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GEL CENTRIFUGATION CHROMATOGRAPHY FOR MACROMOLECULAR SEPARATIONS

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(Received December 16th, 1981)

SUMMARY

A method for chromatographic separation of macromolecules by the use of centrifugation through gel filtration materials is described. The liquid excluded from the gel material is removed by centrifugation; thus, this method offers rapid separation and minimal dilution of samples. Because of the rapid separations obtained, this method is particularly suitable for study of enzyme reactions, ligand binding and formation of intermolecular complexes in general. Within the limitations of the short columns which were employed in the centrifuge, the resolution of macromolecules is equivalent on columns eluted conventionally and columns eluted with the assistance of centrifugation.

INTRODUCTION

Centrifugation methods for desalting macromolecules by gel filtration were first developed for liter volumes, by the use of basket centrifuges[1,2], and later adapted for milliliter volumes[3] and microliter volumes[4], using small centrifugation tubes for support of the gel material. The centrifugation methods have been used both for batch elution[3] and stepwise chromatography[4].

The gel centrifugation technique removes and collects the liquid excluded from the gel by centrifugal force. Its advantages are that separations can be (i) done practically without dilution, (ii) obtained in the order of minutes and (iii) performed on viscous samples.

In the past centrifugation has been applied to gel filtration principally to accelerate simple class separations such as the desalting of macromolecules, on highly cross-linked filtration materials such as Sephadex G-25 (Pharmacia) or Bio-Gel P-2 or P-4 (Bio-Rad Labs.)[5,6]. We have found that it is possible to use less cross-linked materials by which separations of macromolecules can be obtained. The separations can be performed (i) as batchwise separations of large macromolecules from smaller macromolecules or (ii) as stepwise chromatographic analysis of macromolecules. We

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describe both theoretical and practical aspects of the technique in this communication.

MATERIALS AND METHODS

The gel centrifugation column assembly and the principle of gel centrifugation are illustrated in Fig. 1. The gel materials were supported in 1-, 3- or 10-ml plastic syringes (Becton-Dickinson) without pistons. Porous polyethylene discs (Bel-Art Products) were placed in the bottom of the syringes; the syringes were then filled with slurries of the gel material (Sephadex, Sephacryl and Sepharose series; Pharmacia) and placed in a swinging bucket centrifuge (IEC PR6 centrifuge rotor with a radius of 20 cm), hanging by the syringe shoulder in a centrifuge tube. The gel materials from Pharmacia which were utilized are currently supplied as beads in a single size range, except for Sephadex G-25 and G-50. The fine grade of the last two materials was used, with the exception of a test of G-25 beads of different sizes described in Results.

Operation of the columns was performed as illustrated in Fig. 1. (1) The excluded liquid was centrifuged out of the column, (2) sample was applied and (3) the excluded liquid was centrifuged out and collected. Further chromatographic elution of the column was done by repeated application of buffer and recentrifugation of the column.

RESULTS

Gel centrifugation chromatography is related in principle to conventional "wet" gel filtration chromatography, with the difference that in the former the excluded liquid is fully or partly removed from the gel material. Thus in gel centrifu-

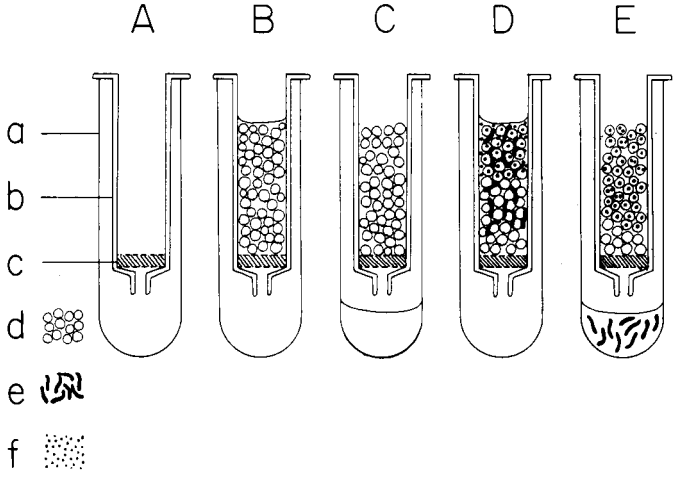

Fig. 1. Centrifugation column assembly and principle of the method. a = Centrifuge tube; b = plastic syringe; C = porous polyethylene disc; d = gel beads; e = large molecules; f = small molecules; the buffer is indicated by the meniscus. A = Assembly; B–E = operation: B, gel slurry applied; C, liquid excluded volume centrifuged out; D, sample applied containing large and small molecules; E, excluded liquid containing the large molecules centrifuged out of the column.

gation chromatography the separations of larger from smaller molecules occur as they pass over the gel beads in a thin layer of excluded liquid.

For a theoretical analysis of gel centrifugation chromatography the gel bed of volume V_t can be divided into the stationary volume included in the gel beads, V_s (gel material volume plus included liquid volume), and the volume excluded from the gel beads, V_0:

$$V_t = V_s + V_0$$

For spherical gel beads the excluded volume is approximately $0.3 \times V_t$ (ref. 7); thus, $V_s = 0.7 \times V_t$. The liquid in the excluded volume is partly removed from the bed by centrifugation

$$V_0 = V_a + V_b$$

where V_a is the volume of the excluded liquid centrifuged out of V_0, and V_b the volume of the excluded liquid remaining in V_0.

A chromatographed molecule is characterized by its elution volume, V_e[7,11], and its partition coefficient K[7,11]. K is a measure of the distribution of macromolecules between the excluded liquid volume and the total included volume; thus, in gel centrifugation chromatography:

$$K = (V_e - V_b)/V_s$$

The elution of a macromolecule in gel centrifugation chromatography can be empirically characterized by the retardation value, R_c, defined as:

$$R_c = V_e/V_t$$

The retardation value for markers excluded from the gel beads ($K = 0$) is therefore a measure of the fraction of the total volume which is contained in the remaining excluded liquid volume, V_b/V_t. Thus, a retardation value equal to zero for such a marker corresponds to removal of all the excluded liquid ("dry gel"), while a retardation value of 0.3 indicates a lack of removal of the excluded liquid ("wet gel").

The force necessary for removal of the excluded liquid from the different gel materials and the maximal force they can withstand is shown in Fig. 2. The maximal force the gel materials are able to resist without collapsing depends on their rigidity. A guideline for the rigidity of the products is given by the manufacturer as the maximal hydrostatic pressure the gel materials are able to sustain[11]. Above the maximal force the gel materials collapse and are pressed out of the centrifugation columns. Sephadex G-10, G-15, G-25, Sephacryl S-200 and Sepharose 6 B can all withstand forces higher than 6000 *g* as indicated in Fig. 2. Materials with increasing molecular weight exclusion limits in the Sephadex and Sepharose series can withstand less force. Thus, Sephadex G-200 can withstand only 120 *g* and Sepharose 2B only 1200 *g*. It must be noted, however, that the maximal force the materials are able to withstand depends on the height of the column, since the stress on the lower part of the column is the total centrifugal pressure of the overlaying material.

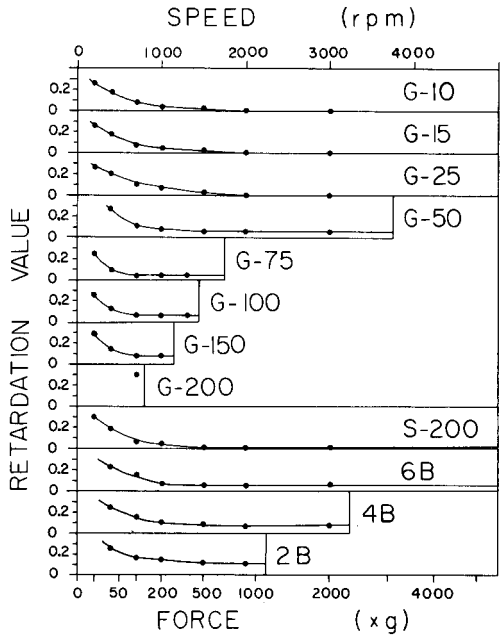

Fig. 2. Properties of gel filtration materials for column centrifugation; the Sephadex, Sephacryl and Sepharose series are indicated with their trade numbers, G, S and B respectively. 10-ml gel beds (55 × 15 mm) were used, in 10-ml syringes. The centrifugation speed above which the gel materials are pressed out of the columns is indicated by the length of the bars. The curves inside the bars indicate the retardation value for Blue Dextran 2000 (for Sephadex and Sephacryl) and bovine milk colloid (for Sepharose), used as excluded markers. The retardation value was calculated from the elution volume, (see text) which was measured by stepwise elution. A 200-μl volume of marker solution was applied and centrifuged for 5 min at the indicated speed, followed by elution steps with 0.5 ml buffer and centrifugation for 5 min at the indicated speed.

At forces less than those causing the gels to collapse, the proportion of the excluded liquid removed by centrifugation is a function of the applied force. In Fig. 2 this is indicated by curves which specify the volume of buffer which must be applied to and step-eluted from the column at any particular centrifugal force level in order to result in the quantitative elution of an excluded marker. For example, using Sephadex G-10, at 40 *g*, 0.2 bed volumes must be applied to the column and removed by centrifugation in order to elute Blue Dextran marker.

Removal of 50% of the excluded liquid (retardation value of 0.15 for excluded markers) requires a force between 40 and 100 *g* for 5 min for the different materials. Sephadex G-200, however, did not release its excluded liquid even when centrifuged at 100 *g* for 5 min.

Nearly total removal of the excluded liquid is obtained with Sephadex G-10, G-15, G-25 and Sephacryl S-200, which show retardation values for excluded markers less than 0.01 at forces above 100 *g*. Eighty percent removal of the excluded liquid (retardation value of 0.06 for excluded markers) is obtained at forces above 200 *g* for Sepharose 6B. Above 200 *g*, 75% removal of excluded liquid was obtained for Sepharose 4B and 60% for Sepharose 2B.

The small amount of excluded liquid phase which remains on these dextran

and agarose beads, including those most tightly cross-linked, at even relatively high centrifugal forces is critical to the separations achieved in gel centrifugation chromatography, as discussed below. We have determined the thickness of this liquid layer on three different sizes of Sephadex G-25 beads, coarse, fine and superfine, whose average diameters in the swollen state we estimated by microscopy to be 190, 75 and 25 μm respectively. To determine the size of the bound, excluded layer we equilibrated columns of 7.8-ml bed volume of each bead type with a solution of Blue Dextran, centrifuged them at 900 *g* for 10 min and then eluted the columns twice with 3.0-ml portions of buffer and measured the absorbance at 280 nm of the eluates. The average thickness of the excluded layer was then calculated from the volume of this liquid retained on the column, the diameter of the beads and the volume of the column occupied by the beads, 70%. The results indicated thicknesses of 0.51, 0.28 and 0.26 μm for the layer on coarse, fine and superfine beads respectively. Thus, this layer is quite thin, and its thickness is relatively independent of the diameter of the Sephadex

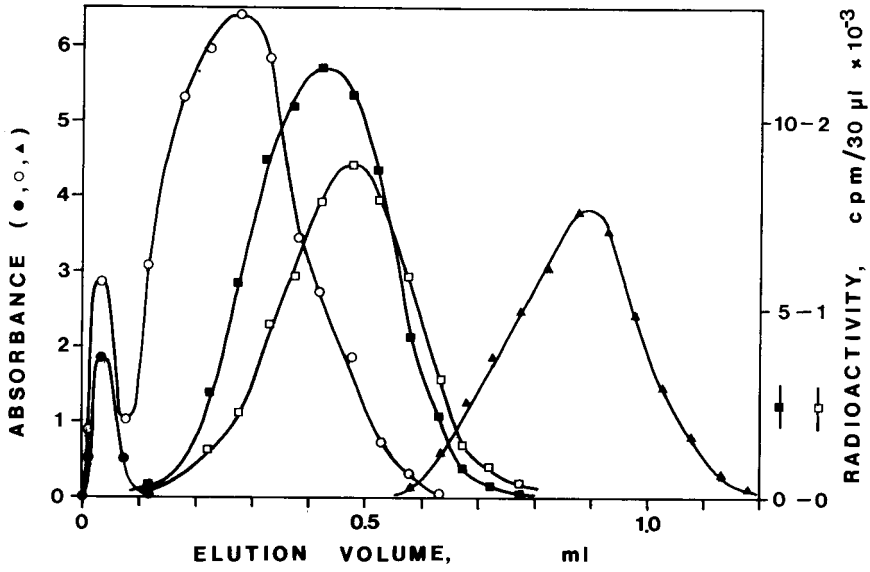

Fig. 3. Stepwise chromatographic separation of a mixture of Blue Dextran, ferritin, albumin, ovalbumin and phenol red on a Sepharose 6B centrifugation column. The column was equilibrated with elution buffer (100 m*M* NaCl, 20 m*M* Tris, pH 7.5) and centrifuged for 3 min for 2000 rpm prior to usage. The column measured 50 mm × 18 mm^2 after centrifugation. An 18-μl volume of a mixture consisting of 120 μg Blue Dextran 2000 (Pharmacia) (●), 250 μg ferritin (Miles). (○), 0.5 μg [^{125}I]bovine serum albumin (120,000 cpm) (Sigma, St. Louis, MO, U.S.A.) (■), 0.2 μg [^{125}I]ovalbumin (19,000 cpm) (Sigma) (□) and 22 μg phenol red (▲) was loaded carefully on the top of the column and centrifuged for 3 min at 2000 rpm. The Blue Dextran was chromatographed prior to the separation on a Sepharose 4B column to exclude molecules with a molecular mass smaller than 2,000,000. The iodinations were performed by the chloramin T method[8]. The column was eluted stepwise with addition of elution buffer and centrifugation for 3 min at 2000 rpm. The first two elution volumes, 16 and 25 μl, were used to wash the mixture carefully into the gel. Thereafter, 50-μl volumes were used. The optical absorption at 630, 430 and 350 nm of the eluates was measured by a Carl Zeiss M4QII spectrophotometer, and the absorptions of Blue Dextran at 630 nm, phenol red at 430 nm and ferritin at 350 nm were calculated from their respective spectra. The amount of ^{125}I-iodinated proteins in the eluates was measured after separation by SDS polyacrylamide gel electrophoresis[9] using a Searle Series 1175 gamma counter.

G-25 beads. This measurement is easily carried out and, as will be discussed, the value of V_b obtained is useful in arranging for optimal separations on any particular column to be used for gel centrifugation chromatography.

An example of the use of centrifugation columns is shown in Fig. 3. This figure illustrates a stepwise chromatographic analysis on a 50 mm × 18 mm^2 Sepharose 6B centrifugation column of a mixture of Blue Dextran (molecular mass larger than 2,000,000), ferritin (molecular mass 470,000), bovine serum albumin (molecular mass 69,000), ovalbumin (molecular mass 46,000) and phenol red (molecular mass 345). Blue Dextran was collected in the excluded volume with an retardation value of 0.04. Phenol red chromatographed at a retardation value of 0.96 and was used as an indication of the total volume of the included plus excluded liquid. As can be seen from the figure, a practically complete separation of albumin and ovalbumin from both Blue Dextran and phenol red was obtained. Ferritin separated totally from the phenol red and well from Blue Dextran. However, ferritin showed a peak in the excluded volume. After analyzing the eluates by SDS-polyacrylamide gel electrophoresis, it was observed that this material mainly consisted of ferritin dimers.

We have compared this separation to a similar separation obtained on an identical column, which was operated by the conventional "wet" technique (not shown). The partition coefficient obtained with the two methods differed less than 0.05 for any of the chromatographed molecules. Furthermore, the resolution obtained with the centrifugation column was similar to that with the "wet" column.

It should be noted that, except for Blue Dextran, the band widths for the compounds were almost identical (0.45–0.50 ml). This similarity of the band widths is normal for gel filtration[10]. The HETP (height equivalent to a theoretical plate)[7] was 0.9 mm for phenol red.

DISCUSSION

The gel centrifugation chromatography method permits a faster separation of macromolecules than conventional "wet" gel filtration chromatography without loss of resolution. A mixture of macromolecules can thus be separated in a few minutes. Enzyme reactions can be stopped and analyzed by gel centrifugation, and binding reactions between macromolecules as well as ligand binding can easily be studied.

The optimal use of the materials in gel centrifugation chromatography is obtained at forces where a maximal volume of the excluded liquid is removed, and the gel bed is not collapsed. These criteria provide a guideline for the optimal use of the materials, as observed in Fig. 2. With the gel materials investigated, separations can be obtained of molecular masses ranging from 500 to 30,000,000 (ref. 11).

Similar gel materials from other sources can also be utilized. As an example we have found that Bio-Gel P-60 (Bio-Rad Labs.) has an optimal usage range in centrifugation chromatography from 100 to 1000 *g*, with a maximal excluded liquid removal of 60%.

The effects of centrifugation on the different gel materials we have utilized vary. With the more tightly cross-linked gels, such as Sephadex G-10, G-15 and G-25, there is very little bed shrinkage on centrifugation, and the excluded liquid which is removed, V_a, is replaced by air. With the other types of gels there is generally some shrinkage on centrifugation at high speed and little or no air appears to enter the

column. Since air has not replaced that portion of the excluded liquid volume removed from these gel types by centrifugation, V_a, there must be a compression of these gel beads. Presumably the individual gel beads lose their spherical shape and pack more tightly, in a manner similar to cells in a tissue. It is also possible that part of the shrinkage of the column might be due to compression of the individual gel beds. This will decrease the pore sizes and thus the exclusion limit. However, although Sepharose 6B columns show some shrinkage on centrifugation, the elution positions of the proteins in Fig. 3 show no significant difference from the positions of the same proteins run by conventional "wet" gel filtration, with respect to the partition coefficients. Thus a compression of the individual gel beads is not likely for this material at least.

The retardation value (0.96) of phenol red in Fig. 3 reflects the tighter packing of the Sepharose 6B beads and smaller air filled volume, V_a. At an excluded volume of $0.04 \times V_t$ the volume of the included liquid becomes $0.92 \times V_t$. V_s which also includes the volume of the gel material is thus slightly larger than $0.92 \times V_t$. As mentioned, V_s is $0.7 \times V_t$ for spherical beads.

A unique practical consideration in gel centrifugation chromatography is the determination of the portion of the excluded volume which is not removed from the column by centrifugation, V_b. This parameter must be taken into account in determining the volume of the sample to be applied to the column and the size of the subsequent elution volumes. These choices will in turn depend upon whether the purpose is to achieve a maximal resolution of a set of different molecular species or a simple class separation, as in desalting a macromolecule.

The role of V_b is best seen by considering the situation under which chromatographic resolution occurs in the gel centrifugation technique. The flow-rate of the moving, excluded liquid phase during centrifugation is very high in comparison to conventional "wet" gel chromatography, and it might thus be expected that only poor resolution would be achieved using centrifugation. However, it has been pointed out[7] that, although high resolution of molecules by "wet" gel filtration requires flow-rates allowing equilibrium partitioning between the mobile, excluded phase and the stationary phase, the limiting diffusion step is not within the gel beads. Instead, the limiting diffusional steps appear to occur *outside* the boundary of the restricted space within the gel particle[7]. We have shown that the average thickness of the liquid layer in V_b on Sephadex G-25 is not more than 0.5 μm or approximately 2% of the radius of the smallest type of G-25 bead. Owing to the small dimensions of this layer, equilibration of molecules partitioning between it and the included, stationary phase should be much more rapid than is the case in "wet" filtration, where the average dimensions of the liquid-filled spaces between beads are relatively large.

Thus, the thinness of the excluded layer in V_b explains the relatively high resolution obtained with gel centrifugation chromatography. For this reason, if high resolution is desired, the volume applied per chromatographic step in this technique should not greatly exceed the value of V_b for a particular column, so as to maintain the thinness of that layer. It should be noted that the discontinuous, step-wise operation of the column in this method is not actually disadvantageous, as it permits equilibration of molecules between the included and excluded phases between elution steps. As in "wet" gel chromatography, careful application of samples and elution buffer, in an even band across the column bed top, is essential if optimal resolution is

to be obtained. Operation of chromatographic columns in normal centrifuge tubes in the present study limited column lengths to a maximum of 65 mm. Within this limitation we have obtained similar degrees of resolution between macromolecules chromatographed on gel filtration columns operated in a conventional wet manner and columns eluted with the aid of centrifugation. While such short columns do not permit full exploitation of the resolving capacity of the filtration media, the rapidity with which they can be operated in the centrifuge should make this technique useful for applications where the macromolecules to be resolved constitute relatively simple mixtures of species differing considerably in molecular weight.

The method of centrifugation chromatography has been examined here with respect to gel filtration materials, but ion exchange and affinity chromatography can also be adapted to obtain many of the advantages of gel centrifugation. The chromatographic materials used in these techniques are often very rigid, and only a little force is necessary for removal of the unbound sample and elution buffer. For example, carboxymethylcellulose and DEAE-cellulose (CM 52 and DE 52 from Whatman) require less than 40 *g* for liquid removal (results not shown).

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DETERMINATION OF PARTITION COEFFICIENTS OF VERY HYDROPHOBIC COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON GLYCERYL-COATED CONTROLLED-PORE GLASS

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SUMMARY

Reversed-phase high-performance liquid chromatography of more than 80 compounds with glyceryl-coated controlled-pore glass as the stationary phase and a mixture of methanol and water as the mobile phase showed that the capacity factor (k') correlates well with the partition coefficient between octanol and water (P_{oct}). This method is very efficient for the determination of P_{oct} for compounds with log P_{oct} > 5. A general method for the determination of P_{oct} by high-performance liquid chromatography in this way is proposed.

INTRODUCTION

The hydrophobicity of biologically active compounds is known to affect their biological responses. The partition coefficient (P) of a compound between a water-immiscible organic solvent and water is a good measure of its hydrophobicity[1]. In investigations on quantitative structure–activity relationships (QSAR), the general rules that govern the hydrophobicity of organic compounds have been studied extensively, and the value of log P between octanol and water has sometimes been determined with the use of the hydrophobic substituent coefficient[2], π, or the hydrophobic fragmental constant[3], f, for simple compounds. However, the value determined in this way is not always correct when there is a strong electronic or steric effect caused by the introduction of a substituent group[4]. Hence it is safer to determine the log P value directly by an experimental procedure. The shaking-flask method, generally adopted as a standard method for the determination of log P, is time consuming and tedious[5], and is unsuitable for the accurate determination of log P values greater than 4 (ref. 6). Therefore, a simple and accurate procedure is required for the measurement of log P values.

Recently, reversed-phase high-performance liquid chromatography (RP-

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HPLC) has been applied to the determination of log P (ref. 7). The capacity factor (k') in RP-HPLC is correlated with the value of log P between octanol and water determined by the shaking-flask method (log P_{oct}) as shown in the equation[8]

$$\log P_{oct} = a \log k' + b \quad (1)$$

where a and b are constants characteristic of a certain partition system. The k' value is determined by RP-HPLC by the relationship

$$k' = (t_R - t_0)/t_0 \quad (2)$$

where t_R and t_0 are the retention times at a certain flow-rate of the biologically active compound and an unretained substance, respectively.

RP-HPLC on an octanol-coated column is very useful, as the properties of the stationary phase are very similar to those of octanol. For this purpose, octanol is adsorbed as the stationary phase on silica gel[9] or on octadecylsilylated silica gel (ODS)[10,11] and octanol-saturated buffer solution is used as the mobile phase. In this instance, the slope (a in eqn. 1) for the linear relationship between log P_{oct} and log k' was found to be very close to unity, indicating that log k' is directly related to log P_{oct}[9–11]. However, there are some experimental limitations to this procedure for determining the exact retention time (t_R) of a compound with a high log P_{oct}, *viz.*, very low solubility in water or the mobile phase, and a long retention time associated with a broad chromatographic peak. Note that increase in one log unit of P_{oct} results in about a 10-fold increase in t_R when a in eqn. 1 is unity. To overcome these difficulties, it is desirable to perform RP-HPLC with a mixture of water and organic solvent such as methanol and acetonitrile as the mobile phase, and with a column giving a slope a in eqn. 1 of less than unity, but still retaining the properties of octanol for a wide variety of compounds. There are two advantages of using a mixture of water and organic solvent: it affords sufficient solubility of highly hydrophobic compounds and a smaller t_R value than that obtained in the absence of an organic solvent.

There have been some reports on the chromatography of chemically bonded hydrocarbons on silica gel, such as ODS. Good linearity was observed between log P_{oct} and log k' with penicillins[12], cephalosporins[12] and propranolols[13]. However, the method has not been used for compounds with log P_{oct} values of more than about 3.

This paper reports the usefulness of glyceryl-coated controlled-pore glass (gly-CPG) as a stationary phase in RP-HPLC for the determination of the log P_{oct} values of compounds with a wide range of such values. A general method for the determination of P_{oct} by RP-HPLC is also described.

EXPERIMENTAL

N-Phenylsuccinimides were kindly supplied by Dr. Chiyozo Takayama, Sumitomo Chemical Co. (Osaka, Japan). 3′-Substituted N-phenylanthranilates were donated by Drs. Shuichi Ikawa and Eiichi Fujihira, Taisho Pharmaceutical Co. (Tokyo, Japan). All other chemicals were commercial products and were used without further purification.

RP-HPLC was carried out with a Tri-Roter-II solvent delivery system (JASCO, Tokyo, Japan) connected with a Uvidec 100-II ultraviolet detector (JASCO)

operated mostly at 210 nm. The column (50 cm × 2.1 mm I.D.) was packed with gly-CPG (Electro-Nucleonics, Fairfield, NJ, U.S.A., Type gly00075, 200–400 mesh). Chromatography of only the neutral forms of acidic compounds was achieved by using an aqueous solution of phosphoric acid (0.03 *M*) of pH 2.2 containing various amounts of methanol as the mobile phase. For determination of partition coefficients test compounds were dissolved in methanol at concentrations of about 0.2 mg/ml and 0.5–4.0 μl of the solution was injected on to the column, together with potassium iodide for determination of t_0, and eluted at a flow-rate of 0.3–2.0 ml/min.

