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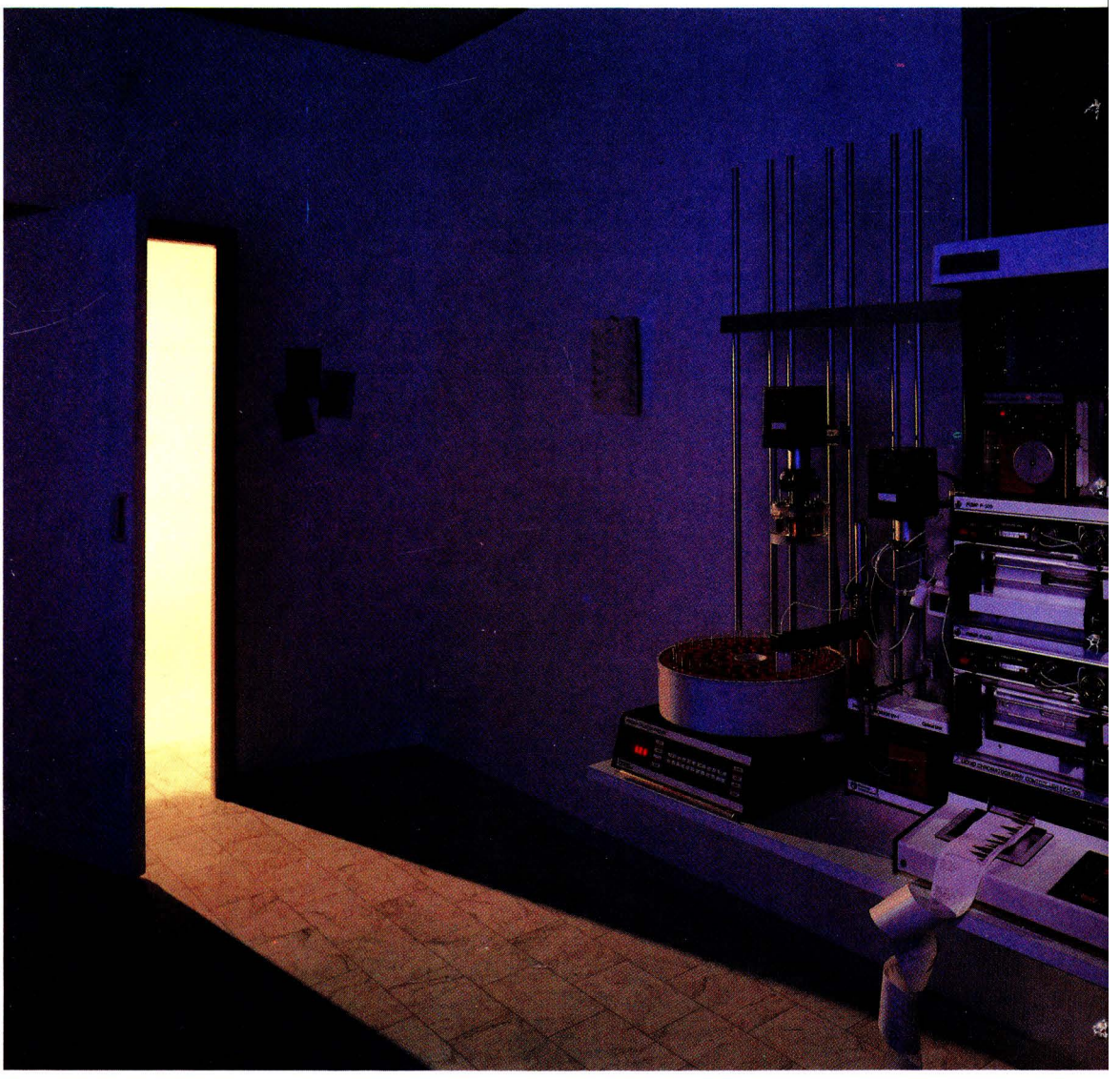

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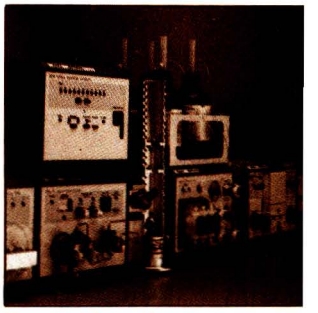

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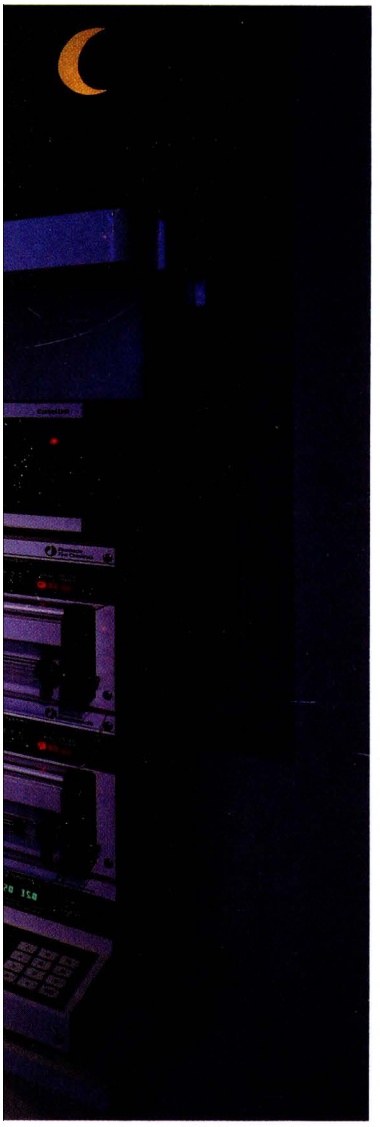

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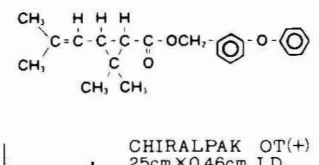

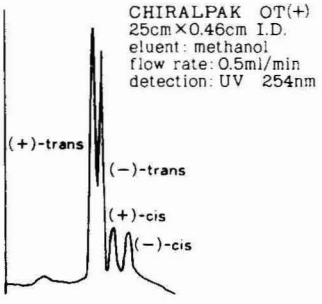

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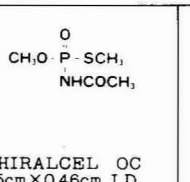

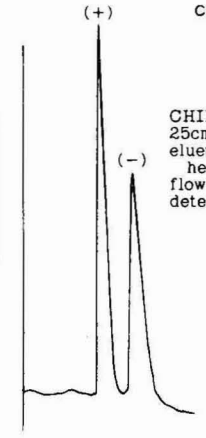

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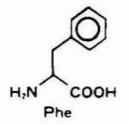

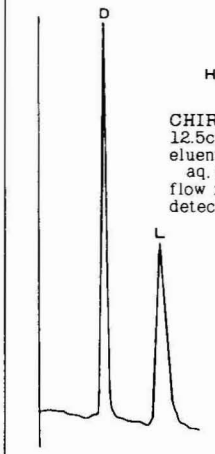

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ELECTROPHORESIS

A Survey of Techniques and Applications

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HIGH-PERFORMANCE ELECTROPHORESIS

ELIMINATION OF ELECTROENDOSMOSIS AND SOLUTE ADSORPTION

STELLAN HJERTÉN
Institute of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala (Sweden)
(Received July 9th, 1985)

SUMMARY

In ideal electrophoresis in free solution neither electroendosmosis nor adsorption of solutes onto the inside of the electrophoresis chamber should occur. In this paper we show that these two disturbing phenomena are negligible in free high-performance electrophoresis when the narrow-bore electrophoresis tube is coated with a mono-molecular layer of non-cross-linked polyacrylamide. The coating procedure is described in detail.

INTRODUCTION

Zone deformation caused by the Joule heat is often negligible in high-performance electrophoresis, since the separations take place in thin-walled, narrow-bore (0.05–0.3 mm I.D.) tubes. However, charges on the inner surface of the tube may give rise to zone distortion due to adsorption of the substances to be separated. (Solute interactions with the tube wall of other than an electrostatic nature are also possible.) These charges also lead to electroendosmosis. In this paper a new method aimed at the elimination of both electroendosmosis and adsorption is described.

MATERIALS AND METHODS

γ-Methacryloxypropyltrimethoxysilane (Pharmacia, Sweden) was covalently bound to the inside of the glass wall of an electrophoresis tube according to the instructions of the supplier. The carrier ampholytes for isoelectric focusing (Pharmalyte®) were also from Pharmacia. Acrylamide, potassium persulphate and N,N,N′,N′-tetramethylethylenediamine (TEMED) were of electrophoresis grade (Bio-Rad Labs., Richmond, CA, U.S.A.). Terephthalic acid, 4-hydroxybenzoic acid and β-naphthylacetic acid were kindly supplied by Dr. K. G. Wahlund, Department of Analytical Pharmaceutical Chemistry, Biomedical Center, Uppsala, Sweden. Human transferrin was a gift from KABI/VITRUM (Stockholm, Sweden). Human

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haemoglobin was prepared from outdated blood (Academic Hospital, Uppsala, Sweden).

EXPERIMENTAL AND RESULTS

Coating of the inner walls of glass or quartz electrophoresis tubes with a monomolecular polymer layer to eliminate electroendosmosis and adsorption of solutes

The method is based on the use of a bifunctional compound in which one group reacts specifically with the glass wall and the other with a monomer taking part in a polymerization process. Examples of such bifunctional compounds are γ-methacryloxypropyltrimethoxysilane, vinyltriacetoxysilane, vinyltri(β-methoxyethoxy)silane, vinyltrichlorosilane and methylvinyldichlorosilane, where one or two of the methoxy, acetoxy, methoxyethoxy or chloro groups react with the silanol groups in the glass wall, whereas the acryl or vinyl groups with acryl or vinyl monomers to form a polymer, *e.g.*, non-cross-linked polyacrylamide, poly(vinylpyrrolidone), poly(vinyl alcohol). Non-covalently attached polymer is then removed simply by rinsing with water. This procedure gives a thin, well defined monomolecular layer of a polymer covalently bound to the glass wall and thus differs from a previously described method[1] which gives a much thicker layer of the polymer (often methylcellulose).

A detailed description of the new procedure used to coat a capillary tube is given below. Although it applies specifically to polyacrylamide coatings, it can with obvious modifications be used for coatings of other polymers, for instance poly(vinyl alcohol) and poly(vinylpyrrolidone).

About 80 μl of γ-methacryloxypropyltrimethoxysilane were mixed with 20 ml of water, which had been adjusted to pH 3.5 by acetic acid. This silane solution was sucked up into the glass capillaries. After reaction at room temperature for 1 h the silane solution was withdrawn. The tubes were washed with water and then filled with a deaerated 3 or 4% (w/v) acrylamide solution containing 1 μl TEMED and 1 mg potassium persulphate per ml solution. After 30 min the excess of (not attached) polyacrylamide was sucked away and the tubes were rinsed with water. Most of the water in the tubes was removed by aspiration and the remainder by drying in an oven at 35°C.

Zone electrophoresis in coated and non-coated tubes

The first experiments were performed with the free zone electrophoresis apparatus where convective disturbances are eliminated by rotating the horizontal quartz electrophoresis tube (380 × 3 mm I.D.)[1]. The sample consisted of an artificial mixture of some aromatic carboxylic acids: terephthalic acid (I), 4-hydroxybenzoic acid (II) and β-naphthylacetic acid (III). The experiments were carried out in a 0.1 *M* Tris–acetic acid buffer (pH 8.6) at 1.840 V (2.6 mA). The rotating tube was scanned with light of wavelengths 280 and 310 nm at the start and after electrophoresis for 5 and 15 min. The ratio between the transmissions at these wavelengths was automatically recorded in order to suppress noise and irregular variations in the baseline of the electropherogram[1]. The experiments were performed in non-coated (Fig. 1a), polyacrylamide-coated (Fig. 1b) and methylcellulose-coated (Fig. 1c) tubes (the methylcellulose treatment is described in ref. 1).

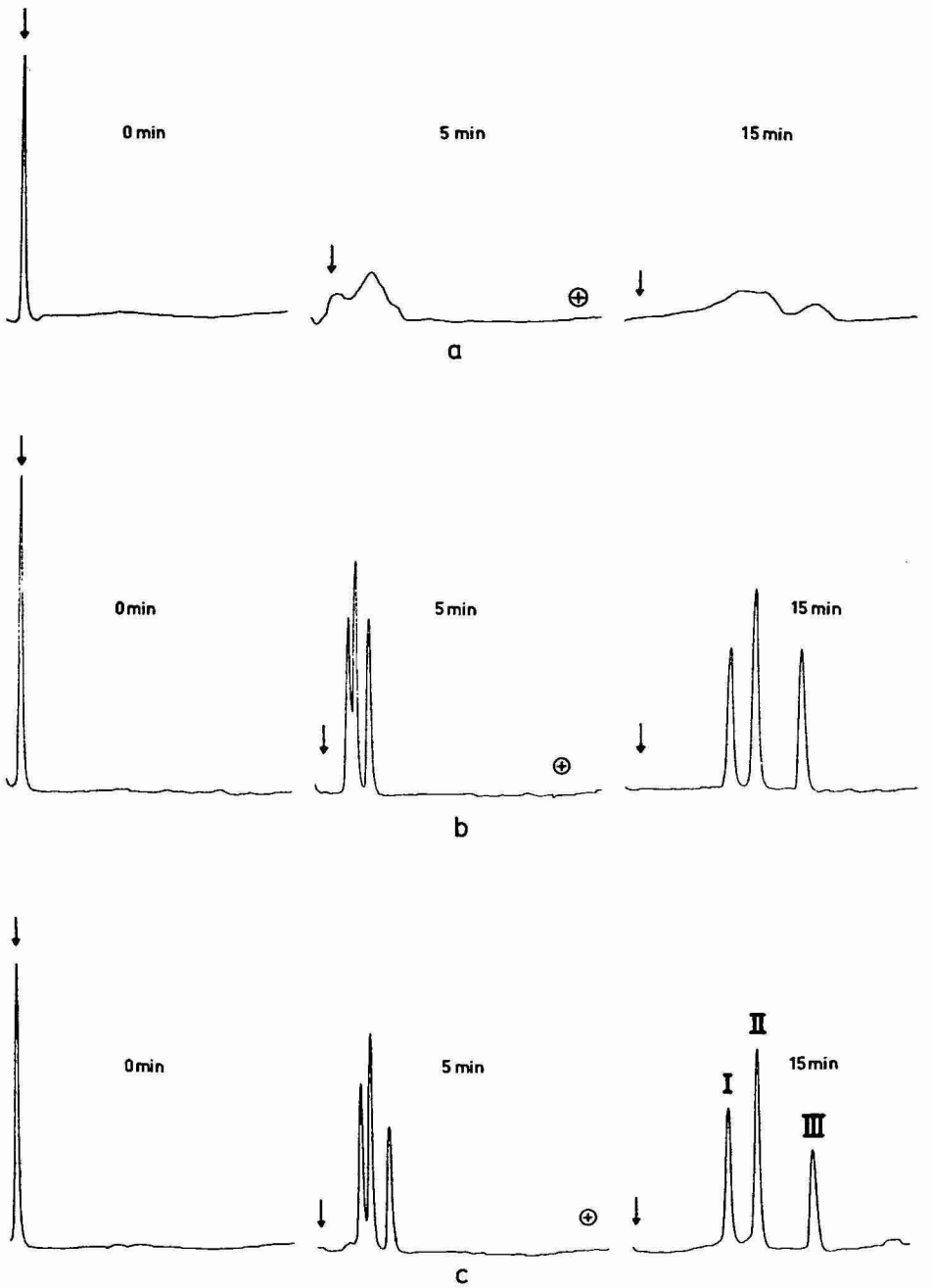

Fig. 1. Zone electrophoresis of aromatic carboxylic acids in a tube of inner diameter 3 mm. The experiments were carried out in the free zone electrophoresis apparatus with a rotating quartz electrophoresis tube of length 380 mm. The experiments were conducted in a non-coated tube (a), in a tube coated with non-cross-linked polyacrylamide (b), as described herein, and in a tube coated with methylcellulose (c), as described in ref. 1. The very marked zone broadening in Fig. 1a is chiefly caused by the hydrodynamic reflow attendent upon the electroendosmosis. No indications of electroendosmosis (or reflow) or adsorption are seen in Fig. 1b and c.

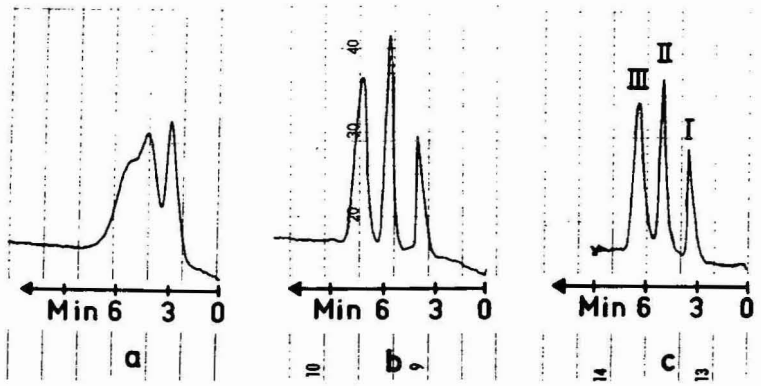

Fig. 2. Zone electrophoresis of aromatic carboxylic acids in a tube of inner diameter 0.2 mm. The experiments were conducted in the high-performance electrophoresis apparatus equipped with a glass tube of length 160 mm, in a non-coated tube (a), in a tube coated with non-cross-linked polyacrylamide (b), as described herein, and in a tube coated with methylcellulose (c), as described in ref. 1.

The above experiments were then repeated in the high-performance electrophoresis apparatus[2–6]. The electrophoresis tube had dimensions 160 × 0.2 I.D. × 0.4 mm O.D. The voltage applied was 2000 V (50 μA). The recording was done by on-tube absorption measurements[2,3] at 280 nm (Fig. 2).

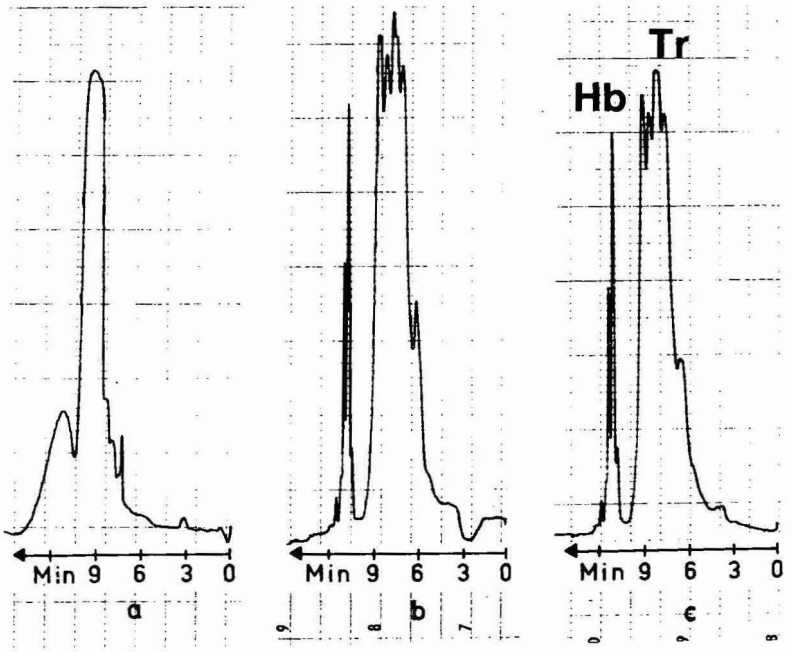

Fig. 3. Isoelectric focusing of proteins in a tube of inner diameter 0.2 mm. The experiments were conducted in the high-performance electrophoresis apparatus in a glass tube of length 120 mm. The sample consisted of transferrin (Tr) and haemoglobin (Hb). Details of tubes a–c as in Fig. 2.

Isoelectric focusing in coated and non-coated tubes

The experiments were performed in glass tubes, 120 × 0.2 I.D. × 0.4 mm O.D., filled with a mixture of human haemoglobin (final concentration: 3 μg/μl), human transferrin (final concentration: 5 μg/μl) and Pharmalyte®, pH 3–10 (final concentration: 1%, v/v). The tube was coated with non-cross-linked polyacrylamide as described above. Focusing was carried out in the high-performance electrophoresis apparatus with on-tube detection at 280 nm at 2000 V for about 15 min with 0.02 *M* phosphoric acid as anolyte and 0.02 *M* sodium hydroxide as catholyte. Elution of the focused protein zones was achieved by replacing the anolyte with 0.02 *M* sodium hydroxide (for details of the elution procedure, see ref. 6). The protein pattern is shown in Fig. 3a. The experiment was repeated in glass tubes coated with non-cross-linked polyacrylamide (Fig. 3b) and methylcellulose (Fig. 3c).

DISCUSSION

Electroendosmosis, which is caused by the presence of fixed charges in the electrophoresis tube (chamber), causes primarily only a displacement of the electrophoretically migrating zones. Although this displacement does not itself cause distortion of the zones, it precludes any possibility of determining accurately the absolute values of the electrophoretic mobilities. Electroendosmosis may, however, be attended by a hydrodynamic reflow, which causes the zones to become strongly parabolically distorted (see Fig. 16 and eqn. 56 in ref. 1). A reflow occurs when the resistance to hydrodynamic flow in the electrophoresis tube is relatively low. For instance, the electroendosmotic reflow is very pronounced in free electrophoresis in a non-coated tube of length 380 mm when the inner diameter is 3 mm (Fig. 1a), but is strongly suppressed when the diameter is reduced to 0.2 mm (Figs. 2a and 3a). Supporting media, such as cellulose powder and gels of agarose or cross-linked polyacrylamide, give too high a flow resistance to allow any reflow.

Electroendosmosis without reflow does not cause significant distortion of a zone, as stated above. A prerequisite is, however, that the zeta potential of the glass wall is the same throughout the length of the tube. This is not always the case. For instance, a non-uniform adsorption of solutes to the charged glass wall may lead to a non-uniform charge distribution on it, which will, in turn, give locally different electroendosmotic migration velocities, resulting in asymmetric zones (peaks).

The surface charges causing electroendosmosis may also give rise to adsorption of the sample solutes onto the glass tube wall. If the wall is coated with a neutral polymer to eliminate electroendosmosis the solutes may be sterically prevented from coming into contact with the wall and therefore from being adsorbed. In this way interactions other than the electrostatic ones are also suppressed. From a comparison of Fig. 1a with b and c, Fig. 2a with b and c and Fig. 3a with b and c, it is obvious that a monomolecular layer of non-cross-linked polyacrylamide (applied by the method described herein) is as efficient as a thicker layer of methylcellulose (applied as previously descriubed[1]) in suppressing zone broadening caused by adsorption or/and electroendosmosis. The absence of adsorption onto the coated glass walls is evident from the fact that the peaks are symmetrical.

Macromolecular substances often show greater tendencies to adsorb to any surface than do low-molecular-weight compounds. The reason is that macromole-

cules have many more binding sites and accordingly can be adsorbed by multipoint attachment[7]. Therefore, it is particularly important to coat the tube when the sample consists of biopolymers, such as proteins, and when the electrophoresis is performed in dilute buffers, since such buffers often do not efficiently suppress the electrostatic interactions between the solute and the glass wall. Although analysis times can be shortened by the use of low buffer concentrations, excessively low concentrations should not be used because they will give rise to relatively large differences in conductivity between a zone and the surrounding buffer, resulting in skewed peaks even in the absence of adsorption; see eqn. 34b and Fig. 12 in ref. 8. In this paper, with the aid of isoelectric focusing experiments, the significance of treating the tube wall with polymers to avoid distortion of protein zones has been demonstrated (Fig. 3). However, the distortions are often more marked in zone electrophoresis, since in this technique the zones are not automatically sharpened as in isoelectric focusing.

Jorgensen and DeArman Lukacs[9] modified the surfaces of silica tubes with glycol groups to decrease the adsorption of proteins. However, if the largest peak in the serum electropherogram in Fig. 9 of ref. 9 corresponds to albumin, electroendosmosis is still very pronounced.

The treatment of the glass tubes with methylcellulose involves baking at elevated temperature and low pH in the presence of formaldehyde[1]. These conditions make the coating more stable because the methylcellulose molecules become cross-linked and perhaps covalently attached to the silanols of the glass wall. In a study of various coating agents for suppression of electroendosmosis, Vanderhoff *et al.*[10] confirmed the high efficiency of methylcellulose. They used γ-glycidoxypropyltrimethoxysilane in an attempt to link the methylcellulose covalently to the tube wall via the epoxide group of the silane molecule. It is, however, very questionable whether this reaction took place under the conditions used (opening of the epoxide ring requires an alkaline pH). The method is very time-consuming, since the authors state that "extensive rinsing of the coated columns for a period of at least 3 days is required to remove the "physically adsorbed" methylcellulose from the surface". In addition, the "physically adsorbed" methylcellulose "may desorb from the cell wall and readsorb onto the colloidal particles" which "would obivate the electrophoretic separation". The coating procedure described herein is rapid and creates a well defined monomolecular polymer layer with so little leakage of polymer material that possible adsorption of released polymer to the substances to be separated will not change their mobilities.

Radola[11] has described isoelectric focusing in gels of cross-linked polyacrylamide covalently bound to glass plates or polyester films pretreated with methacryloxypropyltrimethoxysilane.

The theoretical explanation for the use of a polymer coating to eliminate electroendosmosis is given below. Electrophoresis and electroendosmosis are roughly governed by the classical equations

$$u_{ep} = \varepsilon\zeta_{ep}/4\pi\eta \tag{1}$$

$$u_{eo} = \varepsilon\zeta_{eo}/4\pi\eta \tag{2}$$

where u_{ep} is the electrophoretic mobility, u_{eo} the electroendosmotic mobility, ζ_{ep} the

zeta potential of the solute, ζ_{eo} the zeta potential of the tube wall, ε the dielectric constant and η the bulk viscosity. These equations show that there is no net gain in suppressing electroendosmosis by increasing the viscosity of the buffer, since the electrophoretic mobility will also decrease by the same extent as the electroendosmotic mobility. According to these equations, a feasible way to suppress electroendosmosis, *i.e.*, to get $| u_{eo} | \ll | u_{ep} |$, is to operate under conditions such that $| \zeta_{eo} | \ll | \zeta_{ep} |$. However, in practice, it is very difficult to find materials for the electrophoresis tubes such that this inequality obtains: even the most inert plastic tubes give considerable electroendosmosis[1]. However, the following formula, which I derived several years ago and which is more general than the Helmholz formula (eqn. 2), indicates another way to suppress electroendosmosis[1]

$$u_{eo} = \frac{\varepsilon}{4\pi} \int_0^{\zeta_{eo}} \frac{1}{\eta} \cdot d\psi \tag{3}$$

where ψ is the electric potential. The value of the integral will approach zero when the viscosity, η, in the double layer close to the tube wall approaches infinity. Accordingly, if the inner surface of an electrophoresis tube (chamber) is coated with a polymer solution of high viscosity, electroendosmosis will be virtually eliminated. Any neutral polymer that is soluble or swells in water can be used, for instance methylcellulose or non-cross-linked polyacrylamide. It should be mentioned that these polymers, dissolved in the buffer, will also suppress electroendosmosis[1,2], probably because the polymers tend to adhere to the tube wall and thereby create a thin surface layer of high viscosity. The electrophoretic mobility is, of course, higher in buffer alone than in a polymer-containing (and therefore viscous) buffer (see eqn. 1), which is an obvious advantage when short analysis times are desired.

Since in free solution electroendosmosis causes all electrophoretically migrating zones to be displaced to the same extent, it cannot give rise to any separation of the sample solutes in a carrier-free medium, contrary to what has been stated.

ACKNOWLEDGEMENTS

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COOLING PLATE FOR CELLULOSE THIN-LAYER ELECTROPHORESIS AND ITS APPLICATION TO AMINO ACID ANALYSIS

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SUMMARY

A stainless-steel water-cooled plate has been designed for the electrophoresis of small and intermediate sized molecules on plastic based cellulose thin layers. The upper cooling surface of the plate was formed by milling out five shallow grooves aligned to the direction of electrophoresis. Insulation of the plate was achieved by dipping it in a suspension of electrostatically charged plastic particles. Electrophoresis was carried out by soaking five thin-layer strips in the electrolyte (4%, v/v, formic acid), and laying them with the layer face down along the cooling surface so that the sample spot was sighted over the centre of a groove. Electrophoretic separation then took place along the uncooled zone covering the groove. Adjustment of the electrolyte concentration and the voltage made it possible under these conditions to separate 23 amino acids when developed by chromatography in the second dimension using 15 × 18 cm layers.

INTRODUCTION

Methods using electrochromatography for the separation of charged molecules of small and intermediate size on thin layers have been available for 20 years[1–4]. Although many of these procedures have been subsequently superseded by developments in high-performance liquid chromatography (HPLC), the thin-layer procedures still have an important role to play as an adjunct to HPLC, in studies involving rapid screening of large numbers of samples, and in the analysis of radioisotope tracer distribution in metabolites. One of the reasons why the application of electrophoresis to thin layers has not been used more frequently may be attributed to the fact that equipment specifically designed for the purpose is not generally available. This paper describes an attempt to rectify this deficiency by the development of a simple cooling system which fulfils the requirements of jointly controlling the temperature of the layers, and limiting the diffusion of the solutes. The plate is designed to take five 15 × 4 cm layers or two 15 × 18 cm layers at one time. The device has been applied mainly to the analysis of amino acids. This design is not suitable for application to gel electrophoresis.

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MATERIALS AND METHODS

Materials

Ninhydrin and the amino acids were obtained from Calbiochem (Australia). Tween 20 and the plastic-based cellulose thin-layers (20 × 20 cm) were obtained from Sigma (St. Louis, MO, U.S.A.). Whatman chromatography paper was obtained from W. & R. Balston, U.K. The cooling plate was insulated by Atractaseal (East Brisbane, Australia).

Preparation of the thin layers

The cellulose layers were placed one-by-one in a Pyrex dish and washed successively in 2 *M* acetic acid, and three times each in ethanol and water. The layers were allowed to dry at room temperature. Immediately after drying they were cut into 15 × 4 cm strips and wrapped in thin plastic for storage in the deep freeze. A store of layers ready for immediate use could thus be built-up.

Protein precipitation and determination

All samples containing protein were deproteinised. 12 ml of concentrated acetic acid was diluted to about 50 ml and 5 ml of Tween 20 was added. This solution was then titrated to pH 5.3 with concentrated ammonia. The solution was made-up to 100 ml to give a final approximate concentration of 2 *M* ammonium acetate.

A volume of 0.5 ml (in the case of a dried tissue sample or an acetone powder) or an equal volume (in the case of a physiological fluid) of the above reagent was added to the test sample and the mixture was heated in a boiling water bath for 3 min to bring about protein coagulation. On cooling the solution, two volumes of chloroform were added, and the mixture was emulsified on a vortex mixer to precipitate the remaining protein. The tubes were allowed to stand for 10 min and mixed a second time. The two phases were then separated by centrifugation for 5 min at 3000 rpm. The aqueous phase was removed with a Pasteur pipette, and retained for analysis.

The protein precipitate was digested in 0.1 *M* sodium hydroxide in sealed tubes overnight, and determined by the method of Lowry *et al.*[5].

Application of the samples to the layers

Samples were applied to a point which was 1.5 cm from each side of a selected corner of the layer with an Absoluter Micro-pipettor (Tri-Continental Scientific, U.S.A.) of 2–10-μl capacity. A volume of 2 μl of 2,4-dinitrophenyl (DNP)-lysine (1 m*M*) was first applied as a marker, and was followed by the test samples. All the applications were dried by blowing air at room temperature. Heating of the layers before electrophoresis was strictly avoided. The short side nearest the application point now becomes the anode end of the layer, and the long side nearest the application point becomes the bottom side of the layer.

On completion of the sample applications, all the spots were concentrated by dipping the top side of the layer in 0.01 *M* hydrochloric acid, allowing the fluid front to cross the DNP-lysine ring, and then drying the layer. A second concentrating run was usually required to move the DNP-lysine into a sharp bar about 8 mm from the bottom side of the layer.

Electrophoresis

The design of the apparatus. The design of the apparatus and the arrangements for electrophoresis are shown in Fig. 1.

The stainless-steel cooling plate (235 × 160 × 3 mm) was prepared by milling-out five grooves along the upper surface (140 × 7 × 1.5 mm, approximately) at 37 mm apart and parallel to the short sides of the plate (Fig. 1b and c). Shallow steps (235 × 15 × 0.05 mm, approximately) were then milled-out along the long sides of the plate. The remaining raised surfaces of the plate were ground flat with emery paper, and all corners were smoothed and rounded-off by filing so as to ensure good attachment of the insulating material.

A copper box section (200 × 130 × 11 mm) was then prepared with inlet and outlet pipes silver soldered into one of the short sides. This assembly was then soft soldered onto the under-side of the stainless-steel plate, thereby forming the water circulation compartment for the cooling plate. Cooling water was supplied from a cold tap with the flow-rate set fairly high.

The completed cooling plate was then electrically insulated by dipping it into a fluid containing a suspended electrostatically charged plastic powder. This process provided a hard thin layer of insulating plastic over the entire plate.

A waterproof marker pencil was then used to draw black lines (3 cm) in the middle of each groove at the anode end of the plate.

Preparation of the electrophoresis vessel. The wicks and connecting strips (Fig. 1) were washed three times in the electrolyte (4%, v/v, formic acid), in which they were allowed to soak until required. A volume of 100 ml of the electrolyte was added to each of the electrode compartments. The electrode partitions and the paper connecting strips were then positioned in the electrophoresis vessel. The connecting strips were blotted-off just prior to the addition of the thin layers.

Application of the electrolyte to the thin layers. The anode end of each thin layer was dipped into the electrolyte, and the fluid front was allowed to move as far as the middle of the DNP-lysine spot. The layer was then removed and excess fluid was blotted-off the dipped edge. The opposite end of the layer was then placed in the electrolyte, and the layer fully immersed until the fluid level was about 1 cm from the spot. The two electrolyte fronts were allowed to meet, and the layer was held in this position for about 30 s so as to ensure ample irrigation of the layer. On removal of the layer from the electrolyte, it was given a good shake to remove most of the excess fluid by knocking the plastic backing against the edge of the bench with the cathode end held down. Excess fluid on the cathode end was then blotted-off with tissue and the layers were placed on the cooling plate with the cellulose layer face down, and the DNP-lysine spots centred over the black lines in the grooves. The ends of the layers were pressed firmly against the connecting strips so as to establish good fluid contact with the connecting strips, and soak-off any remaining excess electrolyte on the layers. When all the layers were in position on the cooling plate, the wicks were then squeezed to remove excess fluid and placed in the electrode compartment. The layers were then covered with a piece of clear plastic, followed by a glass plate (225 × 160 × 2 mm) which was placed so that it rested on top of the two wicks. The plate was pressed firmly down so as to ensure a good fluid junction between the layers and the wicks. The apparatus was then closed, and the current turned on. It was usual to run the electrophoresis at 200 V for the first 7 min, and

then at 500 V for the remaining 23 min. With all five of the smaller strips in place on the cooling plate, 500 V gave rise to a constant current of 25 mA. The purpose of an initial run at 200 V is to stabilise the system before applying the full voltage. Occasionally, depending on the source of the sample under test, the DNP-lysine might be seen to form two spots. This characteristic does not normally affect the other amino acids, and is not necessarily an indication of trouble in the electrophoresis. For the final separation of the amino acids, they were subjected to chromatography in the other dimension. This was effected by 2 developments using 42 ml of butanol–0.4% (v/v) pyridine–acetic acid (22:10:10, v/v/v). Between the two runs 0.3 g ninhydrin was dissolved in an equal volume of the developing solvent and added to the chamber immediately prior to the second run. On completion of chromatography, the layers were exposed to cold air in the fume hood for 1 h by which time the ninhydrin colour was well developed. The strips were then placed in a drawer and allowed to complete

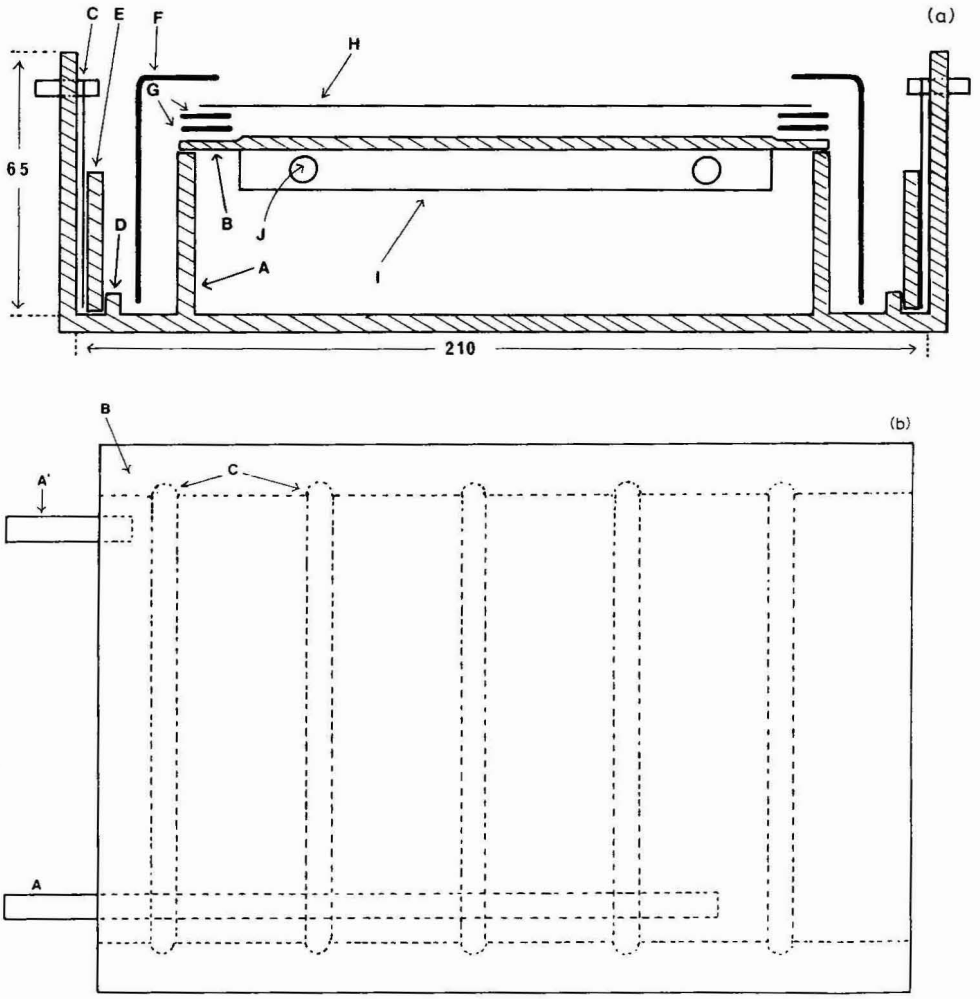

Fig. 1.

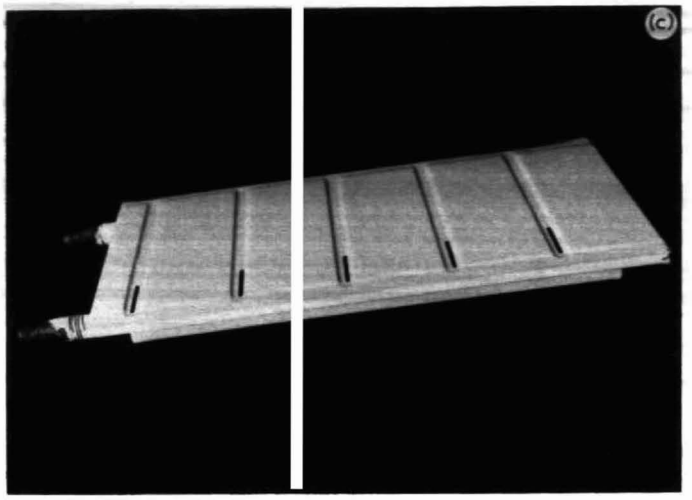

Fig. 1. The design of the electrophoresis vessel and its operation. (a) Cross-sectional side-view of the electrophoresis apparatus. The vessel was made of 4 mm perspex and has inner dimensions 245 × 210 × 65 mm. The electrode compartments at either end of the box are 245 × 25 mm, and the inner walls (A, 40 mm high) support either end of the cooling plate (B, stainless steel, 235 × 160 × 3 mm). The electrodes (C) are triangular pieces of stainless-steel sheet (140 mm at the bottom), which are attached by means of a centrally placed terminal to the top of the end-walls of the electrode compartments. Running along the bottom of each of the electrode compartments (7 mm from each end-wall) is a perspex rib (D, 5 mm high) which provides a retaining step for the electrode compartment partition (E), which consists of a perspex frame (243 × 35 mm) with an open mid-section, fitted into a piece of dialysis tubing (40 mm, flat width). The wicks (F) consist of a pair of 3MM Whatman strips (230 × 70 mm) inserted into dialysis tubing (75 mm flat width). Connection of the current from the wicks to the thin layers is effected at each end by a pair of 3MM Whatman strips (G, 230 × 15 mm). The thin layers (H, 15 × 4 mm) are placed face down on the cooling plate so that both ends overlap onto the connecting strips, and the wicks are then bent to overlap the thin layers. The outline of the copper box for the circulation of cooling water is shown (I), together with the water circulation ports (J). The apparatus is closed by a simple stepped lid. (b) The cooling plate viewed from above showing the inlet (A) and outlet (A′) ports, the slots for the linking paper strips (B), and the grooves (C). (c) Photograph of the cooling plate.

the colour development overnight. When large numbers of samples were being run it was usual not to add ninhydrin for the second chromatographic run, but to apply the ninhydrin on completion of chromatography by dipping the strips in a 0.3% (w/v) solution of ninhydrin in acetone containing 1% (v/v) pyridine. In this procedure the strips were usually placed directly into a drawer for overnight colour development.

The larger format of 15 × 18 cm was used only on those occasions when resolution of the two leucines was important.

The chromatograms can be preserved indefinitely by sealing in thin plastic film and placing in the deep freeze.

