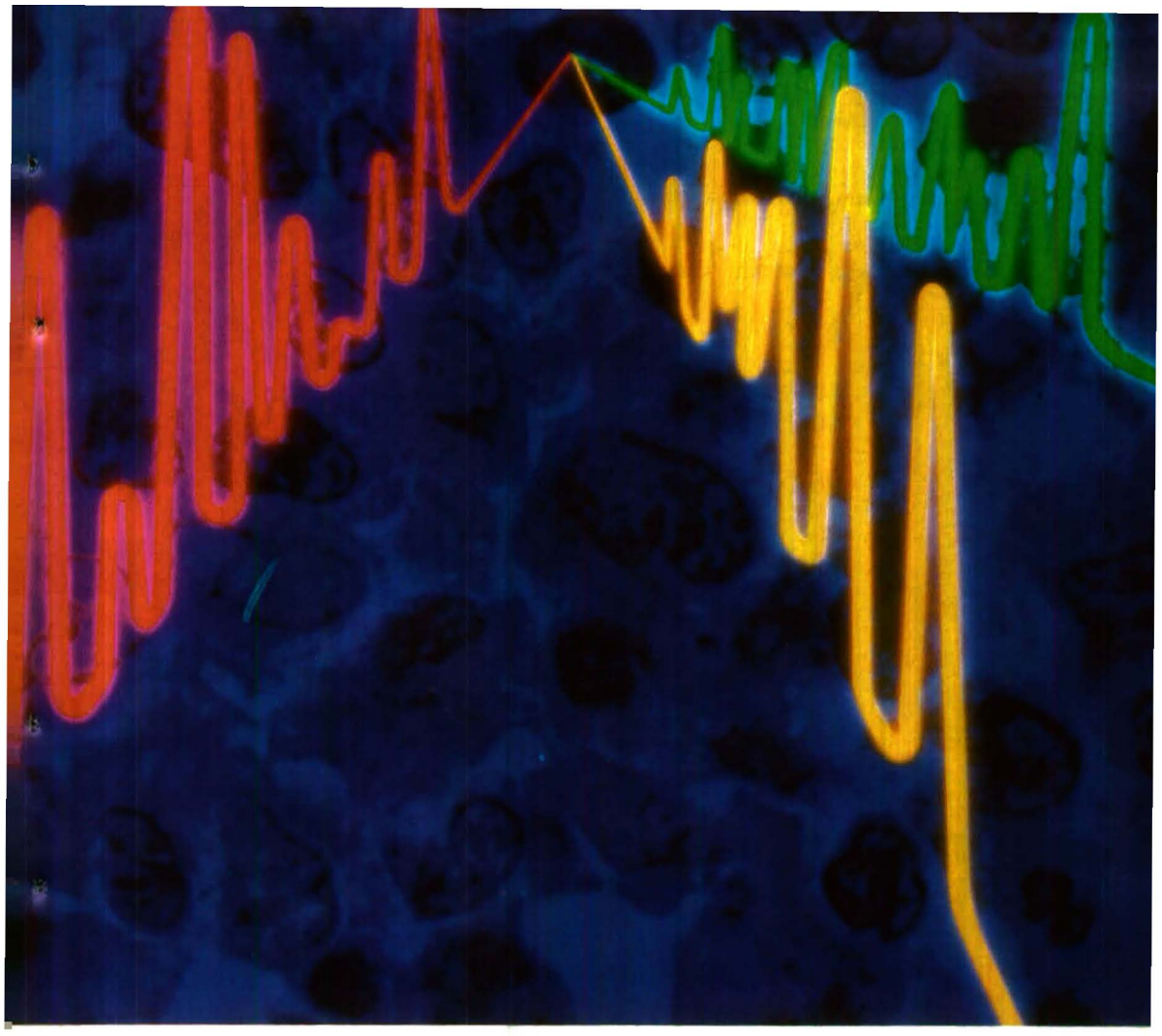

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CHROMATOPHORESIS: A NEW APPROACH TO THE THEORY AND PRACTICE OF CHROMATOFOCUSING

I. GENERAL PRINCIPLES

ANDREW MUREL*, SVETLANA VILDE, MARET PANK, IGOR SHEVCHUK and OSKAR KIRRET

Institute of Chemistry, 200026 Tallinn, Eesti NSV (U.S.S.R.)

(Received June 13th, 1985)

SUMMARY

Accurate mathematical analysis reveals that a smooth pH gradient in a chromatofocusing column cannot be produced by the buffering action of the ion exchanger and running buffer. The generation of the pH gradient is attributed to the mechanism of classical frontal analysis chromatography. Computer simulations predict a dependence of the pH gradient shape on the buffer composition.

Utilization of the displacement technique is expected to produce an improved ampholyte separation. The pattern distribution of steady-state stacked ampholytes has been computed, together with ionic strength and pH of the mobile phase. The term "chromatophoresis" is proposed for this method of pH gradient generation and the conditions for the efficient separation of ampholyte mixtures are discussed.

INTRODUCTION

One of the most modern separation techniques in biochemistry is chromatofocusing[1]. Because of the expensive commercial materials involved, we have searched for a substitute for Polybuffer[2], including a dilute (0.5% w/w) aqueous solution of tetraethylenepentamine adjusted to pH 8.0 with HCl. This polyamine has about the same buffer capacity as polybuffer over a wide pH range (*cf.*, Fig. 1 in ref. 3 and Fig. 8 in ref. 2). Hence, in accordance with chromatofocusing theory[1], a smooth pH gradient of the column effluent has to be produced. Insertion of buffer capacity data into eqns. 3 and 4 in ref. 1 result in an S-shaped pH gradient throughout the entire column, provided the column is ten-sectioned. However, instead of a smooth pH shift from 10.0 to 8.0, in actual experiments the pH first rose to 11.0 and then fell abruptly to 8.0.

There is another observation that cannot be explained in terms of the "buffering mechanism"[1]: in actual experiments with Polybuffer (*e.g.*, Figs. 13, 16 and 17 in ref. 2) a small pH increase precedes the descending pH gradient. However, the addition of more acidic to a less acidic buffer is never expected to increase the pH of the resulting mixture.

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These two observations constituted the starting point of our investigations. The initial ideas were conceived by Dr. Andrew Murel. Dr. Maret Pank and Svetlana Vilde carried out most of the experimental work and Dr. Igor Shevchuk manipulated the mathematical equations for the computer.

THEORETICAL

Buffering action for producing a pH gradient in chromatofocusing

Sluyterman and Elgersma[1] considered the addition of nineteen buffer aliquots to a ten-section column. Assumption that the buffer capacities of one aliquot and column section are equal yields

$$pH_{f,j} = \frac{pH_{f-1,j} + pH_{f,j-1}}{2} \quad (1)$$

where f is the aliquot number and j denotes the section number. In order to approach real conditions, however, the column should be divided into many more sections and smaller aliquots should be considered. Ideally, one section should correspond to the height of one theoretical plate.

Calculated pH values of the effluent from 10 and 100 sectional columns are shown in Fig. 1. A further increase in the theoretical plate number results in an abrupt pH jump that resembles a characteristic sigmoid curve of "breakthrough and total capacity" (Fig. 4.3 in ref. 4) rather than a pH gradient for focusing.

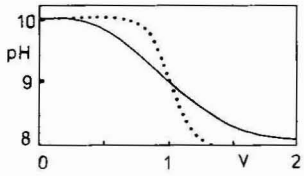


Fig. 1. Buffering mechanism of chromatofocusing: pH of the effluent from the column as a function of the relative elution volume. Solid line, 10-section column; dotted line, 100-section column.

The failed experiment with tetraethylenepentamine as a buffer can now be explained. However, the same data prevent the acquisition of smooth pH gradients with ampholyte mixtures. In attempting to solve this problem, we considered Sluyterman and Elgersma's remark (footnote on page 18, ref. 1) that in the actual mechanism of buffering action a pH increasse results from retention of the more acidic components from the buffer. Although it was stated[1] that the "actual mechanism" does not alter the final equation, we have derived a new equation to reflect the "retention amendment".

Let us assume that the initial buffer aliquot ($j = 0$) consists of equal concentrations of eleven ampholytes with pI values ranging from 3 to 13. The pH of this mixture is $(3+4+5+\ldots)/11 = 8.0$, the initial buffer capacity being $A^m = 1.0$. Suppose one section of column possesses the same capacity as the stationary phase, $A^s = 1.0$, displayed as the ability to retain two ampholyte molecules. Hence, every

column section adsorbs two more acidic components from the first aliquot, and allowance should be made for the loss of buffering material from the mobile phase. The assumption that the buffer capacity of the mobile phase, A^m, is proportional to the ampholyte concentration gives

$$A^m = \frac{(13 - pH_{1,j-1}) \cdot 2 + 1}{11} \tag{2}$$

and eqn. 1 has to be rewritten as

$$pH_{f,j} = \frac{pH_{f-1,j}A^s + pH_{f,j-1}A^m}{A^s + A^m} \tag{3}$$

As far as the first aliquot is concerned, $pH_{0,j}$ and A^s are constant (10.0 and 1.0, respectively):

$$pH_{1,j} = \frac{10 + pH_{1,j-1}A^m}{1 + A^m} \tag{4}$$

Calculated pH values of the first aliquot display an even steeper descent than that based on a "pure" buffering action. Moreover, the "retention amendment" does not elucidate the impact of adsorbed ampholytes on the equilibrium with successive aliquots.

As neither a smooth pH gradient nor a pH jump are explained in terms of the buffering mechanism, we turned to classical frontal development chromatography (for instance, see page 228 in ref. 4).

Frontal development for producing a pH gradient

For the sake of simplicity, let us consider a strong ion exchanger with no buffer capacity in the pH range concerned. The smallest section of the column that still retains the properties of an ion exchanger defines the volume of the buffer aliquot: one aliquot is the buffer volume consisting of a number of ampholyte species equal to the number of "sites of exchange" (page 200 in ref. 4) in one column section, Q (μequiv./ml).

Let $j = 1,2,...$ denote the number of successive column sections, $1j = 0$ being the buffer reservoir. Let the buffer contain L types of ampholyte species, each type i designated by the initial concentration $C_{i,0}$ and by the isoielectric point pI. For one aliquot one can write

$$\sum_{i=1}^{L} C_{i,0} = Q \tag{5}$$

Let us consider an anion exchanger in the OH^- form. The first buffer aliquot is entirely adsorbed in the first column section. The second portion of ampholytes will compete for the "sites of exchange" with the species from the first aliquot. The concentration of type i ampholyte that adheres to the j section, $C^s_{i,j}$, is

$$C_{i,j}^{s} = \frac{C_{i,j}k_i'}{\sum_i (C_{i,j}k_i')} \cdot Q \tag{6}$$

where $C_{i,j} = C_{i,j}^{m} + C_{i,j}^{s}$ is the total concentration of type i ampholytes in section j and k_i' is a capacity factor reflecting the relative strength of the i–ampholyte–exchanger ion complex.

The ampholyte concentration in the mobile phase is calculated as

$$C_{i,j}^{m} = C_{i,j} - C_{i,j}^{s} \tag{7}$$

and pH is given by:

$$\mathrm{pH}_j = \frac{\sum_i (C_{i,j}^{m} \mathrm{p}I_i)}{\sum_i C_{i,j}^{m}} \tag{8}$$

It is assumed here that ampholyte electric charge Z_i is proportional to the slope of the titration curve near its isoelectric point and that all ampholytes have the same charge dependence:

$$Z_i = \mathrm{p}I_i - \mathrm{pH}_j \tag{9}$$

and the ionic strength of the mobile phase is

$$I = \sum_i (C_{i,j}^{m} Z^2) \tag{10}$$

Eqns. 6, 7, 8 and 10 permit the calculation of component composition, pH and ionic strength of aliquots as they pass successively through the column. Similarly, ascending pH gradients can be derived for cation exchangers in the acidic form*.

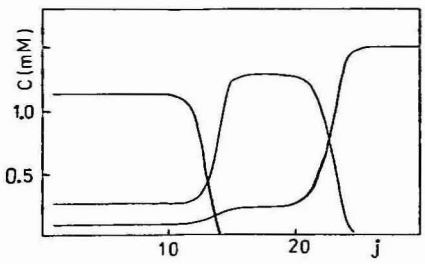
Fig. 2. shows the stationary phase composition addition of 30 infinitely dilute aliquots, the buffer being a three-ampholyte mixture ($k_1'/k_2'/k_3' = 4{:}2{:}1$). Fig. 3 shows the pH of the mobile phase in the nineth section, or the effluent pH from a 9-ml column provided the sizes of the aliquot and the section volumes are 1 ml.

The effluent composition, ionic strength and pH data shown in Fig. 4 correspond to an ampholyte mixture with a lower separation selectivity $\alpha(k_1'/k_2'/k_3' = 2.25{:}1.5{:}1)$.

Displacement development for producing a pH gradient

Suppose that ten ampholyte buffer aliquots are applied to an anion-exchange column and the column is washed with dilute HCl. If the Cl^- ion has a higher affinity for the resin it will displace all other counter ions from exchanger. As calculations

* For computer programs in BASIC and FORTRAN, contact Dr. I. Shevchuk.



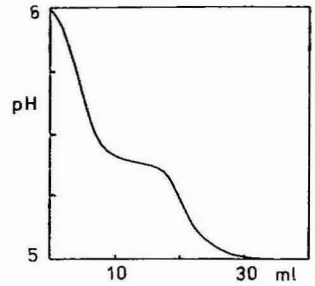


Fig. 2. Chromatofocusing: calculated component distribution inside a column at the moment when 30 infinitely dilute buffer aliquots are loaded. Initial buffer consists of a mixture of three ampholytes; k'_i ratio = 4:2:1; $C_{i,0}$ = 0.5 mM.

Fig. 3. Chromatofocusing: pH of the effluent from the column, V = 9 ml, Q = 1.5 μequiv./ml. Initial buffer consists of a mixture of three ampholytes, $k'_1/k'_2/k'_3$ = 4:2:1; $pI_1/pI_2/pI_3$ = 4:5:6; $C_{i,0}$ = 0.5 mM.

reveal, the displacement technique furnishes zones of pure components and zones of mixed fractions, the quality of the separation being dependent on the experimental conditions. A steady component distribution can be achieved by repetition of the displacement process.

Displacement development is reminiscent of isotachophoretic separation where the counter ions are substituted for the charged matrix of the exchanger and where the stack is driven by a solvent flow instead of electric force. To define this new means of producing a pH gradient via the displacement technique on an ion exchanger we propose the term "chromatophoresis" (CHPH).

Subsequently, a simple equation was conceived to reflect the link between the molarity of the mobile phase, C^m, and that of the displacing (or terminating) constituent, C_t:

$$C^m = \frac{Q_a}{Q_t} \cdot C_t \tag{11}$$

where Q_a and Q_t are the capacities of the exchanger for ampholytes and for the terminating constituent, respectively. This equation gives the effluent concentration provided that steady-state stacking is establishing, *i.e.*, the column is large enough for the given ampholyte load to mould a stable stack. Thus, one act of CHPH caused by the addition of the displacing constituent solution can be calculated mathematically as an addition of the first section stationary phase to the second:

$$C_1^s + C_2^s = C_2^{*s} + C_2^m \tag{12}$$

where the asterisk denotes a new ampholyte composition in the section. Then C_2^m is added to C_3^s to yield C_3^{*s} and C_3^m, etc.

This approach permits the simulation of the CHPH process for the mobile phase concentration range $0 < C^m \leqslant C^s$. For $C^m > C^s$ it is necessary to sum more than two sections. For instance, Fig. 10 depicts the sum of the first three sections:

$$C_1^s + C_2^s + C_3^s = C_3^{*s} + C_3^m \tag{13}$$

where C_3^{*s} is a new stationary phase of the third section, $C_3^m = 2C_3^{*s}$. The next step is $C_3^m + C_4^s = C_4^{*s} + C_4^m$, etc. The second displacement wave is initiated by addition of a new portion of the displacing constituent: $C_3^{*s} + C_4^{*s} + C_5^{*s} = C_5^{**s} + C_5^m$, etc.

Figs. 4–11 show the effluent composition, the top panel representing the pH and ionic strength. The arrows in each instance indicate the point that divides the first and third bands into fractions of equal purity, with the number giving the precentage purity of the first component with respect to the third. Buffer components display their concentration maxima in the column effluent in succession that depends inversely on k': lower affinity patterns are eluted first and the most acidic ampholyte is the last to be displaced from the anion exchanger.

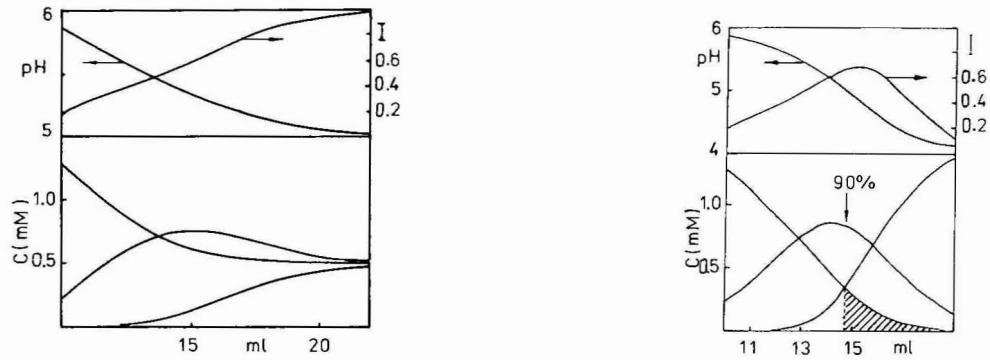


Fig. 4. Chromatofocusing: calculated composition of the effluent. Column: V = 9 ml; Q = 1.5 μequiv./ml. Initial buffer consists of a mixture of three ampholytes, $k'_1/k'_2/k'_3$ = 2.25:1.5:1; $pI_1/pI_2/pI_3$ = 4:5:6; $C_{i,0}$ = 0.5 mM. Top panel: pH and ionic strength of the effluent.

Fig. 5. Chromatophoresis: calculated composition of the effluent. Column: V = 9 ml; $Q_a = Q_t$ = 1.5 μequiv./ml. Initial buffer consists of a mixture of three ampholytes, k'_i ratio = 2.25:1.5:1; pI_i ratio = 4:5:6; $C_{i,0}$ = 0.5 mM. Ampholyte load is 10 aliquots, then the column is washed with 1.5 mM displacing solution. Top panel: pH and ionic strength of the effluent.

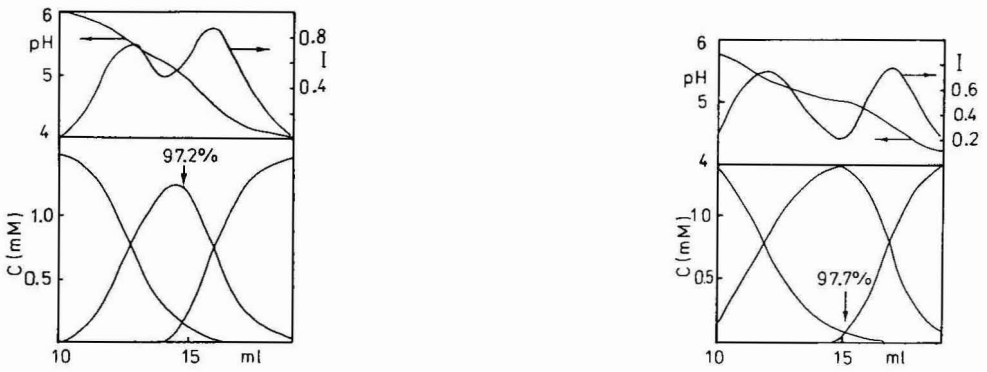
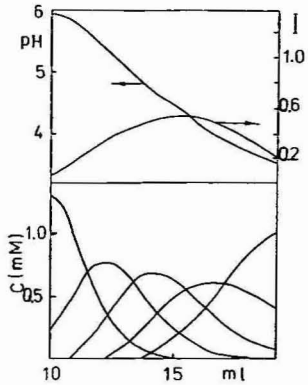


Fig. 6. Chromatophoresis: conditions as in Fig. 5 except for capacity factor ratio, $k'_1/k'_2/k'_3$ = 4:2:1.

Fig. 7. Chromatophoresis: conditions as in Fig. 5 except capacity factor ratio $k'_1/k'_2/k'_3$ and component concentrations in the initial buffer; $C_{i,0}$ ratio = 0.38:0.74:0.38.



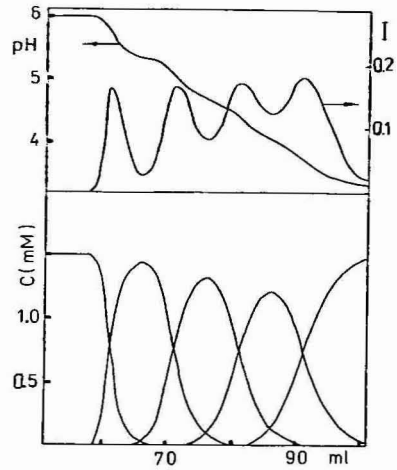
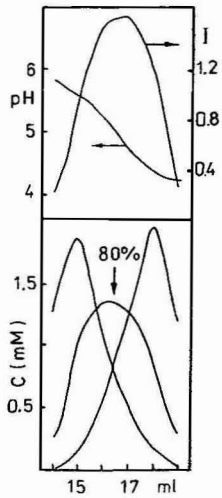


Fig. 8. Chromatophoresis: calculated composition of the effluent. V = 9 ml; Q_a = Q_t = 1.5. μequiv./ml. Initial buffer consists of a mixture of five ampholytes; k_i' ratio = 5:4:3:2:1; pI_i ratio = 3.33:4.00:4.66:5.33:6.00; $C_{i,0}$ = 0.3 mM. Ampholyte load is 10 aliquots, then the column is washed with 1.5 mM displacing solution.

Fig. 9. Chromatophoresis: conditions as in Fig. 8 except column volume = 50 ml and ampholyte load is 50 aliquots.



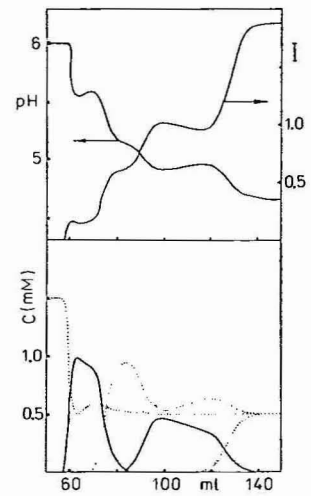


Fig. 10. Chromatophoresis: conditions as in Fig. 5 except column volume = 13 ml and displacing constituent concentration C_t = 3 mM.

Fig. 11. Chromatofocusing: conditions as in Fig. 9 except for the displacing step. Instead of displacement the frontal development is continued with a new buffer composition where the second and the fourth ampholytes are withdrawn. First sample ampholyte with pI = 5.33 is explicitly focused. Embowered in high ionic strength the second sample ampholyte is rather blurred. The divergence between observed and actual pI is *ca.* 1 pH unit for this pI = 4.00 ampholyte.

In order to plot the calculated pH, I and C values *versus* conventional eluent units, it is suggested that one aliquot (except for the data displayed in Fig. 10) consists of a 1-ml volume and one column section has $V = 1$ ml.

DISCUSSION

The frontal development mechanism permits the generation of pH gradients even on a strong ion exchanger with no need for a buffering action of the resin. The larger the column, the higher is the pH gradient volume. While the first ampholyte emerges in a nearly pure state, its successors are more and more contaminated. This contamination is reflected in Fig. 4 as a steady increase in ionic strength and its is supported by experimental data (Fig. 26 in ref. 2).

Note that the equilibrated part of the column (sections 1–10, Fig. 2) represents a component concentration ratio in the stationary phase other than the 1:2:4 that one would expect from the ampholyte affinity k' ratio. This is a good example of how carefully one should approach chromatographic techniques. An increase in the separation selectivity, $k'_{i+1}/k'_i = \alpha$ and in the column size can furnish a stepwise pH gradient (Figs. 3 and 4). Addition of species with intermediate pI and k' values to the initial buffer will have an opposite effect. Finally, the frontal development mechanism elucidated the pH jump in some actual experiments as an elution of less adhesive components at their isoelectric points, provided that the column was initially adjusted to a lower pH than the pI of the leading ampholyte.

Chromatophoretic separations are affected by several factors:

(1) Alteration of the separation selectivity, α; *cf.*, Figs. 5 and 6.

(2) Alteration of the relative "spacer" contents to hold apart components of interest; *cf.*, Figs. 6 and 7.

(3) Alteration of the sample load as reflected by comparison of two five-component mixture separations (Figs. 8 and 9). A reduction in the exchanger capacity, also improves CHPH.

(4) Alteration of the column length, leading to a different CHPH span. For instance, 9- and 22-ml columns furnish purities of 93.4% and 95%, respectively (Fig. 8). However, the effective increase in column length is limited as the component distribution attains the steady state, and the greater separation selectivity the more promptly the stack is trimmed.

(5) Alteration of the ampholyte concentration in the mobile phase with respect to the stationary phase by lowering the displacing constituent concentration (eqn. 11), as shown in Figs. 5 and 10.

The chromatophoretic technique has several advantages over chromatofocusing (CHF):

(1) Utilization of the entire pI range of the buffer ampholytes; *cf.*, Figs. 4 and 5. CHF takes advantage of only half of the available pI values unless the pH of the buffer solution is adjusted with acid to equal the pI of the most acidic ampholyte.

(2) A low ionic strength in CHF has been emphasized (1) as a decisive factor for separation quality. CHPH gives a lower ionic strength of the effluent; *cf.*, Figs. 9 and 11.

(3) The cost of the eluent constitutes the major factor in the experimental costs. While all buffer components are recovered on completion of the run in CHPH, in CHF a column volume of ampholytes is lost.

(4) The sample composition is important in CHF[2] and its should not contain large amounts of salts ($I < 0.05$). CHPH permits the removal of salts from ampholytes and from the sample, provided that the column is large enough to adsorb all counter ions applied.

The sample load in isotachophoresis (ITP) has recently been reported to consist of bare proteins with no need for carrier ampholytes or other spacers[5]. From the analogy between ITP and CHPH, there appears to be no theoretical objection to the possibility of a similar approach in CHPH.

The molecular weight distribution of ampholytes can be obtained by CHPH. The effluent concentration is proportional to the molecular weight of an ampholyte, provided that every "site of adsorption" is occupied by one counter ion, *i.e.*, when $Q_a = Q_t$ in eqn. 11. A pellicular or superficially porous strong ion exchanger with a low capacity is likely to comply with this requirement. Theoretically, displacement development permits the determination of the molecular weights of non-amphoteric electrolytes on ion exchangers, and the same is valid for the displacement technique with other types of adsorption.

Terminology

The term "ampholyte displacement chromatography" has been employed in several papers to define the process of elution of proteins from ion exchangers with ampholyte solutions[6–9]. Other authors[10] have observed the formation of a pH gradient, which was explained as a "retardation phenomenon in which ampholytes, depending on their charge in aqueous solution, are retarded by the ionic groups of the gel", and several column volumes of water are required for complete elution of ampholytes from the exchanger.

However, the "displacement development" denotes washing columns with a solution of the displacing constituent and the term "frontal development" implies passing the sample solution continuously through the column, both terms being conventional terms in liquid chromatography texts[4]. Thus, regarding carrier ampholytes only, "ampholyte displacement chromatography" and CHF should be considered as elution and frontal development techniques, respectively.

The founders of the CHF method[1] distinguish ampholyte and protein separation mechanism, the latter being described as elution with a pH gradient. In fact, unlike continuous ampholyte feeding during frontal development (or CHF), the protein sample is applied to the column in a finite volume. However, owing to the amphoteric nature of both buffer ampholytes and sample proteins, their behaviour should be attributed to a single mechanism (Fig. 11).

In Part II, the frontal development mechanism (for CHF) and the displacement development mechanism (for CHPH) are experimentally verified, and some applications of the new chromatophoretic procedure are presented.

SYMBOLS

$A^{s,m}$	Buffer capacity of the stationary and the mobile phase, respectively;
a	suffix indicating ampholyte constituent;
C	mean concentration of a constituent, mM;
$C^{m,s}$	concentration in the mobile and stationary phase, respectively;

f suffix indicating aliquot number;
I ionic strength;
i suffix indicating a certain pI type of ampholytes;
j suffix indicating column section number;
k' capacity factor reflecting the affinity of a constituent for an ion exchanger;
L total number of ampholyte types;
m suffix indicating mobile phase;
pI isoelectric point;
$Q_{a,t}$ capacity of exchanger for ampholytes and for the terminating constituent, respectively, equiv./ml;
s suffix indicating stationary phase;
t suffix indicating terminating (or washing or displacing) constituent;
V colume volume, ml;
Z electric charge of an ampholyte;
α separation factor, k'_{i+1}/k'_i.

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THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE EVALUATION OF THE LIPOPHILICITY OF ARYLOXOALKANOIC AND ARYLHYDROXYALKANOIC ACIDS

M. KUCHAŘ*, E. KRAUS, M. JELÍNKOVÁ, V. REJHOLEC and V. MILLER
Research Institute for Pharmacy and Biochemistry, 13060 Prague 3 (Czechoslovakia)
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SUMMARY

The series of aryloxoalkanoic and arylhydroxyalkanoic acids were subjected to both reversed-phase thin-layer (TLC) and high-performance liquid chromatography (HPLC) using chemically bonded packing materials. The lipophilicity of these acids seems to be dependent on intramolecular interactions between two hydrophilic fragments and was compared chromatographically with the reference group of arylacetic acids. TLC yielded different linear relationships between log P and R_M for the individual groups of the acids. Calculation of the partition coefficients from the log P *vs.* R_M dependence could thus lead to false results for structurally diverse compounds. No such differences were observed, however, in HPLC in the log P *vs.* log k' relationships derived for arylacetic, aryloxoalkanoic and arylhydroxyalkanoic acids.

INTRODUCTION

Chromatographic methods have been widely used for the evaluation of lipophilicity in quantitative structure–activity relationships (QSAR), especially reversed-phase thin-layer chromatography (RP-TLC)[1–4] and recently also high-performance liquid chromatography (HPLC), using chemically modified stationary phases[4–8]. On the assumption of a prevailing partition mechanism, the values of R_M or log k' are directly proportional to the logarithm of the partition coefficient, P_s, determined in the chromatographic system:

$$R_M (\log k') = \log P_s + \text{constant} \quad (1)$$

and can be used as parameters of lipophilicity in biological correlations.

A close similarity of solvation forces in both partitioning systems[9] is a condition of the validity of the Collander relationship[10] (eqn. 2) between the logarithms of the partition coefficients in the reference (P) and the chromatographic system:

$$\log P = a_1 \log P_s + b_1 \quad (2)$$

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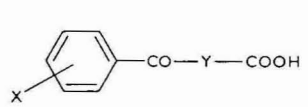
As the nature of the solvation cannot be determined exactly, the extent of the validity of the linear relationships in eqns. 3a and 3b can only be estimated empirically.

$$\log P = aR_M + b \quad (3a)$$

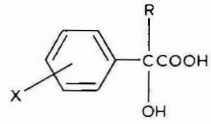
$$\log P = c\log k' + d \quad (3b)$$

Usually, the stastistical significance of these equations is satisfactory when applied to a series of structurally similar compounds. Increasing structural diversity can lead to deviations from linearity, however, as the conditions of similarity of the solvation effects are not longer fulfilled.

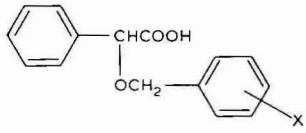
The lipophilicity of the arylaliphatic acids was evaluated[8,11–13] chromatographically on a thin layer of silica gel impregnated with silicone oil with aqueous acetone as the mobile phase. Such a system is similar to the reference system, especially with regard to some intramolecular interactions[8]. This was confirmed in the case of intramolecular hydrophobic interactions in arylalkoxy derivatives of arylaliphatic acids[11,12] and solvation hindrance in 3,4-dialkoxyarylaliphatic acids[8,13]. Similar results were also obtained in the evaluation of lipophilicity by HPLC using chemically bonded stationary phases[8]. Both TLC and HPLC methods were used for the evaluation of the lipophilicity in the series of acids I–VII, where the introduction of a functional group into the connecting chain between the aromatic nucleus and the carboxy group produces so called H/H interactions between the two hydrophilic fragments capable of hydrogen bonding[14]. The acids VIII were used as reference compounds.



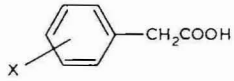
I : Y = $(CH_2)_2$ II : Y = $CH_2CH(CH_3)$
III : Y = $(CH_2)_3$ IV : Y = CH_2CHCH_2 (CH_3)



V : R = CH_3
VI : R = C_2H_5



VII



VIII

Rekker and Nyss[15–17], in their original fragmental method for the calculation of log P values (eqn. 4), characterized the influence of these interactions using the p.e. (proximity effect) correction values

$$\log P = \Sigma a_n f_n \quad (4)$$

Later, the intramolecular interactions were generally treated in the modified fragmental system of Leo and co-workers[14,18] by means of the fragmental factors F. Log

P wass expressed according to eqn. 5 as the sum of the fragmental constants f and the fragmental factors F.

$$\log P = \Sigma a_n f_n + \Sigma b_m F_m \quad (5)$$

We have used the fragmental method for the calculation of log P values, which were then compared with the experimental values obtained both by shake-flask method in the system octanol–water and by the chromatographic methods.

EXPERIMENTAL

TLC

Silanized Kieselgel 60 F_{254} (E. Merck, Darmstadt, F.R.G.) was used as the stationary phase. Impregnation was carried out by washing the glass plates (20 × 10 cm) with a 5% ethereal solution of silicone oil Lukoil 100 (VChZ Kolín, Czechoslovakia); the volatile components were evaporated within 16 h at 20°C. Solutions (1%) of the acids I–VIII in methanol were prepared and 5-μl samples were applied to the plate 3 cm from the lower edge. After evaporating the methanol at 20°C, ascending one-dimensional TLC was carried out using a citrate buffer (pH 3.4) containing 50% acetone as the mobile phase. The chromatographic chamber was equilibrated with the mobile phase for 16 h at 20°C. After migration for 15 cm, the plates were removed and, after the remaining mobile phase had been evaporated, the acids were detected under UV light (254 nm). Each chromatogram contained six compounds, two acids serving as reference samples. In the individual chromatograms the R_F values of the standards did not differ by more than 0.02.

HPLC

Experiments were carried out using a liquid chromatograph assembled from a Model 6000 A pump, a U6K injector, a 440-nm fixed wavelength detector and an M 730 data module (Waters Assoc., Milford, MA, U.S.A.). A commercial μBondapak C_{18} column (30 cm × 3.9 mm I.D.) (Waters Assoc.) was used as the stationary phase. Mixtures of 0.0025 M aqueous phosphate buffer (pH 3.0) with 40% acetonitrile or 50% methanol respectively were used as mobile phases. Doubly distilled water filtered through 0.45-μm Millipore filters was used throughout, and methanol was of LiChrosolv quality (E. Merck). The eluent flow-rate was 1 ml/min. Detection was performed by UV absorption at 254 μm, range 0–0.01 a.u. The retention time of sodium nitrate (0.2% solution) was taken as t_0 and the capacity factor, k', was evaluated from the retention time of the solute, t_R, by the relationship $k' = (t_R - t_0)/t_0$.

Determination of partition coefficients

Partition coefficients, P_{exp}, were determined by the shake-flask method[19] in an octanol–water system at 20°C, with both phases pre-saturated with the other. To eliminate the effect of dissociation, the aqueous phase employed was an acetate buffer (pH 3.4). The concentrations of the acids in the two phases were determined spectrophotometrically and the partition coefficients, P, were calculated as the ratio of concentrations in the octanol and aqueous phases ($P = C_o/C_w$).

Sample preparation

To prepare the acids I–IV we used the Friedel–Crafts reaction of anhydrides of dicarboxylic acids with the appropriately substituted aromatic compounds[20]. The acids V and VI were obtained[21] from the esters of aryloxocarboxylic acids by reaction with methylmagnesium iodide and subsequent hydrolysis. The acids VII were obtained[21] by reaction of 2-phenyl-2-hydroxyacetate with the corresponding benzyl chlorides in the presence of sodium hydride, followed by hydrolysis. The arylacetic acids VIII were prepared[22] by the Wilgerodt reaction or by the hydrolysis of the corresponding arylacetonitriles.

Calculations

Log P values of unsubstituted acids I–V and VII were calculated by the modified fragmental method[18]. Log P values of the substituted acids I–IV were calculated using the parameters π derived[23] for the substituted benzoic acids. For the acids V–VII the parameters π derived for the substituted benzyl alcohols were used, and for the acids VIII the parameters π were taken from those derived for the arylacetic acids[23]. The sum of the π parameters for the 3-chloro-4-alkoxy derivatives was reduced by 0.23, in accordance with the results of partition chromatography of those derivatives of arylaliphatic acids[11,13,22].

The coefficients in the regression equations were calculated from the experimental results by multiple regression analysis. The statistical significances of the regression equations were tested by the standard deviation (s), the coefficient of multiple correlation (r) and the Fischer–Snedecor criterion (F).

RESULTS AND DISCUSSION

The experimental R_F and R_M values of acids I–IV are summarized in Table I. The relationship between the logarithms of partition coefficients and the R_M values is expressed by the equation

$$\log P = 2.715\, R_M + 2.127$$
$$n = 22,\ r = 0.994,\ s = 0.105,\ F = 1676.8 \qquad (6)$$

Comparison of the logarithms of the experimental partition coefficients with the values computed by the fragmental method[14,18] (see Table II) shows that the corrections used describe well the H/H interactions and their effect on the lipophilicity. Eqn. 6 was applied to the acids I–IV with different connecting chains Y and that with different interactions between both hydrophilic fragments. From this equation it can be inferred that the corresponding changes in lipophilicity are similarly reflected both in the chromatographic and in the octanol–water reference system. Eqn. 6 does not include the 4-phenyl derivatives Ii, IIc, IIId and IVc as their lipophilicities differ substantially from the log P values in the octanol–water system.

The differences, however, do not prevent the use of R_M values as parameters of lipophilicity in the correlation with the collagen-induced aggregation of platelets. The equivalence of the log P and R_M values is evident from eqns. 7 and 8.

TABLE I

TLC CHARACTERISTICS OF ω-ARYLOXOALKANOIC ACIDS I–IV

No.	*X*	*Log P**	R_F	R_M	*Log* $(1/C_{exp})$**	*Log* $(1/C_c)$***
Ia	H	1.30*	0.655	−0.28	2.376	2.395
Ib	4-CH_3O	1.38*	0.655	−0.28	2.541	2.395
Ic	3-Cl-4-CH_3O	1.98	0.533	−0.055	2.602	2.648
Id	4-*i*-C_3H_7O	2.18	0.49	0.02	2.640	2.707
Ie	4-Br	2.28	0.517	−0.03	–	–
If	4-*i*-C_3H_7	2.70	0.40	0.18	–	–
Ig	3-Cl-4-*i*-C_3H_7O	2.78	0.365	0.24	2.785	2.803
Ih	4-*i*-C_4H_9	3.20	0.31	0.35	2.836	2.810
Ii	4-C_6H_5	3.20 * (2.70)§	0.38	0.21	2.780	2.797
Ik	4-cyclo-C_6H_{11}	3.76	0.207	0.585	2.775	2.731
Il	4-*n*-$C_6H_{13}O$	3.88	0.167	0.70	–	–
Im	3-Cl-4-cyclo-C_6H_{11}	4.36	0.14	0.79	2.500	2.557
IIa	H	1.62*	0.587	−0.155	2.240	2.352
IIb	4-*i*-C_3H_7O	2.50	0.393	0.19	2.699	2.593
IIc	4-C_6H_5	3.40* (3.13)§	0.30	0.37	2.638	2.610
IId	3-Cl-4-*i*-C_4H_9O	3.60	0.21	0.58	–	–
IIe	4-cyclo-C_6H_{11}	4.08	0.16	0.72	2.417	2.430
IIIa	3-Cl-4-CH_3O	2.17*	0.483	0.03	3.009	3.041
IIIb	4-*i*-C_3H_7	2.89	0.345	0.28	3.167	3.137
IIIc	4-*i*-C_4H_9	3.39	0.234	0.515	3.071	3.095
IIId	4-C_6H_5	3.35* (2.97)§	0.33	0.31	3.185	3.139
IIIe	4-cyclo-C_6H_{11}	3.95	0.172	0.685	2.975	2.986
IVa	3-Cl-4-CH_3O	2.60*	0.414	0.15	2.896	2.907
IVb	3-Cl-4-*i*-C_3H_7O	3.40	0.241	0.50	2.936	2.904
IVc	4-C_6H_5	3.82 (3.43)§	0.248	0.48	2.839	2.912
IVd	4-cyclo-C_6H_{11}	4.38	0.147	0.76	2.762	2.719

* Values marked with asterisks were determined in octanol–buffer (pH 3.5) by the shake-flask method a the others were calculated using the respective π values (see Experimental).

** The anti-aggregating activity was measured by the Born's method[24] and expressed by the concentratior (mol l^{-1}) that produced a 50% inhibition of aggregation.