RESULTS AND DISCUSSION

Effect of methanol during chromatography

RP-HPLC of various compounds was performed using gly-CPG as the stationary phase with a mixture of water (phosphoric acid, pH 2.2) and methanol as the mobile phase. Fig. 1 shows the effect of the methanol concentration in the mobile phase on the capacity factor, k', defined by eqn. 2. In all instances log k' decreased linearly with increase in the concentration of methanol at least up to 30% of methanol. A similar linear relationship has been observed on chromatography on an ODS column eluted with water containing methanol or acetonitrile[14,15]. The results showed that with increase in the concentration of the organic modifier in the aqueous mobile phase, log k' decreased gradually, finally reaching a constant level. Thus, the retention time (t_R) on chromatography with gly-CPG would also become constant when the concentration of methanol is increased much more.

The linear relationship between log k' and the methanol concentration (C) in Fig. 1 is expressed by the equation

$$\log k' = \log k'_0 + m\,C \tag{3}$$

where m is the slope of the straight line in Fig. 1 and k'_0 corresponds to the capacity factor in the absence of methanol from the mobile phase. As shown in Fig. 1, k'_0 for

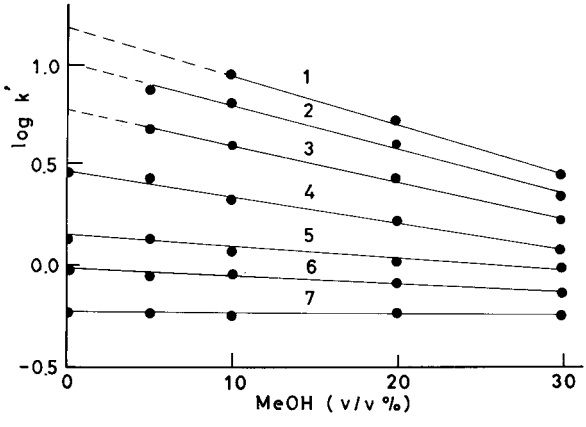

Fig. 1. Effect of methanol (MeOH) concentration on log k'. 1, Flufenamic acid; 2, N-phenylanthranilic acid; 3, diphenyl ether; 4, diphenyl ketone; 5, chlorobenzene; 6, methyl benzoate; 7, phenol.

hydrophobic compounds, such as flufenamic acid, N-phenylanthranilic acid and diphenyl ether, could not be determined directly owing to the low solubility of these compounds in acidic aqueous solutions. For these compounds, we determined k'_0 by extrapolating the straight line to zero methanol concentration.

The capacity factor (k') is defined by the equation[16]

$$k' = \frac{(X)_s}{(X)_m} \cdot \frac{V_s}{V_m} \tag{4}$$

where $(X)_s$ and $(X)_m$ are the concentrations of the solute X in the stationary (s) and mobile (m) phase, respectively, and V_s/V_m is the volume ratio of solvent to the stationary phase. According to the solubility parameter theory[17], the distribution of the solute X between two phases is related to the solubility parameters of the component X (δ_x), stationary phase (δ_s) and mobile phase (δ_m) and also the molar volume of X ($\bar{V}_x$), as shown in the equation

$$\log\left[\frac{(X)_s}{(X)_m}\right] = \bar{V}_x \cdot \frac{(\delta_x - \delta_m)^2 - (\delta_s - \delta_x)^2}{2.3\,RT} \tag{5}$$

In this study, the mobile phase consisted of two components, water (1) and methanol (2). In this instance, the solubility parameter of the mobile phase (δ_m) is expressed by the equation

$$\delta_m = (1 - C')\,\delta_1 + \delta_2\,C' \tag{6}$$

where C' is the volume fraction of methanol in the mobile phase. Under conditions where C' is small, the following relationship applies[18]:

$$\log k' = \log\left(\frac{V_s}{V_m}\right) + \frac{\bar{V}_x}{2.3\,RT}[(\delta_x - \delta_1)^2 - (\delta_s - \delta_x)^2] - \frac{2\bar{V}_x}{2.3\,RT}(\delta_x - \delta_1)(\delta_2 - \delta_1)\,C' \tag{7}$$

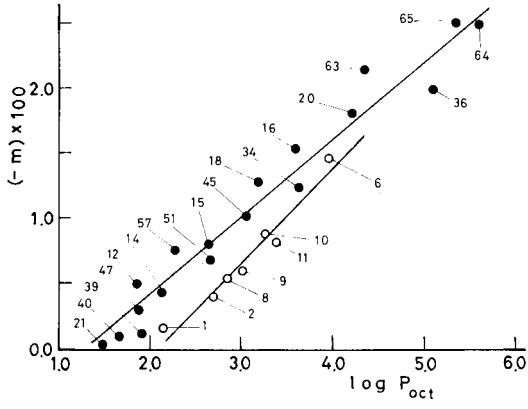

Fig. 2. Relationship between $-m$ in eqn. 3 and log P_{oct}. Numbers correspond to those for the compounds listed in Table I. ○, Non-H-bonders; ●, H-bonders.

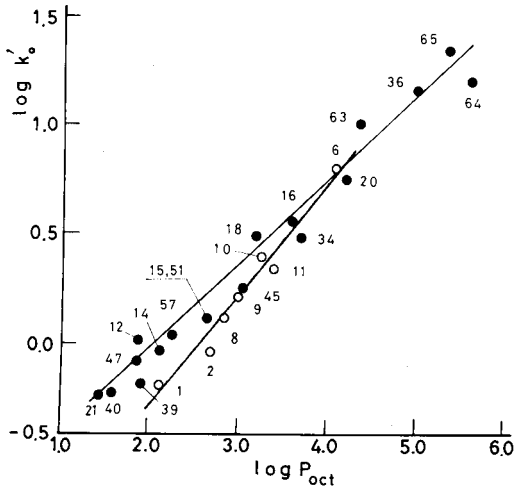

Fig. 3. Relationship between log k'_0 and log P_{oct}. Numbers correspond to those for the compounds listed in Table I. ○, Non-H-bonders; ●, H-bonders.

The first two terms in eqn. 7 are constant in the absence of methanol, and their sum corresponds to the capacity factor (k'_0). The last term in eqn. 7 depends on the concentration of methanol (C') with a coefficient of $2\bar{V}_x(\delta_x - \delta_1)(\delta_2 - \delta_1)/2.3RT$. Thus, eqn. 7 is equivalent to eqn. 3, which explains why log k' decreased linearly with increase in the concentration of methanol up to 30% in the mobile phase, as shown in Fig. 1.

Relationship between chromatographic properties and partition coefficient

From the relationship shown in eqn. 3, k'_0 and the slope m were determined for various compounds, and these values were plotted as a function of the partition coefficient in the octanol–water system (P_{oct}). Fig. 2 shows the relationship between $-m$ and log P_{oct}, and Fig. 3 shows a plot of log k'_0 *versus* log P_{oct}. The compounds used are listed in Table I. It is clear from these figures that both parameters change linearly with log P_{oct}. These linear relationships are expressed by the equations

$$m = -5.81\ (\pm\ 1.07) \times 10^{-3} \log P_{oct} + 7.83\ (\pm\ 3.63) \times 10^{-3} \quad (8)$$
$$(n = 24,\ r = -0.922,\ s = 0.003)$$

and

$$\log k'_0 = 3.90\ (\pm\ 0.04) \times 10^{-1} \log P_{oct} - 8.79\ (\pm\ 1.21) \times 10^{-1} \quad (9)$$
$$(n = 24,\ r = 0.979,\ s = 0.094)$$

where n is the number of compounds, r is the correlation coefficient and s is the standard deviation. The figures in parentheses are the 95% confidence intervals. These relationships are improved when plotted separately for two groups of compounds: compounds capable of forming a hydrogen bond ("H-bonders", *e.g.*, phenols

and benzoic acids) and those incapable of forming a hydrogen bond ("non-H-bonders", *e.g.*, alkyl- and halobenzenes). The relationships are as follows:
For H-bonders:

$$m = -5.57\ (\pm\ 0.99) \times 10^{-3} \log P_{oct} + 5.90\ (\pm\ 3.44) \times 10^{-3} \quad (10)$$
$$(n = 17,\ r = -0.952,\ s = 0.002)$$

$$\log k'_0 = 3.75\ (\pm\ 0.29) \times 10^{-1} \log P_{oct} - 8.07\ (\pm\ 1.00) \times 10^{-1} \quad (11)$$
$$(n = 17,\ r = 0.991,\ s = 0.070)$$

For non-H-bonders:

$$m = -7.12\ (\pm\ 1.81) \times 10^{-3} \log P_{oct} + 1.45\ (\pm\ 0.56) \times 10^{-2} \quad (12)$$
$$(n = 7,\ r = -0.976,\ s = 0.001)$$

$$\log k'_0 = 5.48\ (\pm\ 1.44) \times 10^{-1} \log P_{oct} - 1.42\ (\pm\ 0.45) \quad (13)$$
$$(n = 7,\ r = 0.975,\ s = 0.079)$$

These results indicate that gly-CPG recognizes a difference in the compounds in terms of their ability to form a hydrogen bond. In Figs. 2 and 3, it should be noted that good linear relationships still hold for $\log P_{oct} > 5$. The determination of such high partition coefficients is extremely difficult by the conventional shaking-flask method. From Figs. 2 and 3, $\log k'_0$ and m are expressed as functions of $\log P_{oct}$ by the equations

$$\log k'_0 = a' \log P_{oct} + b' \quad (14)$$

and

$$m = a'' \log P_{oct} + b'' \quad (15)$$

From eqns. 3, 14 and 15, we obtain the following equation:

$$\log k' = (a' + a''C) \log P_{oct} + (b' + b''C) \quad (16)$$

Eqn. 16 corresponds to eqn. 1 at a certain methanol concentration, C. As shown in Fig. 1, $\log k'$ decreases linearly with increase in methanol concentration according to eqn. 3. Hence the relationship between $\log k'$ and the concentration of methanol, and that between $\log k'$ and partition coefficient P_{oct}, can be depicted schematically as shown in Fig. 4. Fig. 4 shows that $\log P_{oct}$ can be determined by RP-HPLC using any of the following calibration graphs: (i) *m versus* $\log P_{oct}$, based on eqn. 15, (ii) $\log k'_0$ *versus* $\log P_{oct}$, based on eqn. 14, and (iii) $\log k'$ determined at a certain methanol concentration ($\log k'_{p\%}$) *versus* $\log P_{oct}$, based on eqn. 1. Of these three methods, the last is the most practically useful, as the solubility of highly hydrophobic compounds is a limiting factor in chromatography. In this instance, the methanol concentration must be as low as possible, because with a lower methanol concentration the $\log k'$ value is larger, as can be seen from eqn. 16.

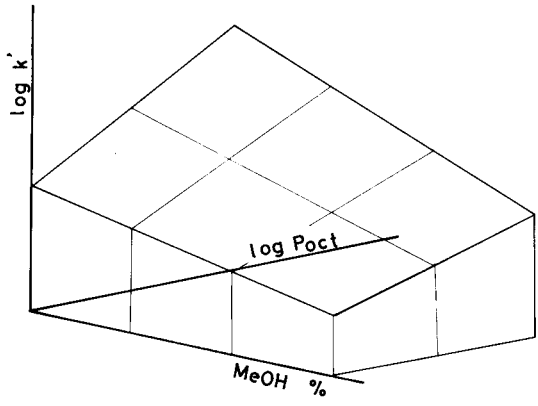

Fig. 4. Dependence of log k' on methanol concentration and log P_{oct}.

Determination of partition coefficient by RP-HPLC

The above results indicate that the partition coefficient (log P_{oct}) can be determined from k' at a certain methanol concentration of less than 30% in the mobile phase by RP-HPLC with gly-CPG as the stationary phase. We measured the k' values at 10% methanol ($k'_{10\%}$) for about 80 compounds. These compounds consisted of phenols, benzoid acids, N-phenylanthranilates, N-phenylsuccinimides, other miscellaneous H-accepting compounds (such as cyano- and nitrobenzene) and non-H-bonding compounds. The hydrophobicities (log P_{oct}) of these compounds ranged between 1.08 and 5.62. Table I shows the log $k'_{10\%}$ and log P_{oct} values of these compounds. The correlations between log $k'_{10\%}$ and log P_{oct} for these compounds are listed in Table II according to their chemical structures.

In all instances, log $k'_{10\%}$ is correlated linearly and highly significantly with log P_{oct}. These relationships are almost the same with all chemical classes except non-H-bonders, which give a regression line with a lower slope. These relationships are shown in Fig. 5.

The correlation for all of the compounds, expressed by eqn. 23, is very high, but it is improved by omission of non-H-bonders (*cf.*, eqn. 24). Note that all the correlations from eqns. 17–24 are significant at more than the 99% confidence level. These results indicate that log P_{oct} can be determined exactly by RP-HPLC. When the partition coefficient is determined by RP-HPLC (the value is referred to as P_{HPLC}), it is more accurate to obtain calibration graphs with compounds belonging to the same class as the sample compounds. In Table I, log P_{HPLC} values calculated by eqns. 17–22 are listed according to chemical structures. These values are almost the same as those determined by the conventional method for all compounds, including very hydrophobic compounds, such as flufenamic acid (log P_{oct} = 5.62), 3′-chloro-N-phenylanthranilic acid (log P_{oct} = 5.57) and mefenamic acid (log P_{oct} = 5.37).

Next, we determined P_{HPLC} for various compounds for which the P_{oct} values were not determined from the $k'_{10\%}$ values on the gly-CPG column. Table III lists the log P_{HPLC} values for these compounds determined from the corresponding correlations in Table II. The P_{HPLC} values are compared with the partition coefficients, log P_{cal}, determined from the hydrophobic substituent coefficients (π). As can be seen, the log P_{HPLC} values are very similar to the log P_{cal} values in all instances.

TABLE I

CAPACITY FACTORS IN RP-HPLC ($k'_{10\%}$) AND PARTITION COEFFICIENTS (P_{oct} AND P_{HPLC}) OF VARIOUS COMPOUNDS

No.	*Compound*	*Log* $k'_{10\%}$	*Log* P_{oct}*	*Log* P_{HPLC}**	Δ***
(I) Non-H-bonders:					
1	Benzene	−0.42	2.13	2.18	−0.05
2	Toluene	−0.13	2.69	2.62	0.06
3	Ethylbenzene	0.22	3.15	3.16	−0.01
4	Isopropylbenzene	0.38	3.66	3.40	0.25
5	Naphthalene	0.42	3.59	3.46	0.12
6	Diphenyl	0.74	3.95	3.95	0.00
7	Fluorobenzene	−0.40	2.27	2.21	0.06
8	Chlorobenzene	0.08	2.84	2.94	−0.10
9	Bromobenzene	0.12	2.99	3.00	−0.01
10	Iodobenzene	0.43	3.25	3.48	−0.23
11	1,4-Dichlorobenzene	0.40	3.38	3.43	−0.05
(II) H-Acceptors:					
12	C_6H_5–NO_2	−0.28	1.85	1.99	−0.14
13	–CN	−0.42	1.56	1.66	−0.10
14	–CO_2CH_3	−0.20	2.12	2.17	−0.05
15	–$CO_2C_2H_5$	−0.05	2.64	2.53	0.11
16	–$CO_2C_6H_5$	0.38	3.59	3.54	0.04
17	–$COCH_3$	−0.46	1.73	1.56	0.16
18	–COC_6H_5	0.28	3.18	3.30	−0.12
19	–OCH_3	−0.26	2.11	2.03	0.07
20	–OC_6H_5	0.65	4.21	4.17	0.03
(III) Phenols:					
21	H–	−0.45	1.48	1.54	−0.06
22	2-CH_3–	−0.23	1.95	1.99	−0.04
23	4-CH_3–	−0.24	1.96	1.97	−0.01
24	3-CF_3–	0.08	2.95	2.61	0.34
25	3-C_2H_5–	−0.11	2.40	2.23	0.17
26	4-C_2H_5–	−0.13	2.26	2.19	0.07
27	4-C_6H_5–	0.32	3.20	3.10	0.10
28	3-F–	−0.21	2.15	2.03	0.12
29	4-F–	−0.23	2.07	1.99	0.08
30	3-Cl–	0.05	2.68	2.55	0.13
31	4-Cl–	0.03	2.65	2.51	0.14
32	4-Br–	0.18	2.86	2.81	0.05
33	2,4-Cl_2–	0.34	3.08	3.14	−0.06
34	2,4,6-Cl_3–	0.51	3.62	3.48	0.14
35	2,3,4,6-Cl_4–	0.90	4.10	4.27	−0.17
36	2,3,4,5,6-Cl_5–	1.30	5.12	5.08	0.04
37	4-CH_3CO–	−0.43	1.35	1.58	−0.23
38	4-C_6H_5CO–	0.23	3.07	2.91	0.16
39	4-NO_2–	−0.19	1.91	2.07	−0.16
40	4-CN–	−0.32	1.66	1.80	−0.14
41	4-CH_3O–	−0.41	1.57	1.62	−0.05
42	3-CH_3CO_2–	−0.59	1.23	1.26	−0.03
43	4-CH_3O_2C–	−0.28	1.96	1.88	0.08
44	4-$C_2H_5O_2C$–	−0.13	2.35	2.19	0.16
45	4-$C_3H_7O_2C$–	0.11	3.04	2.66	0.38
46	2-CHO–	−0.38	1.65	1.68	−0.03

TABLE I *(continued)*

No.	*Compound*	*Log* $k'_{10\%}$	*Log* P_{oct}*	*Log* P_{HPLC}**	Δ***
(IV) Benzoic acids:					
47	H–	−0.26	1.87	1.95	−0.08
48	3-F–	−0.21	2.15	2.05	0.10
49	4-F–	−0.24	2.07	1.99	0.08
50	3-Cl–	0.05	2.68	2.57	0.11
51	4-Cl–	0.03	2.65	2.53	0.12
52	3-Br–	0.18	2.87	2.83	0.04
53	4-Br–	0.18	2.86	2.83	0.03
54	3-I–	0.38	3.13	3.23	−0.10
55	4-I–	0.35	3.14	3.17	−0.03
56	3-CH_3–	−0.02	2.37	2.43	−0.06
57	4-CH_3–	−0.02	2.27	2.43	−0.16
58	3-NO_2–	−0.30	1.83	1.87	−0.04
59	4-NO_2–	−0.33	1.89	1.81	0.08
60	4-CN–	−0.46	1.56	1.55	0.01
61	2-HO–	−0.10	2.25	2.27	−0.02
62	2-CH_3CO_2–	−0.55	1.23	1.36	−0.13
(V) N-Phenylanthranilates:					
63	H–	0.78	4.36§	4.55	−0.19
64	3′-CF_3–	1.21	5.62§	5.47	0.15
65	2′,3′-$(CH_3)_2$–	1.23	5.37§	5.52	−0.15
66	3′-CH_3–	0.87	4.88§	4.74	0.14
67	3′-Cl–	1.27	5.57§	5.60	−0.03
68	3′-NO_2–	0.72	4.57§	4.42	0.15
69	3′-HO–	0.30	3.49§	3.51	−0.02
70	3′-CH_3O–	0.78	4.56§	4.55	0.01
71	3′-CH_3CO–	0.70	4.31§	4.37	−0.06
(VI) N-Phenylsuccinimides:					
72	3-CF_3–	−0.52	1.26§§	1.35	−0.09
73	4-CF_3–	−0.54	1.45§§	1.30	0.15
74	3-n-C_3H_7–	−0.45	1.54§§	1.52	0.02
75	3,5-$(CF_3)_2$–	−0.15	2.46§§	2.25	0.21
76	3,5-$(CH_3)_2$–	−0.60	1.08§§	1.16	−0.08
77	4-Br–	−0.58	1.18§§	1.20	−0.02
78	3-I–	−0.48	1.36§§	1.45	−0.09
79	3,5-Cl_2–	−0.30	1.90§§	1.88	0.02
80	2,3,5-Cl_3–	−0.07	2.40§§	2.44	−0.04
81	3,5-Br_2–	−0.22	2.12§§	2.12	0.00
82	3,4,5-Cl_3–	0.13	2.80§§	2.92	−0.12

* Partition coefficient between octanol and water. Values taken from ref. 19.

** Partition coefficient between octanol and water calculated from log $k'_{10\%}$ with eqns. 17–22 according to chemical class.

*** $\Delta = \log P_{oct} - \log P_{HPLC}$.

§ Taken from ref. 20.

§§ Taken from ref. 21.

The above results show that the RP-HPLC method is useful for the determination of P_{oct}, and can be used for very hydrophobic compounds. Tanaka and Thornton[22] reported that k' values between 0.2 and 25 can be determined accurately by RP-HPLC. If this range is adopted as the efficient range of k' values, log P_{oct} values of

TABLE II

LINEAR REGRESSION DATA FOR PLOT OF LOG $k'_{10\%}$ *VERSUS* LOG P_{oct}

Log $P_{oct.} = a \log k'_{10\%} + b$. Figures in parentheses are the 95% confidence intervals of the corresponding constants.

*No.**	*Chemical class*	*a*	*b*	*n*	*r*	*s*	*Eqn. No.*
I	Non-H-bonders	1.53 (0.26)	2.83 (0.10)	11	0.984	0.110	17
II	H-acceptors	2.35 (0.25)	2.65 (0.09)	9	0.993	0.117	18
III	Phenols	2.08 (0.13)	2.50 (0.06)	26	0.988	0.144	19
IV	Benzoic acids	2.01 (0.19)	2.47 (0.05)	16	0.987	0.092	20
V	N-Phenylanthranilates	2.16 (0.36)	2.87 (0.33)	9	0.983	0.134	21
VI	N-Phenylsuccinimides	2.42 (0.33)	2.61 (0.13)	11	0.984	0.110	22
VII	I–VI	2.26 (0.09)	2.58 (0.04)	82	0.984	0.191	23
VIII	II–VI	2.31 (0.08)	2.56 (0.04)	71	0.990	0.162	24

* See Table I.

up to about 6.5 can be determined from log $k'_{10\%}$ values by gly-CPG column chromatography. For the determination of the P_{HPLC} values of more hydrophobic compounds, the chromatographic conditions must be adjusted so as to reduce k' to within the above range. There are three methods of doing this: (i) to use a higher

TABLE III

COMPARISON OF CHROMATOGRAPHICALLY DETERMINED LOG P_{HPLC} VALUES AND CALCULATED LOG P_{cal} VALUES

Compound	*Log* $k'_{10\%}$*	*Log* P_{HPLC}**	*Log* P_{cal}***	Δ §
Phenols:				
4-C_2H_5–	−0.16	2.12	2.48	0.36
4-C_6H_5–	0.65	3.76	3.46	−0.30
Benzoic acids:				
4-C_2H_5–	0.13	2.73	2.77	0.04
4-i-C_3H_7–	0.27	2.89	3.07	0.18
4-C_2H_5O–	−0.02	2.43	2.25	−0.18
4-C_6H_5–	0.85	4.18	4.23	0.05
H-acceptors:				
$C_6H_5CO_2C_3H_7$	0.10	2.88	3.14	0.26
$C_6H_5CO_2C_4H_9$	0.33	3.42	3.64	0.22

* Capacity factor determined with 10% methanol solution as mobile phase.

** Partition coefficient in RP-HPLC determined from eqns. 18–20 according to chemical class.

*** Partition coefficient determined from the hydrophobic substituent coefficient (π).

§ $\Delta = \log P_{cal} - \log P_{HPLC}$.

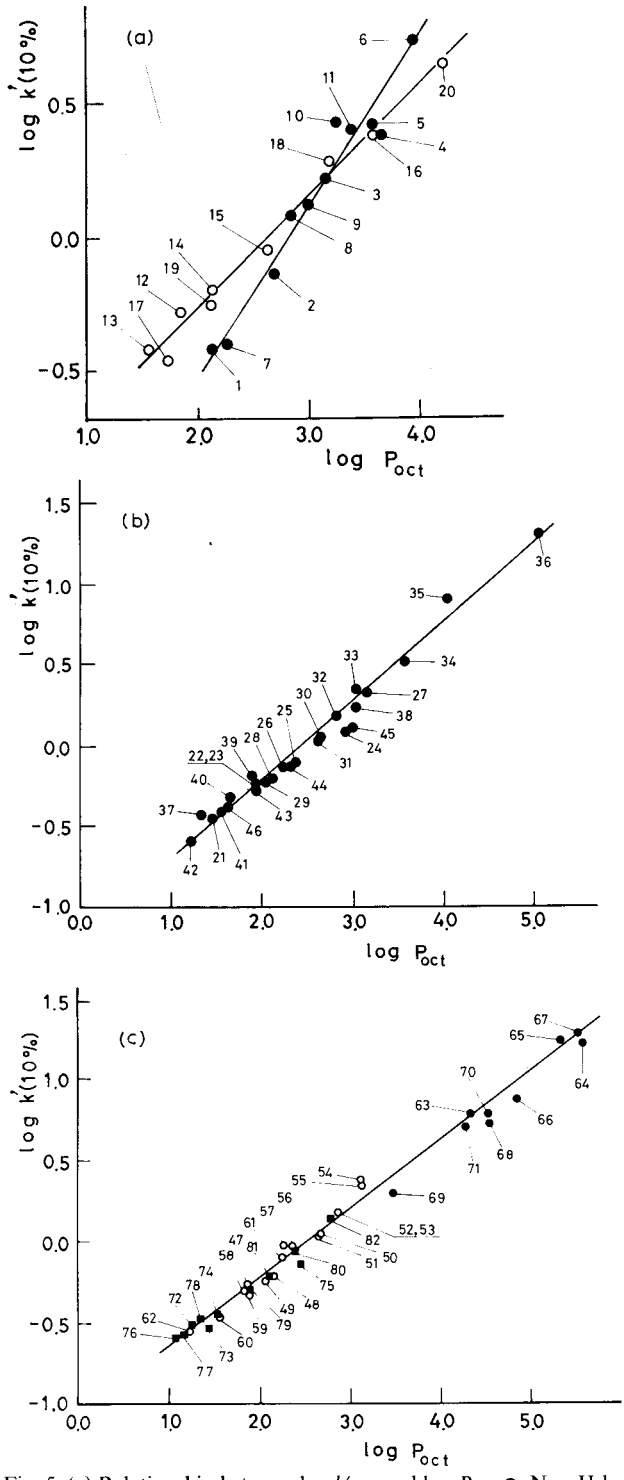

Fig. 5. (a) Relationship between log $k'_{10\%}$ and log P_{oct}. ●, Non-H-bonders; ○, H-acceptors. (b) Relationship between log $k'_{10\%}$ of phenols and log P_{oct}. (c) Relationship between log $k'_{10\%}$ and log P_{oct}. ○, Benzoic acids; ■, N-phenylsuccinimides; ●, N-phenylanthranilates. Numbers correspond to those for the compounds listed in Table I.

flow-rate of the mobile phase, (ii) to use a shorter column and (iii) to use a higher methanol concentration in the mobile phase. Changing the flow-rate or column length to optimize the chromatographic conditions is sometimes difficult, because the t_0 value become too small to determine exactly; in practice, the lower limit of t_0 is about 1 min. In the third method, it is preferable to perform the chromatography with as low a concentration of methanol as possible, as higher k' value gives a more accurate estimate of P_{oct}, as shown in Fig. 4.