Quantitation of the amino acids. When this was required, the ninhydrin spots were removed from the chromatogram by rubbing off with a small steel spike centrally attached by means of a spring clip to the top of a test tube. With the thin layer held facing down the rubbings were quickly collected in the bottom of the test tube

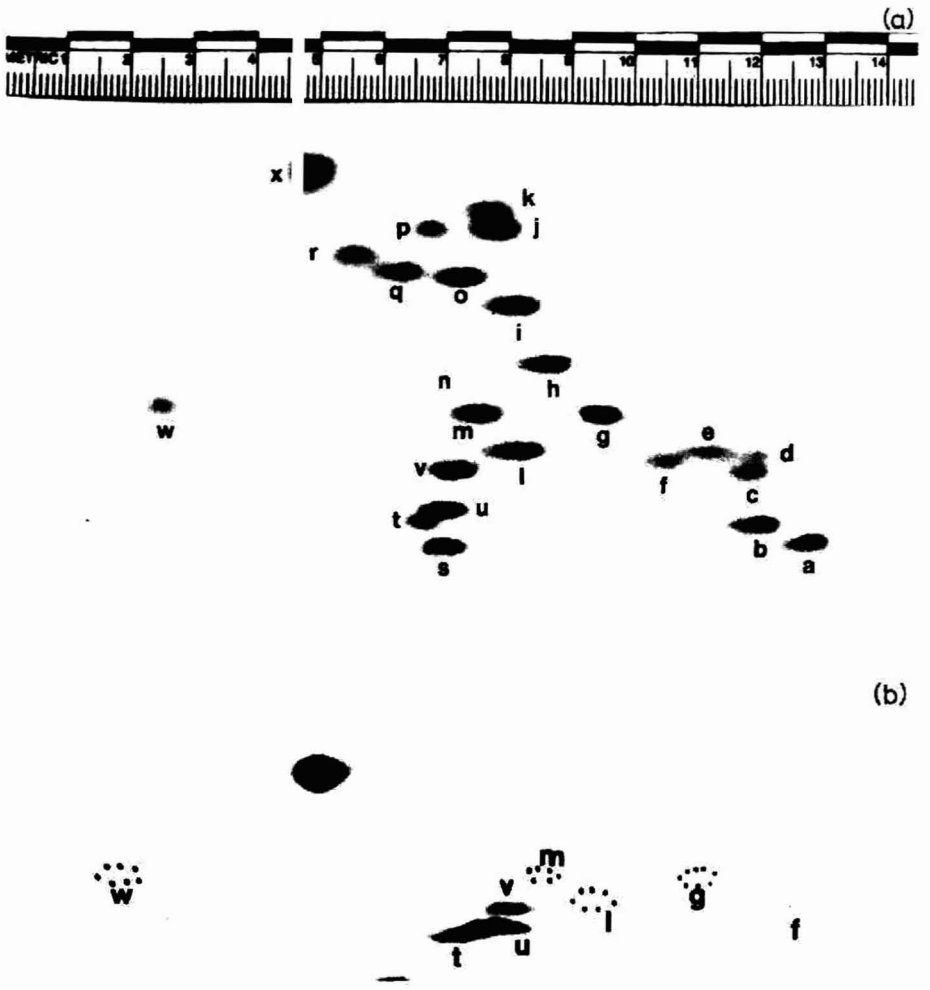

Fig. 2. Electrochromatograms of the amino acids. (a) 2 nmol of each of 23 amino acids were applied to a 15 × 18 cm layer. Letters in the figure refer to the following compounds: a = Lys; b = Arg; c = His; d = β-Ala; e = γ-Abu; f = Gly; g = Ala; h = α-Abu; i = Val; j = Ile; k = Leu; l = Ser; m = Thr; n = Pro; o = Met; p = Phe; q = Tyr; r = Try; s = Cys–Cys; t = Asp; u = Glu; v = Gln; w = Tau; x = DBP-Lys. (b) Separation of free amino acids present in an extract of HeLa cells. The HeLa cell acetone powder (*ca.* 1 mg protein) was extracted with 2 *M* ammonium acetate at pH 5.3, and 9 μl was applied to a 15 × 4 cm layer.

(5 s for each spot). The colour was taken-up in 1 ml butanol–ethanol–water (100:10:20, v/v/v) and measured by difference at 410 (peak) and 455 nm (isosbestic point) in a Cary 219 spectrophotometer. An internal standard consisting of 2 nmol norvaline or α-aminobutyric acid (α-abu) was included. The results were then expressed as norvaline or α-aminobutyric acid units, calculated as the ratio of the colour development of the amino acid to that of the internal standard. The range of measurement was 0.2 3 nmol.

RESULTS

Amino acids

Fig. 2a shows the resolution obtained for 23 amino acids, and Fig. 2b shows the separation of free amino acids present in an extract from HeLa cells. Fig. 3 shows the mean standard curve and standard deviation obtained for the ninhydrin colour reaction for eight of the amino acids on the one chromatogram. The standard colour values are expressed as α-aminobutyric acid units, and the standard deviations refer to the variance of the individual amino acids from the mean colour development for all. The mean coefficient of variation for all the amino acids tested at 2 nmol was 7%. Table I shows the comparison of results obtained for the concentrations of the major amino acids appearing in HeLa cells determined by the present procedure together with those obtained by two other groups of workers[6,7]. In other applications the system is being applied by other groups of investigators in this institution to the resolution of the amino acids and phosphorylated products resulting from photosynthesis in zooxanthellae[8], and the investigation of amino acid metabolism in Na^+-deficient plants[9].

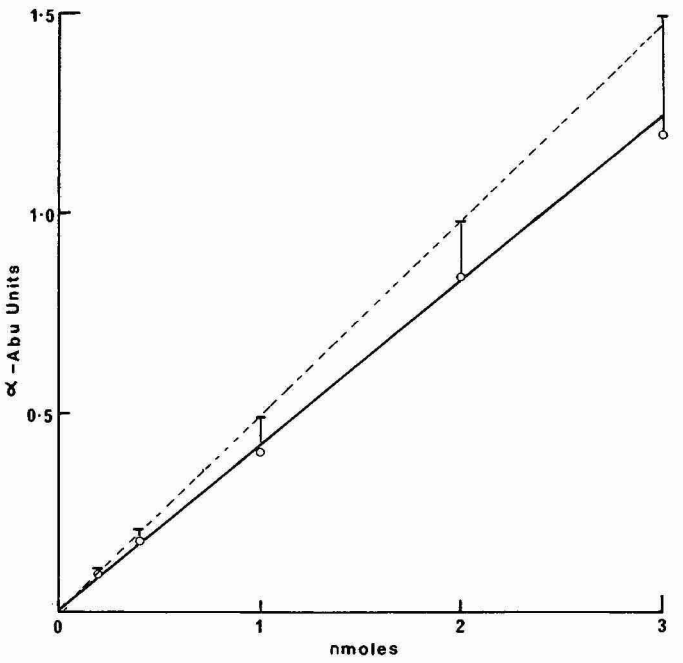

Fig. 3. Amino acid standard curve showing the development of ninhydrin colour. The figure shows the mean colour development and standard deviation for the following amino acids: Gly, Glu, His, Leu, Lys, Met, Phe, Tau. The colour development is expressed in α-Abu units, which was the internal standard in these experiments.

TABLE I

FREE AMINO ACIDS IN HeLa CELLS

	Content ± S.D. (nmol/mg protein)*	*Concentration (mM)***	*Piez and Eagle[6] (mM)*	*Kabus and Koch[7] (mM)****
Asp	101 ± 12	15.0	1.3	16.0
Gln	80 ± 7	12.0	8.1	25.0
Glu	162 ± 12	24.0	10.8	24.0
Gly	37 ± 7	5.5	0.8	5.7
Tau	17 ± 2	2.5	14.3	na[§]
Others	10	–	–	–

* The monolayers on glass were exposed for 3 s to ice-cold isotonic sodium chloride and then fixed with acetone.

** The concentrations in cell water were estimated using the procedure of Piez and Eagle[6].

*** The concentrations in cell water were recalculated from the centrifugation into dibutylphthalate procedure of Kabus and Koch[7].

§ na = Not applicable.

DISCUSSION

Protein precipitation

The combination of heat coagulation and chloroform precipitation in the presence of a surface active agent was more efficient than any of the procedures using strong acids in that it completely eliminated trailing of the amino acids during electrophoresis, which was not the case when the proteins were precipitated by picric, phosphotungstic, trichloracetic or perchloric acids. This procedure has also the added advantage of avoiding the use of low pH values (of great importance in glutamine and asparagine determinations).

The layers

It is important to note that the layers prepared in Germany for Sigma were the only plastic-based thin layers which were found to be fully suitable for electrophoresis. Other layers, also from Germany, had a tendency to float off the plastic base when immersed in 2 *M* acetic acid. This problem can be solved by exposing the layers for 24 h to UV light after dipping in a 3% (v/v) solution of hydrogen peroxide in ethyl alcohol. This produced heavy oxidation in the layer, the products of which served to fix the layer to the plastic base. These layers were then thoroughly washed before use.

The apparatus

The design of the apparatus was dictated by three considerations.

(i) During electrophoresis on cellulose thin layers the separation of small and intermediate sized molecules suffers considerable loss of resolution if another surface (*e.g.* a cooling surface) besides that of the support surface is in contact with the layer. The reason for this is that the capacity of the layers to conduct high currents results in extensive inundation of the layers by the electrolyte, which in its turn gives rise to a second fluid phase (electrolyte only) forming between the cellulose matrix and the

other surface. Thus, solute molecules become distributed between the two phases, and smearing develops because those molecules in the electrolyte only phase migrate and diffuse much faster than the molecules in the cellulose matrix. In the procedure described above the solutes migrate through the cellulose matrix in the zone immediately above the groove along which excess electrolyte is continuously evaporated and condensed at the bottom of the groove. A second electrolyte phase therefore does not develop. Under these conditions smearing was eliminated, resulting in a considerable improvement in resolution compared to that obtained when the entire layer was exposed to a cooling surface.

(ii) Because the cellulose zones which lie over the grooves are not directly cooled, they run at a higher temperature than the surrounding areas of the layers. This gave rise to two effects: (1) the solutes separating along the uncooled zones migrated faster through the cellulose matrix; and (2) the evaporation of electrolyte from the uncooled zones and its condensation at the bottom of the grooves resulted in cooled electrolyte being drawn from the surrounding areas thereby squeezing the solutes in towards the middle of the uncooled zones. In this way lateral diffusion of the solute molecules was reversed to the extent that the longer the run, the greater was the degree of concentration of the solutes along the centre line of the uncooled zone. It was possible to obtain compact spots of uniform shape and size when equal amounts of the amino acids were applied to the chromatogram.

(iii) The final consideration in the development of the cooling plate was the search for a suitable coating which would insulate it electrically, whilst minimally affecting its capacity to transfer heat. The process described here made use of an electrostatic powder coating which provided theoretically an ideal solution to the problem. Two plates coated by this process have now been in operation in this laboratory for two years without any failure in the insulation. All the other plastic coatings tested failed because of the unavoidable presence of microscopic holes which provided charge conducting channels connecting with the metal surface. The continued passage of charge through these holes resulted in local heating which gradually melted away the plastic until the holes were large enough to fully short all charge to the cooling plate.

Quantitation of amino acids

The procedure described is useful in those circumstances where a suitably automated column procedure is not available, or when the procedure more easily resolves a particular mixture of amino acids. The advantage of the procedure over column procedures is that it will resolve most amino acid mixtures together with a large number of other metabolites which can be identified by their position if traced with a radioactive label. The use of dye elution as part of the procedure for the spectrophotometric determination of substances separated on chromatograms generally suffers from one major disadvantage which is that there is usually present large and variable amounts of impurities in the layers which absorb light across a wide band of wavelengths, and this is particularly true of cellulose. These impurities therefore contribute large and variable blanks to the determination depending on the position on the layer and the amount of layer eluted. This problem has been essentially overcome in the present procedure by the use of wavelength difference for the measurement of absorbance, with the chosen wavelengths being only 45 nm apart

and the 455 nm absorbance corresponding to the isosbestic point of the spectra for different concentrations of dye.

In conclusion the cooling plate has been designed specifically for cellulose thin layers, the matrix of which can support the rapid transfer of solute molecules, resulting in relatively high levels of current. For this reason the system is not suitable for polyacrylamide or agarose gels which tend to dry out along the uncooled zone[10]. The apparatus is reliable and flexible in operation and can routinely handle charges of up to 2000 V.

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INFLUENCE OF THE MOBILE PHASE COMPOSITION ON THE REVERSED-PHASE THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR OF A SERIES OF PROSTAGLANDINS

COMPARISON OF THE EXTRAPOLATED R_M VALUES

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SUMMARY

The R_M values of a series of prostaglandins were determined in two reversed-phase thin-layer chromatographic systems, the mobile phase being an aqueous buffer alone or mixed with various amounts of methanol or acetone. The linear relationship between the chromatographic behaviour and the mobile phase composition yielded very similar extrapolated R_M values at 0% organic solvent in both systems. This shows that the extrapolated R_M values are independent of the nature of the organic solvent. In other words, the extrapolated R_M values should be related to the partitioning of the compounds between water and silicone oil in a standard system where all the compounds can be compared.

INTRODUCTION

The lipophilic character of drugs plays a significant rôle in their biological activity[1,2].

The R_M values in reversed-phase thin-layer chromatography (TLC) have been shown to be a reliable measure of the hydrophobicity of molecules[3]. The linear relationship between the chromatographic behaviour and the composition of the mobile phase yields extrapolated R_M values at 0% of organic solvent. Such values might be related to the partitioning of the compounds between water and silicone oil, the latter being the medium impregnating the silica gel G layer in the reversed-phase TLC system[4].

Although few papers have dealt with the relationship between R_M and the mobile phase composition[5–7], this is a very important aspect of chromatography. The

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extrapolation technique might be a way of obtaining R_M values in a standard system, *i.e.*, independent of the nature of the organic solvent in the mobile phase. In a previous study the R_M values of a series of dermorphin-related oligopeptides were determined in two reversed-phase TLC systems[8]. The mobile phase was an aqueous buffer alone or mixed with various amounts of methanol or acetone. The extrapolated R_M values at 0% of organic solvent were shown to be very similar in the two systems.

The purpose of the present study was to measure the R_M values of a series of prostaglandins in the above reversed-phase TLC systems as a further contribution to the assessment of the reliability of the extrapolation technique.

MATERIALS AND METHODS

Compounds investigated

The structures of the prostaglandins are shown in Table I. The compounds were a generous gift from Carlo Erba.

Determination of R_M values

The TLC technique employed has been described previously[4,8]. Glass plates (20 × 20 cm) were coated with silica gel G; in order better to control the pH of the stationary phase a slurry of silica gel G was prepared with 0.09 *M* sodium hydroxide solution. A non-polar stationary phase was obtained by impregnating the silica gel G layer with silicone DC 200 (350 cSt) (Applied Science Labs.). The impregnation was carried out by developing the plates in a 5% silicone solution in diethyl ether. Eight plates could be impregnated in a single chromatographic chamber, containing 200 ml of the silicone solution. The plates were left in the chamber for 12 h, *i.e.*, for several hours after the silicone solution had reached the top of the plates. The chromatographic chamber was saturated with the vapour of the mobile phase.

A migration of 10 cm was obtained on all plates by cutting the layer at 12 cm and spotting the compounds on a line 2 cm from the lower edge of the plate. The mobile phase saturated with silicone was an aqueous buffer (sodium acetate–Veronal buffer, 1/7 *M* at pH 7.0), alone or mixed with various amounts of acetone or methanol.

Two plates were developed simultaneously in a chromatographic chamber containing 200 ml of mobile phase. The dermorphin-related derivatives were dissolved in methanol (1–2 mg/ml) and 1 μl of solution was spotted randomly on the plates in order to avoid any systematic error. The developed plates were dried and sprayed with an alkaline solution of potassium permanganate. After a few minutes at 120°C, yellow spots appeared on an intense pink background. The R_M values were calculated by means of the equation:

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

log P values

Experimental log *P* values for compounds 1, 2 and 6 have been reported by Hansch and Leo[9]. These were used in order to calculate the log *P* values of the remaining compounds, by taking advantage of the additive property of the π values.

RESULTS AND DISCUSSION

R_M values

On the basis of our previous work with reversed-phase TLC or high-performance liquid chromatography (HPLC) and in agreement with data from the literature, we pointed out that the relationship between R_M values and mobile phase composition can generally be described by an S-shaped curve[10]. In fact at the lower organic solvent concentrations the compounds tend not to move from the starting line, while at the higher concentrations they tend to move with the solvent front. It has been suggested that the extrapolation from the linear part of the curve should yield the theoretical R_M values at 0% organic solvent in the mobile phase. In this way one should avoid the physical limitations of the chromatographic system represented by the upper and lower parts of the S-shaped curve. The extrapolated R_M values at 0% could be considered as a measure of the partitioning of the compounds between water or an aqueous buffer and the hydrophobic stationary phase, *i.e.*, in a standard system where all the compounds could be compared on the basis of their lipophilic character. Hydrophilic compounds are supposed to show deviations from linearity only at higher organic solvent concentrations, since even at 0% organic solvent in the mobile phase their chromatographic behaviour yields reliable R_M values. As regards the present work, the test compounds did not move from the starting line when the mobile phase was the aqueous buffer alone. In order to obtain suitable R_M values it was necessary to add an organic solvent to the mobile phase.

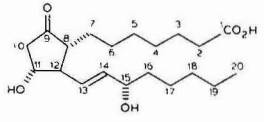
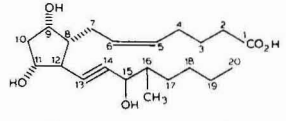
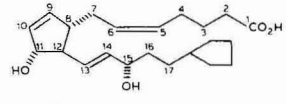
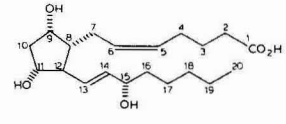
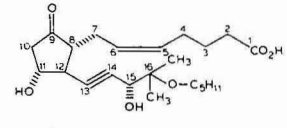
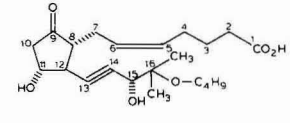
In the methanol system the R_M values reported in Table II were obtained. The plots in Fig. 1 and the equations of Table II show that for each compound the R_M values bear a very good linear relationship to the mobile phase composition over the full range of methanol concentrations yielding reliable R_M values. In Fig. 1 the upper and lower parts of the curves were not reported. At those methanol concentrations the compounds remained so close to the starting line or moved so close to the solvent front that the measurement of the R_M values was unreliable. The intercepts of the equations in Table II represent the theoretical R_M values at 0% methanol in the mobile phase.

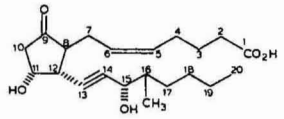
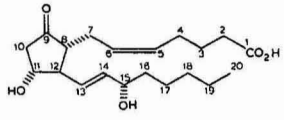
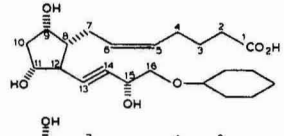
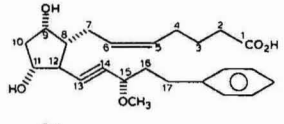
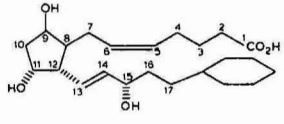
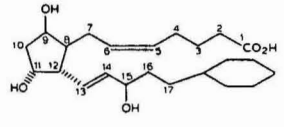
The R_M values obtained similarly in the acetone system are given in Table III. The plots in Fig. 1 show that at acetone concentrations higher than 32–36% all the compounds tend to migrate with the solvent front, *i.e.*, in the lower part of the S-curve. The R_M values obtained at acetone concentrations higher than 48% are not reported. Because of the deviations from linearity, the equations in Table III were calculated by means of the R_M values obtained at acetone concentrations only up to 32–36% as shown in Fig. 1. The theoretical R_M values at 0% acetone are very close to those at 0% methanol.

The analysis of variance did not show any significant difference between the two sets of R_M values. This should mean that the extrapolated R_M values are not dependent on the nature of the organic solvent in the mobile phase. In other words, the extrapolated R_M values should be a measure of the partitioning in the same standard system, *i.e.*, between water and the silicone oil impregnating the silica gel G layer. As a consequence the equation describing the correlation between the R_M values in the two chromatographic systems should be characterized by an intercept and slope close to 0 and 1 respectively. This seems to be the case:

TABLE I

STRUCTURES AND log *P* VALUES OF PROSTAGLANDIN DERIVATIVES

No.	*Compound*	*Structure*	*Empirical formula*	*Molecular weight*	*log P*
1	Prostaglandin E_1 (PGE_1)		$C_{20}H_{34}O_5$	354.492	2.00
2	Prostaglandin E_2 (PGE_2)		$C_{20}H_{32}O_5$	352.476	1.69
3	16δ-Methyl-13,14-didehydro-8,12-diisoprostaglandin E_2		$C_{21}H_{32}O_4$	348.487	3.35
4	13,14-Didehydro-ω-trinor-16-methyl-16-butoxyprostaglandin E_2		$C_{22}H_{34}O_6$	394.514	1.72
5	13,14-Didehydro-ω-trinor-16-methyl-16-pentoxyprostaglandin E_2		$C_{23}H_{38}O_6$	410.557	2.22
6	Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)		$C_{20}H_{34}O_5$	354.492	1.60

7	ω-Trinor-17-cyclopentyl-9-deoxy-$\Delta^{9,10}$-prostaglandin $F_{2\alpha}$		$C_{22}H_{34}O_4$	362.514	3.10
8	13,14-Didehydro-16*R*-methyl-15-epiprostaglandin $F_{2\alpha}$		$C_{21}H_{34}O_5$	366.503	2.10
9	13,14-Didehydro-ω-tetranor-16-cyclohexyloxyprostaglandin $F_{2\alpha}$		$C_{22}H_{34}O_5$	394.514	1.14
10	13,14-Didehydro-ω-trinor-17-phenyl-15-methoxyprostaglandin $F_{2\alpha}$		$C_{24}H_{32}O_5$	400.520	2.92
11	ω-Trinor-17-cyclohexyl-8,12-diisoprostanglandin $F_{2\beta}$		$C_{23}H_{38}O_5$	394.557	2.61
12	ω-Trinor-17-cyclohexyl-8,12-diiso-15-epiprostaglandin $F_{2\beta}$		$C_{23}H_{38}O_5$	394.557	2.61

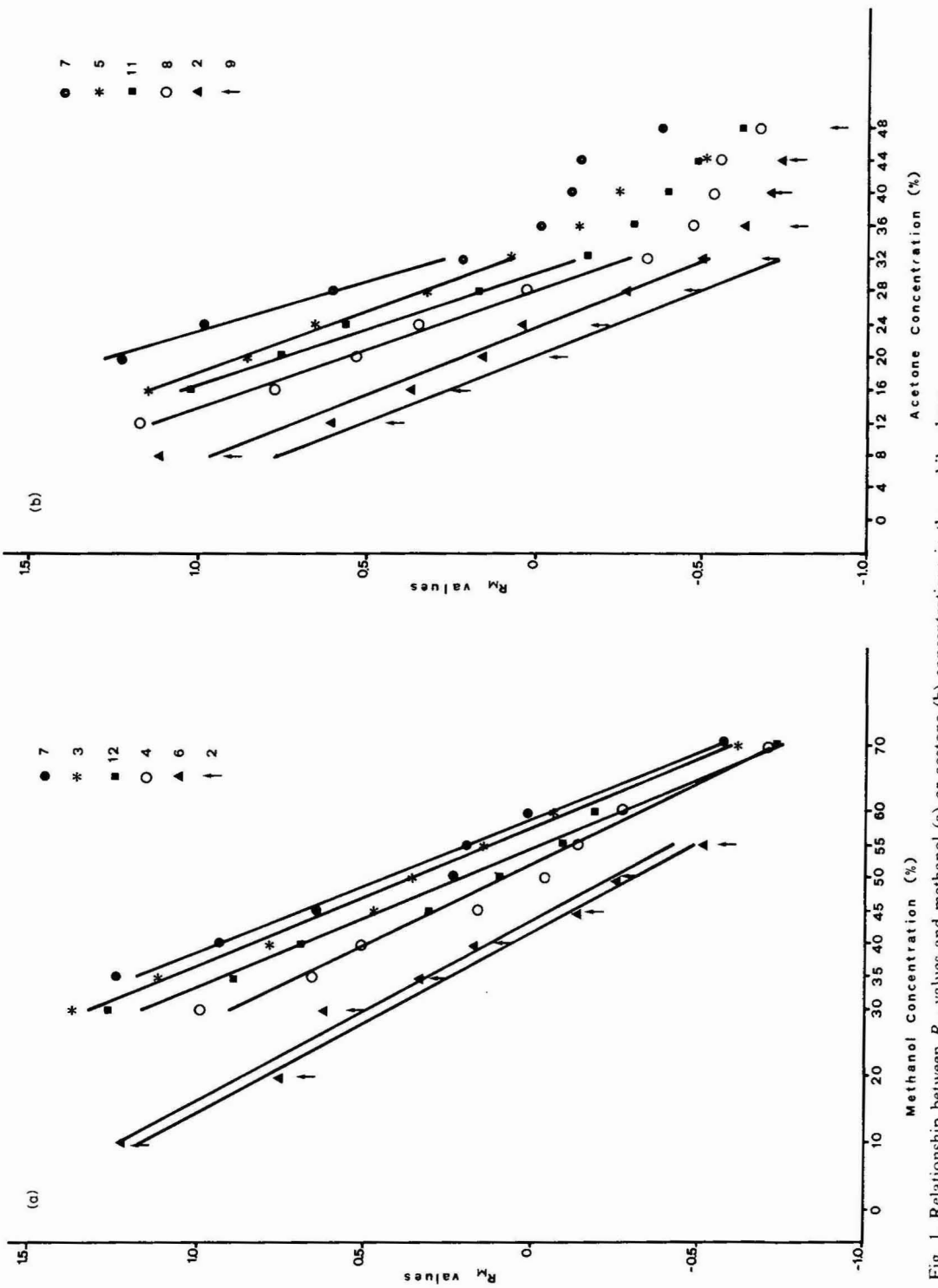


Fig. 1. Relationship between R_M values and methanol (a) or acetone (b) concentrations in the mobile phase.

TABLE II

RELATIONSHIP BETWEEN R_M VALUES OF PROSTAGLANDIN DERIVATIVES AND METHANOL CONCENTRATION IN THE MOBILE PHASE

Compound	*Methanol concentration (%)*													*TLC equation*		
	0	*5*	*10*	*20*	*30*	*35*	*40*	*45*	*50*	*55*	*60*	*70*	*75*	$R_M = a$	*b*	*r*
1	–	–	1.31	0.93	0.69	0.38	0.18	−0.13	−0.26	−0.54	–	–	–	1.788	−0.041	0.994
2	–	–	1.17	0.69	0.54	0.29	0.09	−0.18	−0.27	−0.57	–	–	–	1.542	−0.037	0.992
3	–	–	–	–	1.38	1.12	0.79	0.49	0.37	0.15	−0.06	−0.61	–	2.769	−0.048	0.996
4	–	–	–	–	1.01	0.67	0.52	0.17	−0.03	−0.13	−0.27	−0.70	–	2.136	−0.041	0.990
5	–	–	–	–	1.24	1.01	0.79	0.40	0.12	0.00	−0.13	−0.68	–	2.644	−0.048	0.993
6	–	–	1.22	0.76	0.63	0.34	0.18	−0.12	−0.24	−0.51	–	–	–	1.613	−0.037	0.991
7	–	–	–	–	–	1.25	0.93	0.65	0.24	0.19	0.02	−0.59	–	2.937	−0.050	0.989
8	–	–	–	–	1.03	0.75	0.51	0.12	−0.04	−0.21	−0.26	−0.73	–	2.215	−0.043	0.985
9	–	–	–	0.63	0.55	0.43	0.18	−0.16	−0.24	−0.36	−0.45	−0.80	–	1.377	−0.031	0.981
10	–	–	–	–	–	0.95	0.59	0.10	−0.06	−0.15	−0.43	−0.76	–	2.424	−0.047	0.974
11	–	–	–	–	1.24	0.91	0.56	0.20	0.05	−0.12	−0.21	−0.73	–	2.506	−0.047	0.984
12	–	–	–	–	1.28	0.90	0.70	0.31	0.10	−0.09	−0.18	−0.73	–	2.610	−0.048	0.991

TABLE III

RELATIONSHIP BETWEEN R_M VALUES OF PROSTAGLANDIN DERIVATIVES AND ACETONE CONCENTRATION IN THE MOBILE PHASE

Compound	*Acetone concentration (%)*													*TLC equation*		
	0	*4*	*8*	*12*	*16*	*20*	*24*	*28*	*32*	*36*	*40*	*44*	*48*	$R_M = a$	*b*	*r*
1	–	–	1.41	0.81	0.57	0.28	0.12	−0.28	−0.49	−0.68	−0.76	–	–	1.778	−0.071	0.988
2	–	–	1.12	0.61	0.38	0.16	0.04	−0.28	−0.50	−0.62	−0.71	−0.74	–	1.465	−0.062	0.985
3	–	–	–	–	–	1.30	0.96	0.63	0.30	0.11	0.11	0.05	−0.32	2.788	−0.076	0.995
4	–	–	–	1.09	0.90	0.57	0.40	0.07	−0.26	−0.35	−0.42	−0.57	–	1.940	−0.067	0.995
5	–	–	–	–	1.15	0.86	0.66	0.33	0.07	−0.12	−0.25	−0.50	–	2.228	−0.067	0.998
6	–	–	1.27	0.61	0.48	0.21	0.08	−0.29	−0.43	−0.53	−0.72	−0.90	–	1.579	−0.065	0.975
7	–	–	–	–	–	1.23	0.99	0.61	0.23	−0.03	−0.11	−0.13	−0.37	2.962	−0.084	0.995
8	–	–	–	1.18	0.78	0.54	0.35	0.03	−0.33	−0.47	−0.53	−0.55	−0.67	1.995	−0.071	0.995
9	–	–	0.91	0.44	0.24	−0.06	−0.18	−0.46	−0.71	−0.78	−0.72	−0.77	−0.90	1.286	−0.063	0.990
10	–	–	–	–	1.03	0.86	0.46	0.11	−0.24	−0.46	−0.48	−0.56	−0.72	2.418	−0.082	0.994
11	–	–	–	–	1.03	0.76	0.58	0.17	−0.15	−0.28	−0.39	−0.49	−0.62	2.248	−0.074	0.992
12	–	–	–	–	1.14	0.81	0.56	0.19	−0.16	−0.28	−0.45	−0.53	−0.59	2.440	−0.080	0.998

$$R_{M_{CH_3OH}} = (0.171 \pm 0.177) + (0.975 \pm 0.082)R_{M_{(CH_3)_2CO}} \quad (1)$$
$$n = 12, r = 0.966, \text{S.D.} = 0.141, F = 141.16, P < 0.005$$

Another interesting point arises from the comparison of the slopes of the straight lines describing the relationship between R_M values and the mobile phase composition. The slopes for the methanol and acetone systems have mean values of -0.042 and -0.071, respectively (Tables II and III). The more negative slope in the acetone system is due to the higher eluting power of acetone, when compared with that of methanol. The ratio of 1.69 between the above mean values is very close to the ratio of 1.70 between the solvent-strength parameters, E_0, of methanol (0.95) and acetone (0.56), in a reversed-phase chromatographic system. A very similar result was obtained previously with a series of dermorphin-related oligopeptides[8,11].

Relationship between R_M and log P values

The correlation of the extrapolated R_M values with the log P values in Table I is expressed as

$$R_{M_{(CH_3)_2CO}} = (0.438 \pm 0.171) + (0.734 \pm 0.073) \log P \quad (2)$$
$$n = 12, r = 0.954, \text{S.D.} = 0.163, F = 101.05, P < 0.005$$

$$R_{M_{CH_3OH}} = (0.652 \pm 0.261) + (0.692 \pm 0.111) \log P \quad (3)$$
$$n = 12, r = 0.891, \text{S.D.} = 0.249, F = 38.56, P < 0.005$$

The confidence limits of the intercepts and slopes of eqns. 2 and 3 show that there is no reason to reject the hypothesis that they are from the same population. This is in agreement with eqn. 1, showing that the two sets of R_M values used for calculating eqns. 2 and 3 are not significantly different. Therefore, if in both chromatographic systems the extrapolation yields R_M values which can be considered as a measure of the partitioning of the compounds between water and the hydrophobic stationary phase, *i.e.*, in a standard system, an average R_M value can be calculated for each compound from the extrapolated R_M values in the two different chromatographic systems. Eqn. 4 describes the correlation between the average R_M values and the log P values:

$$R_M = (0.545 \pm 0.209) + (0.713 \pm 0.090) \log P \quad (4)$$
$$n = 12, r = 0.930, \text{S.D.} = 0.199, F = 64.21, P < 0.005$$

The present data seem to provide a further contribution to the use of reversed-phase TLC as a standard system for the measurement of R_M values. Two or three organic solvents might provide extrapolated R_M values for compounds covering a wider range of lipophilicity. In this way one might avoid, at least partially, one of the major disadvantages of the R_M values, *i.e.*, their narrower range when compared with that of the log P values.

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APPLICATION OF DERIVATIVE SPECTROSCOPY TO THE DETERMINATION OF CHROMATOGRAPHIC PEAK PURITY*

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SUMMARY

Derivative spectroscopy was used in the interpretation and exploitation of data obtained from rapid-scan UV–VIS absorbance detectors for ascertaining the purity of chromatographic peaks. Two types of derivatives were examined. The derivative of the elution curve with respect to the specific wavelength at which the major compound has a zero derivative (referred to as the spectral derivative null technique) proved to be rapid and useful for the determination of co-eluting impurity peaks which might form in solution or during chromatography from compounds otherwise known to be pure. The derivatives of the spectral curves obtained during chromatography (referred to as the derivative spectral mapping technique) were also examined both by computer simulation and experimentally and found to have the potential for universal applications in screening compounds for possible overlapping impurities in high-performance liquid chromatographic scans. A novel approach using the derivative spectral mapping technique is described to relate impurity detection limits to chromatographic and spectral resolutions of closely absorbing species which are incompletely resolved chromatographically ($R_c < 0.5$). Using the above techniques, it was possible to detect, under suitable conditions, as little as 0.1% impurity which co-eluted chromatographically with the major compound.

INTRODUCTION

The recent advent of rapid-scanning UV–VIS absorbance detectors in liquid chromatography has made it possible to acquire much more information about a given chromatographic peak. In a sense another dimension has been added to the chromatographic process. In addition to the usual separation on the chromatographic column as a function of time it now becomes possible to observe spectral separation of any given chromatographic peak as a function of wavelength. The question then becomes one of deriving the maximum use from this information. One way of doing

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this is by performing a mathematical manipulation on the data to express it in terms which are readily interpretable in the context of chromatographic peak purity.

The method which was chosen for this study was the one of derivative spectroscopy. There are really two aspects to our approach. The first is one in which the compound under study is of known purity but the processes to which the compound is subject during the chromatographic run are unknown and the formation of impurities which may co-elute with the main band is a possibility. It is necessary to study this problem since the presence of an unresolved component will affect the quantitativeness of our measure of the purity of the main peak. Many workers have used the derivative of the elution curve for various applications[1,2]. In this case, we have monitored the derivative of the elution curve at the wavelength of maximum absorption. The derivative should be ideally zero at this point so that nullification of the main peak is obtained and the presence of other constituents with differing absorption properties is revealed. This procedure will be referred to as the spectral derivative null technique. Two applications of this technique are discussed in the section Applications of the spectral derivative null technique.

The second aspect of the study is the estimation of intrinsic purity of the peak in those cases in which the purity of the compound is not known. This is a question which has occupied many workers recently[3,4], no doubt due to the fairly recent advent of instrumentation which has made it possible to study the problem in greater depth. We chose to approach the problem with the use of complete derivative spectra acquired along the elution curve. Derivative spectroscopy has been applied to the quantitative analysis of mixtures both experimentally and theoretically using computer simulation[5]. The derivative of the spectral curve during chromatography has been studied[6]. Additionally, a slope analysis of the chromatographic peak revealed certain relationships between percent impurity, chromatographic resolution and the ratios of derivative extrema[7]. In this paper, we will combine the various aspects of the problem (including analysis of mixtures, behavior of the derivative, and the changes that take place during chromatography) to develop some measurable parameters which can serve as criteria for peak purity. The development and applications of this technique, which will be referred to as the derivative spectral mapping, are discussed in the section Derivative spectral mapping technique.

EXPERIMENTAL

Reagents and materials

All of the compounds in this study were synthesized at Hoffmann-La Roche and characterized by NMR, IR, mass spectrometry (MS) and UV. Solvents used for liquid chromatography were high-performance liquid chromatographic (HPLC) grade (Fisher).

Equipment

Chromatography was done using a DuPont 870 pump and Waters UK-6 injector. The detector used for peak purity determinations consisted of a Hewlett-Packard (HP) 8450A rapid-scanning UV–VIS spectrophotometer having four cell positions. One of these positions was dedicated as a chromatographic detector and fitted with an adjustable cell holder which held an 8-μl Hellma flow cell that had input

connections for a chromatographic column. The HP8450A was interfaced to an HP82901M disc drive for storage of data as well as an HP7245A plotter. To extend the data processing capabilities of the instrument, it was also interfaced to an HP85A computer.

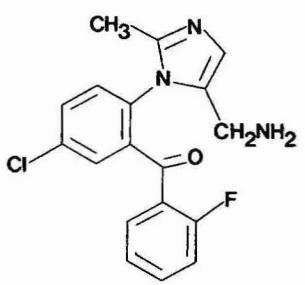
The HP1040A became available for use as a detector. The HP1040A was also interfaced to the HP85A computer as well as the disc drive and plotter.

Software for simulating first and second derivative spectra and their behavior during chromatography was written on the HP85A computer in BASIC.

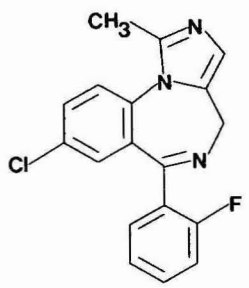
APPLICATIONS OF THE SPECTRAL DERIVATIVE NULL TECHNIQUE

The formation of breakdown products from an otherwise pure compound sometimes occurs during chromatography. The reasons for this may be instability in a solvent system otherwise necessary for dissolution or separation. The compound may be unstable on the column itself. A small number of compounds may be rendered unstable during the photochemical process of detection. Often these changes are revealed by an additional peak in the chromatogram or by tailing of the main component. It is possible however to conceive of a situation in which the breakdown product or products formed are not resolved from the main component. In this situation two sources of error are introduced which might affect the validity of the chromatographic measurement. One source of error is that impurities are present which are masked by the main component. In such situations, the presence of one single chromatographic peak indicates purity that is not actually there. The second source of error is that since quantitative determinations are made at a particular wavelength *versus* a standard, unless the standard is subjected to exactly the same conditions as the sample the presence of other species with different absorption characteristics will affect this determination. The sample may be subjected to different conditions from that of the standard simply by virtue of the fact that it is in a different matrix or that it is analyzed at a different time.