*** Calculated from eqn. 8.

§ Calculated from eqn. 6.

$$\log (1/C) = 0.869 \log P - 0.144 (\log P)^2 + 0.145 E_S + 0.328 I_L + 1.839 \quad (7)$$
$$n = 22, r = 0.973, s = 0.064, F = 75.3$$
$$\log (1/C) = 0.739 R_M - 1.152 R_M^2 + 0.159 E_S + 0.328 I_L + 1.839 \quad (8)$$
$$n = 22, r = 0.972, s = 0.065, F = 73.2$$

Similarity of the solvation forces in the two systems cannot be overestimated, however. The common chromatographic evaluation of selected acids I–VI and VIII yielded the R_M values summarized in Table II. Eqn. 9 and Fig. 1 illustrate the relationship between log P and R_M for all acids.

$$\log P = 2.495 R_M + 2.476 \quad (9)$$
$$n = 21, r = 0.972, s = 0.198, F = 329.7$$

Systematic deviations of the individual structurally different series of acids indicate that these compounds cannot be merged into a single regression equation. In

TABLE II

CHROMATOGRAPHIC BEHAVIOUR OF ACIDS I–VI AND VIII

No.	*X*	*Log P**	R_M	*Log k'***	*Log k'****
Ia	H	1.30* (1.24)§	−0.365	0.201	0.107
Ic	3-Cl-4-CH_3O	1.98	–	0.610	0.365
Id	4-*i*-C_3H_7O	2.18	−0.03	0.815	0.504
Ie	4-Br	2.28	−0.015	0.675	0.445
If	4-*i*-C_3H_7	2.70	0.18	1.039	0.683
Ii	4-C_6H_5	3.20*	–	–	0.795
Ik	4-cyclo-C_6H_{11}	3.76	–	–	1.190
Ih	4-*i*-C_4H_9	3.20	0.30	–	–
IIa	H	1.62* (1.65)§	−0.225	0.437	0.268
IIc	4-C_6H_5	3.40*	–	–	0.964
IIe	4-(2',4'-$F_2C_6H_3$)	3.68	–	–	1.068
IIIa	3-Cl-4-CH_3O	2.17* (2.10)§§	−0.02	0.819	0.452
IIId	4-C_6H_5	3.35*	–	–	0.901
IVa	3-Cl-4-CH_3O	2.60* (2.51)§§	0.145	–	0.650
IVb	3-Cl-4-*i*-C_3H_7O	3.40	–	1.532	–
IVc	4-C_6H_5	3.82*	–	–	1.068
Va	H	0.80* (0.91)§	−0.715	−0.040	−0.144
Vb	4-CH_3O	0.81	−0.71	−0.030	−0.144
Vc	3-Cl-4-CH_3O	1.26	−0.51	–	–
Vd	4-*i*-C_4H_9	2.75*	0.06	1.152	0.653
Ve	4-C_6H_5	2.71*	−0.05	–	0.514
Vf	4-cyclo-C_6H_{11}	3.26	0.215	–	–
Vg	4-C_2H_5	1.78	−0.33	–	–
VIa	4-*i*-C_4H_9	3.37*	0.20	1.432	0.898
VIb	4-C_6H_5	3.37	–	–	0.747
VIIIa	H	1.45*	−0.44	0.226	0.120
VIIIb	4-Cl	2.15	−0.205	0.591	0.379
VIIIc	4-C_2H_5	2.43	−0.03	0.819	0.524
VIIId	4-*i*-C_3H_7	2.85	0.12	1.086	0.710
VIIIe	4-*t*-C_4H_9	3.13	0.26	1.297	0.857
VIIIf	4-*n*-$C_5H_{11}O$	3.46	0.335	1.541	1.052
VIIIg	4-cyclo-C_6H_{11}	3.91	–	–	1.199

* Values marked with asterisks were determined in octanol–buffer (pH 3.5) by the shake-flask method and the others were calculated using the respective π values (see Experimental).

** methanol was used as a modifier in the mobile phase.

*** Acetonitrile was used as a modifier in the mobile phase.

§ Values were calculated using the fragmental constants from ref. 18.

§§ Values were calculated by a combination of the fragmental method[18] (unsubstituted compound) and π parameters from ref. 23.

such a case an attempt to use the equation for the computation of the partition coefficients for structurally diverse compounds could lead to false results. It is evident also from the different linear relationships between log P and R_M for aryloxoalkanoic acids (eqn. 10), arylhydroxyalkanoic acids (eqn. 11) and arylacetic acids (eqn. 12).

$$\log P = 2.765\, R_M + 2.267 \quad (10)$$

$$n = 8,\ r = 0.995,\ s = 0.067,\ F = 565.9$$

$$\log P = 2.632\, R_M + 2.682 \quad (11)$$

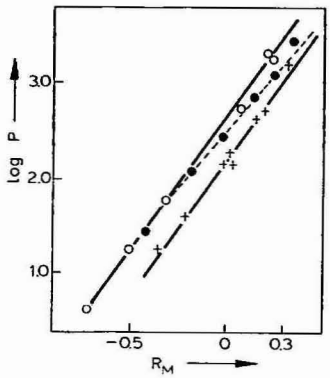


Fig. 1. Relationship between log P and R_M for arylacetic (●), aryloxoalkanoic (+) and arylhydroxyalkanoic (○) acids.

$$n = 7,\ r = 0.998,\ s = 0.066,\ F = 1619.9$$
$$\log P = 2.463\ R_M + 2.562 \quad (12)$$
$$n = 6,\ r = 0.996,\ s = 0.077,\ F = 445.9$$

The capacity factors of selected acids I–VI and VIII are given in Table II. In contrast to previous results there are no significant differences among the individual series of acids, regardless of the modification of the mobile phase by acetonitrile or methanol. The common linear relationships between log P and log k' values are expressed by eqns. 13 and 14.

$$\log P = 2.310 \log k'\ (CH_3CN) + 1.177 \quad (13)$$
$$n = 25,\ r = 0.993,\ s = 0.106,\ F = 1716.9$$
$$\log P = 1.657 \log k'\ (CH_3OH) + 0.949 \quad (14)$$
$$n = 17,\ r = 0.992,\ s = 0.114,\ F = 903.9$$

Eqn. (13) also contains the biphenylyl analogues If, IIc, e, IIId and IVc. These results indicate that for these particular systems the Collander rule holds for all three groups of acids, distiguished by considerable structural changes.

TABLE III

CHROMATOGRAPHIC BEHAVIOUR OF ACIDS VII

No.	*X*	R_M	*Log k'**	*Log k'***	Log P_{exp}***	*Log P_{calc}*		
						A§	*B*§§	*C*§§§
VIIa	H	−0.25	0.875	0.584	2.00	2.71	2.40	2.53
VIIb	3-Cl-4-CH_3O	−0.01	1.158	0.791	2.45	3.32	2.87	3.00
VIIc	4-Cl	−0.09	1.264	0.847	2.60	3.57	3.04	3.13

* Methanol was used as a modifier in the mobile phase.
** Acetonitrile was used as a modifier in the mobile phase.
*** Determined in octanol–buffer (pH 3.5) by the shake-flask method.
§ Calculated by the fragmental method.
§§ Calculated from eqn. 14 using log $k'(CH_3OH)$.
§§§ Calculated from eqn. 13 using log $k'(CH_3CN)$.

The lipophilicity of three derivatives of 2-benzyloxyphenylacetic acid (VII) was also evaluated by HPLC (see Table III). Similarly to the benzyloxy arylaliphatic acids, there was a decrease in the experimental values of log P compared with the values calculated by the fragmental method. This may be due to the intramolecular hydrophobic interaction of both aromatic nuclei connected by a sufficiently flexible three-atom chain. The retention characteristics of these acids do not correspond to the lipophilicity measured in the octanol–water system. The values of log P calculated from eqns. 13 and 14 are considerably higher than those of log P_{exp} (see Table III).

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STUDIES ON LECTINS

LVIII. SUGAR-BINDING PROPERTIES, AS DETERMINED BY AFFINITY ELECTROPHORESIS, OF α-D-GALACTOSIDASES FROM *VICIA FABA* SEEDS POSSESSING ERYTHROAGGLUTINATING ACTIVITY

P. MALÝ, M. TICHÁ and J. KOCOUREK*
Department of Biochemistry, Charles University, Albertov 2030, 128 40 Prague 2 (Czechoslovakia)
(Received June 25th, 1985)

SUMMARY

The interaction of α-D-galactosidases from *Vicia faba* seeds with saccharides was studied by means of affinity electrophoresis on polyacrylamide gel in an acidic buffer system. For the preparation of affinity gels, water-soluble O-glycosyl polyacrylamide copolymers and polysaccharides were used. α-D-Galactosidases interact with immobilized O-α-D-galactosyl residues and glycogen, but no interaction was observed with immobilized O-α-D-mannosyl residues. On the basis of the results of affinity electrophoresis performed in the presence of various free sugars, dissociation constants of the various α-D-galactosidase–free sugar complexes were calculated.

INTRODUCTION

Shannon *et al.*[1] have shown that there are at least two evolutionarily-related, but distinct classes of legume proteins which possess erythroagglutinating activity: the "classic" lectins and α-D-galactosidases possessing erythroagglutinating activity. The sugar specificities of the binding sites responsible for erythroagglutinating activity of α-D-galactosidases are usually different from those of the "classic" lectins. Further, it has been found that not all legume seeds contain α-D-galactosidases with erythroagglutinating activities[1]. Dey and co-workers[2,3] have shown that *Vicia faba* seeds contain three forms of α-D-galactosidase: a high-molecular-weight (I) and two low-molecular-weight species (IIa and IIb); all forms agglutinate only trypsinized rabbit erythrocytes. Contrary to the α-D-galactosidase described by Shannon *et al.*[1], the erythroagglutinating activity of α-D-galactosidase I from *Vicia faba* seeds was inhibited by derivatives of D-mannose and D-glucose.

In our experiments, we have used affinity electrophoresis on polyacrylamide containing poly(glycosyloxyalkenyl–acrylamide) copolymers (O-glycosyl polyacrylamide copolymers)[4] to study the sugar-binding properties of the high- and low-molecular-weight forms of α-D-galactosidase from *Vicia faba* seeds.

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MATERIALS AND METHODS

Seeds of *Vicia faba*, cv. Uran were supplied by State Farm, Prague, Czechoslovakia. Water-soluble poly(glycosyloxyalkenyl–acrylamide) copolymers used for affinity electrophoresis were prepared as described earlier[4]. Mussel glycogen was purchased from Sigma (St. Louis, MO, U.S.A.). Two forms of α-D-galactosidase (α-D-galactosidase I and α-D-galactosidase II) were separated according to Dey *et al.*[2]. For the study of simultaneous interaction of both forms of α-D-galactosidase with saccharides, protein preparation obtained by the precipitation of 0.15 *M* sodium chloride extract with an ammonium sulphate fraction corresponding to 80% saturation, was used.

Affinity electrophoresis

Polyacrylamide gel electrophoresis of α-D-galactosidase was performed in an apparatus designed by Davis[5] in a discontinuous acidic buffer system[6] according to the standard procedure (omitting the large-pore gel layers).

Protein samples (15–30 μg) in 20% glycerol solution (30 μl) were applied to each tube (5 × 75 mm) and electrophoresis was run at 7 mA per tube for 70–100 min. Gels were stained specifically[7]. The migration distances of the zones of α-D-galactosidase were measured with an accuracy of ± 0.5 mm.

The dissociation constants (K_i) of the complexes of α-D-galactosidase I and II with immobilized α-D-galactosyl residues were obtained by a modification of our original method[8,9] from the dependence of $1/d_0 - d$ *vs.* $1/c_1$. The dissociation constants (K) of the complexes of α-D-galactosidase I and II and free sugars were obtained as described in our previous communications[8] from the dependence of $d/d_0 - d$ *vs.* c; c_1 = concentration of immobilized sugar, c = concentration of free sugar, d_0 = mobility on control gel containing water-soluble polyacrylamide without sugar residues, d = mobility on affinity gel at given c_i and c.

Affinity gels were prepared by the addition of an appropriate amount of the solution of O-glucosyl polyacrylamide copolymer or polysaccharide to the polymerization mixture to give a desired concentration c_i of immobilized sugar residues; c_i was used in the range $3.1 \cdot 10^{-3}$–$12.2 \cdot 10^{-3}$ *M*.

For the determination of K, solutions of free sugars were added to the polymerization mixture; the concentration range (c) of free sugars was different for various sugars according to the strength of interaction ($c = 8 \cdot 10^{-3}$–$240 \cdot 10^{-3}$ *M*).

Polyacrylamide gel electrophoresis for the detection of the presence of lectin was performed in discontinuous acidic[6] and discontinuous alkaline[5] buffer systems. Conditions of electrophoresis were the same as described for electrophoresis of α-D-galactosidase or as described by Davis[5]. Gels were stained with Amido Black. Affinity gels were prepared by the addition of O-α-D-mannosyl polyacrylamide copolymer to the polymerization mixture; c_i used was $4.7 \cdot 10^{-3}$ *M*.

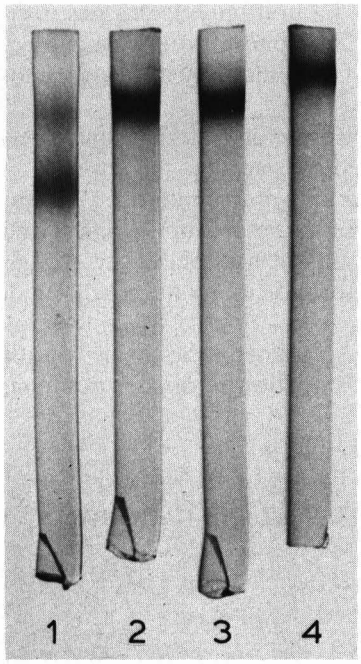
Erythroagglutinating activity

Erythroagglutinating activities of α-D-galactosidase and the lectin and inhibitory activities of sugars were assayed by a test-tube serial dilution method as described previously[10]. Human non-modified erythrocytes (A_1, A_2, B, O) and trypsinized rabbit erythrocytes were used.

RESULTS

High (I) and low molecular (II) forms of α-D-galactosidase present in *Vicia faba* seeds differ in their electrophoretic mobility on polyacrylamide gels in a discontinuous acidic buffer system (Fig. 1). They were separated by means of gel chromatography on Sephadex G-100 as described by Dey *et al.*[3] (Figs. 1 and 2). In the same purification step, the "classic" lectin was removed; it was completely bound to the dextran gel and could be eluted by a 0.2 *M* D-glucose solution. The absence of lectin in the fractions containing α-D-galactosidase was confirmed by affinity electrophoresis on polyacrylamide gels containing O-α-D-mannosyl polyacrylamide copolymer (no protein was retarded at $c_i = 4.7 \cdot 10^{-3}$ *M*) or by an erythroagglutination test with native human erythrocytes (no agglutination of human erythrocytes was observed).

In agreement with finding by Dey *et al.*[2], α-D-galactosidase I and II agglutinated trypsinized rabbit erythrocytes. Erythroagglutinating activity of both forms of α-D-galactosidase was inhibited most effectively by methyl α-D-mannoside, D-mannose and glycogen; D-galactose, methyl α-D-galactoside, D-xylose, N-acetyl-D-galac-



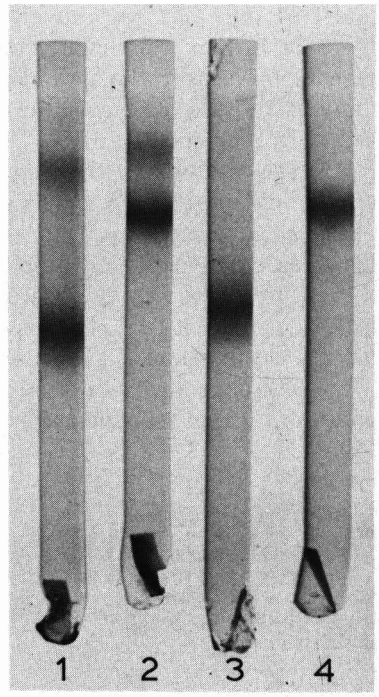


Fig. 1. Affinity electrophoresis of α-D-galactosidase I. 1 = Ammonium sulphate fraction, 2–4 = α-D-galactosidase I, 1, 2 = control gels, 3 = affinity gel containing O-α-D-mannosyl polyacrylamide copolymer ($c_i = 1.9 \cdot 10^{-2}$ *M*), 4 = affinity gel containing O-α-D-galactosyl polyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*).

Fig. 2. Affinity electrophoresis of α-D-galactosidase II. 1, 2 = Ammonium sulphate fraction, 3, 4 = α-D-galactosidase II, 1, 3 = control gels, 2, 4 = affinity gels containing O-α-D-galactosyl polyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*).

tosamine and *myo*-inositol did not affect the erythroagglutinating activity. Contrary to α-D-galactosidase from *Vigna radiata*[1], α-D-galactosidase from *Vicia faba* seeds does not possess the so-called "clot dissolving activity". A clot of agglutinated erythrocytes was observable even after standing for 12 h at laboratory temperature.

Affinity electrophoresis

The addition of O-α-D-galactosyl polyacrylamide copolymer to polyacrylamide gels caused a decrease in the electrophoretic mobility of both forms I and II of α-D-galactosidase (Figs. 1 and 2). The decrease of electrophoretic mobility is dependent on the concentration of immobilized D-galactosyl residues in the polyacrylamide gels. The control gels were prepared by the addition of water-soluble polyacrylamide (containing no saccharide residues) in the same concentration as the O-α-D-galactosyl polyacrylamide copolymer.

From the dependence of electrophoretic mobility on the concentration of immobilized sugar (c_i), the dissociation constant of the complex enzyme-immobilized D-galactosyl residue was calculated (Table I). The electrophoretic mobility of both forms of α-D-galactosidase was not affected by the presence of N-acetyl-O-α-D-galactosaminyl and O-α-D-mannosyl polyacrylamide copolymers at $c_i = 1.98 \cdot 10^{-2}$ *M* and $1.84 \cdot 10^{-2}$ *M*, respectively.

In further experiments, affinity gels containing natural polysaccharides (dextran T-500, yeast mannan and glycogen) in 1% and 2% concentrations were used. Only in the case of glycogen was a marked decrease in the mobility of α-D-galactosidase I observed (Fig. 3).

The addition of free sugars, which interact with the enzyme in affinity gels containing immobilized α-D-galactosyl residues, caused a reversal of the retardation of the enzyme mobility. Only those sugars which are known to inhibit the α-D-galactosidase activity[11,12] (Table II) were able to reverse the retardation (Figs. 4 and 5). In agreement with literature data[10,11], sugars which did not inhibit the α-D-galactosidase activity did not interact with the α-D-galactosidase under the condition of affinity electrophoresis (Table II).

The dependence of electrophoretic mobility of α-D-galactosidase zones on the concentration of free sugar (c) was used for the determination of dissociation con-

TABLE I

DISSOCIATION CONSTANTS OF COMPLEXES OF BOTH FORMS OF α-D-GALACTOSIDASE AND IMMOBILIZED SACCHARIDE

No interaction was observed at $c_i = 1.9 \cdot 10^{-2}$ *M* (D-mannose) and $1.7 \cdot 10^{-2}$ *M* (N-acetyl-D-galactosamine).

Immobilized saccharide	K_i *(M)*	
	α-D-*Galactosidase I*	α-D-*Galactosidase II*
D-Galactose	$11.5 \cdot 10^{-3}$	$6.9 \cdot 10^{-3}$
D-Mannose	–	–
N-Acetyl-D-galactosamine	–	–

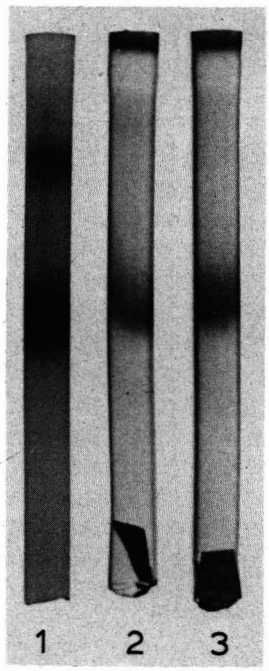


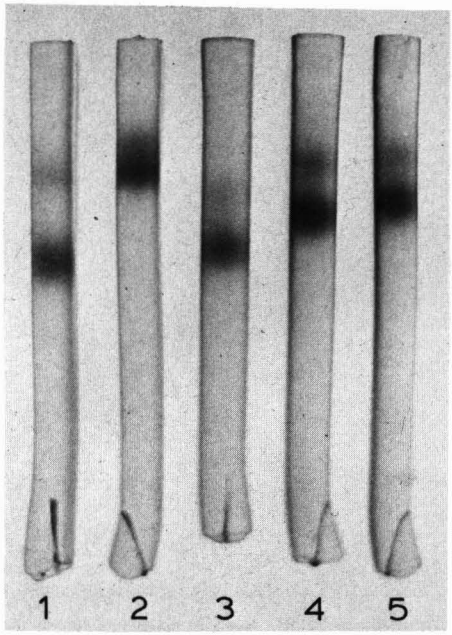
Fig. 3. Affinity electrophoresis of α-D-galactosidase on affinity gel containing glycogen. 1–3 = Ammonium sulphate fraction, 1 = control gel, 2 = affinity gel containing 1% glycogen, 3 = affinity gel containing 1% glycogen and $1.1 \cdot 10^{-1}$ *M* methyl α-D-mannopyranoside.

TABLE II

DISSOCIATION CONSTANTS OF COMPLEXES OF BOTH FORMS OF α-D-GALACTOSIDASE WITH FREE SUGARS

No interaction was observed at the concentration of free saccharide, $c = 0.25$ *M*.

Saccharide	*K (M)*	
	α-D-Galactosidase I	*α-D-Galactosidase II*
D-Galactose	$11.1 \cdot 10^{-3}$	$8.8 \cdot 10^{-3}$
Methyl α-D-galactoside	$8.5 \cdot 10^{-3}$	$4.8 \cdot 10^{-3}$
Ethyl α-D-galactoside	$7.1 \cdot 10^{-3}$	$4.3 \cdot 10^{-3}$
D-Xylose	$31.2 \cdot 10^{-3}$	$30.4 \cdot 10^{-3}$
myo-Inositol	$66.1 \cdot 10^{-3}$	$12.9 \cdot 10^{-3}$
L-Arabinose	–	$73.6 \cdot 10^{-3}$
D-Fucose	–	$64.9 \cdot 10^{-3}$
2-Deoxy-D-galactose	–	–
4-O-Methyl-D-galactose	–	–
N-Acetyl-D-galactosamine	–	–
N-Acetyl-D-glucosamine	–	–
D-Mannose	–	–
L-Mannose	–	–
L-Galactose	–	–
L-Rhamnose	–	–
L-Fucose	–	–
D-Fructose	–	–
Lactose	–	–



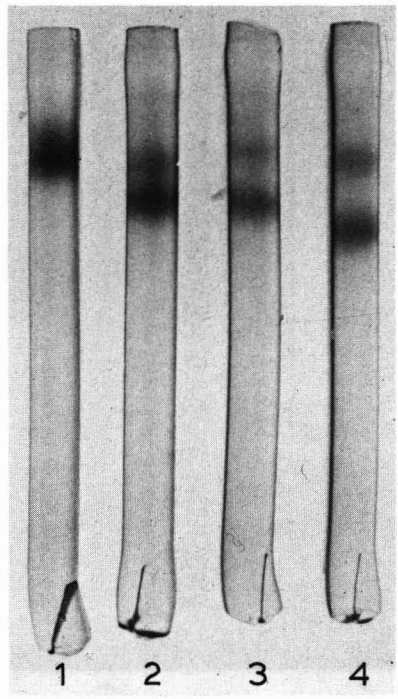


Fig. 4. Affinity electrophoresis of α-D-galactosidase in the presence of free D-galactose. 1–5 = Ammonium sulphate fraction, 1 = control gel, 2–5 = affinity gels containing O-α-D-galactosyl polyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*), 3–5 = affinity gels containing immobilized α-D-galactosyl residues and decreasing concentration of free D-galactose ($c = 7.0 \cdot 10^{-2}$ *M*, $2.0 \cdot 10^{-2}$ and $8.0 \cdot 10^{-3}$ *M*, respectively).

Fig. 5. Affinity electrophoresis of α-D-galactosidase in the presence of D-xylose. 1–4 = Ammonium sulphate fraction, 1–4 = affinity gels containing O-α-D-galactosyl polyacrylamide copolymer ($c_i = 6.1 \cdot 10^{-3}$ *M*), 2–4 = affinity gels containing immobilized α-D-galactosyl residues and increasing concentration of free D-xylose ($c = 4.5 \cdot 10^{-2}$ *M*, $9.0 \cdot 10^{-2}$ *M*, $2.4 \cdot 10^{-1}$ *M*, respectively).

stants of the complexes of α-D-galactosidase I and II and enzyme-free sugars (*K*) (Table II).

DISCUSSION

The method of affinity electrophoresis on polyacrylamide gel has been used to study the interaction of several enzymes, such as amylase, phosphorylase, various dehydrogenases, trypsin, RNAase and D-galactose oxidase, with the respective substrates or inhibitors[13]; but, to our knowledge, this method has not yet been employed to investigate binding properties of glycosidases. The method makes it possible to evaluate simultaneously the strength of interaction of all isoenzymes (as was described in the case of lactate dehydrogenase[14]), as well as all multiple forms of an enzyme, differing in their electrophoretic mobilities (as has been shown in this article).

Vicia faba seeds have been reported to contain multiple forms of α-D-galactosidase: high-molecular-weight I and low-molecular-weight IIa and IIb forms, which

differ in their electrophoretic mobilities[3]. In our experiments, we have observed only the presence of one low-molecular-weight (denoted II) and one high-molecular-weight form (denoted I).

Dey *et al.*[2] have described the isolation and properties of α-D-galactosidase I from *Vicia faba* seeds which possess erythroagglutinating activity. This activity is inhibited by D-glucose and D-mannose derivatives, which are quite ineffective in inhibiting the α-D-galactosidase activity. α-D-Galactosidases from *Vicia faba* seeds differ in erythroagglutinating activity from α-D-galactosidases from *Vigna radiata*[1,15], *Pueraria thunbergiana, Thermopsis caroliniana, Lupinus arboreus* and *Phaseolus lunatus*[1,16] and soybean (*Glycine soja*)[17]. The erythroagglutinating activities of these enzymes are inhibited by the same sugars as those for α-D-galactosidase activity, *e.g.* by derivatives of D-galactose, D-xylose and *myo*-inositol[15–17].

Thus, α-D-galactosidases from *Vicia faba* seeds should possess two types of binding sites for saccharides: one for derivatives of D-galactose and the other for derivatives of D-mannose and D-glucose. In our experiments we have used affinity electrophoresis on gels containing water-soluble O-glycosyl polyacrylamide copolymers to study the interaction of α-D-galactosidases with both types of saccharides. Our results have shown that both forms of α-D-galactosidase from *Vicia faba* seeds interact under the conditions of affinity electrophoresis with immobilized α-D-galactosyl residues, while no interaction was observed in the case of O-α-D-mannosyl residues, even though the erythroagglutinating activity was inhibited by D-mannose and methyl α-D-mannopyranoside (see Dey *et al.*[2] and our results).

An interesting phenomenon has been observed with affinity gels containing polysaccharides; in the case of glycogen, the zone corresponding to α-D-galactosidase I was fully retarded on the affinity gel, but this retardation could not be reversed by addition of methyl α-D-mannopyranoside to the affinity gel; probably much higher concentration or more complex saccharide is needed to inhibit the interaction. The observed interaction of α-D-galactosidase with glycogen under the conditions of affinity electrophoresis is in good agreement with the data described by Dey *et al.*[2], who observed the precipitation of α-D-galactosidase I in the presence of glycogen.

Affinity electrophoresis in the absence and in the presence of free saccharides was used for the determination of dissociation constants of the complexes of α-D-galactosidase with immobilized and free sugars. The determined values are in good agreement with inhibition constants determined kinetically[11,12]. Values of dissociation constants determined separately for individual forms of α-D-galactosidase, or simultaneously in a mixture, did not differ.

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Note

New approach to capillary columns for gas chromatography?

Condensation of hydroxyl-terminated stationary phases

K. GROB* and G. GROB
GC-Laboratory ETH Zürich, EAWAG, 8600 Dübendorf (Switzerland)
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We should make it clear at the outset that the "approach" referred to in the title is new to individual column makers, although its development was started in 1978[1] since when it has received limited attention without influencing the common procedures for column preparation. Owing to the perfect secrecy surrounding commercially applied procedures, we cannot exclude the possibility (we even suspect) that the new approach has become of great importance in industrial column production.

Verzele *et al.*[2] presented a comprehensive survey of the background of the approach, which is characterized by variosus sidetracks for specific purposes that have meanwhile become less attractive. Advanced ideas including the approach among others have been discussed by Lipsky *et al.*[3]. Surprisingly, apart from a short complement by Sandra *et al.*[4], the literature has been silent on the topic for almost 3 years.

Verzele *et al.*[2] described a surface pretreatment consisting of baking a thin deposit of a low-viscosity hydroxyl-terminated phenylmethylsilicone, which is followed by coating with a high-viscosity version of the same phase. Immobilization occurs by mere heat curing (150–250°C, repeatedly). The authors see the strong point of the procedure in the immobilized coating, with well-defined selectivity and polarity that are due to total absence of groups other than phenyl and methyl. As a mechanism of immobilization they assume the formation of very long chains, the terminal silanols of which can be capped by silylation. They also envisage additional cross-linking, if desirable. As an upper temperature limit, 280–300°C was indicated, which was subsequently[4] amended to 370°C.

EXPERIMENTAL

We have studied the following hydroxyl-terminated phases: OV-61 (33% phenyl); OV-17 (50% phenyl); OV-1701 (7%phenyl, 7% cyanopropyl); and an experimental phase with 17% cyanopropyl groups. All phases have been kindly provided, at our request, by Ohio Valley Specialty Chemicals.

DURAN glass was leached and dehydrated as usual[5], but with a wide range of the experimental parameters. Persilylation for OV-61 and OV-1701 was carried out with diphenyltetramethyldisilazane (DPTMDS), for OV-17 with tetraphenyldi-

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methyldisilazane (TPDMDS), and for the 17% cyano phase with the mixture of XF-1150 and DPTMDS, as recently reported[6]. All persilylations occurred at 400°C for 15 h.

For regular static coating, the phases were dissolved in pentane–methylene chloride (1:1, v/v), as described recently[7]. At the end of this procedure, the columns were mounted in a gas chromatograph with the exit connected to a flame ionization detector. At a gas flow-rate roughly three times the normal, and with strong attenuation, the columns were directly heated to 160–180°C, followed by temperature programming to 310°C at a rate determined by the column length (5°/min for 10 m, 2.5°/min for 20 m). During the entire heat treatment (40–100 min) a chromatogram was recorded. When the intense, and complex, elution came to a clear end, a first test was run. Further tests followed after prolonged baking.

THE IMMOBILIZATION PROCESS

Fig. 1 shows a chromatogram obtained during the first heat treatment of a freshly coated column. By the end of the treatment, immobilization was observed, so the chromatogram provides an indirect description of the immobilization process. For a given phase, the corresponding chromatogram is surprisingly typical and reproducible, in terms of the number, shape, and even minor details, such as the shoulders on the rear slope of the peaks. The peak areas correlate reasonably with the total mass of the stationary phase, which depends on the filmthickness and the column length. It seems, therefore, that the thermal process produces a series of defined volatiles, which have not yet been checked mass spectrometrically. Typical for all phases is the step-wise sharp descent, which indicates complete elution of a volatile after chromatography under drastically overloaded conditions. Depending on the phase, the last volatile elutes between 280 and 310°C. Elution is also evidently influ-

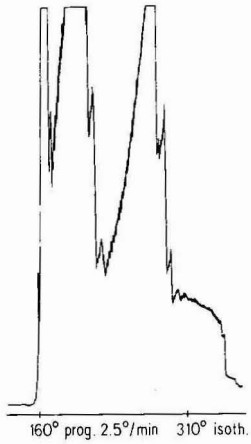

Fig. 1. Chromatogram recorded during the first conditioning of a column (20 m × 0.32 mm I.D.) fresh coated with 0.3 μm OV-61-OH. Immediately after coating the column was connected to a stream of hydrogen at 1.0 bar, and heated directly to 160°C. Flame ionization detection Carlo Erba Model 4160; attenuation, 1024. The three major peaks with the shoulders on the rear slope are typical for OV-61-OH. Note the sharp descent of the baseline after *ca.* 15 min at 310°C isothermal, indicating the end of the gross condensation process.

enced by the column length and the filmthickness. A further influence related to some individual characteristics of the given column is discussed below. Normally, after 40–100 min, the recording suddenly returns to a clean baseline.

POLARITY

The idea that the absence of groups other than methyl or phenyl should ensure a defined polarity works in a less straightforward way than expected. As Fig. 2 shows, the polarity slowly drops during conditioning. A slight decrease may even be observed after several days of continuous heating above 300°C. It seems logical to attribute the change to a gradual loss of terminal silanols, which may no longer play a role in immobilization but still influence the polarity.

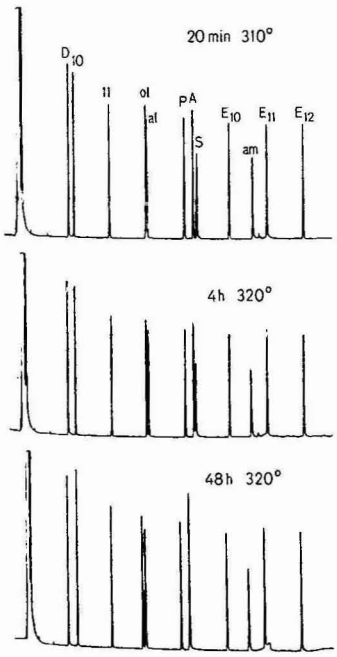

Fig. 2. Decreasing polarity owing to loss of terminal hydroxyl groups. Column, 15 m × 0.32 mm I.D., persilylated with DPTMDS, coated with 0.25 μm OV-1701-OH. First test immediately after the condensation reaction, further tests after prolonged conditioning. Peaks: D = 2,3-butanediol; 10,11 = *n*-alkanes; ol = 1-octanol; al = nonanal; P = 2,6-dimethylphenol; A = 2,6-dimethylaniline; S = 2-ethylhexanoic acid; am = dicyclohexylamine; E_{10}–E_{12} = fatty acid methyl esters. Injection at 25°C, programme 2.5°/min from 40°C. Note the increasing distance of D from 10, and of ol from al, as well as the merging of S with A. Note also the slight loss of inertness at 320°C. Less balanced columns may show far stronger polarity drift. (An unknown impurity influences the baseline of E_{11}.)

Here, we think the following observation to be of importance. As a typical feature of glass, the density of hydroxyls on the silica surface can be varied over a wide range by varying the intensity of leaching (130–185°C, 5–20 h) and of dehydration (150–450°C, 1–4 h), as well as by varying the persilylation conditions. The observation is that the polarity decreases more rapidly on a strongly hydroxylated sur-

face. This, together with further observations (see next paragraph), causes us to suspect that the condensation reaction between silanols on chain-ends and on the surface is faster than the reaction between chain ends. Thus, immobilization may be based primarily on surface bonding, rather than on a big increase of chain length (the latter may occur as well, but with an effect more on the polarity than on immobilization). This interpretation is further supported by the fact that heating the pure phase (to the same temperature, for the same time) is a far less efficient way of producing an insoluble (immobilized) material than heating the phase on the silica surface.

Verzele *et al.*[2] mentioned in passing the possibility of silylating the condensed coating. We confirm, and emphasize, this information. Except for apolar phases, the silylation of silicone coatings generally produces no effect, or even a negative one. In contrast, coatings based on hydroxyl-terminated phases are well suited to silylating. A slow injection, without splitting, of 20–50 μl of hexamethyldisilazane onto a column at 140°C is rather effective. Residual silanols are eliminated, with a corresponding reduction of polarity. Important column-to-column differences in the efficiency of silylation are observed, probably owing to different degrees of silanol consumption by the bonding process.

If reproducibility of the polarity is the major purpose, thorough silylation should be included as the last step of column preparation.

INERTNESS

Coatings obtained from hydroxyl-terminated phases are generally more inert than traditional ones. We attribute this to the elimination of residual surface silanols (not eliminated by persilylation) by the condensation reaction with the phase.

The study of inertness has confirmed a basic idea we have gradually built up over years from scattered observations. The idea is that silanols in the phase increase the polarity without influencing inertness. In contrast, silanols on the surface (besides also affecting the polarity) first of all reduce inertness. Consequently, inertness should not be correlated with the incidence of silanol groups on the column as a whole, but specifically with the silanol groups on the surface of the support.

From a large number of columns produced with widely varying parameters, we deduce an essential role of the balance between residual surface silanols (surviving persilylation) and phase silanols (resulting from the molecular weight of the phase, and film thickness). Columns reaching the balance show perfect inertness and easy conditioning (condensation). Excess phase silanols cause top inertness, but slow immobilization accompanied with a strong and long-lasting polarity drift. Excess surface silanols result in rapid immobilization and quick attainment of the final polarity. However, inertness is modest and never attains the desired level, although it considerably improves during conditioning. Also, it cannot be influenced significantly by silylation.

FILM STABILITY

Here, we face the main general limitation of our coating technique. The immobilized film is absolutely stable. However, we have to deposit it, at least for a short period, non-immobilized. This means that, for this period, stabilization has to be

based on the well-known physical interactions between the phase and the surface that create wetting. In other words, to make the film survive for one critical hour (before it will be stable for years), we have to invest a wealth of perspicacity and care without contributing to the final quality of the product. In fact, our efforts may even limit the final quality. And, as a matter of most regrettable fact, we are unable to make quite a number of columns because we are unable to master the problem of first-hour film stability.

To a certain extent, critical, or even missing, wettability can be replaced by increased phase viscosity (gum phases[8]), but this is not a general way out of the difficulty. The more polar phases (containing more than 50% of cyano groups) in particular are hardly amenable to a gum state, although being able to use them as gums would be especially beneficial. Thus, finding a principle to circumvent wettability as a basis of the first-hour film stability would be a real breakthrough.

Wettability requirements also reduce our freedom to vary the persilylation conditions. Of course, this restriction hinders the free selection of the density of surface silanols. This is a major reason why we are unable to present the above-mentioned balance concept in a more quantitative way.

BLEEDING AND THERMOSTABILITY

A most impressive feature of the new columns is the bleeding, which drops sharply to a very low level after the first condensation (immobilization) reaction and reaches the final level in a few hours, provided that balance has been attached. Bleeding is clearly related to the polarity drift. It may drop during conditioning at 310–330°C for 24–48 h, when polarity also further drops during the treatment.

Bleeding is a reasonable indicator of thermostability. It is difficult, however, to deduce absolute temperature limits from absolute bleed-rates. In most cases, relative indications are more informative. Good coatings based on hydroxyl-terminated phases reach a comparable bleeding behaviour at temperatures 60–80°C higher than conventional coatings, *e.g.* hydroxyl-terminated OV-1701 bleeds as much at 330°C as the conventional phase (vinylated, immobilized) at 250°C.

Our primary purpose of studying both phenyl and cyano phases, was to learn whether the new approach is equally applicable to both types (the earlier work was limited to phenyl phases). Fortunately, we found no dependence on the type of substitution. We agree with the statement of the first authors[4] on that phenyl-containing hydroxyl-terminated coatings reach a critical bleed-rate at 360–380°C. We found the corresponding value for cyano phases to be 330–350°C.

However, we are frequently more interested in knowing at what maximum temperature the inertness is affected within 24 h. Obviously, this temperature limit is below the one based on bleeding. It is here where we find a particularly strong point of the new approach. The difference between the temperatures at which comparable losses of inertness occur is as much as 80–100°C. Hydroxyl-terminated OV-1701 keeps its full inertness during 24 h at 320°C, compared with 220°C for the conventional coating.

CONCLUSIONS

In our view, coatings based on hydroxyl-terminated phases represent an important progress, possibly even a revolution, in column technology. The specific strong points of more defined polarity, and ease of immobilization, are of secondary importance in our experience. The primary advantages are increased thermostability and increased inertness. The final weight of the progress will greatly depend on further advances in film stabilization, which would permit application to more polar phases. An important gap in our evaluation of the new approach is our lack of experience with apolar phases. Some additional information is in preparation[9].

ACKNOWLEDGEMENT

Basic parts of this work have been done in collaboration with W. Blum. He was the first to see the role of condensation between phase and surface silanols.

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Note

Novel gel permeation chromatography packing composed of cross-linked and porous spherical particles of poly-γ-methyl-L-glutamate

CHUICHI HIRAYAMA* and HIROTAKA IHARA
Department of Synthetic Chemistry, Faculty of Engineering, Kumamoto University, Kumamoto 860 (Japan)
(Received June 24th, 1985)

Porous beads composed of dextran and agar for gel permeation chromatography (GPC) in an aqueous system are commercially available and have been widely utilized (Sephadex and Sepharose, respectively)[1]. However, they have the disadvantage that are so soft that they are susceptible to deformation and exhibit a high resistance to the flow-through of fluids. To overcome this, porous beads composed of cellulose, which show a high pressure-resistance superior to that of Sephadex and Sepharose, were prepared by a unique method[2–4]. We consider that the excellent properties of cellulose are due to intermolecular hydrogen bonding.

In this paper, we describe poly-γ-methyl-L-glutamate (PMLG) as a readily available matrix material for GPC packings. PMLG beads can not only withstand a surprisingly high flow-rate, but also show typical GPC behaviour in both aqueous and organic systems. These properties are attributable to the inter- and intramolecular hydrogen bonding produced by the formation of specific conformations (β-structure and α-helix, respectively).

EXPERIMENTAL

Cross-linked and porous spherical particles of PMLG were prepared as follows: 2.5 wt.-% of PMLG (degree of polymerization *ca.* 2000) was dissolved in 1,2-dichloroethane containing 2.5–7.5 wt.-% of diluent (*e.g.*, decahydronaphthalene, diethylbenzene and 1-octanol). This solution was suspended at a concentration of 1.0–3.5 wt.-% in aqueous poly(vinyl alcohol) solution and the mixture was stirred at a fixed speed at 40°C for 12 h. After filtering, the spherical particles produced were washed with water, hot water, ethanol and diethyl ether. Spherical particles with an average diameter of 5–300 μm are obtained. These PMLG particles were placed in a three-necked flask fitted with a condenser for azeotropic distillation. Decahydronaphthalene–chloroform (3:1), 0.2 equiv. of sulphuric acid and 0.5 equiv. of triethylene glycol were added and the mixture was stirred slowly at 65°C with addition of fresh chloroform as necessary. Particles of PMLG cross-linked by triethylene glycol were obtained by filtering and washing with water and methanol. The total yield was 90–100%.

PMLG particles of average diameter 30 μm (range 25–44 μm) were packed in a glass column (30 cm × 5 mm I.D.) and a stainless-steel column (15 cm × 8 mm

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I.D.) and GPC was carried out using polysaccharides and polystyrene, respectively, as standard samples. The porosity and the excluded molecular weight (M_{lim}) were determined from a calibration graph. The degree of pressure resistance was examined from the relationship between pressure drop and flow-rate.

RESULTS AND DISCUSSION

PMLG particles

Fig. 1 shows a typical electron micrograph of PMLG particles of diameter 15 μm. The shape of the particle is spherical and uneven. The average diameter can be regulated by the suspension conditions. For example, when 3 wt.-% of aqueous poly(vinyl alcohol) solution was used as a suspension medium, the diameter of the main products lay in the range 5–15 μm. Our procedure can provide spherical particles with an average diameter in the range 5–300 μm by adjusting the viscosities of the medium and the PMLG solution and the stirring speed.