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CHROM. 14,643

MEASUREMENT OF RELATIVE HYDROPHOBICITY OF AMINO ACID SIDE-CHAINS BY PARTITION IN AN AQUEOUS TWO-PHASE POLYMERIC SYSTEM: HYDROPHOBICITY SCALE FOR NON-POLAR AND IONOGENIC SIDE-CHAINS

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SUMMARY

The effect of ionic strength on the partition of twenty dinitrophenylated amino acids in buffered ficoll–dextran phase system was examined. The relative hydrophobicities of the amino acid side-chains were estimated. The results obtained for apolar side-chains are in agreement with those reported in the literature. It is shown that the relative hydrophobicity of any molecular fragment should be expressed in terms of equivalent number of CH_2 groups and not in terms of the free energy of transfer of a given moiety from one phase to the other of the phase system used in a particular study. A new hydrophobicity scale for apolar and ionogenic side-chains is established and it is shown that this scale depends on the ionic strength and/or ionic composition of the medium.

INTRODUCTION

Proteins are amphiphilic molecules that contain amino acids with hydrophobic side-chains and those with ionic and uncharged polar side-chains. The ratio of hydrophilic to hydrophobic amino acid residues is believed to be an important factor for the tertiary protein structure and to serve as an index of the protein localization and function *in vivo*[1,2]. Several attempts at deriving scales for amino acid hydrophobicity have been reported[3–6]. Nozaki and Tanford[3] measured the solubilities of different amino acids in water and in progressively increasing concentrations of organic solvents, such as ethanol and dioxan in water. The solubilities of the amino acids were extrapolated to pure organic solvents and the free energy of transfer for the amino acid from pure organic solvent to water was calculated. Using glycine as a reference, and subtracting its free energy of transfer from that of all the other amino acids, it was possible to formulate a hydrophobicity scale for amino acid residues with apolar side-chains[3]. Similar data were obtained by Fendler *et al.*[4] using hexane as the organic solvent. Bull and Breese[5] studied the effect of amino acids on the surface tension of water and constructed a hydrophobicity scale comparable to that of Nozaki and

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Tanford[3]. Nandi[6] reported the partition coefficients of N-acetyl ethyl esters of a number of amino acids between water and different organic solvents.

The principal shortcoming of all of these approaches[3–6] is that they cannot be applied to molecular moieties containing polar ionogenic groups. The only promising means for the study of the relative hydrophobicities of polar and ionogenic compounds at present appears to be the technique based on the partition of solutes in an aqueous two-phase ficoll–dextran system[7]. This approach has been used earlier to measure the relative hydrophobicities of proteins[8] and cells[9].

The partition in aqueous two-phase polymeric systems technique was used in this study in order to measure the relative hydrophobicities of various polar and apolar amino acid side-chains. The results are compared with those reported in the literature[3–6] and it is shown that the hydrophobic charachter of a given molecular moiety should be expressed in terms of equivalent number of CH_2 groups. A new combined hydrophobicity scale for hydrophilic and hydrophobic amino acid side-chains is formulated.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade unless indicated otherwise.

Ficoll-400 (lot 11069) was obtained from Pharmacia (Sweden). Dextran (M_w *ca.* 70,000) was obtained from Minmedprom (U.S.S.R.) under the trade-name Polyglucinum (lot 580870).

Dinitrophenylated amino acids (DNP-L-Ala, DNP-L-Arg, DNP-L-Asp, DNP-DL-Glu, DNP-Gly, DNP-L-Ile, DNP-DL-Leu, DNP-L-Phe, DNP-L-Pro, DNP-L-Ser, DNP-L-Thr, DNP-L-Trp and DNP-L-Val) were obtained from Serva (G.F.R.) and DNP-L-Asn, DNP-L-Gln, DNP-DL-Met and mono-O-DNP-L-Tyr from Reanal (Hungary). α-DNP-L-Lys was kindly provided by Dr. S. M. Andreev.

2,4-Dinitrofluorobenzene was obtained from Calbiochem-Behring Corp. (U.S.A.). L-Norleucine was obtained from Reanal, DL-norvaline from Chemapol (Czechoslovakia) and DL-2-amino-*n*-octoic acid from BDH (Great Britain). The amino acids were dinitrophenylated as described in ref. 10. All DNP derivatives of amino acids were checked for purity by thin-layer chromatography and their sodium salts were prepared by titration. The following abbreviations are used: norvaline = NVal; norleucine = NLeu; 2-amino-*n*-octoic acid = NAO.

Methods

Buffered ficoll–dextran aqueous two-phase systems were prepared as described previously[7–9]. All of the phase systems used had the same polymer composition [12.5% (w/w) ficoll and 10.8% (w/w) dextran], but differed in salt composition as indicated in the caption to Fig. 1.

The partition experiments were carried out as described elsewhere[7–9]. The phases were allowed to settle at room temperature for 23–24 h, then aliquots of both phases (0.1–0.15 ml) were carefully pipetted from the phase system and each was diluted by addition of an appropriate volume of water. The absorbance of each diluted aliquot was measured at 360 nm against a correspondingly diluted top or bottom phase blank.

The partition coefficient, K, is defined as the ratio of sample concentration (or absorbance) in ficoll-rich (bottom) phase to sample concentration in the dextran-rich (top) phase.

The partition coefficient for each solute was determined at four or five different ionic strengths as the mean of two measurements on two or more dilutions from each partition carried out two to four times at a given ionic strength. The deviation from the average K value did not exceed 3% for any of the substances studied.

RESULTS

Fig. 1 shows the relationships between the logarithm of the partition coefficient and the ionic strength of the system for some of the compounds studied. It has been shown earlier[7,8] that these relationships can be described by the equation

$$\ln K = A + BI$$

where I is the ionic strength and A and B are constants.

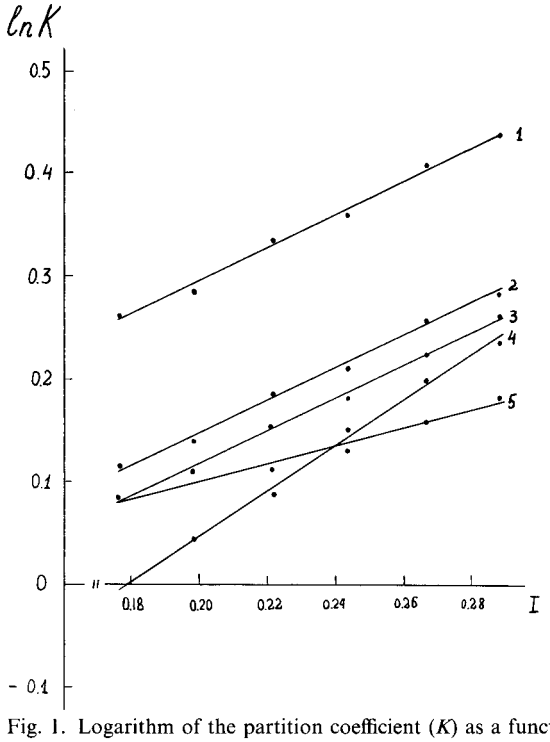

Fig. 1. Logarithm of the partition coefficient (K) as a function of ionic strength (I) in the ficoll–dextran phase system. Polymer composition of the system: 12.5% (w/w) ficoll, 10.8% (w/w) dextran. Amounts of sodium chloride and sodium phosphate buffer (pH 7.4) in the systems can be calculated from the equations $C_{NaCl} = (0.288 - I)/0.75$ and $C_{buffer} = 0.11 - 0.67 \cdot C_{NaCl}$ where C_{NaCl} and C_{buffer} are the sodium chloride and sodium phosphate buffer concentrations, respectively, and I is the ionic strength in the phase system. 1 = DNP-2-amino-*n*-octoic acid; 2 = DNP-glutamine; 3 = DNP-serine; 4 = DNP-glutamic acid; 5 = α-DNP-lysine.

The physical meaning of A and B was discussed earlier[7–9] and it has shown that B reflects the effect of the ionic strength and/or ionic composition on the transfer of a given compound ionogenic group between the system phases[11]. A represents the relative hydrophobicity of the substance under study at zero ionic strength in the medium[7–9,11].

A least-squares treatment of the experimental data according to the above equation led to the A and B values for the solutes listed in Table I. It should be noted that the B value for all of the DNP-amino acids with apolar side-chains was found to be the same within experimental error and it was averaged as indicated in Table I.

TABLE I

CHARACTERISTICS OF THE PARTITION BEHAVIOUR OF DINITROPHENYLATED AMINO ACIDS IN FICOLL–DEXTRAN PHASE SYSTEM

Logarithm of the partition coefficient (K) depends on the ionic strength of the system (I) according to the equation $\ln K = A + BI$ (for details see text). the correlation coefficient exceeded 0.993 for all of the substances examined.

α-DNP-derivative	*A**	*B (kg/mole)***
Gly	−0.190	1.624 ± 0.010
Ala	−0.152	1.624 ± 0.010
Val	−0.125	1.624 ± 0.010
Leu	−0.102	1.624 ± 0.010
Ile	−0.086	1.624 ± 0.010
Phe	0.001	1.624 ± 0.010
Thr	−0.170	1.624 ± 0.010
Ser	−0.203	1.624 ± 0.010
Pro	−0.181	1.624 ± 0.010
Met	−0.107	1.624 ± 0.010
Gln	−0.181	1.624 ± 0.010
Asn	−0.203	1.624 ± 0.010
NVal	−0.107	1.624 ± 0.010
NLeu	−0.087	1.624 ± 0.010
NAO	−0.019	1.624 ± 0.010
Trp	0.275	1.624 ± 0.010
Glu	−0.411	2.277 ± 0.116
Asp	−0.355	1.866 ± 0.061
Arg	−0.089	1.267 ± 0.097
Lys	−0.062	0.829 ± 0.040

* Mean values; standard deviation 0.027 in all instances.
** Mean values ± standard deviation.

In order to calculate the difference in the relative hydrophobicities between the two phases of the system used, the effect of a CH_2 group on the ln K value was established by comparison of the A values for the DNP derivatives of Gly, Ala, NVal, NLeu and NAO. The A values are plotted in Fig. 2 as a function of the number of carbon atoms in the aliphatic side-chain. The slope of the observed linear relationship is a measure of the effect of a CH_2 group on the ln K value. It can be seen from Fig. 2 that the effect of adding an aliphatic CH_2 group to the side-chain increases the ln K value by 0.027 logarithmic units.

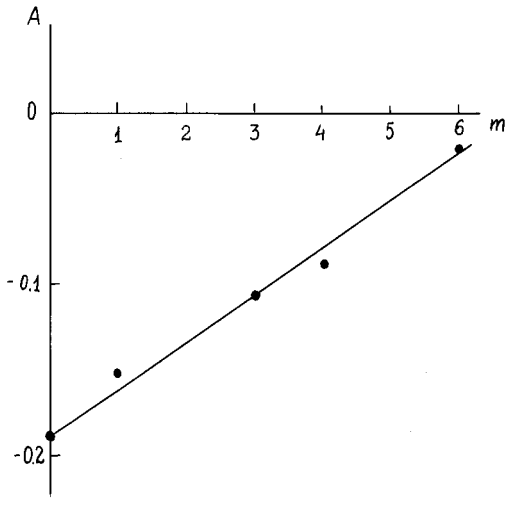

Fig. 2. A as a function of the aliphatic side-chain length (m) of the dinitrophenylated amino acids: glycine, alanine, norvaline, norleucine and 2-amino-n-octoic acid.

DISCUSSION

The unitary free energy of transfer, ΔG_{tr}, of a solute from one to the other phase of a given phase system is related to the partition coefficient, K, by the equation $\Delta G_{tr} = RT \ln K$. Contributions of amino acid side-chains to the unitary free energies of transfer (Δg_{tr}) can be calculated by assuming additivity of the free energy of solvent interactions and using glycine as a reference, as described by Nozaki and Tanford[3].

The range of values of the contributions for the apolar amino acid side-chains as reported in the literature 3–6 is approximately 1000–2500 cal/mole. It can be seen from the data in Table I, however, that the free energy of transfer of the Ile side-chain does not exceed 70 cal/mole, compared with 2300–2900 cal/mole as found by Nozaki and Tanford and other workers[3–6]. The only reasonable explanation of the observed disagreement seems to be related to the fact that the free energy of transfer of a CH_2 group depends on the phase system used in a given study[12]. In order to take account of the probable effect of this dependence we have attempted to estimate the relative hydrophobicity of the amino acid side-chains not in terms of Δg_{tr} but in terms of the equivalent number of CH_2 groups, n, as proposed earlier[7–9,11]. The number n is obviously related to the Δg_{tr} value according to equation

$$n_i = \Delta g_{tr_i} / \Delta g_{tr_{CH_2}}$$

where $\Delta g_{tr_{CH_2}}$ is the free energy of transfer of a CH_2 group in a given phase system and i denotes the amino acid in question. A positive value of n means that the relative hydrophobicity of a given moiety is equal to that produced by n CH_2 groups, and a negative value of n means that the moiety is hydrophilic and its hydrophobicity is the reverse of that produced by n CH_2 groups. The n values calculated from the data in Table I are presented in Table II together with those calculated from the literature data[3–6]. It can be seen that the data are in good agreement. The only exception

TABLE II

RELATIVE HYDROPHOBICITIES OF AMINO ACID SIDE-CHAINS EXPRESSED IN TERMS OF EQUIVALENT NUMBER OF CH_2 GROUPS AT ZERO IONIC STRENGTH IN THE MEDIUM

Side-chain of	*Relative hydrophobicity* ($n = \Delta g_{tr_i}/\Delta g_{tr_{CH2}}$)									
	*Ficoll→ dextran**	*Ethanol, dioxane→ water***	*Hexane→ water****	*Water surface→ bulk water*§	*Isoamyl alcohol→ water*§§	*Hexanol→ water*§§	*Octanol→ water*§§	*$CHCl_3$→ water*§§	*CCl_4→ water*§§	*Dibutyl ether→ water*§§
Trp	17.22§§§	5.77								
Phe	7.06§§§	4.24	3.60	3.64						
NAO	6.33									
Lys	4.74									
NLeu	3.82	4.41			4.01	3.98	3.96		4.04	3.89
Leu	3.30	3.06	3.90	3.84	3.61	3.69	4.20		3.92	3.91
Ile	3.85		3.83	3.53						
Arg	3.74									
Met	3.07	2.21								
NVal	3.07				2.75	2.87	2.89	3.01	2.99	3.23
Val	2.41	2.55	2.43	2.44	2.49	2.66	3.04	3.09	3.00	3.30
Ala	1.41	0.85	−0.61	0.31	0.77	0.81	0.81	1.02	1.07	1.02
Thr	0.74	0.68	−0.06							
Pro	0.33		0.36	1.53						
Gln	0.33									
Gly	0	0	0	0	0	0	0	0	0	0
Ser	−0.48	−0.51	−1.11	−0.61						
Asn	−0.48									
Asp	−6.11									
Glu	−8.19									

* This work, $\Delta g_{tr_{CH_2}} = 16 \pm 1$ cal/mole.

** The data published in ref. 3 were recalculated as indicated in the text using $\Delta g_{tr_{CH2}} = 589 \pm 83$ cal/mole, as found from the results reported in ref. 3 for Gly, Ala, NLeu, Val, Leu and Thr.

*** The data were taken from ref. 4 and recalculated as above using $\Delta g_{tr_{CH_2}} = 692 \pm 60$ cal/mole, as found from the data in ref. 4 for Gly, Val, Leu and Phe.

§ The data were taken from ref. 5 and recalculated as above using $\Delta g_{tr_{CH_2}} = 640 \pm 50$ cal/mole, as found from the results reported in ref. 5 for Gly, Val, Leu and Phe.

§§ The data were taken from ref. 6 and recalculated as above using the following $\Delta g_{tr_{CH2}}$ obtained from the results given in ref. 6 for the derivatives of Gly, Ala, NVal and NLeu: water–isoamyl alcohol, -573 ± 30; water–hexanol, -587 ± 24; water–octanol, -602 ± 20; water–$CHCl_3$, -731 ± 17; water–CCl_4, -784 ± 8; water–dibutyl ether, -631 ± 34 cal/mole.

§§§ The *n* values obtained in this work for the Trp and Phe side-chains seem to be incorrect (for explanation, see text).

appears to be the *n*-values obtained by us for the Trp and Phe derivatives. The observed overestimated relative hydrophobicity of these amino acid side-chains can be attributed to the probable effect of the dinitrophenyl moiety on interactions of these side-chains with water.

Our data make it possible to evaluate the relative hydrophobicity of the ε-amino group in lysine by comparison of the *n* value for DNP-NLeu with that for α-DNP-Lys. It appears that the relative hydrophobicity of the ε-amino group at zero ionic strength in the medium corresponds to that produced by 0.93 CH_2 groups. The hydrophobic character of the side-chain amide group under the same conditions corresponds to −1.5 CH_2 groups and that of the side-chain carboxyl group to −6.6 CH_2 groups. The relative hydrophobicity of an aliphatic OH group can be estimated by comparison of the *n* values for DNP-Ser and DNP-Thr with that for DNP-Ala, and it appears to correspond to *ca.* −1.44 CH_2 groups, compared with −1.19 CH_2 groups established by Nozaki and Tanford[3]. Unlike their results[3], however, our data indicate that the methionyl sulphur atom has no effect on the hydrophobic character of the side-chain, within experimental error.

The data on the relative hydrophobicities of the amino acid side-chains listed in Table II represent a hydrophobicity scale for the side-chains in the absence of salts in the medium. The data given in Table I, however, indicate that in contrast to the apolar side-chains, the hydrophobic character of which similarly depends on the ionic strength and/or ionic composition of the medium (the *B* values are equal), the relative hydrophobicities of the ionogenic side-chains depend on the ionic strength differently. In particular, the ln *K* value for α-DNP-Lys does not depend on the ionic strength as greatly as that for DNP-Arg or DNP-Gly. It can be seen from the *B* values in Table I that the relative hydrophobicity of the side-chain carboxyl group depends on the ionic strength differently to that of the α-carboxyl group. The effect of the ionic strength and/or ionic composition of the medium seems to be related to the position of a carboxyl group in the side-chain. Therefore, the hydrophobicity scale for amino acid side-chains depends on the ionic composition and ionic strength of the medium. In particular, in the presence of 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer (pH 7.4), the relative hydrophobicities of the side-chains of Lys, Arg, Asp and Glu correspond to −0.45, 1.41, −4.53 and −3.92 CH_2 groups, respectively.

It seems that the effect of the ionic strength and/or ionic composition of the medium on the relative hydrophobicity of amino acid side-chains may be of fundamental importance as a factor affecting protein conformation and function *in vivo*.

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CHROM. 14,638

GEL CHROMATOGRAPHIC BEHAVIOUR OF ELUENT IONS ON SEPHADEX G-10 AND EVIDENCE FOR ELECTRONEUTRALITY IN THE GEL PHASE

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SUMMARY

Methods for determining the distribution coefficient, K_d, of sodium, hydrogen, chloride and hydroxide ions in the eluent were examined by using refractometric and potentiometric detection and a radiotracer method on Sephadex G-10 with a 0.1 *M* sodium chloride eluent at several pH values. The K_d values of sodium and chloride ions obtained by using their radiotracers were considerably different from those by both refractometry and potentiometry. It was demonstrated by the use of the K_d values of sodium and chloride ions (radiotracer method) and that of hydrogen ion (refractometry) that electroneutrality for the eluent ions holds in the gel phase over the range pH 1.5–3.

INTRODUCTION

The mechanism of the separation of inorganic ions on tightly cross-linked gels is more complicated than that of organic compounds and has not yet been clarified, although several approaches have been devoted to the interpretation of the behaviour of small inorganic ions in aqueous gel systems[1]. Among them, it has been reported that the volumetric distribution coefficients of sample ions may be correlated chiefly with the properties of the counter ions contained in the eluent[2–6], which is usually regarded as acting as a masking agent for the electrostatic interaction between the sample ions and the charge arising from the ionic dissociation of groups fixed to the gel matrix[7]. Hence, it is very important for studies on the mechanism of the separation of inorganic compounds to obtain detailed information about the behaviour of the eluent ions.

We have previously reported that the K_d values of alkali metal and halide ions[8] and tetraalkylammonium ions[9] vary significantly depending on the pH of the eluent on Sephadex G-10, although these ions do not transform into any other form or species over the range pH 1.5–12 investigated. Moreover, the K_d values of sodium and chloride ions used as an eluting agent are considerably different from each other over this wide pH range, as shown in Fig. 1[8,10]. The pH dependence of the K_d values of

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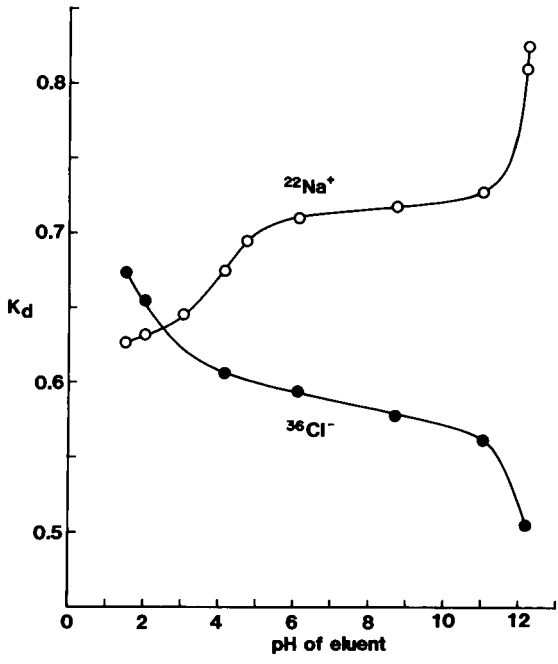

Fig. 1. pH dependence of K_d values of eluent ions obtained by the radiotracer method[8,10]. Gel: Sephadex G-10. Eluent: 0.1 *M* NaCl at various pH values adjusted with HCl or NaOH. Temperature: 20°C.

these ions was interpreted speculatively in terms of the variation in the charge of the gel phase owing to the dissociation of carboxyl and hydroxyl groups fixed to the gel matrix.

Volumetric parameters, such as K_{av} and K_d, of eluting agents have usually been obtained by monitoring the concentration distribution of eluent salts and ions by means of an appropriate chemical analysis[11,12], an atomic absorption flow detector[13], an ion-selective electrode detector[14] or a differential refractometer[5]. In general, it is believed that the K_{av} or K_d values of eluent cations are identical with those of eluent anions.

Therefore, this work was undertaken in order to establish a method for determining the true K_d values of the eluent ions on Sephadex G-10 with a 0.1 *M* sodium chloride eluent at several pH values and for evaluating the charge balance of the eluent ions in the gel phase. It was found that a radiotracer method, rather than refractometry and potentiometry, gives valid K_d values for the eluent ions, and that electroneutrality holds in the gel phase over the range pH 1.5–3, provided that the contribution of hydrogen ion added to adjust the pH of the eluent is taken into account.

EXPERIMENTAL

Sample solutions

All reagents used were of guaranteed reagent grade, unless otherwise stated. Sodium chloride solutions of concentrations 0.1, 0.2 and 0.3 *M* at various pH values

were used as sample solutions (see Figs. 2, 3 and 4). The sample solution contained ^{22}Na or ^{36}Cl (New England Nuclear, Boston, MA, U.S.A.) as a radiotracer to obtain the K_d values of eluent ions in each instance. Dextran T-2000 (Pharmacia, Uppsala, Sweden; 0.04%) and tritiated water (New England Nuclear) were employed as standard materials with $K_d = 0$ and 1, respectively.

Eluents

Eluents used were 0.1 *M* sodium chloride solutions at pH 1.51, 1.83, 2.37, 2.84, 12.05 and 12.50.

Column and elution procedure

Sephadex G-10 (Pharmacia; dry particle size, 40–120 μm) was packed into a column (Pharmacia, K16/100) with flow adaptors at both the top and bottom, as described previously[15]. The dimensions of the gel bed were 73 × 1.6 cm. The eluent was passed through the column before use until the pH of the effluent was identical with that of the eluent.

A 1-cm^3 volume of the sample solution was introduced on to the column with a line sample injector of the loop-valve type (Seishin Pharmaceutical, Tokyo, Japan; VMU-6). The elution was allowed to proceed at a constant flow-rate of 60 cm^3/h under a hydraulic pressure and at a constant temperature of 20 ± 0.5°C. The effluent from the column was monitored continuously with a differential refractometer (Showa Denko, Tokyo, Japan; Shodex RI, SE-11) and then collected in *ca.* 1-cm^3 fractions with an Ultrorac 7000 fraction collector (LKB, Broma, Sweden). Some fractions were chosen arbitrarily and their volumes were measured so that the fraction volume could be determined accurately. The dead volume from the cell of the refractometer to the fraction collector (0.59 cm^3) was taken into account for correction of the elution volumes determined by the potentiometric and radioactivity measurements.