To illustrate the procedure of using the derivative of the elution curve to reveal co-eluting impurities, we give two examples. In both cases, compounds of known purity were made to undergo known chemical reactions and the products formed artificially induced to co-elute with their parent compounds. The first illustration is that of the reversible equilibrium reaction between 8-chloro-6-(2-fluorophenyl)-1-



I



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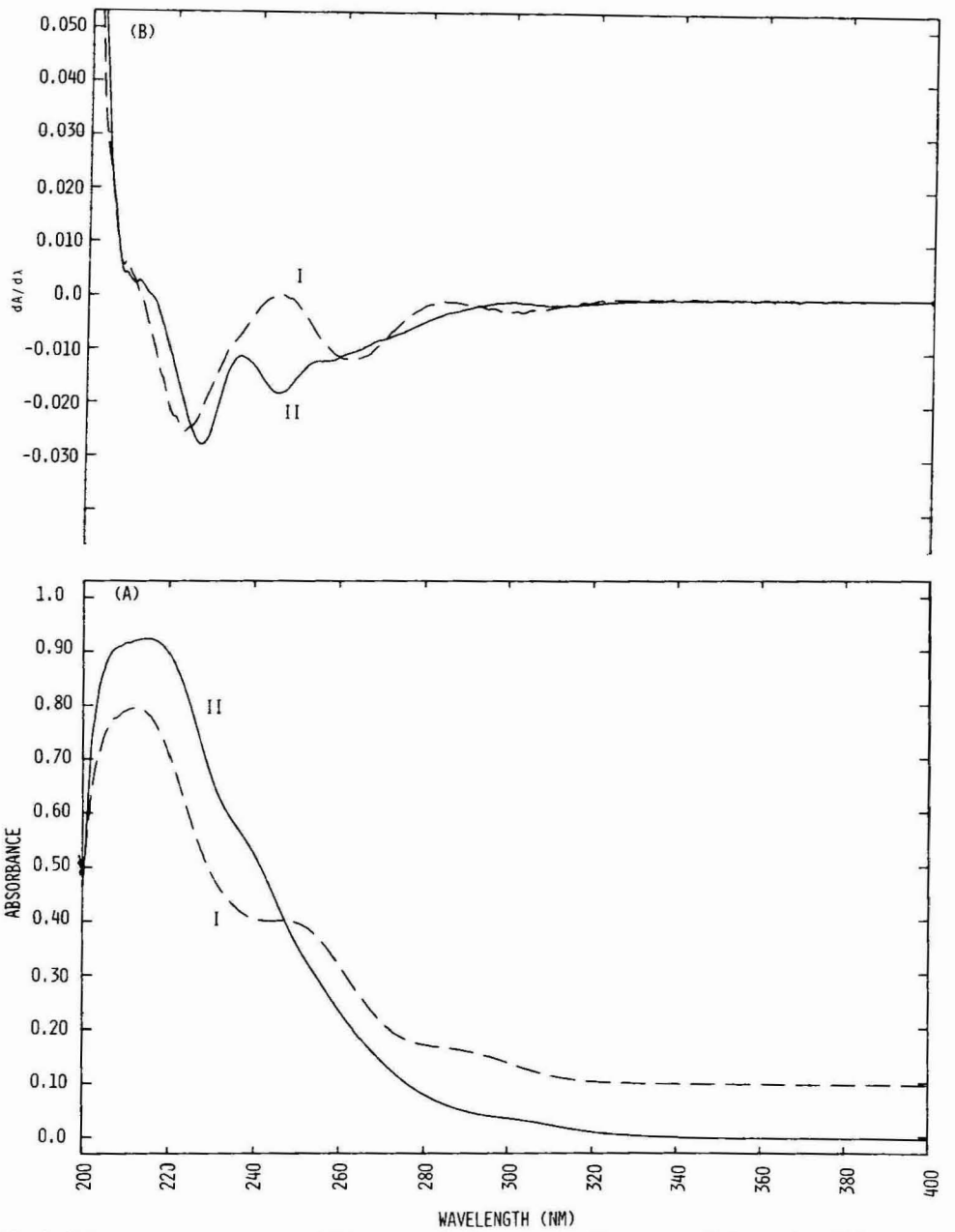


Fig. 1. (A) Absorption spectra and (B) first derivative spectra of 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-*a*][1,4]benzodiazepine (II) and its benzophenone form (I).

methyl-4H-imidazo[1,5-*a*][1,4]benzodiazepine (II) and its benzophenone form (I). A comparison of the first derivative spectral curves of the two compounds (Fig. 1) indicates that, at 245 nm, the derivative of I is approaching zero absorbance while

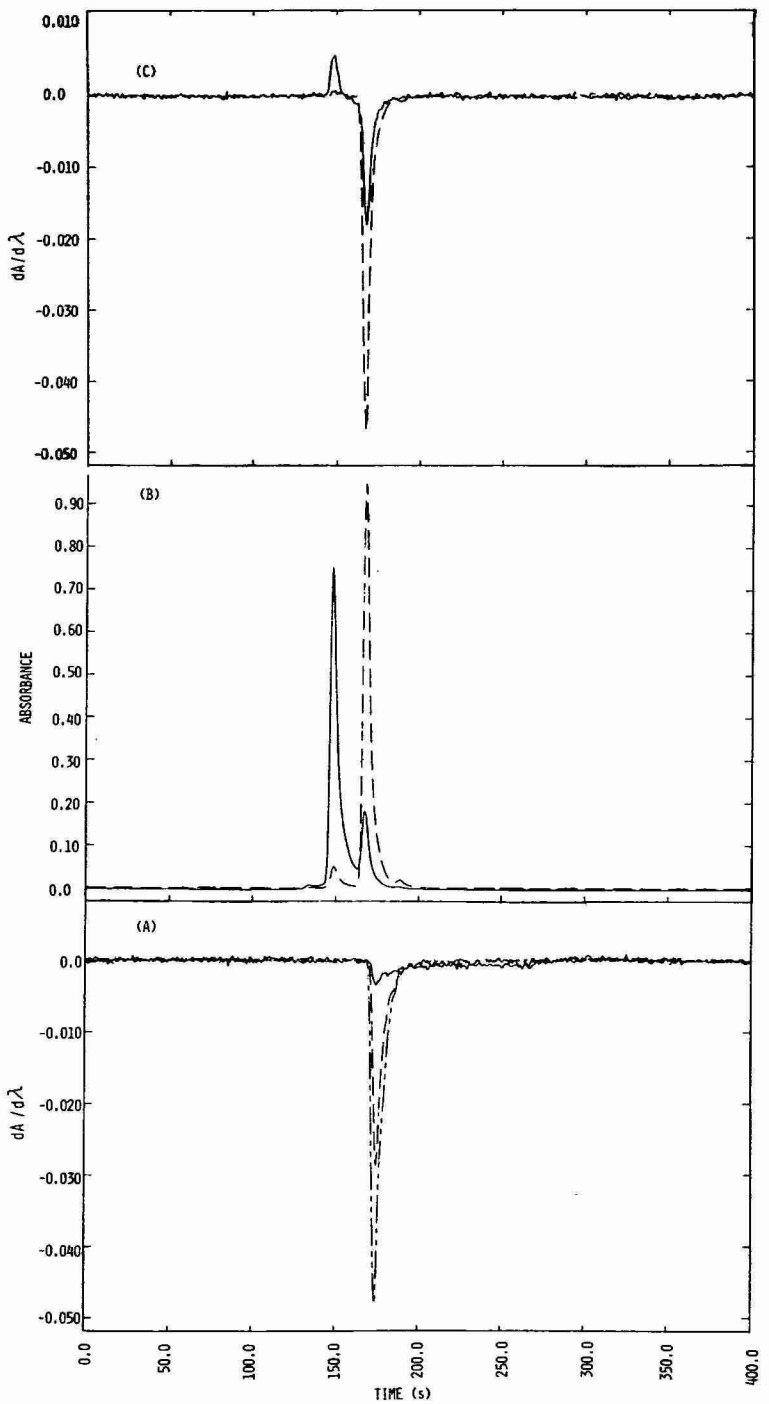
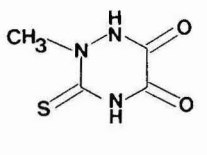


Fig. 2. (A) Spectral derivative of elution curve at 245 nm for the chromatographic system in which compounds I and II co-elute; (B) Elution profile at 245 nm for the system in which I and II are chromatographically separated; (C) Spectral derivative of elution curve for case B. The various curves are identified as follows: zero time (–); 0.5 h (--); final (———).

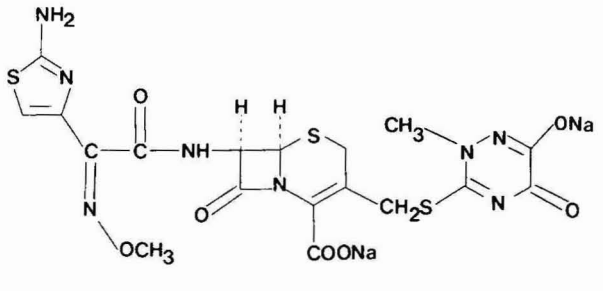
the derivative of II is at a maximum (negative quantity). It can be seen from the zeroth derivative curves that no such differentiation is possible since the curves overlap in all regions of the spectrum. If the derivative of the elution curve is monitored at 245 nm, the formation of II from I can be followed without the necessity for a separation. This situation can be artificially induced to occur by choosing chromatographic conditions such that I and II coelute. Using a reversed-phase column (Ultrasphere 5 μm, 25 cm $\times$ 4.6 mm) and methanol as a mobile phase, the formation of I in methanol solution was followed by making chromatographic injections of a solution of I at various time intervals over an 8-h period and monitoring the chromatographic peak of the first derivative at 245 nm. By measuring peak height at these intervals, the half-life of the combined reactions occurring in solution and on the column is seen to be about 0.5 h (Fig. 2A). Use of the derivative of the elution curve may also yield information about very fast processes which are happening on the chromatographic column. This is observed (Fig. 2B) during the chromatography of I in a mobile phase which is composed of a mixture of phosphate buffer (pH 7)–methanol (1:10).

Fig. 2B shows the formation of II from I at various intervals. This reaction occurs more rapidly than the one in methanol, and monitoring the derivative at 245 nm (see Fig. 2C) reveals a positive inflection in the first peak (compound I) which diminishes with time because of the formation of II (second peak). The most likely interpretation of this observation is that compound I has a slightly positive derivative which is not ordinarily detectable because of the rapid equilibrium of I $\leftrightarrow$ II under most conditions of measurement. It is apparent that this type of technique can be used to reveal other anomalies of a given chromatographic peak, such as tailing.

The second example was chosen to monitor thiotriazinone (III) in the presence of ceftriaxone disodium salt (IV) and is an illustration of the linearity and detection limits possible using the technique of the derivative of the elution curve at a chosen wavelength. From a comparison of the first derivative curves (Fig. 3), it can be seen that monitoring the first derivative of the elution curve at 271 nm should result in a positive inflection for thiotriazinone while the signal for ceftriaxone will be effectively blanked out in an HPLC system in which the two compounds coelute. The result of making various dilutions of thiotriazinone in ceftriaxone and monitoring their chromatography at the derivative of the elution curve at 271 nm is shown in Fig. 4A. In this case, a detection limit as low as 0.1% was achieved (Fig. 4B).



III



IV

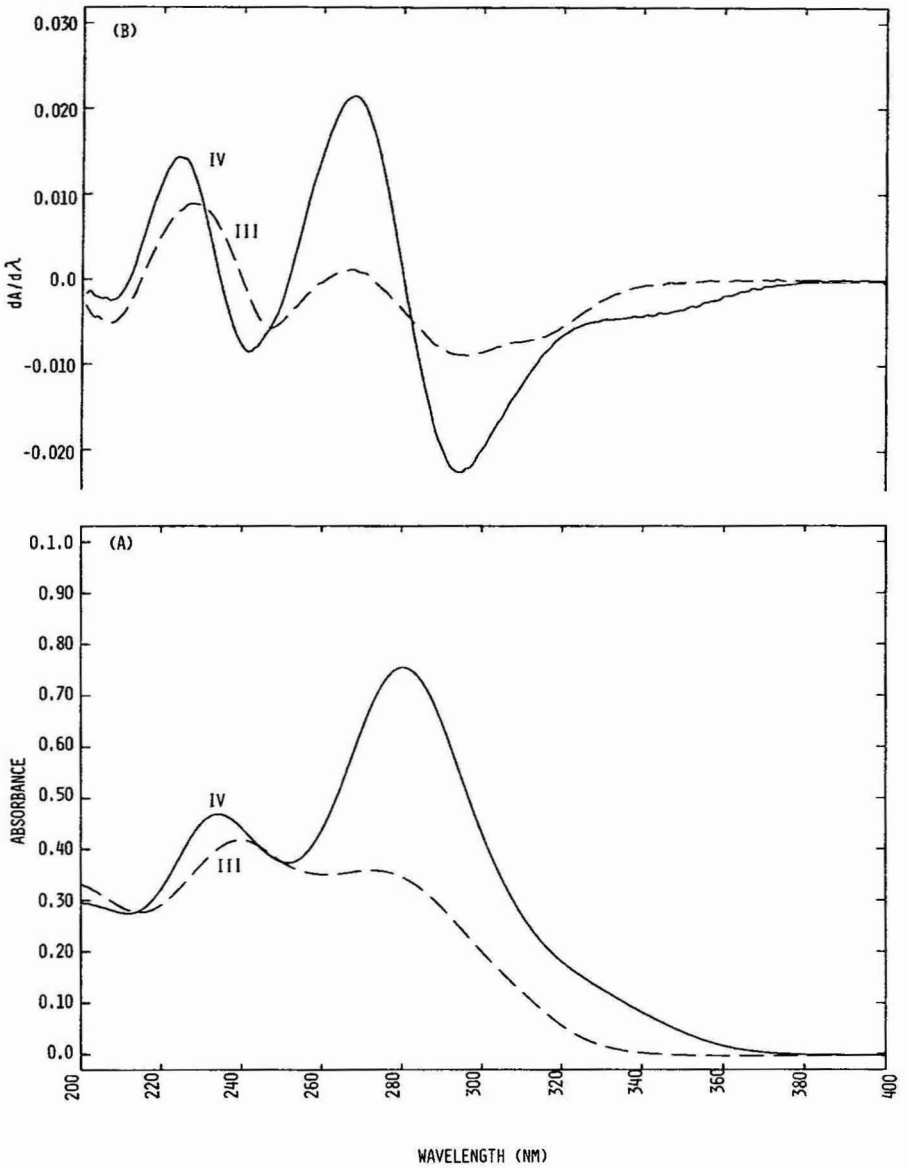


Fig. 3. (A) Absorption spectra and (B) first derivative spectra of thiotriazinone (III) and ceftriaxone disodium salt (IV).

DERIVATIVE SPECTRAL MAPPING TECHNIQUE

Theory

We have arbitrarily imposed certain limits in the development of this technique. We discuss particular cases in which the spectral curve of the suspected impurity falls within the bandwidth of the spectral curve of the main compound. In

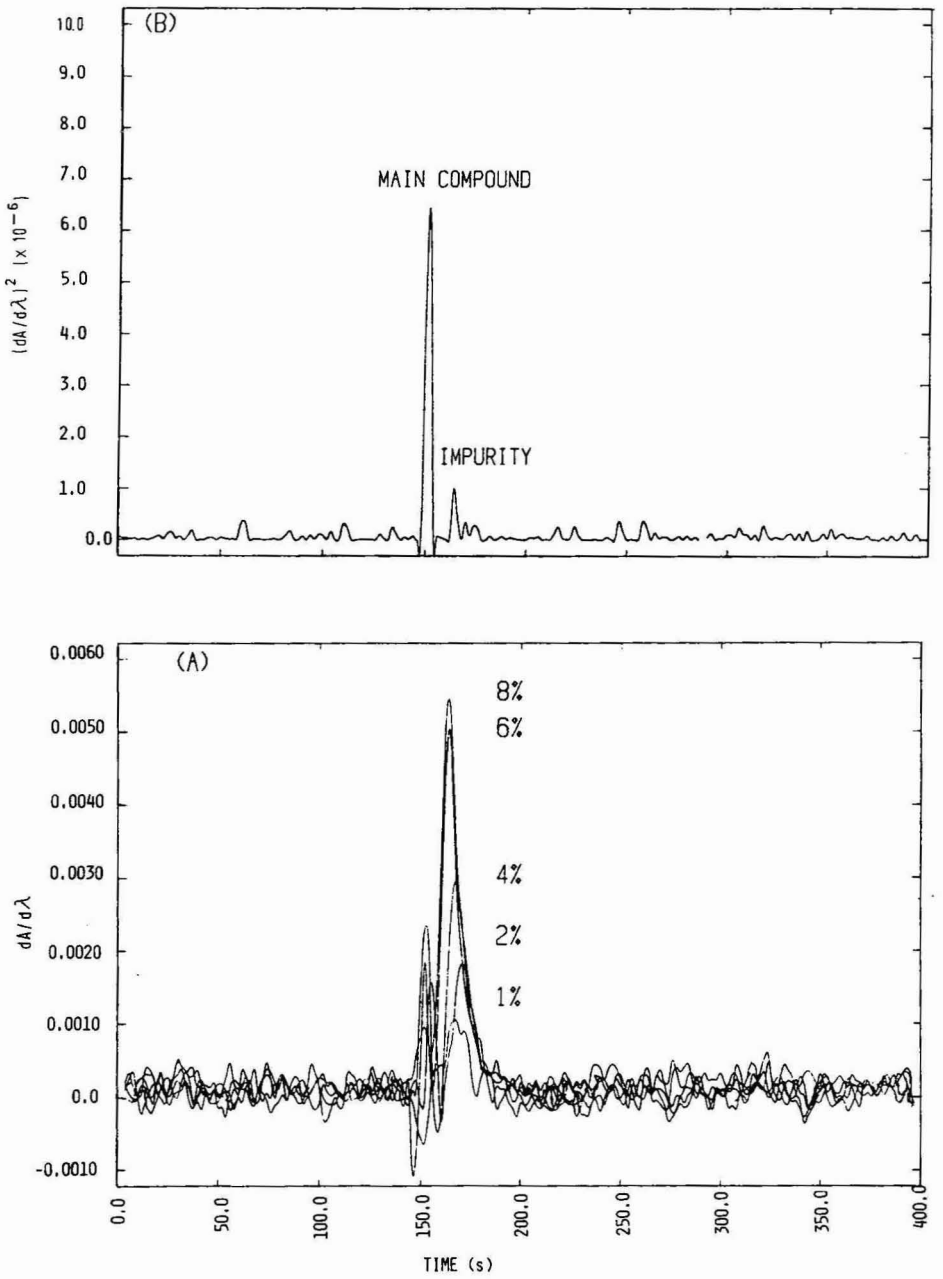


Fig. 4. (A) Investigation of linearity and (B) detection limit (impurity level 0.1%) for the chromatographic system in which the impurity (III) and compound (IV) overlap. The square of spectral derivative is plotted in B for increased sensitivity.

addition, we assume some spectral and chromatographic disparity between the two components. These procedures, while not appearing to be very general, allow us to draw some valuable quantitative relationships between peak purity and spectral and chromatographic resolutions and, in fact, may be applied in a number of real situations.

The equation for a given spectral curve may be expressed in terms of a Gaussian equation:

$$y = A \exp\left[\frac{-(\lambda - C)^2}{2B^2}\right] \tag{1}$$

where y is the absorbance, λ the wavelength, C the wavelength of maximum absorption, A the absorptivity and B the half-bandwidth. The first and second derivatives of this expression are

$$\begin{aligned} \frac{dy}{d\lambda} &= -\frac{A(\lambda - C)}{B^2} \exp\left[\frac{-(\lambda - C)^2}{2B^2}\right] \\ \frac{d^2y}{d\lambda^2} &= \frac{A}{B^2}\left[\frac{(\lambda - C)^2}{B^2} - 1\right] \exp\left[\frac{-(\lambda - C)^2}{2B^2}\right] \end{aligned} \tag{2}$$

The equations for the composite curves are also given:

$$\begin{aligned} Y &= A \exp\left[\frac{-(\lambda - C)^2}{2B^2}\right] + A_1 \exp\left[\frac{-(\lambda - C_1)^2}{2B_1^2}\right] \\ \frac{dY}{d\lambda} &= -\frac{A(\lambda - C)}{B^2} \exp\left[\frac{-(\lambda - C)^2}{2B^2}\right] - \frac{A_1(\lambda - C_1)}{B_1^2} \exp\left[\frac{-(\lambda - C_1)^2}{2B_1^2}\right] \\ \frac{d^2Y}{d\lambda^2} &= \frac{A}{B^2}\left[\frac{(\lambda - C)^2}{B^2} - 1\right] \exp\left[\frac{-(\lambda - C)^2}{2B^2}\right] + \\ &\quad \frac{A_1}{B_1^2}\left[\frac{(\lambda - C_1)^2}{B_1^2} - 1\right] \exp\left[\frac{-(\lambda - C_1)^2}{2B_1^2}\right] \end{aligned} \tag{3}$$

where Y is the absorbance of composite curve, C_1 the wavelength of maximum absorption of added component, A_1 the absorptivity of added component and B_1 the half-bandwidth of added component.

Fig. 5 shows the first and second derivative simulated curves for 1:1 mixtures of a compound with a UV maximum at 360 nm with various other compounds absorbing at longer and shorter wavelengths, all with equal absorptivities. This diagram indicates that the effect of the addition of such impurities will be to shift the zero-crossing wavelength, which is the wavelength where the derivative curve crosses through zero value on the wavelength axis, either to the right or the left of the original curve.

During chromatography, of course, the relative proportions of the two com-

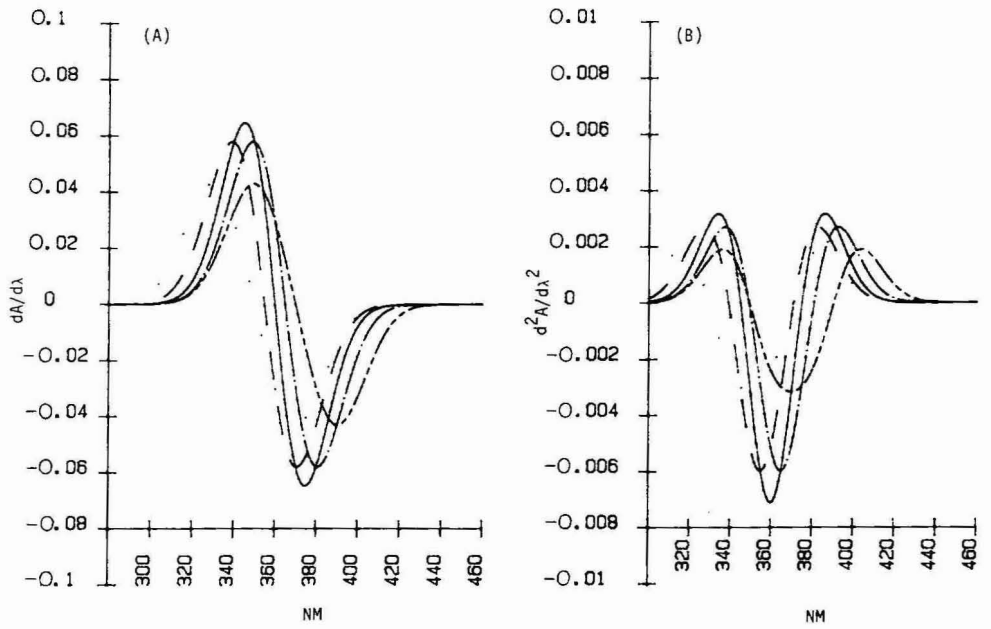


Fig. 5. (A) Simulated first derivative of composite spectra and (B) simulated second derivative of composite spectra of species added in the ratio of 1:1. The mixtures of species with the given UV maxima are identified as follows: 340 and 360 nm (. . . . .); 350 and 360 nm (----); 360 nm only (——); 360 and 370 nm (– . – . – .); and 360 and 380 nm (—·—).

ponents are constantly changing. To represent this situation, an expression is derived for the change of the derivative of the composite spectral curve with respect to time.

The equation of the chromatographic curve may also be approximated by a Gaussian function:

$$z(\lambda,T) = H(\lambda) \exp [-(T - T_0)^2/2D^2] \tag{4}$$

where T is the time, T_0 the retention time, D the bandwidth and $H(\lambda)$ the peak height. Here, $H(\lambda)$ is a function of λ since it is apparent that the magnitude of the chromatographic peak will vary depending on which wavelength we choose to monitor the chromatography. Thus, it will be dependent on the nature of the spectral curve. Therefore, the general expression for the chromatographic curve at any wavelength is given by

$$z(\lambda,T) = A_m \exp (w) \tag{5}$$

where $w = -\dfrac{(\lambda-C)^2}{2B^2} - \dfrac{(T-T_0)^2}{2D^2}$ and A_m is the maximum absorbance for all λ and T values. It would be the absorbance at the peak of the elution curve at λ_{max}.

Finally the expressions for the changes of the derivative spectral curves during

chromatography, *i.e.*, the corresponding derivative chromatographies, would be

$$\frac{dz}{d\lambda} = -\frac{A_m}{B^2}(\lambda - C)\exp(w)$$
$$\frac{d^2z}{d\lambda^2} = \frac{A_m}{B^2}\left[\frac{(\lambda - C)^2}{B^2} - 1\right]\exp(w) \quad (6)$$

The expressions for two co-eluting compounds would be

$$\frac{dZ}{d\lambda} = -\frac{A_m}{B^2}(\lambda - C)\exp(w) - \frac{A_{1m}}{B_1^2}(\lambda - C_1)\exp(w_1)$$
$$\frac{d^2Z}{d\lambda^2} = \frac{A_m}{B^2}\left[\frac{(\lambda - C)^2}{B^2} - 1\right]\exp(w) + \frac{A_{1m}}{B_1^2}\left[\frac{(\lambda - C_1)^2}{B_1^2} - 1\right]\exp(w_1) \quad (7)$$

where w_1 is the corresponding value of w for the added component. Based on the equations derived above, algorithms were developed for programs to be used to simulate the behavior of the first and second derivatives during chromatography and, by using these programs, various simulations were made for the first and second derivative chromatography of two compounds absorbing at 348 and 360 nm respectively (Figs. 6–8). Here, it can be seen that the effect of the added component during chromatography is to change the point of zero-crossing of the derivative spectral curve. The magnitude of this change would depend upon the ratio of the two com-

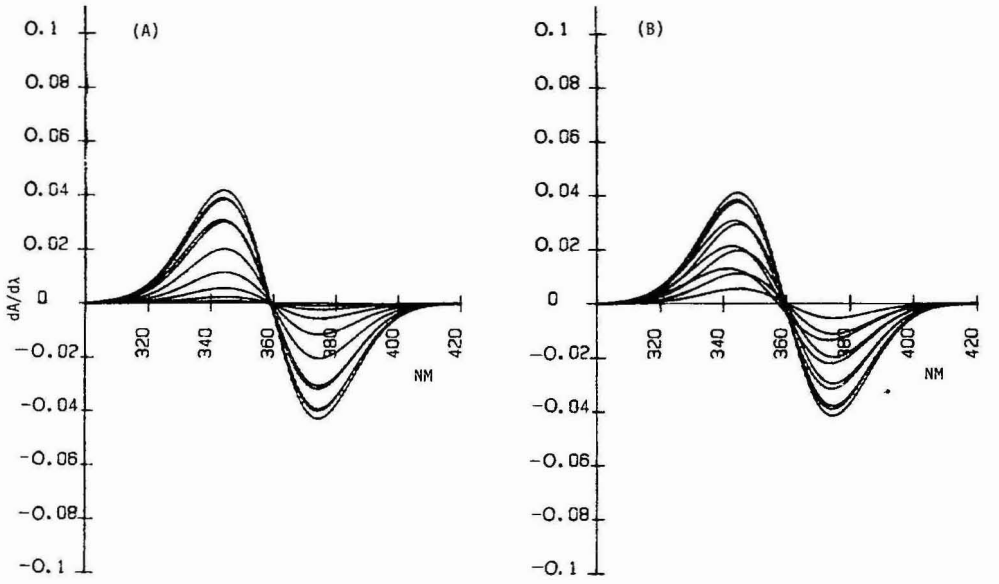


Fig. 6. Simulated first derivative spectra of the elution profile of 1:10 mixture of two chromatographically overlapping compounds absorbing maximally at 348 and 360 nm, respectively, with the chromatographic resolutions (R_c) of 0.1 (A) and 0.3 (B). The various first derivative spectra are shown during the chromatographic elution of the composite peak with a bandwidth at half-height of *ca.* 50 s.

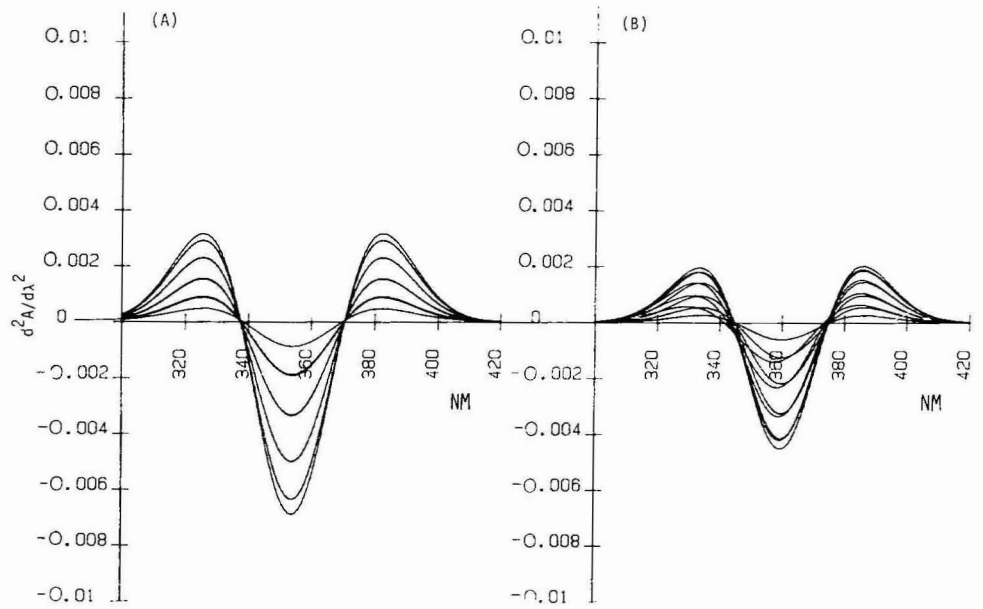


Fig. 7. Simulated second derivative spectra of the elution profile of two compounds absorbing maximally at 348 and 360 nm, respectively, for (A) 1:1 mixture with no chromatographic resolution and (B) 1:10 mixture with a chromatographic resolution of 0.3. The various second derivative spectra are shown during the elution of the composite peak with a bandwidth at half-height of *ca.* 50 s.

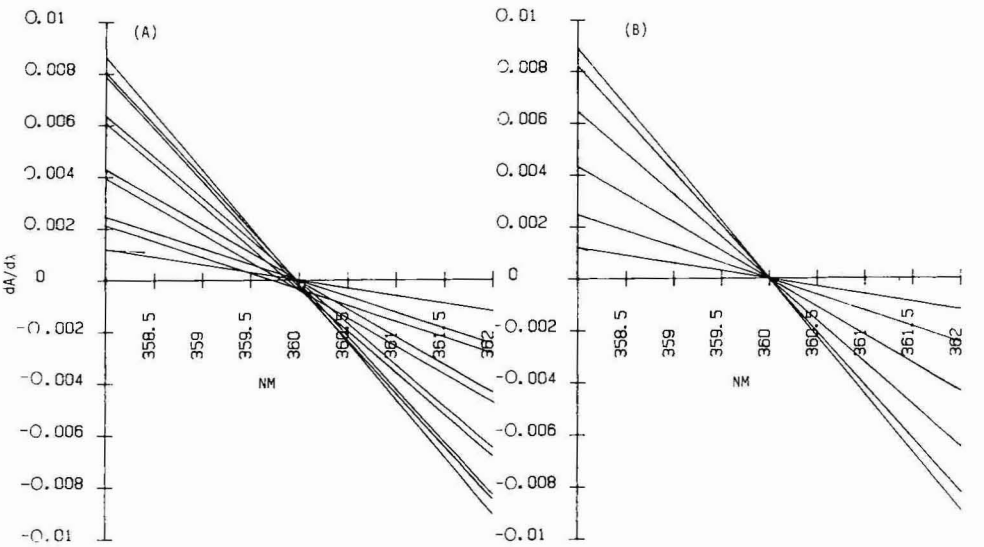


Fig. 8. Zero-crossings in the simulated first derivative spectra of the elution of (A) 1:100 mixture of compounds with the respective UV maxima at 348 and 360 nm (expanded scales); (B) pure compound with the UV maximum at 360 nm.

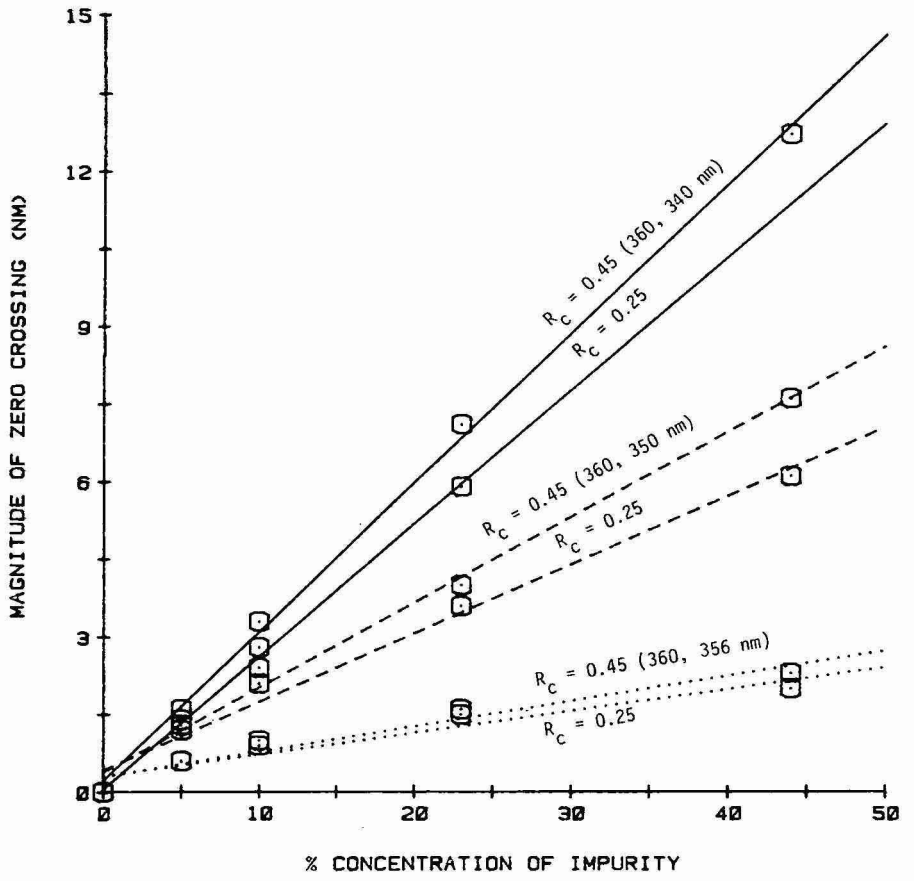


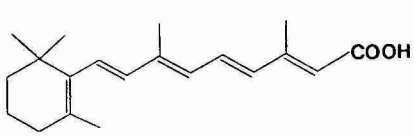
Fig. 9. Computer simulation of the magnitudes of zero-crossings of the first derivative spectra of a major compound (UV maximum at 360 nm) containing various concentrations of impurity as shown. The pairs of lines correspond to different chromatographic resolutions (R_c) between the major compound and impurity with different UV maxima for the latter, as indicated.

ponents and the spectral and chromatographic resolutions between them. These relationships are shown in Fig. 9.

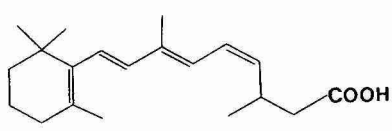
Results and discussion

To test the results obtained by computer simulation, shown in the previous figure, an experiment was performed. The compounds used in this case were all-*trans*-retinoic acid (V) and 11,13-di*cis* isomer of retinoic acid (VI).

The spectra of the two compounds are shown in Fig. 10. The two compounds



V



VI

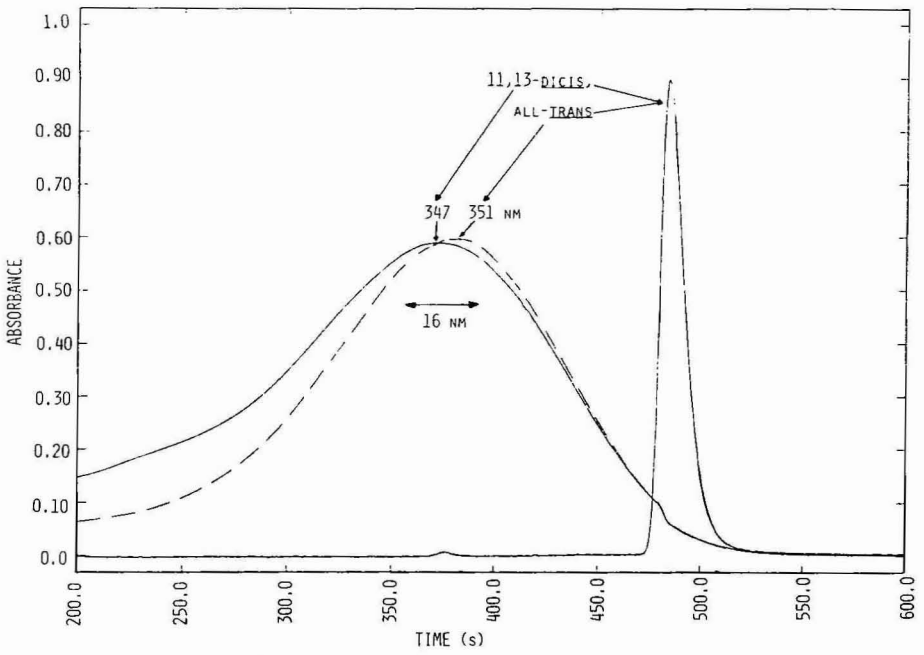


Fig. 10. The HPLC peaks from 1:10 mixture of 11,13-di-*cis*- and all-*trans*-retinoic acid are unresolved (R_c = 0.25) in the system. Corresponding spectral curves are shown to indicate wavelengths for maximum absorptions.

were made to co-elute with two different solvent compositions to give two different chromatographic resolutions. In the first case, a Zorbax Sil column was used with a mixture of ethyl acetate–hexane (5:95) containing 0.1% acetic acid to give a chromatographic resolution (R_c) of 0.45, which was determined by measuring the retention time and bandwidth of each individual chromatographic peak from two separate injections and computing the resolution from the formula:

$$R_c = \frac{T_2 - T_1}{2(\sigma_2 + \sigma_1)} \tag{8}$$

where T_2 and T_1 are the retention times of the two peaks and σ_2 and σ_1, the respective half-bandwidths at the inflection points. (Technically, two peaks with $R_c < 0.5$ are unresolved chromatographically.) By increasing the amount of ethyl acetate in the solvent system to 6%, a resolution of 0.25 was obtained. The chromatogram at this resolution is shown in Fig. 10. At each of these resolutions, various dilutions of the 11,13-di*cis*-retinoic acid in all-*trans*-retinoic acid were made and chromatography was done. Spectra were taken across the entire length of the chromatographic peak at 10-s intervals (the spectra were accumulated for a 10-s period to obtain a better signal-to-noise ratio) and the derivatives of these spectral curves examined. The magnitude of zero-crossing for each experiment was obtained by taking the difference in nanometers between the zero-crossing wavelengths of the derivatives of the first spec-

trum taken and the last spectrum taken. The results of these experiments for a series of dilutions are shown in Fig. 11.

Let us now examine the above illustration of overlapping peaks in which $R_c = 0.25$ and the spectral peaks of the major compound and impurity differ by 4 nm. In this case, using Figs. 9 and 11, it is seen that 5% of the impurity can be detected in the presence of the major compound with which it overlaps. The simulated zero-crossings of composite curves (Fig. 9) were obtained for different compounds having similar absorptivities. Greater sensitivity might be obtained in those situations in which there were greater separation between spectral peaks and the impurity had a greater absorptivity than the main compound. For example, in the exceptional cases in which the spectral peaks of the major compound and impurity are 20 nm apart and the two compounds are just barely unresolved chromatographically ($R_c = 0.45$), it should be possible to see as little as 0.4% impurity for comparable absorptivities. The detection limit drops to 0.1% if the absorptivity of the impurity is four times higher than that of the major compound.

It should be realized that simulating the zeroth derivative spectra of a multi-

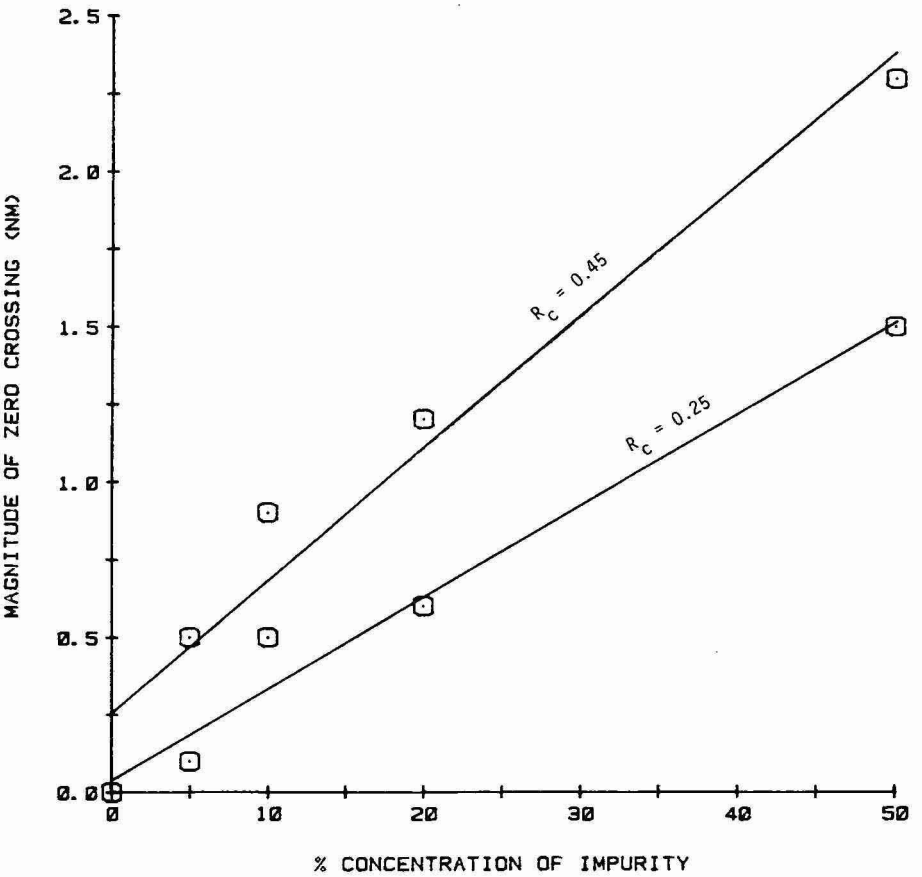


Fig. 11. Experimental determinations of zero-crossing magnitudes of all-*trans*-retinoic acid (tretinoin) doped with various amounts of 11,13-di-*cis* retinoic acid (as impurity) at two different chromatographic resolutions as shown.

component mixture during chromatography should also serve to indicate the effect of an impurity on the composite spectra obtained. There were, however, in spite of lower signal-to-noise ratio of the first derivative relative to that of the zeroth derivative, certain advantages in choosing the first derivative to perform the simulations. One advantage was that choosing the first derivative allowed the point of reference to be zero, which is somewhat less ambiguous than finding the exact wavelength of maximum absorption of a composite, both from an experimental and a theoretical point of view. Experimentally, it is often difficult to choose one single wavelength of maximum absorption on a curve since there may be a number of isoabsorbance points. Additionally, the wavelength may only be determined to 1 nm on the HP8450 and 2 nm on the HP1040, and the peak maximum may be at some intermediate point between these limits. By displacing the maximum through zero with the use of the derivative, one point and one point only is determined which need not be an integral wavelength. Theoretically, it seemed that an easier task was to define the zero-crossing magnitudes with the derivative equations previously given than to locate the composite peak maximum using the Gaussian spectral equations since, in order to do the latter, one would have to compute the resultant bandwidth of the composite curve to characterize the peak maximum properly.

A comparison of the two lower curves of Fig. 9 to that of Fig. 11 shows agreement between experimental and theoretical results in that the magnitude of zero-crossing of the experimental derivative spectra falls within the general range predicted for closely absorbing species at the chromatographic resolution chosen. Exact agreement would depend upon how closely experimental curves conform to ideal Gaussians. Even though this would probably be the exception rather than the rule, the important consideration in terms of peak purity is that a pure compound should have no spread in the magnitude of zero-crossing during chromatography; whereas, an impure compound may exhibit a change depending upon whether some chromatographic and spectral resolutions are present. However, in reality, this statement must be qualified since there is a certain spread in the magnitude of zero-crossing even for a pure compound because of wavelength reproducibility (which is, *e.g.*, 0.05 nm for HP8450), concentration dependence of spectral curves and experimental errors caused by signal-to-noise ratios of derivative spectra obtainable in particular situations. For example, using the chromatographic system of retinoic acid as a model, it was determined that the spread in the zero-crossing of a pure chromatographic peak ranged from 0.3 nm for the HP1040 HPLC detector to 0.5 nm for the HP8450 UV–VIS spectrophotometer. Such spreads in zero-crossing of a pure chromatographic peak would be the limiting factor for the lowest limit of detection of a co-eluting impurity.