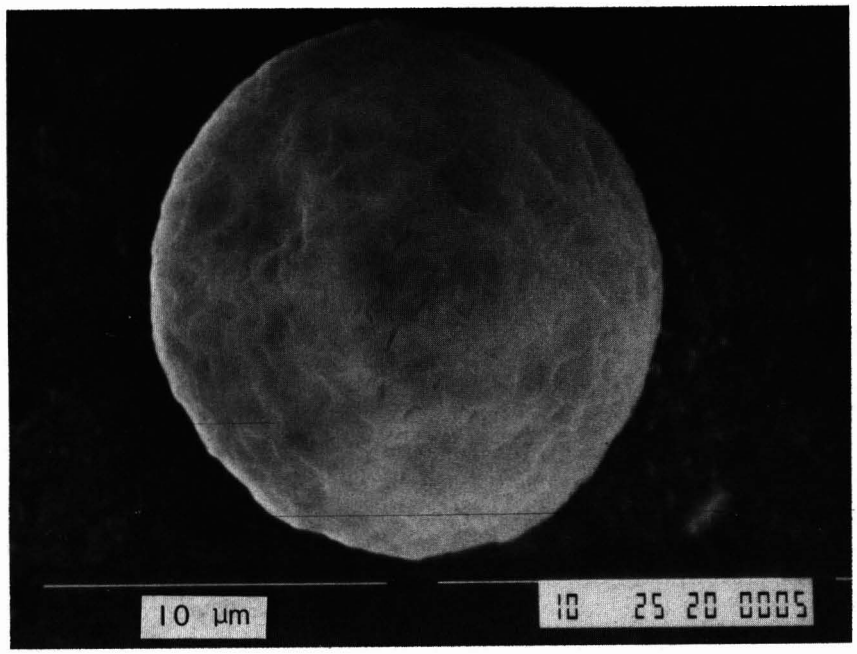

Fig. 1. Electron micrograph of the PMLG particle. Diluent, decahydronaphthalene (100 wt.-%); suspension, 2 wt.-%.

The PMLG particles obtained were insoluble and hardly swelled in the usual solvents used in chromatography, *e.g.*, water, methanol, ethanol, 2-propanol, acetonitrile, tetrahydrofuran, dioxane, benzene, chloroform and hexane. This insolubility must be due to the intermolecular hydrogen bonding with the β-structure of PMLG in addition to cross-linking. Non-cross-linked particles of PMLG are also

insoluble in the above-mentioned solvents, except chloroform. The IR spectra give absorption bands characteristic of the β-structure (1685 and 1630 cm^{-1}) and the α-helix (1650 cm^{-1})[5,6].

These properties produce a remarkably high flow-rate for a chromatographic process. Fig. 2 shows the relationship between the pressure drop and the flow-rate for the particles prepared using 100 wt.-% of decahydronaphthalene as a diluent. The PMLG particles easily reached 30 ml/min · cm^2 (corresponding to *ca.* 6 ml/min at 5 mm I.D.), but the flow-rate in the corresponding commercial packing reached a limit at 2 ml/min (at 5 mm I.D.).

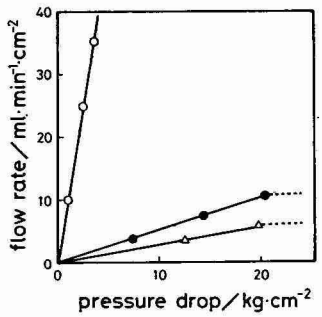

Fig. 2. Relationship between pressure drop and flow-rate in the aqueous system. ○, PMLG particles (M_{lim} 10 000, size 25–44 μm); ●, Sephadex G-25 (M_{lim} 5000, size 44–105 μm); △, Sephadex G-50 (M_{lim} 10 000, size 44–105 μm).

Gel permeation chromatography

The GPC behaviour was examined in aqueous and organic media, using polysaccharides and polystyrene, respectively, as standard samples. Calibration graphs characteristic of GPC were obtained in both mediums (Fig. 3). The excluded molecular weight (M_{lim}) is *ca.* 10 000 in both media. The similarity of the two systems supports the suggestion that cross-linked PMLG particles do not swell and are amphiphillic.

In addition, it was shown experimentally that M_{lim} could be adjusted up or down (from 10^3 to 10^5) by varying the concentration of decahydronaphthalene used as a diluent and by using diethylbenzene or 1-octanol instead of decahydronaphthalene. Typical results are given in Table I. It is very significant for GPC that packings with a series of M_{lim} values can be produced.

The amphiphilic properties of the PMLG particles as shown in Fig. 3 were followed by differential scanning calorimetry (DSC). The DSC thermogram of particles containing water (free water existing around the particles was removed by centrifugation) gives only the melting peak due to water. When this packing was washed with methanol in a column for 10 min, the water peak disappeared. Subsequently, after washing with benzene, the peak for benzene was observed. Even with a change of the medium from water to dioxane, a similar peak transfer was observed. Hence it is clear that the PMLG particles are amphiphilic and that the chromatographic replacement of the medium is very easy.

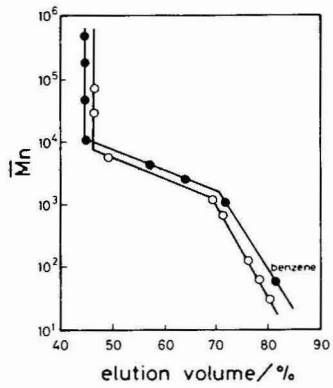

Fig. 3. Typical calibration graphs for porous PMLG particles prepared using decahydronaphthalene as the diluent for GPC. ○, Water; ●, tetrahydrofuran.

In conclusion, PMLG particles constitute an excellent packing for GPC. This is attributed to the fact that PMLG possesses a hydrophilic moiety, –CONH–, and shows hydrophobic properties by forming specific conformations such as the β-structure and α-helix.

TABLE I

M_{lim} OF PMLG PARTICLES

Diluent	*Amount (wt.-%)*	M_{lim}
None	0	200
Decahydronaphthalene	100	8000
	200	40 000
	300	120 000
Diethylbenzene	100	7000
	200	7000
	300	8000
1-Octanol	100	1000
	300	10 000

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Note

Liquefied gases as eluents for thin-layer chromatography

GIORGIO NOTA*, CARLO IMPROTA and ANTONIO CANNATA
Dipartimento di Chimica, Università di Napoli, via Mezzocannone, 4, 80134 Naples (Italy)
(Received July 30th, 1985)

A few studies have been published[1–3] in which liquefied gases were used as eluents for high-performance liquid chromatography (HPLC). These eluents generally have lower viscosity than the common organic solvents, which makes the chromatographic process more efficient and consequently a lower pressure is needed to transport the liquefied gas through the column. Additionally, the physical peculiarities of subcritical fluids, particularly CO_2 and N_2O, favour the use of the most convenient detection systems, such as the mass spectrometer and the flame ionization

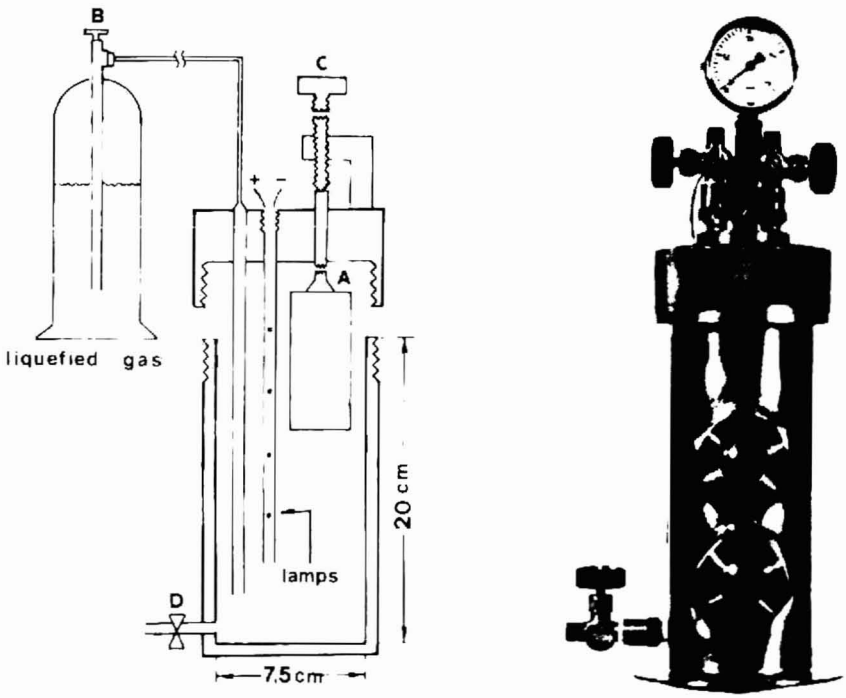

Fig. 1. Schematic diagram and photograph of the chromatographic apparatus employed for TLC using liquefied gases as eluents.

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TABLE I

SPECIFICATIONS OF THE AUTOCLAVE EMPLOYED

Internal height	20 cm
Internal diameter	7.5 cm
Volume	0.95 l
Maximum pressure of use	75 bar
Construction materials	AISI 304, bronze, PTFE, Plexiglass GS 233, GACO rubber

detector, widely adopted in gas chromatography. As the chromatographic process and the problems typical of HPLC are generally similar to those encountered in thin-layer chromatography (TLC), we decided to investigate the possibility of using liquid CO_2 as an eluent in TLC[4]. The interesting results obtained prompted us to extend the investigation to other fluids under subcritical conditions, such as N_2O, Freon 22, propene, propane and *n*-butane.

EXPERIMENTAL

Reagents

The compounds tested were pure products from Fluka (Buchs, Switzerland). Fluoroscent silica gel (60 F_{254}), silica gel 40 and reversed-phase (Stratocrom SI F_{254}

TABLE II

R_F VALUES OBTAINED FOR DIFFERENT COMPOUNDS ON A SILICA THIN LAYER USING LIQUEFIED GASES AND *n*-HEXANE AS ELUENTS

Compound	*N_2O*	*Freon 22*	*Propene*	*Propane*	*n-Butane*	*n-Hexane*
n-Pentadecane	1.00	0.70	0.78	0.80	0.77	0.57
n-Octadecane	1.00	0.70	0.78	0.80	0.77	0.57
1-Decanol	0.06	0.13	0.06	0.02	0.03	0.05
1-Hendecanol	0.06	0.13	0.06	0.02	0.03	0.05
1-Dodecanol	0.06	0.13	0.06	0.02	0.03	0.05
Lauric acid	0.18	0.15	0.16	0.03	0.03	0.00
Myristic acid	0.10	0.05	0.13	0.03	0.03	0.00
Palmitic acid	0.00	0.00	0.05	0.03	0.03	0.00
Methyl laurate	0.15	0.47	0.30	0.05	0.15	0.13
Methyl myristate	0.15	0.44	0.30	0.05	0.15	0.13
Methyl palmitate	0.15	0.41	0.30	0.05	0.15	0.13
o-Cresol	0.17	0.30	0.16	0.00	0.09	0.00
m-Cresol	0.17	0.26	0.14	0.00	0.09	0.00
p-Cresol	0.17	0.28	0.14	0.00	0.09	0.00
o-Xylene	0.00	0.10	0.00	0.00	0.00	0.00
m-Xylene	0.00	0.10	0.00	0.00	0.00	0.00
p-Xylene	0.00	0.10	0.00	0.00	0.00	0.00
Fluorene	0.29	0.49	0.32	0.14	0.18	0.28
Anthracene	0.56	0.50	0.32	0.12	0.17	0.23
Phenanthene	0.33	0.52	0.32	0.15	0.22	0.28
Fluoranthene	0.55	0.51	0.33	0.12	0.15	0.23
Pyrene	0.45	0.51	0.32	0.12	0.22	0.25
Methylpyrene	0.53	0.49	0.34	0.12	0.22	0.
Benzo[*a*]pyrene	0.28	0.47	0.24	0.07	0.13	0.16

$C_{18}W$) thin layers were obtained from Carlo Erba (Milan, Italy). Reversed-phase (C_{18}) HPTLC thin layers were obtained from Merck (Darmstadt, F.R.G.). Liquid N_2O, Freon 22 ($CHClF_2$), propene, propane and *n*-butane cylinders equipped with an eductor tube were purchased from SON (Naples, Italy).

Chromatographic apparatus

A special autoclave (Fig. 1), devised and constructed in collaboration with SON, was used as the chromatographic apparatus. The main characteristics of the autoclave are reported in Table I. The layer bearing the sample is attached to the moveable support A. The lock is applied on the bomb and solvent under subcritical conditions is introduced from a reservoir equipped with an eductor tube, by keeping valve B open. When a sufficient amount of liquid has been introduced, valve B is closed and the lower edge of the layer is introduced into the liquid phase by acting on the support C. When the solvent has reached the upper edge of the layer, it is allowed to evaporate by opening valve D; then the lock is removed and the layer is recovered. Spots are detected by means of iodine or UV light. All the stages of the experiment may be conveniently checked by observing, through two quartz windows, the inside of the adequately illuminated autoclave.

TABLE III

R_F VALUES OBTAINED FOR DIFFERENT COMPOUNDS ON REVERSED-PHASE (C_{18}) THIN LAYERS USING LIQUEFIED GASES AND *n*-HEXANE AS ELUENTS

Compound	*N_2O*	*Freon 22*	*Propene*	*Propane*	*n-Butane*	*n-Hexane*
n-Pentadecane	0.69	0.73	0.80	0.85	0.81	0.62
n-Octadecane	0.49	0.67				
1-Decanol	0.35	0.47	0.24	0.18	0.17	0.32
1-Hendecanol	0.21	0.40	0.18	0.16	0.13	0.25
1-Dodecanol	0.10	0.29	0.14	0.12	0.06	0.14
Lauric acid	0.36	0.42	0.54	0.15	0.24	0.27
Myristic acid	0.27	0.19	0.43	0.14	0.21	0.23
Palmitic acid	0.09	0.08	0.28	0.13	0.16	0.19
Methyl laurate	0.68		0.73	0.22	0.46	0.50
Methyl myristate	0.60		0.70	0.20	0.44	0.46
Methyl palmitate	0.55	0.65	0.68	0.18	0.42	0.42
o-Cresol	0.56	0.59	0.41	0.00	0.24	0.29
m-Cresol	0.50	0.64	0.36	0.00	0.27	0.33
p-Cresol	0.42	0.58	0.41	0.00	0.12	0.30
o-Xylene	0.29	0.34	0.20	0.08	0.00	0.29
m-Xylene	0.20	0.35	0.21	0.09	0.00	
p-Xylene	0.37	0.39	0.24	0.12	0.00	0.21
Fluorene	0.33	0.59	0.53	0.30	0.35	0.49
Anthracene	0.31	0.57	0.53	0.32	0.34	0.49
Phenanthene	0.43	0.59	0.62	0.34	0.43	0.52
Fluoranthene	0.33	0.56	0.53	0.28	0.35	0.47
Pyrene	0.24	0.52	0.47	0.34	0.35	0.50
Methylpyrene	0.24	0.52	0.47	0.32	0.35	0.50
Benzo[*a*]pyrene	0.12	0.39	0.31	0.18	0.22	0.43

TABLE IV

AVERAGE VELOCITIES OBSERVED ON THIN LAYERS OF VARIOUS TYPES OF 10 cm LENGTH

Eluent	*Average velocity (cm/min)*				
	SiO_2-60	*SiO_2-40*	*SiO_2-60 (HPTLC)*	*C_{18}*	*C_{18} (HPTLC)*
Propane	0.59	1.15	0.53	1.56	0.70
Propene	0.9	1.18	0.6	1.26	0.73
n-Butane	0.94	1.40	0.76	1.51	0.88
Freon 22	0.40	0.60	–	0.50	0.44
n-Hexane	0.57	0.87	0.42	0.95	0.45
Methanol	0.27	0.40	0.27	0.56	0.30

RESULTS AND DISCUSSION

The R_F values of various compounds obtained on thin layers of silica gel or the reversed phase, using propane, *n*-butane, propene, N_2O and Freon 22, are reported in Table II and III. R_F values obtained with *n*-hexane, which is a non-polar eluent, are also reported.

As expected, the eluent properties of propane and *n*-butane are similar to those of *n*-hexane, whereas propene, N_2O and Freon 22 behave as polar eluents. In Table IV the average migration velocities of subcritical fluids, *n*-hexane and methanol, observed on thin layers of various types, are reported. All the subcritical fluids tested show a higher average velocity than those exhibited by *n*-hexane and methanol, because of their low viscoisity. On the other hand, N_2O advances only 10 cm during 40 min.

The apparatus described here is easily handled; the use of a transparent autoclave allows one to follow the chromatographic process directly and makes TLC with liquefied gases as simple as normal techniques. The easy removal of the eluent should also be emphasized with respect to the re-elution of the layer and to the use of a flame ionization detector for TLC.

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Note

Unified retention index of hydrocarbons separated on squalane

N. DIMOV
Chemical Pharmaceutical Institute, Sofia 1156 (Bulgaria)
(First received April 11th, 1985; revised manuscript received July 22nd, 1985)

The general identification approach applied over many years in gas chromatographic (GC) practice is to compare the retention of a compound of interest with that of a reference substance or standard. It is not easy, however, to maintain the necessary collection of standards, so comparison is commonly made with literature data. Unfortunately, the published retention data are given in many forms and their usefulness is limited. Even the Kováts retention index, I[1], did not solve the problem, in spite of it being considered to be the most representative characteristic of retention[2,3], as the temperature dependence of I and its high sensitivity with respect to stationary phase composition changes[4,5]. The temperature dependence, dI/dT, is an additional source of information about structure[6] and is used to confirm the identification[7,8]. Hence, the determination of its correct value is as important as the determination of the value of I itself.

Studies on the influence of the GC conditions on the retention of hydrocarbons were completed in about 1973 with the comprehensive work of Rijks[9]. He showed that the repeatability of the experimentally obtained I values could be ± 0.1 index units (i.u.) or even better. This was confirmed later for GC with temperature programming[10,11]. Unfortunately, the inter-laboratory reproducibility remains unsatisfactory, except for a few special cases[9]. The actual discrepancies between the experimental values of I for identical compounds obtained in different laboratories in routine analysis we assume to be ± 1 i.u.[12,13]. Cases in which these discrepancies are up to 10 i.u. or more have been explained[13].

We consider that the observed discrepancies should not be accepted as a disadvantage of the retention index system and it is time to establish better the reliability of this identification approach. The existing uncertainty is connected with the discrepancies between the published data. We assume that the existing numerous experimental data could be unified by using them as inputs for a regression analysis. The values obtained should be more reliable and could be used for a calibration procedure in which the column would be checked in a manner similar to spectral calibration. Then the GC identification could be considered as more reliable than it is at present. This paper gives equations for the calculation of such unified retention indices. They can be used further in the comparison with the experimentally obtained I values at any analysis temperature. The equations given refer to the calculation of unified retention indices of hydrocarbons separated on squalane.

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EXPERIMENTAL

Comparison of the I_{exp} of the same hydrocarbon obtained in different laboratories shows that the discrepancy varied in magnitude and sign. Such comparisons are given in the literature[14], and the differences reported varied from 0.2 to 1.3 i.u. We also compare in Table I I_{exp} values taken from the publications of workers whose activity in the field is well known[13]; even greater differences were found. Hence, the question of which data are the more correct is reasonable. Any practical chromatographer who has to identify the peaks he has obtained is faced with such a question, and so does any theoretician who works on retention index pre-calculation. Thus, Altenburg compared the values of I_{calc}, calculated according to his equations[15], with the I_{exp} values obtained by Evans[16]. Martinov and Vidgergauz[17] and later Randić[18] used in their comparison the I_{exp} values of Tourres[19]. Hammers and De Ligny[20] cited the I_{exp} values of Hively and Hinton[21], and Chrétien and Dubois[22] those of Rijks[9].

We consider that the differences between the I_{exp} values of the different workers result from random errors, because in most instances they have neither equal magnitude nor the same signs. On this basis and in order to remove doubts about the choice of the lierature source for I_{exp} data, and to create a bank of data with known confidence intervals, we treated all of the existing I_{exp} values statistically. If we denote the unified retention index at any analysis temperature T (°C) by UI_T, its value could be calculated by the following equation:

$$UI_T = UI_0 + (dUI/dT)T$$

where UI_0 is the value of UI_T at 0°C. Using the I_{exp} values at the corresponding temperatures and the least-squares approach, we obtain the values of UI_0, dUI_0/dT and the standard deviations. The value of the slope, $-dUI/dT$, is of great interest: it is used for confirmation of the identification made on the basis of I[8,23], it is correlated with the structure of the substance[24,25] or it is used for the prediction the best separation temperature[26]. The general relationship between I and temperature is

TABLE I

I_{SQ}^{70} VALUES OF SOME HYDROCARBONS AND MAXIMAL DISCREPANCES (d) BETWEEN RESULTS OF DIFFERENT WORKERS

No.	*Hydrocarbon*	$I^{Lit.}$	d
1	2,3,3-Trimethyl-1-butene	630.7[9]; 631.3[21]	0.6
2	Methylcyclopentane	630.8[19]; 631.0[9]; 632.7[21]	1.0
3	Benzene	641.8[9]; 642.8[29]; 642.9[32]; 645.3[21]	3.5
4	2,3-Dimethyl-1-pentene	652.2[9]; 653.4[17]	1.2
5	Cyclohexane	666.95[14]; 667.2[9]; 668.0[32]; 668.6[21]	1.6
6	1-Heptene	682.3[9]; 682.5[29]; 682.8[21]	0.5
7	3-Methyl-*cis*-3-hexene	685.3[9]; 691.7[21]	6.7
8	Toluene	750.2[9]; 750.5[32]; 750.7[29]; 751.9[21]	1.7
9	3-Methylheptane	772.3[9]; 772.5[21]; 772.9[14]; 773.6[19]	1.3
10	3-Methyloctane	870.6[17]; 870.7[9]; 871.0[19]	0.4
11	3,3-Diethylpentane	882.4[9]; 882.7[17]; 884.2[19]; 885.4[33]	3.0

TABLE II

VALUES OF UI_0, $(dUI/dT) \cdot 10$, THE STANDARD DEVIATION (s), THE NUMBER (n) OF I_{exp} TAKEN IN THE REGRESSION AND $(dI/dT) \cdot 10$ VALUES TAKEN FROM DIFFERENT REFERENCES

No.	*Hydrocarbon*	UI_0	$(dUI/dT) \cdot 10$	*s*	*n*	*Literature* $(dI/dT) \cdot 10$		
1	*trans*-2-Pentene	501.20	−0.195	0.30	8	−0.10[9];	−0.13[30];	−0.25[21]
2	3,3-Dimethyl-1-butene	503.30	0.741	0.30	4	0.63[30];	0.65[9];	0.75[21]
3	*cis*-2-Pentene	504.48	0.073	0.26	8	0.03[9];	0.06[30];	0.29[21]
4	2-Methyl-2-butene	513.64	0.130	0.17	5	0.06[30];	0.10[9];	−0.27[21]
5	2,2-Dimethylbutane	532.58	0.863	0.27	13	0.82[9];	0.83[21];	0.90[7]
6	4-Methyl-1-pentene	546.60	0.561	0.42	9	0.50[9];	0.58[21];	0.60[30]
7	3-Methyl-1-pentene	547.30	0.834	0.30	9	0.79[9];	0.81[21];	0.86[30]
8	4-Methyl-*cis*-2-pentene	554.49	0.340	0.35	5	0.21[30];	0.24[9];	0.36[21]
9	2,3-Dimethyl-1-butene	556.20	0.490	0.14	8	0.43[9];	0.55[30];	0.69[21]
10	Cyclopentane	558.70	1.475	0.37	14	1.23[30];	1.38[9];	1.54[21]
11	4-Methyl-*trans*-2-pentene	562.25	−0.100	0.16	5	−0.10[30];	−0.13[9];	0.12[21]
12	2,3-Dimethylbutane	562.33	1.055	0.21	9	0.72[7];	0.77[9];	0.90[21]
13	2-Methylpentane	569.19	0.102	0.19	12	0.10[19];	0.12[7];	0.17[9]
14	2-Methyl-1-pentene	579.06	0.210	0.23	8	0.22[9];	0.25[30];	0.34[21]
15	1-Hexene	580.99	0.262	0.35	12	0.24[9];	0.34[30];	0.49[21]
16	3-Methylpentane	581.56	0.568	0.31	14	0.45[9];	0.57[19];	0.58[21]
17	*cis*-3-Hexene	591.80	0.161	0.26	14	0.19[9];	0.24[30];	0.28[21]
18	2-Ethyl-1-butene	592.00	0.008	0.17	9	0.01[30];	0.05[9];	0.29[21]
19	*trans*-3-Hexene	593.40	−0.216	0.40	9	−0.17[30];	−0.22[9];	−0.29[21]
20	*trans*-2-Hexene	597.50	−0.077	0.36	11	−0.07[9];	−0.15[30];	0.02[21]
21	4,4-Dimethyl-1-pentene	600.50	0.876	0.30	8	0.92[21];	0.94[9];	0.95[30]
22	2-Methyl-2-pentene	598.01	−0.020	0.28	10	−0.08[30];	−0.11[9];	0.10[21]
23	3-Methyl-*cis*-2-pentene	600.98	0.432	0.52	6	0.33[9];	0.42[21]	
24	*cis*-2-Hexene	602.45	0.243	0.24	9	0.25[9];	0.35[30];	0.36[21]
25	3-Methyl-*trans*-2-pentene	612.38	0.130	0.37	5	0.08[9];	0.15[21];	0.17[30]
26	3,3-Dimethyl-1-pentene	620.70	1.124	0.45	7	1.17[9];	1.20[30];	1.41[21]
27	Methylcyclopentane	621.12	1.380	0.40	8	1.26[30];	1.52[9];	1.65[34]
28	2,3,3-Trimethyl-1-butene	622.70	1.177	0.24	6	1.12[9];	1.16[30];	1.31[21]
29	2,2-Dimethylpentane	622.80	0.621	0.28	15	0.52[31];	0.65[9];	0.67[19]
30	Benzene	623.01	2.744	0.41	11	2.43[6];	2.47[21];	2.93[1]
31	2,3-Dimethyl-2-butene	623.55	0.326	0.43	8	0.26[9];	0.40[30];	0.42[21]
32	2,4-Dimethylpentane	628.14	0.352	0.28	10	0.32[19];	0.34[21];	0.35[9]

33	4,4-Dimethyl-*cis*-2-pentene	629.94	1.144	0.35	7	1.0[21];	1.05[9];	1.09[30]
34	3,4-Dimethyl-1-pentene	631.8	1.125	0.59	5	1.12[9];	1.22[21]	
35	2,2,3-Trimethylbutane	632.31	1.538	0.30	12	1.44[9];	1.49[21];	1.55[19]
36	2,4-Dimethyl-1-pentene	634.00	0.728	0.24	6	0.65[9];	0.68[30];	0.75[21]
37	2,4-Dimethyl-2-pentene	641.00	−0.100	0.32	6	−0.17[21];	−0.30[9]	
38	3-Methyl-1-hexene	641.84	0.655	0.48	5	0.68[9];	0.93[21]	
39	3-Ethyl-1-pentene	642.45	1.017	0.60	6	1.01[9];	1.15[21]	
40	2,3-Dimethyl-1-pentene	645.90	0.884	0.30	7	0.96[30];	0.99[9];	1.12[21]
41	5-Methyl-1-hexene	647.50	0.517	0.47	8	0.44[9];	0.45[30];	0.51[21]
42	2-Methyl-*trans*-3-hexene	648.10	−0.173	0.40	8	−0.15[21];	−0.16[30];	−0.19[9]
43	3,3-Dimethylpentane	652.22	1.356	0.33	13	1.31[21];	1.32[9];	1.45[19]
44	4-Methyl-*cis*-2-hexene	653.00	0.483	0.60	7	0.60[9];	0.71[21]	
45	Cyclohexane	653.50	1.909	0.64	12	1.89[7];	2.05[31];	2.22[9]
46	4-Methyl-*trans*-2-hexene	654.30	0.463	0.45	9	0.38[9];	0.42[21];	0.44[30]
47	4-Methyl-1-hexene	654.81	0.663	0.45	8	0.63[9];	0.64[21];	0.71[30]
48	1,1-Dimethylcyclopentane	664.70	1.824	0.46	11	1.83[9];	1.84[31];	1.95[21]
49	2-Methylhexane	665.74	0.171	0.24	14	0.15[21];	0.17[9];	0.18[31]
50	2,3-Dimethylpentane	667.90	0.801	0.30	14	0.85[9];	0.86[21];	1.01[19]
51	3,4-Dimethyl-*cis*-2-pentene	668.80	0.428	0.34	7	0.44[9];	0.56[21]	
52	3-Methylhexane	674.38	0.344	0.28	11	0.31[21];	0.35[19];	0.36[9]
53	1-*cis*-3-Dimethylcyclopentane	675.30	1.568	0.38	12	1.32[30];	1.63[9,31];	1.69[21]
54	2-Methyl-1-hexene	677.00	0.200	0.25	3	0.15[21];	0.23[9];	0.25[30]
55	1-*trans*-3-Dimethylcyclopentane	678.10	1.637	0.44	8	1.65[9];	1.66[31];	1.76[21]
56	1-Heptene	680.70	0.248	0.27	12	0.17[21];	0.25[9];	0.27[30]
57	2-Ethyl-1-pentene	681.14	0.237	0.44	7	0.22[21];	0.25[9]	
58	1-*trans*-2-Dimethylcyclopentane	681.30	1.634	0.45	12	1.34[30];	1.59[9];	1.78[21]
59	3-Ethylpentane	683.20	0.580	0.25	12	0.57[9];	0.62[19,21]	
60	2,2,4-Trimethylpentane	684.36	1.168	0.32	13	1.09[9];	1.17[7];	1.18[19]
61	*trans*-3-Heptene	688.10	−0.059	0.28	9	−0.06[9];	−0.11[19]	
62	*cis*-3-Heptene	689.40	0.287	0.43	10	0.34[9]		
63	2-Methyl-2-hexene	691.15	0.36	0.55	10	0.02[9]		
64	3-Methyl-*cis*-2-hexene	691.70	0.406	0.35	6	0.45[9]		

(Continued on p. 370)

TABLE II *(continued)*

No.	*Hydrocarbon*	UI_0	$(dUI/dT) \cdot 10$	*s*	*n*	*Literature* $(dI/dT) \cdot 10$		
65	3-Ethyl-2-pentene	694.90	0.420	0.25	7	0.30[9]		
66	*trans*-2-Heptene	698.60	0.006	0.43	12	0.00[29];	0.01[9]	
67	2,3-Dimethyl-2-pentene	700.50	0.578	0.43	11	0.38[9]		
68	*cis*-2-Heptene	700.61	0.395	0.25	5			
69	2,2-Dimethyl-*cis*-3-hexene	711.70	0.975	0.20	4	1.03[9]		
70	1-*cis*-2-Dimethylcyclopentane	711.70	1.967	0.38	8	2.0[34];	2.09[9];	2.30[19]
71	2,4,4-Trimethyl-2-pentene	712.50	0.546	0.13	5	0.44[9]		
72	2,2,3,3-Tetramethylbutane	713.30	2.552	0.26	5	2.36[7];	2.39[21];	2.62[19]
73	1,1,3-Trimethylcyclopentane	714.00	1.967	0.32	7	1.91[9];	2.00[34,31]	
74	Methylcyclohexane	714.16	2.308	0.38	9	2.2[34];	2.43[9];	2.47[31]
75	2,2-Dimethylhexane	717.00	0.520	0.26	17	0.47[21];	0.52[9];	0.59[19]
76	Ethylcyclopentane	724.44	1.940	0.35	13	1.8[34];	1.83[9];	1.96[25]
77	2,5-Dimethylhexane	726.72	0.336	0.27	15	0.30[19];	0.33[9,7]	
78	2,2,3-Trimethylpentane	728.91	1.698	0.30	11	1.52[9];	1.66[19];	1.72[7]
79	2,4-Dimethylhexane	729.45	0.550	0.28	14	0.51[9];	0.53[19,31]	
80	Toluene	732.98	2.465	0.35	8	2.40[9];	2.45[36];	2.5[31]
81	1-*trans*-2-*cis*-4-Trimethylcyclopentane	733.05	1.676	0.40	7	1.6[34];	1.69[9];	1.72[31]
82	3,3-Dimethylhexane	736.66	1.349	0.33	12	1.30[19];	1.31[31];	1.32[21]
83	1-*trans*-2-*cis*-3-Trimethylcyclopentane	740.20	1.575	0.30	7	1.3[34];	1.65[9]	
84	2,3,4-Trimethylpentane	744.74	1.533	0.33	11	1.38[9];	1.50[19];	1.65[31]
85	2,3,3-Trimethylpentane	748.16	2.287	0.31	9	2.02[9];	2.08[7];	2.14[19]
86	1,1,2-Trimethylcyclopentane	750.64	2.190	5.2	9	2.0[34];	2.29[31];	2.32[9]
87	2-Methyl-3-ethylpentane	754.32	1.437	0.30	9	1.07[9];	1.25[31];	1.60[19]
88	2,3-Dimethylhexane	756.76	0.707	0.25	15	0.70[7];	0.72[9,15]	
89	1-*cis*-2-*trans*-4-Trimethylcyclopentane	761.23	2.674	1.76	6	1.9[34];	2.14[31];	2.18[9]
90	3-Methyl-3-ethylpentane	764.38	1.944	0.31	5	1.95[21];	1.97[31];	1.99[19]
91	2-Methylheptane	764.30	0.128	0.30	15	0.098[7];	0.15[9];	0.27[19]
92	3,4-Dimethylhexane	765.18	1.145	0.32	12	0.99[9];	1.00[21];	1.12[19]
93	4-Methylheptane	765.75	0.290	0.34	14	0.26[9];	0.27[19];	0.30[34]
94	2,2,4,4-Tetramethylpentane	766.00	1.478	0.45	4	1.87[9];	1.90[19]	
95	3-Ethylhexane	769.92	0.535	0.37	9	0.37[19];	0.40[34];	0.61[31]
96	3-Methylheptane	770.63	0.354	0.33	15	0.31[9];	0.34[21];	0.37[19]
97	1,1-Dimethylcyclohexane	772.50	2.916	0.57	7	2.76[31];	2.91[9];	3.2[34]
98	1-*trans*-4-Dimethylcyclohexane	772.96	2.657	0.57	5	2.34[31];	2.6[34]	

99	2,2,5-Trimethylhexane	773.10	0.674	0.25	4	0.62[9];	0.65[19]	
100	1-*cis*-3-Dimethylcyclohexane	773.64	2.363	0.41	7	2.30[31];	2.43[9];	2.6[34]
101	1-Octane	779.40	0.309	0.21	8	0.20[29];	0.28[9]	
102	1-Ethyl-*trans*-2-methylcyclopentane	780.02	2.223	0.51	6	2.2[34]		
103	1-Methyl-1-ethylcyclopentane	782.07	2.539	0.13	5	3.8[34]		
104	2,2,4-Trimethylhexane	782.40	1.358	0.30	4	1.32[9];	1.35[19]	
105	*trans*-4-Octene	782.60	0.158	0.30	6	0.00[29];	0.14[9]	
106	*cis*-3-Octene	786.00	0.386	0.30	4	0.2[29]		
107	*cis*-4-Octene	786.20	0.209	0.08	4	0.2[29]		
108	1-*cis*-2-*cis*-3-Trimethylcyclopentane	788.45	2.850	0.50	4			
109	1-*trans*-2-Dimethylcyclohexane	789.50	2.562	0.45	6	2.8[34];	2.84[31];	2.87[9]
110	*trans*-3-Octene	789.09	−0.090	0.10	4	−0.1[29]		
111	1-*cis*-4-Dimethylcyclohexane	791.64	2.760	0.40	7	2.5[34];	2.67[9];	2.72[31]
112	1-*trans*-3-Dimethylcyclohexane	793.90	2.350	0.43	7	2.45[34];	2.5[31];	2.61[9]
113	*trans*-2-Octene	798.80	−0.130	0.25	5	0.00[9];	−0.1[29]	
114	Isopropylcyclopentane	799.64	2.568	0.27	7	2.35[31];	2.47[9]	
115	*cis*-2-Octene	799.67	0.346	0.19	3			
116	2,2,4-Trimethylhexane	800.23	1.567	0.30	8	1.57[9];	1.60[19]	
117	2,3,5-Trimethylhexane	808.20	0.807	0.25	4	0.84[9];	0.85[19]	
118	2,2,3,4-Tetramethylpentane	808.50	2.260	0.33	5	2.18[9];	2.25[19]	
119	1-Methyl-*cis*-2-ethylcyclopentane	809.20	2.400	0.20	4	2.31[31];	2.33[9]	
120	2,2-Dimethyl-3-ethylpentane	812.03	2.028	0.16	4	2.00[9];	2.10[19]	
121	2,2-Dimethylheptane	813.60	0.425	0.25	5	0.45[19];	0.51[9]	
122	2,2,3-Trimethylhexane	814.00	1.500	0.35	7	1.38[9];	1.60[19]	
123	1-*cis*-2-Dimethylcyclohexane	814.17	3.096	0.40	7	3.08[9];	3.11[31]	
124	2,4-Dimethylheptane	820.60	0.172	0.15	5	0.20[19,21]		
125	Ethylcyclohexane	820.60	2.759	0.53	7	2.69[9];	2.70[31]	
126	*n*-Propylcyclopentane	820.75	1.936	0.25	9	1.84[9];	1.95[31]	
127	Ethylbenzene	821.76	2.575	0.24	8	2.4[29];	2.61[31];	2.62[9]
128	4,4-Dimethylheptane	821.90	1.150	0.10	4	1.15[19]		
129	2-Methyl-4-ethylhexane	822.60	0.319	0.55	4	0.55[19]		
130	2,6-Dimethylheptane	826.20	0.175	0.30	6	0.25[19]		
131	1,1,3-Trimethylcyclohexane	826.84	2.796	0.47	5			
132	1,1,1-Trimethylcyclohexane	827.60	2.688	0.56	6			
133	2,4-Dimethyl-3-ethylpentane	827.70	1.750	0.10	5	1.71[9];	1.80[19]	
134	2,3,3-Trimethylhexane	830.30	1.965	0.20	5	1.85[19]		
135	3,5-Dimethylheptane	831.20	0.501	0.20	4	0.55[19]		
136	3,3-Dimethylheptane	831.30	0.974	0.20	5	1.10[9];	1.15[19]	
137	2,5-Dimethylheptane	831.70	0.258	0.40	7	0.30[19]		

(Continued on p. 372)

TABLE II *(continued)*

No.	*Hydrocarbon*	UI_0	$(dUI/dT) \cdot 10$	*s*	*n*	*Literature* $(dI/dT) \cdot 10$		
138	*p*-Xylene	836.00	2.593	0.45	8	2.3[29];	2.47[9];	2.60[31]
139	2-Methyl-3-ethylhexane	838.50	1.043	0.10	4	1.05[19]		
140	2,3,4-Trimethylhexane	838.60	1.600	0.10	6	1.46[9]		
141	*m*-Xylene	839.42	2.497	0.54	10	2,3[29];	2.55[31]	
142	2,2,3,3-Tetramethylpentane	839.42	2.615	0.50	5	2.70[19];	2.72[9]	
143	3,3,4-Trimethylhexane	842.40	2.144	0.25	4	2.2[19]		
144	3-Methyl-3-ethylhexane	845.30	1.631	0.30	4	1.75[19]		
145	2,3,3,4-Tetramethylpentane	845.52	2.651	0.42	4	2.57[9];	2.65[19]	
146	3-Methyl-4-ethylhexane	847.50	1.455	0.50	4	1.55[19]		
147	2,3-Dimethylheptane	852.30	0.525	0.35	8	0.65[19];	0.80[31]	
148	3,4-Dimethylheptane	853.20	1.018	0.35	5	0.80[19,31]		
149	4-Ethylheptane	855.30	0.450	0.10	5	0.40[9];	0.45[19];	0.50[31]
150	*o*-Xylene	855.61	2.836	0.47	12	2.7[29];	2.85[9];	2.95[31]
151	2,3-Dimethyl-3-ethylpentane	859.11	2.630	0.17	5	2.7[19]		
152	2,2,4,5-Tetramethylpentane	860.70	1.900	0.05	4			
153	4-Methyloctane	861.90	0.174	0.15	7	0.30[19,31]		
154	2,2,4-Trimethylheptane	862.80	2.146	0.25	4			
155	2-Methyloctane	864.00	0.100	0.40	11	0.15[9];	0.30[19]	
156	3,3-Diethylpentane	864.30	2.634	0.15	4	2.62[9];	2.90[19]	
157	3-Ethylheptane	865.00	0.395	0.24	6	0.45[19]		
158	3-Methyloctane	869.00	0.274	0.25	9	0.30[31,9];	0.35[19]	
159	1-Nonene	880.70	0.181	0.05	4			
160	*cis*-4-Nonene	881.25	0.414	0.05	3			
161	*trans*-4-Nonene	883.55	0.071	0.08	3			
162	Isopropylbenzene	883.61	2.373	0.49	9	2.5[29];	2.55[31];	2.65[36]
163	*cis*-3-Nonene	883.73	0.379	0.20	3			
164	*trans*-3-Nonene	886.83	−0.033	0.12	3			
165	*trans*-2-Nonene	895.34	0.117	0.31	3			
166	3,3,5-Trimethylheptane	895.70	2.000	0.05	4			
167	*cis*-2-Nonene	896.36	0.554	0.40	3			

hyperbolic[27,28], but for dI/dT for non-polar substances separated on non-polar stationary phases, the linear regression gives more accurate results[13]. Experimentally obtained values of dI/dT are given in many papers, but the determinations are made over more or less limited temperature intervals[7,19,29–31]. It is evident that the dI/dT values calculated for two or three temperatures cannot be exact. The regression equations given in this paper combine more experimental data and are considered adequate if dUI/dT is calculated from I_{exp} values obtained by at least two groups of workers and at three temperatures. In most instances, however, the data obtained relate to more than five studies. The comparison with the experimental dI/dT values of different workers is given in Table II and the advantage is clear.

The following example explains the calculations made. For cis-2-heptene, retention indices at five temperatures ($n = 5$) from two different sources are as follows: 30°C, 701.9[19]; 50°C, 702.5[19]; 70°C, 703.1[19]; 86°C, 704.3[29]; and 115°C, 705.1[29]. The equation obtained is $UI_T = 700.61 + 0.0395T$ with a standard deviation $s = 0.247$. The calculated values of UI_T agreed well with the corresponding I_{exp} values, the difference varying between +0.30 and −0.27 i.u. in the range 30–115°C. All values obtained satisfied the linear regression. If necessary, however, a statistical exclusion of wrong data should be made.

RESULTS AND DISCUSSION

The values of UI_0, dUI/dT, s and n, the number of I_{exp}, taken in the regression are summarized in Table II. The hydrocarbons are arranged in order of increasing UI_0. The values of dUI/dT are compared with some similar values of dI/dT quoted in the literature. If one now uses the given values for UI_0 and dUI/dT, one could calculate UI_T for the temperature of the analysis. The UI_T value obtained and that calculated according to the given s confidence limit can bwe used further in the identification.

If the experimentally obtained I_T value is statistically equal to UI_T, one could decide with known reliability the peak from the chromatogram to which the hydrocarbon belongs. The UI_T value gives an even better possibility. If one checks the chromatographic column with a limited set of known hydrocarbons and obtains I_{exp} equal to UI_T, one can be confident in using this column for identification purposes. We consider this test of the column to be similar to the adjusting procedure in spectroscopy.

On the basis of the above results and the discussion, we conclude that the proposed unified retention index possesses the following advantages: UI_T is a statistically obtained value and, hence, it is more reliable than any individual I_{exp} value; UI_T is characterized by a standard deviation and the calculation of the confidence interval at any desired level is possible; dUI/dT is a more reliable value than dI/dT for estimating the peak movement with temperature; and both UI_T and dUI/dT can be used as a test to check the reliability of the column for identification purposes.