The radioactivities of tritiated water, $^{22}Na^+$ and $^{36}Cl^-$ of the effluent collected in each fraction were measured as previously[16]. The concentrations of hydrogen, sodium and chloride ions of the effluent were determined by means of a digital potentiometer (Orion Research, MA, U.S.A.; 701A) equipped with a combined electrode of 91-02, 96-11 or 96-17 type.

The procedure for calculating K_d values was as reported previously[16]. Marsden[17,18] reported that the K_d value of tritiated water is 1.091 on Sephadex G-10 owing to an exchange reaction of the hydrogen atoms between the tritiated water and the hydroxyl and/or carboxyl groups of the gel matrix. This isotopic exchange should be taken into account for the evaluation of positive and negative charges in the gel phase. However, the correction of the K_d values was neglected in this study, because the pH dependence of the K_d value of tritiated water was not examined. Even if the factor of 1.091 was introduced, the charge balance in the gel phase would hold over the range pH 1.5–3 within the relative error of 2%.

RESULTS AND DISCUSSION

Identification of elution peaks

Figs. 2a and d show typical chromatograms obtained by means of a differential

refractometer with a 0.1 *M* sodium chloride eluent at pH 1.51 on a Sephadex G-10 column. Peaks 1 and 2 appeared in a positive direction when the concentrations of sodium chloride and hydrogen chloride in the sample solution were larger than those of the eluent, respectively, and *vice versa*. The K_d value of peak 1 and 2 (the values shown in parentheses), however, was almost invariable in each instance. These facts

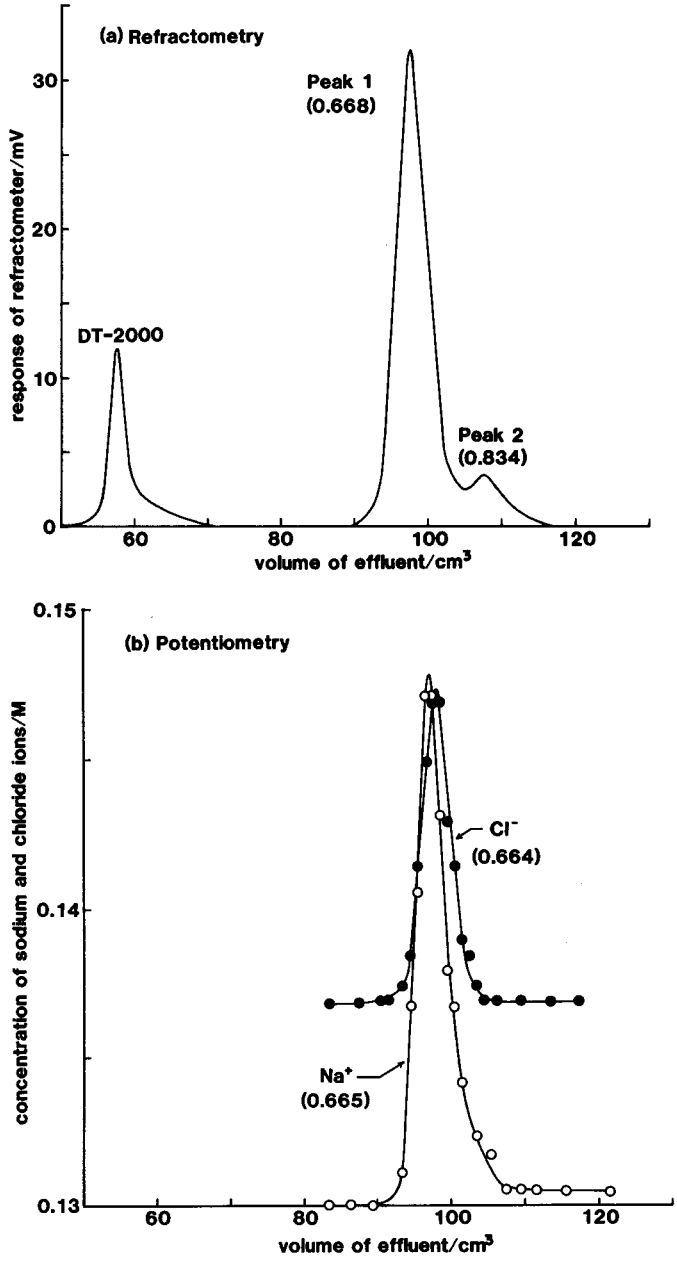

Fig. 2.

suggest that peak 1 corresponds to the variation in the concentration of sodium chloride owing to the introduction of the sample solution, and that peak 2 corresponds to the variation in the concentration of hydrogen chloride.

This was confirmed by the potentiometric detection method using sodium, chloride and hydrogen ion-selective electrodes. Namely, the K_d values of sodium and

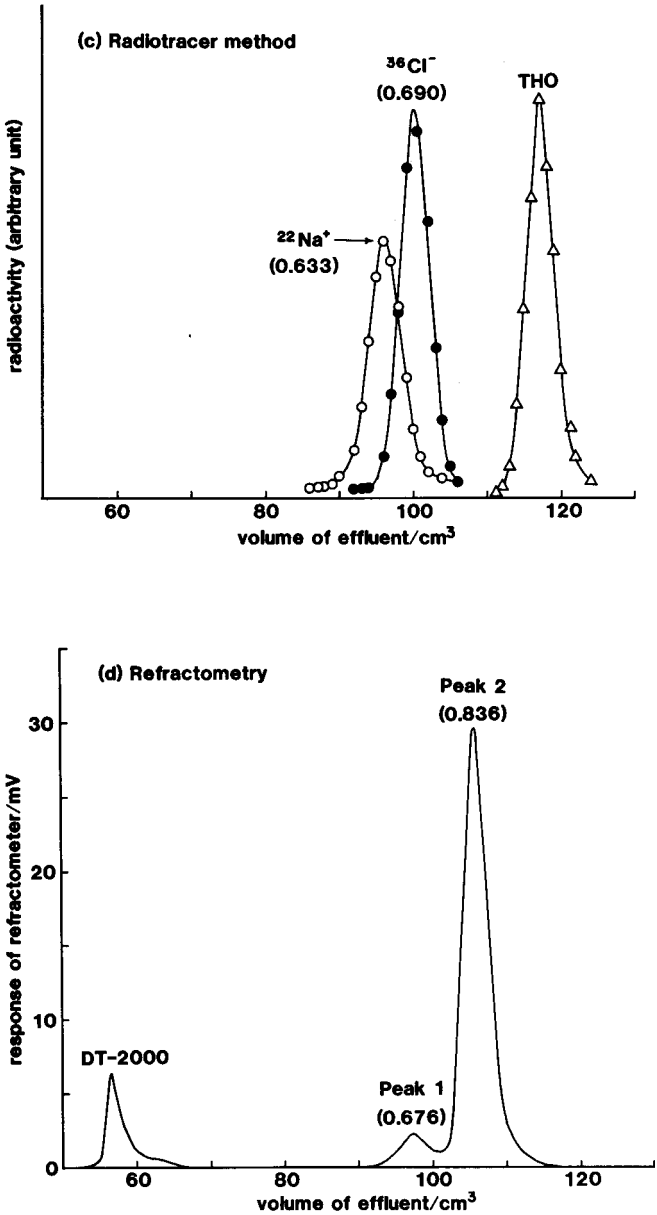

Fig. 2.

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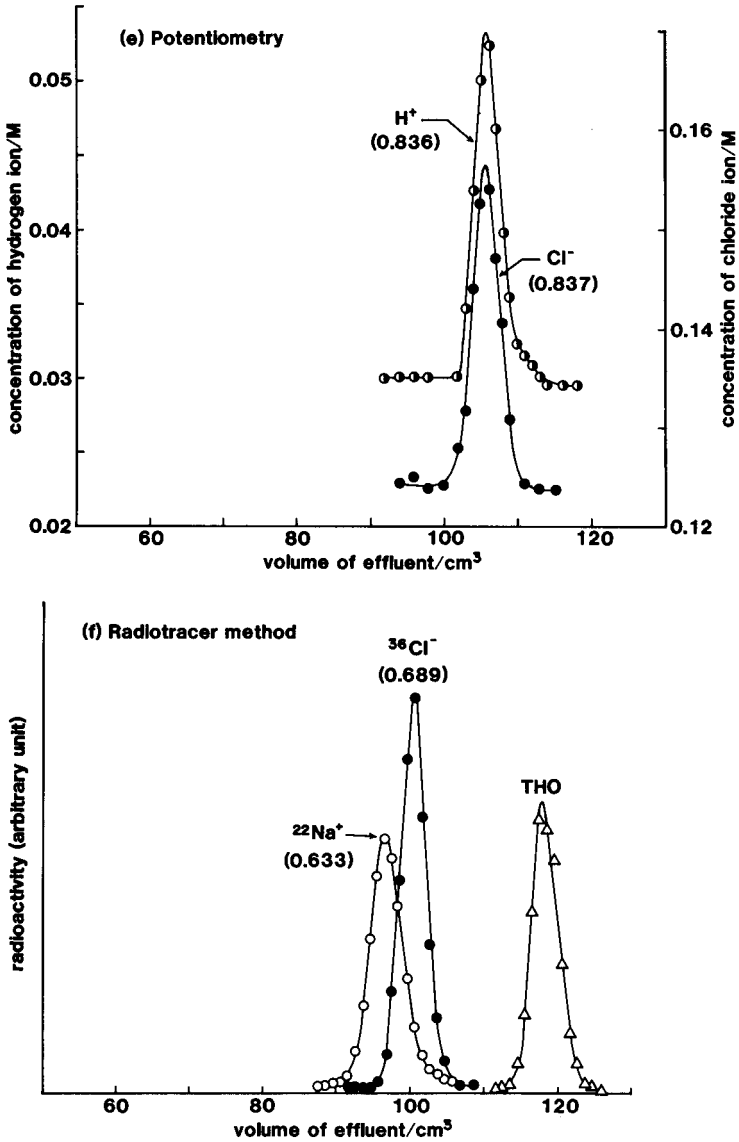

Fig. 2. Chromatograms obtained by refractometry, potentiometry and radiotracer methods with an acidic eluent. Gel: Sephadex G-10, 73 × 1.6 cm. Eluent: 0.1 *M* NaCl at pH 1.51. Sample: (a), (b), (c), 0.2 *M* NaCl at pH 1.51; (d), (e), (f), 0.1 *M* NaCl at pH 0.90. Temperature: 20°C. Attenuation of refractometer: 8. THO = Tritiated water.

chloride ions in Fig. 2b are identical with each other and with those of peak 1 in Fig. 2a and 2d within experimental error. The K_d values of hydrogen and chloride ions in Fig. 2e are also identical with those of peak 2 in Fig. 2a and 2d. The higher baseline for chloride ion than for sodium ion in Fig. 2b is attributable to the higher concentration of chloride ion in the eluent owing to the pH adjustment with hydrogen chloride. The small peak 1 or 2 in Fig. 2d or 2a always appeared in spite of careful adjustment

of the sodium chloride concentration and of the pH in the sample solution. Yóza *et al.*[19] and Deguchi and co-workers[11,12,14] have already reported similar phenomena, *i.e.*, when the sample solution prepared by dissolving electrolytes in the eluent was chromatographed on Sephadex G-15, the peak of the eluting agent appeared at the elution volume corresponding to its K_d value because of the exclusion of the eluent ion from the sample zones.

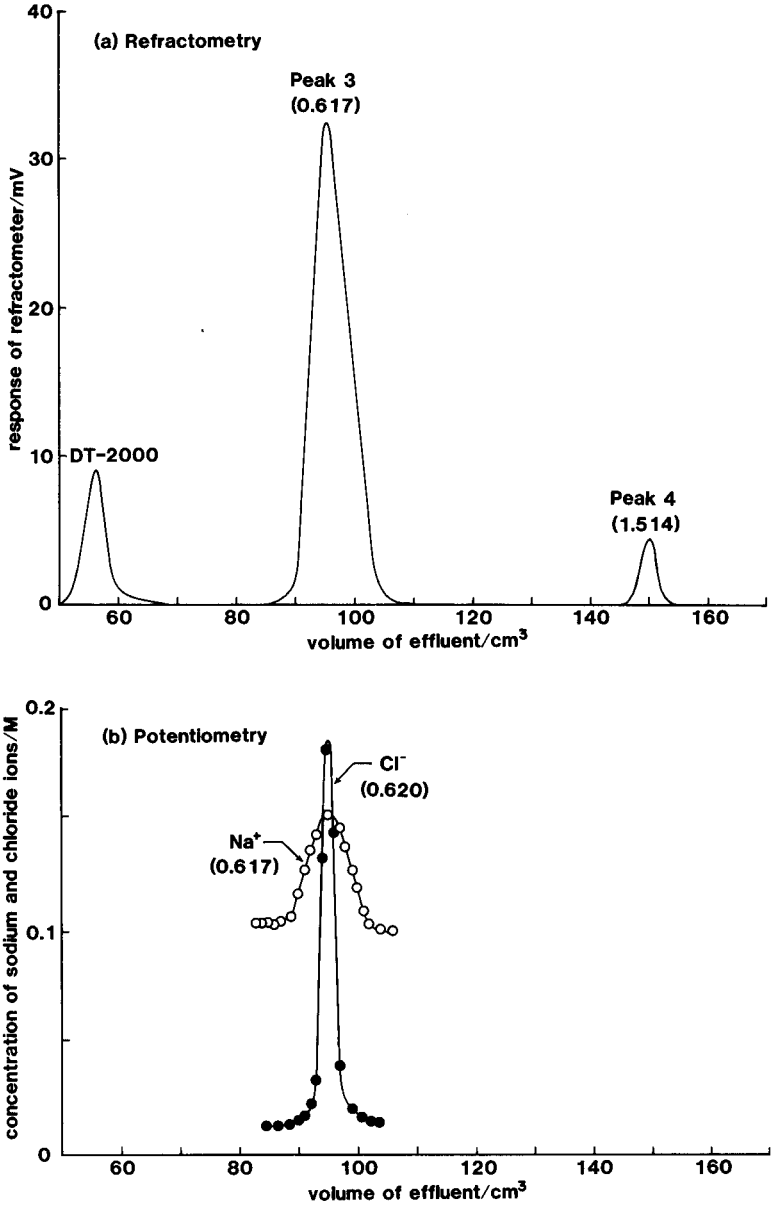

Fig. 3.

(Continued on p. 36)

In contrast to the cases with refractometric and potentiometric detection, the K_d values of $^{22}Na^+$ and $^{36}Cl^-$ in the sample solution were obviously different from each other beyond experimental error, as mentioned in the Introduction.

Fig. 3 shows chromatograms with a 0.1 *M* sodium chloride eluent at pH 12.50. Peaks 3 and 4 were identified as elution peaks of sodium chloride and sodium hydroxide, respectively, by using ion-selective electrodes, as in the case with the acidic eluent.

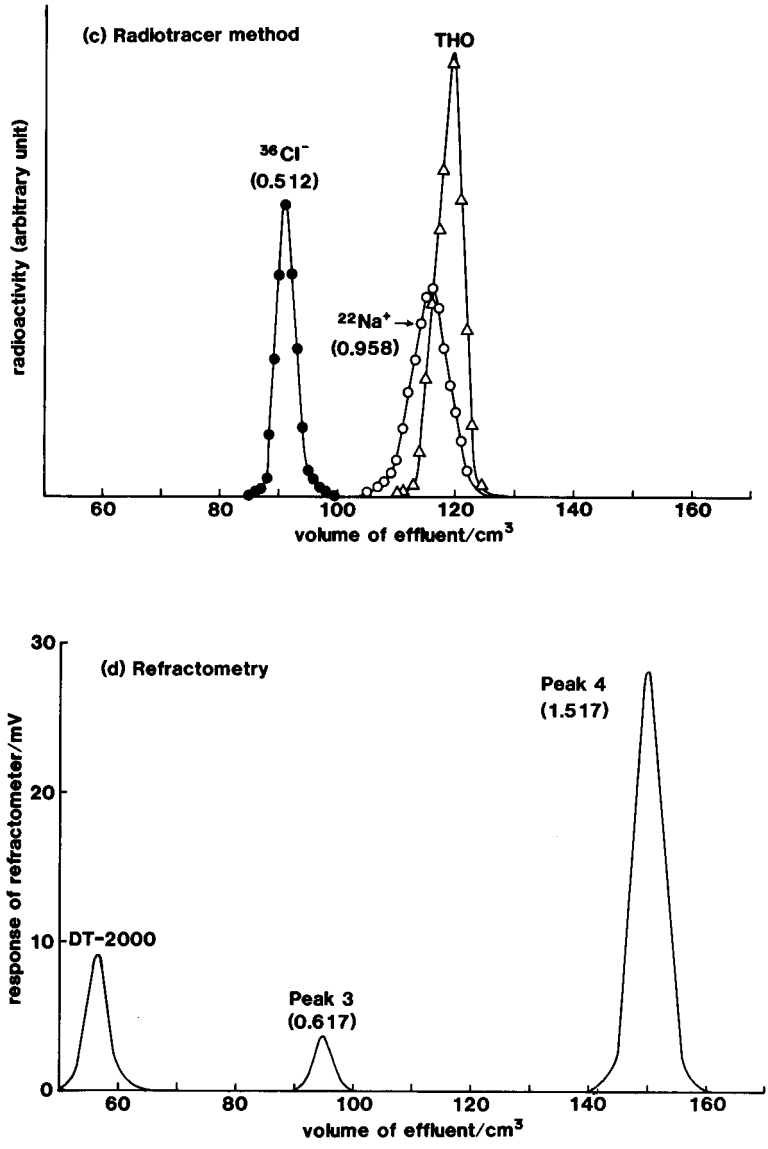

Fig. 3.

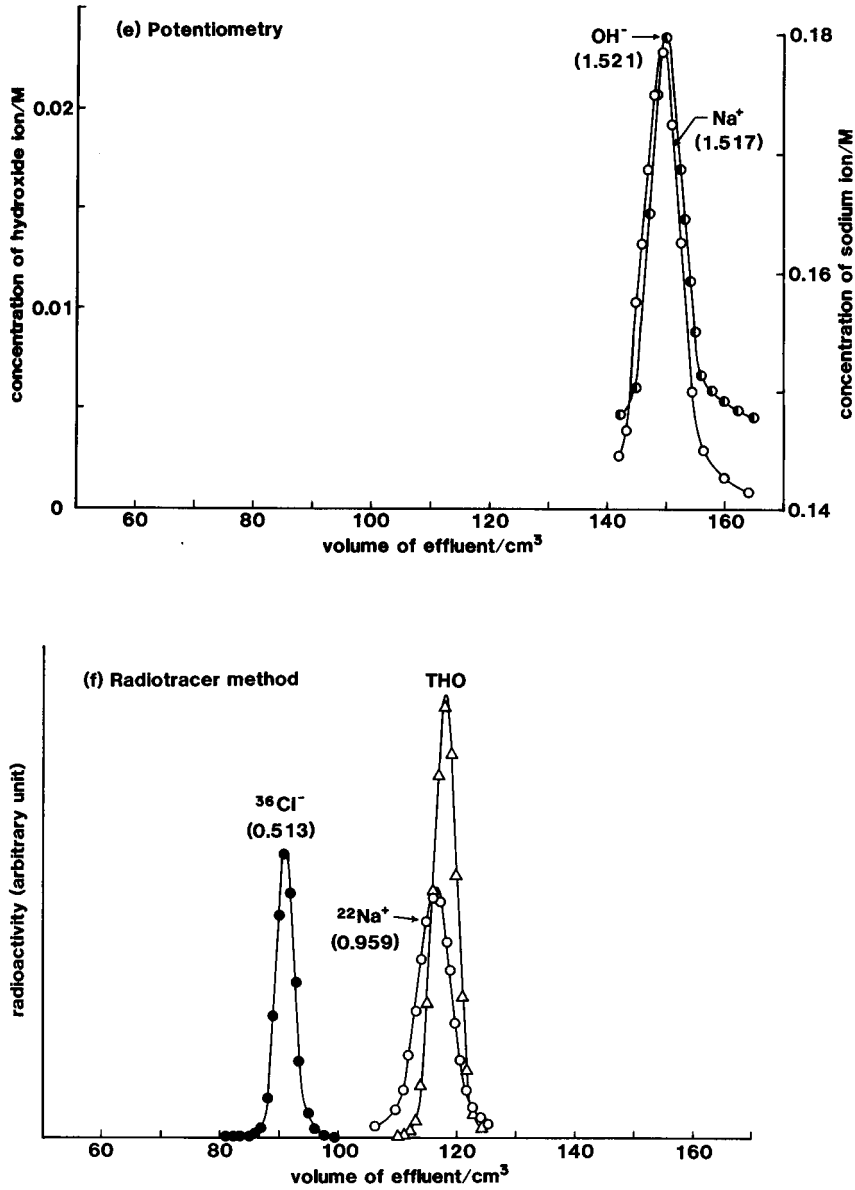

Fig. 3. Chromatograms obtained by refractometry, potentiometry and radiotracer methods with a basic eluent. Gel: Sephadex G-10, 73 × 1.6 cm. Eluent: 0.1 *M* NaCl at pH 12.50. Sample: (a), (b), (c), 0.3 *M* NaCl at pH 12.50; (d), (e), (f), 0.1 *M* NaCl at pH 13.35. Temperature: 20°C. Attenuation of refractometer: 8. THO = Tritiated water.

With the basic eluent, the K_d value of $^{22}Na^+$ increased and that of $^{36}Cl^-$ decreased so significantly that the difference between them became nine times larger than that with the eluent at pH 1.51. This will be discussed in terms of electroneutrality in the gel phase in the following section.

Electroneutrality in gel phase

Concerning only the behaviour of $^{22}Na^+$ and $^{36}Cl^-$, the positive charge does not seem to balance with the negative charge in the gel phase of Sephadex G-10 as shown in Fig. 1, except at pH 2.3, where the K_d values of these ions are identical. Hydrogen and hydroxide ions added to adjust the pH of the eluent must contribute to the charge balance in both the mobile and gel phases, too.

Since the K_d value is generally accepted as a partition coefficient of the solute between the mobile and gel phases in gel chromatography, it is expressed as

$$K_d = C_g/C_m \tag{1}$$

where C_g and C_m denote the concentrations of the solute in the gel and mobile phases, respectively. When the solute is the same as the eluting agent, C_g is obtained according to eqn. 2

$$C_g = K_d \times C \tag{2}$$

because C_m can be regarded as approximately constant and equal to the concentration of the eluent, C, in dynamic experiments using a gel column.

With a sodium chloride eluent at any pH adjusted with hydrogen chloride or sodium hydroxide, the positive and negative charges in the gel phase can be written as follows

$$\text{Positive charge} = K_{d(Na^+)} \times C_{Na^+} + K_{d(H^+)} \times C_{H^+} \tag{3}$$

$$\text{Negative charge} = K_{d(Cl^-)} \times C_{Cl^-} + K_{d(OH^-)} \times C_{OH^-} \tag{4}$$

provided that the gel matrix possesses no electric charge. $K_{d(Na^+)}$ and $K_{d(Cl^-)}$ are the distribution coefficients of sodium and chloride ions determined by using their radiotracers and C_{Na^+}, C_{Cl^-} and C_{OH^-} the concentrations of respective ions contained in the eluent. The radiotracers for hydrogen and hydroxide ions are not available. Therefore, their K_d values, $K_{d(H^+)}$ and $K_{d(OH^-)}$, were assumed to be equal to the K_d values of hydrogen chloride and sodium hydroxide, respectively, obtained by refractometric detection, when both the composition and pH of the sample solution are identical with those of the eluent. The elution peaks of hydrogen chloride and sodium hydroxide, however, are undetectable in such a case. Accordingly, the dependence of the K_d values of hydrogen chloride and sodium hydroxide on their concentrations, *i.e.*, on the pH of the sample solution, was examined, as shown in Fig. 4, where their K_d values are represented as $K_{d(H^+)}$ and $K_{d(OH^-)}$. The $K_{d(H^+)}$ and $K_{d(OH^-)}$ values at the pH values of the eluent were obtained from the plots of Fig. 4. The above assumption proved to be valid since the positive and negative charges in the gel phase balance over the range pH 1.5–3, where the gel matrix seems to possess no electric charge.

Table I gives the results of elucidating the electroneutrality in the gel phase by using the K_d values and the concentrations of the eluent ions. The $K_{d(Na^+)}$ and $K_{d(Cl^-)}$ values in this Table are somewhat different from those shown in Fig. 1 at corresponding pH values, because gels of different lot numbers were used in each series of experiments.