CONCLUSION

It was demonstrated that, in very favorable cases, impurities which chromatographically co-eluted with the major compound could be detected at levels as low as 0.1%, essentially by the suppression of the signal from the major compound. For this purpose, the two techniques of spectral derivative null and derivative spectral mapping were described and utilized. The spectral derivative null technique was found to be especially rapid and useful in the detection of certain chromatographi-

cally overlapping products which could form from pure compounds because of their degradation or instability under certain conditions. It was realized that the spectral derivative mapping technique was much more general and powerful, even though slightly slower, in disclosing the presence of impurities which might overlap in HPLC scans with the major compound of unknown purity. Because of the potential of the latter technique for universal applications such as screening compounds for undetected impurities or decomposition products in HPLC studies, its advantages must be fully exploited.

In our work, conclusions have been drawn based on results obtained by the technique employing ideal systems in which the chromatographic peak maximum of an impurity falls within the bandwidth of the main component. These conclusions should also be valid for non-ideal systems in which the absorption spectrum of the major compound consists of several bands which combine to form the composite spectrum. In such cases, it is possible to have multiple zero-crossings in the derivative spectrum of a pure compound. All of these zero-crossings can then be exploited to our advantage to optimize the determination of the chromatographic peak purity, utilizing techniques similar to that discussed in this paper.

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PURIFICATION OF SYNTHETIC LIPID ASSOCIATING PEPTIDES AND THE MONITORING OF THE DEFORMYLATION OF N^{in}-FORMYLTRYPTOPHAN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A series of five synthetic lipid assocating peptides have been purified by reversed-phase high-performance liquid chromatography (HPLC). Volatile mobile phases were used to allow facile isolation of the purified peptide. These consisted of either 0.1 *M* ammonium bicarbonate, pH 7.9, or 0.5 *M* ammonium formate, pH 6.3, with gradients of 2-propanol and acetonitrile. The columns used were either Radial-Pak C_{18} or Radial-Pak CN. The recovery of the purified peptides was usually in excess of 95%. In addition, this chromatographic system allowed the facile purification of the product isolated from the removal of the N^{in}-formyl protecting group from tryptophan. The use of reversed-phase HPLC has allowed an improvement in both the purity and yield of purified material when compared with the results of low-pressure gel filtration and ion-exchange chromatography. The use of guanidine hydrochloride in the injection mixture and the presence of organic solvents in the mobile phase have allowed the minimization of aggregation of the lipid associating peptides with a consequent improvement in the chromatographic properties of these peptides.

INTRODUCTION

The purification of lipophilic peptides has been shown to be difficult[1] and often characterised by low yields. Recently we have investigated a variety of mobile phases that are designed to facilitate the preparative separation of peptide samples, such as trifluoroacetic acid[2], heptafluorobutyric acid[3] and ammonium bicarbonate[4]. The low yields previously observed with the purification of these lipophilic peptides can be partly attributed to the low solubility of these peptides in aqueous solvents and their tendency to aggregate. As will be shown in this report, semi-preparative reversed-phase high-performance liquid chromatography (HPLC), with a mobile phase which contained ammonium bicarbonate or formate as an ionic modifier, allowed the rapid and high yield purification of lipid associating peptides.

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MATERIALS AND METHODS

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC gradient system was used for the separations (see refs. 4 and 5 for details). Sample injections were made using a microliter 802 syringe or a 10-ml gas-tight 1010 W syringe (Hamilton, Reno, NV, U.S.A.). The Radial-Pak CN and C_{18} cartridges (8 mm I.D.) were also purchased from Waters Assoc. For optimal column life the column was protected with a guard column and an in-line prefilter (Waters Assoc.).

UV spectra were determined using a Shimadzu MPS-5000 instrument.

Chemicals and separation conditions

The solvents and chemicals used in this study were identical to those described in a previous paper[4]. The peptide solutions were prepared as described previously[2], except that all solutions contained 6 *M* urea or 3 *M* guanidine–HCl to prevent sample aggregation.

The ammonium bicarbonate buffer (0.1 *M*, pH 7.7) was prepared freshly each day. The separation was achieved at ambient temperatures and a flow-rate of 1 ml/min was used. The eluted peptides were detected by UV absorbance (generally at 280 nm).

Synthesis procedure

The process used to synthesise the peptides is based on procedures described previously and used the generation of an *in situ* symmetrical anhydride[5]. Syntheses were performed on a scale of 2 g Boc-amino acid resin for all peptides except the synthesis of peptide 199 which was performed on a scale of 3 g Boc-amino acid resin. Each oil obtained from the HF cleavage reaction was dissolved in a minimum volume of 65% acetic acid and loaded onto a Sephadex G-10 gel filtration column (32 cm × 5.5 cm I.D.) at approximately 10°C. The eluted fractions containing the bulk of absorbance at 280 nm were pooled, diluted to approximately 1 l in volume with water and freeze dried.

Deformylation procedure and semi-preparative separations

A sample of 79.1 mg of Trp(CHO)-peptide 208 was dissolved in 100 ml of water and 57.3 g of guanidine hydrochloride. This solution was cooled to 4°C and 14 ml of ethanolamine were added. The solution was stirred for 5 min while the pH was monitored (apparent pH 11.8–11.9). After this time the solution was titrated to pH 7.8 with 6 *M* hydrochloric acid. UV spectra before and after the deformylation showed that the deformylation had been completed (see Fig. 1).

The semipreparative separations used in this study will be illustrated by the following example:

A 60-ml portion of the neutralised deformylation mixture containing approximately 26 mg of peptide 208 was diluted 3-fold with 0.1 *M* ammonium bicarbonate and loaded directly onto a Radial-Pak-C_{18} column via Pump A of the HPLC system. The sample was eluted with a gradient of 2-propanol. The recovery of peptide was 17% of the theoretical yield derived from the initial substitution on the resin. However, the recovery of a sample of the purified peptide rechromatographed on the same

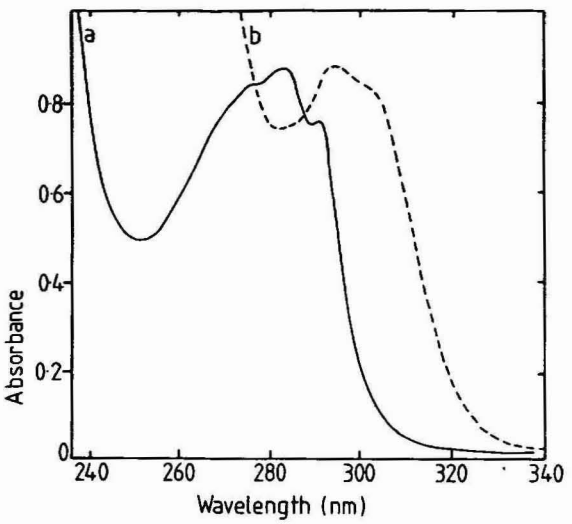


Fig. 1. A comparison of the UV spectra of peptide 208 (curve a) and Trp(CHO)-peptide 208 (curve b).

column was 96% (based on absorbance measurements of the isolated material, $\lambda_{max.}$ = 280 nm; molar absorptivity, ε_M (molar extinction coefficient) = 5200 l mol^{-1} cm^{-1}; mol.wt. 2278). This yield, which was determined here by spectrophotometric techniques, correlated well with corresponding dry weights and amino acid analyses obtained for the samples.

RESULTS AND DISCUSSION

It is now widely appreciated that reversed-phase HPLC offers an excellent procedure for the purification of synthetic peptides[5–7]. Preparative procedures are facilitated by the use of volatile mobile phases such as 0.1% trifluoroacetic acid[8,9] or 0.1 *M* ammonium bicarbonate[4,10]. This paper will demonstrate that reversed phase HPLC with a mobile phase that contains 0.1 *M* ammonium bicarbonate allows the chromatography of lipid associating peptides without the normal problems of aggregation, insolubility, incomplete resolution and low recoveries, often observed during low-pressure gel filtration and ion-exchange chromatography[1]. This system also avoids the tedious work-up of reaction mixtures normally encountered after removing the N^{in}-formyl group used for the protection of tryptophan during solid phase peptide synthesis[5]. The lipid associating peptides used in this study are listed in Table I.

The N^{in}-formyl group was removed with the procedure developed by Sparrow[11,12], which used ethanolamine as the nucleophile to displace the formyl group from the indole ring of tryptophan. Lipid associating peptides are designed to bind to phospholipid micelles and in the absence of lipid tend to self-associate[13]. Since aggregation can inhibit the deformylation reaction, a protein denaturant, guanidine hydrochloride, was added to the reaction mixture. It was previously shown that a peptide could be readily desalted by reversed-phase chromatography, since ionic material such as guanidine hydrochloride is not retarded by the reversed-phase col-

TABLE I

SEQUENCE OF LIPID ASSOCIATING PEPTIDES PREPARED BY THE SOLID PHASE METHOD

Synthesis number	*Peptide sequence*
199	H-Arg-Ala-Leu-Ala-Ser_5-Ser-Leu-Lys-Glu-Tyr_{10}-Trp-Ser-Ser-Leu-Lys_{15} Glu-Ser-Phe-Ser_{19}-OH
202*	H-Leu-Glu-Ser-Phe-Leu_5-Lys-Ser-Trp-Leu-Ser_{10}-Ala-Leu-Glu-Gln-Ala_{15} Leu-Lys-Ala_{18}-OH
203	H-Leu-Glu-Ser-Phe-Lys_5-Val-Ser-Trp-Leu-Ser_{10}-Ala-Leu-Glu-Glu-Tyr_{15} Thr-Lys-Ala_{18}-OH
208	H-Val-Ser-Ser-Leu-Leu_5-Ser-Ser-Leu-Lys-Glu_{10}-Tyr-Trp-Ser-Ser-Leu_{15}-Lys Glu-Ser-Phe-Ser_{20}-OH
209	H-Leu-Glu-Ser-Phe-Leu_5-Leu-Ser-Trp-Leu-Ser_{10}-Ala-Lys-Glu-Gln-Ala_{15} Leu-Lys-Ala_{18}-OH

* This peptide was previously synthesised and named LAP-20 (ref. 13).

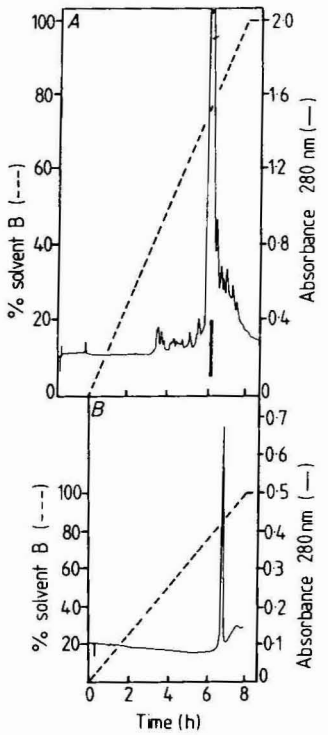


Fig. 2. The purification of peptide 209 on a Radial-Pak C_{18} column with a guard column of μBondapak C_{18}/Porasil B. In this separation solvent A consisted of 0.1 *M* ammonium bicarbonate and solvent B of 2-propanol–solvent A (80:20). A linear gradient from 0–100% solvent B over 8 h was used. The flow-rate was 1 ml/min and 180 ml of the deformylation mix, which contained 24 mg of peptide 209 and had been diluted with solvent A, 1:2 (v:v), was loaded through pump A onto the reversed-phase column. In part B 79 μg of purified peptide was analysed.

umn[10]. Therefore, as is shown in Fig. 2, the reaction mixture from the deformylation reaction could be both purified and desalted by chromatography on a Radial-Pak C_{18} column. However, it was necessary to use an 8-h gradient and include only the back of the main peak in the collected fraction due to a contaminant which eluted immediately before the main peak. The yield of peptide 209 was 16%; however, the recovery of a reinjected sample was 81%.

Geiger and Konig[14] have shown that transformylation from the nitrogen atom of the indole ring to the α-amino group of the peptide or the ε-amino group of lysine can be a significant side reaction in the deformylation procedure. Since the closely eluting contaminant in the separation of peptide 209 could arise from this side reaction, it was decided to study the deformylation reaction on another lipid associating peptide in which the mixture of products was more completely separated. Therefore the deformylation reaction was carried out with a sample of peptide 208 that had been purified by reversed-phase HPLC. Fig. 3A shows the purification of a sample of the N^{in}-formyl derivative of peptide 208. The material that eluted in the center of the peak was collected and analysed for purity in the same chromatographic system (Fig. 3B). In addition, the peptide was shown to be homogeneous by amino acid analysis and further chromatographic analysis (data not shown). The purified peptide was then subjected to the deformylation procedure and the product was again analysed (Fig. 3C). This time the deformylation procedure gave two products, one of

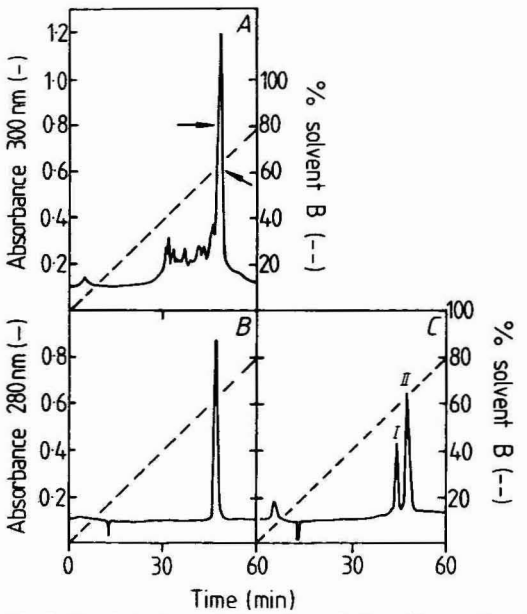


Fig. 3. The detection of a transformylation side reaction during the removal of the N^{in}-formyl group from peptide 208. The chromatographic conditions were as in Fig. 2, except that a linear gradient from 0–80% solvent B over 1 h was used. In part A, 2.5 mg of the peptide dissolved in 10 ml of 6 *M* guanidine hydrochloride was loaded through the solvent manifold of pump A. The purity of the peptide was analysed in the same system (part B). For the deformylation reaction the collected peak from part A (contained in 5 ml, denoted by arrows) was left at 4°C for 5 min after the addition of 2.87 mg of guanidine hydrochloride and 0.7 ml of ethanolamine. After the reaction was complete and the pH of the sample was adjusted, the sample was diluted with buffer A and the sample was loaded following the procedures described in the Materials and methods section. The elution profile is shown in Part C.

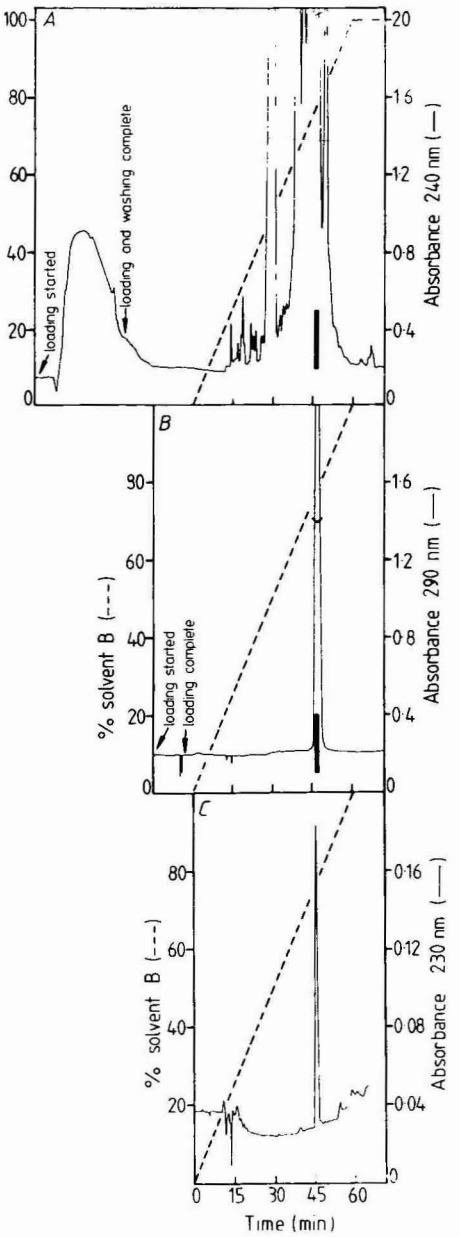
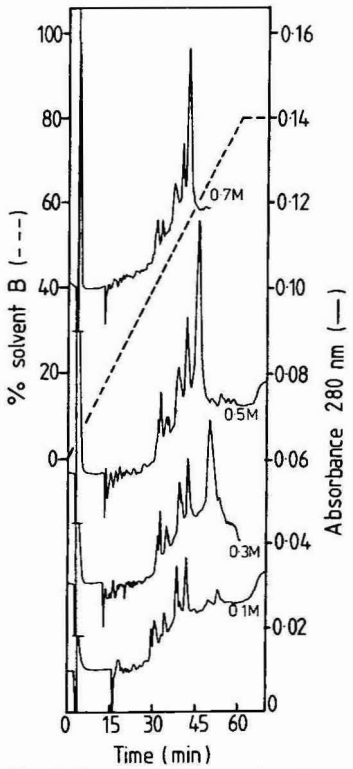


Fig. 4. The semi-preparative reversed-phase HPLC purification of peptide 203. A linear gradient from 0–100% solvent B over 1 h was used to elute the peptide. Solvent B was 2-propanol–acetonitrile–solvent A (3:3:4). A flow-rate of 1 ml/min was used. In part A 11.5 mg of peptide 203 dissolved in 18 ml of eluent was loaded on the Radial-Pak C_{18} column. In part B, 6.1 mg of peptide 203 from part A in 2 ml of eluent was diluted with 8 ml of 0.1 *M* ammonium bicarbonate and then purified. In part C, 5.6 μg of purified peptide was analysed.

which could be attributed to the formation of the α- or ε-amino formyl derivative of the desired peptide. Peaks I and II were found to have identical amino acid analyses, but peak I was identified as a formylated derivative because of its reduced cationic charge at acidic pH values as measured by ion exchange chromatography. Thus these results demonstrated that reversed-phase HPLC could be used in the deformylation procedure both to detect the presence of side reactions and to purify the product in a simple and high yield procedure.

In view of the excellent separations for a number of peptides that have been reported using mobile phases that contained 0.1 *M* ammonium bicarbonate[4,10], it was decided further to test the general applicability of this mobile phase by studying the purification of the peptides listed in Table I. Fig. 4A shows that the complex mixture present in an impure sample of peptide 203 was well resolved. The collected



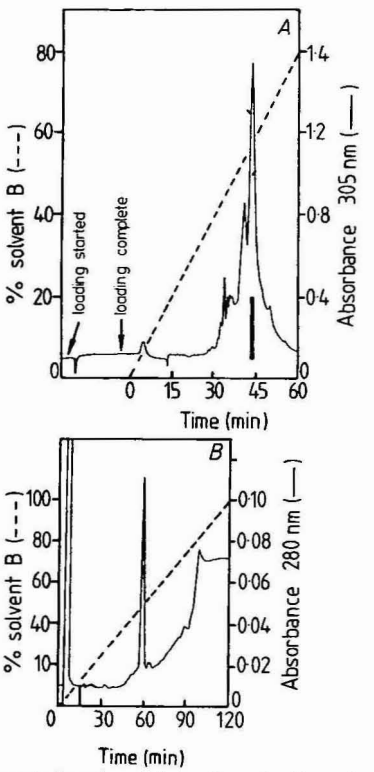


Fig. 5. The effect of increasing the concentration of ammonium formate in solvent A on the retention of Trp(CHO)-peptide 199 in reversed-phase HPLC. The separation was achieved on a Radial-Pak C_{18} column at a flow-rate of 1 ml/min. The concentration of ammonium formate used in solvent A is shown above each trace (pH 6.3). Solvent B (see dotted line) consisted of 2-propanol–solvent A (80:20). The sample loading for each analysis was 120 μg of Trp(CHO)-peptide 199 in 250 μl of 6 *M* guanidine hydrochloride.

Fig. 6. The semi-preparative reversed-phase HPLC purification of peptide 199 using a mobile phase which contained 0.5 *M* ammonium formate (solvent A). The other chromatographic conditions used were as in Fig. 3, except that for part B a linear gradient from 0–100% solvent B over 2 h was used. Part A was achieved with a loading of 9.4 mg of the peptide in 12.5 ml of the neutralised deformylation mixture, diluted to 40 ml with solvent A, filtered and loaded through pump A at 2 ml/min. The loading part B was 60 μg of purified peptide in 800 μl of guanidine hydrochloride.

fraction was diluted 4-fold with solvent A and rechromatographed with the same solvent system (see Fig. 4B). The purity of the peptide after the second chromatographic step is shown in Fig. 4C. The total yield for the two steps was 51%; however, a recovery of 97% was recorded for the rechromatography of the purified peptide.

Peptide 199, the only peptide that contained the strongly basic arginine residue, behaved very differently from the other peptides in this chromatographic system. In the 0.1 *M* ammonium bicarbonate system used for purifying the other peptides a very broad peak was observed upon elution of peptide 199 from a Radial-Pak CN column. With the same solvent system and a Radial-Pak C_{18} column no peak was eluted. It was reasoned that the anomalous behaviour of this peptide was caused by the interaction of ionised silanol groups with the basic groups of the peptide, since an increase in electrolyte concentration in the mobile phase greatly improved the separation (see later). It was also thought that an increased concentration of am-

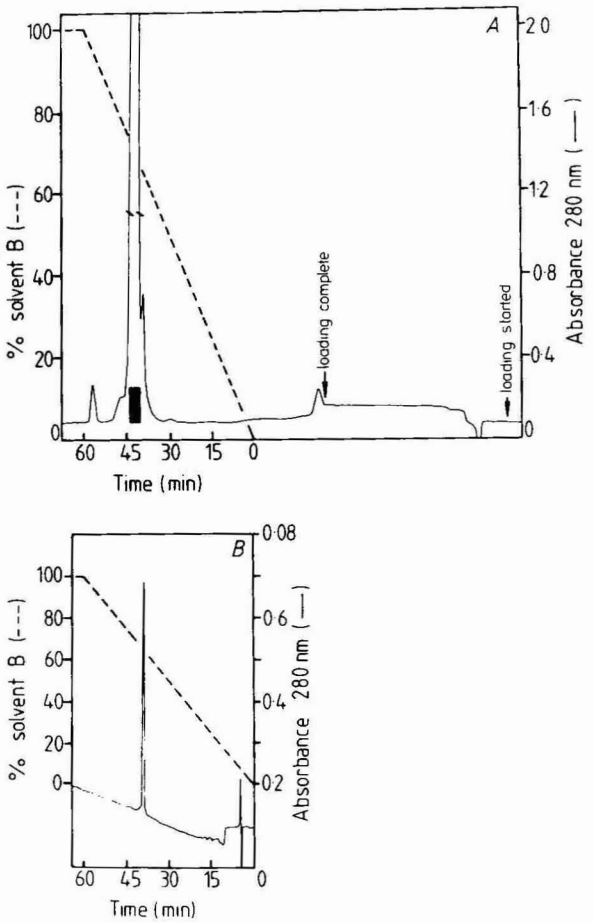


Fig. 7. The use of reversed-phase HPLC for the purification of a lipid associating peptide after purification by gel filtration on Sephadex G-10 and ion-exchange chromatography on SP-Sephadex. In this separation 6.8 mg of peptide 202 was chromatographed on a Radial-Pak CN column using the conditions described in Fig. 4. The purity of the pooled HPLC fractions is shown in part B where 16 μg of peptide was analysed.

monium ions in the mobile phase would reduce this interaction and therefore 0.5 *M* ammonium bicarbonate was added to solvent A. This mobile phase proved very difficult to work with, however, due to formation of carbon dioxide bubbles in the detector and therefore the less volatile salt, ammonium formate was tried. Peptide 199 was retained less and eluted with greater efficiency with increasing concentrations of ammonium formate in the mobile phase. Fig. 5 shows the effect of increasing concentration of ammonium formate in solvent A upon the elution profile of Trp(CHO)-peptide 199. From these results it was decided to use 0.5 *M* ammonium formate in the purification of peptide 199 and Fig. 6 shows a typical separation. The recovery of purified peptide was 27%, but rechromatography resulted in a recovery of 95% and demonstrated that the mobile phase containing ammonium formate had achieved the desired purification. The other peptides (202, 208, 209) behaved similarly to peptide 202 and could be successfully chromatographed with the ammonium bicarbonate containing mobile phase.

The semi-preparative system described in this publication can be used to purify either a partially purified lipid associating peptide (Fig. 7) or a crude preparation (Fig. 8). The general utility of a mobile phase which contains ammonium bicarbonate for the isolation of lipid associating peptides was demonstrated by the successful

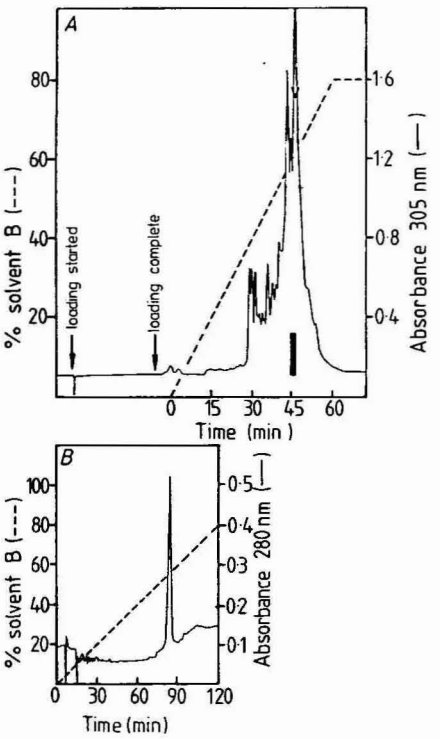


Fig. 8. The use of reversed-phase HPLC for the purification of a crude sample of lipid associating peptide. In this separation 26 mg of peptide 208 was chromatographed on a Radial-Pak C_{18} column using the conditions described in Fig. 3. Part B shows the corresponding analytical chromatogram that was obtained for 48 μg of the purified peptide.

purification of peptides 202 to 209 (see Table I). However, lipid associating peptide 199, which was the only peptide that contained an arginine residue, required a higher concentration of electrolyte in the mobile phase before a satisfactory chromatographic separation was achieved. This result is consistent with the observation of others[15,16] that strongly basic solutes interact significantly with residual silanol groups present on the stationary phase and that the coulombic interaction can be minimised by the addition of electrolyte to the mobile phase. As is shown in Fig. 9

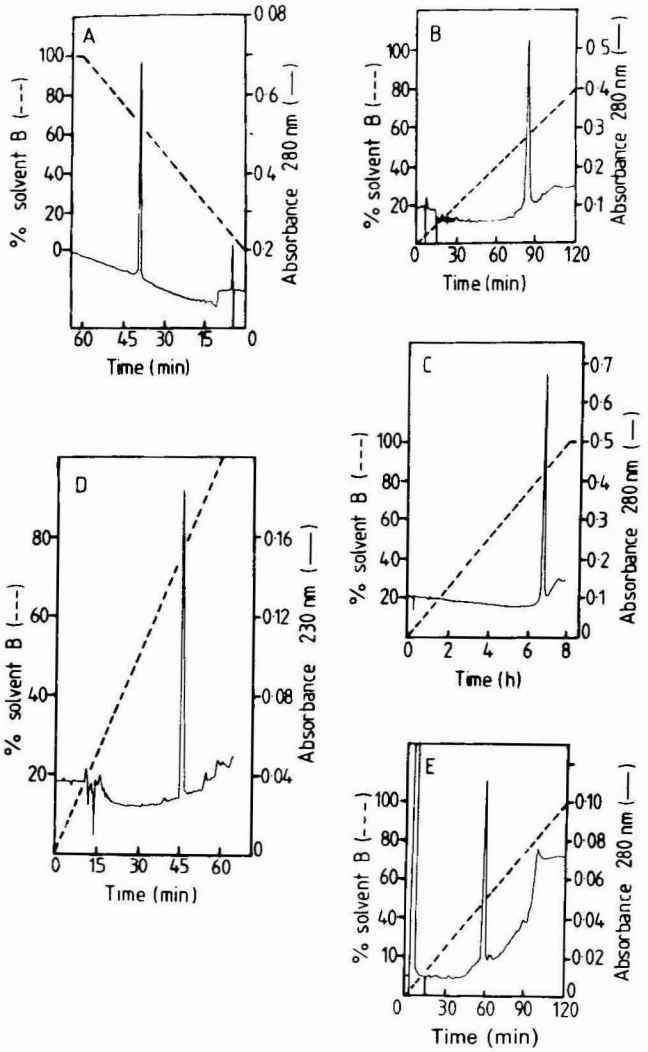


Fig. 9. Analysis of purified peptides by reversed-phase HPLC on a Radial-Pak CN column (A) and a Radial-Pak C_{18} column (B–E) at a flow-rate of 1 ml/min. The following mobile phases were used: A (peptide 202), solvent A: 0.1 *M* ammonium bicarbonate, solvent B: 2-propanol–acetonitrile–solvent A (3:3:4); B (peptide 208), solvent A: 0.1 *M* ammonium bicarbonate, solvent B: 2-propanol–solvent A (80:20); C (peptide 209), same as 208; D (peptide 203), same as 202; E (peptide 199), solvent A: 0.5 *M* ammonium formate, pH 6.3, solvent B: 2-propanol–solvent A (4:1).

TABLE II

AMINO ACID ANALYSIS VALUES FOR PURIFIED LIPID ASSOCIATING PEPTIDES

Conditions of hydrolysis: 6 *M* hydrochloric acid, 22 h, 110°C in evacuated tubes. Values in parentheses are the theoretical ratios of amino acids in the peptides.

Amino acid	*Peptide 202*	*Peptide 203*	*Peptide 208*	*Peptide 209*	*Peptide 199*
Thr*	–	0.8 (1)	–	–	–
Ser*	2.5 (3)	2.3 (3)	5.6 (8)	2.2 (3)	5.9** (6)
Glu + Gln	3.0 (2 + 1)	2.8 (3)	2.0 (2)	3.0 (2 + 1)	2.0 (2)
Ala	3.1 (3)	1.9 (2)	–	3.1 (3)	1.9 (2)
Val	–	0.9 (1)	0.9 (1)	–	–
Leu	5.0 (5)	3.0 (3)	4.0 (4)	5.0 (5)	3.0 (3)
Tyr	1.1 (1)	1.0 (1)	1.2 (1)	1.1 (1)	1.0 (1)
Phe	1.1 (1)	1.0 (1)	1.2 (1)	1.1 (1)	1.0 (1)
Lys	2.0 (2)	1.8 (2)	1.9 (2)	2.0 (2)	2.0 (2)
Arg	–	–	–	–	1.0 (1)
Trp***	N.D. (1)	N.D. (1)	N.D. (1)	N.D. (1)	N.D. (1)

* Values not corrected for oxidation except where stipulated by footnote **.

** Values corrected for oxidation by quantitation of a series of timed hydrolyses and extrapolation back to zero time.

*** N.D. = not determined. Analysis of the UV spectrum of each peptide shows that each peptide contains approximately 1 residue of tryptophan (ε_M = 5200 $lmol^{-1}$ cm^{-1}, $\lambda_{max.}$ = 280 nm).

the peptides purified by use of either system could be shown to be homogeneous by analytical HPLC with mobile phases that contained either ammonium formate or bicarbonate (see Fig. 9) or triethylammonium phosphate (data not shown) or by amino acid analysis (see Table II). In addition the preparative system described here allows the facile isolation and purification of peptides from the reaction mixture after removal of the N^{in}-formyl group that is commonly used to protect tryptophan in solid phase peptide synthesis.

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CHARACTERIZATION OF NEUTRALIZED β-NAPHTHALENESULFONIC ACID AND FORMALDEHYDE CONDENSATES

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SUMMARY

A liquid chromatographic method has been developed to show relative molecular weight distributions for different neutralized condensation products of β-naphthalenesulfonic acid and formaldehyde. This method can be used for quality control in manufacturing and for predicting the relative performance of condensates from different sources.

The separation was achieved on a pellicular, reversed-phase column using ion pairing and solvent programming. The components of the chromatogram were monitored with continuous UV detection at 280 nm. The chromatographic data are used to detect the different components formed during the condensation reaction.

The molecular weight distribution of the condensates can be correlated with their performance as dispersing agents in oil-well cementing services. Ion chromatography and ^{13}C NMR spectrometry provide complementary characterization data.

INTRODUCTION

Sodium salts of the condensation products of β-naphthalenesulfonic acid and formaldehyde have been used extensively as dispersing agents in oil-well cementing services for many years[1]. In addition, the sodium salts of the condensation products constitute the most important class of synthetic tanning agents for hides[2], and are used as dispersants for ceramics and pigments[3].

Previously, the condensates have been studied using electrophoresis, paper chromatography and salting-out chromatography[4]. Unfortunately, these methods gave incomplete results or were very lengthy. A short analysis time is desirable to compare a large number of condensates, or to closely monitor a manufacturing process of a condensate.

This report describes the use of high-performance liquid chromatography (HPLC) to determine relative molecular weight distributions of different condensates. Ion chromatography and ^{13}C NMR spectrometry provide additional characterization of the condensates.

EXPERIMENTAL

Naphthalene, paraformaldehyde, concentrated sulfuric acid, sodium hydroxide, glacial acetic acid, sodium carbonate and sodium bicarbonate were reagent grade. Tetrabutylammonium hydroxide (TBAH), 40% aqueous solution, was obtained from Aldrich. Distilled-in-glass acetonitrile was obtained from Burdick & Jackson Labs. The anion separator resin is a type commonly used in ion chromatography. The suppressor column resin was AG™ 50W-X16, 200–400 mesh in the acid from Bio-Rad Labs. Sodium polystyrene sulfonates were obtained from Pressure Chemical Co.

Apparatus

The liquid chromatograph was a Waters Assoc. Model ALC 202/401 equipped with a second Model M-6000 pump, Model 660 solvent programmer and a U6K injector. The column was a Water Assoc. Bondapak™ Phenyl/Corasil (60 cm × 2.8 mm I.D.). The chromatographic data were obtained using a Supergrator II from Columbia Scientific. The ion chromatograph consisted of a Model 212 conductivity meter and cell from Wescan Instruments, a Model 396-89 Milton Roy miniPump™, a Model R6031SV injector equipped with a 100-μl loop, glass columns, PTFE tubing and associated fittings from Laboratory Data Control. The nuclear magnetic resonance spectrometer was a Varian CFT-20, operating at 20 MHz for ^{13}C.

Condensate preparation

The monomer, β-naphthalenesulfonic acid, was prepared[1] by refluxing 128 g (1 mole) of naphthalene and 118 g (1.2 moles) of concentrated sulfuric acid at 160°C for two h. The monomer solution was cooled below 100°C, then 75 g (2.5 moles) of formaldehyde (as paraformaldehyde) was added. This was refluxed for periods of 24, 28, 72 and 96 h at 100°C and for periods of 4 and 8 h at 130°C. The reaction medium was cooled to room temperature and neutralized with aqueous sodium hydroxide. The condensate products were then dried at 110°C and crushed to a fine powder.

Solvent preparation

Solvent A, for the solvent programming, consisted of 0.01 *M* of TBAH and 1% (v/v) of glacial acetic acid in deionized water. Solvent B consisted of 0.01 *M* TBAH and 1% (v/v) of glacial acetic acid in acetonitrile. The TBAH went into solution faster when dissolved in the acetic acid, then added to the solvent. All solutions were filtered through a 0.5-μm filter.

Procedure for condensate ion-pair chromatogram

The condensates were dissolved in solvent A, at a concentration of 5.0 mg/ml. The solutions were filtered through a 0.5-μm filter. Initial conditions for the solvent program were 60% solvent A and 40% solvent B. After a 3-μl injection of the condensate solution, the solvent programmer and electronic integrator were started. The integrator was operated in the area normalization mode. The chromatograms were obtained using a 10-min linear gradient with a flow-rate of 1 ml/min and continuous UV monitoring at 280 nm. The final condition was held for 2 min. A 5-min reversed linear program was used to return to the initial conditions. The column was equilibrated for 5 min before another injection. Seven successive injections of a condensate

gave reproducible retention times with a 0.4% relative deviation and reproducible peak areas with a 0.7% relative deviation for the larger and longer retained peak.

Procedure for sulfate ionogram

After dissolving a condensate in the eluant of 0.003 *M* sodium bicarbonte and 0.002 *M* sodium carbonate, 100 μl was injected onto a 150 × 3 mm anion separator column. The suppressor column was 500 × 3 mm I.D. A flow-rate of 1 ml/min was used to obtain a retention time of 5.4 min.

RESULTS AND DISCUSSION

Anion-exchange chromatography

The initial study of the condensates was done within the Dow Chemical Company using anion-exchange chromatography[5]. With this methodology, the retention times of the components of a condensate increase as the number of sulfonate groups per molecule increases. The components of the condensates appear to be well resolved in Fig. 1, but failure to return to the baseline suggests that additional components are present, which elute very slowly. This was found to be the case.

By collecting injections with the column in-line and not in-line, it was found for one condensate that only 35%, based upon UV absorbance at 280 nm, had eluted within the 12-min solvent program. It is believed that the peaks are due to the monomer, dimer, trimer and higher oligomers of polynaphthalenesulfonates. The first peak had the same retention time as the monomer (β-naphthalenesulfonate). Since the condensate did not elute in an acceptable manner, this procedure was abandoned and a different approach was taken.

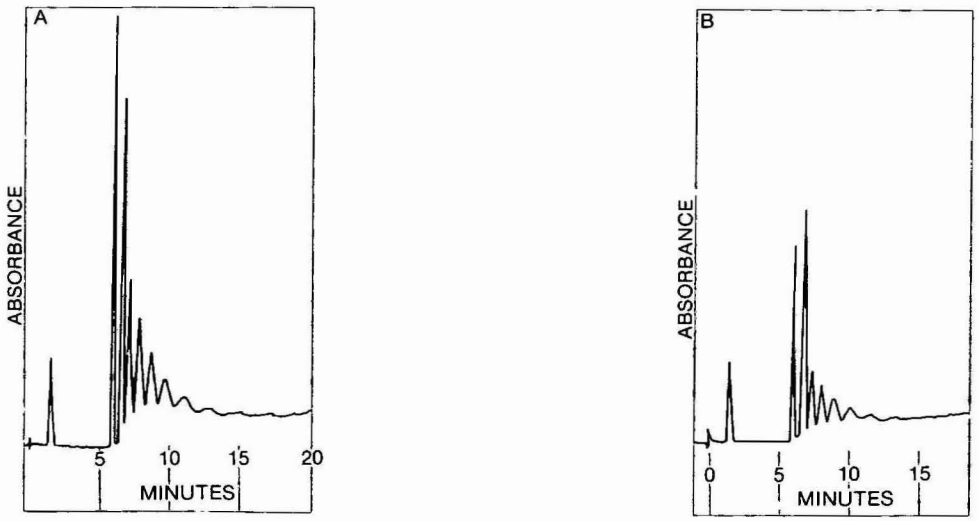

Fig. 1. Anion-exchange chromatograms of 100°C condensation reaction products of neutralized β-naphthalenesulfonic acid and formaldehyde: A, 48-h reaction; B, 96-h reaction.

Ion-pair chromatography

Probably the most powerful technique to separate highly ionic organic compounds is ion-pair chromatography[6]. Therefore, this methodology was investigated for a separation of the condensate components. Since a difference in the condensate components would be varying numbers of naphthyl rings per molecule, a phenyl-silica stationary phase was chosen over the more popular octadecyl column. Tetrabutylammonium cation was the pairing ion for the sulfonate anion. With the phenyl-base column, ion pairing and solvent programming, a chromatogram for the total condensate was obtained. Without the pairing ion in the mobile phase, the condensates eluted unretained.

Fig. 2 shows the chromatograms from several condensation products. At first glance, the two-peak chromatograms may not appear to show good separation, since the anion-exchange chromatograms of Fig. 1 contain several distinct peaks. However, a closer study of the chromatograms yields a vast amount of information about the reaction and the condensation product. The first peak in Fig. 2 is strongly believed to be the peak shown in Fig. 1. This is supported by the fact that the monomer has the same retention time as the front of the first peak of the chromatograms in Fig. 2 and the first peak in Fig. 1. In addition, the area of the first peak in Fig. 2 decreases with increasing reaction time at constant temperature, as does the area of the group of peaks in Fig. 1. Chromatographic data for Fig. 2 are shown in Table I.

The chromatograms in Fig. 2 strongly indicate that the products formed early in the reaction undergo significant changes as the reaction time increases. Since this is a polymerization reaction, components with varying molecular weights are expected. It is apparent from the trend of the ion-pair chromatograms that the second peak is produced, to some extent, from the components in the first peak. The condensation reaction is analogous to an acid-catalyzed phenol–formaldehyde conden-

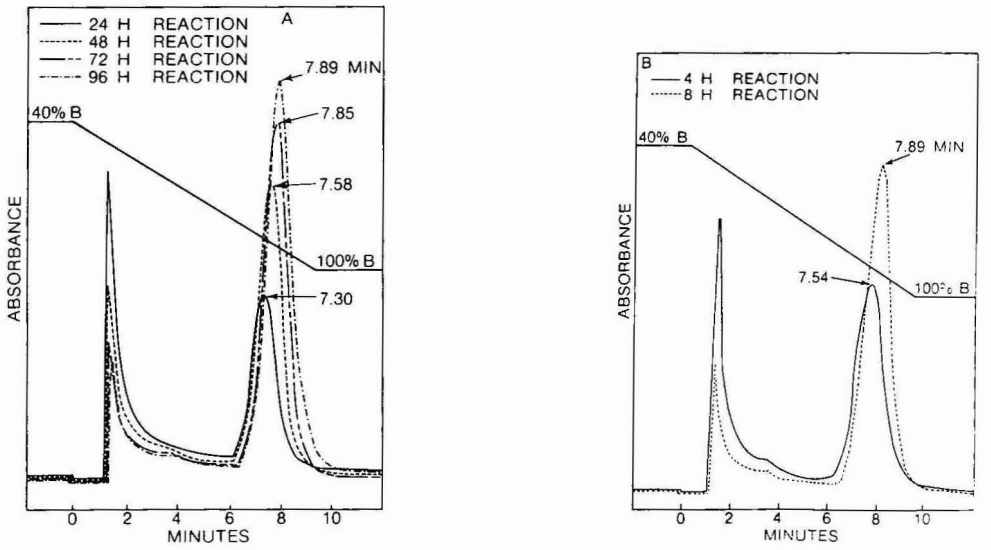

Fig. 2. Ion-pair chromatograms of neutralized β-naphthalenesulfonic acid and formaldehyde condensation products: A, 24-, 48-, 72- and 96-h reaction at 100°C; B, 4- and 8-h reaction at 130°C.