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Note

Selectivity of a diol phase high-performance liquid chromatographic system in trace analysis of anabolic compounds

EUGÈNE H. J. M. JANSEN*, HENK VAN BLITTERSWIJK, PAUL W. ZOONTJES, REINSKJE BOTH-MIEDEMA and RAINER W. STEPHANY

Laboratory for Endocrinology, National Institute of Public Health and Environmental Hygiene, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

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In the Netherlands the control for the illegal use of hormonal anabolics in cattle has been focused for the past few years on the presence of the "stilbenes" diethylstilbestrol (DES), dienestrol (DE) and hexestrol (HEX) in urine. Detection methods have been developed, including radioimmunoassay (RIA)[1], thin-layer chromatography (TLC)[2] and gas chromatography–mass spectrometry (GC–MS)[3]. To increase both the specificity and the sensitivity by a substantial purification of the urine extract, a selective reversed-phase high-performance liquid chromatographic (HPLC) step was introduced[4] prior to all detection techniques.

The same isocratic HPLC system was also used in an investigation of illegal preparations isolated from application sites of slaughtered cattle[5]. In this multi-residue monitoring study, ten different anabolic compounds were found more or less frequently. Because of the high concentrations and relatively clean matrix, on-line detection with a diode array was possible and turned out to be very specific for identification purposes also. For monitoring and screening purposes in urine, however, there is still an urgent need for fast multi-residue detection methods using HPLC–UV, TLC or GC–MS. The HPLC clean-up with reversed-phase chromatography was not satisfactory. On testing other HPLC columns, which were described in the literature for separation of androgenic[7,8] and/or estrogenic[8–12] steroids, it was found that normal-phase HPLC columns substantially improved the purification of steroids from urine samples[6].

A practical and simple prepurification step that uses a diol phase HPLC column, in combination with various detection methods, is presented in this report.

MATERIALS AND METHODS*

The HPLC equipment consisted of an automatic injector (WISP, Waters Assoc.), a solvent delivery system (Model 2150, LKB), a variable-wavelength detector

* Reference to a company and/or product is for purposes of information and identification only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environmental Hygiene, to exclusion of others which may also be suitable.

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operated at 210 nm (Model 773, Kratos) and a printer–plotter–integrator (Model 3390A, Hewlett-Packard). The chromatographic column (150 mm × 4.6 mm I.D.), obtained from Chrompack, was packed with LiChrosorb Diol, 5 μm (Merck) using a Column Packing Instrument (Shandon). Elution conditions were isooctane–ethanol (95:5, v/v or 93:7, v/v) at a flow-rate of 2.0 ml/min.

All solvents were of analytical grade (Merck). Anabolic standards were checked for purity by melting point, HPLC and infrared spectroscopy.

RESULTS AND DISCUSSION

Characteristic retention times of some endogeneous steroids and some frequently found anabolics on a LiChrosorb Diol (5 μm) column are listed in Table I, from which it can be seen that the anabolic compounds and some of their metabolites can be divided into two groups. From 2 to 7 min the androgens methyltestosterone (MT), testosterone (T), 19-nortestosterone (NT) and Trenbolone (TB), and the gestagens progesterone (P) and medroxyprogesterone (MP), are eluted and from 10 to 15 min the phenolic estrogens estradiol (E), *meso*-HEX, Zeranol (Z), ethynylestradiol (EE_2), *trans*-DES and alpha-DE.

TABLE I

RETENTION TIMES OF VARIOUS ANABOLIC COMPOUNDS AND SOME OF THEIR METABOLITES ON A COLUMN OF LICHROSORB DIOL

Compound	t_R *(min)*
Progesterone (P)	2.1
17α-Methyltestosterone (MT)	3.7
17α-Testosterone (α-T)	4.4
Medroxyprogesterone (MP)	4.5
17β-Testosterone (T)	4.6
19-Nortestosterone (NT)	5.1
17β-Trenbolone (TB)	6.5
17α-Trenbolone(α-TB)	6.7
17α-Estradiol (α-E_2)	10.0
17β.Estradiol (E_2)	10.4
meso-Hexestrol (HEX)	12.0
Zeranol (Z)	12.4
17α-Ethinylestradiol (EE_2)	12.5
trans-Diethylstilbestrol (DES)	13.3
α-Dienestrol (DE)	14.4

For practical purposes the composition of the mobile phase can be changed in order to collect the androgen–gestagen and estrogen fractions in a smaller volume (Fig. 1). In addition, this HPLC step causes a substantial purification of the urine extract, since the majority of the matrix components are retarded on normal-phase columns[6].

The separation in an androgen–gestagen fraction and an estrogen fraction is of great importance for the final detection methods. For two-dimensional TLC analy-

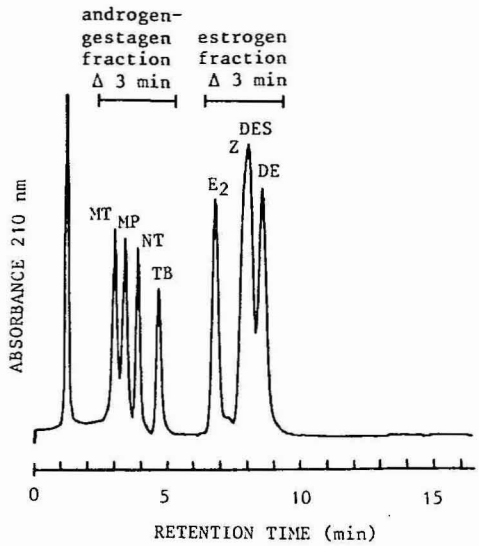

Fig. 1. HPLC group separation of a standard mixture on a LiChrosorb diol column after elution with isooctane–ethanol (93:7, v/v) at a flow-rate of 2.0 ml/min. The total volume of the combined androgen–gestagen and estrogen fractions is 6.0 ml each under these conditions.

sis both the elution system and the detection system methods are different for both groups of anabolics. Androgens are generally detected on the TLC plate by *in situ* fluorescence after acid treatment[13], whereas the estrogens E_2, HEX, Z and EE_2 are also convertable into their dansyl derivatives prior to TLC analysis[14].

For on-line UV detection the separation between androgens and estrogens has a very practical application. In subsequent HPLC analysis, the androgens and gestagens can be detected in general most sensitively at their maximum absorbances (λ_{max} 242–244 nm), whereas the estrogens have their maximum absorbances at substantially lower wavelengths (195–215 nm). 3-Ketotrienic steroids such as trenbolone are best detected at 350 nm.

Another application in which the separation between the two groups is very important for further analysis is GC–MS. The derivatization to trimethylsilyl derivatives can be performed on estrogens without further precautions. For androgens, however, the 3-keto steroids have to be converted into their 3-carboxymethoxime derivatives prior to derivatization of the 17-hydroxyl groups.

In this report a fast and simple separation method is presented for the fractionation of androgen and gestagen anabolics and estrogen anabolics, which has implications for the use of final detection methods, Such as TLC, UV and GC–MS analysis. Further work is in progress to integrate this purification method into the routine detection procedures of hormonal anabolics in urine.

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Note

Screening of parts-per-billion levels of diethylstilbestrol in bovine urine by high-performance liquid chromatography with ultraviolet detection

E. H. J. M. JANSEN*, P. W. ZOONTJES, R. BOTH-MIEDEMA, H. VAN BLITTERSWIJK and R. W. STEPHANY

Laboratory for Endocrinology, National Institute of Public Health and Environmental Hygiene, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

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In the Netherlands the screening of bovine urine for the presence of the illegal anabolic estrogen diethylstilbestrol (DES) is performed by radioimmunoassay (RIA) after chromatographic purification steps[1,2]. Confirmation of the identity of DES is performed by gas chromatography–mass spectrometry (GC–MS)[3] after purification of the urine extract by high-performance liquid chromatography (HPLC)[4]. After introduction of this procedure in a National Control Program, the use of the carcinogen DES for fattening purposes stopped almost completely in the Netherlands[5]. For practical reasons we are interested in screening methods other than RIA. Detection procedures for DES in urine using HPLC with various detection principles, such as post-column photochemical detection[6,7], dansylation with fluorescence detection[8], electrochemical detection[9] and detection with GC–MS[10] have been reported recently. None of these detection methods proved to us to be suitable for large scale screening, owing to laborious procedures or insufficient practical evaluation or validation.

To control for residues of the synthetic androgen trenbolone (TB) in bovine urine a fast screening method has been developed using HPLC with on-line UV detection at 350 nm[11]. Here a rather simple detection method using HPLC with UV detection at 240 nm is presented and evaluated for large scale screening of bovine urine samples for the anabolic DES.

MATERIALS AND METHODS*

Apparatus

The HPLC equipment consisted of an automatic injector (WISP, Waters Assoc.), a solvent delivery system (Model 2150, LKB) equipped with a solvent switch (Model PSV-3, Pharmacia), a variable-wavelength detector operated at 240 nm

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(Model 773, Kratos) and a printer–plotter–integrator (Data module, Waters Assoc.). The chromatographic columns (150 × 4.6 mm I.D.), obtained from Chrompack, were packed with LiChrosorb Diol 5 μm (Merck) or Hypersil ODS 5 μm (Shandon) using a Column Packing Instrument (Shandon). The DES fractions were collected with a modified fraction collector (Redirac, LKB), equipped with a electric three-way valve (Model PSV-3, Pharmacia). Both fraction collector and valves were operated by the timed events of the integrator mediated by a laboratory-made interface (Model SE 459). The columns were thermostated at 30°C by a laboratory-made metal holder. Elution conditions for the diol column were 8 min isooctane–ethanol (97:3, v/v) and 2 min isooctane–ethanol (60:40, v/v) to clean the column of tightly bound urine matrix components. Elution conditions for the reversed-phase column were 8 min methanol–water (60:40, v/v) and 2 min methanol to clean the column of tightly bound compounds. A constant flow-rate of 2.0 ml/min was used. DES was quantified using a 3390A integrator (Hewlett-Packard).

All solvents were of analytical grade (Merck). Anabolic standards were checked for purity by melting point, HPLC and infrared spectroscopy.

Sample clean-up and detection

Bovine urine (2 ml) was hydrolysed enzymatically[1] during 2 h at 37°C with glucuronidase/sulfatase (*Suc d'Helix pomatia,* IBF, France). After *n*-hexane extraction (10 ml) of the urine hydrolysate, the organic layer was evaporated under nitrogen and the dry residue was dissolved in 0.300 ml of the HPLC mobile phase of the diol column. After application of 0.250 ml of the extract to a column with LiChrosorb diol the *trans*-DES fraction was collected automatically for 1.5 min around the retention time of *trans*-DES. The eluting solvent of the DES fraction was evaporated and the dry residue was redissolved in 0.300 ml of the HPLC mobile phase of the reversed-phase column. During the HPLC separation on Hypersil ODS of 0.25 ml of the extract, *trans*-DES was detected by on-line UV absorption at 240 nm. With this procedure 40 urine samples can be processed and analysed by a single technician within 48 h. Both HPLC purification of urine extracts on LiChrosorb diol and HPLC detection on Hypersil ODS can be performed automatically during two sequential nights.

RESULTS AND DISCUSSION

The chromatographic behaviour of *trans*-DES on an isocratic reversed-phase HPLC system is shown in Fig. 1. From this figure it has been calculated that the absolute detection limit of standard *trans*-DES is 0.4 ng, with a signal-to-noise ratio of 2.

Early attempts to monitor nanogram amounts of DES in purified urine extracts with UV detection failed on account of the interfering absorption of matrix components. Also results obtained with combinations of two HPLC columns in series were not fully satisfactory. Only for trenbolone, which can be monitored at 350 nm, could UV detection be performed in urine extracts using one normal-phase HPLC column[11].

In these procedures the first purification step was an extraction with diethyl ether. On testing other organic solvents, it appeared that extraction of DES from

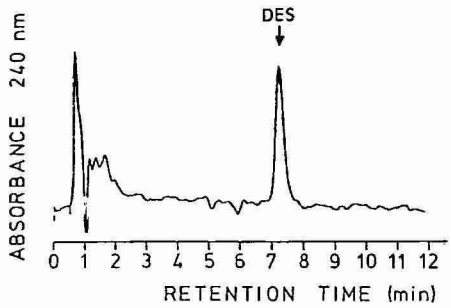

Fig. 1. HPLC chromatogram of 5 ng of *trans*-DES standard on Hypersil ODS (5 μm) in methanol–water (6:4, v/v) at a flow-rate of 2 ml/min.

urine hydrolysates with *n*-hexane was as efficient as with ether, but in addition the extracts appeared to be much cleaner. Application of *n*-hexane extracts in a double HPLC separation and clean-up procedure resulted in very "clean" chromatograms in which *trans*-DES can be detected at microgram per litre (ppb) concentrations. Detection was performed at 240 nm rather than 195 or 200 nm. In Figs. 2 and 3 two examples are shown of chromatograms of extracts of bovine urine samples from practice containing DES in concentrations of 1.2 and 8.5 μg/l, respectively, according to HPLC–RIA[1]. The identity of DES in these samples was confirmed by HPLC–GC–MS. It can be concluded from these Figures that DES can be identified and quantified at the ppb level based on its characteristic retention time. At present, after

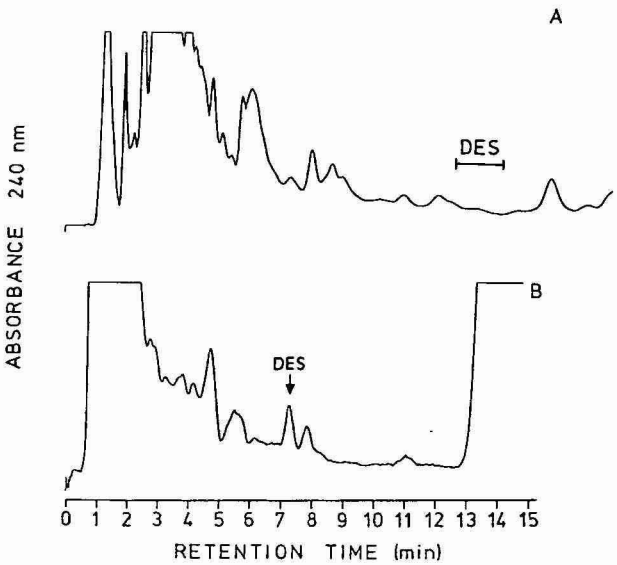

Fig. 2. HPLC chromatogram (A) of an extract of 1.67 ml of urinary DES containing bovine urine (H 146743) on LiChrosorb Diol (5 μm). The *trans*-DES fraction was collected automatically for a total of 1.5 min as indicated and applied (5/6 part, an aliquot of 1.39 ml of urine) to a Hypersil ODS (5 μm) column (B). From Figure B an amount of 2.6 ng of *trans*-DES (1.9 μg/l) has been calculated. The retention time of *trans*-DES is indicated by the arrow. In Figure B the absorbance scale was increased 20-fold.

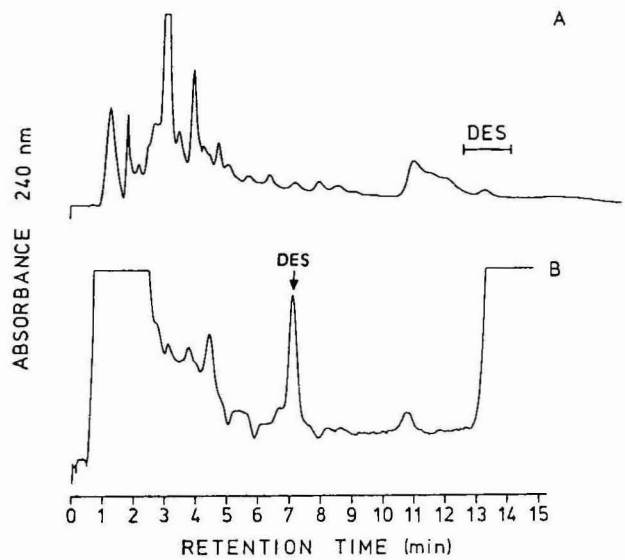

Fig. 3. HPLC chromatograms of an extract of 1.67 ml of urinary DES containing bovine urine (H 146841) on (A) LiChrosorb diol (5 μm) and (B) Hypersil ODS (5 μm). Further conditions as in Fig. 2. From Figure B an amount of 8.5 ng of *trans*-DES (6.1 μg/l) has been calculated.

preliminary analysis of 20 blank urine samples, no false positive responses were observed. Using this clean-up procedure the recovery of *trans*-DES was 73.3% ± 9.7% (S.D.) at the level of 5 μg/l (N = 15). In addition, 24 samples of bovine urine from the control programme of 1983 have been analysed and the quantitative results (X) compared with those obtained with the HPLC–RIA (Y) for DES. After orthogonal regression analysis a good correlation was found with a correlation coefficient of 0.987, a slope of 1.57 and an intercept of 0.10 ($Y = 1.57X + 0.10$).

The reported detection method is a fairly simple, relatively fast and partially automated screening method for the detection of DES in bovine urine in concentrations down to the 1 ppb level. Therefore this screening method can be considered as an alternative to RIA in certain applications. If necessary the reliability of the identification can be improved by co-chromatography of standard *trans*-DES in the reversed-phase run. In the case of urinary DES only an increase of peak height should be observed without peak broadening. At present similar methods are under development for monitoring other anabolics, such as xenobiotic androgens and other stilbene derivatives such as hexestrol and dienestrol.

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Note

Improved staining procedure for nucleic acids in polyacrylamide gels after complexing with nitroso compounds

R. EBERMANN*
Institut für Chemie , Arbeitsgruppe für Lebensmittel-, Umwelt- und Naturstoffchemie, Universität für Bodenkultur, Gregor Mendelstrasse 33, A-1180 Vienna (Austria)
and
E. LICKL
Ludwig Boltzmann Institut für klinische Onkologie, Wolkersbergenstrasse 1, A-1130 Vienna (Austria)
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Dyes such as methylene blue, acridine orange, ethidium bromide and pyronine Y are used for the detection of unlabelled nucleic acids in polyacrylamide gels[1–5]. Usually the only method applied for immobilizing the nucleic acids is to insert the gels in acid in order to convert the salts into the free acids. Many species of nucleic acids, especially those having a low molecular weight, are fairly soluble in water or dilute acids, so that the stained bands of nucleic acids fade soon after the necessary destaining procedure has been completed. This paper describes an alternative fixation procedure that allows more stable staining of nucleic acids to be achieved.

EXPERIMENTAL

Ribonucleic acids were prepared from *Saccharomyces cerevisiae* according to Holley[6]. Crude extracts were purified over DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden). The elution buffer system was a gradient of Tris–chloride (0.15–1.0 *M*). Fractions containing ribonucleic acids were pooled, ice-cold ethanol (96%) was added and the precipitate was freeze-dried. The lyophilized samples of ribonucleic acids were used for polyacrylamide gel electrophoresis (PAGE). Ribonucleic acid purchased from Serva (Heidelberg, F.R.G.) could not be used because of their low molecular weight, all molecules migrating with the front.

PAGE was carried out as described earlier[7]. In a block gel apparatus, a separation gel with 20% acrylamide (Merck, Darmstadt, F.R.G.), cross-linked 1:30 with bismethyleneacrylamide (Bis) (Merck), pH 7.5, was used. This gel is very suitable for nucleic acids of low molecular weight. The electrophoresis buffer was a Tris–glycine system[7]. For a run of 4 h a voltage of 300 V and a starting current of 100 mA were used. The apparatus was cooled with running water.

Samples of lyophilized nucleic acids were dissolved in an aqueous solution of acrylamide, Bis and Tris in the same proportions as used for preparing the separation gel.

On each lane $1 \cdot 10^{-4}$ g of ribonucleic acid were separated. To the samples of

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ribonucleic acids 8% of one of the following solutions were added for complexing: 0.1% Amido Black 10B (Merck) in methanol–glacial acetic acid–water (5:1:5); 1% 4-nitroso-N,N-dimethylaniline (Merck) in the same solvent; 1% disodium 2-hydroxy-1-nitrosonaphthalene-3,6-disulphonate (nitroso-R salt) (Merck) in the same solvent; or 1% 2-nitroso-1-naphthol (Merck) in aqueous solution.

The dyed samples and the control were separated by PAGE. The slab gels were stained with methylene blue[1] or acridine orange[3]. Running water was used for destaining in every instance. For documentation the gels were photographed.

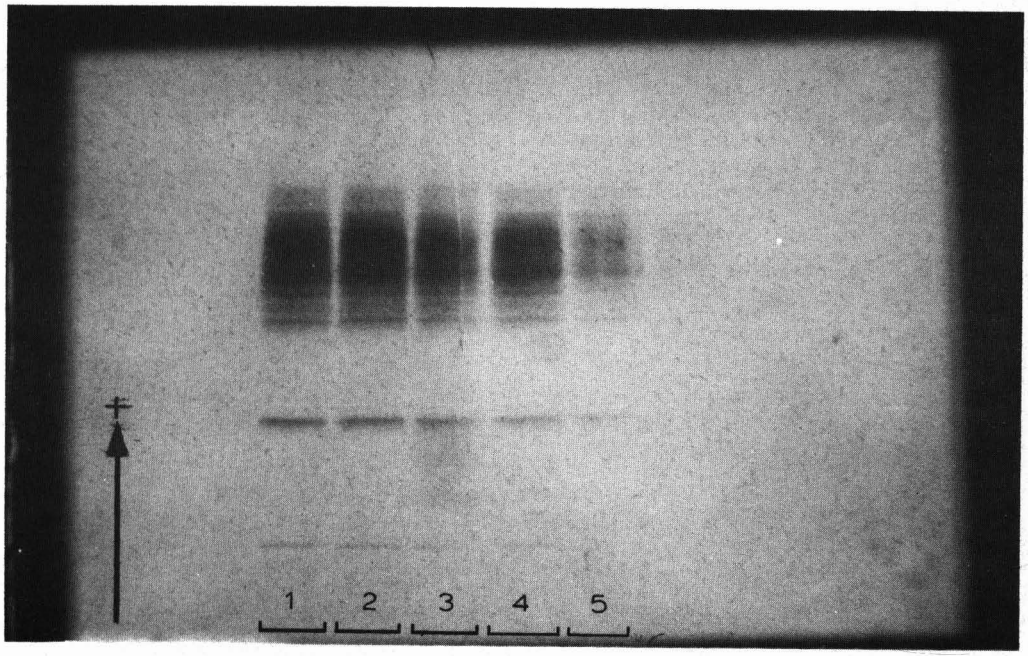

Fig. 1. Electropherogram of RNA from *Saccharomyces cerevisiae*. 1, RNA + Amido Black 10B; 2, RNA + nitroso-R-salt; 3, RNA + 2-nitroso-1-naphthol; 4, RNA + 4-nitroso-N,N-dimethylaniline; 5, RNA in aqueous solution. Staining was carried out with methylene blue and destaining with water. Each lane contains $1 \cdot 10^{-4}$ g of RNA.

RESULTS

Fig. 1 shows the staining results for yeast RNA separated on 20% polyacrylamide gel with methylene blue, destained in running water for 18 h. Easily the most intense dye–RNA interaction is obtained when the RNA had been pre-incubated with nitroso compounds. Further destaining results in the early disappearance of the RNA that had not been treated with the nitroso compounds before. No differences in staining intensity depending of the kind of nitroso compound used could be observed. Amido Black 10B, 4-nitroso-N,N-dimethylaniline, nitroso-R salt and 2-nitroso-1-naphthol gave essentially the same staining results. As these reagents had been dissolved in different solvents, the effect of the solvent seems unimportant.

Therefore, it seems that the presence of the nitroso group and its interaction with the nucleic acid is solely responsible for the improved staining.

Fig. 1 also shows that exactly the same electrophoretic bands appeared with or without addition of the nitroso compound. The interaction of the fixation agent therefore does not influence the electrophoretic mobility of the RNA.

Essentially the same staining results were obtained with acridine orange. We suggest that the staining of RNA in polyacrylamide gel is improved in general by the addition of organic compounds carrying a nitroso group.

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CHROM. 18 134

Note

Use of radio gas chromatography for monitoring the *in vivo* labelling of postmortem [^{3}H]choline production

R. M. DICK*, J. J. FREEMAN and J. W. KOSH
Department of Basic Pharmaceutical Sciences, University of South Carolina, Columbia, SC 29208 (U.S.A.)
(First received June 18th, 1985; revised manuscript received August 26th, 1985)

Many methods exist for the quantitation of endogenous acetylcholine and choline. A few examples include bioassay[1], fluorometry[2], chemiluminescence[3], paper or thin-layer chromatography, gas chromatography[4,5], high-performance liquid chromatography (HPLC)[6], and column chromatography–radioenzymology[7]. However the simultaneous measurement of choline, acetylcholine and tracer isotopes of these compounds is more complex and is basically limited to chromatographic procedures. Radio gas chromatography[8] has been used to separate ^{14}C isotopes of choline and acetylcholine from non-radioactive analogues. However the procedure was limited by low sensitivity and long retention times ($\approx$20 min). Gas chromatography–mass spectrometry[9] (GC–MS) has been used for the separation of deuterated isotopes but requires a major equipment expenditure. More recently Potter *et al.*[10] used HPLC for the separation of tritiated isotopes of choline and acetylcholine from non-radiolabelled analogues. The procedure was sensitive but required several enzymes, post column mixing and reactions.

Using a bioassay procedure, it was established that more choline left the brain than was supplied by the arterial blood[11]. In addition, a marked production of choline occurred in the brain after death[11,12]. The identity of the precursors responsible for the postmortem production of choline at present is incomplete but probably results from the sequential hydrolysis of acetylcholine, glycerophosphorylcholine, and phospholipids[13]. It has also been observed that the specific activity of deuterated choline decreased with postmortem incubation[13], indicating significant dilution with unlabelled choline. However, the postmortem production of deuterated choline was not evident.

It was the purpose of the present study to inexpensively modify an existing GC method of analysis to simultaneously measure choline, acetylcholine, and their tritiated isotopes. The modification was then used to demonstrate *in vivo* labelling of the postmortem production of choline.

MATERIALS AND METHODS

Animals, chemicals and radioisotopes

Male Sprague Dawley albino rats weighing 200–250 g were used for determining incorporation and release of [^{3}H]choline. Tritiated choline and acetylcholine were

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obtained from New England Nuclear. Paraterphenyl crystals used for isotope trapping were obtained from Aldrich.

Synthesis of [^{3}H]hexanoylcholine

[^{3}H]hexanoylcholine was synthesized for use as an internal standard by adding 0.3 ml hexanoyl chloride (Aldrich) to 200 μCi of [^{3}H]choline dissolved in 1.0 ml of 5 m*M* silver *p*-toluene sulfonate in acetonitrile. The mixture was incubated at 80°C for 45 min, centrifuged and the supernatant transferred and evaporated to dryness. After vacuum dessication, the residue was then reconstituted in 1.0 ml sodium acetate buffer (pH 4.0, 0.05 *M*). Radiochemical purity (98%) was determined with cellulose thin-layer chromatography using *n*-butanol–water–ethanol–acetic acid (100:33:70:17) as the eluent.

Simultaneous detection of choline, acetylcholine and tritiated variants

The simultaneous assay of non-radioactive and tritiated choline and acetylcholine was accomplished using a modification of the nitrogen–phosphorus GC procedure of Kosh and Freeman[14]. Brain tissue was homogenized in 4 ml 1 *M* formic acid–tetrahydrofuran (15:85) together with 5 nmol of propionylcholine and $1.3 \cdot 10^6$ dpm of [^{3}H]hexanoylcholine as internal standards. Butyrylchloride was used to convert choline and [^{3}H]choline to the respective butyryl esters.

Several GC modifications were required for the quantitation of the tritiated choline esters. The column packing material used was Chromosorb 750 coated with 10% OV-17 and 10% Triton X-100. During a chromatographic run, oven temperature was 130°C during the initial 3 min, and was then increased to 150°C for the remainder of the run. The gas flow-rates for helium (carrier), hydrogen and air were 40, 3.0 and 50 ml/min, respectively. The column effluent was split using SGE micro valves so that 80% of the flow was diverted to the mass (nitrogen–phosphorus) detector and 20% to a heated side port containing trapping cartridges (see Fig. 1A). The cartridges were held in place by O-rings inserted into a Swagelok (6.25 mm to 1.56 mm) adapter assembly (Fig. 1B). The trapping cartridges were prepared similar to the method of Karmen *et al.*[15] using glass tubing (4.8 cm × 6.5 mm O.D. × 2 mm I.D.) with a glass wool plug at one end, packed with 0.15 g of *p*-terphenyl crystals coated with 10% OV-17 and 10% Triton X-100. Cartridges were changed at 3, 6, and 9 min during the chromatographic run, which corresponded to the acetyl-, propionyl-, and hexanoylcholine radioactivity areas. After trapping, the cartridges were placed in scintillation vials containing 10 ml of scintillation fluid, shaken until the *p*-terphenyl was suspended in the fluid, and counted for radioactivity. Radioactivity was converted to dpm using a computer program which utilized matrix calculations to correct for radioactivity bleed between peaks, similar to the method utilized for correction of deuterated isotope spill-over[9]. Standard curves were prepared using $1.3 \cdot 10^6$ of [^{3}H]hexanoylcholine as the internal standard and known quantities of [^{3}H]choline or [^{3}H]acetylcholine. The ratios of the corrected dpm for [^{3}H]choline or [^{3}H]acetylcholine to [^{3}H]hexanoylcholine were then plotted *versus* the amount of tritiated compound (pmol) used. A suction trapping system was mounted above the detector to prevent the exhausting of radioactivity into the atmosphere. The system relied on a vacuum source to pull air and detector exhaust sequentially through a drying tube containing molecular sieve and then through a solution of mineral oil and water.

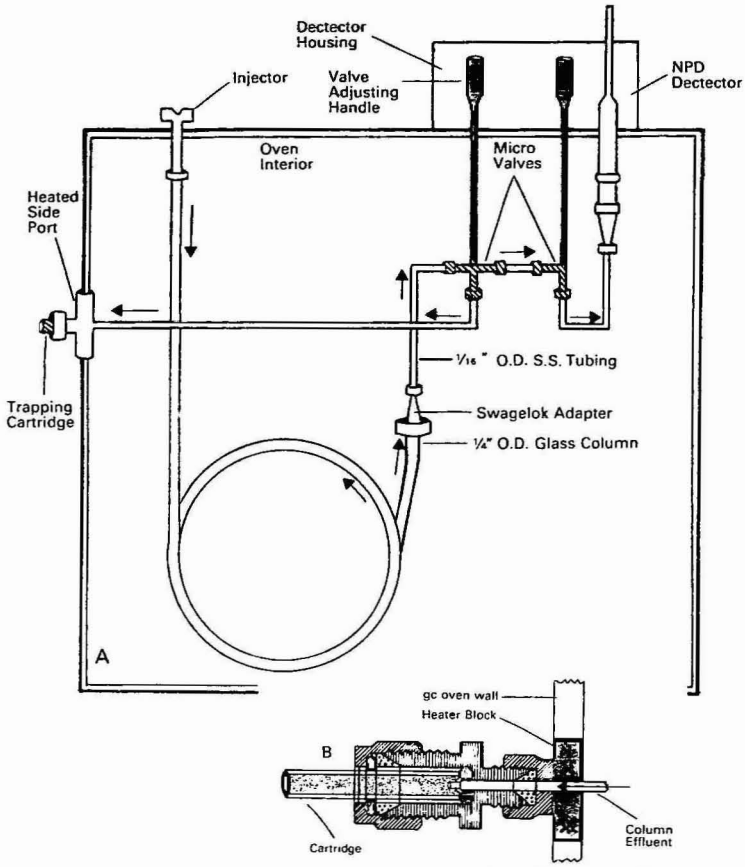

Fig. 1. (A) Plumbing schematic of gas chromatograph modified for split stream operation. Twenty percent of the column effluent was diverted to the heated side port for collection of tritiated compounds in the trapping cartridge. (B) Enlargement of cartridge–Swagelok assembly showing column effluent and O-ring seals.

In vivo incorporation and release of [^{3}H]choline

Male rats were surgically implanted with polyethylene canulas into the lateral ventricle according to the method of DeBalbian–Verster *et al.*[16]. After 24–36 h recovery, the conscious animals were injected through the cannula with 2 μCi [^{3}H]choline (80 Ci/mmol, 1 μCi/μl) at 10 A.M., 2, 6, and 10 P.M. each day for a total of six days. Thirty gauge wire was used to seal the cannula after each injection. Animals were sacrificed by decapitation 12 h after the last injection, the brain removed, and split in half longitudinally. One half was then homogenized 2 min following decapitation and the second half homogenized after 17 min incubation in a water bath at 37°C. The homogenates were then analyzed for tritiated and non-tritiated choline and acetylcholine content as described above.

RESULTS

Quantitation of choline and acetylcholine

The GC assay changes made in the present method did not affect the resolution nor the quantitation of endogenous acetylcholine and choline obtained with our previously published method[14]. One normal formic acid–tetrahydrofuran (15:85) rather than 1 *M* formic acid–acetonitrile (15:85) was used for tissue homogenization to reduce losses of quaternary compounds. Recovery of tritiated acetylcholine, choline and hexanoylcholine through the homogenization procedure averaged 98–99%. When formic acid–acetonitrile was used, recovery ranged from a low of 45–50% for hexanoylcholine to a high of 85–90% for choline. An interesting characteristic observed by homogenizing in tetrahydrofuran was complete dissolution of the brain tissue.

Various solid supports and phase coatings were examined as packing material for the glass cartridges used to trap the tritiated compounds. Maximum trapping efficiency was obtained with a 1 7/8 inch cartridge packed with *p*-terphenyl crystals coated with 10% OV-17 and 10% Triton X-100. Comparison of dpm obtained following direct injection of the tritiated compounds onto the cartridge with total dpm obtained after elution from the GC produced recoveries ranging from 90–95% for all compounds.

The collection time "windows" for acetylcholine (0–3 min), choline (as butyrylcholine, 3–6 min) and the internal standard hexanoylcholine (6–9 min) allowed adequate separation with a minimum amount of radioactive bleed between fractions. Since radioactive bleed was proportionally constant, matrix corrections were utilized to obtain the corrected dpm. A plot of the ratio of tritiated choline or acetylcholine to tritiated hexanoylcholine *versus* molar quantity is shown in Fig. 2. The minimum detectable quantity taken through the assay was 150 (± 83) fmol choline ($2.6 \cdot 10^4$

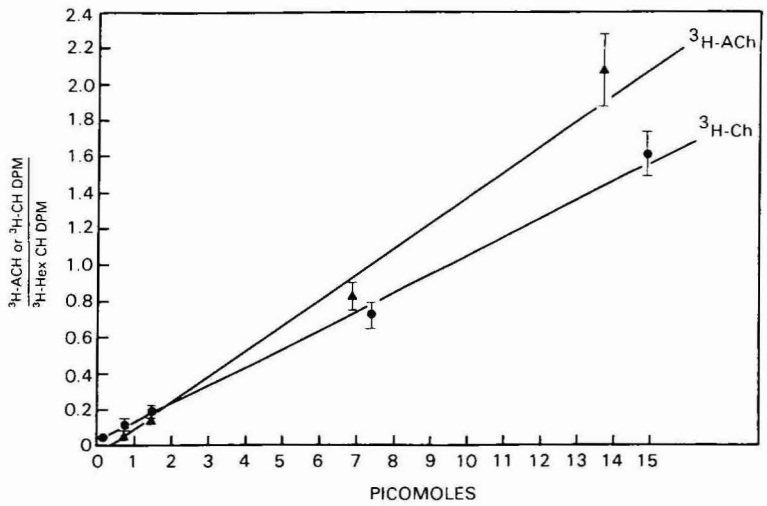

Fig. 2. Standard curve showing linearity of dpm ratio *versus* molar quantity for [^{3}H]choline and [^{3}H]acetylcholine. [^{3}H]Hexanoylcholine (0.6 μCi) was used as internal standard. Samples were analyzed for [^{3}H]choline and [^{3}H]acetylcholine as described in Materials and methods.

TABLE I

POSTMORTEM PRODUCTION OF [^{3}H]CHOLINE AND CHOLINE BY RAT BRAIN TISSUE

*Treatment**	*[^{3}H]Choline (dpm/g)*	*[^{3}H]Choline (fmol/g)*	*Choline (nmol/g)*	*Sp. act.*** (dpm/nmol)*	*Acetylcholine (nmol/g)*
Incubated 2 min	120 806 ± 58 211	636.4 ± 330	96.7 ± 3.0	1249	14.1 ± 1.0
Incubated 17 min	774 800 ± 191 522***	4402.3 ± 1088***	448 ± 28.3***	1729	9.6 ± 0.8§

* Rats were injected intraventricularly at 10 A.M., 2, 6, and 10 P.M. for six days with [^{3}H]choline (2 μCi/injection, 80 Ci/mmol). Brain tissue was homogenized in formic acid–tetrahydrofuran after incubating at 37°C for 2 or 17 min and then assayed for tritiated and endogenous choline and acetylcholine content.

** Sp. act. = specific activity, defined as (dpm[^{3}H]choline)/(nmol choline).

*** $p \leqq 0.001$, compared to 2-min incubated tissue.

§ $p \leqq 0.005$, compared to 2-min incubated tissue.

dpm) and 730 (± 102) fmol acetylcholine ($1.3 \cdot 10^5$ dpm). Linearity was observed up to 15 pmoles ($2.6 \cdot 10^6$ dpm) and was not examined further. Recovery of tritiated choline and acetylcholine through the complete procedure (chemical assay and GC separation) ranged between 50 and 60%. The value obtained for recovery was good, considering that 33% of the radioactivity is lost during the chemical demethylation step.

Incorporation and postmortem production of [^{3}H]choline

The purpose of this experiment was to utilize the radio gas chromatographic procedure developed above to determine if the postmortem production of choline in brain tissue could be labelled following chronic administration of labelled choline. Tritiated choline (intraventricular) was administered four times a day for six days to rats and the brain tissue examined for endogenous and tritiated choline and acetylcholine content 12 h after the last injection. Brain tissue analyzed at 2 min produced ≈121 000 dpm/g of [^{3}H]choline, whereas 17-min incubation produced ≈775 000 dpm/g of [^{3}H]choline (Table I). As expected non-labelled choline increased 4–5 fold and acetylcholine decreased approximately 24%. Tritiated acetylcholine could not be detected at either time. The specific activities for choline at 2 and 17 min postmortem were 1249 and 1729, respectively.

DISCUSSION

The GC method of Kosh and Freeman[14] was modified in the present study for the purpose of simultaneously quantitating choline, acetylcholine, and tritiated choline and acetylcholine. The modification was accomplished for less than $500 and enabled rapid GC processing (< 10 min /sample) for both non-labelled and tritiated compounds. The combined assay required the use of propionylcholine and [^{3}H]hexanoylcholine as internal standards for the non-labelled and labelled analogues, respectively. It was found necessary to change the intial homogenizing medium to formic acid–tetrahydrofuran to provide recoveries of 98–99% for all of the quaternary compounds during homogenization.

Residual isotope bleed from the column between sample injections was mini-

mal for the tissue samples. However, in preparing the standard curve, the amount of bleed was found to be proportional to the amount of tritiated choline and acetylcholine used. Even with the highest amounts of radioactivity used, one hour of continued column elution decreased the background to baseline values. The reduced background bleed of radioactivity is an advantage since other methods have required oxidation reduction combustion trains[15] or disposable columns[17] to minimize background bleed from tritium or ^{14}C isotopes respectively.

A high sensitivity for both choline and acetylcholine (150 and 730 fmol, respectively) was obtained in the present assay. The method compares favorably with other assays for labelled choline and acetylcholine in which the limit of sensitivity has been in the pmol range[7,18]. The present assay utilized only a 20% column effluent split for radioactivity trapping. Sensitivity could be further increased if a greater column split ratio were utilized and carrier make-up gas added.

After six consecutive days of intraventricular [^{3}H]choline administration, postmortem production of both non-labelled and [^{3}H]choline was observed in rat brain tissue. While the time course of labelling of phospholipids can vary from minutes[19], to hours[20], to days[13], the present data demonstrates that much longer pretreatment times are required to label the choline precursor(s) involved in postmortem production of choline, suggesting incorporation of choline into phospholipids with long turnover times. In the present study (Table I), labelling of the postmortem production of choline was not observed with less than six days of chronic [^{3}H]choline administration. The specific activity of the [^{3}H]choline produced after 17 min incubation was greater than after 2 min incubation. This may reflect the inability of six days of pretreatment to uniformly label all phosphatidylcholine pools, resulting in the rapidly turning over pool contributing a greater proportion of labelled choline to endogenous choline produced during postmortem incubation.

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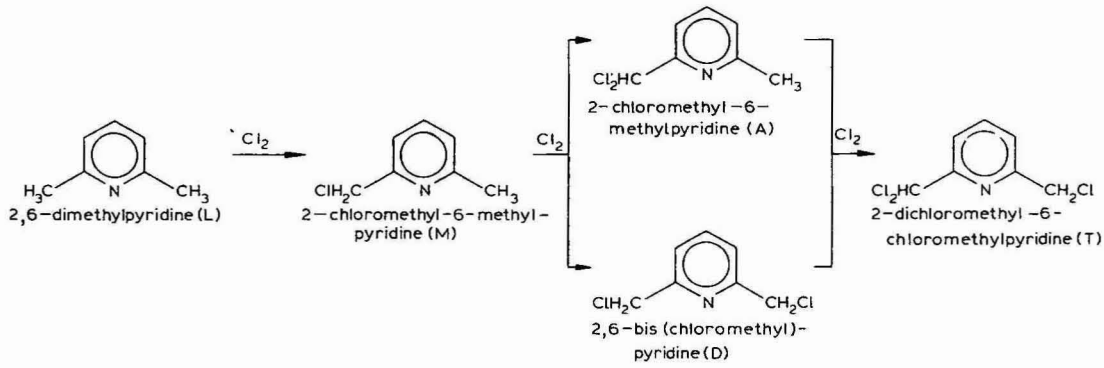
Note

Gas chromatographic separation of 2,6-dimethylpyridine and its chloro derivatives

A. SIKLÓSI*, S. KOVÁCS and L. SZOTYORY
Research Institute for Technical Chemistry of the Hungarian Academy of Sciences, P.O. Box 125, H-8201 Veszprém (Hungary)
(First received May 21st, 1985; revised manuscript received July 22nd, 1985)

2,6-Bis(chloromethyl)pyridine, a pharmaceutical intermediate, is prepared by free radical chlorination of 2,6-dimethylpyridine (2,6-lutidine)[1]. Owing to parallel and consecutive side-reactions there are several components in the reaction mixture:



As it was necessary to follow the course of the reaction, we developed an optimized, rapid gas chromatographic (GC) separation to quantify lutidine and its chloro derivatives.