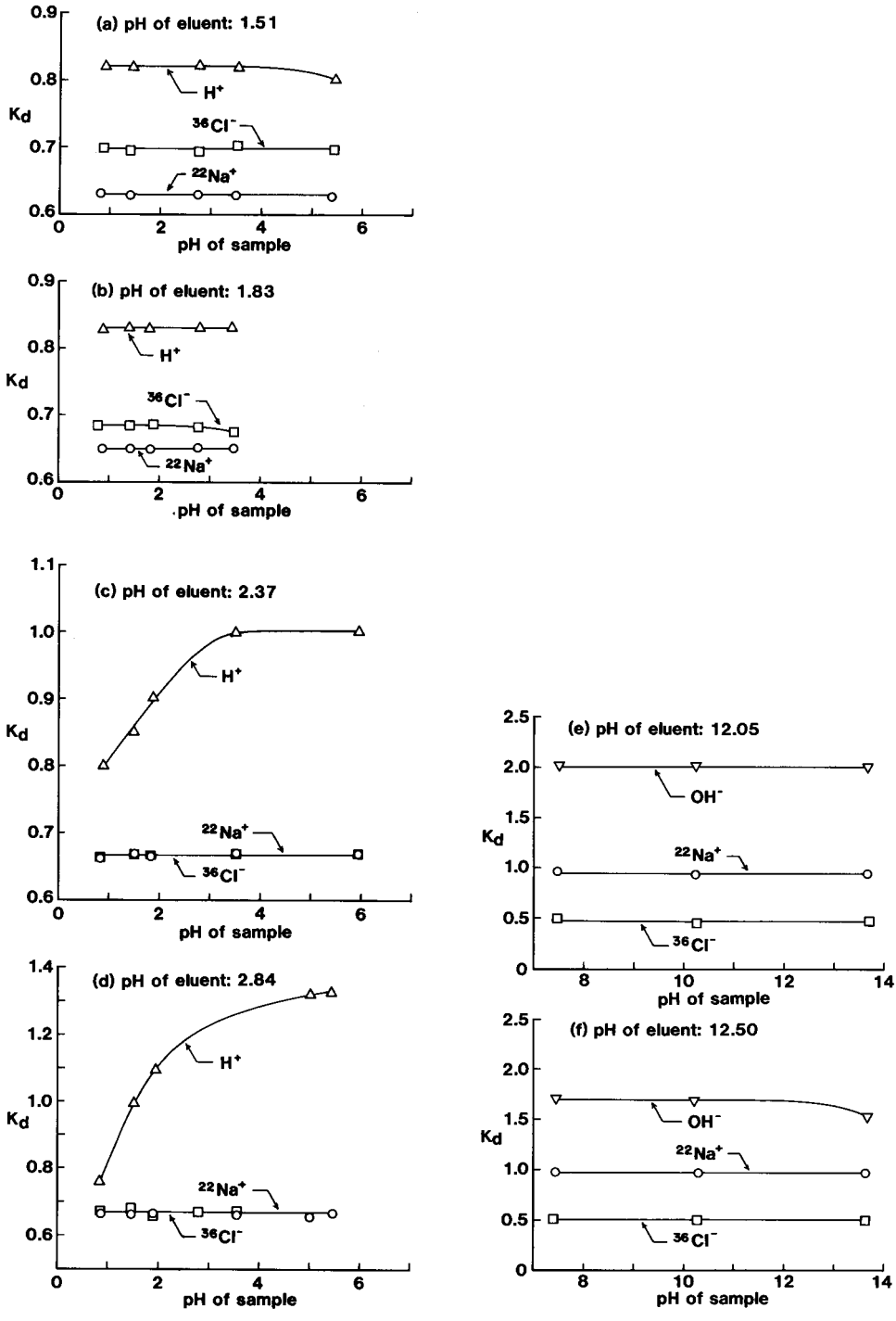

Fig. 4. Dependence of K_d values of eluent ions on pH of sample solutions. Gel: Sephadex G-10, 73 × 1.6 cm. Eluent: 0.1 *M* NaCl at pH 1.51, 1.83, 2.37, 2.84, 12.05 and 12.50. Sample: 0.1 *M* NaCl at various pH values shown in this figure. Temperature: 20°C.

TABLE I

EVALUATION OF ELECTRONEUTRALITY IN GEL PHASE

Gel: Sephadex G-10. Eluent: 0.1 *M* NaCl at various pH values. Temperature: 20°C. The K_d values of NaCl and of H^+ and OH^- were obtained by refractometry, those of Na^+ and Cl^- by the radiotracer method. C = Coulomb; *M* = molar concentration.

Eluent pH	K_d					*Positive charge* (10^{-20} *CM*)	*Negative charge* (10^{-20} *CM*)
	NaCl	Na^+	Cl^-	H^+	OH^-		
1.51	0.664	0.636	0.686	0.833	–	1.45	1.44
1.83	0.673	0.651	0.683	0.838	–	1.25	1.26
2.37	0.674	0.660	0.665	0.952	–	1.12	1.11
2.84	0.674	0.660	0.660	1.230	–	1.08	1.07
12.05	0.656	0.872	0.543	–	2.067	1.55	1.24
12.50	0.615	0.964	0.512	–	1.578	2.30	1.62

With eluents in the low pH region, the values of the positive and the negative charges agree well with each other within experimental error. Moreover, as shown in Fig. 5, the charge of the gel phase increases linearly with the ionic strength of the eluent in the gel phase, *I*, which was calculated according to:

$$I = \tfrac{1}{2}\left[K_{d(Na^+)} \times C_{Na^+} + K_{d(H^+)} \times C_{H^+} + K_{d(Cl^-)} \times C_{Cl^-} + K_{d(OH^-)} \times C_{OH^-}\right] \quad (5)$$

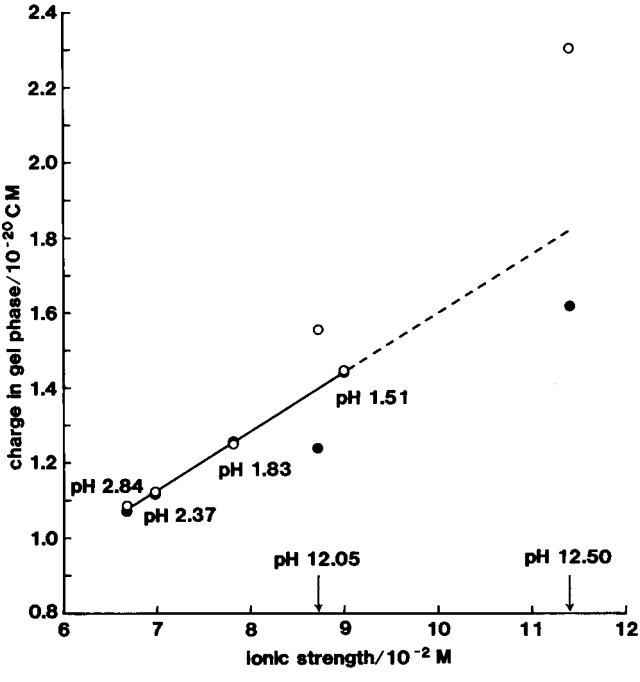

Fig. 5. Relationship between charge of eluent ions and ionic strength in gel phase. Data taken from Table I. ○, Positive charge; ●, negative charge.

These facts strongly support the approach used in this study for elucidating the electroneutrality in the gel phase in gel chromatography.

In contrast, the positive charges at pH 12.05 and 12.50 were markedly larger than the negative charges. These points for the positive charge deviate above and those for the negative charge below the linear plot of the charge *vs.* I in Fig. 5. This suggests that the negative charge of the gel matrix should be taken into account in evaluating the charge balance in the high pH region, as discussed later.

The pH dependence of the K_d values of $^{22}Na^+$ and $^{36}Cl^-$ can be explained in terms of the variation in the charge of the gel phase. In the acidic region the K_d value of hydrogen ion is considerably larger than those of sodium and chloride ions, as shown in Table I. Therefore, Na^+ tends to be repelled and Cl^- to be attracted by the gel phase. The carboxyl groups of the gel matrix may dissociate above this pH region. The negative charge increases markedly above pH *ca.* 11 possibly due to the adsorption of hydroxide ions[20] and/or to proton dissociation from hydroxyl groups of the gel matrix[16]. In fact, preliminary experiments revealed that the pH profile of the cation exchange capacity of a Sephadex G-10 column resembles closely that of the K_d value of $^{22}Na^+$ in Fig. 1 (ref. 21). Hence, the variation of charge of the gel phase with pH may cause the pH dependence of the K_d value of sodium and chloride ions.

The K_d values of both $^{22}Na^+$ and $^{36}Cl^-$ differ appreciably from those of sodium chloride obtained by refractometric detection at corresponding pH values, as shown in Table I. We may speculate that after the sample is introduced on to a gel column, sodium chloride including $^{22}Na^+$ and $^{36}Cl^-$ equilibrates instantaneously on a plate between the mobile and gel phases according to plate theory. When the gel phase carries an electric charge, the Donnan equilibrium also should hold between two phases. For example, on a gel with negative charge, the concentration of sodium ions in the gel phase may be larger than that of chloride ions. Such an equilibrium is successively attained in each plate due to the movement of the mobile phase. Then the elution peak obtained by refractometric detection arises according to the concentration distribution of sodium chloride, regardless of the sample or the eluent, since the cold sodium and chloride ions in the sample solution cannot be differentiated from those in the eluent. On the other hand, the elution peaks of sodium and chloride ions of the sample, represented by $^{22}Na^+$ and $^{36}Cl^-$, appear at different positions from that of cold sodium chloride because of the Donnan effect mentioned above.

In conclusion, the K_d value of an eluent ion should be determined by the use of its tracer, at least on Sephadex G-10 which is tightly cross-linked and has dissociable groups fixed to the gel matrix.

ACKNOWLEDGEMENTS

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STUDIES ON LECTINS

LIII. AFFINITY ELECTROPHORESIS IN THE STUDY OF THE EFFECT OF DETERGENTS ON THE INTERACTION OF LECTINS WITH CARBOHYDRATES

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SUMMARY

The effect of various types of detergents (Triton X-100, sodium dodecyl sulphate, cetyltrimethylammonium bromide) on the carbohydrate-binding activity of lectins was investigated by affinity electrophoresis on polyacrylamide gel. The non-ionic detergent Triton X-100 (0.5–2 %) did not cause dissociation of any of the lectins tested nor did it significantly affect the interaction of lectins with immobilized sugars. Application of the anionic detergent sodium dodecyl sulphate (0.1 %) resulted in the rapid dissociation of lectins into subunits. Subunits of none of the lectins studied interacted specifically with carbohydrates. If the dissociation of a lectin was incomplete, the carbohydrate-binding activity of undissociated lectin remained preserved. The cationic detergent cetyltrimethylammonium bromide (0.1 %) brought about a complete or partial dissociation into subunits. In the presence of cetyltrimethylammonium bromide neither subunits nor the undissociated molecules of most of the lectins studied interacted with sugars. Also, in the presence of this detergent, α-D-glucosyl and α-D-mannosyl ligands in polyacrylamide copolymers or mannan showed an enhancing effect in the dissociation into subunits of D-mannose-binding lectins (concanavalin A, seed lectins of *Pisum sativum*, *Lens esculenta* and *Lathyrus sativus*).

INTRODUCTION

Affinity chromatography using immobilized lectins is an effective procedure in the fractionation of glycoproteins and glycopeptides including cell surface glycoproteins[1]. However, membrane glycoproteins are not easily solubilized and separated in neutral aqueous solutions; therefore, chaotropic agents or detergents are commonly used for the solubilization of glycoproteins and as components of buffers used in affinity chromatography[2,3]. Chaotropic agents and detergents may influence the properties of the immobilized lectins in two ways: (i) modify and/or change the carbohydrate binding sites of lectins: (ii) bring about dissociation of the native lectin molecules into subunits which are held together by non-covalent forces.

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In spite of the increasing use of immobilized lectins in the isolation of membrane glycoproteins in detergent solutions, only Lotan *et al.*[3] have studied the effects of several commonly used detergents on the specific binding of asialo-[^{3}H]fetuin to immobilized lectins and on lectin-mediated agglutination of formalin-fixed erythrocytes. In this way they compared the effects of detergents on the ability of lectins, immobilized or free in a solution, to interact with carbohydrates. Dodeur and Jacquet[4] investigated the effect of detergents on the binding of hepatoma cell surface [^{3}H]galactoglycoproteins to concanavalin A or *Ricinus communis* lectin conjugated to Sepharose 4B.

In this study, affinity electrophoresis was used in the investigation of the effect of various types of detergents on the interaction of lectins with carbohydrates and on the stability and dissociation of lectin molecules.

EXPERIMENTAL

Water-soluble O-α-D-mannosyl and O-α-D-galactosyl polyacrylamide copolymers were prepared according to Hořejší *et al.*[5].

Lectins from the seeds of *Lens esculenta*[6], *Pisum sativum*[7], *Lathyrus sativus*[8] and *Canavalia ensiformis* (concanavalin A)[9] were isolated by affinity chromatography on Sephadex G-150. *Glycine soja* lectin was isolated from the seeds by affinity chromatography on α-D-galactosyl derivative of Separon (Spheron)[10]. Lectins from seeds of *Erythrina indica* and *Momordica charantia* were isolated by affinity chromatography on O-α-D-galactosyl polyacrylamide gel[11]. A mixture of lectins (toxin and agglutinin) from *Ricinus communis* seeds isolated by affinity chromatography on O-β-lactosyl polyacrylamide gel[12] was kindly provided by Dr. V. Hořejší.

Affinity electrophoresis was performed essentially as described previously[13]. The gel rods (0.5 × 8 cm) were prepared from a mixture containing 7% polyacrylamide, 0.2% N,N′-methylenebisacrylamide, an appropriate buffer system, water-soluble O-α-D-mannosyl or O-α-D-galactosyl polyacrylamide copolymer in a concentration giving the desired concentration of immobilized ligand (c_i) and an appropriate concentration of a detergent. Large-pore gels were omitted. Gels containing no specific glycosyl copolymer served as a control for checking the possible lectin dissociation. To prove the specific binding of lectins to immobilized sugar, either free D-mannose or D-galactose was added to the polymerization mixture to give a final concentration of 2%.

Affinity electrophoresis in the presence of Triton X-100

To study the effect of Triton X-100 the acidic buffer system described by Reisfeld *et al.*[14] was used. Triton X-100 was added to the polymerization mixture and sample solution in such an amount as to obtain a final concentration in the gel of 0.5–2%.

Affinity electrophoresis in the presence of sodium dodecyl sulphate (SDS)

The effect of SDS on the interaction of lectins with saccharides was studied in two systems: (i) in an alkaline buffer system[15] and (ii) in a buffer system of pH 7.2 according to Weber and Osborn[16]. SDS was added to the polymerization mixture, the electrode buffer and sample solution in amounts yielding final concentrations of 0.05–0.7%.

Affinity electrophoresis in the presence of cetyltrimethylammonium bromide (CTAB)

For the investigation of the effect of CTAB on the interaction of lectins with saccharides, the buffer system and conditions of electrophoresis according to Eley *et al.*[17] were used. The final concentration of CTAB in the polymerization mixture and in the electrode buffer was 0.1–0.2%.

Freshly prepared protein samples (50 μg) in 20% glycerol solution containing an appropriate amount of detergent (20 μl) were applied to the top of the gels. Alkaline discontinuous electrophoresis[15] was run for 1.5 h at a current density of 4 mA per tube, acidic discontinuous electrophoresis[14] for 2 h at 7 mA per tube, electrophoresis according to Weber and Osborn[16] for 2–2.5 h at 7 mA per tube and electrophoresis in the presence of CTAB[17] for 3–4 h at 8 mA per tube.

The gels were stained with Amido Black 10B, except for the gels obtained after electrophoresis in the presence of CTAB, which were stained, after fixation with hot 10% trichloroacetic acid, with Coomassie Blue R-250 according to Eley *et al.*[17].

The migration distances of the protein zones were measured with an accuracy of ±0.5 mm after staining. Dissociation constants were estimated graphically from the dependence of $1/d_0 - d$ on $1/c_i$ (for definitions see ref. 18).

RESULTS

For the investigation of the effect of detergents on the interaction of lectine with saccharides, three types of detergents were chosen: non-ionic (Triton X-100), anionic (SDS) and cationic (CTAB). With SDS, the interaction was studied at pH 7.2 and in an alkaline buffer system[15].

The interaction of following lectins with immobilized saccharides (given in parentheses) was tested: concanavalin A (D-Man), lectins from the seeds of *Pisum sativum* (D-Man), *Lens esculenta* (D-Man), *Lathyrus sativus* (D-Man), *Ricinus communis* (D-Gal), *Erythrina indica* (D-Gal), *Glycine soja* (D-Gal) and *Momordica charantia* (D-Gal).

Non-ionic detergent: Triton X-100

The presence of Triton X-100 at concentrations of 0.5–2% did not influence the electrophoretic mobility of the lectins studied. None of the lectins dissociated under these conditions.

The results of affinity electrophoresis showed that at concentrations of 0.5–2% the non-ionic detergent affected the interaction of the lectins with immobilized saccharides only very slightly. With concanavalin A, a slight decrease in the binding activity of this lectin to immobilized D-mannosyl residues was observed; the determined value of the dissociation constant of the lectin–immobilized α-D-mannosyl residues complex was higher ($7.9 \cdot 10^{-5}$ *M*) than that determined in the absence of detergent ($5.3 \cdot 10^{-5}$ *M*). On the other hand, the interaction of soybean lectin and both lectins from *Ricinus communis* seeds (toxin and agglutinin) with immobilized α-D-galactosyl residues was enhanced in the presence of Triton X-100; the dissociation constants of complexes of these lectins with the specific immobilized saccharide (soybean lectin $2.9 \cdot 10^{-4}$ *M*, agglutinin $4.1 \cdot 10^{-4}$ *M*, toxin $1.0 \cdot 10^{-3}$ *M*) were slightly lower than those determined in the absence of the detergent ($5.0 \cdot 10^{-4}$ *M*, $6.0 \cdot 10^{-4}$ *M* and $1.8 \cdot 10^{-3}$ *M*, respectively). Interaction of the lectins from seeds of *Pisum*

sativum, *Lens seculenta* and *Lathyrus sativus* with immobilized α-D-mannosyl residues was not influenced by the presence of the non-ionic detergent.

Anionic detergent: sodium dodecyl sulphate (SDS)

In contrast to Triton X-100, SDS, even at a low concentration (0.1 %), very easily caused dissociation of several of the lectins into subunits, as was revealed by polyacrylamide gel electrophoresis at pH 7.2 and in the alkaline buffer system.

Affinity electrophoresis on polyacrylamide gel containing immobilized specific sugars has shown that subunits of none of the lectins interacted specifically with sugars. An interaction with saccharides in the presence of SDS was observed only when the lectin was not dissociated into subunits. The concentration of SDS at which a lectin molecule dissociated into subunits was different for different lectins and was slightly dependent on the pH and composition of the buffers (ionic strength) used for the electrophoresis.

Concanavalin A was very easily dissociated into subunits even with 0.1 % SDS under the conditions of electrophoresis described by Weber and Osborn[16] (pH 7.2). When polyacrylamide gel electrophoresis was carried out in an alkaline buffer system according to Davis[15], with 0.1 % SDS concanavalin A was not completely dissociated and undissociated lectin molecules interacted with immobilized α-D-mannosyl residues. At SDS concentrations higher than 0.1 %, the concanavalin A molecule was completely dissociated and the subunits did not interact with the sugar ligand.

Lectins from *Pisum sativum*, *Lens esculenta* and *Lathyrus sativus* seeds were characterized by similar electrophoretic behaviours in the presence of SDS. Under the conditions of alkaline electrophoresis[15], these lectins were not completely dissociated into subunits at SDS concentrations of 0.1–0.3 %. A portion of the lectin molecules undissociated at these SDS concentrations interacted with immobilized α-D-mannosyl residues. Under the conditions of electrophoresis at pH 7.2 (ref. 16), the amount of undissociated lectins was greatly decreased and only at 0.1 % SDS was an interaction with immobilized saccharides observable (mostly with *Pisum sativum* lectin); even at this low SDS concentration most of the lectin was dissociated and neither of the two types of subunits interacted with the immobilized specific saccharide.

The interactions of both lectins from *Ricinus communis* seeds, which are not dissociated in the absence of mercaptoethanol, with immobilized α-D-galactosyl residues were very sensitive to SDS. Under conditions of alkaline electrophoresis, only at a 0.1 % SDS concentration were interactions of both lectins with immobilized saccharides observed. At pH 7.2, at the same SDS concentration, no interaction could be detected.

The least sensitive to the action of SDS were D-galactose-binding lectins from seeds of *Glycine soja*, *Erythrina indica* and *Momordica charantia*. Very similar results were obtained both in the alkaline buffer system[15] and in the system according to Weber and Osborn[16]. With 0.1–0.5 % SDS lectins from soybean and *Erythrina indica* seeds were not completely dissociated; part of the lectins did not dissociate into subunits and was specifically bound to the immobilized D-galactose residues (see Fig. 1). The lectin from *Momordica charantia* seeds, which does not dissociate in SDS medium in the absence of mercaptoethanol, interacted with α-D-galactosyl residues even at a 0.7 % SDS concentration. In the alkaline buffer system containing SDS, isolectins present in the preparation of *Momordica charantia* lectin were detected even on con-

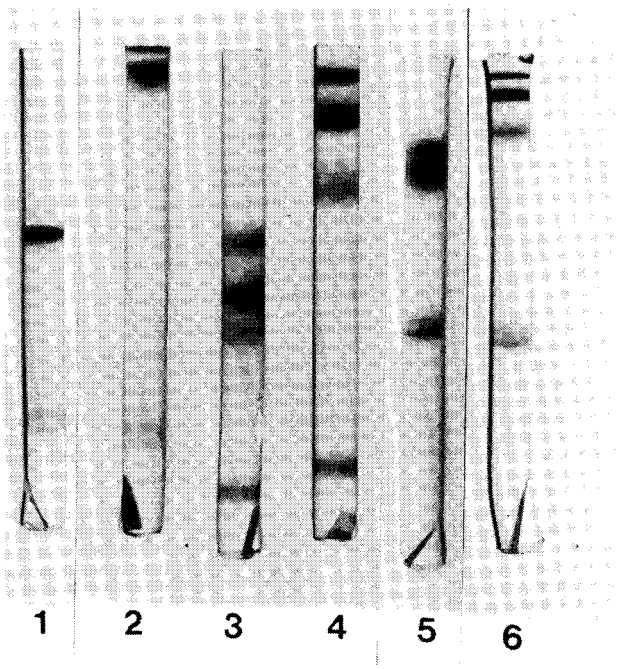

Fig. 1. Affinity electrophoresis of lectins from seeds of *Glycine soja* and *Erythrina indica* in the presence of 0.3% SDS. 1, 2 = *Glycine soja* lectin; 3–6 = *Erythrina indica* lectin. 1, 2, 3, 4: Electrophoresis carried out in the alkaline buffer system[15]; 5, 6: electrophoresis carried out in the system according to Weber and Osborn[16]. 1, 3, 5: Control gels containing no glycosyl copolymer ($c_i = 0$); 2, 4, 6: affinity gels containing immobilized α-D-galactosyl residues ($c_i = 3.5 \cdot 10^{-3}$ *M*).

trol gels, whereas in the absence of the detergent these multiple forms could be separated only on affinity gels[11].

Cationic detergent: cetyltrimethylammonium bromide (CTAB)

As with SDS, the cationic detergent CTAB even at a 0.1% concentration caused the dissociation of several lectins, subunits of which were held by non-covalent bonds. In some lectins the dissociation was incomplete.

In contrast to SDS medium, none of the lectins, which were not fully dissociated in the presence of CTAB, interacted specifically with immobilized saccharides. The mobilities of zones corresponding to undissociated lectins and the lectin subunits were the same both on affinity and the control gels in the presence or absence of the free specific sugar. The only exception was the lectin from *Erythrina indica* seeds: in the presence of 0.1% CTAB, the undissociated lectin interacted with immobilized α-D-galactosyl residues; the interaction was inhibited by the addition of free D-galactose to the affinity gel.

The soybean lectin was partially dissociated in the presence of 0.1–0.2% CTAB, but neither undissociated lectin nor its subunits interacted with immobilized specific sugar. *Ricinus communis* lectins did not interact with immobilized α-D-galactosyl residues even at a 0.1% concentration of CTAB.

As with D-galactose-binding lectins, lectins binding α-D-mannosyl residues

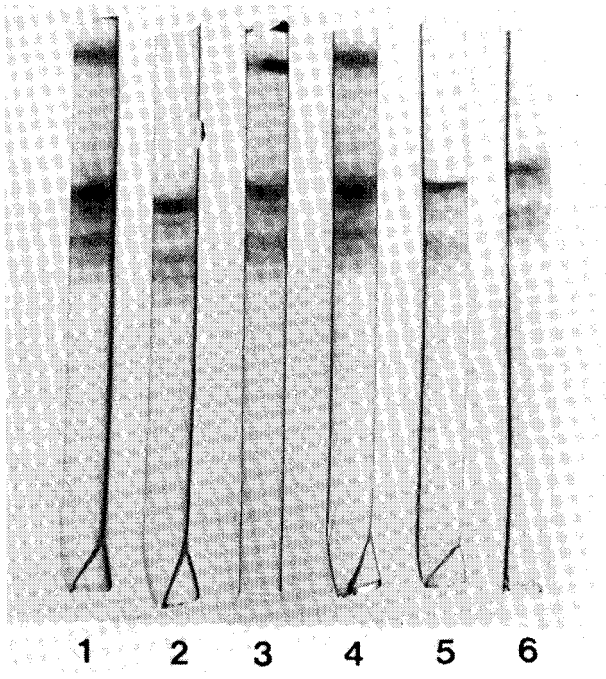

Fig. 2. Affinity electrophoresis of concanavalin A in the presence of 0.1 % CTAB. 1 = Control gel without any glycosyl copolymer (c_i = 0); 2 = affinity gel containing immobilized α-D-mannosyl residues (c_i = $2.5 \cdot 10^{-3}$ *M*); 3 = affinity gel containing immobilized α-D-mannosyl residues (c_i = $2.5 \cdot 10^{-3}$ *M*) and free D-mannose (c = $1.1 \cdot 10^{-1}$ *M*); 4 = gel containing immobilized α-D-galactosyl residues; 5 = control gel; in contrast to other gels, the sample solution applied to the gel contained α-D-mannosyl polyacrylamide copolymer in the same concentration as in affinity gels; 6 = affinity gel containing mannan (2 %).

(concanavalin A, lectins from seeds of *Pisum sativum*, *Lens esculenta* and *Lathyrus sativus*) were dissociated of the major part into subunits even at a 0.1 % CTAB concentration. However, on affinity gels these lectins showed interesting behaviour.

In the presence of 0.1 % CTAB on affinity gels containing immobilized α-D-mannosyl residues, zones corresponding to undissociated lectins could not be detected, whereas on control gels without D-mannosyl or D-glucosyl copolymers or gels containing α-D-galactosyl copolymer under the same conditions incompletely dissociated lectins were found. In the presence of a free specific sugar (2 % D-mannose) in the affinity gel, the electrophoretic behaviour of all of these lectins did not differ from that on a control gel. This phenomenon was observed for all of the α-D-mannose binding lectins studied in the presence of 0.1–0.2 % CTAB. Subunits of none of the studied lectins interacted specifically with immobilized α-D-mannosyl residues (see Fig. 2).