TABLE I

DATA FOR ION-PAIR CHROMATOGRAMS

Sample	*Retention time (min)*		*Area (%)*	
	Peak No. 1	*Peak No. 2*	*Peak No. 1*	*Peak No. 2*
Monomer	1.35			
24–100	1.38	7.30	43.9	56.1
48–100	1.35	7.58	22.8	77.2
72–100	1.36	7.85	14.5	85.5
96–100	1.33	7.89	10.9	89.1
4–130	1.37	7.51	35.3	64.7
8–130	1.33	7.89	13.0	87.0

sation reaction[7]. Initially, the reaction products would be expected to consist of linear, low-molecular-weight molecules, producing chromatograms as observed in Fig. 1. These small molecules could react with more monomer, or each other. When most of the monomer has been depleted and additional formaldehyde is present, the small molecules can react with each other to produce larger molecules. In turn, the larger molecules can react with each other to form the largest molecules. This stepping of molecular size would account for the absence of a continuum of oligomers in the condensation reactions, as observed in Fig. 2. If the larger molecules did not condense with each other, the second peak in Fig. 2 would not increase in retention time but only grow in percent area. As observed in Fig. 2, the retention time and percent area of the second peak increase with longer reaction times. The argument that the increased retention time of the second peak represents a higher molecular weight is supported by the increasing retention time and molecular weight for a set of polystyrene sulfonate standards using the ion-pair procedure. Table II listst these data.

As with phenol–formaldehyde condensates[7], the branching of the products is controlled by the amount of the formaldehyde. Since the formaldehyde is in excess of the naphthyl rings, branching of the condensate molecules would be expected. Thus, the second peak in Fig. 2 is likely not only higher molecular weight molecules, but also molecules having branched structures. Fig. 3 illustrates possible molecular structures for the condensate products.

TABLE II

RETENTION TIME OF SODIUM POLYSTYRENE SULFONATE MOLECULAR WEIGHT STANDARDS

Molecular weight	*Retention time (min)*
1 800	1.57
4 600	6.15
8 000	6.72
18 000	7.30

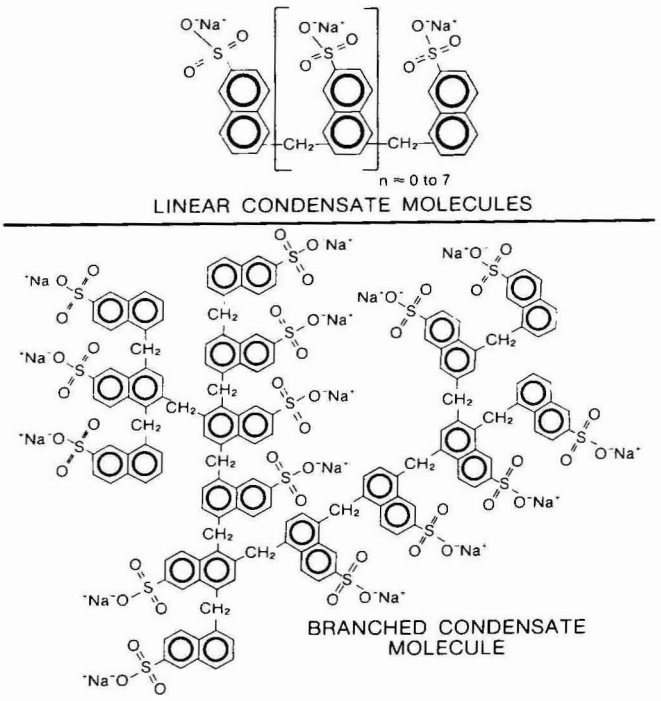

Fig. 3. Models of linear and branched condensate molecules.

^{13}C *NMR*

^{13}C NMR spectra support the argument that the first peak consists essentially of linear molecules and the second peak consists of branched molecules. ^{13}C NMR spectra show a broadening of peaks as the reaction time increases. This is expected for a branching polymer. If both peaks represented linear molecules, the spectra should have reasonably well-resolved peaks, as observed for the monomer[8]. Viscosity of the samples should not be the cause of the broadness, since the NMR solutions did not show any appreciable increase of viscosity. The spectra show the aromatic carbons (*ca.* 142 to 120 ppm) and the bridging methylene carbons (*ca.* 35 ppm). The peaks at *ca* 140 ppm and higher represent the sulfonated naphthyl carbons.

Sulfate and moisture

After the condensation reaction, neutralization forms a significant amount of sodium sulfate. This may affect the performance of the polymer by decreasing its purity. Thus, a large excess of sulfuric acid in the initial reaction may be undesirable. The sulfate content can be determined by ion chromatography, as illustrated in Fig. 4. The large condensate molecules elute in front of the sulfate in the chromatogram and do not interfere with the determination. The condensates were dissolved and diluted in the eluent to eliminate the "water dip" commonly occurring in ion chromatography. Also, the hygroscopic nature of the condensates causes a significant quantity of water to be present, which further reduces the purity of the polynmer. The water content can be determined by drying 1 g of the condensate product at

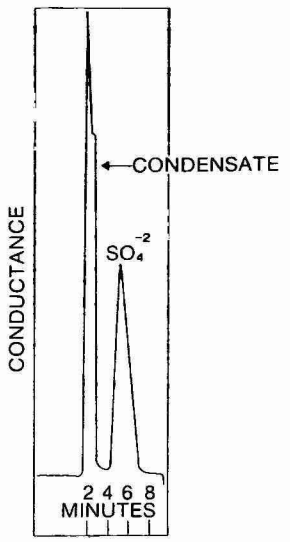

Fig. 4. Chromatogram showing sulfate in condensate product.

110°C for 1 h. The sulfate and water contents of the condensates in this report were not taken into consideration. The contents would remain nearly constant with the same reactant ratios in all reactions. The water and sulfate content in condensation products from different sources should be determined to predict their relative performances with a greater degree of confidence.

CONCLUSIONS

The utility of ion-pairing and solvent programming provides an informative and fast method for the chromatographic analysis of β-naphthalenesulfonic acid–formaldehyde condensation products. The chromatograms show the various components formed during the reaction and yield a relative molecular weight distribution. This method could easily be used to determine when a condensation reaction has reached a desired point by comparing chromatograms to that of a selected standard. In addition, different sources of condensates can be compared based upon the percent area and the retention time of the second peak. A condensation product can be further characterized by determining the moisture and sulfate content. The use of anionic pairing reagents and solvent programming should allow chromatographic analysis of cationic polymers.

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CAPILLARY COLUMN GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF TABUN

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SUMMARY

A sample of munitions grade tabun, known to contain several impurities, was studied by combined capillary column gas chromatography–mass spectrometry under both electron-impact and chemical ionization conditions. Five impurities, three of which were previously unreported in tabun, were identified and characterized based on their mass spectral data. Ammonia chemical ionization was particularly useful since this technique provided molecular ion information for all the organophosphorous impurities.

INTRODUCTION

The alleged use of the chemical warfare agent tabun in the Iran/Iraq conflict emphasizes the need for specific detection and identification methods for this and other nerve agents[1–3]. Recent gas chromatographic (GC) analysis of a munitions grade tabun sample in our laboratory indicated the presence of a number of impurities. The identity of these compounds, estimated to comprise about 25% of the organic content, would be valuable for future chemical event analyses.

A capillary column GC–mass spectroscopic (MS) study using both electron-impact (EI) and chemical ionization (CI) conditions was initiated with the principle objective being the identification of the tabun impurities. Initial EI investigation led to the positive identification of only one impurity, triethyl phosphate. As a result CI, using methane and ammonia as reagent gases, was employed in order to resolve the ambiguous EI data.

CI-MS using methane, isobutane, ethylene and methanol has been used recently for the analysis of organophosphorus chemical warfare agents[4] and pesticides[5–7]. Both the unique character of the CI data and the molecular ion information often afforded have been exploited for identification and characterization purposes.

Methane CI and in particular ammonia CI, an ionization technique previously unreported for chemical warfare agent determination and used sparingly in pesticide study[5], provided the molecular ion and fragmentation information necessary for the identification of the five tabun impurities. The mass spectral data acquired, along with GC retention index data, are reported for tabun and the other organophosphorus compounds identified in the munitions grade sample.

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EXPERIMENTAL

Standards

All tabun samples were prepared in HPLC-grade chloroform (Fisher Scientific, Edmonton, Canada) and stored in PTFE-lined screw-capped glass vials prior to GC analysis. The munitions grade tabun, used for impurity identification, was taken from a sample wich had been in a steel container for at least 30 years.

Distillation of munitions grade tabun

Munitions grade tabun was fractionally distilled in an all glass apparatus at 3 Torr using a Vigreux column (20 cm × 1.8 cm I.D.) and the fraction boiling at 97–97.5°C (3 Torr) was analysed by combined GC–MS.

Tabun synthesis

Tabun was prepared according to the method of Holmstedt[8] using benzene instead of chlorobenzene as the solvent. Crude tabun was purified by fractional distillation at 0.6 Torr using a vacuum-jacketted and silvered Hempel column (30 cm × 1.8 cm I.D.) filled with glass rings (5 × 5 mm). The fraction boiling at 57°C (0.6 Torr) was collected as pure tabun and used for combined GC–MS analysis.

Thermal decomposition of pure tabun

Pure tabun (9 ml) was heated in glass at 200° for 20 min. After cooling a sample of the resulting dark material was used for capillary column GC–MS analysis.

Ethyl isopropyl dimethylphosphoroamidate synthesis

Ethyl dimethylphosphoramidochloridate (8.24 g, 0.048 mole) was added to a solution of sodium isopropoxide (made from 1.2 g of sodium) in 2-propanol (75 ml). After the addition was complete the reaction mixture was heated for 30 min at 60°C. Excess 2-propanol was removed *in vacuo*, water (50 ml) was added and the aqueous layer was extracted with dichloromethane (3 × 50 ml). The organic layers were combined, dried over anhydrous sodium sulphate, filtered and the excess solvent removed leaving a clear, colourless oil. Distillation afforded 7.8 g (83%) of product b.p. 56–57°C/0.2 mm.

^1H NMR (C^2HCl_3). δ 1.3 (3H, t, CH_3), δ 1.3 (6H, d, $J = 12H_z$, CH_3), δ 2.6 (6H, d, $J_{PNCH_3} = 20\ H_z$), δ 4.0 (2H, m, $J = 14H_z$, CH_2), δ 4.6 (1H, m, $J = 12H_z$, CH).

^{13}C NMR (C^2HCl_3). δ 15.43 (1C, d, $J_{POCC} = 6.7\ H_z$, CH_3), δ 22.92 (2C, d, $J_{POCC} = 2.7\ H_z$, CH_3), δ 35.84 (2C, d, $J_{PNC} = 3.4\ H_z$, CH_3), δ 60.98 (1C, d, $J_{POC} = 5.6\ H_z$, CH_2), δ 69.62 (1C, d, $J_{POC} = 5.7\ H_z$, CH).

IR (thin film). 1460, 1387, 1310, 1260, 1180, 1112, 1054, 1000, 960, 891, 784, 702 cm^{-1}.

Analysis. Calculated: $C_7H_{18}NO_3P$; C, 43.10%; H, 9.30%; N, 7.20%. Found: C, 43.10%; H, 9.20%; N, 6.95%.

EI mass spectrum [m/z (% relative intensity)]. 39 (4); 41 (7); 42 (13); 43 (11); 44 (100); 45 (13); 82 (2); 108 (18); 109 (2); 110 (4); 124 (15); 125 (4); 126 (4); 136 (3); 152 (3); 153 (8); 154 (3); 195 (3).

Ethyl n-propyl dimethylphosphoroamidate synthesis

Ethyl dimethylphosphoramidochloridate (15 g, 0.09 mole) was added to a solution of sodium propoxide (made from 2.3 g of sodium) in 1-propanol (100 ml). After the addition was complete the reaction mixture was heated for 30 min at 60°C. Excess 1-propanol was removed *in vacuo*, water (100 ml) was added and the aqueous layer was extracted with dichloromethane (3 × 75 ml). The organic layers were combined, dried over anhydrous sodium sulphate, filtered and the excess solvent removed leaving a clear, colourless oil. Distillation afforded 17 g (74%) of product b.p. 62–63°C/0.2 mm.

^1H NMR (C^2HCl_3). δ 0.95 (3H, t, J = $7H_z$, CH_3), δ 1.30 (3H, t, J = $7H_z$, CH_3), δ 1.64 (2H, m, J = $7H_z$, CH_2), δ 2.70 (6H, d, J_{PNC} = $9H_z$, CH_3), δ 3.73–4.30 (4H, m).

^{13}C NMR (C^2HCl_3) δ 9.39 (1C, s, CH_3), δ 15.48 (1C, d, J_{POCC} = 6.5 H_z), δ 23.09 (1C, d, J_{POCC} = 6.8 H_z, CH_2), δ 35.97 (2C, d, J_{PNC} = 3.2 H_z, CH_3), δ 61.39 (1C, d, J_{POC} = 5.6 H_z, CH_2), δ 66.92 (1C, d, J_{POC} = 5.6 H_z, CH_2).

IR (thin film). 1460, 1392, 1311, 1258, 1194, 1168, 1071, 1048, 1000, 963, 855, 787, 754, 700 cm^{-1}.

Analysis. Calculated: $C_7H_{18}NO_3P$; C, 43.10%; H, 9.30%; N, 7.20%. Found: C, 42.85%; H, 9.20%; N, 7.06%.

EI mass spectrum [m/z (% relative intensity)]. 39 (2); 41 (4); 42 (10); 43 (11); 44 (100); 45 (14); 82 (3); 83 (3); 108 (21); 109 (2); 110 (5); 111 (3); 124 (20); 125 (3); 126 (15); 136 (3); 152 (3); 153 (3); 154 (17); 195 (3).

Instrumental analysis

A Varian (Georgetown, Canada) Model 3700 gas chromatograph equipped with a flame ionization detector was used for all capillary column GC–flame ionization detection (FID) analyses. Data were recorded on a Varian 4270 integrator.

Capillary column GC–MS analyses were performed with a VG Micromass 70/70H double focusing mass spectrometer (VG Analytical, Wythenshawe, U.K.).

TABLE I

CAPILLARY COLUMN GC–MS OPERATING CONDITIONS

Operating parameters	*EI*	*Methane* CI*	*Ammonia** CI*
Accelerating voltage (kV)	4	4	4
Electron energy (eV)	70	70	70
Emission (μA)	200	1000	1000
Source temperature (°C)	200	110	100
Source pressure (Torr)	$2 \cdot 10^{-6}$	*ca.* 0.1–0.5***	*ca.* 0.1–0.5***
Resolution (10% valley defination)	500	500	500
GC–MS interface	direct (230°C)	direct (230°C)	direct (230°C)
Scan function and rate	350 to 20 a.m.u., exponential down, 1 s/decade.		

* Pure grade (99.5%) (Liquid Carbonic, Scarborough, Canada).

** Anhydrous grade (99.99%) (Liquid Carbonic).

*** Pressure inside the source was estimated from a pressure reading of $5 \cdot 10^{-5}$ Torr near the source, since the VG 70/70H does not read the pressure within the source.

The EI and CI operating conditions used during GC–MS study are listed in Table I. Details of CI optimization have been reported[9].

GC analyses were performed on three J + W 15 m × 0.32 mm I.D. capillary columns (J + W Scientific, Rancho Cordova, CA, U.S.A.) coated with 0.25 μm DB-1 (100% dimethylpolysiloxane), DB-5 (95% methyl-(5%)-diphenylpolysiloxane) and DB-1701 (86% dimethyl-(14%)-cyanopropylphenylpolysiloxane) films. An on-column injector of our own design was used for sample introduction[10]. Injections were made at 50°C. This temperature was maintained for 2 min and followed by a 10°C/min temperature program to a maximum of 300°C. High-purity helium, at a linear velocity of 35 cm/s (methane injection at 50°C), was used as the carrier gas.

RESULTS AND DISCUSSIONS

GC–MS analysis

Fig. 1 illustrates the ammonia CI capillary column GC–MS chromatogram obtained for the munitions grade tabun sample. Similar total-ion-current chromatograms were obtained under EI and methane CI conditions. Tabun and five other organophosphorus compounds, listed in Table II, were identified in the sample based on mass spectral interpretation. Tabun, the major sample component, accounted for 76% of the organic content while diethyl dimethylphosphoramidate and triethyl phosphate contributed 12% and 1%, respectively.

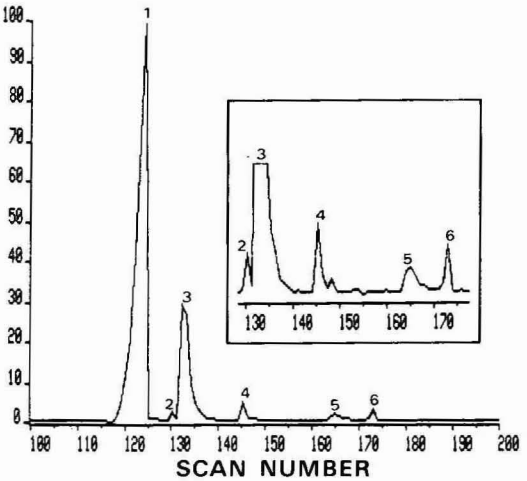


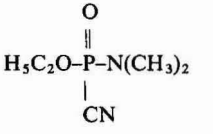
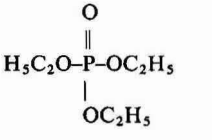
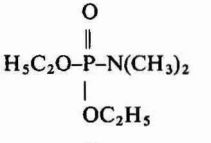
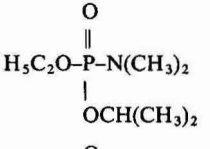
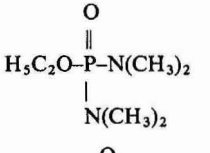
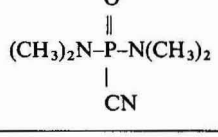
Fig. 1. Ammonia chemical ionization capillary column GC–MS chromatogram of munitions grade tabun. Compounds are identified in Table II (15 m × 0.32 mm I.D. J + W DB-5 column).

Similar percentage contributions were found in an alleged liquid chemical warfare sample analysed in Sweden[2]. Their analyses indicated the presence of tabun, diethyl dimethylphosphoramidate and triethyl phosphate at 75%, 3–10% and, 1–4% levels, respectively. Chlorobenzene, often used as a solvent during tabun synthesis, was also found. Two phosphorus-containing compounds remained unidentified.

Our application of CI-MS with both ammonia and methane as reagent gases enabled the identification of three previously unreported organophosphorus com-

TABLE II

COMPOUNDS IDENTIFIED IN MUNITIONS GRADE TABUN

Compound No.	*Compound identity**	*Structure*	*Molecular weight*	*% of sample***	*CA registry No.*
1	Ethyl dimethylphosphoramidocyanidate (Tabun)		162	76	77-81-6
2	Triethyl phosphate		182	1	78-40-0
3	Diethyl dimethylphosphoramidate		181	12	2404-03-7
4	Ethyl isopropyl dimethylphosphoramidate		195	5	
5	Ethyl tetramethylphosphorodiamidate		180	5	2404-65-1
6	Tetramethylphosphorodiamidic cyanide		161	1	14445-60-4

* Based on EI and CI mass spectral data.

** Based on capillary column GC–FID chromatogram peak areas.

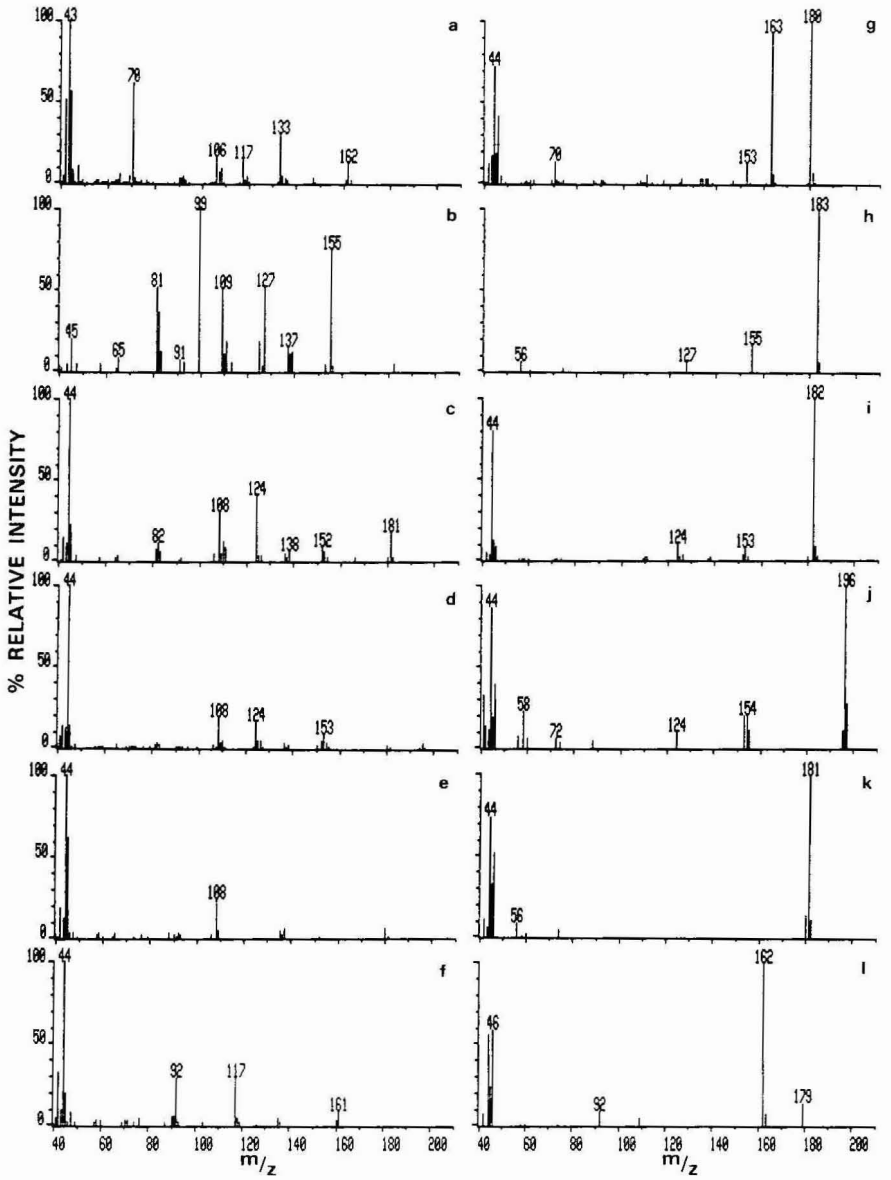


Fig. 2. (a) to (f) EI mass spectra of compounds 1 to 6 respectively. (g) to (l) Ammonia CI mass spectra of compounds 1 to 6, respectively. Compounds are identified in Table II.

pounds in munitions grade tabun. CI, a much softer ionization technique than EI, allowed us to positively identify the molecular ions of the tabun impurities. Pseudo-molecular $(M + H)^+$ ions were found for all components under both ammonia and methane CI conditions. In addition the $(M + NH_4)^+$ complex ion was found for tabun and another component under ammonia CI conditions. This molecular ion information was used with the acquired EI fragmentation data to identify the organophosphorus impurities.

Both the EI and ammonia CI mass spectra of the six organophosphorus compounds are shown in Fig. 2. The considerable fragmentation observed during EI analysis, due primarily to simple homolytic or heterolytic cleavage(s) and one or two hydrogen rearrangements (*e.g.*, McLafferty rearrangement), was not observed during ammonia CI operation.

Those compounds containing an ethoxy substituent were generally characterized by $(M - C_2H_3)^+$, $(M - C_2H_5)^+$ or $(M - OC_2H_5)^+$ EI fragmentation ions. Multiple ethoxy substitution may lead to ions with the following possible ion structures: $(M - C_4H_7)^+$, $(M - C_4H_9)^+$, $(M - OC_4H_9)^+$, $(M - C_6H_{11})^+$ or $(M - OC_6H_{13})^+$. Ethyl isopropyl dimethylphosphoramidate produced fragmentation ions with possible structures $(M - C_3H_6)^{+\cdot}$, $(M - OC_3H_6)^{+\cdot}$, $(M - C_5H_{11})^+$ and $(M - OC_5H_{11})^+$ due to the presence of both an ethoxy and isopropoxy substituent.

Ethyl isopropyl dimethylposphoramidate was tentatively identified based on similarities in EI fragmentation to diethyl dimethylphosphoramidate. Concern arose as to the presence of a isopropoxy substituted compound since the synthesis scheme specifies the use of ethanol. It was thought that the ethanol, used in the synthesis, could be contaminated with trace levels of isopropanol. The presence of this impurity could and probably did result in production of ethyl isopropyl dimethylphosphoramidate during synthesis. This compound and the *n*-propyl analogue, previously unreported in the literature, were synthesized and used to confirm the presence of ethyl isopropyl dimethylphosphoramidate in the munitions grade tabun.

The cyano and dimethylamino substituted compounds often produced $(M - CN)^+$ and $(M - C_2H_6N)^+$ EI fragmentation ions, respectively. Significant *m/z* 44 ions, observed for compounds containing the dimethylamino substituent, were probably due to $(C_2H_6N)^+$ ions.

Fragmentation was more extensive under methane CI than ammonia CI for all the compounds studied. This was due to the more energetic ion–molecule reactions occurring under methane CI. The fragmentation ions were similar to those observed under EI operation with the exceptions being the presence of $(M + H)^+$ and in some cases $(M + C_2H_5)^+$ pseudo molecular ions. Table III summarizes the principle methane CI ions observed for the tabun components.

GC retention indices

The GC retention indices of the compounds identified, relative to a homologous series of *n*-alkane standards under temperature programming conditions, were determined using capillary columns coated with DB-1, DB-5 and DB-1701 films according to a previously described method[10]. These data (Table IV), along with the mass spectra acquired should aid other researchers confronted with the analysis of samples alleged to contain tabun.

Retention indices were found to increase with column polarity. Small index changes (ΔRI) of 45 to 66 units were observed between the DB-1 and DB-5 stationary phases. However, the relative ordering of the six components remained the same. A different retention order and considerably larger ΔRI values (142 to 270) were observed between the DB-5 and the more polar DB-1701 column. Most notable were the large ΔRI values of 208 and 270 obtained for tabun and tetramethylphosphorodiamidic cyanide. It appears that the cyano functional group of the DB-1701 film exerted considerable influence on these, the only two compounds, with a cyano substituent.

Distillation of the munitions grade tabun

The munitions grade tabun was distilled and tthe fraction boiling at 97–97.5°C was collected in an unsuccessful attempt to obtain pure tabun. Tabun still accounted for about 75% of the GC–FID chromatogram obtained. Components 2 through 5 (refer to Table II) were present at 1%, 12%, 6% and 6%, respectively. The sixth component, tetramethylphosphorodiamidic cyanide, was not detected during capillary column GC–FID analysis.

TABLE III

METHANE CHEMICAL IONIZATION MASS SPECTRA OF COMPOUNDS IDENTIFIED IN MUNITIONS GRADE TABUN

Compound name	*Molecular weight*	*m/z*	*Relative intensity*
Ethyl dimethylphosphoramide-cyanidate (tabun)	162	163	34
		136	100
		135	56
		108	61
		70	40
Triethyl phosphate	182	183	73
		155	100
		127	51
		113	24
		99	40
Diethyl dimethylphosphoramidate	181	210	4
		182	100
		154	40
		140	15
		126	20
		124	20
		108	20
Ethyl isopropyl dimethyl-phosphoramidate	195	196	10
		195	13
		182	11
		154	100
		153	26
		140	10
		126	14
		124	13
		108	16
Ethyl tetramethyl-phosphorodiamidate	180	209	5
		181	100
		180	44
		153	25
		137	23
		135	21
		108	20
Tetramethylphosphoro-diamidic cyanide	161	162	63
		135	100
		117	11
		92	19

TABLE IV

GC RETENTION INDICES FOR COMPOUNDS IDENTIFIED IN MUNITIONS GRADE TABUN ON DB-1, DB-5 AND DB-1701 FUSED-SILICA CAPILLARY COLUMNS

Compound name	*Retention index**		
	DB-1	*DB-5*	*DB-1701*
Ethyl dimethylphosphoramido-cyanidate (tabun)	1077.9 ± 0.3	1131.6 ± 0.5	1339.8 ± 0.2
Triethyl phosphate	1090.5 ± 0.1	1137.2 ± 0.4	1308.5 ± 0.8
Diethyl dimethylphororamidate	1096.6 ± 0.6	1145.2 ± 0.1	1295.6 ± 0.2
Ethyl isopropyl dimethyl-phosphoramidate	1121.4 ± 0.4	1166.6 ± 0.5	1308.5 ± 0.8
Ethyl tetramethylphosphoro-diamidate	1158.7 ± 0.3	1216.6 ± 0.6	1392.8 ± 0.6
Tetramethylphosphorodiamidic cyanide	1179.5 ± 0.6	1245.7 ± 0.3	1514.7 ± 0.5

* Retention indices expressed as a mean ± S.D. (n = 3).

Tabun synthesis

In order to obtain pure material, tabun (compound I) was synthesized[8] according to the following scheme.

$$POCl_3 + (CH_3)_2NH \cdot HCl \rightarrow (CH_3)_2NPOClp_2 + 2\ HCl$$

$$(CH_3)_2NPOCl_2 + C_2H_5OH + 2\ KCN \rightarrow (CH_3)_2NPO(CN)\ (OC_2H_5) + 2\ KCl + HCN \quad (I)$$

Tabun was isolated by fractional distillation and unlike the munitions grade material was found to constitute 100% of the organic content based on capillary column GC–FID analysis.

The impurities identified in the munitions grade tabun, probably resulted from the original large scale synthesis or from decomposition on storage. In order to investigate this possibility pure tabun was heated in glass at 200°C for 20 min. This material was cooled and examined by capillary column GC–MS. All the tabun impurities, except ethyl isopropyl dimethylphosphoramidate, thought to be due to the presence of isopropanol in the ethanol used during munitions synthesis, were observed.

Tabun accounted for 80% of the organic content in the GC–FID chromatogram and, compounds 2, 3, 5 and 6 contributed 0.4%, 5%, 0.2% and, 3%, respectively in the thermally treated sample. The remaining FID signal was due to two components of molecular weight 269 and 288. Pseudo molecular $(M + H)^+$ ions at m/z 270 and 289, obtained during ammonia CI analysis, confirmed the molecular weights of these thermolysis products. These two compounds, as yet unidentified, are probably two sets of diastereomers. The EI and CI data were identical for both contributing GC peaks observed for each component. Pyrophosphate structures are being considered.

Based on the results described above it appears that a proportion of the components found in the munitions grade tabun resulted from disproportionation of the tabun either during synthesis or in storage.

CONCLUSIONS

We have reported the application of ammonia CI for the detection and identification of organophosphorus chemical warfare agents. This technique provides excellent molecular ion information with minimal fragmentation and aided in the identification of five impurities, three of which were previously unreported, in munitions grade tabun.

The GC and mass spectral data provided are sufficient for the identification of tabun and its impurities. The relative proportions of tabun impurities may also provide analysts with information on origin or synthesis method when dealing with samples contaminated with this chemical warfare agent.

Tabun was found to disproportionate upon heating. All the impurities observed except ethyl isopropyl dimethylphosphoramidate can be accounted for by this mechanism. This compound, thought to be formed due to isopropanol impurities during origin synthesis of the munitions grade tabun, was synthesized along with the *n*-propyl analogue for confirmation purposes.

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DETERMINATION OF ETHYL PHOSPHITE, PHOSPHITE AND PHOSPHATE IN PLANT TISSUES BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY

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SUMMARY

A sensitive method of analysis of some phosphorus oxyanions (ethyl phosphite, phosphite and phosphate) in plant material was developed. The plant extracts were purified by anion-exchange high-performance liquid chromatography. The oxyanions were then converted into their *tert.*-butyldimethylsilyl derivatives and evaluated by capillary gas chromatography using a flame ionization detector. This method allowed the quantitation of amounts as low as 40 nmol of phosphite and phosphate and 100 nmol of ethyl phosphite per gram of fresh plant tissue.

INTRODUCTION

Aluminium ethyl phosphite (fosetyl-Al), a systemically active compound known as Aliette®, is used increasingly to control diseases caused by Phycomycetes[1,2]. Ethyl phosphite and its degradation product in plant tissues, phosphite (Fig. 1), have a greater effect on fungal growth *in vivo* than *in vitro*. In view of the low fungitoxicity *in vitro*, ethyl phosphite has been reported to stimulate the defence responses of infected grape leaves, tomato leaflets[3] and tobacco stems[4]. Moreover, this induced defensive reaction is highly dependent on the phosphate content of the treated tissues[5].

In order to obtain precise information about the location and concentration of this compound in healthy and infected plant tissues, its rate of uptake by host or fungal cells, its rate of degradation and the exact mode of competition with phosphate

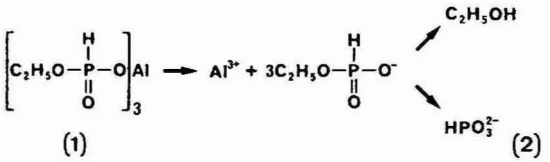

Fig. 1. Structural formulae and metabolic pathway of aluminium ethyl phosphite (1) and phosphite (2) in plant tissue.

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ions, we developed a sensitive and quantitative analysis of the three anions ethyl phosphite, phosphite and phosphate.

The speed, accuracy and sensitivity afforded by gas–liquid chromatography (GLC) offers definite advantages over other analytical methods. Among the derivatization techniques for oxyanions already reported for GC using a flame ionization detector, silylation is the most appropriate. Hashizume and Sasaki[6] first reported the GC detection of the trimethylsilyl derivative of orthophosphate. Similar studies have employed other silylating agents[7–10]. Recently, Mawhinney[11] reported the conversion of inorganic oxyanions into their *tert.*-butyldimethylsilyl derivatives by using N-methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide (MTBSTFA), a stable silylating agent.

However, application of this method to the analysis of biological materials necessitated purification before derivatization and GC analysis. Anion-exchange chromatography using a standard high-performance liquid chromatographic (HPLC) apparatus with UV detection, which has been described as a suitable method for separation of inorganic anions in water[12], was used for the purification step.

The purpose of this study was to determine the applicability of GC in combination with anion-exchange chromatography to the quantification of fosetyl-Al, its metabolite phosphite and phosphate in plant material.

EXPERIMENTAL

Standards and reagents

Standard samples. Phosphorous and phosphoric acids of analytical grade (Fluka, Buchs, Switzerland) were used to prepare aqueous stock solutions. Ethylphosphorous acid was obtained from fosetyl-Al (technical grade; Rhône-Poulenc Agrochimie, Lyon, France) after removal of the aluminium cations by a batch technique using a Dowex 50W-X8 cation-exchange resin (hydrogen form) (Bio-Rad, Glattbrugg, Switzerland).

HPLC buffers. Potassium biphthalate or sodium salicylate of analytical grade (Prolabo, Paris, France) were used to prepare buffered solutions (10 m*M*, pH 4.0). Water of HPLC grade (Fisons, Loughborough, U.K.) was used throughout. The buffers were prepared daily.

Silylating agents. Dimethylformamide (DMF) for gas chromatography was purchased from Prolabo and MTBSTFA was obtained from Chrompack (Middelburg, The Netherlands).

Extraction of biological material

Tomato and cowpea leaves were first frozen in liquid nitrogen and extracted twice by cold 0.1 *M* trichloroacetic acid (TCA) (1:5, w/v). TCA was eliminated by five washings with diethyl ether.

High-performance liquid chromatography

Aliquots of 2 ml (equivalent to 200 mg fresh tissue) of plant extract previously filtered on a 0.45-μm filter (Millipore, Molsheim, France) were injected in the HPLC apparatus.

The system used consisted of an U6K injector (Waters Assoc., Milford, MA,

U.S.A.), a single M 6000 A HPLC pump (Waters Assoc.) and a M 450 UV–visible detector (Waters Assoc.). The column (250 × 4.6 mm I.D.) packed with Ionospher™ A (Chrompack) was operated at ambient temperature with a flow-rate of 2 ml/min and a pressure of 1320–1360 p.s.i. The optical density was monitored at 308 nm and recorded on a 730 Data Module (Waters Assoc.).

After HPLC purification, the fractions were passed through a microcolumn system consisting of three C_{18} Sep-Pak cartridges (Waters Assoc.) and a microcolumn containing a 1-ml resin bed of Dowex 50W-X8 (hydrogen form). The microcolumn employed was a pipette tip containing a small plug of glass wool at the bottom. The columns were connected in series, the three C_{18} cartridges being above the cation-exchange microcolumn. Samples were eluted with distilled water and evaporated to dryness at 45°C under vacuum. The final residue was redissolved in methanol and transferred to a reaction vial.

Derivatization

Samples placed into reaction vials were first evaporated to dryness with a stream of nitrogen gas. To these vials were then added successively 100 μl of DMF and 300 μl of MTBSTFA. Aliquots were then subjected to GC analysis.

Gas chromatography

A Girdel 30C gas chromatograph equipped with a flame ionization detector was used. The column was a fused-silica capillary (25 m × 0.22 mm I.D.) wall coated with CP™-Sil 5 (Chrompack). The injector and detector temperatures were 250 and 270°C respectively and the oven temperature was programmed from 100 to 200°C at a rate of 3°C/min. The carrier gas was helium with a column-head pressure of 0.7 bar. The splitless injection technique was used with a purge activation time of 0.5 min and an injection volume of 1 μl.

A SP-4200 computing integrator (Spectra-Physics, Orsay, France) was used to record and quantify the peaks.

RESULTS AND DISCUSSION

GC separation and quantitation of oxyanions

The separation of the *tert.*-butyldimethylsilyl (tBDMS) derivatives of ethyl phosphite, phosphite and phosphate in their acid forms is shown in Fig. 2. Each oxyanion derivative yielded a sharp chromatographic peak without tailing. No visual evidence of decomposition of the derivatized compounds could be found during GC analysis. Phosphite appeared to be stable toward oxidation, giving a peak distinct from phosphate. A small peak corresponding to phosphite was obtained when a reaction mixture of ethyl phosphite was injected. As was confirmed by anion-exchange chromatography, this is due to traces of phosphite (3%) present in the fosetyl-Al technical material.

The tBDMS derivatives of the three compounds were eluted in order of increasing molecular weight on CP™-Sil 5.

The sensitivity proved to be excellent; the minimum detectable amounts of phosphite and phosphate were 20 pmol and for ethyl phosphite, 50 pmol per injection volume (1 μl). The response curves for each compound were linear over the range of

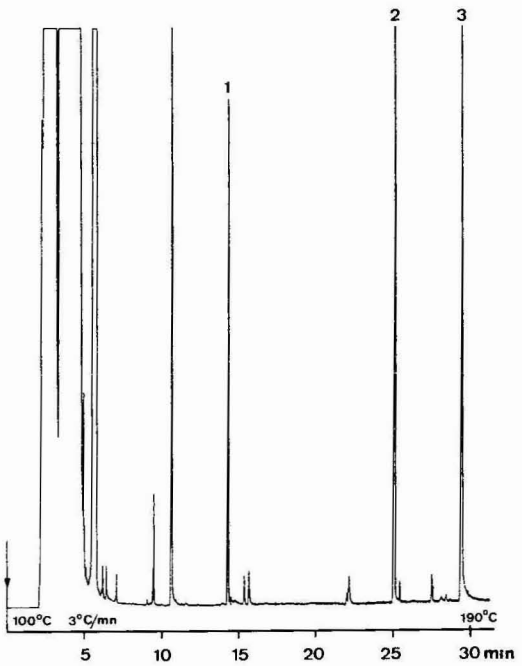

Fig. 2. Chromatographic separation of ethyl phosphite (1), phosphite (2) and phosphate (3) tBDMS derivatives on a fused-silica capillary column wall coated with CP^{lm}-Sil 5 (25 m × 0.22 mm I.D.). Temperatures: column, 100 to 200°C at 3°C/min; injector, 250°C; detector, 270°C. Carrier gas: helium (0.7 bar). Splitless sampling: volume injected, 1 μl. Each peak represents 200 pmol of each oxyanion.

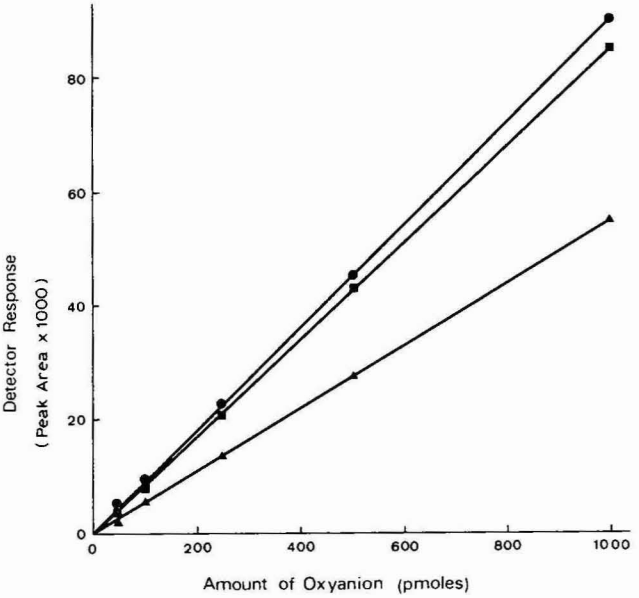

Fig. 3. Calibration curves: ▲—▲, ethyl phosphite; ■—■, phosphite; ●—●, phosphate.

20 pmol to 1 nmol (Fig. 3). In contrast to the derivatives of phosphite and phosphate which possess three tBDMS groups[11], the response to the ethyl phosphite derivative (lower molecular weight) is less strong.

Effect of plant material on derivatization

The formation of the tBDMS derivatives of the free acid forms of the three oxyanions was complete in less than 5 min. However, before application of this GC method to biological material, it was necessary to study its influence on the yield of the derivatization.

A known amount of phosphorous acid was added to a tomato leaf extract and, after evaporation to dryness and silylation by MTBSTFA, different aliquots were injected into the gas chromatograph. The presence of plant material reduced the peak areas of the derivatized phosphite and the response was inversely proportional to the amount of vegetable material (Fig. 4). Many interfering peaks were introduced, and repeated injections led to severe contamination of the injection port. These results showed that a rigorous purification is necessary before the quantification of the silylated oxyanions by flame ionization detection.