The window diagrams proposed by Purnell and co-workers[2–8] permit the optimization of the composition of mixed GC phases resulting in the best possible separation of all the components of the sample.

The separation of 2,6-lutidine and the solvent proved difficult, because the capacity factor of 2,6-lutidine is small and the excess of solvent is large. Therefore, the first task was the complete separation of these two compounds. As a second optimization criterion, the retention time of the last eluted component (T) had to be kept at a minimum.

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EXPERIMENTAL

Apparatus

Experiments were carried out with a Sigma 2 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.), equipped with a flame ionization detector, a Minigrator integrator (Spectra-Physics, Santa Anna, CA, U.S.A. and a Model 6 recorder (Perkin-Elmer).

Column

Columns were packed with 100–120-mesh Chromosorb W AW DMCS, coated with 5% (w/w) of stationary phase. Pyrex glass columns (2 m × 2 mm I.D.) were used. The following stationary phases were selected to represent the fundamental intermolecular interactions: OV-101, dispersion; OV-25, induction; OV-275, orientation, electron donor; OV-330, orientation, hydrogen bonding.

RESULTS AND DISCUSSION

Using the most polar of the four stationary phases selected, the lowest temperature at which the extent of tailing was acceptable was determined. Using this temperature (120°C), the optimum gas velocity leading to the minimum plate height was determined (26 ml/min).

The chromatograms of all the lutidine derivatives were obtained on all four columns using identical separation conditions (injector, detector and oven temperature, carrier gas velocity, phase loading, support, column length and efficiency). The distribution coefficients used in the window diagrams were calculated from the retention data.

The window diagrams of the non-polar–selective and selective–selective phase pairs were constructed from the measured values. The elution order was L, M, A, D and T on each column. Only the values of the immediate neighbours are shown in the window diagrams because the separation of the other components is easy. From the window diagrams it is apparent[9] that the highest selectivity for the benzene–lutidine pair is obtained on pure OV-330 stationary phase. The retention time of the last component, T, however, is almost twice as large on OV-330 as on OV-101, while the benzene–lutidine selectivity is only slightly lower on OV-101 than on OV-330. The window diagrams revealed that the use of mixed stationary phases does not offer any advantage in this instance. the selectivities offered by the windows were either smaller than on OV-101 or, if comparable, the retention time of T was much higher.

It was concluded that pure OV-101 is optimal for the separation of benzene, lutidine and its chloro derivatives. Therefore, the separation temperature for the OV-101 phase was optimized using the window diagram method[10]. The other temperatures used for the optimization were 100 and 130°C. The window diagram obtained is shown in Fig. 1. The separation selectivity for the benzene–lutidine pair (LB) increases only slightly as the temperature is lowered, but the retention time of the last eluted component increases rapidly. There is a window at 115°C, where the selectivity is optimal.

The optimal separation conditions are as follows: stationary phase, 5% (w/w) OV-101; column temperature, 115°C, isothermal; carrier gas (nitrogen) velocity, 26

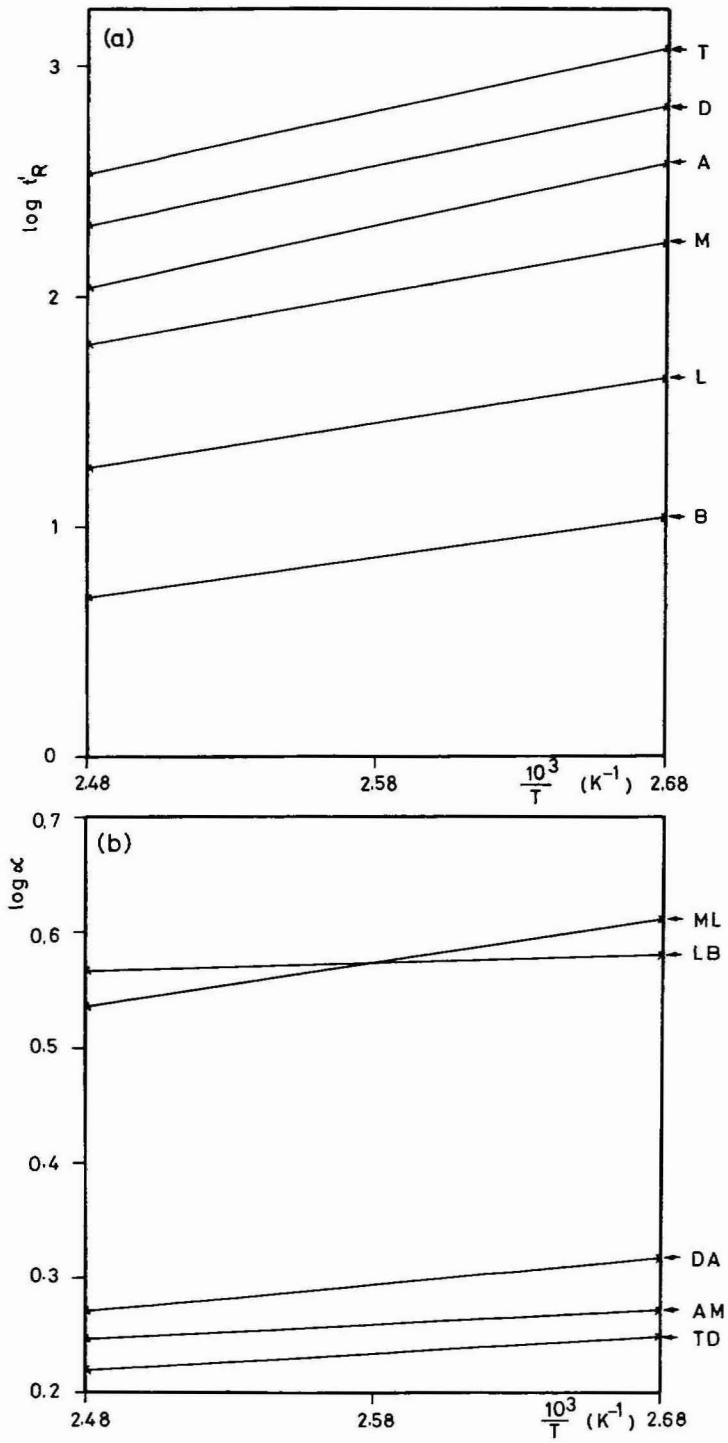

Fig. 1. (a) Log t'_R versus $10^3/T$ diagram for the six components and (b) the window diagram constructed from it (B = benzene).

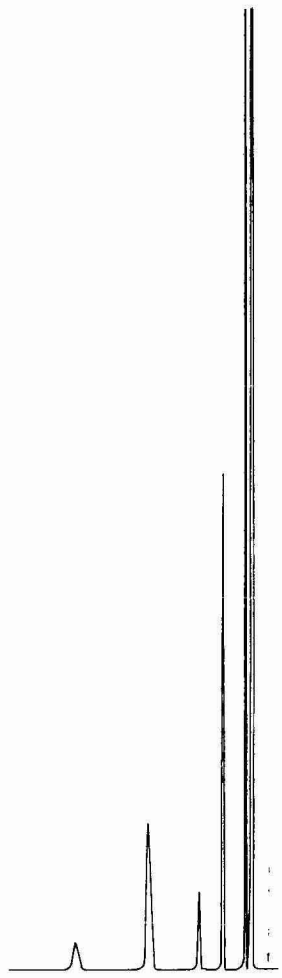

Fig. 2. GC separation of 2,6-lutidine and its chloro derivatives on an OV-101 column under optimized conditions. Injector temperature, 190°C; column temperature, 115°C; detector temperature, 200°C; carrier gas velocity, 26 ml/min.

ml/min; detector temperature, 200°C; and injector temperature, 190°C. The chromatogram obtained using these conditions is shown in Fig. 2. It can be seen that all six components are well separated and the analysis time is sufficiently short.

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Note

Improved method for the simultaneous determination of morphine, codeine and dihydrocodeine in blood by high-performance liquid chromatography with electrochemical detection

K. R. BEDFORD* and P. C. WHITE*
The Metropolitan Police Forensic Science Laboratory, 109 Lambeth Road, London SE1 7LP (U.K.)
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The determination of morphine and other opiates in small samples of blood is a problem commonly encountered by forensic toxicologists. Radioimmunoassay (RIA) can be used for screening samples and quantitation of morphine is possible but the assay is not specific due to cross-reaction with the other opiates. A high-performance liquid chromatographic (HPLC) method utilising electrochemical detection of morphine was developed in this Laboratory several years ago[1]. For accurate quantitation of the opiates complete chromatographic separation is required, but unfortunately this system did not permit resolution between codeine and morphine or dihydrocodeine and the internal standard dextrorphan. Discrimination between the former pair of compounds can be achieved by comparing detector responses at applied potentials of +0.6 V and +0.8 V, but this method is time consuming and requires additional amounts of sample.

To overcome these problems a chromatographic system has been developed to produce complete separation of these compounds. Additional improvements to the original method, which have removed negative peaks and reduced co-extractive levels, are also reported. This modified system also permits the separation of a number of other drugs of toxicological interest.

EXPERIMENTAL

Reagents

Acetonitrile and methanol, both HPLC grade, were obtained from Fisons (Loughborough, U.K.), perchloric acid (60%) from May and Baker (Dagenham, U.K.) and β-glucuronidase Type H5 from Sigma (Poole, U.K.).

The pH 5.0 acetate buffer was prepared by dissolving sodium acetate (5.74 g) and glacial acetic acid (1.74 ml) in distilled water (500 ml). The pH 8.9 borate buffer was prepared by mixing 0.05 *M* borax (70 ml) with 0.2 *M* boric acid (30 ml).

All glassware was silanized with a 5% (v/v) solution of dichlorodimethylsilane in toluene.

* Present address: Chemistry Division, DSIR, P.O. Box 2224, Auckland, New Zealand.

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HPLC conditions

The analyses were performed on a 25 cm × 4.9 mm I.D. stainless-steel column packed with Spherisorb S5W silica (5 μm: Phase Separations, Queensferry, U.K.). To prevent dissolution of the silica in the analytical column a guard column containing 40-μm silica was placed between the pump (Model 400; Applied Chromatography Systems, Macclesfield, U.K.) and the sample injection valve that was fitted with a 20-μl sample loop (Model 7125; Rheodyne, CA, U.S.A.).

The eluent consisted of a mixture of 3 parts of a solution of perchloric acid (0.05 *M*) adjusted to pH 9.0 with sodium hydroxide solution (1.0 *M*), and 7 parts of acetonitrile–methanol (9:1). The pH was measured with a pH meter (Model 6000; Jenway, Essex, U.K.) that had been calibrated against aqueous buffer solutions. Analyses were performed at a flow-rate of 1.5 ml min^{-1}.

The details of the electrochemical cell and associated electronics were similar to those described previously[1]. The glassy carbon working electrode (V25 grade; Le Carbone, Portslade, U.K.) was maintained at an applied potential of +1.1 V *versus* a silver–silver chloride reference electrode.

Extraction procedure

The extraction procedure was similar to that described by White[1]. Blood samples (500 μl) were dispensed into 8-ml screw-topped test-tubes, and the internal standard (50 μl; 5 μg ml^{-1} dextrorphan tartrate in water), acetate buffer (1 ml; 0.1 *M* pH 5.0), and β-glucuronidase (3.8 mg; Type H5) were added. After mixing, four drops of chloroform were added to activate the enzyme, and the mixture was incubated overnight at 37°C. The hydrolysed blood was made basic with borate buffer (1.5 ml; 0.8 *M*, pH 8.9), saturated with sodium chloride and then extracted with ethyl acetate–isopropanol (9:1, 2 × 4 ml) for 10 min on a rotary mixer. After evaporation to a small volume the extract was transferred to an agglutination tube and evaporated to dryness. Prior to analysis the residue was dissolved in methanol (100 μl). For calibration standards blank blood samples were spiked with an aqueous solution (5 μg ml^{-1}) of the opiate to be determined, and then treated as described above. To determine free (unconjugated) as opposed to total (conjugated) opiate levels the addition of the enzyme, and the incubation period are omitted from the procedure.

RESULTS AND DISCUSSION

Optimisation of chromatographic separation

The chromatographic method developed originally in this Laboratory for the detection of morphine in blood was performed on a silica column with methanol–aqueous ammonium nitrate eluents, but failed to separate morphine from codeine and dihydrocodeine from the internal standard dextrorphan[1]. It was considered that greater selectivity could be achieved with this system by the addition of acetonitrile to increase the solvent strength of the eluent. Solvents based upon ammonium nitrate buffer–organic mixtures (10:90) in which the organic component consisted of varying proportions of methanol and acetonitrile were investigated. The buffer was prepared by adjusting the pH of a 0.1 *M* solution of ammonium nitrate to pH 9.5 with ammonia (sp. gr. 0.88). As shown in Fig. 1 separations were obtained by the addition of acetonitrile, and an increase in acetonitrile content increased the separation between morphine and codeine, and dextrorphan and dihydrocodeine.

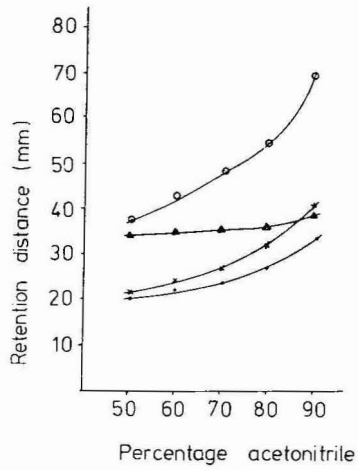

Fig. 1. Effect of changing the percentage of acetonitrile in the organic portion of the eluent on opiate retention. Eluent: 10% ammonium nitrate (0.1 *M*) adjusted to pH 9.5 with ammonia (sp. gr. 0.88) and 90% acetonitrile–methanol (X : 100 − X). ● = codeine, × = morphine, △ = dextrorphan and ○ = dihydrocodeine.

To achieve optimal conditions, changes in the aqueous content, pH, and ionic strength were studied using eluents containing methanol–acetonitrile (20:80). For an increase in aqueous content from 10 to 20%, retention of all compounds was reduced by 75%, and a level of 10% was considered to be the most favourable in terms of peak shape and overall analysis time.

The effects of changing ammonium nitrate concentration and pH are shown

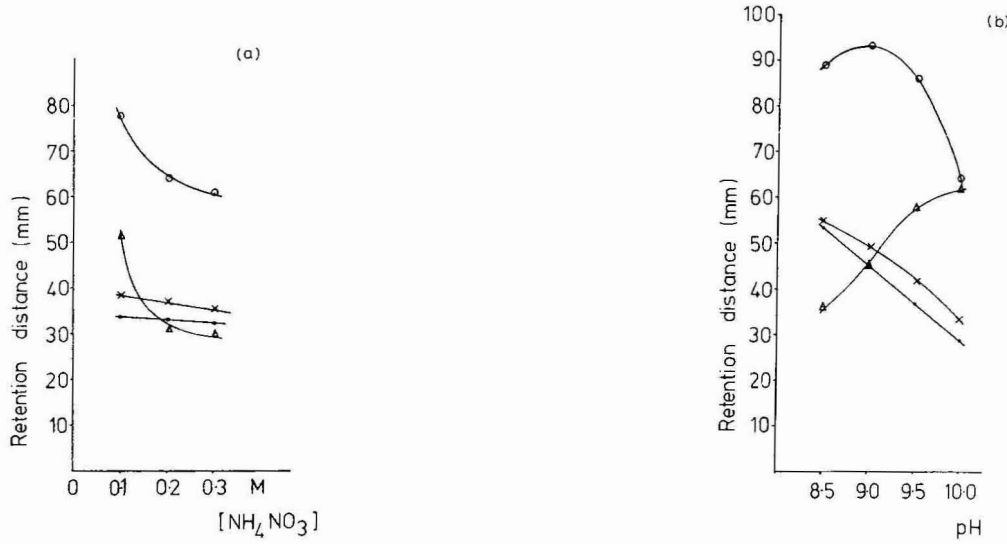

Fig. 2. (a) Effect of changing ammonium nitrate concentration on opiate retention. Eluent: 10% ammonium nitrate adjusted to pH 9.5 with ammonia (sp. gr. 0.88) and 90% acetonitrile–methanol (80:20). (b) Effect of changing pH on opiate retention. Eluent: 10% ammonium nitrate (0.1 *M*) pH varied by addition of ammonia (sp. gr. 0.88) and 90% acetonitrile–methanol (80:20). ● = codeine, × = morphine, △ = dextrorphan, and ○ = dihydrocodeine.

in Figs. 2a and 2b, respectively. An ammonium nitrate concentration of 0.1 *M* was considered to be most favourable as it produced the best separation between components and still permitted dextrorphan to be used as the internal standard. From the pH study it was evident that optimum separation was produced when the aqueous portion of the eluent was adjusted to pH 9.5.

These studies indicated that the eluent with the following composition was the most appropriate, *viz.* 10% ammonium nitrate (0.1 *M*) adjusted to pH 9.5 with ammonia (sp. gr. 0.88) and 90% acetonitrile–methanol (80:20). An excellent separation of a standard containing these compounds was observed under these conditions, but when a blank blood sample was analysed, a negative peak with a retention time similar to that of morphine was detected. Following further studies it was established that when a UV detector was used to monitor the separations, no negative peaks were observed. These results indicate that with the eluent–electrochemical system described, the negative peak effect is similar to that generated and used in indirect photometric detection[2].

When the aqueous portion of the eluent was prepared from either sodium acetate or nitrate solutions buffered with sodium hydroxide, no negative peaks were observed in chromatograms of blood extracts. However, with these eluents a further problem of variable retention of the opiates was observed, and this was attributed to their poor buffering capacity. The phenomena of negative peaking and variable retention time data were finally overcome by buffering the eluent with an aqueous solution of perchloric acid and sodium hydroxide.

To provide optimum resolution of the opiates with the perchloric acid mobile phase some modifications to the eluent described earlier were required, and the final conditions were; a mixture of 3 parts perchloric acid (0.05 *M*) adjusted to pH 9.0 with sodium hydroxide (1.0 *M*) and 7 parts of acetonitrile–methanol (9:1). A chro-

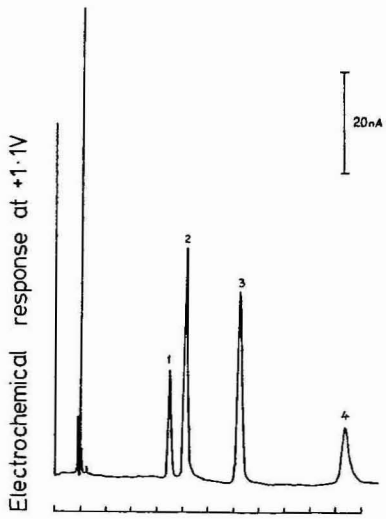

Fig. 3. Illustration of the separation of a mixed opiate standard using the finalised eluent conditions; 30% of a solution of perchloric acid (0.05 *M*) adjusted to pH 9.0 with sodium hydroxide (1.0 *M*) and 70% acetonitrile–methanol (90:10). 1 = Codeine, 2 = morphine, 3 = dextrorphan and 4 = dihydrocodeine. Scale graduations represent 2-min intervals and all other conditions are as described in the text.

matogram showing the separation of a standard containing the opiates analysed with the eluent is shown in Fig. 3.

With this eluent reproducible retention data were obtained from freshly prepared columns after pumping eluent for a period of 24 h. Thereafter columns performed with no loss in performance on systems that were run continually for one month. Retention data for the opiates and some possible interfering compounds obtained with this chromatographic method are given in Table I.

TABLE I

RETENTION TIME DATA FOR OPIATES AND POSSIBLE INTERFERENCES

NR = Not retained.

Compound	*Relative retention time**	*Compound*	*Relative retention time**
Buprenorphine	NR	Codeine	0.88
Dextromoramide	NR	Morphine	1.00
Diphenoxylate	NR	Dextromethorphan	1.34
Nalorphine	0.31	Dextrorphan	1.42
Dextropropoxyphene	0.33	Normorphine	1.58
Pentazocine	0.37	Pholcodeine	1.63
Methadone	0.48	Dihydrocodeine	2.22
Acetylcodeine	0.48	Dihydromorphine	2.49
6-Monoacetylmorphine	0.54		

* Relative to morphine (retention time = 10 min).

Detection conditions

The background current, and hence sensitivity of any electrochemical detector is very dependent on the eluent used and the potential applied to the working electrode. With the introduction of acetonitrile to the eluent it was found that large variations in background currents were observed with various grades produced by several suppliers. At an applied potential of +1.1 V background currents varied from greater than 1000 nA for reagent grade down to 85–95 nA for the UV grade. The HPLC grade supplied by Fisons, which is not recommended for low-wavelength UV detection, produced a level of 110 nA. Based upon these values and the relative costs, the latter material was found to be the most acceptable.

To determine the optimum applied potential for the low-level detection of codeine, morphine, dihydrocodeine and dextrorphan these compounds were analysed over a range of potentials from +0.6 to +1.3 V, and the detector response and eluent background currents were compared. An applied potential of +1.1 V was considered to offer the best sensitivity, and it was also observed that for the same concentration of each of the opiates very similar responses were obtained. Under these conditions detection limits based upon the amount of material injected were, 250 pg for morphine and codeine and 500 pg for dihydrocodeine (signal-to-noise ratio = 3).

Analysis of blood samples

Hydrolysed and unhydrolysed blood samples were analysed for opiate content following the extraction method that was originally developed in this Laboratory, except that the acid back-extraction was omitted. Experimental results have shown that when using the new eluent, there were no advantages to be gained by including this stage in the procedure.

The type of β-glucuronidase enzyme preparation used to hydrolyse opiate conjugates was found to influence the final chromatogram. Several of these preparations produced compounds which eluted under the described chromatographic conditions, and in some instances they produced a strong electrochemical response. A comparative study of six enzyme preparations was conducted, and based upon hydrolysis efficiency and extract purity, it was found that the Type H5 preparation gave the most satisfactory results. A comparison of chromatograms of the same blood sample containing a very high level of morphine (free = 2.0 μg ml^{-1}, total = 5.6 μg ml^{-1}) hydrolysed with Type H2 and H5 β-glucuronidases is shown in Fig. 4.

An example of the use of the system to detect opiate mixtures in a single chromatographic run is shown in Fig. 5. This hydrolysed blood sample was found to contain a high therapeutic level of codeine (290 ng ml^{-1}) and morphine (95 ng ml^{-1}), and the morphine level in this sample was attributed to the *in vivo* metabolism by the person who has been prescribed codeine.

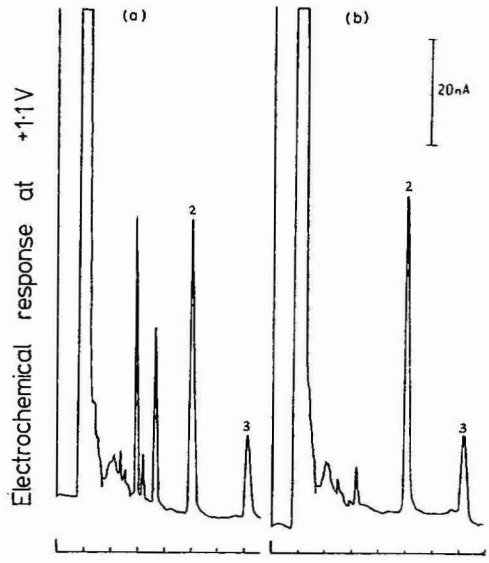

Fig. 4. A casework blood sample containing morphine hydrolysed with (a) Sigma Type H2 β-glucuronidase and (b) Sigma Type H5 β-glucuronidase. Eluent conditions as described in Fig. 3 and scale graduations represent 2-min intervals.

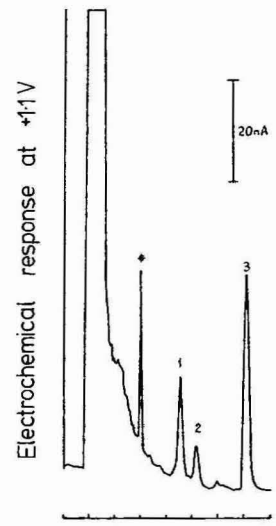

Fig. 5. A casework blood sample containing codeine and morphine after hydrolysis with the Type H5 β-glucuronidase. Eluent conditions as described in Fig. 3 and scale graduations represent 2-min intervals. 1 = Codeine, 2 = morphine, 3 = dextrorphan and * = unidentified.

CONCLUSIONS

The described chromatographic method permits excellent separation and quantitation of the opiates commonly encountered in blood samples. Through the judicious choice of HPLC reagents and β-glucuronidase employed, negative peaks are not detected and interference problems can be minimised, thereby increasing the sensitivity and selectivity of the technique.

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Note

High-performance liquid chromatography with electrochemical detection for the determination of nicotine and N-methylnicotinium ion

S. MOUSA and G. R. VAN LOON
Veterans Administration Medical Center and Department of Medicine, University of Kentucky, Lexington, KY 40511-0847 (U.S.A.)
and
A. A. HOUDI and P. A. CROOKS*
Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0053 (U.S.A.)
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The determination of nicotine and its metabolites is of particular interest in studying the correlation between the biological effects of tobacco smoking and the pharmacodynamics and pharmacokinetics of nicotine, the principal pharmacologically active component of tobacco.

Previous studies have shown that nicotine is primarily metabolized in humans to cotinine and nicotine-N′-oxide[1], both metabolites being formed via independent oxidation pathways[2]. However, nicotine methylation has also been reported[3–6], and recent studies indicate that N-methylnicotinium ion, the major methylated urinary metabolite of nicotine, is formed from an S-adenosylmethionine-dependent methyltransferase enzyme system that is widely distributed in tissues[7–9]. Also N-methylnicotinium ion has the potential to function as an *in vivo* methylating agent[10], and there is evidence that this metabolite is subsequently biotransformed *in vivo* in both mouse and rat, to nicotine[11]. Thus, we were interested in the development of a sensitive and selective assay for the determination of both nicotine and N-methylnicotinium ion.

Several analytical techniques for the quantitation of nicotine and its oxidation metabolites in biological fluids have been described in the literature. However, these methods generally lack the required sensitivity and selectivity for measuring the low levels of these substances in the urine and plasma of smokers, and no current method is available for the determination of the low levels of polar, water-soluble N-methylated quaternary metabolites of nicotine. The most commonly used techniques employed involve solvent extraction of nicotine followed by gas chromatography–mass spectrometric analysis[12–19], or liquid chromatography with ultraviolet spectrophotometric detection[20,21]. However, these procedures are not suitable for the determination of the non-volatile, non-organic soluble, N-methylated quaternary nicotine metabolites.

The use of liquid chromatography coupled with electrochemical detection (ED) has been widely and successfully applied to the detection of oxidizable or reducible

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compounds such as catecholamines and other amines[22]. We have utilized the chemistry of nicotine and its metabolites for the selective analysis of nicotine and its N-methylated metabolite by coulometric ED. We now report an analytical method for the determination of both nicotine and N-methylnicotinium ion by high-performance liquid chromatography (HPLC) and coulometric detection with sensitivity in the picogram range.

MATERIALS AND METHODS

Reagents and standards

S(−)-Nicotine (Fig. 1, structure 1) was purchased from Aldrich (St. Louis, MO, U.S.A.) and was distilled under vacuum before use; the N-methylnicotinium iodides (Fig. 1, structures 2–4) (X = I) were prepared by the procedure of Seeman and Whidby[23].

HPLC-grade acetonitrile, methanol, 85% phosphoric acid and sodium dihydrogen phosphate were obtained from Fisher Scientific (Pittsburg, PA, U.S.A.); sodium octyl sulfate was obtained from Kodak (Rochester, NY, U.S.A.). All buffers were prepared in preboiled, double distilled water. The solutions were then cooled and filtered under vacuum through a 0.22-μm filter (type GS) followed by degassing using sonication under vacuum.

Equipment

Analyses were carried out on a HPLC system (Waters Assoc., Milford, MA, U.S.A.) comprising a Model 6000A solvent delivery system, a Model U6K Universal injector unit and data module recorder and integrator, Model 730. Chromatographic separations were carried out on a reversed-phase μBondapak C_{18} column (30 × 0.39 cm I.D., 5 μm particle size) (Waters Assoc.). Elution of solutes was monitored with a Coulometric Electrochemical Detector, Model 5100A fitted with a dual electrode assembly (ESA, Bedford, MA, U.S.A.).

Chromatographic conditions

Separations were effected using an isocratic mobile phase consisting of a pri-

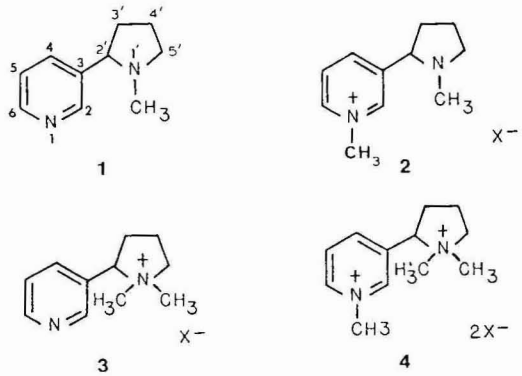


Fig. 1. Structural formulae for nicotine and N-methylated derivatives: 1 = nicotine; 2 = N-methylnicotinium ion; 3 = N′-methylnicotinium ion; 4 = N,N′-dimethylnicotinium ion.

mary mobile phase of 2 m*M* sodium dihydrogen phosphate containing 0.25 m*M* sodium octyl sulfate (92.5–95.0%) and a secondary mobile phase of acetonitrile–methanol (3:1) (7.5–5.0%), adjusted to pH 3.0 with phosphoric acid; for appropriate conditions see legend to Fig. 2. Analyses were performed at room temperature and the analytical column was equilibrated with mobile phase before connecting to the detector. After connecting the detector, the system was equilibrated overnight before applying samples.

Optimization of chromatographic performance

The optimum parameters selected for the use of the coulometric ED system were: an applied reduction potential of −0.50 V and an oxidation potential of +0.75 V. A pulse dampener, a good grounding, a low molarity of electrolyte, and the application of an oxidation potential in excess of +1.00 V through the guard cell for oxidation of the mobile phase and reduction of background noise, afforded improved performance and enhanced detector sensitivity and stability.

RESULTS AND DISCUSSION

Chromatographic separation of nicotine and N-methylnicotinium ion from the oxidation metabolites cotinine, 3-hydroxycotinine and nicotine-N′-oxide, was easily achieved (see Table I and Fig. 2) using mixtures of 90–95% primary buffer, and 5–10% secondary buffer in the isocratic mode. A convenient system was developed using 92.5% primary mobile phase and 7.5% secondary mobile phase at a flow-rate of 1.20 ml/min, isocratically (see Table I and Fig. 2).

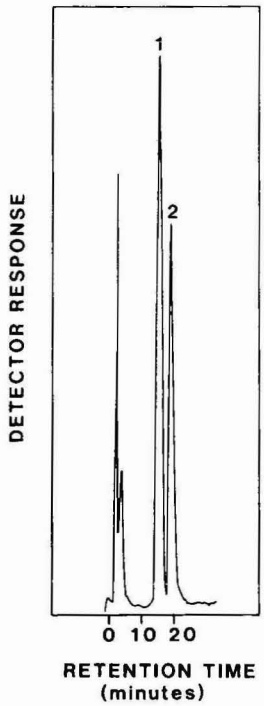
In the analysis of nicotine and N-methylnicotinium ion by ED, it is likely that the electrochemical reaction involves a one electron transfer with an irreversible secondary reaction involving solvent, as has been observed for other tertiary amino compounds[24–26]. It is postulated that nicotine initially loses a lone pair electron from the pyrrolidine ring N to afford a cation radical, which then rapidly extracts a proton from the solvent (see Fig. 3). The presence of acetonitrile as a solvent component appears to be important, as has been previously reported[27], since it is likely that it

TABLE I

ELECTROCHEMICAL DETECTABILITY AND RETENTION TIMES OF NICOTINE AND RELATED COMPOUNDS

Compound	*Electrochemical detectability*	*Retention time (min) (UV 254 nm)*
Nicotine	Yes (0.1 ng)*	16.1
Cotinine	No	4.5
Nicotine N′-oxide	No	2.5
3-Hydroxycotinine	No	2.5
N-Methylnicotinium ion	Yes (0.2 ng)*	18.0
N′-Methylnicotinium ion	No	–
N,N′-Dimethylnicotinium ion	No	–

* Limits of detection at a signal-to-noise ratio of 4:1.



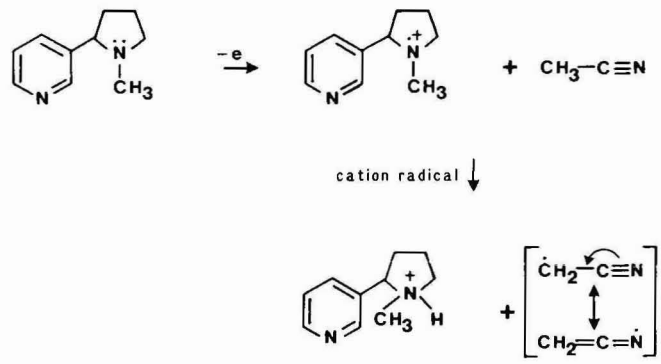


Fig. 2. Separation of nicotine (1) and N-methylnicotinium ion (2) by reversed-phase HPLC with ED. Conditions: primary buffer, 92.5% 2 m*M* $NaHPO_4$ containing 0.25 m*M* sodium octyl sulfate; secondary buffer, 7.5% methanol–acetonitrile (3:1) adjusted to pH 3.0 with phosphoric acid; flow-rate 1.20 ml per min; column, reversed-phase μBondapak C_{18}, 5 μm particle HPLC (30 × 0.39 cm I.D.). See Materials and methods section for electrochemical detector parameters.

Fig. 3. Electrochemical oxidation of nicotine.

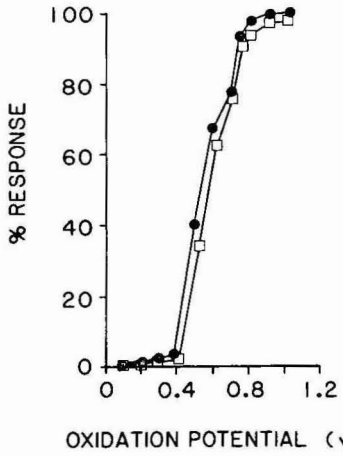


Fig. 4. Relationship between applied oxidation potential and detector response for nicotine (●) and N-methylnicotinium ion (□).

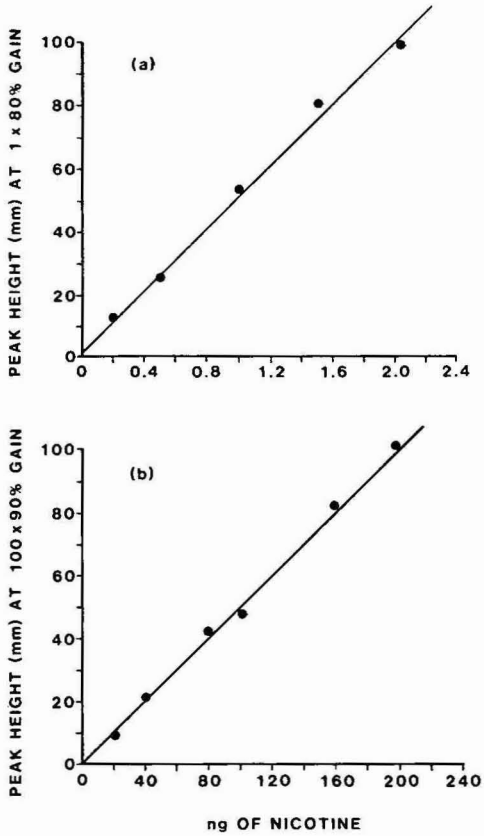


Fig. 5. Nicotine standard curves in the range (a) 0.2–2.0 ng, and (b) 20–200 ng. Values are means of at least three determinations; correlations of 0.9965 and 0.9987 were obtained for curves (a) and (b) respectively.

is the active species in the solvent reaction, generating a resonance stabilized radical. The resulting protonated amine is not reducible and is electroinactive. The observation that N-methylnicotinium ion (Fig. 1, structure 2) affords a level of detection almost comparable with nicotine, whereas N′-methylnicotinium ion (Fig. 1, structure 3) and N,N′-dimethylnicotinium ion (Fig. 1, structure 4) are both insensitive to ED using the conditions described above (see Table I), is strong evidence to support the involvement of the pyrrolidine N, and not the pyridine N lone pair of electrons in the oxidation mechanism.

Fig. 4 illustrates the relationship between the oxidation potential applied and the percentage response obtained after injection of a constant amount of nicotine and N-methylnicotinium iodide. For optimum conditions, an oxidation potential of +0.75 V was found to be most suitable.

Calibration curves for nicotine and N-methylnicotinium ion showed linearity over the concentration range 0.2 ng to 5 μg. Nicotine standard curves are illustrated in Fig. 5a and b, over the range 200 pg to 200 ng. Good reproducibility was obtained for repeat injections; similar sensitivity and wide range linearity were observed with N-methylnicotinium ion (data not shown). The oxidation metabolites, cotinine, 3-

hydroxycotinine and nicotine N'-oxide were not observed in the above sensitivity range by ED using the conditions employed for the nicotine analysis.

CONCLUSION

A sensitive and selective HPLC system with ED, has been developed for the analysis of nicotine and N-methylnicotinium ion down to the upper picogram level. This analytical procedure should be of utility in quantitating the levels of the above compounds in the blood, tissue and urine of animals and humans that have been exposed to nicotine in tobacco smoke.

ACKNOWLEDGEMENTS

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Note

Analysis of buprenorphine by high-performance liquid chromatography

I. R. TEBBETT
Forensic Science Unit, University of Strathclyde, Glasgow (U.K.)
(Received September 3rd, 1985)

Buprenorphine (temgesic) is a derivative of the opiate thebaine. It is a long-acting analgesic with both narcotic agonist and antagonist actions[1]. Buprenorphine is often prescribed for the treatment of chronic post-operative pain and for terminal cancer patients[2]. It has also been used recently for the treatment of heroin addicts[3]. Buprenorphine is available as an injection for intramuscular or intravenous administration, and as sublingual tablets. The usual recommended doses are 200–600 μg by slow intravenous or intramuscular injection repeated every 6–8 h or 400 μg sublingually also every 6–8 h.

However, there have been recent reports of buprenorphine being abused by opiate addicts and doses of 3 mg per day sublingually or by injection have been commonly encountered[4]. This drug has a similar effect to morphine and pethidine but is more readily obtainable since it is not subject to the stringent restrictions associated with controlled drugs. The problem of buprenorphine abuse is apparently on the increasse and this situation has necessitated the search for a rapid method for the analysis of buprenorphine both in the form of pharmaceutical preparations and in blood samples of addicts. This paper describes a high-performance liquid chromatographic (HPLC) method which goes some way towards fulfilling these requirements.

EXPERIMENTAL

HPLC conditions

A Perkin-Elmer Series 4 liquid chromatograph was used to deliver solvent at 1 ml/min. The eluent was monitored at 290 nm with a Perkin-Elmer LC-75 variable-wavelength ultraviolet detector. The column was 20 cm × 4.5 mm I.D. RP-18, 5 μm (Supelco) fitted with a Rheodyne injection system incorporating a 20-μl loop. Separation was achieved with a mobile phase of 0.05 *M* sodium pentanesulphonic acid–acetonitrile–methanol (30:15:55) to pH 2.0 with orthophosphoric acid.

Sample preparation

Human serum was prepared by centrifugation and kept frozen at −20°C until required for analysis. Serum (2 ml) was made alkaline with 1 *M* sodium hydroxide (0.1 ml) and extracted by shaking with 3-ml aliquots of diethyl ether. The serum

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sample was extracted three times and the extracts combined. The organic phase was evaporated to dryness under a stream of nitrogen and the residue redissolved in 100 μl of methanol. Samples of 20 μl were injected onto the column.

A straight line calibration graph was obtained for buprenorphine based on peak area measurements for concentrations of 2.5, 5.0, 10, 20, 50 and 100 ng/ml by addition of the drug to control serum and extraction by the procedure described above. Each point was taken as the average of two determinations.

Materials

Buprenorphine hydrochloride was supplied by Reckitt and Colman. The free base was prepared by dissolving the hydrochloride in water making alkaline with 1 *M* sodium hydroxide and extracting into diethyl ether. The ether extract evaporated to dryness under a stream of nitrogen and the residue redissolved in methanol and used for analysis. Tablets and injections of buprenorphine (Reckitt and Colman) were similarly subjected to a base/ether extraction prior to analysis by HPLC. Opiate standards were of pharmaceutical grade (MacFarlan Smith). All solvents were HPLC grade (Rathburn). Blood samples were obtained from hospitalised patients receiving high doses of buprenorphine by the sublingual route.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram of buprenorphine together with the commonly abused opiates morphine, codeine and heroin. Thebaine and papaverine, whilst not showing good peak shape in this system, do not interfere with the analysis of buprenorphine. Basic ether extracts of serum samples containing buprenorphine yielded clean extracts with between 98 and 100% recovery. A straight line calibration graph was determined for buprenorphine and found as $y = 0.91x - 0.464$ with a correlation coefficient of 1.0. Limit of detection (signal-to-noise ratio >2) was approximately 2 ng on column.

Fig. 1. Separation of commonly used opiates. Peaks: m = morphine; c = codeine; h = heroin; b = buprenorphine.

Fig. 2. (a) Chromatogram of a blank serum extract; (b) serum extract from an individual receiving buprenorphine.

The chromatogram of a blank serum extract (from a control subject who had not taken buprenorphine) is shown in Fig. 2a and the chromatogram of an extract of a serum sample from an individual taking high doses of buprenorphine is shown in Fig. 2b.

The method outlined is suitable for the analysis of buprenorphine in pharmaceutical preparations and for the detection of the drug in body fluids. Buprenorphine is rapidly metablished by the liver. Sublingual administration of 0.4–0.6 mg is reported to produce peak levels of 1–4 ng/ml after about 2 h as determined by radioimmunoassay[1,5]. These values are close the limit of detection of the described system (1 ng/ml) and sensitivity would need to be improved to enable the analysis of blood samples taken from patients receiving therapeutic doses of buprenorphine. However, sensitivity was found to be adequate for the detection of buprenorphine in patients receiving high doses of the drug. This method should therefore be suitable for the analysis of blood samples taken from abusers of buprenorphine.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. L. Rivier for the use of facilities at the Institut de Médecin Légale, Lausanne, Switzerland and the Carnegie Trust for the Universities in Scotland, U.K.