The same effect was observed, even when α-D-mannosyl copolymer was added only to the sample solutions prior to application to the gel. No zones corresponding to undissociated lectins were observed on affinity gels. A similar effect, *viz.*, an enhancement of dissociation of lectins in the presence of 0.1 % CTAB, was also brought about in affinity gels containing the α-D-glucosyl copolymer or mannan. On the other hand, addition of the α-D-galactosyl copolymer to the sample solution did not result in complete dissociation of the studied lectins.

DISCUSSION

In the experiments described above we used affinity electrophoresis to establish the effect of detergents directly on lectin molecules. This method enabled us also to study the possible interactions of lectin subunits with specific saccharides.

Dodeur and Jacquet[4] found that the interaction of lectins with saccharides depends on the ionic strength and pH of the medium in which affinity chromatography is carried out. We have also observed the dependence on composition of buffers and pH with SDS when the experiments were carried out in a buffer system of pH 7.2 (ref. 16) and in an alkaline buffer system[15].

In agreement with results of Lotan *et al.*[3] and Dodeur and Jacquet[4], our results have shown that the non-ionic detergent Triton X-100, even at a 2% concentration, had no effect on the interaction of any of the lectins with saccharides; with some lectins the interaction in the presence of Triton X-100 was sligthly higher than in its absence. Subunits of none of the studied lectins, even in trace amounts, were detected.

As reported previously[3,4], the anionic detergent SDS strongly affected the interaction of lectins with carbohydrates in affinity chromatographic experiments. Our results on affinity electrophoresis have shown that the anionic detergent SDS brought about rapid dissociation of most of the lectins to subunits. However, if under the given conditions the dissociation was incomplete, the undissociated part of some lectins interacted specifically with the sugar ligands even at relatively high SDS concentrations (0.5%). This phenomenon was observed especially with D-galactose binding lectins. Contrary to Lotan *et al.*[3], who described a very easy dissociation of soybean lectin in the presence of SDS, we have observed that this lectin belongs to the group of lectins that are the most insensitive to the action of this anionic detergent. Even though the presence of SDS does not inhibit the interaction of undissociated lectins with immobilized sugar ligands in affinity gels, no saccharide-binding activity of subunits of any of the studied lectins could be detected.

In the presence of the cationic detergent CTAB, we observed no specific interactions with sugars of most of the lectins even at low detergent concentrations (0.1%); the only exception was the lectin from *Erythrina indica* seeds. Our results are not in agreement with those of the affinity chromatographic experiments described by Lotan *et al.*[3]; the interaction of the glycoprotein with immobilized lectins in some instances was not affected, in some instances was decreased (depending on the deteregent concentration), but with 0.1% CTAB the interaction was still detectable. The results of their hemagglutination tests also showed that CTAB affected lectins less than SDS. These differences in the results could probably be explained by different experimental conditions under which examination of the detergent effect was carried out.

The behaviour of D-mannose-binding lectins in the presence of CTAB was very interesting; the presence of a high-molecular-weight substance specifically interacting with these lectins, either synthetic (D-glucosyl or D-mannosyl polyacrylamide copolymers) or a natural polysaccharide (mannan) caused an easier dissociation of D-mannose-binding lectins. The effect could be reversed by addition of a free specific sugar to the affinity gel. With D-galactose-binding lectins, this phenomenon was not observed.

In addition to the previously reported studies on the effect of ionic strength[19] and pH[20] on binding activities of lectins, this paper gives another example of the

applicability of affinity electrophoresis to the investigation of the effect of different factors on the interaction of proteins with specific ligands.

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HIGH-BACK-PRESSURE LIQUID CHROMATOGRAPHY

III. OPEN-TUBULAR MICROCAPILLARY LIQUID CHROMATOGRAPHY USING LIQUEFIED ALKANES AS THE MOBILE PHASE

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(Received December 30th, 1981)

SUMMARY

Open-tubular microcapillary liquid chromatography using lower alkanes as the mobile phase was examined. The diffusivity of the solutes increased with decreasing carbon number of the solvents, leading to higher column efficiency. Some typical separations of standard solutes are demonstrated.

INTRODUCTION

Lower alkanes such as propane and butane can be easily liquefied at relatively low pressure (5–10 atm). If the pressure of the entire chromatograph is kept high enough to exceed the vapour pressure of the now liquid solvents, even such low-boiling solvents can be employed as the mobile phase in liquid chromatography (LC)[1]. In previous work[1], micro-high-performance liquid chromatography (MHPLC) using C_3–C_6 alkanes was investigated and useful results were obtained owing to their low viscosity, which encouraged us to use low-boiling alkanes as the mobile phase in LC. In addition, the resolution increased with decreasing carbon number of the alkanes for the separation of polynuclear aromatic hydrocarbons on a silica gel column.

The larger difference in diffusion speed in the liquid and gaseous states is the main reason why open-tubular capillary LC has not been so successful as capillary gas chromatography[2]. Thus the employment of liquefied alkanes of low viscosity as the mobile phase will be advantageous in capillary LC.

The basic equation expressing column performance in open-tubular capillary LC is

$$H = \frac{2D_m}{u} + \frac{2k'd^2u}{3(1 + k')^2D_s} + \frac{(11k'^2 + 6k' + 1)r_c^2u}{24(1 + k')^2D_m} \quad (1)$$

where H is the height equivalent to a theoretical plate, u is the linear velocity of the mobile phase, k' is the capacity factor, d is the thickness (or depth) of the stationary

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phase, r_c is the radius of an open tube and D_m and D_s are the diffusion coefficients of a solute in the mobile phase and the stationary phase, respectively.

For non-retained solutes ($k' = 0$), eqn. 1 is simplified under normal conditions to

$$H = \frac{2D_m}{u} + \frac{r_c^2 u}{24D_m} \approx \frac{r_c^2 u}{24D_m}$$

From plate theory, H is represented by the following equation:

$$H = \frac{L}{N} = \frac{L}{16}\left(\frac{V_w}{V_t}\right)^2 \tag{3}$$

where L is column length, N is theoretical plate number, V_w is the peak width in volume units and V_t is the retention volume.

Combining eqns. 2 and 3 and rewriting, we obtain

$$V_w^2 = \frac{2V_t^2 r_c^2}{3L} \cdot \frac{u}{D_m} \tag{4}$$

The first term in eqn. 1 is negligible under the usual conditions. Hence the selection of a system with large D_m and D_s as well as the use of a narrow-bore capillary column is necessary in order to attain high efficiency. As the third term in eqn. 1, based on the resistance to mass transfer in the mobile phase, is dominant in open-tubular capillary LC[3,4], particular attention should be paid to the viscosity of the mobile phase and the column bore. It is relatively easy to deal with the former problem, whereas the latter suffers from technical problems associated with the preparation of narrow-bore columns.

The viscosity of the mobile phase generally decreases with increasing column temperature. The influence of column temperature on column efficiency and the retention of solutes was examined in open-tubular capillary LC[5]. It was found that operation at higher temperature had a tendency to give a higher column efficiency owing to the decrease in the viscosity of the mobile phase.

This paper examines the use of lower alkanes as the mobile phase in open-tubular capillary LC in order to attain higher column efficiencies using low-viscosity mobile phases.

EXPERIMENTAL

All reagents were supplied by Wako (Osaka, Japan) or Tokyo Chemical Industry (Tokyo, Japan).

A liquid chromatograph was assembled from a Micro Feeder (Azumadenki Kogyo, Tokyo, Japan) equipped with a gas-tight syringe, Model MS-GAN 025 (250 μl) or 050 (500 μl) (Terumo Co., Tokyo, Japan), as a pump, a three-way valve [Japan Spectroscopic Co. (JASCO), Tokyo, Japan], a guard column, a saturation column, a micro valve injector (JASCO), a gas cylinder and a back-pressure pump. A diagram of the apparatus is shown in Fig. 1.

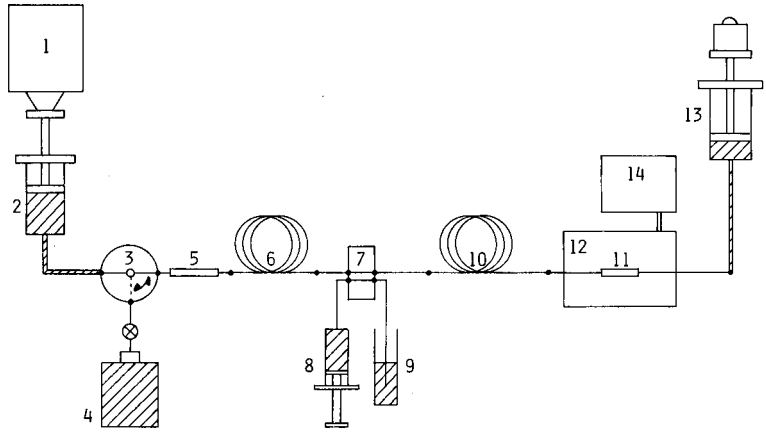


Fig. 1. Diagram of the apparatus. 1 = Micro feeder; 2 = gas-tight syringe; 3 = three-way valve; 4 = gas cylinder; 5 = guard column; 6 = saturation column; 7 = micro valve injector (0.02 μl); 8 = sample; 9 = waste reservoir; 10 = separation column; 11 = micro flow cell; 12 = UV detector; 13 = back-pressure pump; 14 = recorder.

A low-boiling solvent was collected by the method described previously[1]. The guard column (3 cm × 0.5 mm I.D.) was made of PTFE tubing and was packed with Develosil spherical porous silica (Nomura Chemical, Seto-shi, Japan), which removed impurities from the solvent. β,β'-Oxydipropionitrile (BOP) columns were generally employed in this work. The preparation method was the same as described previously[3]. When a physically coated column is employed as the separation column, the mobile phase should be saturated with the stationary phase. An open-tubular capillary saturation column, *ca.* 5 m × 60 μm, physically coated with BOP worked well for saturating the mobile phase with BOP *in situ*. Samples could be loaded with good reproducilibity using the micro valve injector, in spite of the small volume (0.02 μl).

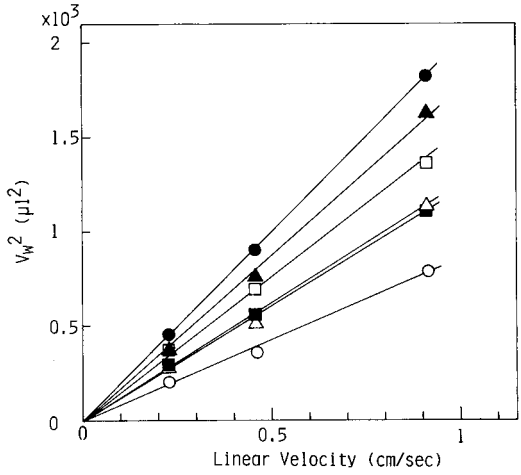


Fig. 2. Dependence of the square of the band broadening on linear velocity of the mobile phase. Column: 3.8 m × 0.35 mm I.D. Mobile phases: ● = *n*-hexane; ▲ = neopentane; □ = *n*-pentane; ■ = *n*-butane; △ = isobutane; ○ = propane. Sample: benzene. Temperature: 18°C.

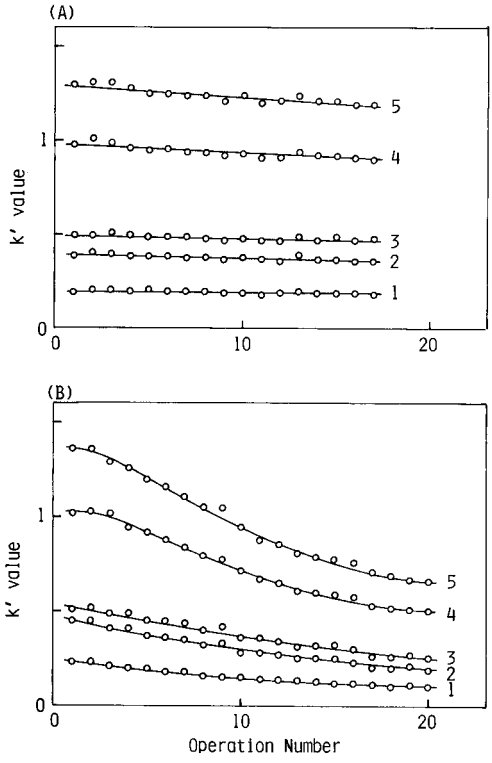


Fig. 3. Durability of column performance: (A) with saturation column; (B) without saturation column. Separation column: BOP, 7.4 m × 44 μm I.D. Saturation column: BOP, 5 m × 60 μm I.D. Mobile phase: *n*-butane. Flow-rate: 2.1 μl/min. Samples: 1 = N-phenyl-α-naphthylamine; 2 = N-phenyl-β-naphthylamine; 3 = aniline; 4 = α-naphthylamine; 5 = β-naphthylamine.

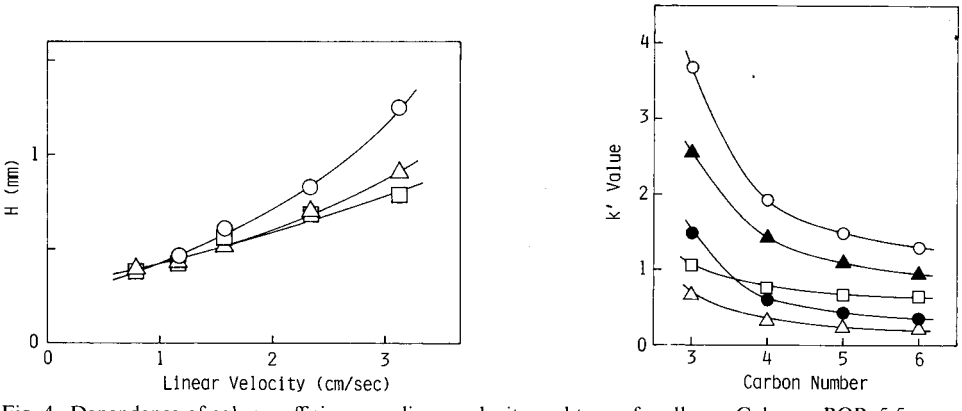


Fig. 4. Dependence of column efficiency on linear velocity and type of *n*-alkane. Column: BOP, 5.5 m × 44 μm I.D. Mobile phases: ○ = *n*-hexane; △ = *n*-pentane; □ = *n*-butane. Sample: β-naphthylamine. *k'* values: 0.64 (*n*-hexane); 0.79 (*n*-pentane); 1.05 (*n*-butane).

Fig. 5. Relationship between retention of aromatic amines and carbon number of *n*-alkanes. Column as in Table I. Samples: △ = N-phenyl-α-naphthylamine; ● = N-phenyl-β-naphthylamine; □ = aniline; ▲ = α-naphthylamine; ○ = β-naphthylamine. Temperature: 25°C.

The pressure in the detector and the column was kept sufficiently high to exceed the vapour pressure of the solvent by applying pressure with the back-pressure pump, consisting of a gas-tight syringe and a weight, as described previously[1,6].

RESULTS AND DISCUSSION

As described in the preceding section, the diffusion coefficient of solutes in the mobile phase plays an important role in open-tubular capillary LC. In other words, the viscosity of the mobile phase directly affects the column efficiency. Eqn. 4 indicates that the square of the peak width of a non-retained solute is proportional to the linear velocity of the mobile phase and inversely proportional to the diffusion coefficient of a solute in the mobile phase. Hence, if the peak width of a solute flowing through a capillary tubing is measured, D_m can be calculated.

Fig. 2 illustrates relationship between V_w^2 and u for various low-boiling alkanes using benzene as the test solute. The slope of these plots decreases with decreasing carbon number of the alkanes, *i.e.*, the lower the carbon number, the smaller is the viscosity. Diffusion coefficients of benzene in various solvents can be calculated from the data in Fig. 2, *viz.*, $4.4 \cdot 10^{-5}$ cm^2/sec in *n*-hexane, $4.8 \cdot 10^{-5}$ cm^2/sec in neopentane, $5.5 \cdot 10^{-5}$ cm^2/sec in *n*-pentane, $6.9 \cdot 10^{-5}$ cm^2/sec in *n*-butane, $7.0 \cdot 10^{-5}$ cm^2/sec in isobutane and $1.0 \cdot 10^{-4}$ cm^2/sec in propane. These results encouraged us to employ lower alkanes as the mobile phase in open-tubular capillary LC.

Physically coated columns were generally employed as separation columns, necessiating saturation of the mobile phase with the stationary phase. Solvents that are liquid under ambient conditions can be easily pre-saturated with the stationary phase in the batch method[3], whereas liquefied solvents should be saturated with the stationary phase in the closed system, as described in the experimental section. Fig. 3

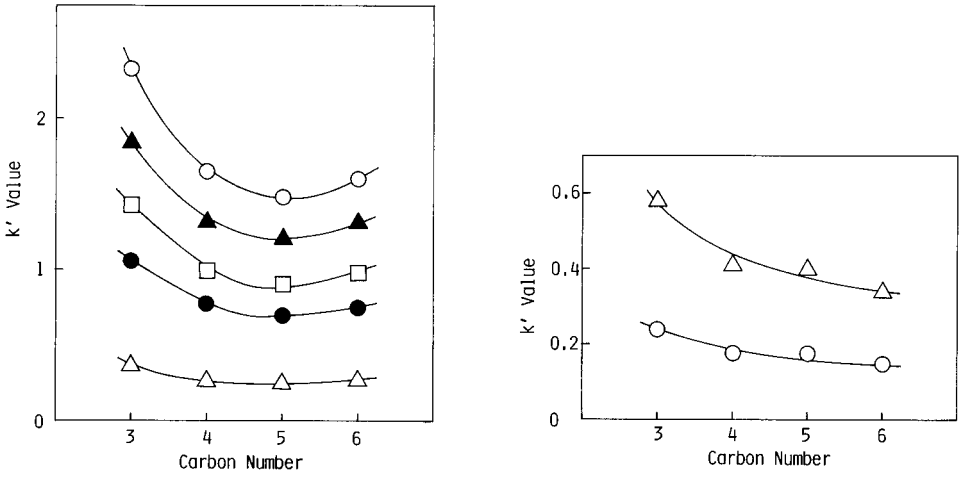


Fig. 6. Relationship between retention of xylenol isomers and carbon number of *n*-alkanes. Column: BOP, 5.1 m × 42 μm I.D. Samples: △ = 2,6-xylenol; ● = 2,5-xylenol; □ = 2,3-xylenol; ▲ = 3,5-xylenol; ○ = 3,4-xylenol. Temperature: 27°C.

Fig. 7. Relationship between retention of N-alkylanilines and carbon number of *n*-alkanes. Column: BOP, 5.4 m × 44 μm I.D. Samples: ○ = N-ethylaniline; △ = N-methylaniline. Temperature: 30°C.

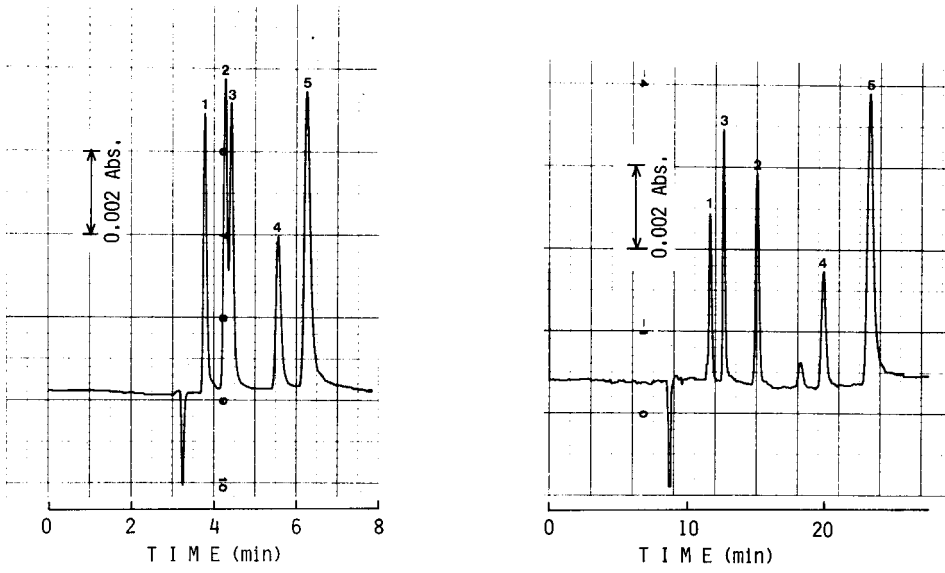


Fig. 8. Separation of aromatic amines. Column: BOP, 5.5 m × 44 μm I.D. Mobile phase: *n*-butane. Flow-rate: 2.8 μl/min. Samples: 1 = N-phenyl-α-naphthylamine; 2 = N-phenyl-β-naphthylamine; 3 = aniline; 4 = α-naphthylamine; 5 = β-naphthylamine. Wavelength of UV detection: 235 nm.

Fig. 9. Separation of aromatic amines. Column: BOP, 7.4 m × 44 μm I.D. Mobile phase: propane. Flow-rate: 1.4 μl/min. Samples as in Fig. 8. Wavelength of UV detection: 235 nm.

illustrates the variation of the retention of solutes in successive operations during a few days. The k' value of each solute decreases slightly with the saturation column, whereas the decrease in k' is significant without the saturation column. Chemically bonded open-tubular columns will solve the above problems.

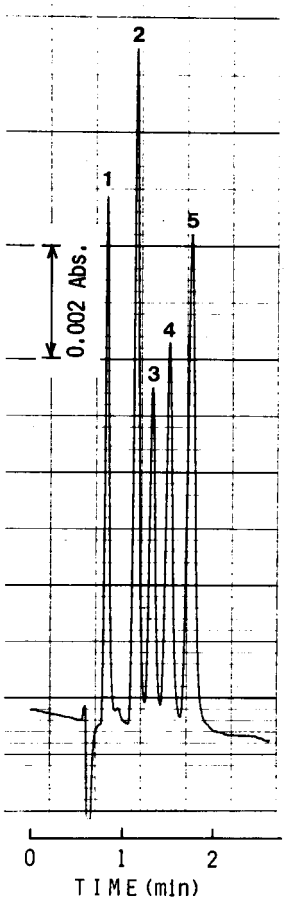
The results obtained for non-retained solutes suggest that a higher column efficiency can be attained for retained solutes with lower alkanes as the mobile phase. Fig. 4 shows H *versus* u relationships obtained by using *n*-alkanes with 4–6 carbon atoms as the mobile phase. As the carbon number decreases a lower dependence of H on u is observed, in spite of the larger retention. Eqn. 1 indicates that H increases with

TABLE I

RETENTION OF AROMATIC AMINES ON A BOP COLUMN

Column: BOP, 5.1 m × 42 μm. Temperature: 25°C. NPαNA = N-phenyl-α-naphthylamine; NPβNA = N-phenyl-β-naphthylamine; A = aniline; αNA = α-naphthylamine; βNA = β-naphthylamine.

Mobile phase	*Sample*				
	NPαNA	*NPβNA*	*A*	*αNA*	*βNA*
n-Hexane	0.18	0.34	0.65	0.94	1.30
n-Pentane	0.24	0.42	0.66	1.10	1.48
Neopentane	0.50	0.99	0.94	2.08	2.86
n-Butane	0.32	0.61	0.75	1.42	1.92
Isobutane	0.50	0.99	0.93	2.05	2.78
Propane	0.70	1.49	1.06	2.55	3.69



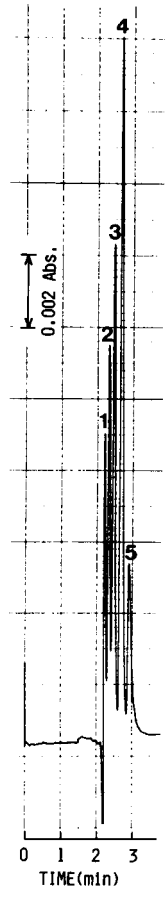


Fig. 10. Rapid separation of xylenol isomers. Column: BOP, 3.4 m × 33 μm I.D. Mobile phase: propane. Flow-rate: 5.6 μl/min. Samples: 1 = 2,6-xylenol; 2 = 2,5-xylenol; 3 = 2,3-xylenol; 4 = 3,5-xylenol; 5 = 3,4-xylenol. Wavelength of UV detection: 280 nm.

Fig. 11. Separation of aromatic hydrocarbons on a silica gel column. Column: 5.4 m × 44 μm I.D., prepared by treatment with 1 *N* sodium hydroxide solution for 2 days at 50°C. Mobile phase: isobutane. Flow-rate: 4.2 μl/min. Samples: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene; 5 = pyrene. Wavelength of UV detection: 254 nm.

increasing k' of a solute when the third term is dominant. Hence the above results strongly recommend the use of lower alkanes as the mobile phase and indicate their advantages in open-tubular capillary LC.

The retention behaviours of typical aromatic compounds were examined using BOP columns. The retention of the solutes was dependent on the concentration of BOP in the coating solution, the coating speed of BOP or conditions of pre-treatment with sodium hydroxide solution. The retention of solutes increased with increasing BOP concentration, coating speed of BOP and pre-treatment temperature. The coating solution and pre-treatment temperature adopted in this work were 15% (w/w) of BOP in dichloromethane containing 0.15% (w/w) of sodium tetraphenylborate and

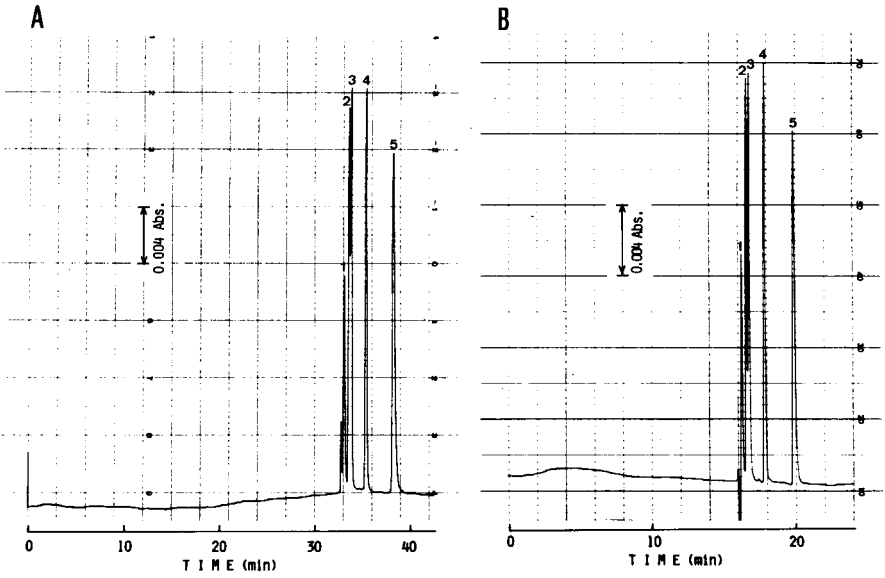


Fig. 12. Separations of N-alkylanilines. Column: BOP, 19.2 m × 37 μm I.D. Mobile phases: (A) *n*-butane; (B) propane. Flow-rates: (A) 0.69 μl/min; (B) 1.4 μl/min. Samples: 1 = N,N-diethylaniline; 2 = N,N-dimethylaniline; 3 = N-*n*-butylaniline; 4 = N-ethylaniline; 5 = N-methylaniline. Wavelength of UV detection: 235 nm.