Anion-exchange purification

The purification of the biological material was performed by anion-exchange removal of the oxyanions of interest from the biological background. The analysis was accomplished in less than 30 min using a relatively low concentration of an UV-absorbing buffer. Fig. 5 shows the separation profile of a standard mixture of the three oxyanions. The concentration of buffer (10 m*M*) was adjusted in order to reduce the volume of the collected fraction.

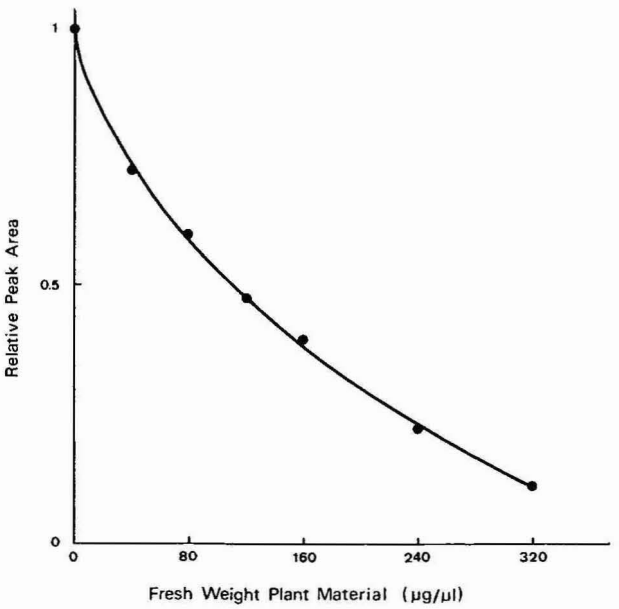

Fig. 4. Dependence of the $(tBDMS)_3PO_3$ peak area on the presence of plant material in the sample. The peak areas were normalized on the $(tBDMS)_3PO_3$ peak area obtained with a standard sample.

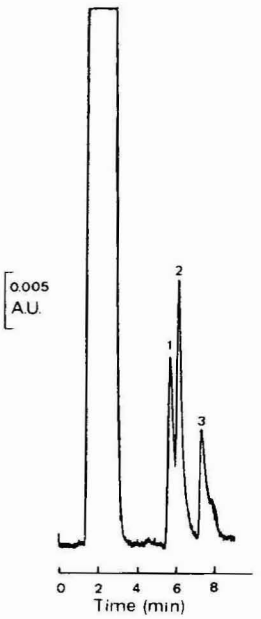

Fig. 5. HPLC separation of a standard mixture of oxyanions: 1 = phosphate; 2 = phosphite; 3 = ethyl phosphite. Column: 10-μm anion-exchange material (Ionospher^{tm} A), 250 × 4.6 mm I.D. Mobile phase: 10 m*M* biphthalate buffer (pH 4.0). Flow-rate: 2 ml/min. UV detection at 308 nm, 0.05 a.u.f.s.

Of the two tested buffers, sodium salicylate and potassium biphthalate, the second was more appropriate, giving better separation and repeatability. Prolonged utilization of sodium salicylate dramatically decreases column performance.

Silylation of a purified HPLC fraction of tomato leaf extract (200 mg) with MTBSTFA gave relatively smaller peaks of each derivative. This is due to the excess of potassium biphthalate and the formation of potassium salts of the oxyanions which are not soluble in the reaction mixture[10].

In order to optimize the yield of silylation, a further clean-up procedure was necessary. Elimination of biphthalate and conversion of the oxyanions into their free acid forms was achieved by a microcolumn system consisting of three C_{18} cartridges and a microcolumn of cation-exchange resin (hydrogen form). The utilization of the C_{18} cartridges allowed the elimination of *ca.* 80% of the biphthalate, and the cation-exchange resin removed the potassium cations.

Known amounts of ethyl phosphite and phosphite were added to plant material previously purified by HPLC and the microcolumn system just before derivatization. GC analysis of the micropreparation showed no decrease in the response of each tBDMS derivative, and the complete absence of interfering peaks.

A series of standard mixtures of ethyl phosphite, phosphite and phosphate were analysed by this total procedure (HPLC purification, C_{18} cartridges + cation-exchange column, silylation and GC analysis). A recovery of the three oxyanions of 85 ± 5.8% (S.D.) was found.

TABLE I

OXYANION CONTENTS OF TOMATO AND COWPEA LEAVES

Leaves were analysed after floating for 48 h on aluminium ethyl phosphite solution (0.56 μmol/ml). Each value represents duplicate analyses of an independent sample. n.d. = Not detected.

Oxyanion	*Amount (μmol/g)*	
	Tomato	*Cowpea*
Ethyl phosphite	n.d.	0.2
Phosphite	1.2	2.0
Phosphate	3.6	12.5

Application to the analysis of plant extracts

Detached tomato and cowpea leaves were placed in petri dishes with the lower surface in contact with an aqueous solution of aluminium ethyl phosphite (0.56 μmol/ml) containing benzimidazole (50 μg/ml). After 48 h under fluorescent light, the leaves were rinsed, washed and extracted as described above. Table I gives the oxyanion contents of the treated leaves.

CONCLUSION

The combined use of HPLC and GC in the analysis of ethyl phosphite, phosphite and phosphate yields an excellent sensitivity and the results are accurate and repeatable. Further application of this method to the quantification of other oxyanions of biological origin or pollutants is possible, therefore providing valuable information about their rôles in the physiology of living organisms.

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SEPARATION OF GERANYLGERANIOL ISOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND IDENTIFICATION BY ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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SUMMARY

The geometric isomers of geranylgeraniol were prepared by isomerization of *trans,trans,trans*-geranylgeraniol by UV irradiation in the presence of thiobenzoic acid. The resulting isomers were separated by high-performance liquid chromatography on styrene–divinylbenzene gel as the stationary phase. The mixture of eight isomers was separated into two fractions according to the geometric isomerism of the hydroxylated terminal unit using 2,2,4-trimethylpentane as the eluent. Each fraction was further separated into four fractions in methanol as the eluent. The isomers were identified by ^{13}C NMR spectroscopy. Pure *cis,cis,cis*-, *cis,trans,trans*-, *trans,cis,cis*-, *trans,cis,trans*- and *trans,trans,trans*-geranylgeraniols were obtained together with three other isomers in 75–87% purity.

INTRODUCTION

Geranylgeraniol (3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-ol) is a linear diterpene isolated from linseed oil[1] and the wood of *Cedrela tonna*[2]. The natural form exists as only one isomer comprising *trans,trans,trans* double bonds. It is widely recognized that *trans,trans,trans*-geranylgeranyl pyrophosphate plays a significant role as a precursor in the biosynthesis of cyclic diterpenes, carotenes, polyterpenoids and polyprenols. Although the other geometric isomers have never been isolated from natural sources, they would be expected to possess some activity in biological systems. The effects of saturation and chain length were determined for various polyprenyl phosphates employed as lipid acceptors for sugars[3–5]. However, very little evidence is available about the geometric isomerism of polyprenyl compounds. For example, undecaprenol phosphate consisting of two *trans*- and eight *cis*-isoprene units has been shown to act as a sugar carrier in the synthesis of peptidoglycan and polysaccharide in microorganisms, whereas the role of the isomeric undecaprenol containing three *trans*- and seven *cis*-isoprene units, which exists predominantly in the leaf tissues of angiosperms, has not yet been clarified[6]. It seems likely that a particular geometric arrangement offers advantages in certain biological situa-

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tions or a geometric isomer may simply result from biosynthesis sequences that provide no functional advantage to the final product. The synthesis of the geranylgeraniol isomers may provide a way to elucidate the biological significance of geometric isomerism in isoprenoid compounds.

Several methods have been proposed for the synthesis of linear terpenes. The *trans,trans,trans-* and *cis,trans,trans*-geranylgeraniols can be prepared by chain extension from *trans,trans*-farnesol[7]. Similarly, the other geranylgeraniol isomers may be synthesized using the other farnesol isomers as starting materials, although the desired products are always accompanied by geometric isomers. In a previous paper, we reported[8] the separation of the geometric isomers of farnesol by use of high-performance liuid chromatography (HPLC). In the present investigastion we undertook to prepare all of the geranylgeraniol isomers by separation of isomerized geranylgeraniol by means of preparative HPLC. The isomers were then characterized by ^{13}C NMR spectroscopy.

EXPERIMENTAL

Isomerization of geranylgeraniol

A solution of the geranylgeraniol isomer having the 2-*trans*,6-*trans*,10-*trans* configuration (I) (Kuraray Co.), abbreviated as t,t,t-GG, in benzene (2 g per 100 ml) was irradiated with a high-pressure mercury lamp in the presence of 0.01–0.15 g of thiobenzoic acid per 1 g of geranylgeraniol, for 7–10 h at 10°C under a nitrogen atmosphere and with magnetic stirring. The resulting solution was washed with aqueous 2 *M* sodium hydroxide to remove thiobenzoic acid. The 2-*cis*,6-*trans*,10-*trans* isomer (II) (Kuraray Co.), abbreviated as c,t,t-GG, was isomerized using the same procedure. The resulting geranylgeraniol isomers were separated by HPLC.

Liquid chromatography

HPLC was carried out using a JASCO Trirotar II as a high-pressure pump and a Waters R 401 differential refractometer as a detector. For analytical purposes, a 500 × 7.5 mm I.D. stainless-steel column was used at a flow-rate of 1 ml/min, and for preparative separations, a 600 × 21 mm I.D. column at a flow-rate of 6 ml/min. The columns were packed with a high-resolution styrene–divinylbenzene copolymer gel having an exclusion limit of 3000 in gel permeation chromatography. The gel was prepared by conventional suspension polymerization[9]. Analytical columns packed with silica gel (5 μm) or ODS-silica gel (5 μm) were also used.

Gas chromatography

Gas chromatography (GC) wass carried out using a Hitachi 163 gas chromatograph equipped with a 30-m glass capillary column coated with free fatty acid polyester (FFAP).

^{13}C NMR measurements

The ^{13}C NMR spectra were obtained at 50.1 MHz using a JEOL FX-200 spectrometer. Measurements were made at room temperature in deuterochloroform solution (about 5%, w/v). Chemical shifts were referred to tetramethylsilane added as an internal standard. The accuracy of the chemical shifts was ± 0.01 ppm.

RESULTS AND DISCUSSION

Preparation of geranylgeraniol isomers

Isomerized I showed four peaks in its gas chromatogram, corresponding to the $(cis)_3$, $(cis)_2$ (*trans*), (*cis*) $(trans)_2$ and $(trans)_3$ isomers in order of increasing retention time. Fig. 1 shows the total amount of *trans* units in the mixture during the isomerization reaction. The amount levelled off after 2–7 h depending upon the amount of thiobenzoic acid. The final content of 62–64% *trans* units was presumed to be an equilibrium value, because the same value was obtained by isomerization starting from compound II. This equilibrium value is in good agreement with that found in the isomerization of *cis*- and *trans*-polyisoprenes using a similar procedure[10].

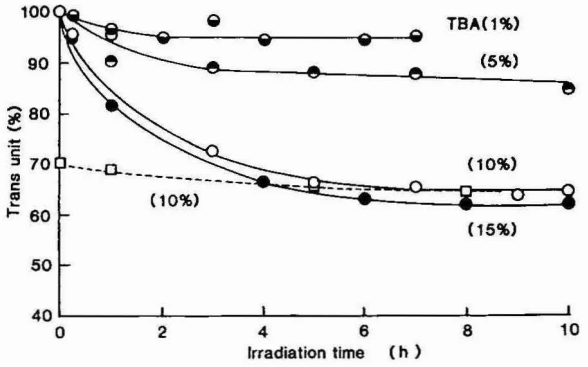


Fig. 1. The content of *trans*-isoprene units in isomerized geranylgeraniol from the t,t,t-GG isomer (——) and from the c,t,t-GG isomer (– – –). TBA = Thiobenzoic acid.

The observed relative intensities of these GLC peaks were in good agreement with the theoretical values obtained on the assumption of random isomerization of the isoprene units.

Separation of geranylgeraniol isomers by HPLC

The use of HPLC was proven to be effective in separating 2-*trans*,6-*trans*- and 2-*cis*,6-*trans*-farnesol on silica gel as the stationary phase[11]. We have demonstrated that a complete separation of the farnesol isomers can be achieved by HPLC using styrene–divinylbenzene gel as the stationary phase[8]. However, little is known about the separation of geranylgeraniol isomers. In order to find the most suitable separation conditions, the elution behaviour of isomers I and II was determined on silica gel, ODS-silica gel and styrene–divinylbenzene gel with various eluents.

Table I lists the observed elution volumes for compounds I and II. Columns packed with silica gel or ODS-silica gel yielded the same elution volume for both isomers independent of the eluent polarity. An unresolved peak was observed for isomerized I on the silica gel and ODS-silica gel columns. On the other hand, a marked difference in elution volumes was observed between I and II on styrene–divinylbenzene gel with 2,2,4-trimethylpentane, cyclohexane or methanol as an

TABLE I

ELUTION VOLUMES (ml) OF GERANYLGERANIOL ISOMERS

I = t,t,t-GG isomer; II = c,t,t-GG isomer.

Eluent	*Polystyrene gel**		*ODS-silica gel***		*Silica gel***	
	I	*II*	*I*	*II*	*I*	*II*
2,2,4-Trimethylpentane	79.0	71.0	19.5	19.3	28.4	28.4
Hexane	–	–	–	–	26.0	26.0
Cyclohexane	40.8	38.4	18.0	17.3	–	–
Diisopropyl ether	33.1	33.6	–	–	–	–
Chloroform	14.5	14.5	–	–	–	–
Tetrahydrofuran	–	–	13.5	13.3	16.6	16.4
Acetone	34.6	34.6	–	–	–	–
Acetonitrile	70.7	69.9	38.6	34.1	18.3	18.0
Methanol	91.0	83.5	20.2	19.5	16.6	16.5

* Measured on a 500 × 10 mm I.D. column at a flow-rate of 1 ml/min.
** Measured on a 500 × 7.5 mm I.D. column at a flow-rate of 1 ml/min.

eluent. This elution behaviour is in accord with that of farnesol isomers, and indicates that the separation of geranylgeraniol isomers proceeds according to two types of mechanisms; one reflects the number of *cis* units in the isomers in methanol as eluent, and the other reflects the geometric isomerism of the hydroxylated terminal (α-terminal) units in 2,2,4-trimethylpentane or cyclohexane as eluent, as in the case of farnesol isomers[8].

Fig. 2 shows the chromatograms of isomerized I in 2,2,4-trimethylpentane and in methanol as eluent. The first and second peaks in 2,2,4-trimethylpentane were tentatively assigned to the isomers having the *cis* α-terminal unit (c,c,c-, c,t,c-, c,c,t- and c,t,t-GG) and those having the *trans* α-terminal unit (t,t,t-, t,t,c-, t,c,t- and t,c,c-GG), respectively. In methanol as eluent the isomers were separated into four peaks, which were tentatively assigned to the $(cis)_3$, $(cis)_2$ $(trans)$, (cis) $(trans)_2$ and $(trans)_3$ isomers in order of increasing elution volume.

The preparative separation of the isomers was carried out under similar conditions. With a sample of 250 mg in 1 ml of solution, fractions A and B were obtained by recycling three times in 2,2,4-trimethylpentane as eluent, as shown in Fig. 3a. The

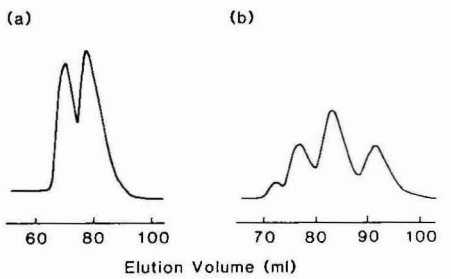


Fig. 2. Liquid chromatograms of isomerized geranylgeraniol in 2,2,4-trimethylpentane (a) and in methanol (b) as eluent.

purity of both fractions was confirmed to be 100% by comparison of the ^{13}C NMR signals characteristic of *cis* and *trans* α-terminal units. Fractions A and B were then subjected to further chromatography in methanol as eluent as shown in Fig. 3b and c. The fraction A was separated into three peaks after three cycles. After removing the first and the last fractions, A-1 and A-4, the central peak was recycled 23 times and separated into two fractions, A-2 and A-3. Similarly, fraction B was separated into four fractions, B-1 to B-4. According to the results mentioned above, fractions A-1, A-4, B-1 and B-4 can be unequivocally assigned to c,c,c-, c,t,t-, t,c,c- and t,t,t-GG, respectively. However, it is difficult to identify fractions A-2, A-3, B-2 and B-3 only from their elution behaviours. The fractions A-2 and A-3 correspond to either c,c,t-GG or c,t,c-GG, and B-2 and B-3 to either t,c,t-GG or t,t,c-GG. These fractions were identified on the basis of the ^{13}C NMR analysis.

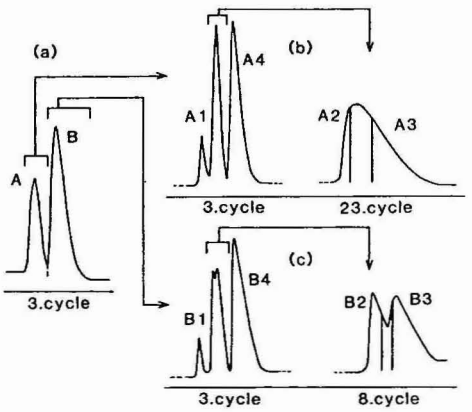
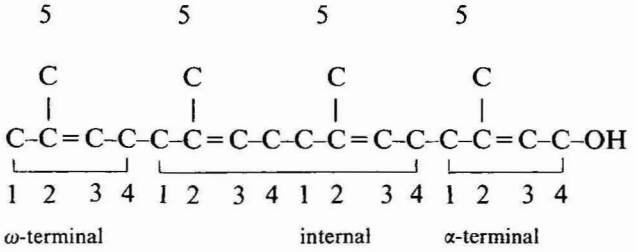


Fig. 3. Preparative HPLC separation of isomerized geranylgeraniol in 2,2,4-trimethylpentane (a) and in methanol (b and c) as eluent.

^{13}C *NMR analysis of geranylgeraniol isomers*

The ^{13}C NMR spectra of the isomers were assigned by considering the corresponding assignments for geraniol, nerol and farnesol isomers and solanesol[12], and the spin–lattice relaxation times, T_1[13]. The aliphatic and olefinic carbon atoms in the isomers showed signals characteristic of the alignment of the *cis* and *trans* units as well as of the geometric isomerism of the internal and α-terminal units. The chemical shifts and assignments of the signals are listed in Tables II–IV.

The carbon atoms are designated as follows by considering the similarity of the chemical and steric environments of the corresponding carbons in repeating isoprene units:



The geometric isomerism of the internal and α-terminal units can be determined from the chemical shifts of the methyl carbon signals listed in Table II. The C-4 methylene carbon atom in the *trans* and *cis* α-terminal units showed characteristic signals at 59.4 and 59.1 ppm, respectively as listed in Table III.

TABLE II

CHEMICAL SHIFTS OF METHYL CARBON SIGNALS FOR GERANYLGERANIOL ISOMERS

The abbreviations t and c correspond to *trans* and *cis* units, respectively.: (*cis*) and (*trans*) correspond to the methyl carbon of the ω-terminal unit in *E* and *Z* configurations, respectively.

Fraction	*Isomer*	*ω-Terminal*		*Internal*		*α-Terminal*	
		(cis)	*(trans)*	*cis*	*trans*	*cis*	*trans*
A-1	c,c,c-GG	25.71	17.64	23.39		23.44	
A-2	c,c,t-GG	25.68	17.68	23.37	15.98	23.45	
A-3	c,t,c-GG	25.71	17.63	23.37	16.00	23.43	
A-4	c,t,t-GG	25.68	17.67		16.01	23.44	
B-1	t,c,c-GG	25.70	17.64	23.40			16.28
B-2	t,c,t-GG	25.69	17.69	23.40	16.00		16.30
B-3	t,t,c-GG	25.70	17.64	23.38	16.03		16.29
B-4	t,t,t-GG	25.68	17.68		16.02		16.28

The C-1 methylene carbon atom in the *trans* and *cis* units showed signals around 40 and 32 ppm, respectively, reflecting the geometric isomerism of the unit linked to the C-1 carbon atom, *i.e.*, 39.6 ppm [*trans-trans*(α)], 39.7–39.8 ppm (*ω-trans* and *trans-trans*), 39.9 ppm [*cis-trans*(α)], 40.0 ppm [*cis-trans*(α)], 32.0–32.1 ppm [*ω-cis*, *trans-cis*, and *trans-cis*(α)] and 32.3–33.4 ppm [*cis-cis* and *cis-cis*(α)]. It was observed that the ω-terminal unit has the same shielding effect on the subsequent C-1 methylene carbon atom as does an internal *trans* unit[12]. The fractions A-2, A-3, B-2 and B-3 were unequivocally identified on the basis of these signal assignments.

The C-2 carbon atom in the ω-terminal unit in the *ω-trans* linkage gave a signal around 131.3 ppm, while that in the *ω-cis* linkage showed a signal around 131.5 ppm as listed in Table IV. These signals reflecting the sequence structure of the isoprene units were used to determine the geranylgeraniol isomers. The other olefinic carbons and C-4 methylene carbons showed complicated signals reflecting the diad or triad sequences of the isoprene units. The relationship between the chemical shifts and the structure of the isoprene units has been observed for various linear isoprenoid compounds, independent of their molecular weights[12].

On the basis of these ^{13}C NMR assignments, the purity of the fractions A-1 to A-4 and B-1 to B-4 were determined by considering the relative intensities of the signals characteristic of each isomer as listed in Table V. The results show that the pure c,c,c-, c,t,t-, t,c,c-, t,c,t- and t,t,t-GG isomers can be obtained by this preparative HPLC method. The purity of the other three isomers was found to be 74.7–87.4%, which is expected to be improved by injecting smaller samples or by further recycling.

TABLE III

CHEMICAL SHIFTS OF METHYLENE CARBONS IN GERANYLGERANIOL ISOMERS

Fraction	*Isomer*	*C-1 (CH_2) (internal, α-terminal)*						*C-4 (CH_2)*				
		*trans**				*cis**		*ω-Terminal*	*Internal*		*α-Terminal*	
		$C\underline{T}$	$C\underline{T}_\alpha$	$T\underline{T}$ $\omega\underline{T}$	$T\underline{T}_\alpha$	$C\underline{C}_\alpha$ $C\underline{C}$	$T\underline{C}_\alpha$ $T\underline{C}$ $\omega\underline{C}$				*trans*	*cis*
A-1	c,c,c-GG					32.24	31.94	26.68	26.40	26.34		59.04
A-2	c,c,t-GG			39.75		32.27	31.94	26.74	26.58	26.35		59.03
A-3	c,t,c-GG	39.99					31.99	26.64	26.54	26.55		59.06
A-4	c,t,t-GG			39.70			32.02	26.79	26.60	26.47		59.00
				39.73								
B-1	t,c,c-GG		39.84			32.29	31.98	26.70	26.39	26.22	59.42	
B-2	t,c,t-GG		39.85	39.77			31.99	26.75	26.53	26.22	59.42	
B-3	t,t,c-GG	40.00			39.57		32.02	26.65	26.55	26.35	59.41	
B-4	t,t,t-GG			39.75	39.60			26.82	26.68	26.39	59.35	

* The signals reflect diad sequences of *cis* and *trans* units. The observed unit is underlined.

TABLE IV

CHEMICAL SHIFTS OF OLEFINIC CARBONS IN GERANYLGERANIOL ISOMERS

Fraction	*Isomer*	*C-2 (=CH)*						*C-3 (=C)*			
		*ω-Terminal**		*Internal*		*α-Terminal*					
		ωC	*ωT*			*cis*	*trans*				
A-1	c,c,c-GG	131.50		136.45	135.11	139.91		124.88	124.53	124.47	124.31
A-2	c,c,t-GG		131.51	136.29	135.23	140.03		124.48	124.48	124.36	124.05
A-3	c,t,c-GG	131.51		136.27	135.22	139.91		124.92	124.47	124.37	123.61
A-4	c,t,t-GG		131.23	135.04	135.96	139.87		124.45	124.41	124.12	123.62
B-1	t,c,c-GG	131.53		135.37	135.50		139.76	124.99	124.60	124.35	123.41
B-2	t,c,t-GG		131.32	135.22	135.60		139.80	124.56	124.38	124.13	123.41
B-3	t,t,c-GG	131.50		135.37			139.76	125.02	124.41	123.83	123.40
B-4	t,t,t-GG		131.22	134.96	135.38		139.64	124.45	124.23	123.85	123.48

* Abbreviations as in Table III.

TABLE V

PURITY (%) OF GERANYLGERANIOL ISOMERS

Fraction	*c,c,c-GG*	*c,c,t-GG*	*c,t,c-GG*	*c,t,t-GG*	*t,c,c-GG*	*t,c,t-GG*	*t,t,c-GG*	*t,t,t-GG*
A-1	100	0	0	0				
A-2	0	87.4	12.6	0				
A-3	0	25.3	74.7	0				
A-4	0	0	0	100				
B-1					100	100	0	0
B-2					0	100	0	0
B-3					0	17.9	82.1	0
B-4					0	0	0	100

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Note

Chromatography of bis-quaternary amino steroids

II*. Separation on chemically bonded silica phases

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In Part I[1], new thin-layer chromatographic (TLC) and high-performance liquid chromatographic (HPLC) methods were introduced for the separation of bis-quaternary amino steroids on silica stationary phases. It was found that the retention of the compounds investigated depends on the number of quaternary amino groups and also on the presence or absence of ester groups in the molecule. The HPLC system developed for the separation of pipecuronium bromide and its related steroids could be transferred to the TLC separation of the same components.

The HPLC separation of bis-quaternary amino steroids on chemically bonded silica phases has now been investigated. The main aim of this work was to study the retention behaviour of quaternary amines on chemically bonded phases in order to obtain more information about the possible interactions between the mobile phase composition (salt concentration, type of organic solvent, etc.) and sample molecules to elucidate the separation mechanism.

EXPERIMENTAL

A Liquochrom 2010 high-performance liquid chromatograph equipped with a variable-wavelength UV detector and a loop-type injector (Labor MIM, Esztergom-Budapest, Hungary) was used. The separations were performed on pre-packed Nucleosil CN, 10 μm; Nucleosil $(CH_3)_2N$, 10 μm, and Nucleosil 10 C_{18} (250 × 4.6 mm I.D.) columns (Chrompack, Middelburg, The Netherlands). The compounds were detected at 218 nm.

The solvents used for the preparation of eluents were of HPLC grade and were obtained from E. Merck (Darmstadt, F.R.G.). All other reagents and solvents were of analytical-reagent grade and were obtained from Reanal (Budapest, Hungary).

The compounds investigated were prepared at the Chemical Works of Gedeon Richter (Budapest, Hungary) and were of the highest possible quality.

* For Part I, see ref. 1.

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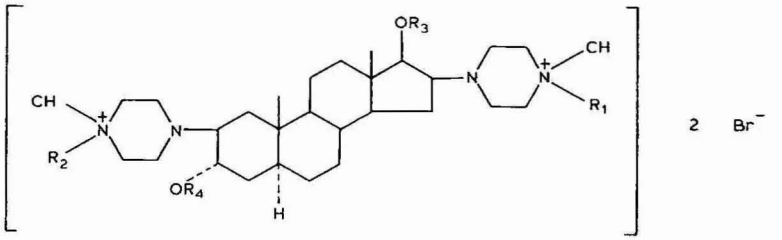
RESULTS AND DISCUSSION

The compounds investigated were the same as used in Part I[1] and are shown in Table I. To investigaste the separation of the model compounds, a medium polarity chemically bonded cyanopropylsilica stationary phase was used.

TABLE I

STRUCTURES OF THE COMPOUNDS INVESTIGATED



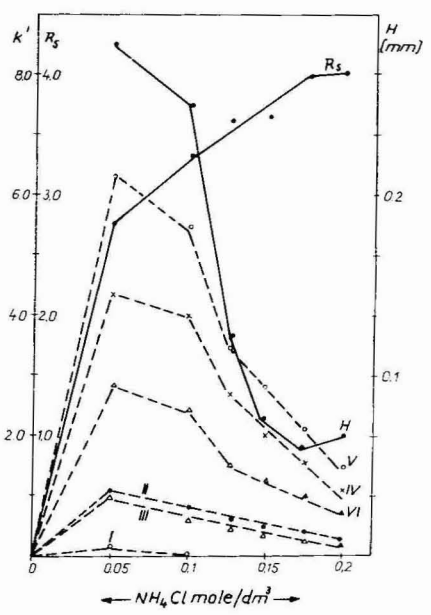
Compound	R_1	R_2	R_3	R_4
I	H	H	CH_3CO	CH_3CO
II	CH_3	H	CH_3CO	CH_3CO
III	H	CH_3	CH_3CO	CH_3CO
IV	CH_3	CH_3	H	CH_3CO
V	CH_3	CH_3	H	H
VI (pipecuronium bromide)	CH_3	CH_3	CH_3CO	CH_3CO

Effect of ammonium chloride and ammonium carbonate used in the eluent

Fig. 1 shows the change in the retention of the compounds investigated with variation in the ammonium chloride concentration of the eluent. In the absence of ammonium chloride, no separation was obtained. On increasing the salt concentration, the retention of the compounds showed a maximum (the maximum appeared at the lowest salt concentration applied), while the resolution calculated for VI and IV increased slightly. A satisfactory separation between the two mono-quaternary compounds II and III was achieved. The column efficiency is highly dependent on the salt concentration.

A similar dependence of retention on ammonium carbonate concentration in the eluent was observed, as illustrated in Fig. 2. When ammonium carbonate was used in the eluent, higher capacity ratios for bis-quaternary compounds and resolutions were obtained.

The best separation conditions were achieved when both ammonium chloride and ammonium carbonate were used in the eluent, in a 1:1 molar ratio. The influence of the total salt concentration on the chromatographic separation is demonstrated in Fig. 3. It can be seen that the eluent containing ammonium chloride and ammonium carbonate in 0.125–0.125 moles/dm^3 concentrations seems to be the most suitable.



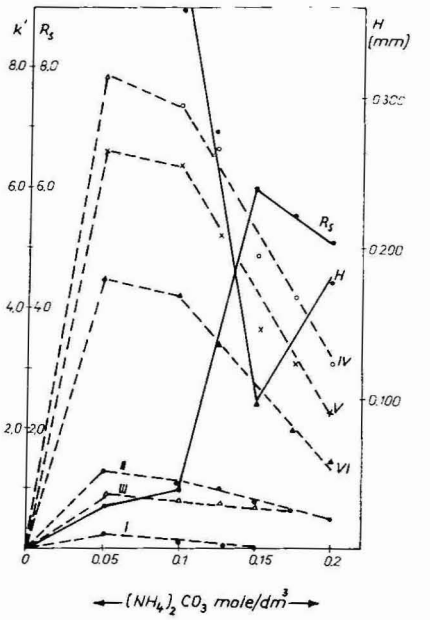


Fig. 1. Effect of ammonium chloride in the eluent on retention. Column, Nucleosil CN, 10 μm (250 × 4.6 mm I.D.); eluent, mixture of A and B (86:14), where A is methanol–acetonitrile (64:22) and B is ammonia solution (sp.gr. 0.880); flow-rate 1 cm³/min; detection, 218 nm. The R_s values were calculated for IV and VI and the H value for VI. Compound as in Table I.

Fig. 2. Effect of ammonium carbonate in the eluent on retention. Conditions as in Fig. 1.

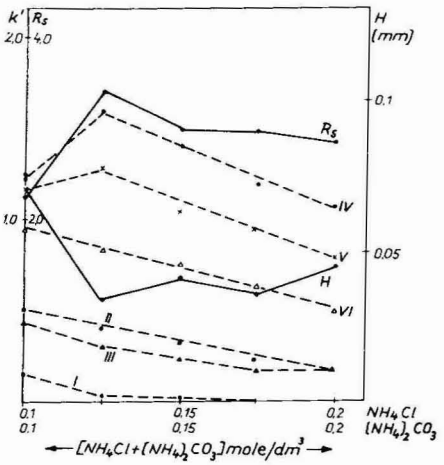


Fig. 3. Effect of ammonium chloride and ammonium carbonate in the eluent on retention. Conditions as in Fig. 1.

Effect of the nature and concentration of organic solvents on the selectivity and efficiency of the separation

Fig. 4 shows the dependence of capacity ratios, resolution and column efficiency on the ratio of methanol to acetonitrile in the eluent at a constant organic solvent concentration. The retention of the compounds shows a minimum (Fig. 5).

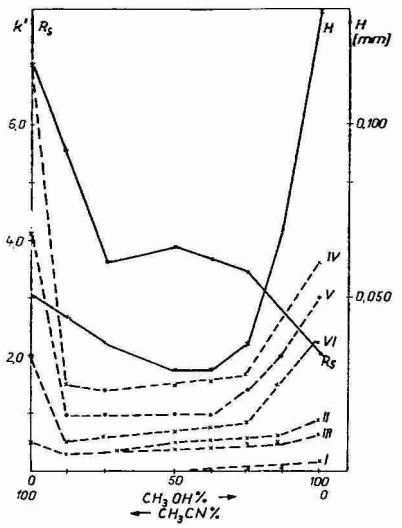


Fig. 4. Dependence of the retention on the ratio of methanol to acetonitrile. Eluent: mixture of A and B (86:14), where A is methanol–acetonitrile in different ratios and B is ammonia solution (sp.gr. 0.880) containing ammonium chloride and ammonium carbonate at concentrations of 0.125–0.125 moles/dm³. Other conditions and compounds as in Fig. 1.

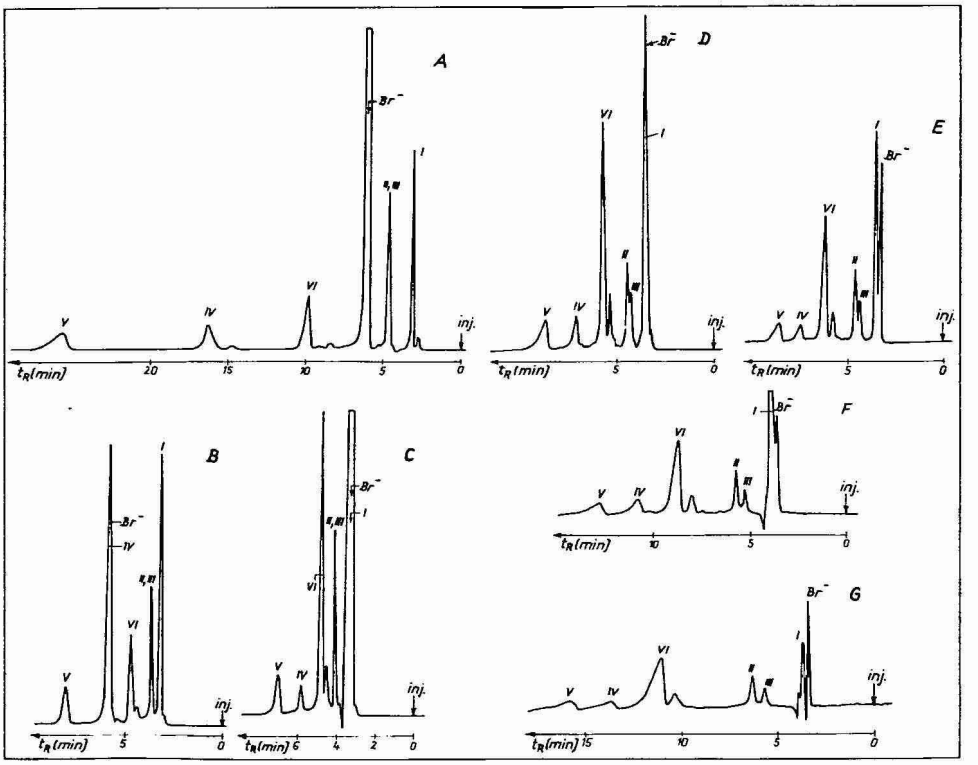


Fig. 5. Dependence of the separation on the ratio of methanol to acetonitrile. Conditions as in Fig. 4, the methanol concentrations relative to the total organic solvent concentration being (A) 0%, (B) 12.8%, (C) 25.6%, (D) 50%, (E) 74.4%, (F) 87.2% and (G) 100%.

When only acetonitrile is used in the eluent as an organic solvent, no separation of the mono-quaternary compounds II and III was achieved. Bromide ion also had a significant retention in this eluent system (Fig. 5A). On increasing the methanol concentration to 11% (Fig. 5B) the retentions of the bis-quaternary compounds (IV, V and VI) decreased dramatically and IV could not be separated from the bromide ion. The retention of bromide ion did not change significantly.

When the methanol concentration was 22% (Fig. 5C), the separation was more effective, except for II and III. With a 1:1 methanol to acetonitrile ratio (Fig. 5D) the mono-quaternary compounds could be distinguished from each other; with decreasing acetonitrile concentration the separation of mono-quaternary compounds was much better, but the peak shape of the bis-quaternary compounds deteriorated (Fig. 5E and F). When only methanol was used in the eluent the chromatogram was completely unsuitable for analytical applications (Fig. 5G).

From the chromatograms shown in Fig. 5, it can be concluded that the best separation can be achieved with an acetonitrile to methanol ratio in the range 1:1–1:2.

Fig. 6 illustrates the influence of the organic solvent concentration on the retention of the compounds (with a ratio of methanol to acetonitrile of 1:1). The best separation was achieved when 86% of organic solvent was used in the eluent.

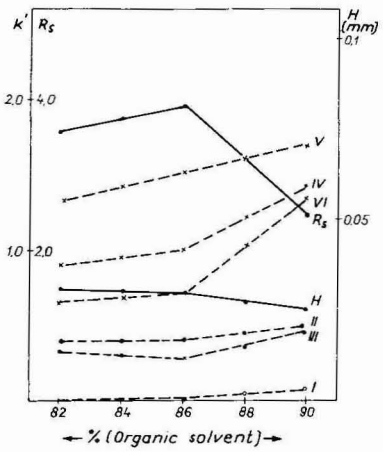


Fig. 6. Influence of organic solvent concentration on retention. Conditions as in Fig. 4; ratio of methanol to acetonitrile, 1:1.

Influence of stationary phases on the separation

Separations on octadecylsilica and dimethylaminosilica columns were also studied. On octadecylsilica, no successful separation was obtained when a reversed-phase eluent (less than 80% of organic solvent) and an aqueous buffered system (between pH 2 and 10) were used. Acceptable separations were obtained only when similar eluent systems were applied to those used on cyanopropylsilica, as shown in Fig. 7.

It can be seen that a reversed elution order was obtained; first the bis-quaternary compounds (VI, V and IV), then the mono-quaternary compounds (III and

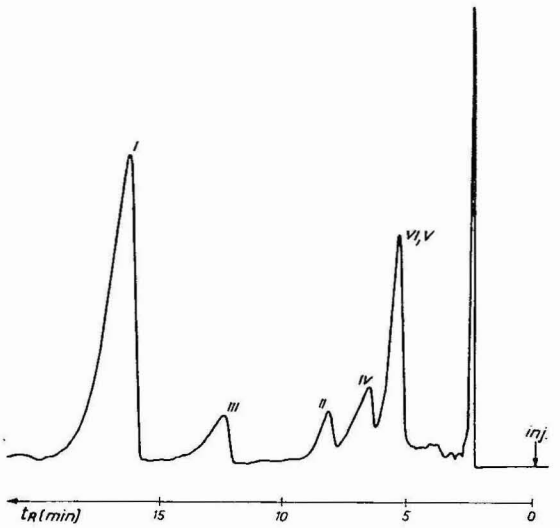


Fig. 7. Separation of bis-quaternary amino steroids on octadecylsilica stationary phase. Column, Nucleosil 10 C_{18} (250 × 4.6 mm I.D.); eluent, mixture of A and B (86:14), where A is methanol–acetonitrile (64:22) and B is ammonia solution (sp.gr. 0.880) containing ammonium chloride and ammonium carbonate at concentrations of 0.125–0.125 moles/dm^3. Other conditions and compounds as in Fig. 1.

II) and finally the tertiary amino derivative were eluted. However, the separation efficiency was significantly lower than on cyanopropylsilica or bare silica columns.

We found that on dimethylaminosilica stationary phase no successful separation can be obtained. However, if the column is decomposed by using an adverse solvent system, the remaining silanol groups can help the separation and similar results were achieved to those on a silica column.

Retention principles

Regarding the separation mechanism, from the experiments reported here and in Part I[1], the following conclusions can be drawn.

An eluent system containing a high concentration of organic solvents (acetonitrile and methanol) and ammonium salts dissolved in ammonia solution is suitable for the separation of bis-quaternary amino steroids on both silica and chemically bonded silica phases. First an ion-pair adsorption mechanism based on the migration of the uncharged ion pair formed by using chloride ion was assumed. This concept was similar to that described by de Zeeuw and co-workers[2,3]. However, this premise was in contrast to some of our experiences, as the use of ammonium carbonate instead of ammonium chloride results in a similar separation, the separation can be performed not only on a silica column, etc.

Based on the results obtained, we consider that the retention principle may be similar to "salting-out" chromatography. The salts used in the eluent should have combined effects on the separation. On the one hand, the salts can decrease the polarity of a quaternary amino group, supressing its dissociation. This assumption seems to be supported by the significant dependence of the retention on the nature and concentration of the organic solvents used in the eluent. On the other hand, the

presence of an inorganic salt in the eluent can decrease the silanophilic effect of the stationary phase supported by the influence of the salt concentration on the retention of the compounds. Both effects of the inorganic salts can increase the hydrophobic interaction of the compounds with the stationary phase.

Based on this concept, we assume that on bare silica or cyanopropylsilica stationary phases the retention mechanism is normal-phase hydrophobic interaction chromatography and on octadecylsilica it is reversed-phase hydrophobic interaction chromatography.

CONCLUSION

The best separations can be obtained on bare silica and cyanopropylsilica stationary phases. On cyanopropylsilica more efficient separations were obtained and on unbonded silica an increased selectivity could be achieved. A reversed elution order was obtained by using octadecylsilica but the selectivity and efficiency of the separation were significantly lower. Dimethylaminosilica itself cannot be used to separate bis-quaternary amino compounds unless it is partly hydrolysed. The separation characteristics obtained in the presence of a high concentration of silanol groups is similar to that obtained unbonded silica. Based on the results obtained, hydrophobic interaction is suggested as the separation mechanism.