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Note

Quantitation of hydralazine hydrochloride in pharmaceutical dosage forms by high-performance liquid chromatography

RONALD J. MOLLES Jr. and YVES GARCEAU*
Department of Analytical Chemistry, Research and Development Division, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT 06877 (U.S.A.)
(First received July 29th, 1985; revised manuscript received August 19th, 1985)

Hydralazine hydrochloride is a widely prescribed anti-hypertensive[1]. Several high-performance liquid chromatographic (HPLC) methods have been developed, which utilize the derivatization product of hydralazine with *p*-hydroxybenzaldehyde[2] or *p*-anisaldehyde[3–5]. Derivatization methods are generally time consuming and less precise. A method which could chromatograph hydralazine hydrochloride underivatized would be preferable. Only a few HPLC methods have been found in the literature, which chromatograph hydralazine hydrochloride in the underivatized form[6–8]. Two of these methods[6,7] were not validated as stability indicating. For our purposes, stability-indicating methods are defined as those which are capable of monitoring peak decomposition. The other method[8] was an ion-exchange HPLC procedure, which is less commonly used than reversed-phase HPLC[9].

This note describes a reversed-phase HPLC method for the quantitative analysis of hydralazine in pharmaceutical dosage forms. The method is precise, accurate, and stability indicating.

EXPERIMENTAL

Materials

Hydralazine hydrochloride reference standard was obtained from USP (Rockville, MD, U.S.A.) and phthalazine from Aldrich (Milwaukee, WI, U.S.A.). The methanol, acetic acid, and water were HPLC grade.

Equipment

Two separate liquid chromatographs were used in the analyses. Both systems contained a WISP Model 710B (Waters, Milford, MA, U.S.A.) and a Column Compartment Oven Model 860 (Dupont, Wilmington, DE, U.S.A.). The system used for most of the experimentation utilized a reciprocating pump Model 590 (Waters) and a Spectroflow UV–VIS absorbance detector Model 757 (Kratos, Ramsey, NJ, U.S.A.). The second system was used for the interlaboratory evaluation of the method. This system utilized a reciprocating pump Model 510 and a UV–VIS absorbance detector Model 440 (both from Waters). The hydralazine capsule powder was stressed in a Sunlighter 150 (Engler, Jersey City, NJ, U.S.A.) to generate deg-

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radation products for a proof of method specificity. All data were collected and processed by a Model 3357 laboratory data system (Hewlett-Packard, Sunnyvale, CA, U.S.A.). Spectral scans of chromatographic peaks were done "on the fly" with a 1040A photo diode array detector (Hewlett-Packard).

HPLC method

The chromatographic parameters were as follows; analytical column: μBondapak Phenyl, 10 μm (Waters) 30 cm × 3.9 mm I.D., mobile phase: methanol–2% acetic acid solution (60:40, v/v); column temperature: 35°C; flow-rate: 1.0 ml/min; wavelength: 295 nm; injection concentration: 20 μg/ml; injection volume: 50 μl.

Standard preparation

Approximately 20 mg of hydralazine hydrochloride reference standard was accurately weighed into a 10-ml volumetric flask. Methanol was used to dissolve and dilute the sample to volume. A 1.0-ml aliquot was transferred to a 100-ml volumetric flask and diluted to volume with water. This solution was used for injection.

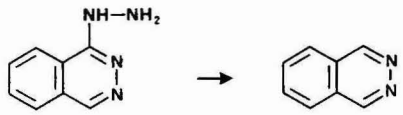
Sample preparation

A number of capsule contents were quantitatively transferred to an appropriate volumetric flask, such that the theoretical concentration of hydralazine hydrochloride in the solution was about 1 mg/ml. About 2/3 volume of methanol was added. The sample was sonicated 5 min. This was followed by 10 min of mechanical shaking. The sample was then diluted to volume with methanol and centrifuged. A 1.0-ml aliquot was transferred to a 50-ml volumetric flask and diluted to volume with water. This solution was used for injection. (This sample preparation was used for hydralazine hydrochloride capsules. Other dosage forms such as tablets or solutions could be used with the appropriate modifications.)

RESULTS

Specificity

Hydralazine hydrochloride was shown to separate from its primary breakdown product (phthalazine):



hydralazine phthalazine

A chromatogram of this separation is shown in Fig. 1. Hydralazine capsule powder was extracted into methanol and exposed to intense light for one week. Several degradation products formed and they were all separated from the hydralazine peak (Fig. 2). As illustrated, the retention time of the hydralazine peak in Fig. 2 (7.4 min) was greater than that in Fig. 1 (5.5 min). Presumably, this difference in retention time was due to column ageing, as all operating conditions for both Figs. 1 and 2

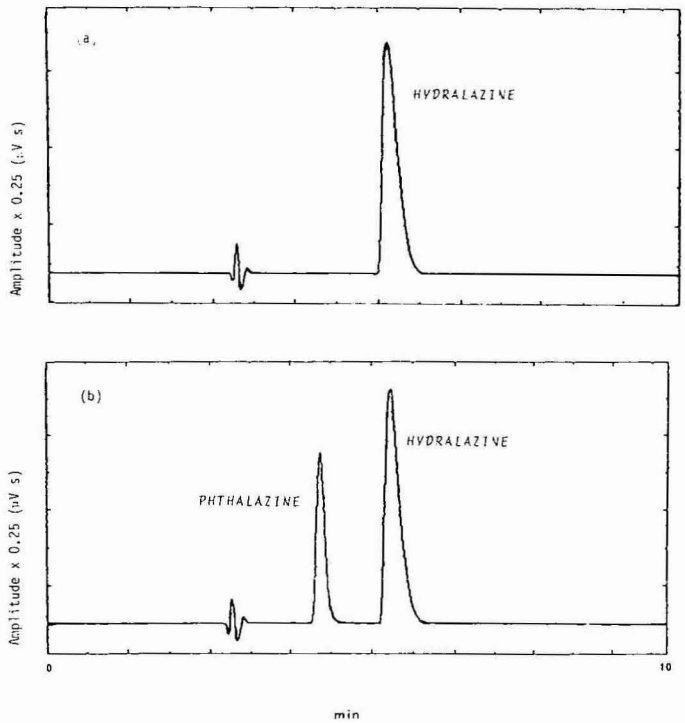


Fig. 1. (a) Chromatogram of hydralazine hydrochloride in water. (b) Chromatogram of hydralazine hydrochloride spiked with phthalazine (in water).

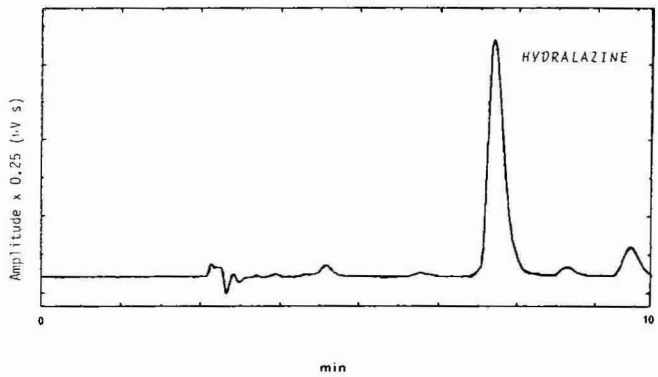


Fig. 2. Chromatogram of hydralazine capsule powder extracted into methanol and exposed to intense light one week.

were identical. The identity and purity of the hydralazine peak were ascertained by spectral overlay using a photo diode array detector (Fig. 3).

Linearity

The linearity of the calibration curve was investigated over the hydralazine

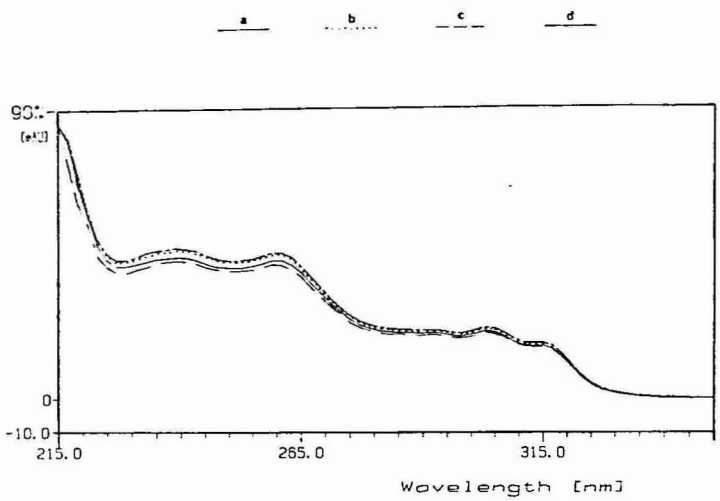


Fig. 3. UV spectra of hydralazine hydrochloride reference standard (d), and of upslope (a), apex (b), and downslope (c) of hydralazine peak shown in Figure 2.

hydrochloride concentration range of 9.7 to 29.1 μg/ml. The following results were obtained; correlation coefficient = 0.999, y-intercept = −1740, slope = 2686.

Precision

The precision of the method was demonstrated by replicate assays performed by two individual laboratories. Both laboratories reported a relative standard deviation (R.S.D.) of 0.8% on five replicates each.

Accuracy

Standard addition was used as a means of accuracy. The powder from the dosage form was spiked with accurately weighed amounts of hydralazine hydrochloride standard material (10%, 20%, 30% over claim). A recovery of 100.9% with an R.S.D. of 0.8% was achieved (Table I).

TABLE I

RESULTS OF THE STANDARD ADDITION EXPERIMENTS

Amount of hydralazine hydrochloride per capsule (mg)		*Recovery (%)*
Theoretical (including spike)	*Actual*	
11.49	11.54	100.4
11.20	11.32	101.1
12.19	12.20	100.1
12.28	12.29	100.1
13.20	13.38	101.4
13.14	13.42	102.1
Mean		100.9
R.S.D. (%)		0.8

DISCUSSION

From the data shown, the method described above for the quantitation of hydralazine hydrochloride is specific, accurate, precise, and easy to use. Derivatives of hydralazine used in other methods mentioned in this paper are perhaps more stable than hydralazine itself. However, we found that if hydralazine was extracted from the dosage form in methanol and diluted with water within 2 h, no significant degradation occurred. Solutions of hydralazine hydrochloride in water are stable for several days[10]. In clinical and stability studies where multiple sample assays are needed, this method is adequate and more convenient than derivatization procedures.

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Note

Rapid determination in water of chloride, sulphate, sulphite, selenite, selenate and arsenate among other inorganic and organic solutes by ion chromatography with UV detection below 195 nm

R. G. GERRITSE* and J. A. ADENEY
CSIRO Division of Groundwater Research, P.O. Box, Wembley, W. Australia 6014 (Australia)
(Received August 22nd, 1985)

Single-column ion chromatography, using silica bonded ion exchangers[1], is a well established technique for the separation of cations and anions[2]. The ions are detected indirectly through pairing[3] or marker[4–6] ions by UV absorption, conductivity or refractive index. Typical limits of detection are about 1 mg/l. Sensitivities down to μg/l level are in principle obtainable by pre-concentration methods[4]. For optimal sensitivity all these methods require a good control of the temperature of the detector and/or column. High-performance liquid chromatography (HPLC) of ions with direct photometric detection at wavelengths of 195–220 nm is much less influenced by temperature. Also broad vacancy peaks or system peaks due to the slow elution of dips in the concentration of the pairing or marker ions after sample injections, do not occur. This wavelength range is suitable for the direct detection of a number of anions (*e.g.* bromide, nitrite and nitrate)[6–9] at the μg/l level and many organic compounds *e.g.* organochlorines[9] and carboxylic acids[10–12] are easily detected. Chloride and sulphate among many other inorganic anions do not absorb sufficiently in the 195–220 nm range to be detectable[6,7,9]. However if the UV detector can be operated at wavelengths below 195 nm, chloride and sulphate become increasingly detectable and the sensitivity increases strongly for most other ions detectable in the 195–220 nm range.

Requirements for successfully operating the detector in this wavelength range are a sufficiently transparent eluent, displacement of dissolved oxygen and a low-UV-absorbing column bleed. These conditions can easily be met when using a silica-bonded anion exchanger[1] and eluting with a phosphate buffer. The simplicity, versatility and sensitivity of high-performance ion chromatography with direct UV direction below 195 nm is demonstrated in this paper for a range of inorganic and organic compounds.

EXPERIMENTAL

A stainless-steel (SS316) column of 25 × 0.4 cm I.D. containing a silica-bonded quaternary amine (Vydac 302 ion chromatography column; Vydac Separations Group, Hesperia, CA, U.S.A.) was used for the separations. A new column was eluted with 0.1 *M* phosphate at pH 5.5 until the baseline of the detector was within

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back-off range at the most sensitive setting (0.0025 AU) at 190 nm. This took about 48 h at a flow-rate of 2 ml/min. Eluent was pumped through the system with an ETP-Kortec K35 M HPLC pump (ETP-Kortec, Sydney, Australia). Samples were injected with a Rheodyne 7125 high-pressure sampling valve. All samples were passed through a 0.45-μm filter (type SM11306, Sartorius, Göttingen, F.R.G.) immediately before injection. The effluent was monitored at between 170 and 200 nm with an ETP-Kortec K95 variable-wavelength UV detector. Organic acids were determined simultaneously with chloride, bromide, nitrite, nitrate as well as selenite and arsenate, eluting with 0.01 – 0.03 *M* phosphate at pH 3.8. Iodide, sulphate, sulphite and selenate were determined in *ca.* 0.02 *M* phosphate at pH 4.8. pH was adjusted by adding phosphoric acid to solutions of dipotassium hydrogen phosphate or potassium dihydrogen phosphate. All chemicals were of high-purity grade (BDH Aristar). Solutions were made up in double quartz distilled water.

RESULTS AND DISCUSSION

In Fig. 1 the simultaneous separation of trace amounts of carboxylic acids and inorganic anions is shown for a detector wavelength of 190 nm. Optimization for these ions with regards to the required time of separation, resolution, background signal, detector noise and maximum salinity allowed in the sample, was done by varying the flow-rate, pH and phosphate concentration of the eluent and the detector wavelength. A compromise was reached using a wavelength of 190 nm, flow-rate of 2 ml/min, phosphate concentration of 0.01–0.03 *M* and a pH range of 3–5. The Vydac

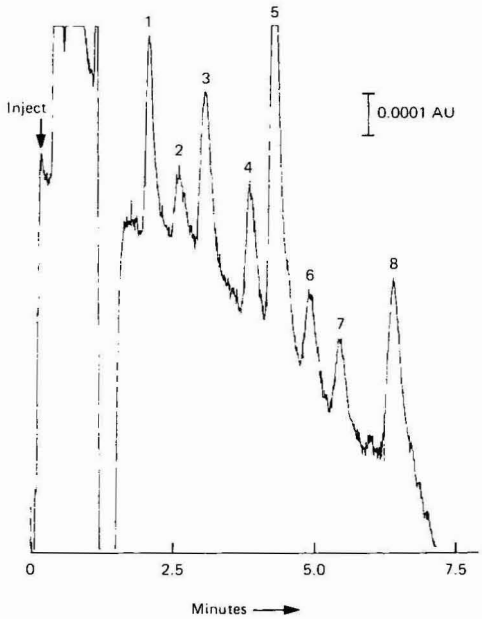

Fig. 1. Separation of 10^{-5} *M* each of acetate and propionate (1), butyrate (2), lactate (3) and formate (4), 0.72 mg/l chloride (5), 10 μg N/l of nitrite (6) and nitrate (8), 20 μg/l bromide (7). Conditions: column Vydac, 302-IC 4.6; eluent, 0.02 *M* potassium dihydrogen phosphate pH 3.8; flow-rate, 2 ml/min; UV detector wavelength, 190 nm; detector attenuation, 0.00125 AU; injector volume, 100 μl.

column gave a low-UV-absorbing bleed up to a pH of 5, above which it increased exponentially.

The effect of flow-rate is shown in Fig. 2. The decrease in resolution with increasing flow-rate is clearly seen for selenite and arsenate (peaks 5 and 6) and for formate and succinate (peaks 7 and 8). The effects of wavelength on noise and sensitivity of the detector is shown in Fig. 3 for a wavelength range of 170–210 nm. Due to the decreasing energy output of the deuterium lamp the noise level increases rapidly below 190 nm. The sensitivity decreases with increasing wavelength for all compounds, especially for acetone (peak 1 in Fig. 3), selenite (peak 5), chloride (peak 9) and bromide (peak 11).

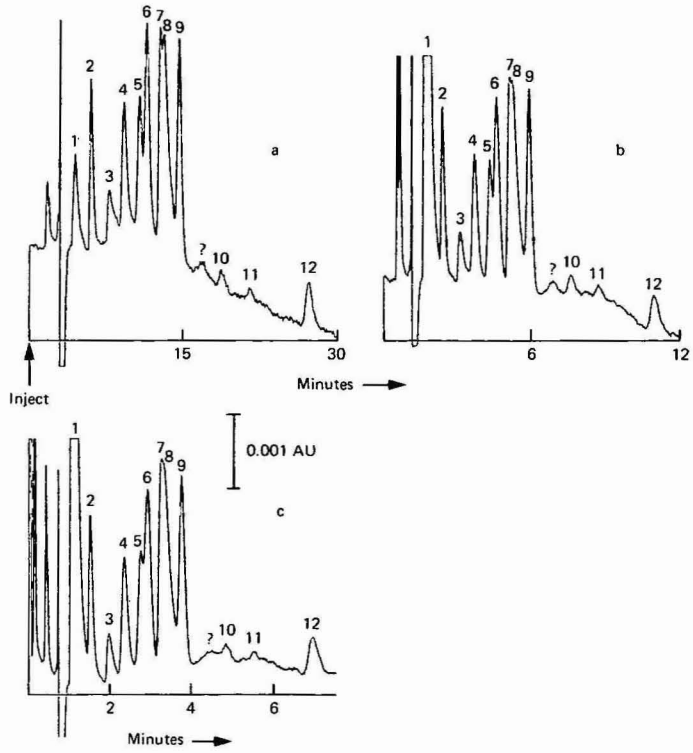

Fig. 2. Effect of flow-rate on the separation of a mixture of 0.01% *n*-butanol (peak 1 in a), 0.01% acetone (peak 1 in b and c), 10^{-4} *M* each of acetate plus propionate (2), butyrate (3), lactate (4), formate (7) and succinate (8), 1 mg/l Se as selenite (5), 1 mg/l. As as arsenate (6), 5 mg/l chloride (9), 10 μg N/l each of nitrite (10) and nitrate (12), 10 μg/l bromide (11). Conditions: eluent, 0.01 *M* phosphate (pH 3.7); wavelength, 175 nm; detector attenuation, 0.005 AU; injection volume, 100 μl; flow-rate, 0.8 ml/min (a), 2 ml/min (b) and 3.2 ml/min (c).

The effect of pH on the retention times and resolution is shown in Fig. 4 for succinate and a range of inorganic anions. The retention of selenate and succinate is strongly influenced by pH. For some anions and all organic acids used sensitivity decreases with decreasing pH, shown in Fig. 4 for succinate (peak 3), nitrite and

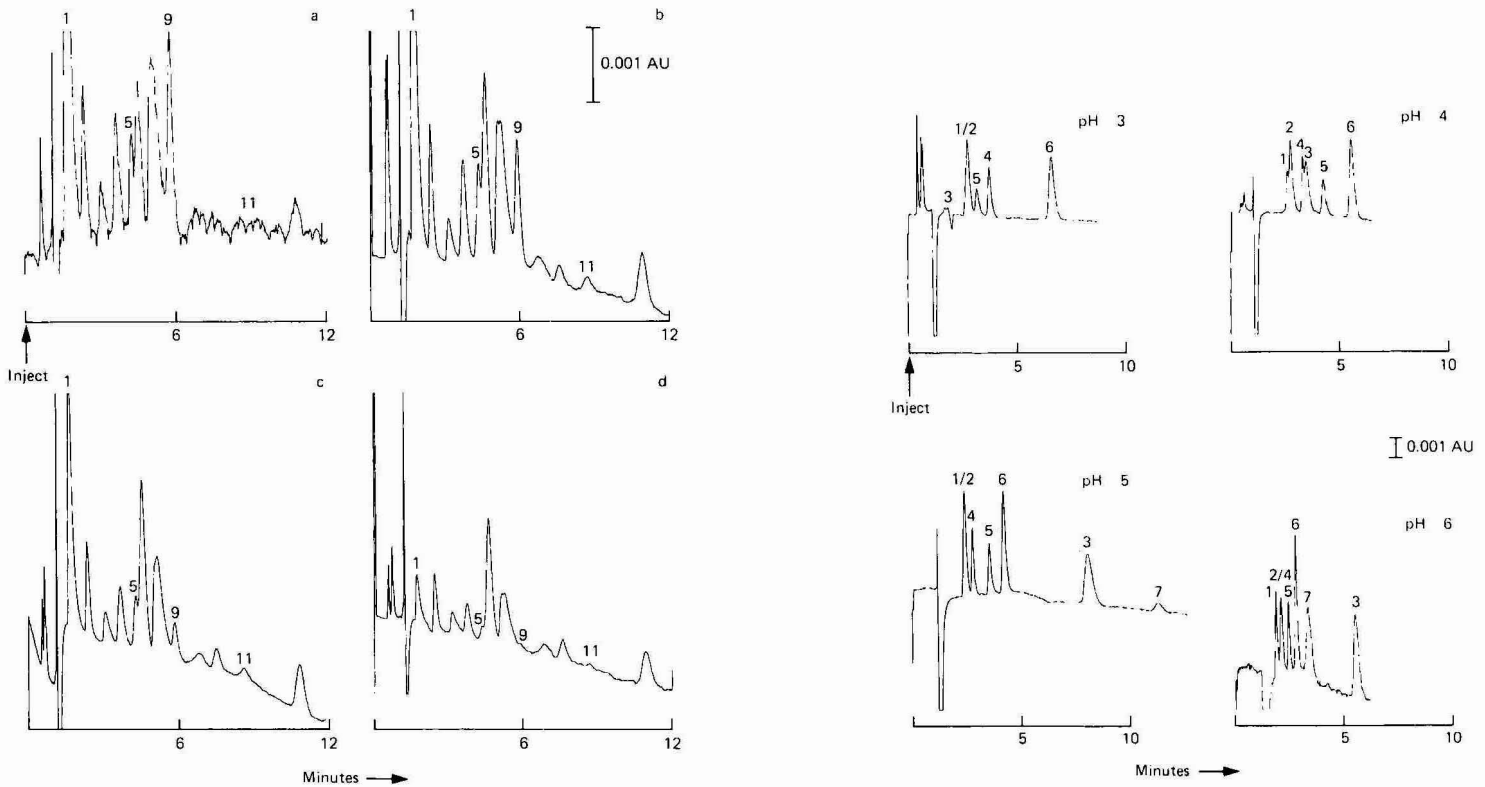

Fig. 3. The effect of wavelength on detector noise and sensitivity. The mixture of Fig. 2 was run at 170 nm (a), 190 nm (b), 200 nm (c) and 210 nm (d). Conditions and peak identification as in Fig. 2b.

Fig. 4. The effect of pH on the resolution and retention times of 1 mg/l Se as selenite (1), 1 mg/l As as arsenate (2), 10^{-4} *M* succinate (3), 5 mg/l chloride (4), 0.1 mg N/l each of nitrite (5) and nitrate (6), 1 mg/l Se as selenate (7). Conditions: eluent, 0.025 *M* phosphate (pH 3.6); wavelength, 180 nm; detector attenuation, 0.02 AU; flow-rate, 2 ml/min; sample volume, 100 μl.

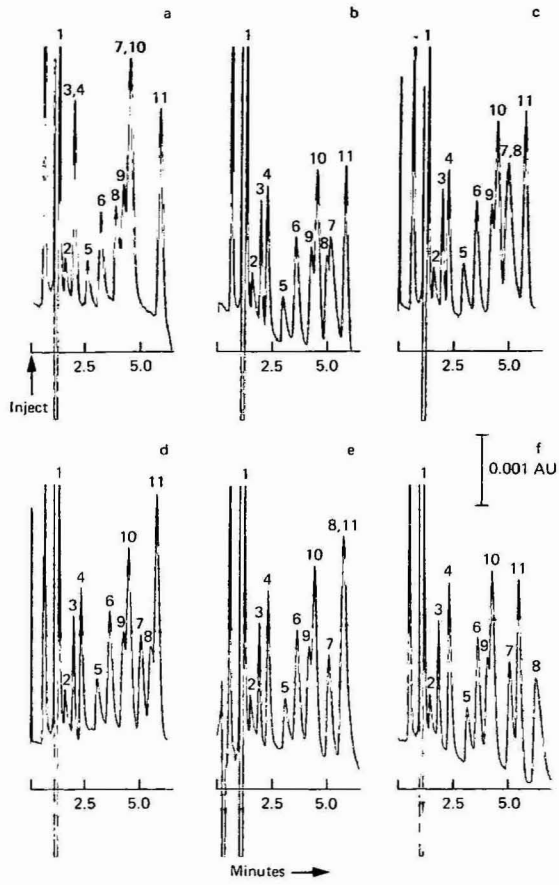

Fig. 5. The effect of pH on the resolution of 10^{-3} *M* glycerol (1), 0.01% *n*-butanol (2), 10^{-6} *M* phenol (3), 10^{-4} *M* phenol (3), 10^{-4} *M* each of acetate plus propionate (4), butyrate (5), lactate (6), formate (7), succinate (8), 1 mg/l Se as selenite (9), 1 mg/l As as arsenate (10), 5 mg/l chloride (11). Conditions: eluent, *ca.* 0.02 *M* phosphate, pH increasing from 3.55 (a) to 3.90 (f); wavelength, 180 nm; detector attenuation, 0.005 AU; flow-rate, 2 ml/min; sample volume, 100 µl.

selenate (peaks 5 and 7). At pH ≈3.8 most organic compounds and inorganic anions are reasonably resolved. This is shown in Fig. 5 for a pH range of 3.5–3.9. In this pH range iodide, sulphate, selenate and sulphite are however strongly retained (*e.g.* selenate in Fig. 4, peak 7) but can be better separated at a pH ≈ 4.8 (Fig. 6).

In Fig. 7 the elution of iodide at pH 4.8 is shown. Iodide could not be determined below 0.1 mg/l, which was surprising considering the sensitivity measured at the mg/l level. Removal of oxygen and nitrite from the sample gave some improvement, but below 0.1 mg/l iodide seemed to disappear rapidly from solution. The action of exo-enzymes in water samples, catalyzing the conversion of iodide offers a possible explanation for this[13], although traces of metals and organics in the HPLC system could be involved in the conversion of iodide.

Some organic acids, other than the acids shown in Figs. 1–5, were also inves-

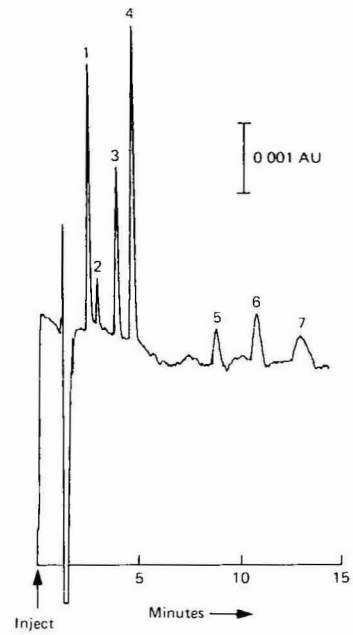

Fig. 6. Chromatogram of a sample containing 1 mg/l each of Se as selenite (1), chloride (2), S as sulphate (5), Se as selenate (6) and S as sulphite (7), 0.05 mg N/l nitrite (3) and nitrate (4). Conditions: eluent, 0.015 *M* phosphate (pH 4.8); wavelength, 190 nm; detector attenuation, 0.01 AU; flow-rate, 2 ml/min; sample volume, 100 μl.

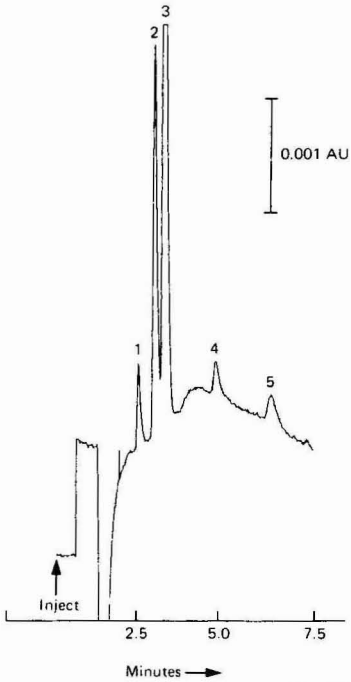

Fig. 7. Separation of 1 mg/l chloride (1) and S-sulphate (5), 0.1 mg N/l of nitrite (2) and nitrate (3), 0.1 mg/l iodide (4). Conditions as for Fig. 1, but pH 4.8 and attenuation 0.005 AU.

tigated. It was found that benzoate, phthalate, oxalate, citrate, tartrate as well as humic and fulvic acids were adsorbed very strongly within the pH range of 3–6. For this reason benzoate and phthalate are often used as marker ions[4,5] in ion chromatography.

The applicability of the method to water samples varying greatly in organic matter content and salinity was tested with organically polluted groundwater, seawater and tap water.

Results of applying the separation method to samples of groundwater taken from boreholes adjacent to a liquid waste disposal site are shown in Fig. 8A and B. In Fig. 8A one major peak at the start of the chromatogram can be seen and only traces ($\leqslant 10^{-6}$ *M*) in the carboxylic acid region. The major peak was not identified. Many organics elute in this region *e.g.* acetone, *n*-butanol, glycerol, phenol (Figs. 2 and 5). Urea and thio-urea also elute in this region and are detectable to 10^{-6} *M* at 190 nm.

Bromide (Fig. 8A and B) and iodide (Fig. 8B) are present in the groundwater at relatively high concentration (2.1 and 1.0 mg/l, respectively). In Fig. 9 results are shown (A) for a sample drawn from the water table under soil that had been sprayed with septage. From the chromatogram of the sample spiked with carboxylic acids (B) it is probable that acetate and/or propionate are present at a total concentration of about 5000 μM (peak 2). Acetate and propionate could not be resolved on the Vydac column. Peak 1 (Fig. 9) was not identified.

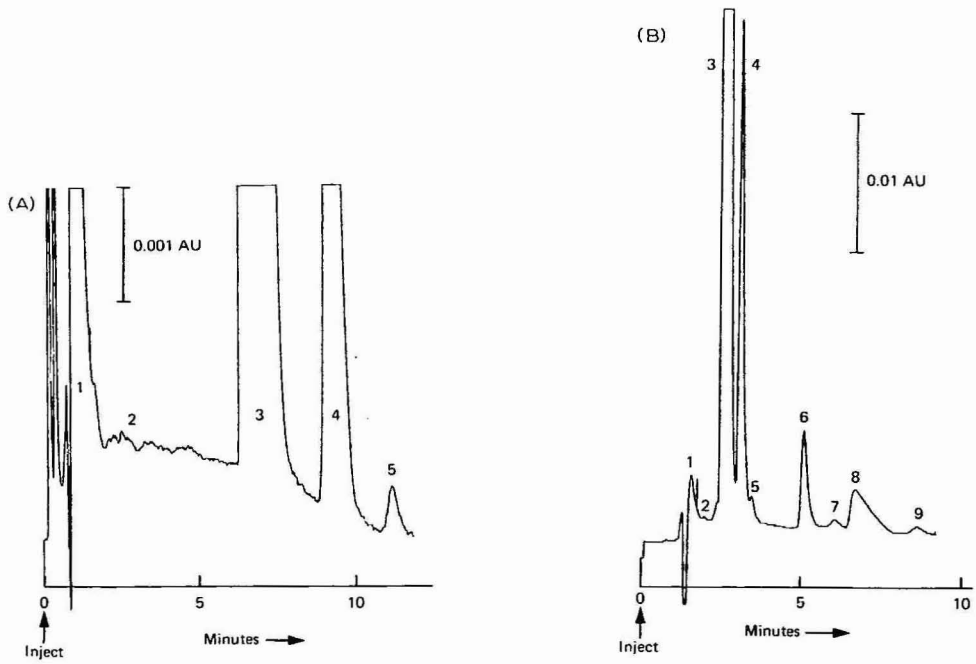

Fig. 8. (A) Chromatogram of a sample taken from a water table polluted by septage. Eluent, 0.01 *M* phosphate (pH 3.8); attenuation, 0.005 AU; other conditions as in Fig. 1. Peaks: 1 = unidentified; 2 = traces of organic acids (?); 3 = 290 mg/l chloride; 4 = 2.1 mg/l bromide; 5 = 0.012 mg N/l nitrate. (B) As for A, but 0.02 *M* phosphate (pH 4.6) as eluent and attenuation 0.04 AU.

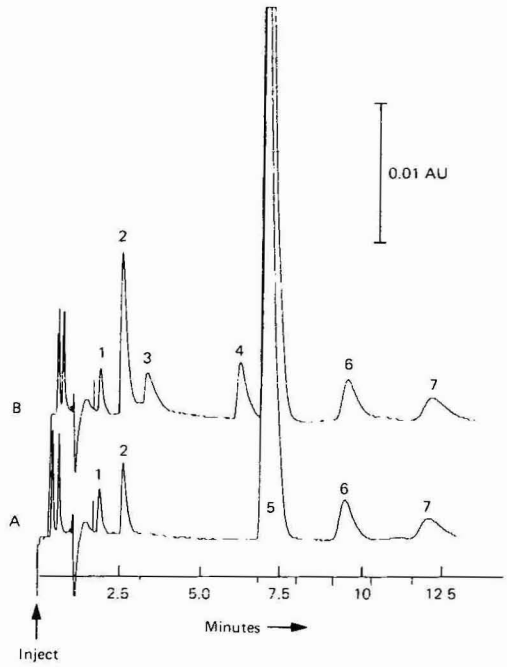

Fig. 9. Chromatograms of a sample of groundwater polluted by septage. The sample was diluted 1:10. Attenuation, 0.04 AU, other conditions as for Fig. 8A. Trace A: 1 = unidentified; 2 = acetate plus propionate (450 μM), 5 = 70 mg/l chloride; 6 = 0.54 mg/l bromide; 7 = 0.1 mg N/l nitrate. Trace B: 2, 3, 4 = 500 μM each of added acetate plus propionate (2), butyrate (3) and formate (4).

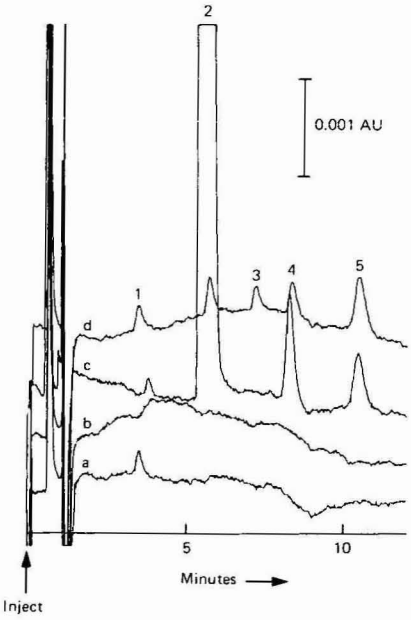

Fig. 10. Traces of bromide and nitrate in tapwater. Eluent, 0.02 M phosphate (pH 3.75); wavelength, 180 nm. Other conditions and peak numbering as in Fig. 8A. Traces; a = double quartz distilled demineralized water; b = double quartz distilled tap water; c = tap water; d = as a but spiked with 0.5 mg/l chloride (peak 2), 40 μg/l bromide (4), 20 μg N/l nitrite (3) and nitrate (5). Peak 1 was not identified.

In Fig. 10 a chromatogram of Perth (Wembley, Australia) tap water (3) is shown and compared with double distilled water prepared from deionized water (1) or tap water (2). Levels of chloride, bromide and N-nitrate (4) in the tap water were found to be 160, 0.13 and 0.02 mg/l, respectively.

Analyses of anions in the presence of high levels of chloride are easily accomplished. In Fig. 11A and B results are shown for bromide and sulphate in seawater diluted 1:10. The chloride, bromide and S-sulphate concentratuions were found to be 19 000, 70 and 750 mg/l, respectively. Above about 2000 mg/l chloride the resolution on the Vydac column was adversely affected.

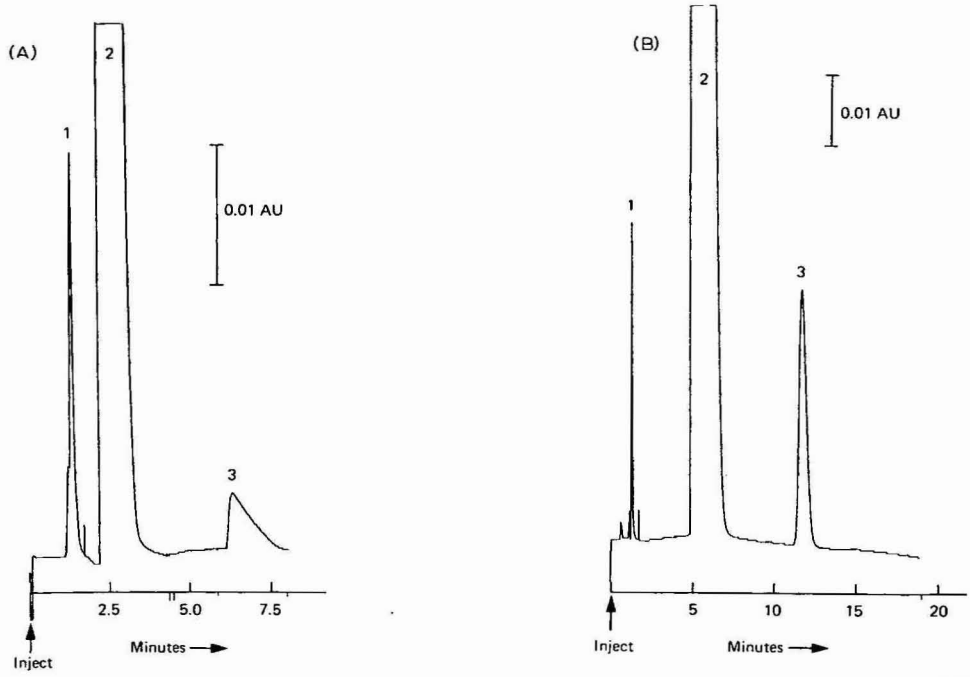

Fig. 11. (A) Chromatogram of seawater diluted 1:10. Conditions as for Fig. 8B, attenuation, 0.04 AU. Peaks: 1 = unidentified; 2 = chloride plus bromide; 3 = 75 mg S/l sulphate. (B) Chromatogram of seawater diluted 1:10. Eluent, 0.01 *M* phosphate (pH 3.6). Attenuation, 0.08 AU, other conditions as in Fig. 1. Peaks: 1 = unidentified; 2 = 1900 mg/l chloride; 3 = 7 mg/l bromide.

CONCLUSIONS

Single-column ion chromatography with UV detection below 195 nm has great potential for direct trace analysis of many organic and inorganic anions in surface and groundwater. Detection levels at the μg/l level or less should be attainable by improvement of the stability and energy output of UV sources below 195 nm and, if possible, concentrating the sample on a pre-column before injection.

When analysing anions that are easily oxidized special attention must be given to both sample preservation and catalytic effects of the HPLC materials in contact with the sample. Especially for iodide large losses occur when the concentration is less than 0.1 mg/l.

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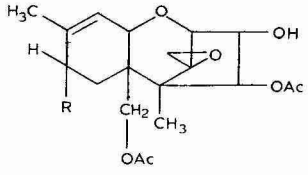
Note

Analysis of some trichothecene mycotoxins by liquid chromatography

R. MAYCOCK and D. UTLEY*
Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire SP4 0JQ (U.K.)
(First received March 25th, 1985; revised manuscript received August 5th, 1985)

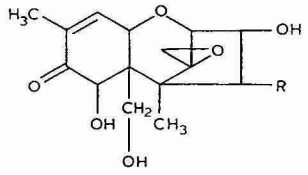
Trichothecene mycotoxins are toxic metabolites of Fusarium and other fungi which are capable of growing on agricultural products before harvest and during storage. Foodstuffs contaminated with these materials can cause severe effects in humans and animals and may result in death[1].

Trichothecenes in foodstuffs and body fluids have been extensively analysed by gas chromatography[2] combined gas chromatography–mass spectrometry[3,4], thin-layer chromatography[5,6], polarography[7], radioimmunoassay[8], and to a limited extent by column liquid chromatography (LC)[9–14]. This paper describes simple LC methods for the analysis of the four trichothecene mycotoxins: T2-toxin; diacetoxyscirpenol (DAS), nivalenol (NIV), and deoxynivalenol (DON).



T2, R = $-OOCCH_2CH(CH_3)_2$

DAS, R = H



NIV, R = −OH

DON, R = H

Analysis of these compounds at trace levels by LC with UV detection is difficult because of their low intensity of absorption and the need to use low wavelengths. The analysis of the toxins containing the conjugated enone (NIV, DON) has however been described[9–11]. T2 and DAS have been analysed at higher levels by using a refractive index detector[12–14]. A more suitable approach would seem to involve the derivatisation of the –OH groups, which are present in all the compounds, with a strongly UV absorbing group. Such an approach has been suggested for T2 and DAS but no experimental details are available[15]. In this study the preparation of the *p*-nitrobenzoyl derivatives of T2, DAS, NIV and DON and their separation by LC is described.

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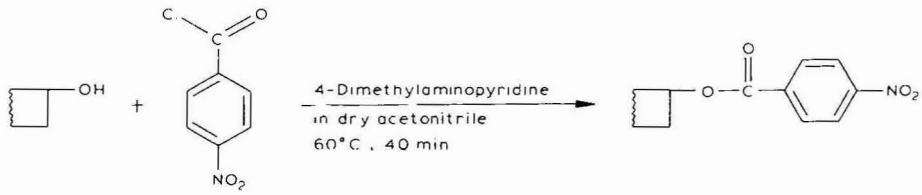

EXPERIMENTAL

Materials

Trichothecenes were obtained from Sigma (U.K.), Mycolabs (U.S.A.) and Wako (Japan).

The LC solvents were prepared from acetonitrile far UV grade (Fisons), organic free water from an Elgastat Spectrum and analytical reagent quality potassium dihydrogen phosphate and phosphoric acid.

The derivatising reagents were *p*-nitrobenzoyl chloride (puriss) and 4-dimethylaminopyridine (purum) from Fluka.

Instrumentation

UV spectra were recorded on a Shimadzu spectrophotometer Model UV-240. LC system comprised: pumps, Waters Model 6000A; detectors, Waters Model 480 and 440; injector, Rheodyne 7125; integrator/recorder, Spectra Physics SP4270, Waters M730 data module; gradient controller, Waters 720 system controller; column, 12.5 × 0.5 cm I.D. packed with 3-μm Hypersil ODS (Hichrom); column heater, Jones Chromatography Model 7910.

Methods

Derivatisation. Solutions containing the trichothecenes in the ng to μg range were dispensed into micro-vials and evaporated to dryness at 60°C under a stream of nitrogen. To the residue were added solutions of 3 mg/ml *p*-nitrobenzoyl chloride (20 μl) and 10 mg/ml 4-dimethylaminopyridine in acetonitrile (10 μl). The tubes were capped with PTFE lined septa and heated at 60°C for 40 min. The handling of the *p*-nitrobenzoyl chloride and preparation of solutions were carried out in a dry box. Calibration solutions were prepared over the range 2–300 ng for NIV and DON and 8 ng–2 μg for T2 and DAS.