4% (w/w) methanol, and 20–30°C, respectively, unless indicated otherwise.

Table I shows the retention of aromatic amines on a BOP column. The retention of each solute generally increased with decreasing carbon number but the order of elution of N-phenyl-β-naphthylamine and aniline changed, depending on the carbon number or structure of the solvent. In addition, a branched-chain solvent gave a larger k' value than the corresponding straight-chain solvent, these results being similar to those obtained in MHPLC employing packed columns[1].

The relationship between the k' values of aromatic amines and the carbon number of *n*-alkanes is shown in Fig. 5.

Figs. 6 and 7 show the relationships between k' and carbon number of *n*-alkanes for xylenol isomers and N-alkylanilines, respectively. In the former instance *n*-pentane gives the minimum k' values, whereas the k' values decrease monotonously with increasing carbon number in the latter.

Figs. 8 and 9 show typical separations of aromatic amines on a BOP column using *n*-butane and propane, respectively, as the mobile phase. Symmetrical chromatographic peaks are observed.

A low viscosity of the mobile phase facilitates rapid separations. Fig. 10 demonstrates the rapid separation of xylenol isomers on a BOP column. The linear velocity of propane was about 11 cm/sec.

Fig. 11 shows the separation of aromatic hydrocarbons on a silica gel column, prepared by treatment with 1 *N* sodium hydroxide solution for 2 days at 50°C. The retention of solutes generally increased with decreasing carbon number also for silica gel columns.

Liquefied alkanes with low viscosity favour the operation of longer columns.

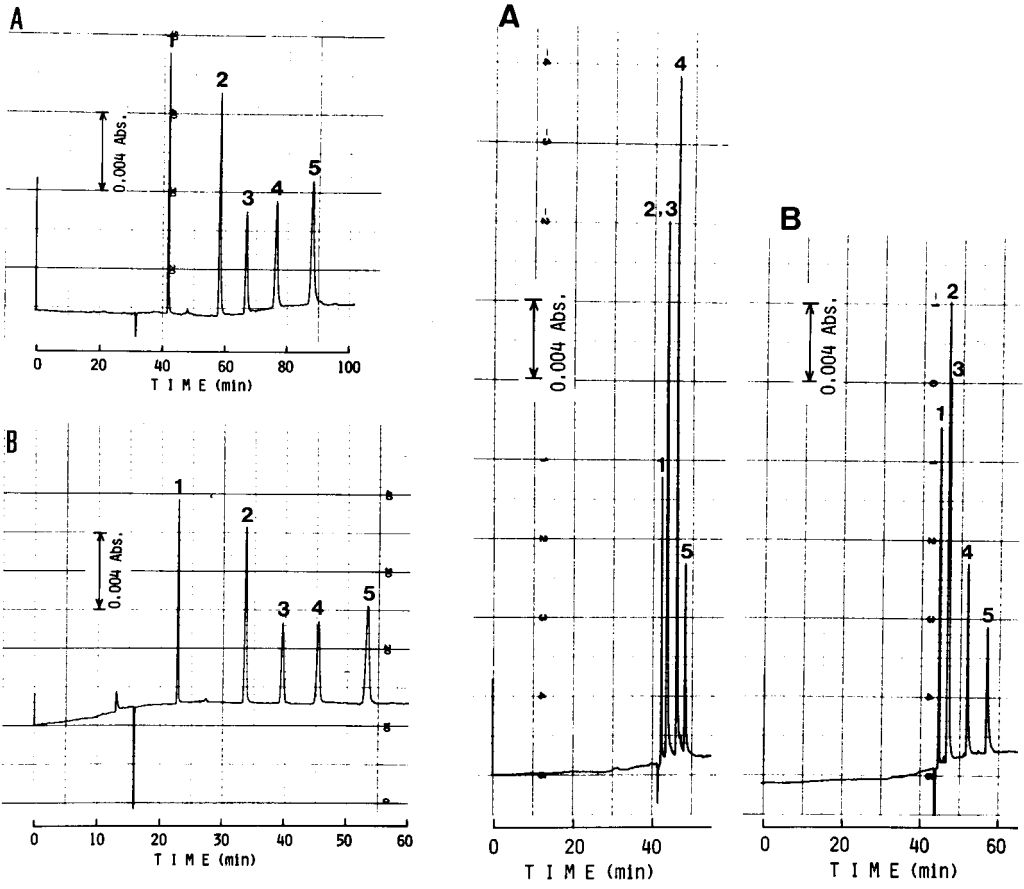


Fig. 13. Separations of xylenol isomers. Operating conditions, except samples and wavelength of detection, as in Fig. 12. Samples: 1 = 2,6-xylenol; 2 = 2,5-xylenol; 3 = 2,3-xylenol; 4 = 3,5-xylenol; 5 = 3,4-xylenol. Wavelength of UV detection: 280 nm.

Fig. 14. Separations of aromatic hydrocarbons. Column: BOP, 30.6 m × 34 μm I.D. Mobile phases: (A) *n*-butane; (B) propane. Flow-rate: 0.69 μl/min. Samples as in Fig. 11. Wavelength of UV detection: 254 nm. Efficiency: (A) (solute, k', N): benzene, 0.02, 300,000; anthracene, 0.12, 190,000; pyrene, 0.17, 140,000. (B) (solute, k', N): benzene, 0.02, 340,000; anthracene, 0.19, 120,000; pyrene, 0.31, 94,000.

Figs. 12 and 13 show separations of N-alkylanilines and xylenol isomers, respectively, on a BOP column (19.2 m × 37 μm). Fig. 14 shows separations of aromatic hydrocarbons on a BOP column (30.6 m × 34 μm) in which 100,000–300,000 theoretical plates are attained within 1 h.

CONCLUSION

The use of liquefied alkanes as the mobile phase in open-tubular capillary LC led to higher column efficiency; 100,000–300,000 theoretical plates were easily attained on BOP columns. The development of chemically bonded capillary columns with high efficiency and the use of various kinds of low-viscosity solvents as the mobile phase will advance this field.

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INVESTIGATION OF THE EFFECT OF ADDED ORGANIC AMINE ON THE CHROMATOGRAPHY OF TRICYCLIC ANTIDEPRESSANT DRUGS USING REVERSED-PHASE CHROMATOGRAPHY ON OCTADECYLSILICA WITH SODIUM LAURYL SULPHATE AS PAIRING ION

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SUMMARY

The chromatography of a group of tricyclic antidepressant drugs has been investigated using reversed-phase chromatography on ODS Hypersil with sodium lauryl sulphate as pairing ion both in the presence and absence of various organic amines. The amines investigated ranged in hydrophobicity from methylammonium to cetrimide. The general effect of such added amines is to decrease retention of basic solutes and to alter the selectivity of the stationary phase. The results are interpreted on the basis of the ion-exchange desolvation process previously proposed and optimised systems for the separation of clinically relevant solute pairs in this drug class are demonstrated.

INTRODUCTION

Currently, the most widely used high-performance liquid chromatographic (HPLC) mode is that of reversed phase using chemically bonded stationary phases with octyl- and octadecylsilanes being far the most commonly used modifiers of the silica surface[1]. Such stationary phases, together with suitable choice of aqueous mobile phase incorporating methanol or acetonitrile as organic modifier, will produce retention and usually excellent separation in most chromatographic problems involving electrically uncharged solutes.

The retention of ionogenic species can be achieved in the case of acidic solutes by control of pH[2,3]. Ion suppression, at low pH, will often allow chromatography of weak acids in the undissociated state. For basic solutes ion suppression is less advantageous due to deterioration of the silica support material at high pH[4,5].

Both types of electrolyte have been successfully chromatographed by addition of hydrophobic pairing ions, opposite in charge to the solute, to the mobile phase[6–9].

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In such cases the pH is adjusted so that the solute species is completely in the ionised condition. This procedure has been studied by very many workers and various terms have been coined to describe the processes leading to retention and separation[6–12]. In spite of the confusion produced by terminology, this technique has augmented the ion suppression method for the chromatography of acids and has extended chromatographic possibilities to strong acids where the pH required for complete suppression would be so low as to adversely affect the silica support[6]. Its major application has been to the chromatography of organic bases at low pH. Using such systems the chromatographic conditions can be controlled extensively by modification of the mobile phase, in particular the type and concentrations of pairing ion and associated counter ion used[13]. Recently the variation of retention and separation of several solutes has been shown to be complex with respect to mobile phase pairing ion concentration; the capacity factors of all solutes studied showing a maximum with pairing ion concentration. These results have been shown to be general for groups of both acidic and basic solutes and have been interpreted on a quantitative basis by an ion exchange model which also involves desolvation on the silanised silica surface[8,9]. On this model, counter ion concentration, *i.e.*, an ion of similar charge to that of the solute, is also involved in the retention equilibrium. It has been shown that the effect of increasing ionic strength and thus counter ion concentration has the effect of reducing retention in such systems[13,14]. Previous work on such counter ion dependence has been limited to inorganic counter ions where interaction is largely confined to the mobile phase.

The literature contains several reports of organic amines being added to the mobile phase during the chromatography of basic drugs, in particular the tricyclic antidepressant group of bases[4,15–18]. The effect of such addition is generally to reduce tailing of eluted peaks. This has been explained on the basis of masking active silanol groups[19] or of suppressing dissociation of ion pairs in the organic phase[17,18]. Most of the data on such separations have been obtained in liquid–liquid systems in which higher alcohols have been used to modify the C-18 or C-8 surfaces. Little information is available on the effect of such amine addition on the chromatography of basic drugs on modern systems involving an octadecylsilica surface without the addition of higher alcohols. Neither has there been any systematic study as to the relative effectiveness of different organic bases in improving the chromatography of basic solutes.

Because of the clinical importance of the tricyclic antidepressant drugs together with the poor chromatography generally associated with these compounds[20], they are used as a model set of compounds in the present investigation. It is intended to examine the effect of several amines and quaternary ammonium salts on the chromatography of the major drugs in this group and to attempt to explain the mode of action in the light of the ion exchange desolvation model of hydrophobic pairing ion retention previously proposed[8,9].

EXPERIMENTAL

Chromatography was carried out using a variety of equipment including Altex (Model 110A) and Waters Associates (M6000A) constant flow pumps. Detection at 254 nm was by Cecil (CE2012) and Pye Unicam (LC3) detectors. The wavelength of

measurement was not optimised in this study. Injection was by a Rheodyne 7125 valve fitted with 20- or 100-μl loops. Columns of conventional design and incorporating Swagelok fittings were either 100 mm or 70 mm long (4.6 mm I.D.) slurry packed at 600 bar with 5-μm ODS Hypersil (Shandon Southern Products). Retention time data were measured directly from chromatograms as recorded on a Servoscribe potentiometric recorder.

The tricyclic antidepressant drugs amitriptyline, nortriptyline, imipramine, desipramine, doxepin, maprotiline, mianserine, nomifensine, protriptyline, trimipramine, clomipramine and dothiepin were kindly donated by their manufacturers. The drugs were used as the hydrochlorides at a concentration of 50 μg cm^{-3}. Sodium lauryl sulphate (SLS), dimethylamine (DMA) and triethylamine (TRIEA) were obtained from Fisons and used as supplied. Methylammonium chloride (META), trimethylamine (TRIMA) and propylamine (PPLA) were obtained from BDH and tetramethyl-(TMA), tetraethyl-(TEA) and tetrabutylammonium (TBA) bromides were obtained from Aldrich. Cetrimide (CETA) was obtained from ICI Pharmaceuticals. Water used in chromatography was distilled before use and acetonitrile (HPLC grade) was obtained from Rathburn Chemicals and Fisons. All other reagents were of AnalaR or similar grade.

RESULTS

The separation obtained among twelve tricyclic antidepressant drugs is summarised in Fig. 1, which shows the variation of capacity factor, k', with the mobile phase concentration of sodium lauryl sulphate used as highly adsorbed pairing ion. While other pairing ions of lower hydrophobicity would affect the magnitude of the k' values obtained, previous work indicated that no substantial improvement in resolution could be obtained[9]. Fig. 1 shows that the capacity factors go through the expected maxima and that for most solutes maximum k' and thus optimum separation is obtained at a SLS mobile phase concentration of 80 mM. While considerable separation is obtained among several of the test compounds at the maximum in k', the clinically relevant separations, namely between imipramine and desipramine and between amitriptyline and nortriptyline, are not adequate. The separations achieved are further demonstrated in Fig. 2 which shows representative chromatograms of selected groups of drugs from among the compounds shown in Fig. 1. Fig. 2 indicates that the addition of pairing ion alone to the mobile phase is adequate to separate certain but not all compounds in the class. It also indicates that good chromatographic peak symmetry is achieved with little evidence of the peak tailing problems claimed to be associated with such compounds[17,18].

In order to evaluate the effect of counter ion type and concentration, various concentrations of different organic base salts, ranging in hydrophobicity from methylammonium chloride to cetrimide, were added to the mobile phase of 80 mM SLS in acetonitrile–buffer (50:50). Fig. 3 shows that the effect of such counter ion addition was general for a given test compound, namely to decrease the capacity factor. This effect was found to be general for all other compounds in the series but the extents of the decrease differed. Fig. 3 also indicates that the effect is greater the greater the apparent hydrophobicity of the added counter ion. The inverse relationship between k' and counter ion concentration previously noted for inorganic counter ions and

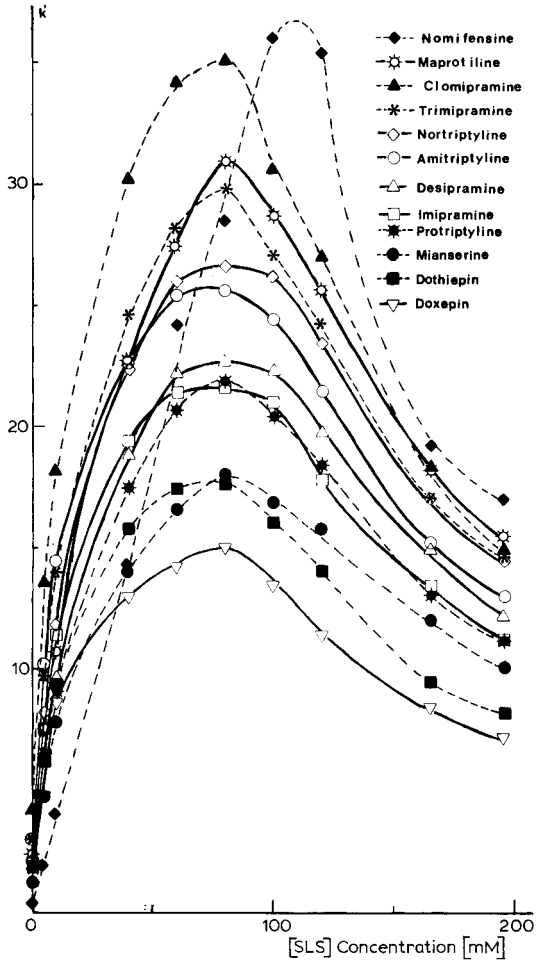

Fig. 1. Plots showing the variation of capacity factor, k', with mobile phase pairing ion (SLS) concentration for twelve tricyclic antidepressant drugs. Chromatographic conditions: column, 100 × 4.6 mm; mobile phase, acetonitrile–10 mM sodium dihydrogen phosphate (50:50) at pH 2; stationary phase, 5-μm ODS Hypersil; flow-rate 2.0 cm^3 min^{-1}.

predicted by the ion-exchange interpretation of ion pairing is not apparent from the present work with hydrophobic counter ions. It is also noted that while peak sharpening occurs, it is as a consequence of the decreased retention time. No real increase in plate number is obtained as a result of organic counter ion addition. Of greater significance is the effect of organic counter ion on the resolution between the pair amitriptyline and nortriptyline as shown in Fig. 4. The effect of adding amine to the mobile phase is to increase the resolution for this pair of compounds. The improvement is most marked in the case of cetrimide and least for methylammonium chloride. The effect of inorganic counter ion was observed to be minimal. These effects were identical for the imipramine–desipramine pair of compounds.

Although cetrimide shows by far the most noticeable effect on resolution both

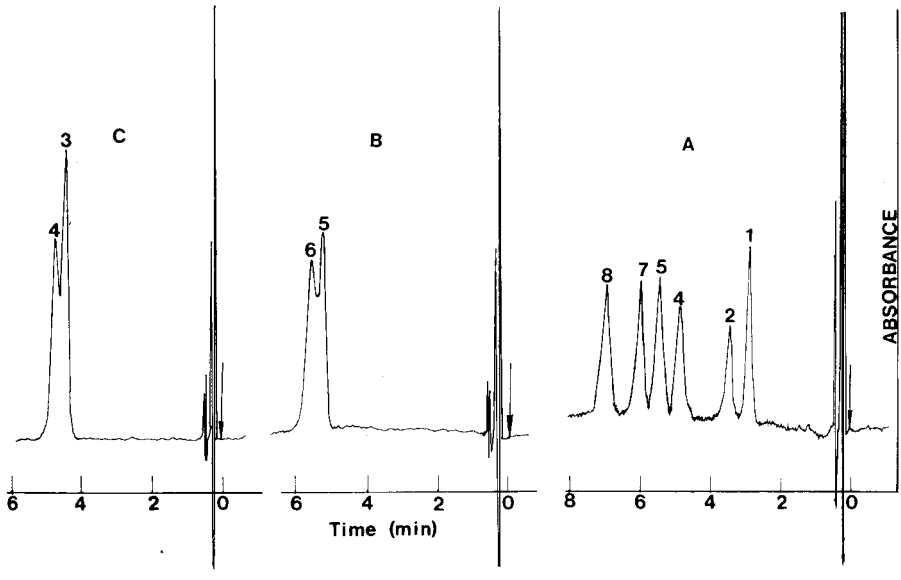

Fig. 2. Representative chromatograms showing complete and incomplete resolution obtained among certain tricyclic antidepressant drugs. Chromatographic conditions: column, 70 × 4.6 mm; mobile phase as in Fig. 1 with SLS concentration 80 m*M*. Compounds: 1 = doxepin; 2 = mianserine; 3 = imipramine; 4 = desipramine; 5 = amitriptyline; 6 = nortriptyline; 7 = trimipramine; 8 = clomipramine.

in the degree of resolution obtainable and in the minimum concentration required to achieve this, the retention times become very short and thus unacceptable for application to assay situations. Tetrabutylammonium (TBA) ion provides a better compromise between retention time and resolution for the above pair of compounds. Representative chromatograms are shown in Fig. 5, indicating the separations that can be achieved among selected members of the tricyclic antidepressant group of compounds by the addition of 5 m*M* TBA to 80 m*M* SLS. Comparison of Fig. 5 with Fig. 2 shows that although retention times are shorter, the peaks are considerably sharper and in fact measured plate numbers are comparable. It is also observed that resolution among the compounds has changed and that compounds previously separable without addition of added counter ion are now unresolved and *vice versa*. Complete data for the retention of all twelve compounds as a function of mobile phase pairing ion concentration in presence of a fixed TBA concentration of 5 m*M* are shown in Fig. 6. The maximum in k' with pairing ion concentration is still apparent but the relative magnitude of the capacity factors at the maximum is radically altered.

DISCUSSION

Previous explanations of the effect of added amine during the separation of basic drugs have suggested that the improvement in chromatographic behaviour is either as a result of decreased tailing due to the inactivation of unreacted silanol groups or alternatively, as a result of the suppression of secondary equilibria, namely the dissociation of ion pairs desolvated on the C-18 surface. Both of these expla-

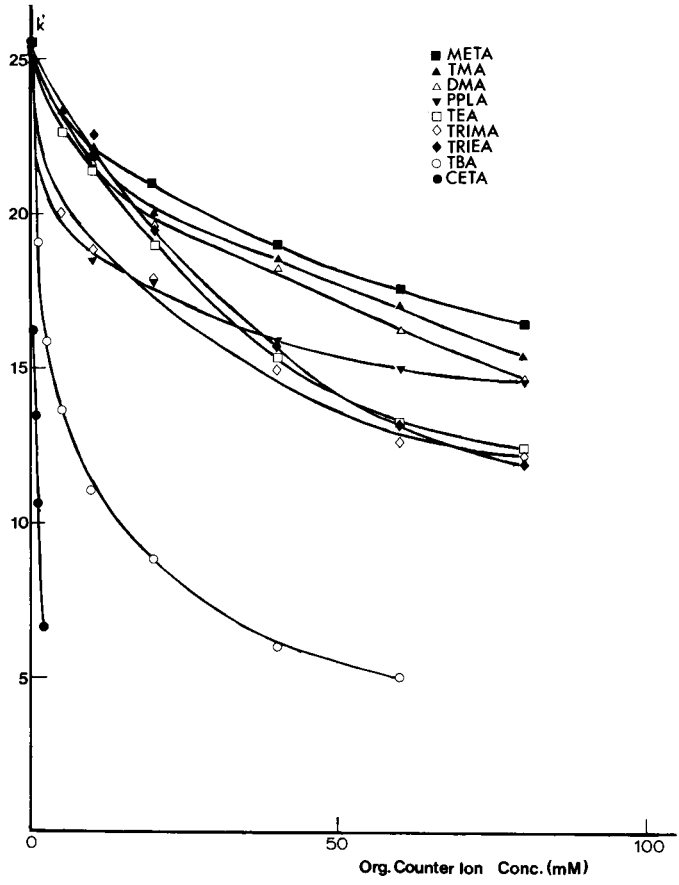

Fig. 3. Plots showing the variation in capacity factor, k', for amitriptyline as a function of various added counter ion concentrations. Chromatographic conditions as in Fig. 2, using a 100 × 4.6 mm column.

nations would require that the effect be general for all solutes and would result in a measurable increase in column efficiency for all basic solutes.

Any increase in efficiency in such systems involving added amine would be as a result of an increased rate of mass transfer. The mass transfer coefficient, measured as the slope of the HETP *vs.* mobile phase linear velocity curve at high linear velocities, was compared in presence and absence of 5 m*M* TBA for four solutes in a mobile phase of acetonitrile–water containing 80 m*M* SLS. Fig. 7 shows such plots for amitriptyline. No significant alteration in gradient was observed on addition of amine for any of the four solutes. This was taken as indicating constancy in the mass transfer rate and as further verifying the lack of real increase in column efficiency.

While the efficiency of the column is unaffected by addition of organic counter ion, resolution among the different solutes is markedly altered. The quantities contributing to resolution among any pair of compounds are included in the equation[21]

$$R = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1 + k'}\right)N^{\frac{1}{2}}$$

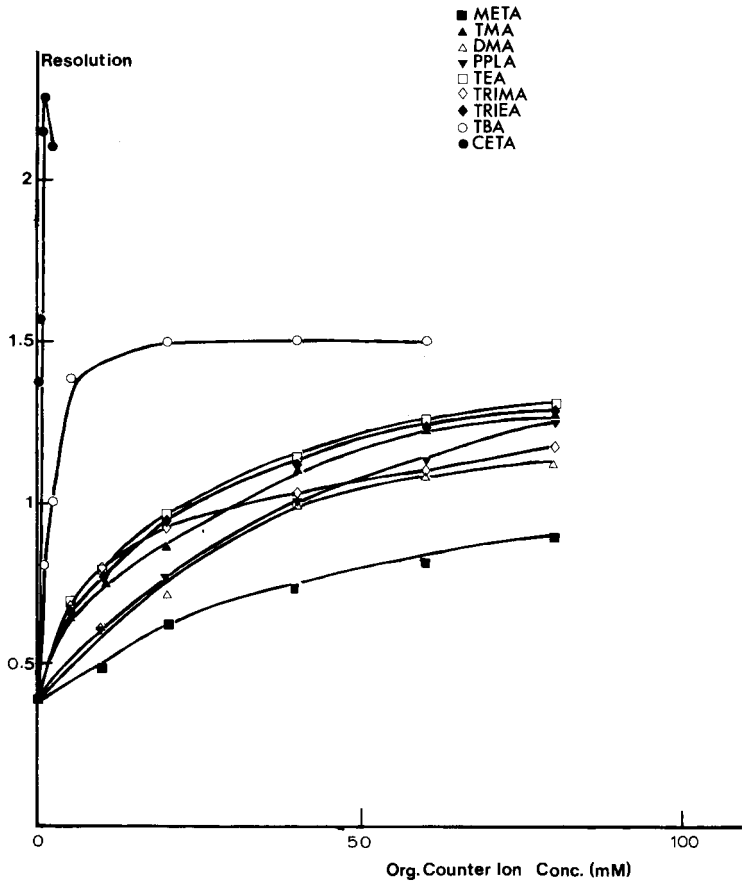

Fig. 4. Plots showing resolution between amitriptyline and nortriptyline as a function of various added counter ion concentrations. Chromatographic conditions as in Fig. 3.

where α represents the relative retention of the pair of compounds, k' the larger capacity factor and N the measured number of theoretical plates. In the present investigation, N is constant and the effect of added amine is to reduce k' so that any increase in R would appear to be as a consequence of a change in the selectivity term $(\alpha - 1)/\alpha$. The variation of $(\alpha - 1)/\alpha$ with concentration of added counter ion is shown in Fig. 8 and the overall shape of the plots appears to parallel that of the resolution as shown in Fig. 4. The ion exchange desolvation model formulated for hydrophobic pairing ions on octadecylsilica surfaces using aqueous mobile phases[8] can be used to explain the present findings.