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Note

Polyethylene oxide: a ligand for mild hydrophobic interaction chromatography?

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Since the initial paper by Er-el *et al.*[1] in 1972, much work has been carried out on the fractionation of protein mixtures by means of hydrophobic interaction chromatography. Denaturation problems encountered during the elution under the severe conditions frequently required have made it necessary to seek less hydrophobic ligands liable to afford retention of the activity, together with good purification ratios.

In this respect, the use of mild hydrophobic stationary phases prepared by immobilization of polyethylene oxide either on soft gels[2,3] or, very recently, on more rigid supports, suitable for high-performance liquid chromatographic (HPLC) studies[4,5], appears to be a promising alternative. However, as mild hydrophobic interactions are involved, much care should obviously be taken not to introduce strongly interacting hydrophobic spacer arms on the gels, as these would lead to erroneous interpretations of the phenomena observed. In our opinion, the previously mentioned papers[2–5] do not give this particular point all the attention we think it deserves. In fact, we recently demonstrated[6] that the purification of $\Delta_{5\rightarrow4}$-3-oxosteroid isomerase by elution of the initial complex mixture on polyethylene oxide-bound Sepharose 6B was mainly due to hydrophobic interactions with the spacer arm itself, *i.e.*, 1,4-butanediol diglycidyl 1-ether.

In this paper, we report the synthesis of various stationary phases, obtained by unambiguous immobilization of polyethylene oxide on to Sepharose 6B by means of spacer arms totally devoid of hydrophobic moieties strong enough to interfere in the fractionation process.

EXPERIMENTAL

Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol monomethyl ether (MW 750) and 1,4-butanediol diglycidyl 1-ether were supplied by Janssen Chimica (Belgium). Epichlorohydrin and boron trifluoride etherate were purchased from Fluka (Buchs, Switzerland). Ethylene glycol diglycidyl ether, (±)-1,3-butadiene diepoxide and N,N′-carbonyldiimidazole (CDI) were obtained from Aldrich Chimie (F.R.G.). As previously[6], the term potassium phosphate implies a mixture of K_2HPO_4 and KH_2PO_4 with a ratio of 306.9 g of K_2HPO_4 to 168.6 g of KH_2PO_4.

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$\Delta_{5\to4}$-3-oxosteroid isomerase (E.C. 5.3.3.1) (referred to as isomerase in the following text) was extracted from *Pseudomonas testosteroni* acetonic powder as described previously[6]. The chromatographic conditions (flow-rate, sample loading, isomerase activity measurements, total protein concentration, etc.) were as previously reported[6].

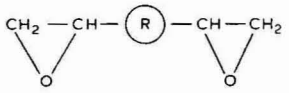
The amount of polyethylene glycol monomethyl ether immobilized on the various gels prepared was measured according to the procedure described by Drevin and Johansson[7] using a Girdel 300 gas chromatograph, an Icap 5 integrator and the same GC packings and experimental conditions, except for the oven temperature (60°C).

RESULTS AND DISCUSSION

Synthesis of the stationary phases

Previously reported results[6] showed that the fractionation of isomerase from the initial protein mixture can be obtained by elution on a gel merely substituted by 1,4-butanediol diglycidyl 1-ether. Various stationary phases were prepared in order to study the influence of the spacer arm structure on the hydrophobic interactions observed and to find out the extent to which polyethylene oxide can act as a mild hydrophobic ligand.

Three "blank gels" were obtained by reaction of Sepharose 6B, under conditions similar to those described by Sundberg and Porath[8], with various diepoxides represented schematically by the general formula



(a)

Table I gives the amounts of epoxy groups immobilized on the three different gels prepared. The final stationary phases were complete after deactivation of the epoxy groups by treatment at pH 14 for about 36 h at room temperature.

Two stationary phases were prepared by the immobilization on Sepharose 6B of polyethylene glycol monomethyl ether [HO-$(CH_2CH_2O)_n$-CH_3, MW 750], using two different synthetic routes:

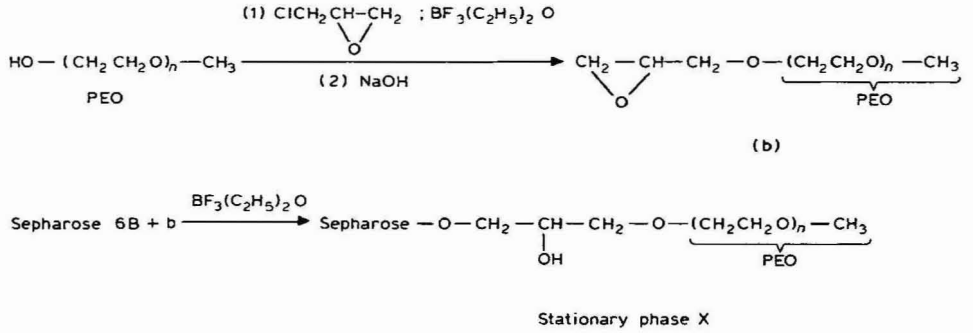
TABLE I

IMMOBILIZATION OF DIEPOXIDES OF GENERAL FORMULA (a) ON TO SEPHAROSE 6B

Parameter	*Stationary phase*		
	A	*B*	*C**
Reagent	(±)-1,3-Butadiene diepoxide	Ethylene glycol diglycidyl ether	1,4-Butanediol diglycidyl 1-ether
®	None	CH_2–O–$(CH_2)_2$–O–CH_2	CH_2–O–$(CH_2)_4$–O–C
Epoxy groups per gram of dry gel (μmol/g)	910	100	850

* Gel C is identical with that described in ref. 6.

Route I. The stationary phase X was prepared according to the following procedure, previously described by Hjertén *et al.*[9] and Ulbrich *et al.*[10]:

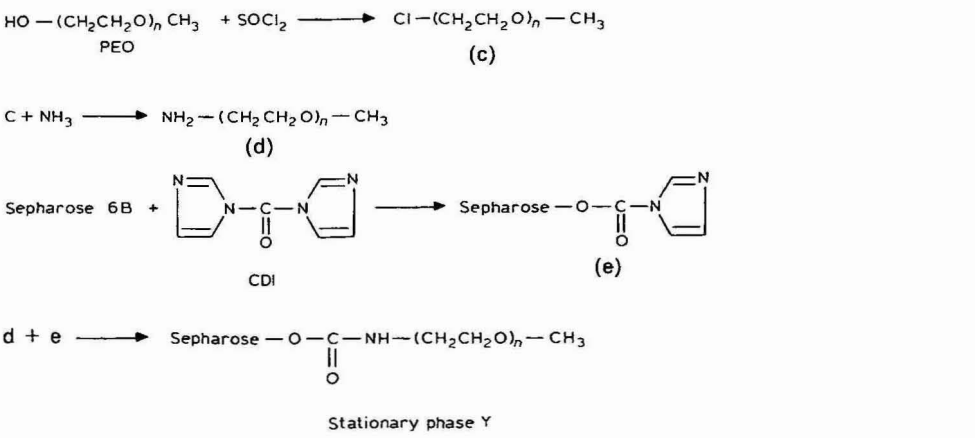


Immobilization of ligands on chromatographic supports is usually carried out after preliminary activation of the matrix. In route I a reverse procedure was used, so as not to introduce on to the Sepharose 6B undesirable unreacted spacer arms that might interfere in the fractionation process.

Polyethylene glycol monomethyl ether (PEO) was first treated with epichlorohydrin in the presence of $BF_3(C_2H_5)_2O$ as a catalyst at 55°C for 4 h with vigorous stirring. The intermediate obtained was subsequently reacted with NaOH at room temperature for 4 h to yield the epoxy-activated derivative b. This compound contained 800 μmol of epoxy groups per gram of polymer, corresponding to about 0.6 mol of epoxy groups per mol of PEO.

This partially epoxy-activated polymer was reacted with Sepharose 6B under rotation for 1 h at 50°C in dry dioxane. The gel was then extensively washed with dioxane, dioxane–water mixtures and finally with water in order to remove unreacted PEO. The amount of PEO immobilized on stationary phase X was 70 μmol per gram of dry gel as determined according to the procedure described by Drevin and Johansson[7].

Route II. The stationary phase Y was prepared according to the following procedure:



In a preliminary step, Sepharose 6B was activated with carbonyldiimidazole (CDI) according to the procedure reported by Bethell *et al.*[11]. The amount of imidazolyl carbamate groups immobilized on Sepharose 6B (e) was 1700 μmol per gram of dry gel.

In order to avoid the formation of water-sensitive carbonate groups, which the reaction of (e) with PEO itself would yield, the hydroxy function of this polymer was converted into NH_2 by successive treatment of PEO with thionyl chloride and then NH_3 at 100°C under pressure, according to a procedure reported earlier[12].

Subsequent reaction of (d) with (e) was carried out in dry dioxane at 40°C under rotation for 48 h. The gel obtained was extensively washed with dioxane, dioxane–water mixtures and finally with water to remove unreacted amino–PEO. Unreacted imidazolyl carbamate groups were subsequently deactivated by treatment at pH 3 overnight. Stationary phase Y thus obtained contained about 520 μmol of PEO per gram of dry Sepharose, as determined according to Drevin and Johansson[7]. Nitrogen microanalysis (1.2%) led to a higher result (800 μmol/g).

Chromatographic experiments

The synthesis of three different gels (A, B and C), differing in the number of CH_2 units, allowed us to investigate the role of the spacer arm structure on the hydrophobic interaction observed.

As was pointed out earlier[6], purification of isomerase from the initial complex mixture can be achieved with a good recovery of its activity (*ca.* 75%) together with elimination of most of the contaminants (>99.5%) by elution at high ionic strength (10% potassium phosphate) on Sepharose 6B merely substituted by 1,4-butanediol diglycidyl 1-ether (gel C). Our assumption was that the fractionation is based on hydrophobic interactions with the $(CH_2)_4$ units of this immobilized spacer arm.

Total retention of isomerase can also be achieved on gel B, provided that the experiment is carried out at even higher ionic strength (15% *vs.* 10% potassium phosphate). This difference is probably due to the stronger hydrophobic character of the $(CH_2)_4$ moieties compared with that of $(CH_2)_2$, together with the substitution ratio of interacting groups, which is higher on gel C (850 μmol/g) than on gel B (100 μmol/g).

In contrast, no fractionation of isomerase from the bulk of contaminants can be obtained on gel A [$(CH_2)_0$].

Chromatographic experiments carried out on either gel X or Y, obtained by immobilization of PEO on Sepharose 6B by means of spacer arms ($-CH_2-CHOH-CH_2$ or $-CO-NH$) not liable to interfere in the hydrophobic interaction, gave very similar results. Fig. 1 shows the elution profile obtained on either stationary phase X or Y. Isomerase is strongly retained on gels equilibrated with 15% potassium phosphate and subsequently eluted stepwise with 10% potassium phosphate, once the bulk of contaminants has been removed. This result can be compared with those obtained on gels B and C, and indicates that polyethylene oxide exhibits a lower hydrophobic character than that of $(CH_2)_4$ moieties, but similar to that of $(CH_2)_2$ units. On the other hand, an increase in the amount of PEO bound on the stationary phase does not seem to influence the strength of the hydrophobic interaction involved, as no major changes in the elution profiles are observed whether the experiment is carried out on gel X or gel Y (500 *vs.* 70 μmol/g).

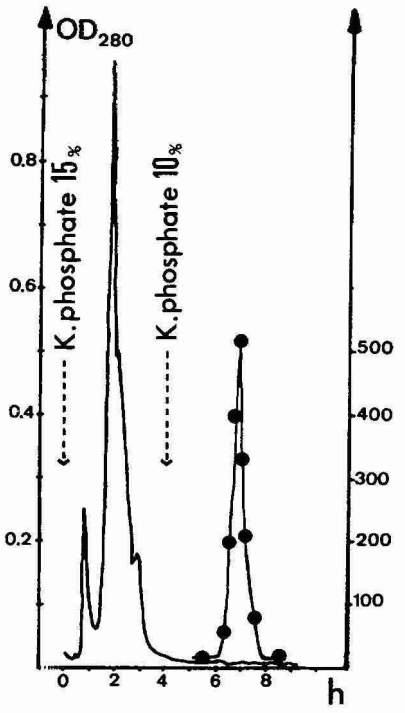


Fig. 1. Stepwise elution of the isomerase-containing sample (0.4 ml) successively with 15% and 10% potassium phosphate (pH 7.0) on gel X (see text). Other experimental conditions as described under Experimental. ●, Isomerase enzymatic activity in arbitrary units.

Further experiments are currently in progress to explore the field in more detail. Nevertheless, the present results already clearly demonstrate without any ambiguity the capacity of polyethylene oxide to act as a mild hydrophobic ligand, able to afford a convenient alternative to traditional hydrophobic interaction chromatography.

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Note

Separation of isomeric halogenobicyclononene carbonitriles

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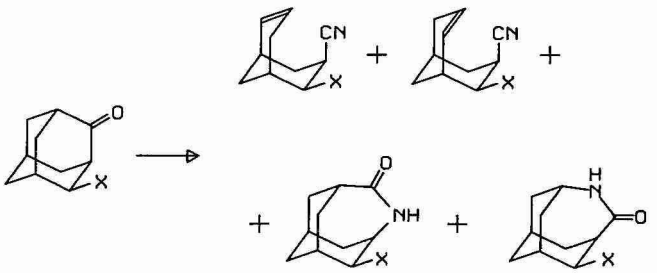
The Schmidt reaction of 4-substituted adamantanones yields a mixture of two 4-halogenobicyclononene carbonitriles and two halogenoazahomoadamantanones[1] (see Scheme 1); the halogenoazahomoadamantanones were separated by medium-pressure liquid chromatography using silica gel and a light petroleum–acetone mixture[2]. We have applied both analytical and preparative high-performance liquid chromatography (HPLC) to the analysis and preparative separation of isomeric halogenobicyclononene carbonitriles and methanesulphonatobicyclononene carbonitriles.



Scheme 1.

EXPERIMENTAL

Analytical HPLC

The retention times and analyses of the fractions obtained from the preparative separation were carried out on a Varian 8500 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.). A 250 × 4 mm I.D. column packed with silica gel (8 μm) (Sila-

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TABLE I

ANALYTICAL CHROMATOGRAPHY

Mobile phase: *n*-pentane–acetone (*a*:*b*).

No.	*Compound*	*a = 99, b = 1*			*a = 97, b = 3*		
		T_r *(s)*	*k'*	α	T_r *(s)*	*k'*	α
1	6-*endo*-Chlorobicyclo[3.3.1]non-2-en-7-carbonitrile	1996	23.3	1.15	565	8.11	1.09
2	6-*endo*-Chlorobicyclo[3.3.1]non-3-en-7-carbonitrile	2286	26.7		609	8.82	
3	6-*endo*-Iodobicyclo[3.3.1]non-2-en-7-carbonitrile	1672	19.4	1.14	493	5.85	1.07
4	6-*endo*-Iodobicyclo[3.3.1]non-3-en-7-carbonitrile	1892	22.1		521	6.24	
	Unidentified	1126	12.7	1.53	362	4.84	1.35
	Unidentified	1678	19.5	1.15	468	6.55	1.28
5	6-*endo*-Bromobicyclo[3.3.1]non-2-en-7-carbonitrile	1918	22.4	1.14	581	8.37	1.09
6	6-*endo*-Bromobicyclo[3.3.1]non-3-en-7-carbonitrile	2182	25.6		630	9.16	
		a = 90, b = 10					
7	6-*endo*-Methanesulphonoxybicyclo[3.3.1]non-2-en-7-carbonitrile	998	11.5	1.02	–	–	–
8	6-*endo*-Methanesulphonoxybicyclo[3.3.1]non-3-en-7-carbonitrile	1021	11.8		–	–	–

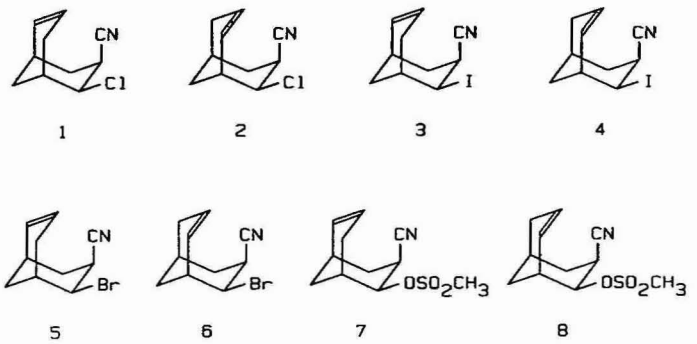


Fig. 1. The structures of the investigated compounds.

sorb; Lachema, Brno, Czechoslovakia), an Optilab 902 B refractive index detector (Tecator, Sweden) and a TZ 4221 strip-chart recorder (Laboratorní přístroje, Prague, Czechoslovakia) were used. Sample injection was performed by the stop flow technique with a 5-μl syringe (Hamilton, Bonaduz, Switzerland). Mixtures of *n*-pentane and acetone in different proportions (see Table I) were used as the mobile phase at a flow-rate of 100 ml/h.

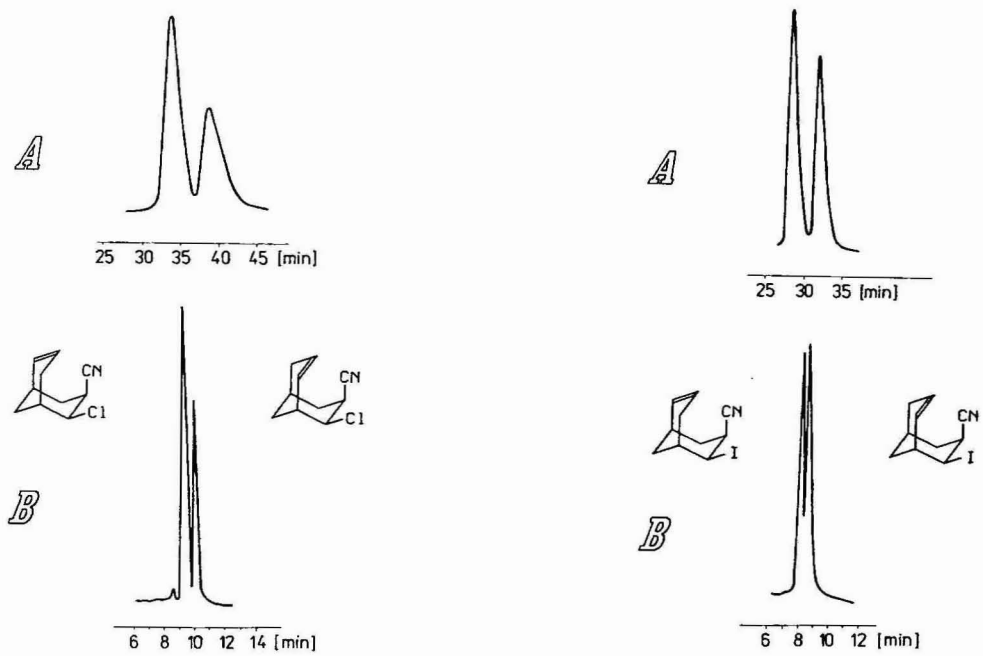


Fig. 2. Analytical separation of chlorobicyclononene carbonitriles. Column: 250 × 4 mm I.D., packed with 8-μm silica. Mobile phase: (A) *n*-pentane–acetone (97:3, v/v); (B) *n*-pentane–acetone (99:1, v/v). Flow-rate: 100 ml/h. Refractive index detector.

Fig. 3. Analytical separation of iodobicyclononene carbonitriles. Conditions as in Fig. 2.

TABLE II

PREPARATIVE SEPARATION

Mobile phase: *n*-pentane–acetone (97:3, v/v).

No.	*Compound*	*Amount separated (mg)*	*Yield (mg)*	*Purity (%)*
1	6-*endo*-Chlorobicyclo[3.3.1]non-2-en-7-carbonitrile	300	179	95
2	6-*endo*-Chlorobicyclo[3.3.1]non-3-en-7-carbonitrile		110	88
3	6-*endo*-Iodobicyclo[3.3.1]non-2-en-7-carbonitrile	400	212	92
4	6-*endo*-Iodobicyclo[3.3.1]non-3-en-7-carbonitrile		169	82

Separation of individual compounds

The preparative separations were carried out on a 300 × 17 mm I.D. stainless-steel column, with a conical inlet part (designed and manufactured in our laboratory[3,4]), slurry packed with 8.5-μm irregular silica gel (Silasorb). The pump was an LC-XPD (Pye Unicam, Cambridge, U.K.), operated at 600 ml/h. A refractive index detector (Varian) was used. The mobile phase used was *n*-pentane–acetone (97:3, v/v). Sample injection was performed using a loop injector (Rheodyne, U.S.A.) equipped with a 200-μl sample loop. A 500-μl injection syringe (Hamilton) was used. The fractions were collected manually according to the shape of the chromatographic curve.

RESULTS AND DISCUSSION

Analytical retention data of the separated bicyclononene derivatives are listed in Table I. The structures of the investigated compounds are shown in Fig. 1.

Although the compounds were not separated satisfactorily by thin-layer chro-

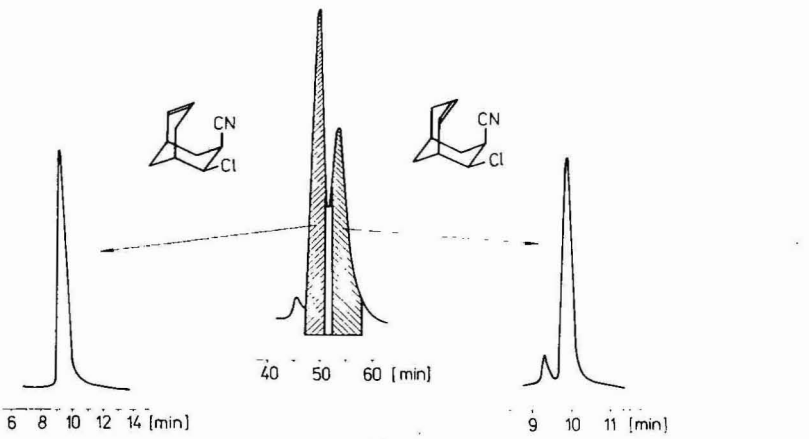


Fig. 4. Preparative separation of chlorobicyclononene carbonitriles and analysis of the fractions obtained. Preparative: column, 300 × 17 mm I.D., packed with 8.5-μm silica; mobile phase, *n*-pentane–acetone (97:3, v/v); flow-rate, 600 ml/h. Analytical: column, 250 × 4 mm I.D., packed with 8-μm silica; mobile phase, *n*-pentane–acetone (97:3, v/v); flow-rate, 100 ml/h.

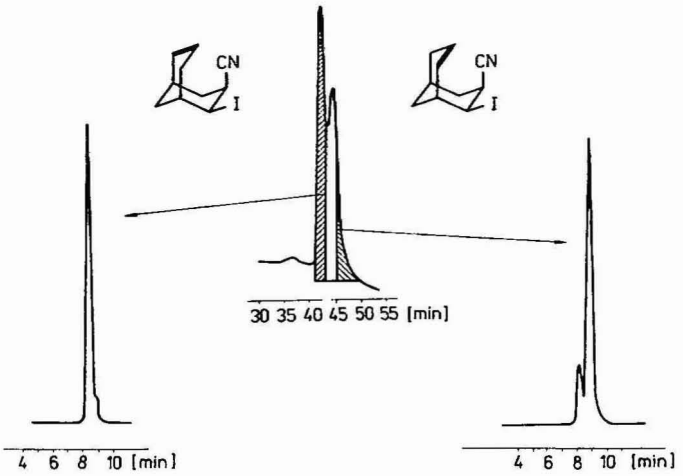
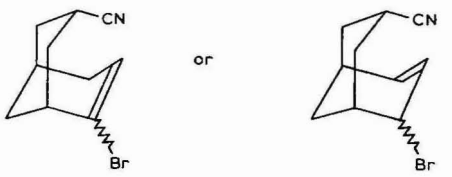


Fig. 5. Preparative separation of iodobicyclononene carbonitriles and analysis of the fractions obtained. Conditions as in Fig. 4.

matography (TLC)[2], the mobile and stationary phases used in TLC were chosen for HPLC.

The analytical separations of isomeric chlorobicyclononene carbonitriles, using two different mobile phases, are shown in Fig. 2. The separation of isomeric iodobicyclononene carbonitriles, using the same mobile phases, are shown in Fig. 3. The identifications of the structures of both iodoisomers were confirmed by NMR spectroscopy[5] after their separation by preparative HPLC. Other compounds were identified on the basis of their chromatographic behaviour.

Two compounds with unidentified structures accompanying the bromobicyclononene carbonitriles are considered to have one of the following structures:



The amounts separated, yields and purities of compounds obtained by preparative HPLC are listed in Table II. Fig. 4 shows the preparative separations of chlorobicyclononene carbonitriles, including a description of the collected fractions and their HPLC analysis. Fig. 5 shows similar results for iodo derivatives.

Although analytical HPLC using a mobile phase with a lower content of acetone yields better separations, owing to the lability of the compounds a mobile phase with a higher content of acetone (3%) was chosen for the preparative separation. The purity of the individual compounds obtained was sufficient for the subsequent NMR investigation[5].

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Note

High-performance thin-layer chromatography of phenylethylamines and phenolic acids on silanized silica and on ammonium tungstophosphate

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The phenylethylamine group includes natural and synthetic compounds which are used in drug formulations, catecholamines and other biogenic amines which are excreted in the urine together with phenolic acids and glycols. The separation of these compounds by thin-layer chromatography (TLC) is very important, as shown by the numerous papers dealing with the determination of phenylethylamines in pharmaceutical preparations[1], with their identification as drugs of abuse[2,3] and with the determination of catecholamines, their metabolites and precursors in urine[4–7]. These studies were carried out on silica gel or cellulose thin layers, taking only one group of compounds and/or a restricted number of them into account.

Using reversed-phase TLC, good results were obtained in the separation of some primary phenylethylamines[8] of the three most important catecholamines[9] and of some phenolic acids[10,11].

We therefore considered it useful to study a large number of phenylethylamines and phenolic acids and glycols on different ready-for-use plates of silanized silica gel untreated and impregnated with anionic and cationic detergents.

The chromatographic behaviour of these compounds has also been studied on layers of ammonium tungstophosphate (AWP), an inorganic synthetic ion exchanger, which was already been used in the separation of primary aromatic amines[12] and other nitrogen compounds[13].

EXPERIMENTAL

Standard solutions of amines and phenolic acids and glycols (1–2 mg/ml) were prepared by dissolving the compounds in water–methanol (1:1), except 3,4-dihydroxymandelic acid, which was dissolved in water.

The amount deposited on the layer was between 0.2 and 0.5 μg for silanized silica gel and between 0.5 and 1 μg for ammonium tungstophosphate.

The amines were rendered visible by spraying the layers with 1% ninhydrin solution in pyridine–acetic acid (5:1) and heating the plates for 5 min at 100°C. Hordenine, phenolic acids and glycols were detected by the Boute[14] reaction, exposing the layers successively to nitrogen dioxide and ammonia vapours.

The silanized silica gel layers (Sil C_{18}-50, Macherey, Nagel & Co., Düren,

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TABLE I

R_F VALUES OF PHENYLETHYLAMINES, PHENOLIC ACIDS AND GLYCOLS ON DIFFERENT PLATES WITH THE FOLLOWING ELUENTS (a) 1 *M* CH_3COOH + 3% KCl IN WATER; (b) 1 *M* HCl + 3% KCl IN WATER; (c) 1 *M* CH_3COOH IN WATER–METHANOL (20%); (d) 1 *M* CH_3COONa IN WATER–METHANOL (30%); (e) 0.5 *M* Na_2CO_3 IN WATER–METHANOL (30%); (f) 1 *M* CH_3COOH + 1 *M* HCl IN WATER–METHANOL (40%); AND (g) 1 *M* NH_4NO_3 IN WATER

No.	*Compound**	*OPTI-UP* C_{12}		*Sil* C_{18}*-50*	*Sil* C_{18}*-50 + 4% N-DPC*		*RP-18 + 4% HDBS*	*AWP +* $CaSO_4$ *(4:2)*
		a	*b*	*c*	*d*	*e*	*f*	*g*
1	1-Phenylethylamine	0.29	0.28	0.54	0.65	0.26	0.30	0.42
2	2-Phenylethylamine	0.29	0.27	0.51	0.56	0.18	0.24	0.28
3	Tyramine	0.54	0.47	0.68	0.70	0.44	0.66	0.42
4	Dopamine	0.66	0.61	0.74	0.68	0.16	0.75	0.42
5	3-Methoxytyramine	0.44	0.26	0.66	0.72	0.46	0.62	0.21
6	Nor-adrenaline	0.84	0.84	0.83	0.67	0.25	0.87	0.57
7	Adrenaline	0.70	0.68	0.77	0.67	0.26	0.88	n.d.**
8	Amphetamine	0.26	0.18	0.48	0.55	0.14	0.24	0.17
9	β-Hydroxyphenethylamine	0.50	0.47	0.62	0.62	0.38	0.44	0.46
10	3,4-Dimethoxyphenetylthylamine	0.14	0.10	0.50	0.67	0.34	0.44	0.06
11	Nor-metanephrine	0.69	0.55	0.79	0.78	0.70	0.77	0.40
12	Octopamine	0.78	0.74	0.79	0.76	0.70	0.78	0.56
13	Nor-ephedrine	0.39	0.28	0.55	0.56	0.29	0.32	0.41
14	Nor-ψ-ephedrine	0.34	0.23	0.54	0.56	0.21	0.32	0.44
15	Ephedrine	0.26	0.16	0.53	0.55	0.16	0.29	0.27
16	ψ-Ephedrine	0.22	0.13	0.52	0.55	0.15	0.31	0.26
17	Synephrine	0.63	0.54	0.72	0.75	0.52	0.74	0.48
18	Metanephrine	0.53	0.35	0.69	0.75	0.52	0.72	0.27
19	Hordenine	0.29	0.16	0.60	0.52	0.13	0.54	0.17
20	3,4-Dihydroxy-MA	0.89	0.73	0.95	0.56	0.62	0.90	0.95
21	*p*-Hydroxy-MA	0.82	0.57	0.90	0.56	0.62	0.87	0.94
22	*m*-Hydroxy-MA	0.75	0.46	0.83	0.46	0.53	0.87	0.92
23	4-Hydroxy-3-methoxy-MA	0.64	0.39	0.88	0.59	0.64	0.85	0.83
24	Homogentisic acid	0.64	0.37	0.70	0.30	0.47	0.87	0.90
25	Homovanillic acid	0.19	0.07	0.41	0.44	0.52	0.75	0.69
26	3,4-Dihydroxy-PG	0.74	0.61	0.77	0.59	0.66	0.89	0.91
27	4-Hydroxy-3-methoxy-PG	0.45	0.28	0.65	0.61	0.65	0.85	0.74
28	*o*-Hydroxy-PA	0.33	0.19	0.35	0.11	0.23	0.52	0.72
29	*m*-Hydroxy-PA	0.34	0.19	0.43	0.27	0.44	0.65	0.72
30	*p*-Hydroxy-PA	0.33	0.19	0.53	0.36	0.55	0.77	0.74

* MA = mandelic acid; PG = phenylglycol; PA = phenylacetic acid.

** n.d. = not determined.

F.R.G.; OPTI-UP C_{12}, Antec, Bennwil, Switzerland; RP-18, E. Merck, Darmstadt, F.R.G.) were impregnated with N-dodecylpyridinium chloride (N-DPC) or with dodecylbenzene sulphonic acid (HDBS) according to a previous work[15] and the AWP and its layers were prepared as described previously[12,16].

The migration distance was 6 cm for the ready-for-use plates and 10 cm with the inorganic exchanger, unless stated otherwise. The chromatographic measurements were carried out at 25°C.

RESULTS AND DISCUSSION

Untreated layers of silanized silica gel

The behaviour of the amines and phenolic acids and glycols was studied on layers of OPTI-UP C_{12}, Sil C_{18}-50 and RP-18. The RP-18 plates are ill-suited, as eluents containing at least 60% of methanol must be used and under such elution conditions most compounds are only very slightly retained.

Table I lists the chromatographic characteristics of nineteen phenylethylamines and eleven phenolic acids and glycols on OPTI-UP C_{12} and Sil C_{18}-50 plates eluted with aqueous solutions at different pH values (columns 1 and 2) and with a 20% methanol content (column 3), respectively.

The presence of potassium chloride in the aqueous eluents accounts for the compactness of the spots. Eluent (a) allows the separation of most amines, with the exception of the two phenylethylamine isomers.

The presence in the molecule of one or more hydroxy groups decreases the retention of the compounds considerably, while an opposite behaviour occurs with replacement of a phenolic group with a methoxy group, *i.e.*, dopamine (R_F = 0.66)/3-methoxytyramine (R_F = 0.44)/3,4-dimethoxyphenetylamine (R_F = 0.14); 3,4-dihydroxymandelic acid (R_F = 0.89)/4-hydroxy-3-methoxymandelic acid (R_F = 0.64); 3,4-dihydroxyphenylglycol (R_F = 0.74)/4-hydroxy-3-methoxyphenylglycol (R_F = 0.45).

Secondary amines are more strongly retained than primary amines. Among the diastereomers, the differences in the retention allow the separation of nor-ephedrine from nor-ψ-ephedrine.

Differing behaviour depends on the position of the phenolic group, which causes different retentions of the two isomers of hydroxymandelic acid but does not affect the behaviour of the three isomers of hydroxyphenylacetic acid.

On the basis of the R_F values obtained with eluent (a), many interesting separations can be achieved; we separated compounds 2, 3, 4, 7, 10 and 5, 9, 11, 12, the four mandelic acid derivatives and homovanillic acid from vanylmandelic acid (these two compounds are generally excreted in human urine) and also from all the other phenolic acids and glycols.

On eluting with hydrochloric acid solution (eluent b), the behaviour of the compounds is connected, in addition to the sharp acidity increase, with the lack of an organic solvent, such as acetic acid, in the eluent. The strongest retention observed for most compounds can be ascribed more to the absence of acetic acid than to the acidity increase and/or to the higher ionic strength of the eluent.

In fact both amines, which are in an ionized form in the two eluents, and the un-ionized compounds, such as the two glycols, are more strongly retained with

eluent (b). Furthermore, elution with 2 *M* hydrochloric acid + 3% potassium chloride in water does not lead to substantial R_F differences for most compounds. With eluent (b), some separations that cannot be carried out with eluent (a) can be effected, such as that between 2-phenylethylamine and amphetamine and that among the three most important catecholamines. The separation of the three catecholamines and of two other biogenic amines on OPTI-UP C_{12} layers is shown in Fig. 1.

Eluting with alkaline solutions (*i.e.*, 1 *M* sodium acetate in water) the amines are more retained, while the phenolic acids migrate more and in a different sequence. The strongest retention of the amines must be ascribed to their smaller degree of protonation, as shown also from the further decrease in their R_F values on eluting with more alkaline solutions (*i.e.*, 0.5 *M* sodium carbonate in water). Fig. 2 shows the separation on OPTI-UP C_{12} plates of the most important phenolic acids and glycols that are excreted in urine.

On Sil C_{18}-50 plates (see column 3 in Table I) the following differences can be seen with respect to the behaviour on OPTI-UP C_{12} layers: (1) a smaller retention of the compounds, which can be attributed both to the higher hydrophobicity of the layer and to the presence of methanol in the eluent; (2) a levelling of the R_F values of the phenylethylamines as the chromatographic behaviour of the ephedrine diastereomers shows; and (3) a good separation among the three isomers of hydroxyphenylacetic acid.

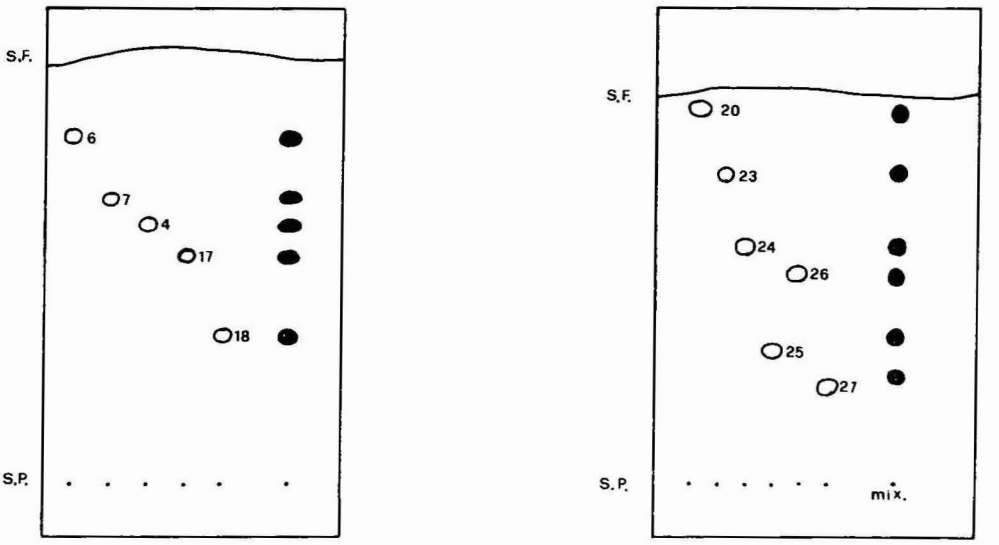

Fig. 1. Thin-layer chromatogram of biogenic amines on OPTI-UP C_{12} plates. Migration distance, 8 cm. Eluent: 1 *M* HCl + 3% KCl in water. Spots: 6, nor-adrenaline; 7, adrenaline; 4, dopamine; 17, synephrine; 18, metanephrine.

Fig. 2. Thin-layer chromatogram of urinary phenolic acids and glycols on OPTI-UP C_{12} plates. Migration distance, 7.5 cm. Eluent: 1 *M* CH_3COONa in water. Spots: 20, 3,4-dihydroxy-MA; 23, 4-hydroxy-3-methoxy-MA; 24, homogentisic acid; 25, homovanillic acid; 26, 3,4-dihydroxy-PG; 27, 4-hydroxy-3-methoxy-PG.

Sil C_{18}-50 and RP-18 plates impregnated with 4% N-DPC or 4% HDBS

We impregnated Sil C_{18}-50 and RP-18 plates only, as on OPTI-UP C_{12} plates the detergents are very weakly adsorbed, and migrate during elution with aqueous or aqueous–organic solutions, giving rise to very irregular solvent fronts.

Impregnation of the Sil C_{18}-50 plates with 4% N-DPC solution allows the retention of both acids and amines to be increased on eluting with alkaline solutions.

Table I (columns 4 and 5) gives the data obtained on eluting with 1 *M* sodium acetate (eluent d) and 0.5 *M* sodium carbonate (eluent e) in water–methanol (30%). With respect to the untreated layers, considerable differences are observed both in the retention and in the affinity sequence of the classes of compounds examined. This indicates that the detergent adsorbed on the layer not only gives rise to anion-exchange reactions but also modifies the hydrophobic characteristics of Sil C_{18}-50.

Under these elution conditions, a good separation between the two phenylethylamine isomers and an improvement, with respect to the untreated layers, in the separation of the three hydroxyphenylacetic acids are achieved.

Elution with 0.5 *M* sodium carbonate in water–methanol (30%) gives a further increase in the retention of amines with respect to eluent (d) owing to the stronger adsorption of the unprotonated species.

With the acids, the smaller retention can be attributed to the higher ionic strength of the eluent. With this eluent, the separation of nor-metanephrine and octopamine from all the other amines and that between the two diastereomers of nor-ephedrine should be noted.

The layers of RP-18 were impregnated with a 4% HDBS solution in order to increase the retention power and the selectivity of the stationary phase. On impregnated layers, the percentage of organic solvent may be decreased to 40%. In Table I (column 6) are reported the R_F values obtained on eluting with a mixture of 1 *M* acetic acid + 1 *M* hydrochloric acid in water–methanol (6:4) (eluent f). With respect to the trend observed on the two above-mentioned supports, on RP-18 + 4% HDBS a greater influence of the number and position of the hydroxy groups on the chromatographic behaviour of phenylethylamines can be noted (*cf.*, R_F values of compounds 2, 3, 4 and 6 in Table I). With acid compounds, the differences among the

TABLE II

R_M VALUES OF PHENYLETHYLAMINES ON THIN-LAYERS OF OPTI-UP C_{12} AND SIL C_{18}-50, ELUTING WITH (a) 1 *M* ACETIC ACID + 3% KCl IN WATER, (b) 1 *M* HYDROCHLORIC ACID + 3% KCl IN WATER AND (c) 1 *M* ACETIC ACID IN WATER–METHANOL (20%)

Amine	*OPTI-UP C_{12}*				*Sil C_{18}-50*	
	$R_{M(a)}$	$\Delta R_{M(a)}(OH)$	$R_{M(b)}$	$\Delta R_{M(b)}(OH)$	$R_{M(c)}$	$\Delta R_{M(c)}(OH)$
Nor-adrenaline	−0.720	0.432	−0.720	0.526	−0.689	0.235
Dopamine	−0.288		−0.194		−0.454	
Nor-metanephrine	−0.347	0.452	−0.087	0.541	−0.575	0.287
3-Methoxytyramine	0.105		0.454		−0.288	
Octopamine	−0.550	0.480	−0.454	0.506	−0.575	0.248
Tyramine	−0.070		0.052		−0.327	

R_F values are less marked than on the other two layers, even though the separation of the three isomers of hydroxyphenylacetic acid is still possible.

The retention of phenylethylamines depends on the concentration of hydrochloric acid in the eluent and can be ascribed to the cation-exchange process between the protonated amino group and the detergent adsorbed on the layer. In fact, if the R_M values of the amines are plotted as a function of the logarithm of the hydrochloric acid activity in the eluent, straight lines are obtained for most amines; the slopes are between 0.7 and 0.9 and are, therefore, in accordance with the theoretical values[8]. Among the different separations that can be achieved on this layer, of interest is that between the two phenylethylamine isomers, which is not feasible on home-made layers of silanized silica gel (C_2) impregnated with 4% DBS[8].

Retention mechanism

On layers of silanized silica gel the retention of the compounds is probably controlled by a liquid–liquid partition mechanism. We wanted to see whether, for the phenylethylamine derivatives also, the introduction into the molecule of a functional group, such as a hydroxy or N-methyl group, might give rise to a specific contribution to the retention on untreated OPTI-UP C_{12} and Sil C_{18}-50 plates.