Chromatographic analysis. Separation of the underivatised toxins was achieved with a solvent gradient from 5–80% acetonitrile mixed with water at a flow-rate of 1.0 ml/min. Detection was by UV at 220 nm.

The *p*-nitrobenzoyl derivatives of the toxins were analysed by the direct injection (5 μl) of the derivatising solution. A mobile phase of acetonitrile–aqueous buffer (0.2 *M* potassium dihydrogen phosphate plus 0.2 *M* phosphonic acid) (65:35) was used with a column temperature of 35°C. Solvent flow-rate was 1.0 ml/min; pressure 2000 p.s.i. UV detection was at 254 nm.

RESULTS AND DISCUSSIONS

The separation of the underivatised toxins with low-wavelength detection, Fig. 1, shows that they can all, even T2 and DAS, be handled directly by LC–UV. The sensitivity is not great and the analysis is subject to interference at this low wavelength. The method is however suitable for the analysis of cultures and for following the reaction of the toxins at high concentrations.

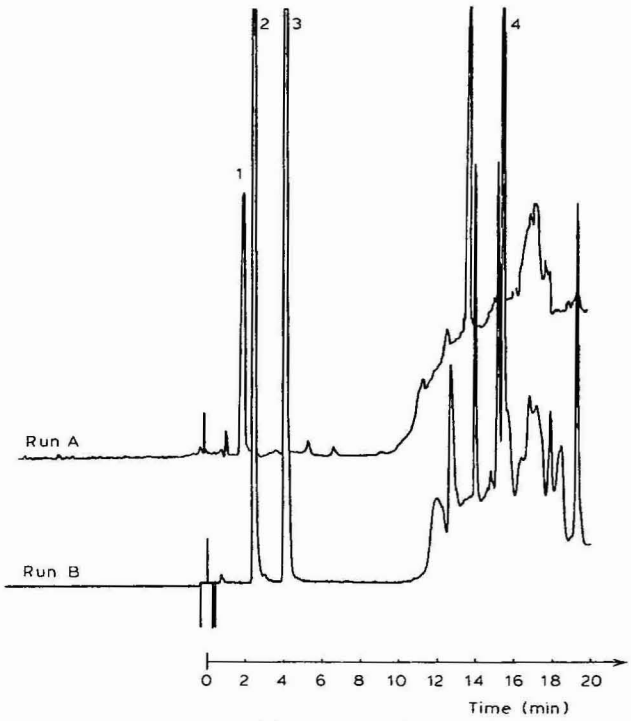

Fig. 1. Chromatogram of trichothecenes using a gradient programme, detection at 220 nm (0.50 a.u.f.s.). Run A: 1 = DAS 75 μg injected in acetonitrile. Run B: 2 = NIV 18 μg; 3 = DON 18 μg; 4 = T2-toxin 55 μg. All injected in methanol–water (10:90). Peaks other than the tabulated toxin peaks are due to the gradient background and impurities.

For the trace analysis of the trichothecenes in foodstuffs the stronger and longer wavelength UV absorption of the *p*-nitrobenzoyl derivatives, Fig. 2, is more suitable. The chromatogram, Fig. 3, shows that all four toxins can be well separated with good peak shape in a single isocratic run. Calibration is linear from the low nanogram detection limit to the limit of the linear range of the detector. The sensitivity to DON and NIV is higher because of the existence of several –OH groups which can be derivatised.

It was found that most samples of *p*-nitrobenzoyl chloride commercially available were of low purity and contained large quantities of *p*-nitrobenzoic acid. Furthermore, the reagent degraded rapidly when opened. The most reliable procedure

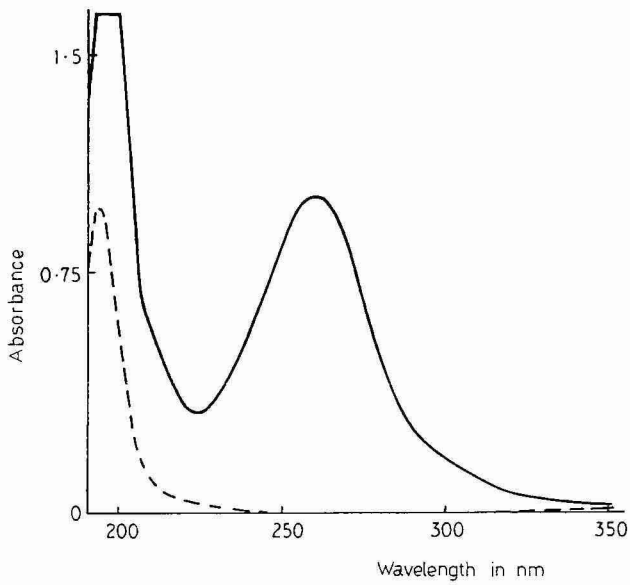

Fig. 2. UV spectra of T2-toxin ($2 \cdot 10^{-6}$ *M*) and its *p*-nitrobenzoate derivative ($8 \cdot 10^{-7}$ *M*).

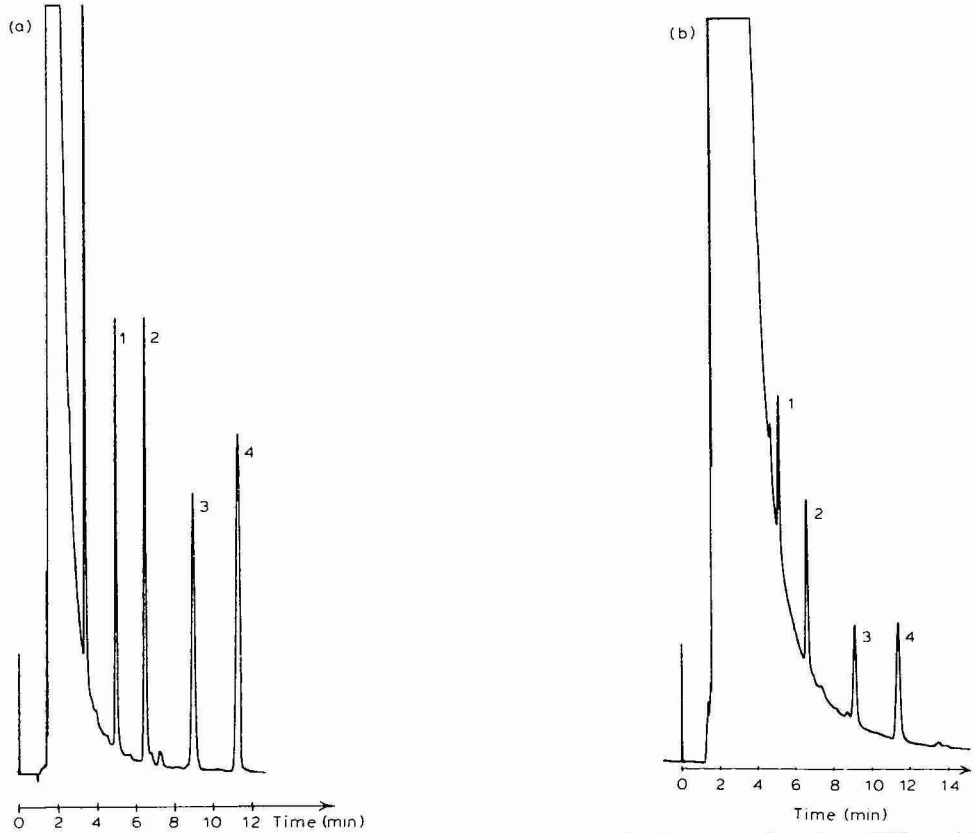

Fig. 3. Chromatogram showing the separation of trichothecene *p*-nitrobenzoates, detection at 254 nm. (a) 0.2 a.u.f.s. Peaks: 1 = DAS 600 ng; 2 = DON 166 ng; 3 = T2-toxin 800 ng; 4 = NIV 116 ng. (b) 0.02 a.u.f.s. Peaks: 1 = DAS 25 ng; 2 = DON 6.3 ng; 3 = T2-toxin 30 ng; 4 = NIV 6.3 ng.

was to prepare sealed ampoules of the reagent and carry out all operations in a dry box. The toxin derivatives were however quite stable to hydrolysis with normal laboratory handling for several days.

Phosphate buffer was included in the mobile phase to ensure the elution of the *p*-nitrobenzoic acid as a sharp peak. With a simple mobile phase of acetonitrile–water alone the acid was eluted as a series of broad peaks which interferred with subsequent chromatograms.

The esterification catalyst, 4-dimethylaminopyridine, has been used previously in the derivatisation of sterically hindered alcohols[16] and in this work it was found to give reliable results.

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Note

Separation of three dimers of α-tocopherol by high-performance liquid chromatography

J. CILLARD, J. GOBAILLE and P. CILLARD*
Laboratoire de Botanique et Biologie Cellulaire, 2 avenue Léon-Bernard, U.E.R. "Médicament", 35043 Rennes Cedex (France)
(First received June 25th, 1985, revised manuscript received August 2nd, 1985)

The oxidation of α-tocopherol (vitamin E, α-T) leads to the formation of different compounds, particularly dimeric products, which result from divalent oxidation of α-T.

Dimers can be formed either by alkaline ferricyanide oxidation of α-T[1–5] or during the autoxidation of unsaturated fatty acids with α-T[6–9]. A dimer has also been considered as the major metabolite of Vitamin E[2,10].

Nevertheless, there were conflicting proposals on the final structure of the dimer. It was suggested that the dimer was a mixture[11]. In earlier studies, the purification of the dimer of α-T was achieved by column chromatography on magnesium silicate[1] or neutral alumina[2,4]. In our laboratory, we separated a dimer from α-T and α-tocopherylquinone by reversed-phase high-performance liquid chromatography (HPLC)[12].

The purpose of this paper is to describe a rapid method for purification and separation of a crude alkaline ferricyanide oxidation product of α-T into three dimers, using both analytical and preparative normal-phase high-performance liquid chromatography.

EXPERIMENTAL

Reagents and chemicals

α-T was a gift from Hoffmann-laRoche (France). Potassium ferricyanide, petroleum ether (40–60°C), isopropanol and di-isopropyl ether were supplied by Merck (Darmstadt, F.R.G.), sodium hydroxide by Prolabo and *n*-heptane (chromasol) by S.D.S.

Instrumentation

An LDC high-performance liquid chromatograph was purchased from Sopares-France and equipped with a Valco 7000 p.s.i. injector and a constametric III pump which can be modified for preparative chromatography. The spectromonitor III detector was set at 300 nm.

The UV absorption spectra were recorded in *n*-heptane on a Pye-Unicam SP 8-400 spectrophotometer. Mass spectra were obtained on a Varian MAT 311 instrument.

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Preparation of a crude oxidation product of α-T

The crude alkaline ferricyanide oxidation product of α-T was prepared according to the procedure of Skinner and Alaupovic[4]. A sample of α-T (2.2 g) was dissolved in 100 ml of light petroleum (b.p. 40–60°C) and shaken vigourously in a separating funnel with a solution of 6.4 g of potassium ferricyanide in 64 ml of 0.2 *M* sodium hydroxide for 3 min. The petroleum ether layer was separated, washed with water and dried over anhydrous sodium sulphate. The solvent was removed *in vacuo* leaving *ca.* 2 g of a yellow oily oxidation product of α-T.

Chromatography of the crude oxidation product of α-T

Chromatographies were performed either on a 25 × 0.49 cm I.D. column for analytical chromatography or on a 25 × 2.5 cm I.D. column for preparative chromatography. Both columns were packed with Lichrosorb Si 60 (particle size 5 μm).

Two solvent systems were used: *n*-heptane–isopropanol (99.85:0.15) at a flow-rate of 2 ml/min for analytical chromatography; *n*-heptane–diisopropyl ether (97.5:2.5) at a flow-rate of 22.5 ml/min for preparative chromatography.

The crude oxidation product of α-T was dissolved in *n*-heptane. Samples of 20 μl (*ca.* 10 μg of crude oxidation product) and 2 ml (*ca.* 10 mg) were injected into the chromatograph for analytical and preparative chromatography, respectively.

RESULTS AND DISCUSSION

The crude oxidation product of α-T was separated into four fractions by both analytical and preparative chromatography (Fig. 1). It should be noted that the elut-

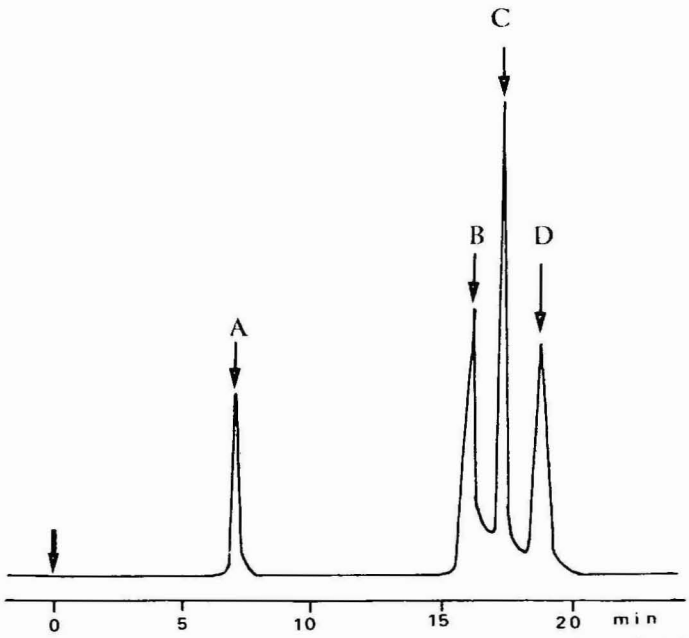

Fig. 1. Separation of the alkaline ferricyanide oxidation product of α-T by preparative HPLC using *n*-heptane–diisopropyl ether (97.5:2.5) as solvent. Peak A: unidentified compound; peaks B, C and D: dimers of α-T.

ing solvent used for analytical chromatography does not allow an efficient separation of fractions B, C and D by preparative chromatography. So, in this latter case, we shall have to use a solvent that is slightly less polar.

Fraction A is a colourless product, which showed a maximum absorption spectra at 294 nm (Table I).

TABLE I

RETENTION TIMES AND UV ABSORPTION OF THE FRACTIONS ISOLATED FROM THE CRUDE ALKALINE FERRICYANIDE OXIDATION PRODUCT OF α-T

Fraction	*Retention time (min)*	*UV absorption maximum (nm)*
A	7	294
B	16	300–337
C	17.5	300–337
D	19	300–337

Fractions B, C and D, which are eluted between 16 and 19 min, have a bright yellow colour and exhibited the same absorption spectra with two maxima at 300 and 337 nm (Table I). An identical UV, absorption was mentioned for the dimer of α-T by various authors[1,2,4].

Mass spectra of fractions B, C and D showed, for each fraction, a peak for a molecular ion at *m/e* 858 which corresponded to the dimerization of α-T and the removed of two hydrogen atoms.

In the literature, there is much controversy about the structure of the dimer of α-T. Nelan and Robeson[1] postulated that the dimer is a spirenone ether with a molecular weight of 856. Csallany[13] carried out a reappraisal of the structure of the dimer and concluded that dimerization entails the formation of a nine-membered chelate ring through strong intramolecular hydrogen-bonding. The resulting compound has a molecular weight of 858.

To compare our results to those previously found by other workers, we have also chromatographed the crude oxidation product of α-T according to the procedure of Skinner and Alaupovic[4] using a neutral alumina column (Brockmann activity I) and a solvent composed of petroleum ether–diethyl ether (9:1) at low pressure.

This procedure leads to the elution of a single yellow fraction which corresponded to a dimer of α-T according to the authors. This fraction, rechromatographed by HPLC according to the method described in the present paper, gives three fractions B, C and D. Fraction A was not detected in this case.

Considering the physical properties (UV spectra and mass spectra) of fractions B, C and D, we can conclude that these fractions correspond to three distinct dimers of α-T which can be separated by normal-phase HPLC. Further investigations will be undertaken to elucidate the difference of structure between these three dimers. We have noted that fraction C is quite stable, while fractions B and D both lead rapidly to a mixture of B + D.

Fraction A, which is eluted before the three dimers, could presumably be the trimer of α-T described by Skinner and Alaupovic[4].

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Note

Analysis of isoflavones in *Puerariae radix* by high-performance liquid chromatography with amperometric detection

YOSHIMI KITADA*, MUNEHIKO MIZOBUCHI and YASUYUKI UEDA
Nara Prefectural Institute of Public Health, 57-6 Ohmori-cho, Nara 630 (Japan)
and
HIROYUKI NAKAZAWA
The Institute of Public Health, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108 (Japan)
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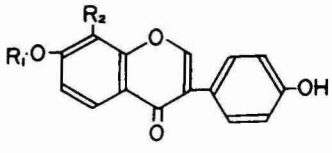
The *Puerariae radix*, which is a major component of Kakkontou, is used as one of the important herbs in traditional chinese medicine. Isoflavones such as puerarin [8-β-D-glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], daidzin [7-(β-D-glucopyranosyloxy)-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], and daidzein [7-hydroxy-3-(4-hydroxyphenol)-4H-1-benzopyran-4-one] have been isolated from the herb[1–3]. Pharmaceutical studies on the biological action of these compounds, *e.g.* antipyretic action[4], papaverin-like action[5,6], cholinergic action[7], the hydrothermic effect, the spasmolitic effect[8], etc. have been reported. Also, in the course of our isolation study on biologically active substances in this herb, we found that daidzin shows anti-inflammatoric action and depresses the bleeding volume[9].

Previously, a high-performance liquid chromatography (HPLC) technique based on a reversed-phase system with UV and/or fluorescence (FL) for detecting isoflavones in *Puerariae radix*[10,11], soybean[12–15] and others[16–18], has been reported. A highly sensitive, analytical method for detecting isoflavones is important for studies on their metabolism. Considering the nature of their phenolic group (see Fig. 1) in electrochemical oxidations, they should be highly sensitive to amperometric detection[19,20]. We found that amperometric detection is very useful for perceiving the three isoflavones in *Puerariae radix*. The proposed method is highly sensitive compared to other methods.



Puerarin: R_1=H, R_2=glucosyl
Daidzin : R_1=glucosyl, R_2=H
Daidzein: R_1=R_2=H

Fig. 1. Structures of isoflavones.

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EXPERIMENTAL

The three isoflavones were isolated from commercial *Puerariae radix*. *Puerariae radix* was extracted with methanol and water (1:1) under reflux, and the extract was partitioned with *n*-butanol and water. The butanol fraction was subjected to silica gel column chromatography (74–149 μm, Wako, Osaka, Japan) with a solvent system of chloroform–methanol–water (X:1:0.1, where X = 9–5) to give the following three compounds[1–3].

(1) Puerarin (recrystallized from acetic acid); m.p. 206–208°C; $C_{21}H_{20}O_9 \cdot H_2O$. IR$\nu_{max}^{KBr}$($cm^{-1}$): 3400, 3250, 1703, 1635, 1618, 1580, 1265. UVν_{max}^{MeOH}mμ(log ε): 304(3.93), 249(4.40). MS(*m/e*): 395, 362, 293, 256. NMR (in d_6-DMSO) δ(ppm): 9.50(1H,br), 8.32(1H,s), 7.93(1H,d,J=8.7 Hz), 7.39(2H,d,J=8.4 Hz), 6.97(1H,d,J=8.7 Hz), 6.79(2H,d,J=8.4 Hz), 4.35–5.03(5H,m), 3.90–4.08(2H,m).

(2) Daidzin (recrystallized from methanol); m.p. 247°C (234–236°C)[21]; $C_{21}H_{20}O_9 \cdot H_2O$. IR$\nu_{max}^{KBr}$($cm^{-1}$): 3420, 3270, 3000, 1635, 1443, 1270, 1095. UVν_{max}^{MeOH}mμ(log ε): 259(4.78), 250(sh)(4.76), 231(sh)(3.67). MS(*m/e*): 416, 254, 225, 137. NMR (in d_6-DMSO) δ(ppm): 9.50(1H,bs), 8.34(1H,d,J=8.8 Hz), 7.40(2H,d,J=8.3 Hz), 8.00–8.32(2H,m), 5.35–5.45(1H,m), 4.83–4.20(5H,m), 4.50–4.63(1H,m).

(3) Daidzein (recrystallized from 50% ethanol solution); m.p. 320–321°C; $C_{15}H_{10}O_4$. IRν_{max}^{KBr}(cm^{-1}): 3420, 3270, 3000, 1635, 1445, 1270, 1095. UVν_{max}^{MeOH}mμ(log ε): 299(sh)(3.95), 248(4.36). MS(*m/e*): 254, 225, 195, 137. NMR (in d_6-DMSO) δ(ppm): 9.50(1H,br), 8.20(1H,s), 7.96(1H,d,J=8.4 Hz), 7.17(2H,d,J=8.2 Hz), 6.60–7.00(4H,m).

The mobile phase for HPLC was prepared by mixing acetonitrile and 0.1 *M* potassium dihydrogen phosphate solution acidified with phosphoric acid to pH 4.0 (15:85).

A Shimadzu HPLC model LC-3A (Shimadzu Seisakusho, Kyoto, Japan) equipped with a column oven (Shimadzu CTO-2A) was used to deliver the mobile phase at a flow-rate of 1.0 ml/min. Two reversed-phase columns, LiChrosorb RP-8 (5 μm, 250 × 4 mm I.D., Merck, Darmstadt, F.R.G.) and IRICA ODS (5 μm, 250 × 4 mm I.D. IRICA-Kogyo, Kyoto, Japan) were used, which were octylsilane and octadecylsilane-bonded columns, respectively. The columns were thermostated with an oven at 50°C.

A IRICA E-502 amperometer operated at a potential setting of +1.00 V *vs.* Ag/AgCl, a Shimadzu SPD-1 spectrophotometer at a wavelength 254 nm, and a Shimadzu RF-510LC spectrofluorometer with excitation at 320 nm and emission at 470 nm, were used in series.

The sample solution was prepared as follows, by Hayakawa's method[11]; *Puerariae radix* (1 g) was extracted with 200, 150 and, finally, 150 ml of methanol under reflux. The combined methanol extracts were concentrated to less than 10 ml by evaporation. The concentrate was treated with a Waters Sep-Pak C_{18} cartridge (Millipore, Bedford, MA, U.S.A.) and filled to 500 ml with methanol. A 1-ml aliquot of this methanol solution was diluted to 50 ml with methanol.

RESULTS AND DISCUSSION

To determine the optimum applied voltage of the amperometric detector, the peak heights of puerarin, daidzin and daidzein were measured at various potentials in the range from +0.60 to +1.10 V *vs.* Ag/AgCl. Fig. 2 shows that the peak heights of these compounds increased with applied potential, and the curves of hydrodynamic voltammograms of puerarin, daidzin and daidzein were sigmoidal. Taking into consideration the intensity of the dark current and the interference of the component from the herb, the applied potential of the amperometric detector was set at +1.00 V *vs.* Ag/AgCl.

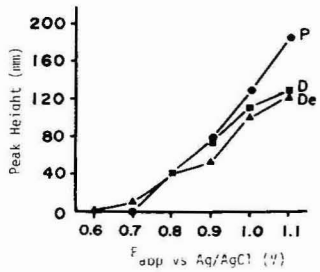


Fig. 2. Hydrodynamic voltammograms of puerarin, daidzin and daidzein: P, puerarin (10 ng); D, daidzin (10 ng); De, daidzein (10 ng). Conditions: column, LiChrosorb RP-8 (5μm, 250 × 4 mm I.D.); mobile phase, 0.1 *M* KH_2PO_4 (pH 4.0)–acetonitrile (85:15); flow-rate, 1.0 ml/min; column temperature, 50°C.

In order to determine the effect of pH on the capacity factors of these compounds, the pH was changed in the range 3–8 by using phosphate buffer. The pH was adjusted with 0.1 *M* KH_2PO_4 and phosphoric acid. The capacity factors of puerarin and daidzein were constant in the pH range 3–6 but decreased at a pH of more than 7, while that of daidzin remained constant in the studied pH range (Fig. 3). On this basis, acidic phosphate buffer set at pH 4.0 was used as the mobile phase. The effect of the concentration of the phosphate buffer on the peak heights and their capacity factors in the mobile phase was examined. As seen in Fig. 4, the maximum

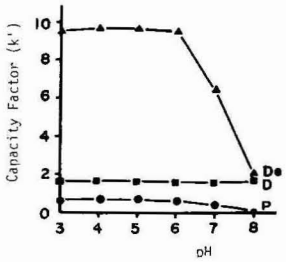


Fig. 3. Effect of pH of phosphate buffer mobile phase on the capacity factor. Conditions: applied voltage, +0.80 V *vs.* Ag/AgCl; others as in Fig. 2.

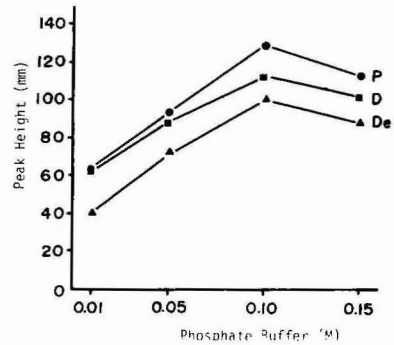
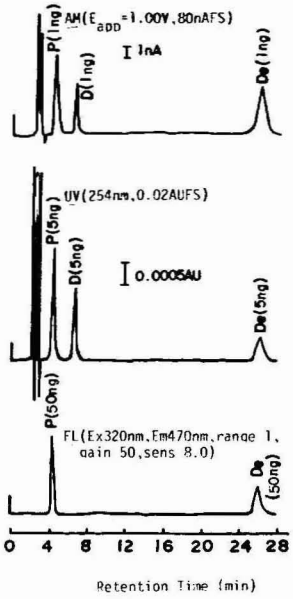


Fig. 4. Effect of concentration of phosphate buffer mobile phase on peak height. Conditions: applied voltage, +1.00 V *vs.* Ag/AgCl; others as in Fig. 2.



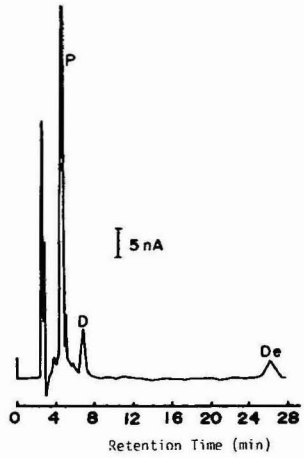


Fig. 5. Comparison of sensitivity with different methods of detection: AM, amperometric; UV, ultraviolet; FL, fluorescence. Conditions: as in Fig. 2.

Fig. 6. Chromatogram of methanol obtained from *Puerariae radix* extract. Conditions: applied voltage, +1.00 V *vs.* Ag/AgCl; range, 160 nA.f.s.

peak heights of these compounds were obtained with 0.1 *M* buffer, and the capacity factors were constant in the examined range.

Under the conditions described above, these compounds were well separated and completely eluted within 28 min in a LiChrosorb RP-8 column and within 35 min in a IRICA ODS column. They were detected at a level of 1.0 ng. As illustrated in Fig. 5, each compound was detected with the highest sensitivity when using amperometric detection as opposed to the other two methods of detection. In a chromatogram of sample solution (Fig. 6), the contents of puerarin, daidzin and daidzein in the extract of *Puerariae radix* were 2.96, 0.32 and 0.13%, respectively (Table I).

In conclusion, the simultaneous analysis of puerarin, daidzin and daidzein in *Puerariae radix* by HPLC was achieved. These compounds were perceived with high sensitivity by using amperometric detection as compared with UV and FL detection. The method described here is applicable to the study of the metabolism of *Puerariae radix*, other *Puerariae* spp. and their components.

TABLE I

DETERMINATION OF PUERARIN, DAIDZIN AND DAIDZEIN IN THE EXTRACT OF *PUERARIAE RADIX*

Isoflavone	*n*	*Mean (%)*	*Standard deviation (%)*	*Coefficient of variation (%)*
Puerarin	3	2.96	0.079	2.67
Daidzin	3	0.32	0.009	2.81
Daidzein	3	0.13	0.005	3.85

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Note

Reversed-phase high-performance liquid chromatography of 5-hydroxyflavones bearing tri- or tetrasubstituted A rings

F. A. T. BARBERAN*, F. TOMAS, L. HERNANDEZ and F. FERRERES
Laboratorio de Sustancias Orgánicas Naturales, C.E.B.A.S., C.S.I.C., Apdo. 195, Murcia 30003 (Spain)
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In the last few years, high-performance liquid chromatography (HPLC) has been applied to flavonoid aglycones both with silica columns[1–4] and with reversed-phase columns[5–10]. However, to our knowledge no work on the HPLC of 5-hydroxyflavones with a tri- or tetrasubstituted A ring has been published.

In the course of our research on the flavonoid aglycones from Labiatae species, we have isolated and identified more than thirty 5-hydroxyflavones, most of them bearing a tri- or tetrasubstituted A ring. These compounds have been used in this work as a model to study the effect of hydroxy or methoxy groups located at different positions on the flavone nucleus on the reversed-phase HPLC behaviour of these interesting compounds.

EXPERIMENTAL

HPLC analyses were carried out with a Perkin-Elmer liquid chromatograph, equiped with a 2/2 pump module, a Model LC85B UV–visible variable-wavelength detector and a Sigma-15 data station.

A Perkin-Elmer C_{18} reversed-phase column with 3-μm particles was used (10 cm × 2.7 mm I.D.). Working solutions contained approximately 1 mg of flavone per 2 ml of methanol. Runs were carried out for 25 min. The elution solvents were water–formic acid (19:1) from pump B (formic acid was added to prevent "tailing") and acetonitrile from pump A. The flow-rate was 2 ml/min with pump A providing 23% and pump B 77% isocratically for 11 min. A gradient increasing at the rate of 2%/min of acetonitrile was then applied for 25 min. Samples of 6 μl were injected, and peaks were detected at 340 nm.

RESULTS AND DISCUSSION

The retention times (t_R) and capacity factors (k') of the different 5-hydroxyflavones analysed are shown in Table I. The elution sequence of the individual compounds can be interpreted by assuming that the hydrophobic interaction increases the retention times and the formation of hydrogen bonds with the mobile phase decreases them.

As a general rule, the lower hydroxy/methoxy group ratio, the higher is the

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TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF FLAVONES

Column, C_{18} (3 μm); solvent system, acetonitrile/water–formic acid (19:1); flow-rate, 2 ml/min.

Common name	*Systematic name*	*OH/OCH₃*	t_R *(min)*	*k'*
6-Hydroxyluteolin	5,6,7,3',4'-Pentahydroxyflavone	5:0	1.01	1.02
Hypolaetin	5,7,8,3',4'-Pentahydroxyflavone	5:0	1.10	1.20
Scutellarein	5,6,7,4'-Tetrahydroxyflavone	4:0	1.73	2.46
Isoleucanthogenin	5,6,3',4'-Tetrahydroxy-7,8-dimethoxyflavone	4:2	2.20	3.40
Luteolin	5,7,3',4'-Tetrahydroxyflavone	4:0	2.44	3.88
Nepetin	5,7,3',4'-Tetrahydroxy-6-methoxyflavone	4:1	2.58	4.16
Leucanthogenin	5,8,3',4'-Tetrahydroxy-6,7-dimethoxyflavone	4:2	2.84	4.70
–	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone	3:2	3.99	6.98
Apigenin	5,7,4'-Trihydroxyflavone	3:0	4.25	7.50
Thymusin	5,6,4'-Trihydroxy-7,8-dimethoxyflavone	3:2	4.44	7.88
Hispidulin	5,7,4'-Trihydroxy-6-methoxyflavone	3:1	4.70	8.40
Chrysoeriol	5,7,4'-Trihydroxy-3'-methoxyflavone	3:1	4.97	8.94
Isothymusin	5,8,4'-Trihydroxy-6,7-dimethoxyflavone	3:2	5.09	9.18
Thymonin	5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone	3:3	5.55	10.10
Cirsiliol	5,3',4'-Trihydroxy-6,7-dimethoxyflavone	3:2	5.71	10.42
Isothymonin	5,8,4'-Trihydroxy-6,7,3'-trimethoxyflavone	3:3	6.05	11.10
Sideritoflavone	5,3'.4'-Trihydroxy-6,7,8-trimethoxyflavone	3:3	7.79	14.58
–	5,6-Dihydroxy-7,3',4'-trimethoxyflavone	2:3	9.10	17.20
Cirsimaritin	5,4'-Dihydroxy-6,7-dimethoxyflavone	2:2	10.87	20.74
Eupatorin	5,3'-Dihydroxy-6,7,4'-trimethoxyflavone	2:3	13.62	26.84
Cirsilineol	5,4'-Dihydroxy-6,7,3'-trimethoxyflavone	2:3	13.86	26.72
–	5,6-Dihydroxy-7,8,3',4'-tetramethoxyflavone	2:4	13.92	26.84
Xanthomicrol	5,4'-Dihydroxy-6,7,8-trimethoxyflavone	2:3	14.64	28.28
Ladanein	5,6-Dihydroxy-7,4'-dimethoxyflavone	2:2	15.23	29.46
Acacetin	5,7-Dihydroxy-4'-methoxyflavone	2:1	16.17	31.34
Gardenin D	5,3'-Dihydroxy-6,7,8,4'-tetramethoxyflavone	2:4	16.23	31.46
Genkwanin	5,4'-Dihydroxy-7-methoxyflavone	2:1	16.85	32.70
8-Methoxycirsilineol	5,4'-Dihydroxy-6,7,8,3'-tetramethoxyflavone	2:4	16.95	32.90
–	5,6-Dihydroxy-7,8,4'-trimethoxyflavone	2:3	16.98	32.96
–	5-Hydroxy-6,7,3',4'-tetramethoxyflavone	1:4	19.07	37.14
5-Demethylnobiletin	5-Hydroxy-6,7,8,3',4'-pentamethoxyflavone	1:5	20.35	39.70
Salvigenin	5-Hydroxy-6,7,4'-trimethoxyflavone	1:3	20.72	40.44
Gardenin B	5-Hydroxy-6,7,8,4'-tetramethoxyflavone	1:4	22.25	43.50

retention time. Hence the highly hydroxylated flavones 6-hydroxyluteolin, luteolin, nepetin, etc., show shorter t_R and the highly methylated 5-desmethylnobiletin, gardenin B, salvigenin, etc., elute with higher t_R values. However, when pairs of compounds are studied, some surprising t_R can be found. These results can be explained on the basis of the capacity for the formation of hydrogen bonds between the flavonoid phenolic hydroxy groups and the mobile phase. Therefore, internal hydrogen bonding usually decreases the capacity for interaction with the solvent and increases retention times.

The strongest hydrogen bond acceptor in a flavone is the carbonyl group at C-4, which, owing to resonance, bears a partial negative charge. If a hydroxy group is present at position 5 (as in all the flavones studied in this work), a strong internal hydrogen bond is formed between this group and the carbonyl group, and therefore

the latter can no longer interact with the solvent[5,7]. In this work, we observed that 6-hydroxy compounds elute with shorter t_R than the 8-hydroxy isomers. This could be explained by internal hydrogen bonding between the hydroxy groups at C-6 and C-5, which decreases the interaction described above between the latter hydroxy and the 4-keto group, and so decreases t_R (Fig. 1). This also could explain the fact that 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone and 5,6-dihydroxy-7,3′,4′-trimethoxyflavone elute with shorter t_R than 8-methoxycirsilineol (5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone) and cirsilineol (5,4′-hidroxy-6,7,3′-trimethoxyflavone), respectively. This is the only case in which an internal hydrogen bond between two hydroxy groups decreases t_R.

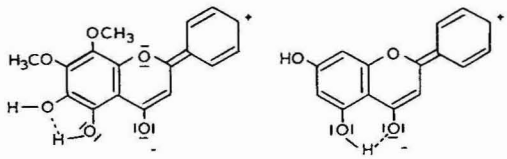

Fig. 1. Structures of 5-hydroxyflavones showing internal hydrogen bonding.

On the other hand, the internal interaction between hydroxy groups at C-3′ and C-4′ increases t_R when compounds bearing such groups are compared with those bearing the same substitution pattern and OH/OCH_3 ratio but with isolated hydroxy groups. Therefore, sideritoflavone and cirsiliol elute with higher t_R than isothymonin and 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, respectively.

In addition, it has been observed that the size of the molecule affects t_R. This is important in the hydrophobic interaction, as the smallest molecules can interact easily with the C_{18} branches of the stationary phase. Thus, flavones bearing a single methoxy group on ring B (5,6-dihydroxy-7,8,4′-trimethoxyflavone, ladanein, gardenin B, salvigenin) interact more strongly with the stationary phase than their counterpart bearing two methoxy groups on ring B, although the latter compounds have a higher number of methoxy groups, which should increase t_R. The 4′-methoxy compounds, with their small size, penetrate more easily into the C_{18} matrix than do the 3′,4′-dimethoxy compounds, and therefore, the former can interact more strongly with the stationary phase (Fig. 2). These results are in accord with those reported previously for permethylated flavones[6]. This size effect is not observed when a hydroxy group is present at C-4′ (xanthomicrol and cirsimaritin elute faster than 8-methoxy cirsilineol and cirsilineol, respectively), possibly owing to the fact that hydrogen bond formation, which is the main effect in these compounds, is less affected by the molecular size than the hydrophobic interaction effect.

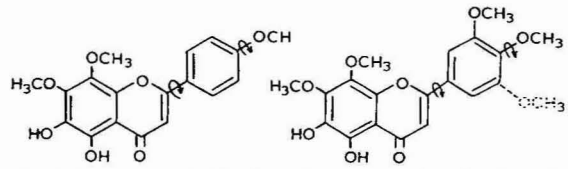

Fig. 2. Structures of 5-hydroxyflavones methoxylated on the B ring.

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Note

Identification of natural red dyes in old Indian textiles

Evaluation of thin-layer chromatographic systems

B. V. KHARBADE and O. P. AGRAWAL*
National Research Laboratory for Conservation of Cultural Property, C-257, Nirala Nagar, Lucknow-226 007 (India)
(Received July 24th, 1985)

The art of textile dyeing in India goes far back into antiquity, with the development of fixing dyes on fabrics with the use of mordants. Many old dyed textiles are now housed in various museums, but are subject to deterioration owing to progressive fading of the dyes and decay of the support.

The two phenomena are linked with the nature of the dyes used and with the reactions between the dyes and the support. In order to resolve these problems, it was considered necessary to acquire a greater understanding to the dyes present on the fabric. First, work on the identification of red and yellow dyes has been undertaken, and this paper deals with red dyes.

A survey of literature on natural dyes and dyeing[1–10] reveals that those listed in Table I were commonly used in India to produce red and related colours. The

TABLE I

NATURAL RED DYES COMMONLY USED FOR DYEING OLD INDIAN TEXTILES

Dyestuff	*Common name*	*Part employed*	*Colouring principle*
Rubia cordifolia Linn.	Munjistha or munjeet	Roots and stem	Munjisthin, purpurin
Morinda citrifolia Linn.	Al or soranji	Root and root bark	Morindone soranjidiol
Morinda spp.	Al or soranji	Root bark and heart wood	
(a) *Morinda tinctoria* Roxb.			
(b) *Morinda umbellata* Linn.	Al or soranji	Root bark and stems	Morindone soranjidiol
Oldenlandia umbellata Linn.	Chay root	Root	Alizarin
Lawsonia alba Lam.	Mehndi henna	Leaves	Lawsone
Ventilago Madraspatana Gaertn.	Ventilago	Root and root bark	Ventilagone
Arnebia nobilis Rach.	Ratanjot	Root and root bark	Alkannin
Carthamus tinctorius Linn.	Safflower	Flowers (red part)	Carthamone
Laccifera lacca Kerr.	Lac insect	Female insect	Laccaic acid
Kermococcus illicis	Kermes	Wingless female insect	Kermesic acid
Dactylopius coccus	Cochineal	Female insect	Carminic acid
Caesalpinia sappan Lin.	Sappan wood	Heart wood	(Brasilin) brasilein
Pterocarpus Santalinus Linn. F.	Red sanders	Wood	Santalin

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colouring principles from most of these dyes were extracted from dyestuffs in weakly alkaline media of sodium hydrogen carbonate or sodium carbonate.

Much research on natural pigments has been carried out and comprehensive texts[11–15] are available. It is clear that most red dyes are derivatives of quinones, and anthraquinones, naphthoquinones and benzoquinones (Figs. 1–3) give strong, red mordant dyes. The red colouring principle of safflower (*Carthamus tinctorius*), previously known as carthamin or carthamic acid[11], was renamed carthamone. Perkin and Hummel[16] were the first to study the chemistry of the red dye principle from the root bark of ventilago (*Ventilago madraspatana*). They named the compound ($C_{15}H_{14}O_6$) ventilagin but could not establish the structure, which is still unknown.

It is also interesting that the wood of sappan (*Caesalpinia sappan*) was used to obtain red dye. The colouring principle is brazilin, a neoflavinoid. Brazilin is unstable and is easily oxidised by atmospheric oxygen to brazilein, a quinone methide[17], the structure of which is similar to that of quinone (Fig. 4).

Thin-layer chromatography (TLC) has been applied to the identification of dyes in European textiles of artistic and historical value[18–21]. The TLC systems reported have limited scope for identification of natural red dyes of Indian origin, based on a variety of plants and animals (*e.g.* insects such as *Kermococcus illicis* and *Lacciferra lacca*). Therefore, in the first instance it was felt necessary to establish the TLC system that would be most suitable for their identification.

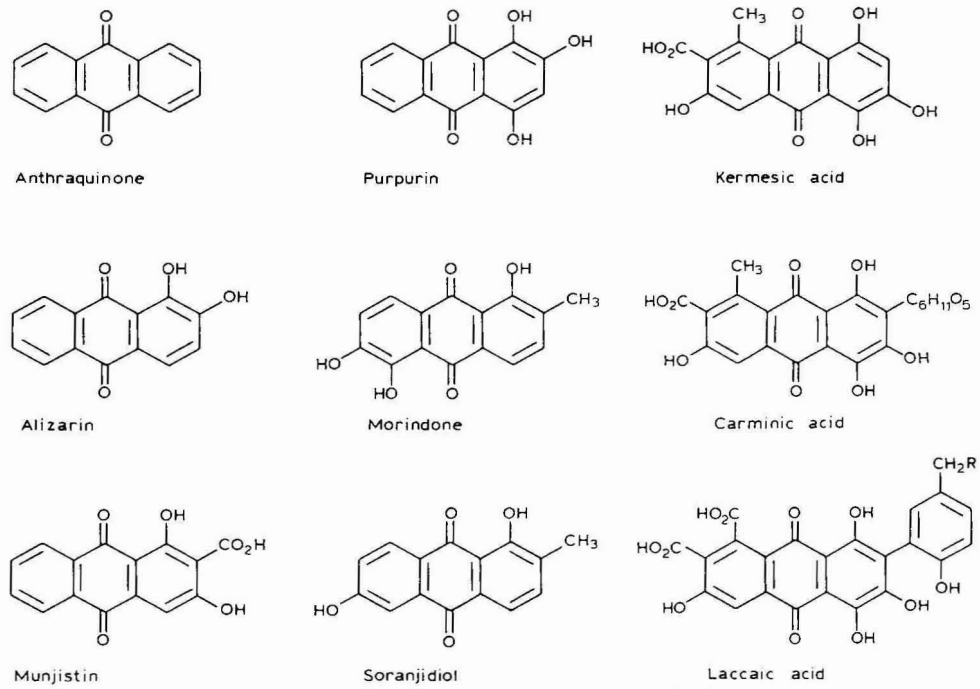

Fig. 1. Dye structures: the basic structure of anthraquinone is found in the following natural red dyes: alizarin from chayroot (*Oldenlandia umbellata*); purpurin and munjistin from munjeet (*Rubia cardifolia*); morindone and soranjidiol from Al or soranji (*Morinda citrifolia* or other species such as *tinctoria* and *umbellata*); kermesic acid from kermes (female insect of *Kermococcus illicis*); carminic acid from cochineal (*Dactylopius coccus* insect); laccaic acid from lac (*Lacciferra lacca* female insect).