Table II gives the ΔR_M(OH) values, calculated on the basis of the chromatographic data in Table I. The ΔR_M(OH) values are substantially constant for the three pairs of phenylethylamines. The mean value of ΔR_M(OH) is 0.525 for eluent (b), 0.455 for eluent (a) and 0.256 for eluent (c). Such a decrease further demonstrates the R_F levelling caused by an increase in the percentage of organic solvent in the eluent.

Table III gives the ΔR_M(N-CH_3) values calculated on OPTI-UP C_{12} plate with eluents (a) and (b) from Table I. The data on Sil C_{18}-50 plates are not reported because, owing to the presence of 20% of organic solvent in the eluent, the differences between the R_M values are too small. The ΔR_M(N-CH_3) valuews refer to six pairs of

TABLE III

R_M VALUES OF PHENYLETHYLAMINES ON THIN LAYERS OF OPTI-UP C_{12}, ELUTING WITH (a) 1 *M* CH_3COOH + 3% KCl IN WATER AND (b) 1 *M* HCl + 3% KCl IN WATER

Amine	$R_{M(a)}$	$\Delta R_{M(a)}$(*N-CH₃*)	$R_{M(b)}$	$\Delta R_{M(b)}$(*N-CH₃*)
Nor-adrenaline	−0.720	0.352	−0.720	0.393
Adrenaline	−0.368		−0.327	
Nor-metanephrine	−0.347	0.295	−0.087	0.356
Metanephrine	−0.052		0.269	
Octopamine	−0.550	0.319	−0.454	0.384
Synephrine	−0.231		−0.070	
Nor-ephedrine	0.194	0.260	0.410	0.310
Ephedrine	0.454		0.720	
Nor-ψ-ephedrine	0.288	0.262	0.525	0.301
ψ-Ephedrine	0.550		0.826	
Tyramine	−0.070	0.459*	0.052	0.668*
Hordenine	0.389		0.720	

* This value is relative to the dimethylamino group and is double that for N-CH_3.

amines and are consistent among them. The mean values of $\Delta R_M(\text{N-CH}_3)$ are 0.344 (eluent b) and 0.278 (eluent a).

Ammonium tungstophosphate plates

On layers of this exchanger, eluting with aqueous solutions of 1 *M* ammonium niotrate (column 7 in Table I), a peculiar behaviour is observed with amines. Such behaviour, as has already been pointed out in previous papers[12,13], depends on the structural characteristics of the compounds. In fact, the steric hindrance of the phenyl group on the carbon atom bound to the amino group allows a good separation between 1- and 2-phenylethylamine. The N-methyl groups increase the affinity of the compound towards the exchanger; the following sequence of R_F values is observed: primary amine > secondary amine > tertiary amine. The replacement of a phenolic group by a methoxy group also results in an increase in retention; this increase is more marked than on the other supports.

The acid compounds, as was predictable, are retained less than on silanized silica gel; some separations, however, can be effected, such as that concerning compounds 20, 23 and 25 in Table I.

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Note

Isoelectric focusing separation of *Gliocladium* enzyme components

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Cellulase is a complex of enzymes containing mainly exoglucanases and endoglucanases plus cellobiase[1,2]. The cellulase components are often present as isoenzymes, differing only slightly in isoelectric pH, and are difficult to separate. Fractionation studies reported on cellulases of different fungal origin have involved the use of standard protein separation techniques such as gel filtration, ion-exchange chromatography, affinity chromatography and isoelectric focusing[2–6], high-performance liquid chromatography (HPLC)[7] and polyacrylamide gel electrophoresis (PAGE)[8].

This work was concerned with the separation and characterization of isoenzymes from a culture filtrate obtained by fermentation of the fungus *Gliocladium* sp.

EXPERIMENTAL

Enzyme source and enzyme activity determination

A culture filtrate of the cellulolytic fungus *Gliocladium* sp. was used as the enzyme source. The culture filtrate was obtained by courtesy of the Institute of Technical Chemistry of the Academy of Science of the G.D.R., Leipzig[9].

Filter-paper degrading activity (FPA) was assayed according to the method of Mandels *et al.*[10] and the cotton-hydrolysing C_1 activity by a slight modification of the method of Mandels and Weber[11], using dinitrosalicylic acid (Merck, Darmstadt, F.R.G.)[12]. Carboxymethylcellulose (CMC) (Sigma, St. Louis, MO, U.S.A.) degrading C_x activity and β-glucosidase activity were measured by the release of glucose from a CMC solution[13] and from a cellobiose solution[14], respectively.

Proteolytic activity was determined by the Anson[15] method, using haemoglobin as substrate.

Protein content and amino acid composition

Soluble protein was measured by the phenol procedure[16] after precipitating with trichloroacetic acid (5%, w/v) using bovine plasma albumin as standard (Browning Chemical Corp., New York, NY, U.S.A.).

Amino acid composition was determined with the Biotronik LC 2000 Amino Acid Analyzer.

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Methods of separation

The culture filtrate was prepared for isoelectric focusing by concentration (10-fold) in a rotational evaporator and dialysis through a 4-22/32 in. membrane (Medicell, London, U.K.). Slab gels for analytical isoelectric focusing (IF) were prepared according to the Pharmacia manual on IF[17], using Ampholine (LKB, Bromma, Sweden) in the pH range 3–10. Samples, containing 2 mg of protein, were focused at 50, 100, 200, 600 and 1000 V each for 30 min in the Pharmacia flat-bed electrophoresis system. After completion of focusing, proteins were fixed and stained for proteins with Servablau G (Serva, Heidelberg, F.R.G.) and for glycoproteins with Schiff's reagent[17].

Preparative IF was performed on 5-mm Sephadex IEF layers (Pharmacia, Uppsala, Sweden) according to the Pharmacia manual[17].

The Ampholine was the same as used in analytical IF. The separated proteins were recovered with the fractionation grid, pressing 27 strips into the gel at the positions determined by the paper replica. The gel strips, containing proteins of interest, were removed, the pH values were determined and the proteins eluted with distilled water.

The homogeneity of the separated components was monitored by analytical IF. The pH of the fractions was determined using Protein Test Mixture 9 (Serva, Heidelberg, F.R.G.).

Determination of molecular weight

The molecular weights of the separated fractions were determined by SDS-PAGE using the Weber and Osborn[18] SDS–phosphate continuous buffer system system and 125 mm wide × 110 mm long × 1.0 mm thick gel slabs. The samples, containing 1% (w/v) protein, 0.01 *M* sodium phosphate buffer (pH 7.2), 3% (w/v) SDS (Serva) and 5% (w/v) β-mercaptoethanol (Reanal, Budapest, Hungary) were boiled for 2 min and mixed with 36% (w/v) urea (Serva) and 20% (w/v) saccharose. The electrophoresis was pre-run at 50 V for 1 h at room temperature and developed at 100 V for 4 h at 4°C. The gel was stained with silver nitrate stain[19].

RESULTS AND DISCUSSION

The isoelectric points of the cellulolytic enzyme components determined by analytical flat-bed IF were found to cover the pH range 9.3–3.0 (Fig. 1). A total of 28 protein fractions was obtained. The fractions in the pH range 4.1–6.1 and two fractions of pH 8.6 and 9.0 gave the strongest protein stains. Most of these proved to be glycoproteins.

Preparative IF confirmed the above results (Fig. 2). Most (85%) of the soluble protein content was found in fractions 14–28 with p*I* values in the range 5.9–9.8.

The C_1 activity was mainly located in the acidic p*I* range, while C_x covered the p*I* range 4.1–6.6. Two fractions of considerable cellobiase activity were on the cathodic side (p*I* 9.0 and 9.3). The remainder of the cellobiase fractions more or less coincided with the endoglucanase activities. The FPA activity spread practically over the whole p*I* range, which was to be expected as this substrate is acted on by several cellulolytic activities.

While the C_x and cellobiase activities could be recovered almost quantitatively

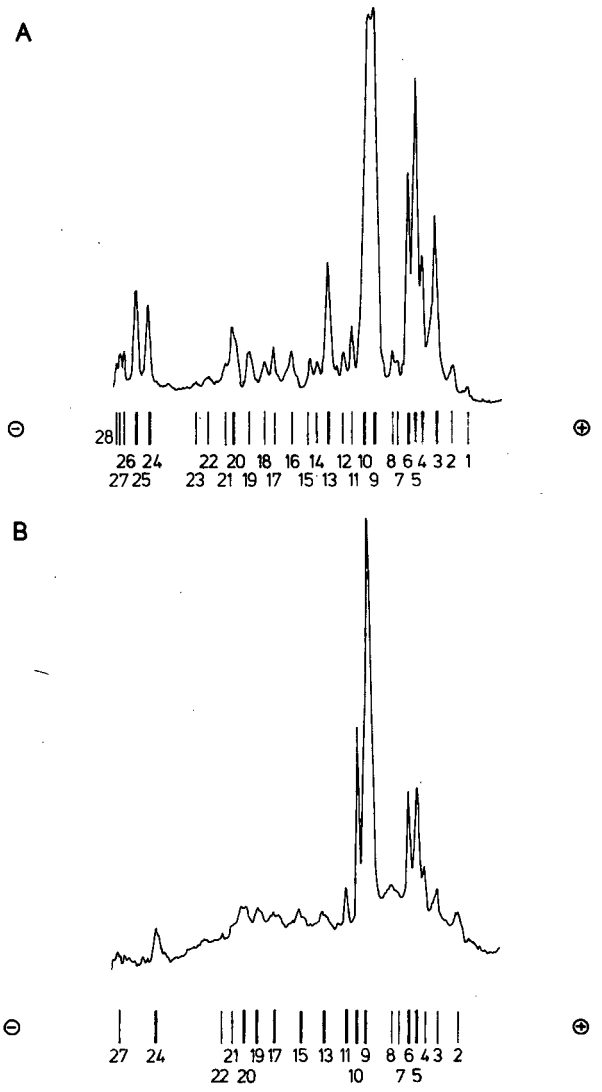

Fig. 1. Isoelectric points (p*I*) of the cellulase enzyme components. A = *Gliocladium* enzyme components; protein staining with Servablau G (Serva). B = *Gliocladium* enzyme components; glycoprotein staining with Schiff's reagent. Separation was performed by analytical IF of the concentrated and dialysed culture filtrate on slab gels according to the Pharmacia Manual[17] using Ampholine, pH 3–10 (LKB). Slab gels were evaluated with a Shimadzu Model CS-930 dual-wavelength thin-layer chromato-scanner. Enzyme component (p*I*): 1 (3.0), 2 (3.2), 3 (3.5), 4 (3.8), 5 (4.0), 6 (4.1), 7 (4.4), 8 (4.7), 9 (4.8), 10 (5.0), 11 (5.3), 12 (5.5), 13 (5.8), 14 (6.1), 15 (6.6), 16 (7.1), 17 (7.3), 18 (7.4), 19 (7.5), 20 (7.6), 21 (7.7), 22 (8.0), 23 (8.3), 24 (8.4), 25 (8.6), 26 (9.0), 27 (9.1), 28 (9.3).

(94% and 99%, respectively) after the purification procedures, only low levels of FPA and C_1 activities (41.9% and 5.7%, respectively) could be detected in the fractions. The protein recovery was 77%. The recombination of the 28 fractions resulted in the recovery of the total FPA and C_1 activities.

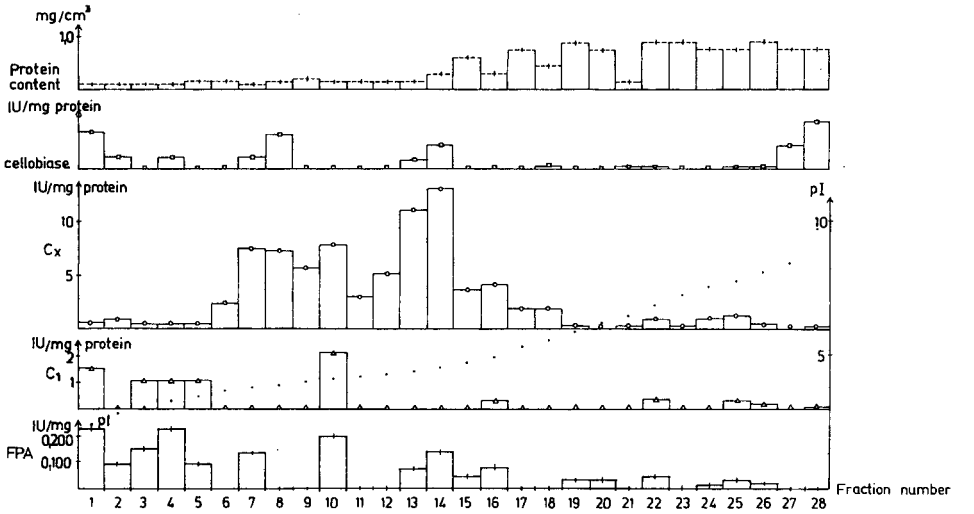

Fig. 2. Activities of the cellulase components separated by preparative isoelectric focusing (IF). Protein content according to Lowry *et al.*[16]; cellobiase activity according to Sternberg[14]; C_x = carboxymethylcellulose-degrading activity; C_1 = cotton-hydrolysing activity according to Mandels and Weber[11]; FPA = filter-paper degrading activity according to Mandels *et al.*[10]; 1 IU = released glucose equivalent, mg from 100 mg of cellulose in 24 h at pH 4.8 and 50°C.

Consequently, the separation losses are only apparent and might be due to the fact that the FPA and C_1 activities are the result of the complex action of endo- and exoglucanases plus cellobiase. These results seem to support the cellulase multienzyme theory, which means that the C_1 and FPA components (mainly cellobiohydrolase) play important initial roles in the overall process of enzymatic cellulose degradation, but for total hydrolysis the other enzyme components are also required[20].

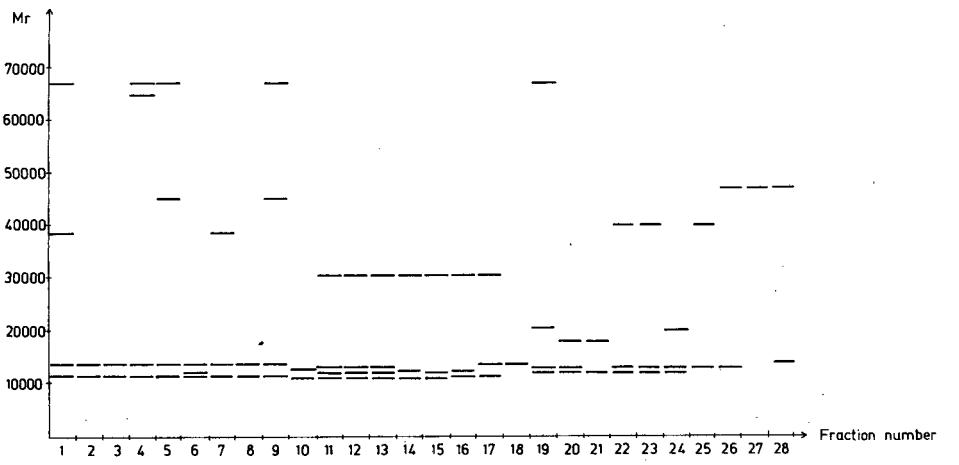

Fig. 3. Molecular weights (M_r) of the fractions separated by preparative isoelectric focusing. The determinations were carried out by SDS vertical slab electrophoresis according to Weber and Osborn[18]; proteins were stained with the silver nitrate method[19].

The presence of more than ten enzymes (or isoenzymes) was reported for some *Trichoderma* cellulases[2]. The great number of active fractions in our enzyme complex suggested this to be the result of proteolytic fragmentation. However, no proteolytic activity could be detected in the culture filtrate by the Anson method.

The molecular weights of the fractions were found to range from 10000 to 70000 (Fig. 3). The yellow colour obtained with the silver stain for 19 fractions indicate these to be glycoproteins. This is in agreement with IF findings.

The amino acid analysis of the separated 28 fractions can be seen in Fig. 4. All the fractions contained glutamic acid and aspartic acid (except fractions 18 and 25), serine (except fractions 1 and 3) and threonine (except fractions 1-4). Only a few fractions contained cysteine (1, 11 and 26), methionine (2, 11 and 26), lysine (1, 3, 6, 17, 18, 25 and 28) and tyrosine (2, 3, 4, 11, 16, 21 and 27).

Components with similar molecular weights, amino acid contents and isoelectric points were found to have similar activities.

The relatively large number of isoenzymes of similar activities might be the result of a proteolytic activity which splits the cellulase enzyme components from proenzymes of higher molecular weight during the fermentation process. As no proteolytic activity could be detected in the culture filtrate, such proteases must be endocellular. Investigations into this problem are in progress.

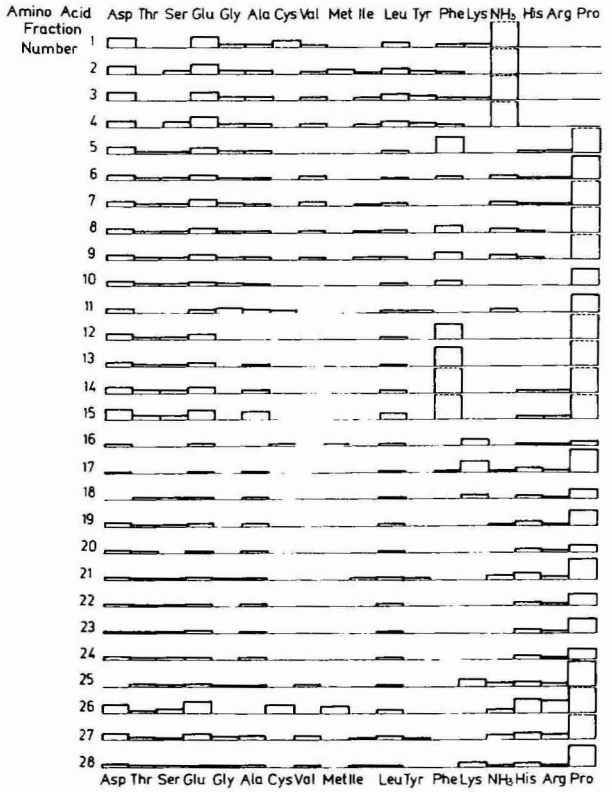

Fig. 4. Amino acid analysis of the fractions separated by preparative IF. Analysis was performed after acid hydrolysis in a Biotronik LC 2000 Amino Acid Analyzer.

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Note

Purification of D-amino acid oxidase apoenzyme by affinity chromatography on Cibacron Blue Sepharose

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(Received July 18th, 1985)

In recent years, there has been a marked increase in the use of affinity chromatography for protein purification[1]. Thus, enzymes requiring cofactors with an adenylate moiety can be purified on nucleoside or nucleotide affinity columns. It has also been shown that polyaromatic dyes can bind at the active sites of globular proteins that have a cofactor possessing a nucleotidyl residue[2]. For example, Cibacron Blue has an affinity for several dehydrogenases, kinases and phospho- or acetyltransferases[3]. Flavine adenine dinucleotide (FAD) belongs to the adenylate cofactor group and it should, therefore, be possible to purify flavoproteins by affinity chromatography on Cibacron Blue resins, especially when the cofactor is not too tightly bound to the protein. We could not find a reference to this technique in the literature. It appeared to us that such a technique would be of great value, as we needed to obtain an apoprotein in a chemically pure form in order to assay flavin analogues as electron transfer moieties.

We report here a simple and efficient method of purification of D-amino acid oxidase apoenzyme (E.C. 1.4.3.3).

EXPERIMENTAL

Blue Sepharose CL6B was obtained from Pharmacia. The disodium salt of nicotinamide adenine dinucleotide in reduced form (NADH) and the enzymes D-amino acid oxidase (15 U/mg) from hog kidney (D-AAO), catalase (65 000 U/mg) (CAT) from beef liver and lactate dehydrogenase (500 U/mg) (LDH) from rabbit muscle were obtained from Boehringer (Mannheim, F.R.G.). Flavine adenine dinucleotide was a product of Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade.

Apo-D-amino acid oxidase was prepared either by ammonium sulphate precipitation at acidic pH[4] or by extensive dialysis against 1 *M* potassium bromide solution[5].

The enzymatic activity was measured as follows. The concentration of pyruvic acid produced during the enzymatic reaction was continuously measured by lactate dehydrogenase activity [0.05 *M* sodium pyrophosphate buffer (pH 8.5), 0.035 *M* D-alanine, $1.8 \cdot 10^{-4}$ *M* NADH, 13 U/ml CAT, 8 U/ml LDH, D-AAO (2 μg) or D-amino acid oxidase apoenzyme (20 μg or more)] at 340 nm on a Cary 210 spectrometer.

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The chromatograms were obtained at 4°C on Blue Sepharose columns equilibrated with 0.01 *M* sodium pyrophosphate (pH 8.5) prior to the application of crude D-AAO apoenzyme. The elution was performed first with 0.01 *M* sodium pyrophosphate buffer (pH 8.5) until a peak was eluted (void volume), then the purified D-AAO apoenzyme was eluted with 1 *M* potassium bromide in 0.01 *M* sodium pyrophosphate buffer (pH 8.5). The protein fractions were collected and concentrated by dialysis against solid polyethylene glycol (PEG 20M). The protein was then subjected to extensive dialysis against 0.05 *M* sodium pyrophosphate buffer (pH 8.5). The apoenzyme solution was stored at −20°C. The protein concentration was determined by the method of Bradford[6], using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

As we needed chemically pure D-AAO apoenzyme to assay different flavins for electron transfer experiments, we tried an affinity technique to purify the apoenzyme, which was contaminated with up to 5% of FAD. The holoenzyme was expected to elute in the void volume of the column, whereas the apoenzyme would be retained on the matrix. As Cibacron Blue is assumed to mimic an adenine moiety, Blue Sepharose appeared to be suitable for our purpose.

FAD was first dissociated from its D-AAO apoenzyme either by ammonium sulphate precipitation[4] or by extensive dialysis[5]. About 5% of the cofactor was retained on the final protein preparation. Such a biological preparation was not suitable for our electron transfer investigations and was, therefore, used as starting material for our chromatographic experiments. Chromatography on Blue Sepharose allowed its dissociation into two peaks (Fig. 1). The first peak (A), which represented about

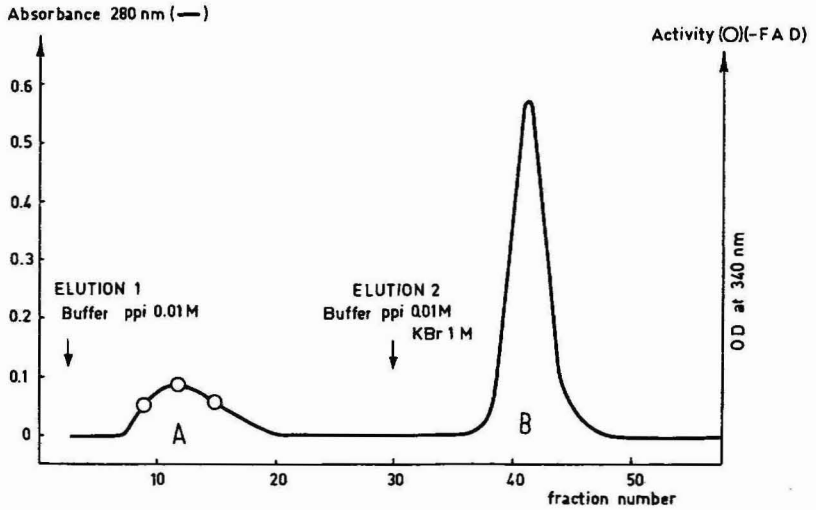

Fig. 1. Chromatography of D-amino acid oxidase holo- and apoenzymes, obtained by dialysis against 1 *M* KBr, on Blue Sepharose. The column (10 × 1 cm I.D.), equilibrated with 0.01 *M* pyrophosphate buffer (ppi) (pH 8.5), was loaded with 1 mg of proteins. The elution profile was obtained by absorbance at 280 nm. The column was eluted with 0.01 *M* sodium pyrophosphate buffer pH 8.5 (arrow 1), followed by 0.01 *M* sodium pyrophosphate buffer pH 8.5 containing 1 *M* KBr (arrow 2). Fractions of 1 ml were collected. The activity was measured (O) as described under Experimental in the absence of FAD.

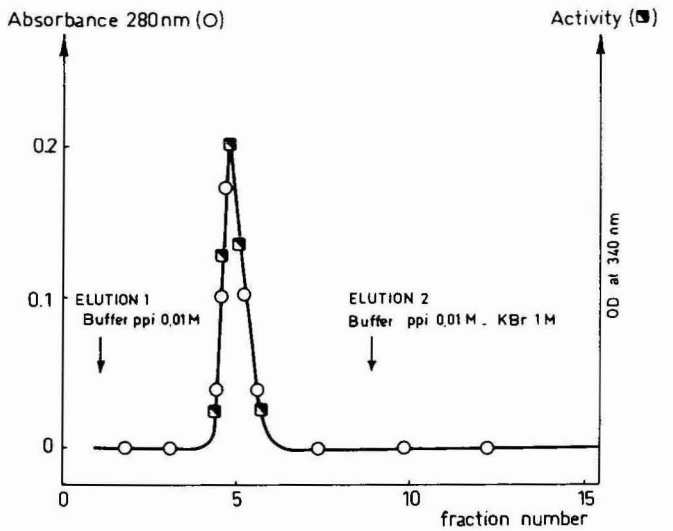

Fig. 2. Chromatography of D-amino acid oxidase holoenzyme on Blue Sepharose. The column (5 × 0.8 cm I.D.), equilibrated with 0.01 *M* sodium pyrophosphate buffer (pH 8.5), was loaded with 0.5 mg of holoenzyme. Arrow 1 indicates the beginning of elution with 0.01 *M* sodium pyrophosphate buffer (pH 8.5) followed, at arrow 2, by the same buffer containing 1 *M* KBr.

5% of the total material loaded on the column, was eluted in the void column of the column. The second peak (B), representing 95% of the material, was eluted with 1 *M* potassium bromide in 0.01 *M* sodium pyrophosphate buffer.

Peak A had the physico-chemical properties and enzymatic activities of the D-AAO holoenzyme, and presented the same elution profile (Fig. 2) obtained when

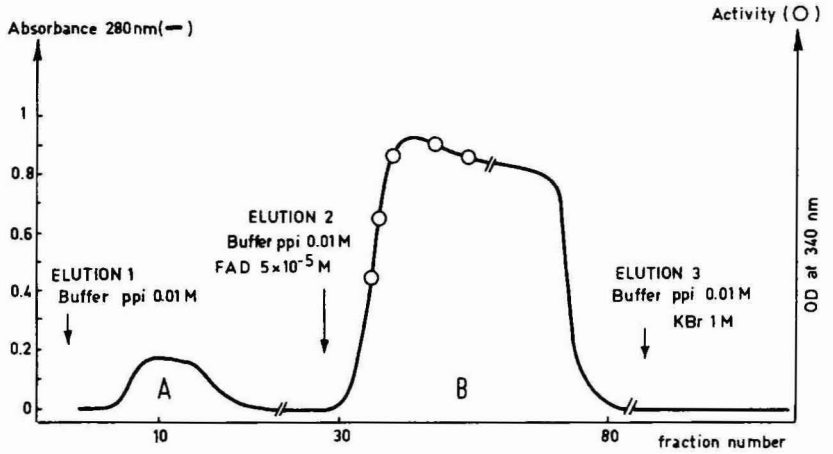

Fig. 3. Displacement of D-amino acid oxidase apoenzyme from Blue Sepharose with FAD. The column (10 × 1 cm I.D.), equilibrated with 0.01 *M* pyrophosphate buffer (pH 8.5), was loaded with a mixture D-AAO holo- and apoenzymes (2 mg). The elution profile was obtained from the absorbance at 280 nm. The column was eluted first with 0.01 *M* sodium pyrophosphate buffer (pH 8.5) (arrow 1), followed by 0.01 *M* sodium pyrophosphate buffer containing $5 \cdot 10^{-5}$ *M* FAD (arrow 2) and by 0.01 *M* sodium pyrophosphate buffer containing 1 *M* KBr (arrow 3). Fractions of 1 ml were collected.

D-AAO holoenzyme was submitted to the same type of chromatography under the same experimental conditions.

Peak B represented the apoenzyme, as demonstrated by the experiments reported below. The material of peak B was a protein but had no absorption band in the visible spectrum, demonstrating the absence of FAD in the structure. It had no D-AAO activity. The enzymatic activity could be completely restored, however, by incubation with FAD. The specificity of the binding to the Blue Sepharose column was demonstrated as follows. The crude apoenzyme preparation was absorbed on a column equilibrated with 0.01 *M* sodium pyrophosphate buffer (pH 8.5) and the elution (Fig. 3) was carried out first with the same buffer. A first peak (A) was immediately eluted. When the UV absorbance at 280 nm reached zero, the elution was developed with the same buffer containing $5 \cdot 10^{-5}$ *M* FAD, and a second peak (B) was eluted. The same buffer, this time containing 1 *M* potassium bromide, was then applied. No additional material was desorbed from the column. The analysis of the fractions showed that peak A had D-AAO activity. Its specific activity was lower than expected, probably owing to some denaturation of the protein. Peak B had, as expected, a fully restored enzymatic activity.

This work demonstrates that the use of Cibacron Blue Sepharose for chromatography readily provides a flavoapoenzyme in a chemically pure form. The binding between Cibacron Blue Sepharose and D-AAO apoenzyme may also be an excellent tool for studying cofactor binding. Other experiments are under way to explore the potential of such resins.

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Note

Determination of amino acids in the brain by high-performance liquid chromatography with isocratic elution and electrochemical detection

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Several methods are available for the analysis of the naturally occurring amino acids by high-performance liquid chromatography (HPLC)[1–5]. The formation of the *o*-phthaldehyde (OPA)–mercaptoethanol derivatives, their separation on a reversed-phase column, and detection by fluorescence is one of the primary methods used[4,5]. The latter methodology requires a gradient maker, usually two pumps and careful attention to details of timing because of the instability of the derivatives formed[5,6]. During a study of amino acids in the brain, we sought a simpler and less expensive method which avoided the use of unstable derivatives. We devised a procedure for quantitating amino acids in the brain which only requires a single HPLC pump and a step gradient. This procedure is a modification of that devised by Allison *et al.*[6] for the electrochemical detection and quantitation of OPA–*tert.*-butyl thiol derivatives of amino acids. Separation of the amino acids derivatives is achieved by varying the organic phase or by including the cationic ion pair tetrabutylammonium phosphate (TBAP) with a step gradient for elution.

EXPERIMENTAL

Equipment and materials

The equipment consisted of a Bioanalytical Systems (BAS) Model PM30A pump, a Rheodyne 7125 injector, a BAS Model 4B electrochemical detector and a Houston Instruments Omniscribe D-5000 chart recorder. The column was stainless steel (25 cm × 4.6 mm I.D.) filled with Biophase ODS 5-μm (BAS) and protected with a Spheri-10, RP-18, 10-μm guard column (Brownlee Labs.). Similar results were obtained with a Waters μBondapak C_{18} reversed-phase column (10 cm × 4.6 mm I.D.). A low volume electrochemical cell containing a glassy-carbon electrode with a potential of 700 mV was used for derivative detection. A three-way slider valve (Rainin Instruments) was used for switching between solvents. The gradient controller (Autochrom Model III OPG/S), used in a limited number of experiments, pre-mixed the solvents before entering the dual piston pump. This arrangement avoided the necessity of a second solvent pump.

The mobile phases consisted of 50 m*M* potassium phosphate, pH 7.0, 1 m*M* EDTA, 25% acetonitrile, 0–2 m*M* TBAP and 0–10% tetrahydrofuran (THF). The

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pumping rate was 2.5 ml/min. All the organic solvents were HPLC quality (Fisher) and used without filtration. TBAP and *tert.*-butyl thiol were purchased from Aldrich. All other solutions were filtered through nylon 66 filters (Rainin Instruments). The chemicals were of reagent grade and used without further purification.

Peak identification of glutamate, glutamine and γ-aminobutyric acid (GABA) was confirmed by enzyme digestion. Glutamate was converted to GABA with glutamate decarboxylase (Sigma). To 15 μl of sample were added 60 nanomoles magnesium sulfate, 3 nanomoles EDTA, 0.1 unit of enzyme and 750 nanomoles citrate buffer, pH 5.0 in a total volume of 30 μl. The sample was incubated at 37°C for 30 min and then reacted with 90 μl of the OPA–thiol reagent. The GABase preparation (Sigma) contains glutaminase activity, therefore, the results with pure glutaminase are not presented. GABA and glutamine in the rat cerebellum were digested with 0.1 unit GABase, 600 nanomoles α-ketoglutarate and 750 nanomoles borax buffer, pH 8.5 in a total volume of 30 μl as above. Control samples lacked the enzyme or replaced the brain extract with pure amino acid standards.

Sample preparation

The amino acid standards were dissolved in water and diluted to 1 m*M* with methanol–water (1:1) containing 1 m*M* EDTA and kept at 4°C. The brain samples were sonicated in 1 *M* perchloric acid with α-aminobutyric acid (AABA) added as an internal standard. The solution was centrifuged and the supernatant was neutralized with two volumes of 0.5 *M* potassium bicarbonate. The precipitate was removed by a second centrifugation and the neutralized supernatant filtered before analysis.

The amino acids derivatives were prepared by the method of Allison *et al.*[6]. The OPA–thiol reagent was made as follows: 27 mg of OPA was dissolved in 2 ml of methanol and 20 μl of *tert.*-butyl thiol (2-methyl-2 propanethiol) was added followed by the addition of 4.5 ml of 100 m*M* sodium tetraborate decahydrate (borax), pH 9.5. The OPA–thiol reagent was prepared fresh each week and kept tightly sealed to prevent evaporation of the thiol. Heating was required to solubilize the sodium tetraborate decahydrate. Both solutions were stored at room temperature. A volume of 20 μl the amino acid solution was mixed gently in a 500-μl plastic centrifuge tube with 100 μl of the OPA–thiol reagent using a plastic pipet tip. The tube was capped until injection. Excessive shaking resulted in a diminished aspartate and glutamate peak and the appearance of an additional peak prior to the first amino acid peak. After 2 min an aliquot was injected into the high-performance liquid chromatograph. Injection of the reagent solution without amino acids resulted in a peak labeled RSH. The RSH corresponds to the free thiol. Its retention times was less affected by the concentration of organic phase or by the addition of the ion pair reagent than were amino acids derivatives.

RESULTS AND DISCUSSION

The separation of the OPA–*tert.*-butyl thiol derivatives of the most common amino acids found in the brain is shown in Fig. 1. The inclusion of the ion pair reagent, TBAP, was necessary to separate aspartate and glutamate. Separation of aspartate and glutamate in the absence of TBAP was achieved if tetrahydrofuran

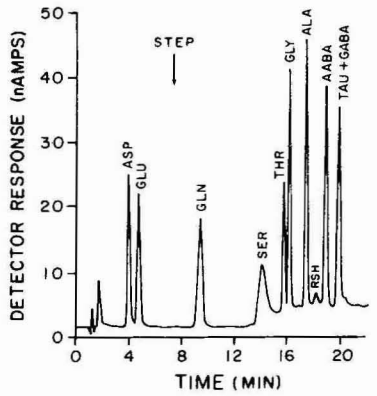

Fig. 1. Separation of OPA–*tert.*-butyl thiol derivatives of amino acids with a step gradient. The column was initially equilibrated with 50 m*M* potassium phosphate, pH 7.0, 1 m*M* EDTA, 25% acetonitrile, 2% THF and 1 m*M* TBAP. At 7 min the mobile phase was changed to include 10% THF. The quantity of each amino acid injected was 166 picomoles.

was deleted from the mobile phase and the concentration of acetonitrile was decreased to 20% or less. However, the retention time of glutamine increased to 16 min with the remaining amino acids eluting even later. Therefore, the step gradient used in Fig. 1 achieved both separation of aspartate from glutamate as well elution of the remaining derivatives in about 20 min.

The methodology is amenable to alteration of either the ion pair or the organic solvent phase. In Fig. 2 a simple gradient, in the absence of TBAP, resolved all compounds of interest except the amino acid pair threonine/glycine.

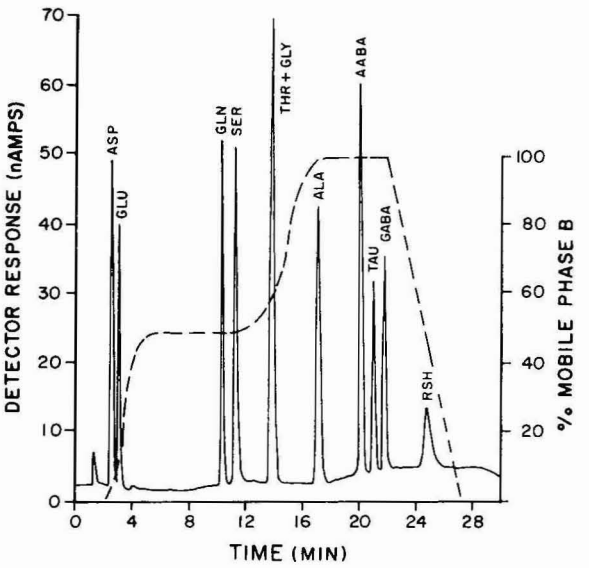

Fig. 2. Elution of OPA–*tert.*-butyl thiol amino acid derivatives with a step gradient in the absence of TBAP. The organic component of mobile phase A was 20% acetonitrile and for mobile phase B, 20% acetonitrile and 10% THF.

The application of the step gradient from Fig. 1 with an extract of rat cerebellum is shown in Fig. 3. Treatment of the rat brain extract with glutamate decarboxylase resulted in a 90–100% decrease in the glutamate peak height with concomitant increase in the taurine–GABA peak. Fig. 4A shows the cerebellum chromatogram using isocratic elution with an elevated concentration of organic solvent in the mobile phase. Under this third set of conditions taurine and GABA are resolved in less than 8 min, but two double peaks are observed: aspartate-glutamate and threonine-glycine. Treatment of the brain extract with glutaminsae plus GABAse resulted in the disappearance of the glutamine and GABA peaks and an increase in the aspartate-glutamate peak.

In an extension of the findings of Allison *et al.*[6], we tested the stability of ten OPA–*tert.*-butyl thiol derivatives for 60 min before injection into the HPLC. No decrease in peak height was observed unlike that reported for mercaptoethanol derivatives[5,6]. When eluted under the conditions used in Fig. 1, the OPA–mercaptoethanol derivatives are eluted as overlapping peaks in less than 4 min. If should be noted that Allison *et al.*[6] reported that although the electrochemical response was similar for both classes of thiol derivatives, the intensity of fluorescence of the

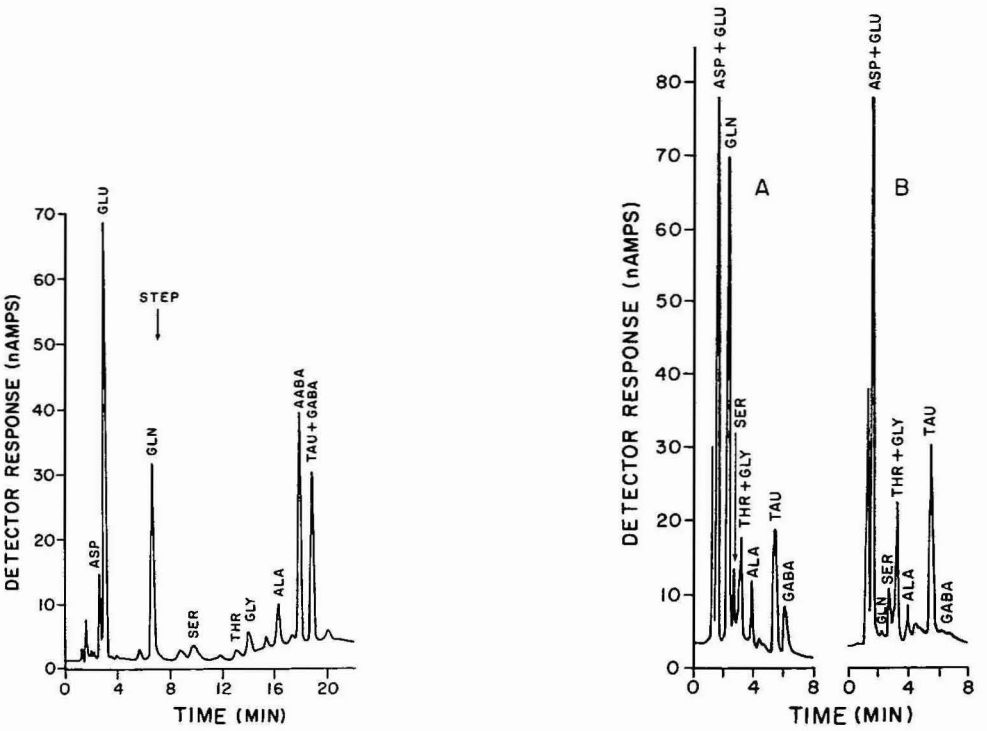

Fig. 3. Amino acid pattern in rat cerebellum. The conditions as in Fig. 1. The volume injected corresponded to 330 ng of tissue, wet weight.

Fig. 4. Rat cerebellum extract treated with GABAse and glutaminase. The composition of the mobile phase was 50 m*M* potassium phosphate, pH 7.0, 1 m*M* EDTA, 25% acetonitrile and 10% THF. (A) Untreated rat cerebellum extract. (B) Rat cerebellum extract treated with GABAse containing glutaminase activity. The volume injected corresponded to 375 ng of tissue, wet weight.

OPA–*tert.*-butyl thiol derivatives was only 10–15% as great as that of the OPA–mercaptoethanol derivatives.

This methodology is applicable for quantitating amino acids below 20 picomoles per injection. The sensitivity is limited by the shift in base line observed at 15 min (see Fig. 1) which prevented using full scale ranges below 20 nA for peaks eluting after this time. The sensitivity for the first five amino acids can be increased several fold if the subsequent amino acid peaks are allowed to go off scale or no step gradient is utilized. The regression coefficients of the lines found by plotting nanoamp response versus pmoles injected for nine test compounds was 0.991 ± 0.007 (mean ± S.D.).

In conclusion, a method for separating and quantitating amino acids by HPLC is described. It utilizes electrochemical detection of stable amino acid derivatives separated by isocratic or simple step gradient thereby eliminating the need for a gradient maker and a second pump. Asparate and glutamate are separated either by inclusion of the cationic ion pair TBAP or by reducing the proportion of the organic phase. These methods were utilized to separate amino acids in rat brain tissue. The glutamate, glutamine and GABA peaks were verified by enzymatic digestion.

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