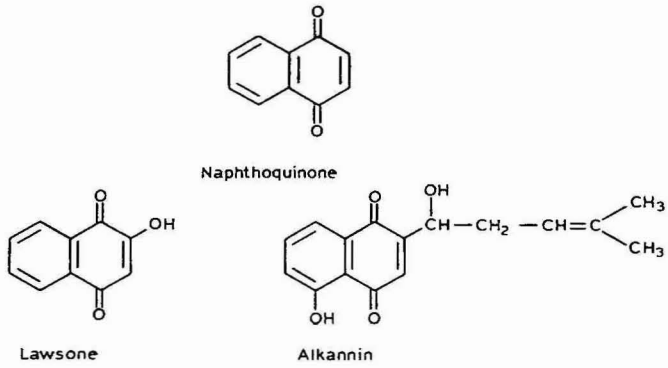

Fig. 2. Dye structures: the basic structure of naphthoquinone is found in lawsone, the red dye from henna leaves (*Lawsonia alba*), and in Alkanin from root and root bark of rantanjot (*Arnebia nobilis*).

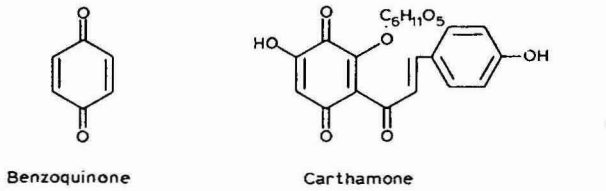

Fig. 3. Dye structures: the basic structure of benzoquinone is found in carthamone, the red dye from the floret of safflower (*Carthamus tinctorius*).

Several TLC systems for the separation of naturally occurring quinones have been reported[22–27]. Many solvent systems were screened initially and those in which dye samples migrated were used for this study.

The purpose of this study was to find a suitable TLC system for the identification of natural red dyes in old Indian textiles.

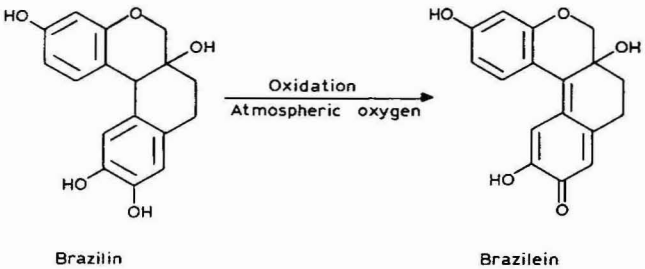

Fig. 4. Brazilin, the colouring principle of sappan wood (*Caesalpinia sappan*). It is oxidized in atmospheric oxygen to brazilein, responsible for the red colour in textiles.

EXPERIMENTAL

Preparation of samples

Five red dyes, namely (i) munjeet (*Rubia cordifolia*), (ii) lac (*coccus lacca*), (iii) *ventilago madraspatana* (all supplied by the Regional Technical Development and

Design Centre, Bangalore), (iv) sappanwood (*Ceasalpinia sappan*) and (v) henna (*Lawsonia alba*) and (both purchased in the local market in Lucknow) were studied.

Amounts of 5 g of powdered dyes were mixed in a beaker with 0.5 g of sodium hydrogen carbonate and extracted with a sufficient volume of water on a water-bath at about 80°C for 1–2 h. The extract was filtered into a separate beaker. The procedure was applied to all the dyes except the sappan wood, the extraction of which was carried out only with water. Wool fibres were mordanted in alum solution and dyed with the extracts of the dyes.

A fibre approximately 1–2 cm long and 0.2 cm in diameter, removed from a textile sample, was placed in a small test-tube, 2–3 ml of 10% hydrochloric acid were added and the tube was boiled on water-bath for about 30 min. The tubes were then placed in a vacuum dessicator to remove any traces of hydrochloric acid. After complete removal of hydrochloric acid, the dye from the fibre was extracted with 1–2 ml of analytical-reagent grade methanol.

Standards

Solutions were prepared by dissolving 1-mg amounts of standards in 1 ml of analytical-reagent grade methanol.

TLC solvent system

A silica gel layer was preferred in this study and solvent systems were chosen according to the literature[22–27] and screened initially to ensure the suitability of the systems for the separation of the dye samples on a glass microscope slide coated with silica gel. Fourteen solvent systems were selected for detailed study (Table II).

TABLE II

TLC SOLVENT SYSTEMS USED IN THE EVALUATION OF SYSTEMS FOR THE IDENTIFICATION OF NATURAL RED DYES

No.	*Components*	*Composition*
I	Ethyl acetate–methanol–water	100:16.5:13.5
II	Isopropanol–ethyl acetate–water	40:40:30
III	Benzene–carbon tetrachloride–acetic acid	50:75:0.8
IV	Benzene–acetic acid	66:33
V	Chloroform–95% ethanol–water	60:30:2
VI	Benzene–methanol	90:10
VII	Chloroform–methanol	90:10
VIII*	*n*-Propanol–ethyl acetate–water	40:40:30
IX	Light petroleum (b.p. 60–80°C)–ethyl acetate	70:30
X	*n*-Butanol–*n*-propanol*–2 *N* Ammonia solution	10:60:30
XI	Benzene–ethyl formate–formic acid	74:24:1
XII	Chloroform (saturated with 25% ammonia)–methanol	70:30
XIII	Toluene (saturated with 25% ammonia)–methanol	40:10
XIV	Toluene–ethyl acetate–methanol	85:10:5

* *n*-Propanol of laboratory-reagent grade was used because of the non-availability of analytical-reagent grade material.

TLC procedure

A 24-g amount of silica gel G was placed in a conical flask with 48 ml of distilled water. The mixture was shaken throughly for 2–3 min to prepare a slurry, which was applied on glass plates with the help of an applicator. The thickness of the layer was 0.25 mm. The plates were allowed to dry at room temperature for about 2–3 h, then activated in an oven at 110°C for about 30 min. The plates were stored in a wooden cabinet over blue silica gel as drying agent.

The solutions containing the extracted dye and standards were spotted with a 5-μl microcapillary on to silica gel. A total volume of approximately 5–10 μl was spotted. Drying was effected with the help of a hand-held dryer. After spotting, the plates were kept in an oven for 10 min and developed until the solvent front reached the 10-cm mark. The plates were sprayed with 10% potassium hydroxide solution and viewed under ultraviolet light at 254 nm. The thin-layer chromatograms were recorded by the graphical copying method.

RESULTS AND DISCUSSION

The resolving power of the individual solvent systems was evaluated by their ability to resolve the samples and standards. The results were interpreted visually and results were expressed as good separation, very good separation, trailing at the start, moved with the solvent front or no separation. A comparison of the resolving powers of the fourteen selected solvent systems is given in Table III. It can be seen that system XI (benzene–ethyl format–formic acid, 74:24:1) has the best resolving power and gives a clear separation of all five natural red dyes.

A thin-layer chromatogram is shown in Fig. 5. In sample 1, there were three major bands; the middle band can be compared with standard 7 (purpurin), but the other two bands could not be identified. Likewise, sample 4 of sappanwood can be

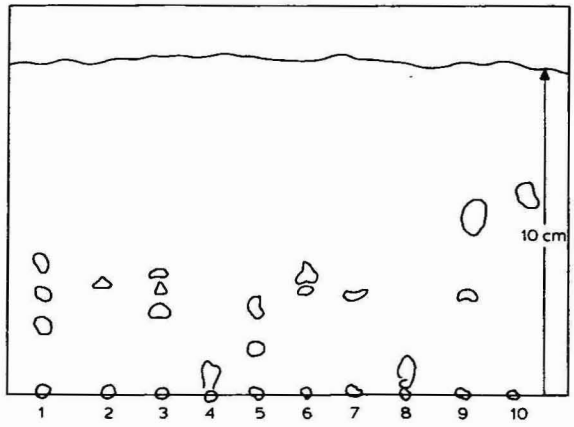

Fig. 5. Thin-layer chromatogram of natural red dyes. Adsorbent, silica gel G; solvent system, XI (Table II), benzene–ethyl formate–formic acid (70:24:1); run, 10 cm; detection, spraying with 10% methanolic potassium hydroxide and observation under UV light. 1 = Munjeet or munkjistha (*Rubia cordifolia*); 2 = lac (*Laccifera lacca* female insect); 3 = ventilago (*Ventilago madraspatana*); 4 = sappan wood (*Caesalpinia sappan*); 5 = henna, mehndi (*Lawsonia alba*); 6 = alizarin; 7 = purpurin; 8 = brazilin; 9 = lawsone; 10 = emodin.

TABLE III

COMPARISON OF THIN-LAYER CHROMATOGRAMS DEVELOPED WITH THE 14 SOLVENT SYSTEMS USED IN THE EVALUATION STUDIES FOR THE IDENTIFICATION OF FIVE NATURAL RED DYES

+ + + = very good separation; + + = good separation; + = trailing at the start; + – = moved with the solvent front; – = no separation.

Dye	*Solvent system**													
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*	*XIII*	*XIV*
Munjeet	–	+ –	+	–	+	–	–	+ –	–	+ –	+ + +	+ –	+ –	+ +
Lac	–	+ –	–	–	+	–	–	+	–	–	+ + +	+ –	+ –	+ +
Ventilago	–	+ –	+	–	–	–	+	–	+	–	+ + +	–	–	–
Sappan wood	–	+ –	–	+	–	–	–	–	–	–	+ +	–	–	–
Mehndi	–	–	–	–	–	–	+	–	+	–	+ + +	–	–	–
Standards:														
Alizarin	–	+ –	–	+	+	–	–	+ –	–	+ –	+ + +	+ –	+ –	+ +
Purpurin	–	+ –	–	–	+	–	–	+ –	–	+ –	+ + +	+ –	+ –	+ +
Brazilin	+ +	–	–	+	–	–	–	–	–	–	+ +	–	–	–
Lawsone	+ +	–	–	+ +	–	+	+	–	+	–	+ + +	–	–	–
Emodin	+ +	–	–	+ +	–	+ +	+ +	–	–	–	+ + +	–	–	–

* For the compositions of solvent systems I–XIV, see Table II. The adsorbent for all the solvent system used was silica gel G

compared with standard 8 (brazilin). Sample 5 had two bands, one of which could be identified with the lower band of standard 9 (lawsone).

CONCLUSIONS

A silica gel layer and the solvent system benzene–ethyl formate–formic acid (74:24:1) can resolve minor and major components in textile dyes, with the formation of definite patterns on the TLC plate. It is necessary to spot the extracted dyes from the questioned and standard fibre samples on the same plate so that a side-by-side comparision of the separated dye components can be made.

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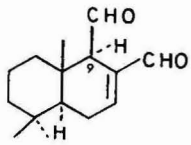
Note

Analytical separation of enantiomeric polygodials by gas chromatography of pyrrole derivatives

CHARLES J. W. BROOKS*, DAVID G. WATSON and W. JOHN COLE
Chemistry Department, University of Glasgow, Glasgow G12 8QQ, Scotland (U.K.)
(Received September 13th, 1985)

The natural drimenedial, (−)-polygodial[1,2] (1), is of strong interest by virtue of its biological activities[3,4]. The racemic dialdehyde is accessible by total synthesis[5]



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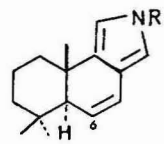

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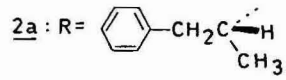

and has been resolved[6], but no productive partial synthesis of compound 1 has been reported. The phytotoxic (+)-enantiomer has to be excluded from samples of compound 1 used in biological tests[6]. We report a convenient method of analysis of the enantiomers via reactions that yield diastereomeric pyrroles. Studies of such reactions of compound 1 in aqueous media have been described earlier[3,7]. We find that polygodial (100 μg) in ethyl acetate (50 μl) at 20°C reacts very rapidly with primary amines (5 molar proportions). Gas–liquid chromatography (GLC) of an aliquot of the solution leads to conversion of the initial products[3,7] into less polar 6-enes (2) which afford good GLC peaks. The reaction products from polygodial and (−)-amphetamine showed on thin-layer chromatography (cyclohexane–ethyl acetate 70/30) spots of R_F *ca.* 0.15 and 0.42. (Vacuum sublimation yielded a new major spot, R_F 0.67, due to compound 2a.) The more polar components, on GLC , gave the same peak as compound 2a: for analytical studies, aliquots of the reaction mixtures were directly suitable. The gas chromatograms in Fig. 1 show (a) separation of derivatives formed from (±)-polygodial and (−)-*R*-amphetamine; (b) characterisation of the product (compound 2a) of treatment of a leaf extract of *Polygonum hydropiper* L. with (−)-amphetamine; and (c) complete separation of diastereomeric pyrroles

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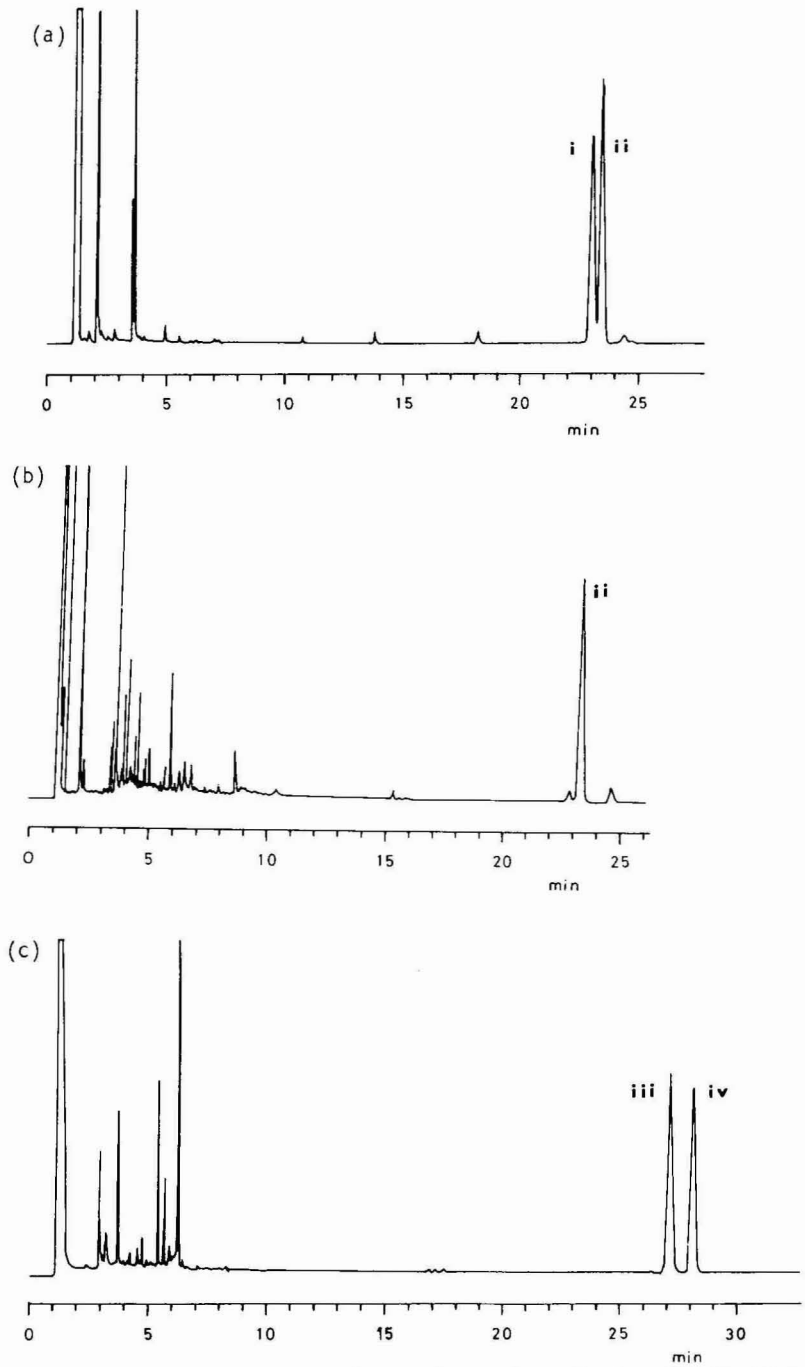

Fig. 1. Gas chromatographic traces for reaction products of polygodial with chiral amines. (a) Peak i (retention index, I, = 2475) from (+)-polygodial/(−)-amphetamine, peak ii (I = 2480) from (−)-polygodial/(−)-amphetamine; (b) reaction products from treatment of extract of mature *Polygonum hydropiper* leaf with (−)-amphetamine; (c): products of reaction of (±)-polygodial with (±)-*p*-chloroamphetamine; peak iii, I = 2664; peak iv, I = 2676 (sequence of diastereomers not yet known). Column, 25 m × 0.32 mm I.D. CP Sil 5CB (bonded phase) fused silica (Chrompack, Middelburg, The Netherlands); column temperature, 190°C (a and b), 200°C (c); helium flow-rate, 3 ml/min (flame ionisation detector).

formed from polygodial and *p*-chloroamphetamine. Mass spectra (electron impact) of diastereomers were almost identical. The reactions described show promise for precise quantitative enantiomer analysis of polygodial and related dialdehydes.

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Book Review

Organic trace analysis, by K. Beyermann, translated from the German: Organische Spurenanalyse, R. A. Chalmers, translation editor, Ellis Horwood, Chichester, 1984, 365 pp., price £ 35.00, ISBN 0-85312-638-0 (Ellis Horwood), ISBN 0-470-20077-4 (Halsted Press).

Modern organic trace analysis is an extremely varied and quickly developing subject with many bonds with other branches of science, such as biology, medicine, ecology and economics, and compiling the problems of such a dynamic discipline into a monograph is a difficult task. It seems that the author of this book has succeeded, thanks to his strategy of giving a broad survey of the techniques of organic trace analysis without going into too many details.

In Chapter 2 (Chapter 1 constitutes the Introduction) the general aspects of organic trace analysis are dealt with, such as the basic definitions, the importance, difficulties and aims of organic trace analysis, and methods of calibration and checking the results, followed by treatments of collaborative inter-laboratory studies and good laboratory practice. This chapter contains a fine treatment of the use of statistics in trace analysis, contributed by S. Gorbach. Chapters 3 and 4 cover sampling and the treatment of samples before analysis, respectively. Chapter 5 is devoted to separation methods and sample-enrichment steps, with treatments of the evaporation of solvents and distillation techniques, liquid extraction, chromatography, zone refining, precipitation and coprecipitation, separation with the aid of membranes, and dialysis. Chapter 6 deals with the methods of detection and determination of compounds, containing subchapters on UV and visible-light, infrared and Raman spectroscopy, techniques based on fluorescence and phosphorescence, optoacoustic spectroscopy, mass spectrometry, chromatographic detectors, chemical detection, enzymatic and immunological reactions, protein binding and biological methods. In the last chapter, special topics are discussed, such as the determination of groups of substances, determination of trace compounds without separation, methods of lowering the detection limit and increasing the specificity of detection, screening methods, local and surface analysis, and the use of trace analysis for the recognition of diseases. There is a comprehensive index of compounds.

It is difficult to find something that might be criticized in the book. The only comment that could be made relates to the scheme demonstrating the ranges of analyte concentrations covered with different detection methods on p. 222. With GC detectors, it should have been specified whether the ranges refer to detectors alone or to GC column–detector combinations, and whether the analyte concentrations refer to gaseous or liquid samples. However, this is a detail that does not detract from the merit of the book.

This book provides a useful introduction to trace organic analysis, ideal for those who want to scan quickly the state-of-the-art of the field. Those who seek a method to solve their particular analytical problem will appreciate the numerous

substance-oriented tables backed up by the index of compounds and a wealth of up-to-date references to original papers. The book makes pleasant reading, for which congratulations are due to the translation editor. All chemists involved both directly and indirectly with the problems of trace analysis and also chemistry students will find the book a valuable source of information.

Brno (Czechoslovakia) JOSEF NOVÁK

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Book Review

Topics in forensic and analytical toxicology. Proceedings of the Annual European Meeting of the International Association of Forensic Toxicologists, Munich, August 21–25, 1983 (Analytical Chemistry Symposia Series, Vol. 20), edited by R. A. A. Maes, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 214 pp., price Dfl. 150.00, *ca.* US$ 57.75, ISBN 0-444-42313-3.

This book is a collection of papers presented at the 20th Annual European Meeting of the International Association of Forensic Toxicologists, held in Munich in 1983. The introductory lecture, by Allan S. Curry, describes how the considerable advances in and refinements of analytical techniques have contributed to the rapid progress in forensic toxicology over the past 30 years. Twenty-nine contributions of very varied quality follow, ranging from the very poor to the good and informative. Most of them deal with analytical methodology, screening procedures for drugs and poisons and case data.

Cases are diverse and feature the toxic oils and anilides found during the toxic oil syndrome scare in Spain in 1981, a review of fatal cases involving propoxyphene and paracetamol, the study of a death by tetrahydrofuran poisoning, the determination of arsenic poisoning by the analysis of hair samples and the interpretation of *post mortem* tissue concentrations of amiodarone and metabolites.

Analytical methodologies emphasize the preparation of samples (liquid–liquid extraction, column extraction, subtilisin digestion and preservation of standards by lyophilization) and the application and extensions of some modern techniques [radioimmunoassay, enzyme multiplied immunological technique (EMIT), thin-layer chromatography, high-performance liquid chromatography, gas chromatography–mass spectrometry, dual mass spectrometry, atomic absorption spectrometry, headspace analysis and the use of computers in toxico-kinetics].

Screening procedures are evaluated for their reliability in drug dependence programmes or for therapeutic drug monitoring in clinical practice. General discus-

sions of the problems of drugs and driving, data collection and toxicology in developing countries complete the plenary lectures.

Finally, the round table discussions of "quality control in analytical toxicology", "documentation" and "education in forensic toxicology" contributed more substance to an otherwise bland book with too many generalities and an abundance of obvious and well known statements which, in any case, had been well described in the introductory lecture.

The volume is well presented and contains an author and a subject index. However, it is certainly below the standard of other books in this series, and editing seems to have been limited to collecting and collating the articles without attempting to select worthy papers and to correct the English, even where the meaning is ambiguous, totally obscure or unintentionally funny!

At its stated price this book will have limited appeal, but analytical toxicologists and clinical chemists may find it useful.

Canberra (Australia) P. MARGOT

Author Index

Errata

J. Chromatogr., 325 (1985) 111–126
Page 112, 10th line, "Gly-D/LLys-LLYyy" should read "Gly-D/LLys-LYyy".

J. Chromatogr., 330 (1985) 193–201
Page 193, the footnote with the present address refers to Dr. Nobuto Bhwada, correspondence should be addressed to Dr. Kunishige Naito.
Page 194, last line of the legend of Fig. 1 should read "modified alumina; ○, 50°C, Chromosorb P; ◐, 70°C, modified alumina; ◑, 70°C, Chromosorb P".
Page 201, first line should read "We continue to take an interest in these aspects and are studying GLC systems".

J. Chromatogr., 330 (1985) 356–359
Page 357, eqns. 4 and 5 should read

$$C' = \frac{\exp(-t_{ad})\ \{[\varphi(t_{ad}+t',rs)+I_0[2\sqrt{rs(t_{ad}+t')}]\}\ -\ [\varphi(t',rs)+I_0(2\sqrt{rst'})]}{\exp(-t_{ad})\ \{\varphi(t_{ad}+t',rs)+I_0[2\sqrt{rs(t_{ad}+t')}]+\varphi[s,r(t_{ad}+t')]\}+[\varphi(rt',s)-\varphi(t',rs)]} \quad (4)$$

$$Q' = \frac{\exp(-t_{ad})\ \varphi(t_{ad}+t',rs)\ -\ \varphi(t',rs)}{\exp(-t_{ad})\ \{\varphi(t_{ad}+t',rs)+I_0[2\sqrt{rs(t_{ad}+t')}]+\varphi[s,r(t_{ad}+t')]\}+[\varphi(rt',s)\ -\ \varphi(t',rs)]} \quad (5)$$

corrected
ch.S
27 Feb 86

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journal of chromatography news section

NEW BOOKS

Trace residue analysis (*ACS Symposium Series 284*), edited by D.A. Kurtz, American Chemical Society, Washington, DC, 1985, X + 284 pp., price US$ 59.95 (U.S.A. and Canada), US$71.95 (rest of world), ISBN 0-8412-0925-1.

QSAR in toxicology and xenobiochemistry (*Proceedings of a Symposium, Prague, Czechoslovakia, September 12–14, 1984; Pharmacochemistry Library Vol. 8*), edited by M. Tichý, Elsevier, Amsterdam, New York, 1985, X + 474 pp., price Dfl. 295.00, US$ 109.25, ISBN 0-444-42483-0.

ANNOUNCEMENTS OF MEETINGS

I CONGRESSO LATINO-AMERICANO DE CROMATOGRAFIA, RIO DE JANEIRO, BRASIL, MARCH 17–19, 1986

The 1st Latin-American Congress on Chromatography (COLACRO) will be held in Rio de Janeiro, Brasil, March 17–19, 1986.

COLACRO will cover both pure and applied aspects of chromatographic science (GC, HPLC, SFC, TLC, GC–MS, etc.). The programme includes plenary lectures by invited scientists, poster sessions of submitted papers, workshop seminars and short courses. In conjunction with the congress there will be an exhibition of instruments and accessories by local and international companies. The congress languages will be English and Spanish. No simultaneous translation system will be provided.

If you are interested in the event and wish to receive further information, please contact the organizing committee at the following address: Dr. Fernando M. Lanças, University of São Paulo, Institute Fís. Quím. São Carlos, 13.560 - São Carlos - SP, Brasil. Tel.: (0162) 72-5935, telex: (0162) 165122 - FQSC - BR.

WORKSHOP ON SIZE EXCLUSION CHROMATOGRAPHY, NEW YORK, NY, U.S.A., APRIL 12–13, 1986

The purpose of this workshop, sponsored by the ACS Division of Polymeric Materials: Science and Engineering, is to provide the attendee with a knowledge base in size exclusion chromatography (SEC). The topics to be covered are listed below and will cover basic theory, instrumentation data analysis and interpretation and application examples.

The topics to be covered include: basic molecular weight statistics, mechanism of the SEC separation, calibration methods (primary, broad standard, universal calibration etc.), data treatment and instrumental broadening corrections, multicomponent polymer analysis – use of multiple detectors, aqueous SEC, oligomer and small molecule analysis – operational variable considerations, analysis of branching and cross-linked networks.

Lecturers will be well known experts in the field of size exclusion chromatography. Instrument demonstrations are planned.

There is limited space for registrants, and early registration is encouraged. The final registration deadline is February 4, 1986. For more information or a workshop registration form, contact: Dr. Theodore Provder, Glidden Coatings & Resins, Division of SCM Corporation, 16651 Sprague Road, Strongsville, OH 44136, U.S.A. Tel.: (216) 826-5289.

ADVANCES IN SIZE EXCLUSION CHROMATOGRAPHY, NEW YORK, NY, U.S.A., APRIL 13–18, 1986

The forthcoming symposium on "Advances in Size Exclusion Chromatography" will be held at the April 1986 National ACS Meeting in New York City. Over the last several years improvements have been made in column technology, detectors, solvent delivery and data analysis systems, etc. This symposium is intended to call forth the latest developments in the field of size exclusion chromatography (SEC) and related methods, both in experimental and data analysis methodology as well as specific applications.

Proposed subjects include: Theoretical considerations of the mechanism of SEC, data treatment and instrumental broadening effects, polymer chain branching (theory, viscometry and LALLS detectors, etc.), multicomponent polymer analysis (use of multiple detectors), aqueous SEC, oligomer and small molecule analysis, significant advances in column technology, applications of SEC to R & D and problem solving, related techniques (HDC, SFFF, TFFF, FFFF, SFC, etc.), use of SEC for kinetic and reaction engineering studies.

The key deadline dates are: receipt of preliminary title, as soon as possible; receipt of final paper title and ACS abstract, October 1, 1985; receipt of preprint paper, November 1, 1985; receipt of paper for symposium monograph, April 1, 1986.

Please send your title as soon as possible to insure a place on the programme. Upon acceptance of your paper, the appropriate ACS forms will be sent to you. For further information please contact: Theodore Provder, Ph.D., Principal Scientist, Polymer Research & Computer Science, Glidden Coatings and Resins, Division of SCM Corporation, 16651 Sprague Road, Strongsville, OH 44136, U.S.A. Tel.: (216) 826-5289, or Dr. C. Kuo, tel.: (216) 826-5346.

1986 HEWLETT-PACKARD ANALYTICAL SYMPOSIUM, HARROGATE, U.K., JUNE 23–27, 1986

Following the considerable success of the 1985 Analytical Symposium held in Stratford-upon-Avon last June, Hewlett-Packard Limited are pleased to announce that their 1986 Analytical Symposium has been booked for June 23–27, 1986 at the Majestic Hotel, Harrogate, U.K.

The 1986 Symposium will repeat this year's successful formula, with distinguished speakers from Europe and the U.S.A. presenting original work on the techniques and applications of UV–visible spectroscopy, gas and liquid chromatography, chromatography–mass spectrometry and laboratory automation and information management. Delegates will be able to register for individual sessions as appropriate.

Further details may be obtained from Tina Mears, Hewlett-Packard Limited, Analytical Instrumentation Group, Miller House, The Ring, Bracknell, Berkshire, RG12 1XN U.K. Tel.: Bracknell (0344) 424898, telex 848733.

A SYMPOSIUM ON NEW ADVANCES IN LIQUID CHROMATOGRAPHY, SZEGED, HUNGARY, SEPTEMBER 4–8, 1986

General papers and post sessions are planned to be presented at the above-mentioned symposium in all areas of chromatography, including HPLC, classical column liquid chromatography, TLC, HPTLC, GC, GC–MS and electrophoretic techniques. Special sessions will be devoted to preparative and analytical separations of amines, amino acids, peptides, proteins, nucleic acids as well as chromatography in biotechnology. Authors wishing to present research papers should send a one-page 250-words abstract

along with the author's indication of preference for lecture or poster presentation not later than April 15, 1986.

To obtain the forms and additional information contact: Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, Budapest 1445, Hungary.

THE SILVER JUBILEE EASTERN ANALYTICAL SYMPOSIUM, NEW YORK, NY, U.S.A., OCTOBER 6–10, 1986

A limited number of oral and poster presentations on new developments in analytical chemistry will be accepted for the Silver Jubilee Eastern Analytical Symposium. These contributed presentations will be grouped into several sessions to complement the invited technical sessions at this, the celebration of the twenty-fifth annual EAS. Prospective authors should submit a 50–100 word abstract on the proposed presentation before the deadline (*February 15, 1986*), indicating preference of oral or poster format, to Concetta M. Paralusz, EAS Program Chairman, Permacel/Avery International, P.O. Box 671, New Brunswick, NJ 08903, U.S.A., tel.: (201) 524-5633. Care should be exercised in considering the title and authors of the proposed presentation; if the presentation is accepted, both title and authors will be considered final and not subject to change. Authors of accepted presentations will receive forms for submission of a 200–300-word abstract which will appear in the final programme. The Silver Jubilee EAS will be moving to a new home at the New York Hilton Hotel.

For further information about the symposium, contact: Dr. S. David Klein, EAS Publicity, 642 Cranbury Cross Road, North Brunswick, NJ 08902, U.S.A. Tel.: (201)846-1582.

22nd INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY, MODERN TECHNIQUES AND BIOMEDICAL APPLICATIONS, TOKYO, JAPAN, OCTOBER 7–9, 1986

The 22nd International Symposium on Advances in Chromatography will be held in Tokyo, Japan on October 7–9, 1986. The scope of the meeting will cover papers, poster sessions and informal discussion groups by outstanding researchers from throughout the world in all fields of chromatography. Particular emphasis will be given to biomedical applications. New developments in gas, liquid, supercritical and thin-layer chromatography will be included. A commercial exhibition of the latest instrumentation and books is also scheduled. Participation in the symposium will be on the basis of invited papers as well as unsolicited contributions. Authors desiring to present papers or posters must submit 200-word abstracts by April 15, 1986. Complete manuscripts of accepted authors will be due on October 7, 1986 at the meeting in Tokyo.

All correspondence pertaining to the symposium and exhibition space should be directed to: Prof. N. Ikekawa, Department of Chemistry, Tokyo Institute of Technology, O-Okayama, Meguro-Ku, Tokyo, Japan.

CALENDAR OF FORTHCOMING MEETINGS

Jan. 15–17, 1986 Paris, France	**1st International Symposium on Preparative and Up-Scale Liquid Chromatography** Contact: Société Francaise de Chimie (SFC), 250, rue Saint Jacques, 75005 Paris, France. (Further details published in Vol. 330, No. 2.)
Jan. 20–24, 1986 Bilthoven, The Netherlands	**EUCHEM Conference: Sampling Strategies and Techniques in Environmental Analysis** Contact: RIVM, Secretariat Sampling Conference, Dr. C.E. Goewie, Laboratory for Organic Chemistry, P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Tel.: (030) 742851, telex: 47215 RIVBH.

March 10–14, 1986 Atlantic City, NJ, U.S.A.	**37th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Alma Johnson, Program Secretary, 12 Federal Drive, Suite 322, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 330, No. 2.)
March 17–19, 1986 Lausanne, Switzerland	**16th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Dr. Ernest Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland.
March 17–19, 1986 Rio de Janeiro, Brasil	**I Congresso Latino-Americano de Cromatografia** Contact: Dr. Fernando M. Lanças, University of São Paulo, Institute Fís. Quím. São Carlos, 13560 São Carlos SP, Brasil. Tel.: (0162) 72-5935, telex: (0162) 165122-FQSC-BR. (Further details published in Vol. 331, No. 2.)
March 25–June 6, 1986 Uppsala, Sweden	**Biochemical Separation Methods, Uppsala Separation School** Contact: Ingela Strömberg, Institute of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden. (Further details published in Vol. 331, No. 2.)
April 13–18, 1986 New York, NY, U.S.A.	**Advances in Size Exclusion Chromatography** Contact: Theodore Provder, Ph. D., Principal Scientist, Polymer Research & Computer Science, Glidden Coatings and Resins, Division of SCM Corporation, 16651 Sprague Road, Strongsville, OH 44136, U.S.A. Tel.: (216) 826-5289, or Dr. C. Kuo, tel.: (216) 826-5346.
April 22–24, 1986 Noordwijkerhout The Netherlands	**Anatech '86 – An International Symposium on Applications of Analytical Chemical Techniques to Industrial Process Control** Contact: Prof. W.E. van der Linden, Laboratory for Chemical Analysis, Department of Chemical Technology, Twente University of Technology, P.O. Box 217, 7500 AE Enschede, The Netherlands. (Further details published in Vol. 322, No. 3.)
April 23–25, 1986 Vienna, Austria	**Liquid Chromatography in Gene Technology** Contact: Mrs. Romana Zeilinger, Hewlett-Packard GmbH, Lieblgasse 1, Postfach 72, A-1222 Vienna, Austria. (Further details published in Vol. 330, No. 2.)
May 11–14, 1986 Nagara, Japan	**7th International Symposium on Capillary Chromatography** Contact: Dr. D. Ishii, Department of Applied Chemistry. Nagoya University, Nagoya 464, Japan. (Further details published in Vol. 331, No. 2.)
May 12–14, 1986 Washington, DC, U.S.A.	**2nd Symposium on Preparative Liquid Chromatography** Contact: Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 330, No. 2.)
May 18–23, 1986 San Francisco, CA, U.S.A.	**HPLC '86. New Frontiers in HPLC. 10th International Symposium on Column Liquid Chromatography** Contact: Ms. Shirley Schlessinger, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A. (Further details published in Vol. 331, No. 2.)
May 26–29, 1986 Lerici, Italy	**III CAC – Meeting of the Chemometrics Society** Contact: Prof. M. Forina, Istituto di Analisi e Tecnologie Farmaceutiche ed Alimentari, Via Brigata Salerno (ponte), I-16147 Genova, Italy. Tel.: (010) 3993656. (Further details published in Vol. 330, No. 2.)

May 30–31, 1986 East Lansing, MI, U.S.A.	**1st International Symposium for Metabolic Profiling of Organic Acids and Steroids** Contact: Professor Charles C. Sweeley, Department of Biochemistry, Michigan State University, Biochemistry Building, East Lansing, MI 48824-1319, U.S.A.
June 3–6, 1986 Munich, F.R.G.	**Analytica 86, 10th International Trade Exhibition and 10th International Conference 'Biochemical Analytics'** Contact: Dr. Rosemarie Vogel, Nymphenburgerstrasse 70, D-8000 München 2, F.R.G. (Further details published in Vol. 330, No. 2.)
June 23–27, 1986 Harrogate, U.K.	**1986 Hewlett-Packard Analytical Symposium** Contact: Tina Mears, Hewlett-Packard Ltd., Analytical Instrumentation Group, Miller House, The Ring, Berkshire, RG12 1XN, U.K. Tel.: Bracknell (0344) 424898, telex 848733.
July 20–26, 1986 Bristol, U.K.	**SAC 86 – International Conference and Exhibition on Analytical Chemistry** Contact: Miss P.E. Hutchinson, Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, U.K. Tel.: (01) 734-9971. (Further details published in Vol. 299, No. 1.)
Aug. 25–29, 1986 Antwerp, Belgium	**10th International Symposium on Microchemical Techniques** Contact: Dr. R. Dewolfs, University of Antwerp, Department of Chemistry, Universiteitsplein 1, B-2610 Wilrijk, Belgium. Tel.: 03/828.25.28 (ext. 204), Telex: 33646.
Aug. 27–30, 1986 Brussels, Belgium	**12th International Congress of the European Association of Poison Control Centres (EAPCC)** Contact: Administrative Secretariat, Mrs. D. Shanni, SDR Associated, Rue Vilain XIIII, 17a, B-1050 Brussels, Belgium. Tel.: (02) 647 87 80. Telex: 61434 SDRBRU B.
Aug. 27–30, 1986 Brussels, Belgium	**3rd World Congress of the World Federation of Associations of Clinical Toxicology and Poison Control** Contact: Administrative Secretariat, Mrs. D Shanni, SDR Associated, Rue Vilain XIIII, 17a, B-1050 Brussels, Belgium. Tel.: (02) 647 87 80. Telex: 61434 SDRBRU B.
Sept. 4–8, 1986 Szeged, Hungary	**A Symposium on New Advances in Liquid Chromatography** Contact: Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, Budapest 1445, Hungary.
Sept. 8–10, 1986 Freiburg, F.R.G.	**4th International Symposium on Bioluminescence and Chemiluminescence** Contact: Dr. J. Schölmerich, Medizinische Universitätsklinik, D-7800 Freiburg, F.R.G.
Sept. 9–12, 1986 London, U.K.	**5th Meeting of the International Electrophoresis Society, "Electrophoresis '86"** Contact: Dr. M.J. Dunn, Muscle Research Unit, Royal Postgraduate Medical School, DuCane Road, London W12 0HS, U.K. Tel.: 01-743-2030 ext. 338.
Sept. 21–26, 1986 Paris, France	**16th International Symposium on Chromatography** Contact: GAMS, 88 bd Malesherbes, 75008 Paris, France. Tel.: (1) 563-9304. (Further details published in Vol. 331, No. 2).
Oct. 6–10, 1986 New York, NY, U.S.A.	**The Silver Jubilee Eastern Analytical Symposium** Contact: Dr. S. David Klein, EAS Publicity, 642 Cranbury Cross Road, North Brunswick, NJ 08902, U.S.A. Tel.: (201) 846-1582.

Oct. 7–9, 1986 Tokyo, Japan	**22nd International Symposium Advances in Chromatography** Contact: Prof. N. Ikekawa, Department of Chemistry, Tokyo Institute of Technology, Ohokayama, Meguro-ku, Tokyo 152, Japan; or Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.
Oct. 12–16, 1986 Washington, DC, U.S.A.	**100th Annual AOAC International Meeting** Contact: Margaret Ridgell, AOAC, 1111 North 19th Street, Suite 210, Arlington, VA 22209, U.S.A. Tel.: (703) 522-3032.
May, 1987 Ghent, Belgium	**2nd International Symposium on Quantitative Luminescence Spectrometry Biomedical Sciences** Contact: Dr. W. Baeyens, State University of Ghent, Laboratory of Pharmaceutical Chemistry and Drug Quality Control, Harelbekestraat 72, B-9000 Ghent Belgium.
June 21–26, 1987 Toronto, Canada	**XXV Colloquium Spectroscopium Internationale** Contact: Mr. L. Forget, Executive Secretary CSI XXV, National Research Council of Canada, Ottawa, K1A 0R6 Canada. Tel.: (613) 993-9009, telex: 053-3145. (Further details published in Vol. 330, No. 2.)
June 28–July 4, 1987 Amsterdam, The Netherlands	**HPLC '87, 11th International Symposium on Column Liquid Chromatography** Contact: Organisatie Bureau Amsterdam bv, Europaplein, 1078 GZ Amsterdam, The Netherlands. Tel.: (31) 20-440807, telex: 13499 raico nl. (Further details published in Vol. 331, No. 2.)
August 17–21, 1987 Oberammergau, F.R.G.	**7th International Symposium on Affinity Chromatography and Interfacial Macromolecular Interactions** Contact: Prof. Dr. H.P. Jennissen, Institut für Physiologie, Physiologische Chemie und Ernährungsphysiologie, Universität München, Veterinärstr. 13, D-8000 München 22, F.R.G.

PUBLICATION SCHEDULE FOR 1986

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

MONTH	O 1985	N 1985	D 1985	J
Journal of Chromatography	346 347/1	347/2 347/3 348/1	348/2 349/1 349/2 350/1 350/2	351/1
Chromatographic Reviews				
Bibliography Section				
Biomedical Applications				374/1 374/2

The publication schedule for further issues will be published later

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 329, No. 3, pp. 449–452. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

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