This model represents retention of a basic solute in a completely ionised form by a hydrophobic anionic pairing ion adsorbed to an equilibrium extent on the C-18 surface. The basic equilibrium is one of ion exchange between the solute and the counter ion reinforced by desolvation of the solute on the C-18 surface. In the present investigation the hydrophobic nature of the counter ions will modify the behaviour from that observed with inorganic counter ions such as buffer salts, the interaction of which with the stationary phase pairing ion is purely electrostatic. When counter ions

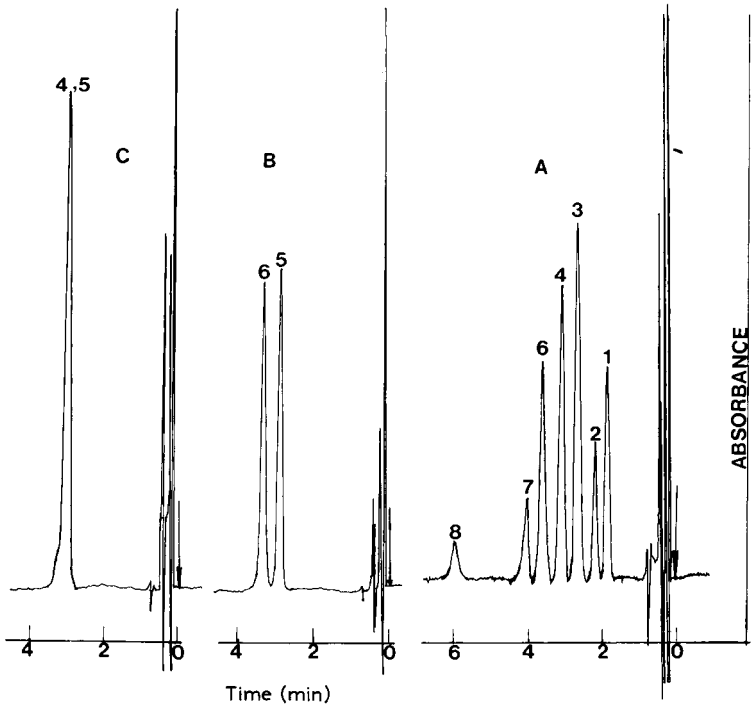

Fig. 5. Representative chromatograms showing complete and incomplete resolution obtained among certain tricyclic antidepressant drugs in presence of added organic counter ion. Chromatographic conditions as in Fig. 2 with the addition of 5 m*M* TBA to the mobile phase. Compounds: 1–6 as in Fig. 2; 7 = maprotiline; 8 = nomifensine.

differing in hydrophobic character are used, the counter ion, as well as being bound to the pairing ion by coulombic forces, is also desolvated on the adjacent C-18 surface. This equilibrium situation is identical in nature with the transient equilibria, producing retention of any hydrophobic solute. For retention of a solute to be achieved when organic counter ions are employed, not only must ion exchange occur between solute and counter ion, but the counter ion must be resolvated into the mobile phase. Thus, the more hydrophobic the counter ion, the more difficult will the resolvation process be and the greater will be the reduction in retention for a given concentration of added counter ion. Fig. 3 is interpreted on this basis as representing the effect of gradual replacement of inorganic counter ion by organic amine. The equilibrium reaction producing retention in the situation involving organic counter ion may be represented as before by the ion exchange reaction

$$(P^-C^+)_{org} + A^+_{aq} \rightleftharpoons (P^-A^+)_{org} + C^+_{aq} \quad (1)$$

where $(P^-C^+)_{org}$ represents the adsorbed pairing ion together with its associated counter ion and A^+ represents the fully ionised solute. The equilibrium constant, K_{IE}, will depend upon which species predominates as the counter ion C^+. That is, K_{IE} will vary from a maximum when the organic counter ion concentration is negligible to a

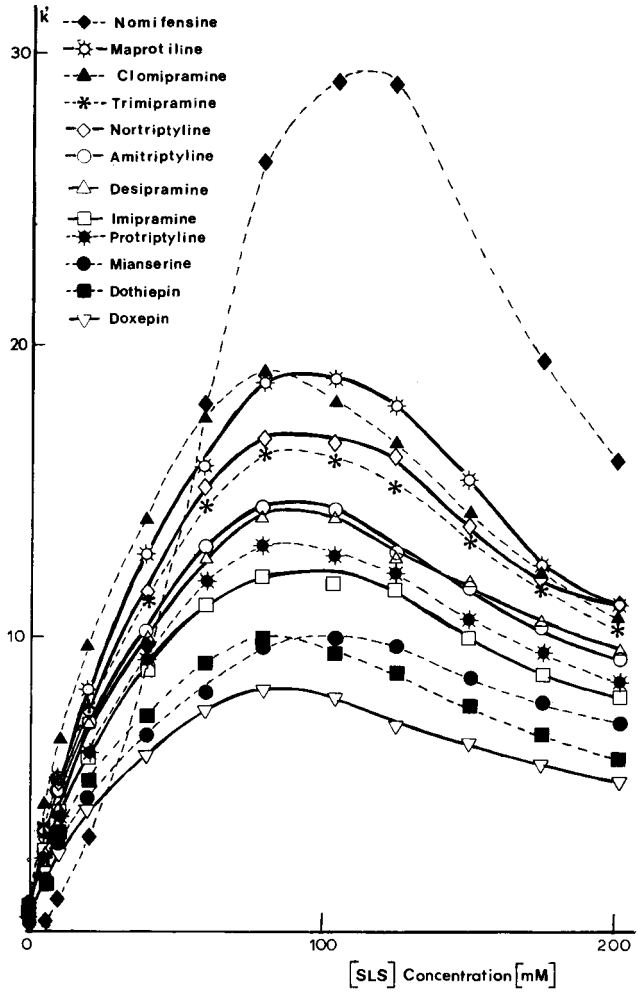

Fig. 6. Plots showing variation in k' with mobile phase pairing ion (SLS) concentration for antidepressant drugs. Chromatographic conditions as in Fig. 1 with the mobile phase modified to include 5 mM TBA.

minimum when all of the inorganic counter ion has been replaced by the added amine. Thus two limiting forms of the above equilibrium may be written:

$$(P^-Na^+)_{org} + A^+_{aq} \rightleftharpoons (P^-A^+)_{org} + Na^+_{aq} \ (K_{IE} \text{ large}) \quad (2)$$

$$(P^-TBA^+)_{org} + A^+_{aq} \rightleftharpoons (P^-A^+)_{org} + TBA^+ \ (K_{IE} \text{ small}) \quad (3)$$

At intermediate conditions, where not all of the sodium has been replaced due either to the limited hydrophobicity of the added amine, or to its low concentration, the value of K_{IE} will be intermediate between the two extremes.

The equation derived previously relating capacity factor to adsorbed pairing

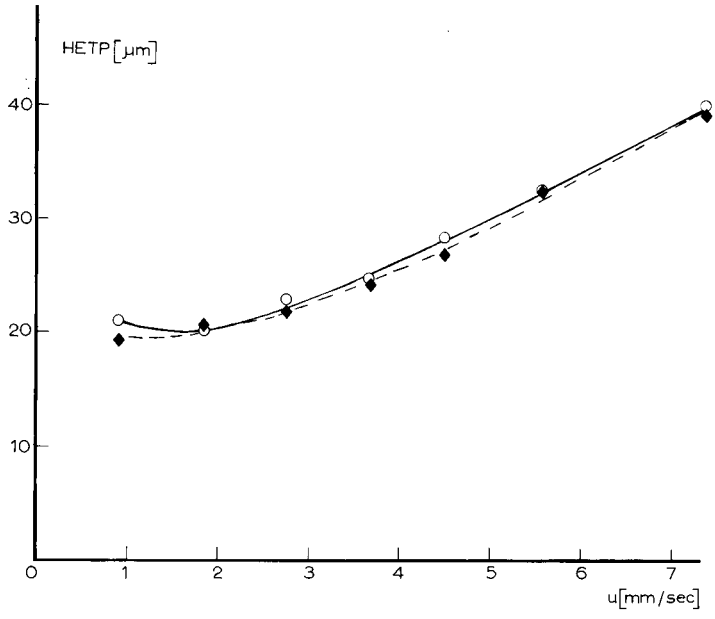

Fig. 7. Plot showing variation of HETP with linear mobile phase velocity for amitriptyline. Chromatographic conditions: O—O, as in Fig. 2, *i.e.*, with no added counter ion; ◆ ---◆, as in Fig. 5, *i.e.*, in presence of 5 m*M* TBA.

ion and aqueous counter ion concentration[8,9] will still apply for the condition where the counter ion concentration is held constant, *i.e.*:

$$k' = \frac{1}{V_m}\left(A_s K_1 - K_1 [P^- C^+]_{org} A_p + K_2 K_{IE} \frac{[P^- C^+]_{org}}{[C^+]_{aq}} - K_2 K_{IE} A_p \frac{[P^- C^+]^2_{org}}{[C^+]_{aq}} \right. \quad (4)$$

Eqn. 4 represents an improvement over the purely ion exchange processes represented above in that the ion exchange and desolvation tendency of a particular solute is represented by a combined constant K_2K_{IE}. The term K_1 refers to the desolvation constant of the solute in the non-ion pairing situation and will be small in comparison with the K_2K_{IE} term when separation is obtained. A_s and A_p are the areas of the stationary phase and pairing ion on a molar basis respectively, and V_m the void volume of the column.

This form of equation is seen to apply both in the situation of pairing ion and buffer only for all the tricyclic antidepressant drugs investigated as shown by Fig. 1 and also for the case of a fixed concentration of TBA as organic counter ion in presence of SLS pairing ion as shown in Fig. 6. In this equation in the case of organic counter ion the K_2K_{IE} term will have the increased significance that it will reflect not only the desolvation of a given solute subsequent to ion exchange but it will provide an estimate of the desolvation tendency of a solute displacing an already desolvated organic counter ion. This is seen as providing an additional parameter of selectivity in such systems and may account for the alteration in elution order in Fig. 6 compared with Fig. 1. It is emphasised that while in this case the selectivity alteration has been

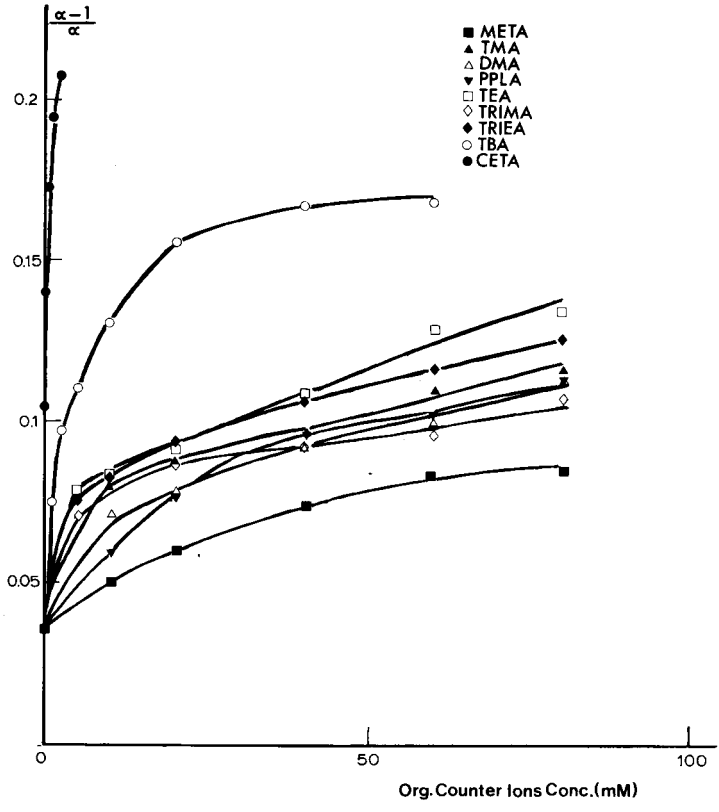

Fig. 8. Plots of the variation of selectivity $(\alpha - 1)/\alpha$ between amitriptyline and nortriptyline as a function of different organic counter ion concentrations. Chromatographic conditions as in Fig. 3.

to improve the resolution for the pairs desipramine and imipramine and amitriptyline and nortriptyline, its effect is also to reduce the resolution between other pairs of compounds as shown in Fig. 5C for desipramine and amitriptyline.

The applicability of the above equation on a quantitative basis for the above pairs of compounds can be assessed by determining the K_2K_{IE} constants for each compound in presence and absence of organic counter ion. The K_2K_{IE} constants were evaluated by fitting the curves for the appropriate compound as shown in Figs. 1 and 6 to the above equation by the method of least squares using the Hooke software system previously employed[8,9]. $[P^-C^+]_{org}$ values were obtained by interpolation of values from previous isotherms[9] measured at 30% and 60% acetonitrile concentrations and it is assumed on this model that the area available for desolvation of solutes will be unaffected by the presence of the more bulky organic counter ion. If the equation is applicable to such systems involving added organic counter ion, the numerical values of K_2K_{IE} should be seen to decrease in the presence of organic counter ion. In addition the separation factors, α, which can be measured by direct observation of the relative retention times between pairs of compounds should be identical with the ratio of the calculated K_2K_{IE} values for that pair of compounds obtained by the above curve fitting procedure. The results of such a comparison are shown in

TABLE I

OBSERVED SEPARATION FACTORS AND CALCULATED RATIOS OF ION EXCHANGE DESOLVATION CONSTANTS, K_2K_{IE}, DERIVED FROM EQN. 4 IN PRESENCE AND ABSENCE OF ORGANIC COUNTER ION FOR SELECTED PAIRS OF COMPOUNDS

Compound	*SLS (80 mM)*			*SLS (80 mM) + TBA (5 mM)*		
	K_2K_{IE}	K_2K_{IE} *ratio*	α	K_2K_{IE}	K_2K_{IE} *ratio*	α
Imipramine	0.179	1.04	1.05	0.0955	1.188	1.129
Desipramine	0.185			0.114		
Amitriptyline	0.208	1.06	1.04	0.109	1.250	1.123
Nortriptyline	0.219			0.136		
Desipramine	0.185	1.12	1.13	0.114	1.05	1.03
Amitriptyline	0.208			0.109		

Table I. It is seen that the expected decrease in K_2K_{IE} is observed and that good agreement is obtained between α values and K_2K_{IE} ratios calculated both for the situation where resolution has been improved and for that in which resolution has been decreased.

CONCLUSIONS

The present investigation using the closely related group of tricyclic antidepressant drugs as a model system indicates that the action of an organic amine when added to a mobile phase containing hydrophobic anionic pairing ion is that of a counter ion involved in an ion exchange desolvation process. Such species, because of their desolvation on the C-18 surface, act to reduce capacity factors for all basic solutes. They may also provide an additional degree of selectivity over that observed in such systems in absence of added amine. The effect may be utilised to increase resolution among certain compounds but may also act to reduce resolution among others. It has been found that the systems discussed can provide the required selectivity and sensitivity for the measurement of these drugs in serum at therapeutic levels. The quantitative results of such an assessment will be the subject of a future communication.

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CHROM. 14,617

EVALUATION OF POLYETHYLENE GLYCOL-HT AS A STATIONARY PHASE FOR CAPILLARY COLUMN GAS–LIQUID CHROMATOGRAPHY OF TRIMETHYLSILYL ETHERS OF BILE ACID METHYL ESTERS

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SUMMARY

Polyethylene glycol-HT was introduced as a polar stationary phase for bile acid analysis in capillary column gas–liquid chromatography. It has an excellent thermal stability compared with polyethylene glycol 20,000. Helium can be used as carrier gas. These characteristics show the superiority of this liquid phase to polyethylene glycol 20,000 which is of similar polarity. The relative retention times of some bile acid derivatives on a polyethylene glycol-HT capillary column are reported.

INTRODUCTION

Capillary column gas–liquid chromatography is a well established method for bile acid analysis[1,2]. Several non-polar or weakly polar stationary phases such as SE-30, OV-1, OV-101 and SP-2250 have been used for this purpose[2–5]. Although an attempt was made to produce polar capillary columns[6], polyethylene glycol 20,000 (PEG 20,000) is the only polar stationary phase available commercially for bile acid analysis[1]. Hydrogen is used as a carrier gas for the PEG 20,000 capillary column, which is potentially hazardous. Furthermore, in our experience, satisfactory results are not obtained using a PEG 20,000 capillary column to analyze trimethylsilyl (TMS) ethers of bile acid methyl esters, which indicates the possible instability of this stationary phase for bile acid analysis by gas chromatography.

Polyethylene glycol-HT (PEG-HT) is a derivative of polyethylene glycol having a molecular weight higher than 20,000. It has the similar polarity as PEG 20,000 and has been used as a stationary phase for fatty acid and sugar analyses[7]. We have evaluated PEG-HT for bile acid analysis in capillary column gas–liquid chromatography. The relative retention times of such derivatives as TMS ethers of bile acid methyl esters and ketonic bile acid methyl esters on both PEG-HT and OV-1 capillary columns are described.

MATERIALS AND METHODS

PEG-HT and OV-1 support-coated open tubular glass capillary columns were obtained from Gasukuro Kogyo Co. Ltd. (Tokyo, Japan). The sources of standard

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bile acids used in this study were as reported before[8]. In addition, 3α,6β,7β-trihydroxy-5β-cholanoic acid was purchased from Steraloids (Wilton, NH, U.S.A.). 3-Keto-5β-cholanoic acid and 7-keto-5β-cholanoic acid were synthesized by chromium oxidation from their parent hydroxycholanoic acids[9]. All compounds were confirmed by combined gas chromatography–mass spectrometry (GC–MS). Pyridine, hexamethyldisilazane and trimethylchlorosilane were obtained from Pierce (Rockford, IL, U.S.A.), and the remaining reagents and solvents were of analytical grade from Wako (Osaka, Japan).

The standard bile acids were dried at room temperature in vacuum overnight. They were methylated with freshly distilled diazomethane, and trimethylsilylation of hydroxycholanoic acid methyl esters was performed with pyridine–hexamethyldisilazane–trimethylchlorosilane (3:2:1) at 45°C for 30 min[10]. Immediately before the application of bile acid derivatives on to the capillary column, the TMS reagent was removed with a stream of nitrogen and the samples were redissolved in *n*-hexane. The purity of such standard bile acids as lithocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and cholic acid was checked by thin-layer chromatography and gas–liquid chromatography using a QF-1 packed column. All except lithocholic acid had no appreciable amount of impurity. Lithocholic acid had about 4% of impurity, but was used without further purification.

A gas chromatograph (Shimadzu GC-7A) was equipped with a flame ionization detector and an all-glass solid injector (Shimadzu, Kyoto, Japan). Helium was used as a carrier gas at a flow-rate of about 1.5 ml/min. The temperature of the oven was kept at 230°C during the isothermal analysis. The injector and detector temperatures were 280°C. The signals from the detector were processed by an integrator (Model 7000A; System Instruments Co., Ltd., Tokyo, Japan). Combined GC–MS was performed using Hitachi 063 and Hitachi M-60 instruments (Hitachi Ltd., Tokyo, Japan). The temperatures of the molecular separator and ion source were 250 and 200°C, respectively.

The relative retention times were determined in triplicate using the standard bile acids. The detector response and reproducibility were studied using mixtures of lithocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and cholic acid. 5α-Cholestane was used as an internal standard. 0.2-nmol amounts of all compounds were injected. The results were calculated from the areas of the peaks obtained from the successive injections of five mixtures, the area of 5α-cholestane being taken as 1.00 in each case.

RESULTS AND DISCUSSION

A PEG-HT capillary column is made by coating with a specially pretreated polyethylene glycol which has a molecular weight higher than 20,000. Both support-coated open tubular glass and silica capillary columns are available having maximum operating temperatures of 240 and 250°C, respectively. Thus, their thermal stability is superior to that of the PEG 20,000 capillary column. Generally, bile acid analysis by gas–liquid chromatography requires a high column temperature, from which point of view the thermal stability of PEG-HT is of particular use. The major fragment ions of PEG-HT were *m/z* 207, 281, 355, 429, 503 and 577 by mass spectrometry. The most intense one was at *m/z* 207. Thus, the background ions of PEG-HT would not inter-

fere with the MS analysis of bile acid TMS ether derivatives. The use of hydrogen as a carrier gas can be hazardous. In this study helium was used for the PEG-HT capillary column, which would be safe and available in most laboratories without any additional change of gas chromatographic systems.

With regard to the column performance of PEG-HT, as shown in Fig. 1 the analysis time was short and a baseline separation was nearly obtained for the main TMS ethers of bile acid methyl esters under the isothermal conditions. The separation of these compounds will be improved by the temperature programming technique. The absolute retention times of these compounds did not change over a period of at least 5 months on the same capillary column. Therefore, it was not necessary to alter

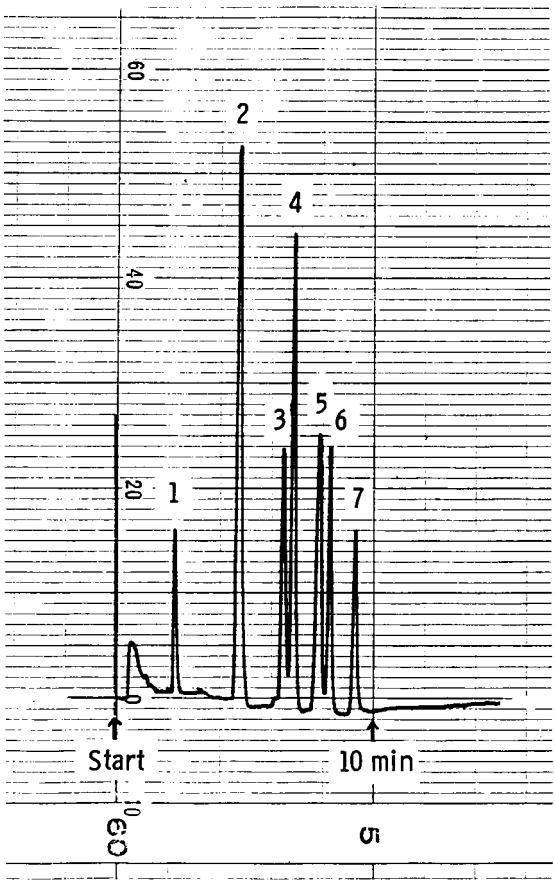

Fig. 1. Gas chromatogram of trimethylsilyl ethers of standard bile acid methyl esters and 5α-cholestane as internal reference on a 20-m PEG-HT support-coated open tubular glass capillary column. Peaks: 1 = 5α-cholestane; 2 = cholic acid and 7α-monohydroxy-5β-cholanoic acid; 3 = deoxycholic acid; 4 = chenodeoxycholic acid; 5 = hyodeoxycholic acid; 6 = lithocholic acid; 7 = ursodeoxycholic acid. Note the overlapping of the trimethylsilyl ethers of cholic acid methyl ester and of 7α-monohydroxy-5β-cholanoic acid methyl ester. The internal diameter of the column was 0.28 mm. Temperatures: oven, 230°C; injector and detector, 280°C. Carrier gas (helium) flow-rate, about 1.5 ml/min. A Van den Berg solventless injector was used.

the parameters of retention times for the integrator during those 5 months in order to analyze biological samples.

The relative retention times of several TMS ethers of bile acid methyl esters and ketonic bile acid methyl esters on PEG-HT are shown in Table I together with those on OV-1. The polarity of PEG-HT is almost the same as that of PEG 20,000. Accordingly, they afford similar relative retention times for some of the bile acid derivatives[1]. A major characteristic of PEG-HT was the retardation of ketonic bile acid methyl esters. For instance, 3,7,12-triketo-5β-cholanoic acid methyl ester was not eluted from a 10-m PEG-HT capillary column within 3 h. This was also the case for ketonic TMS ethers of cholanoic acid methyl esters, whose retention times were increased remarkably by the presence of ketonic group(s). Since the advantage of the PEG-HT capillary column was a quick elution of the compounds, the analysis of di- or triketocholanoic acid methyl esters did not seem practical. Another disadvantage was the overlapping of the TMS ethers of cholic acid methyl ester and of 7α-monohydroxy-5β-cholanoic acid methyl ester. When GC–MS was used, however, this overlapping could easily be resolved by the selective ion monitoring technique.

The detector response and reproducibility are shown in Table II. The detector response for the PEG-HT capillary column did not correspond with the weight of the

TABLE I

RELATIVE RETENTION TIMES OF BILE ACID METHYL ESTER DERIVATIVES ON A 20-m PEG-HT AND A 10-m OV-1 SUPPORT-COATED OPEN TUBULAR GLASS CAPILLARY COLUMN

The oven temperature for OV-1 was 240°C; other operating conditions as in Fig. 1.

*Functional groups of bile acid methyl ester**	*RRT***	
	PEG-HT	*OV-1*
None	0.35	0.41
3α-TMS	1.27	0.85
3β-TMS, 5-cholenoic acid	1.66	0.98
3-Keto	4.08	0.86
7α-TMS	0.72	0.61
3α,6α-Di-TMS	1.21	1.14
3α,7α-Di-TMS	1.06	1.07
3α,7β-Di-TMS	1.46	1.21
3α,12α-Di-TMS	1.00	1.00
3α-TMS, 7-keto	4.06	1.36
3,7-Diketo	13.10	1.30
3,12-Diketo	12.92	1.30
3α,6α,7α-Tri-TMS	0.95	1.40
3α,6β,7β-Tri-TMS	1.14	1.49
3α,7α,12α-Tri-TMS	0.72	1.11
3α,7α-Di-TMS, 12-keto	2.97	1.70
3α,12α-Di-TMS, 7-keto	3.21	1.64
3α-TMS, 7,12-Diketo	13.56	1.93
3,7,12-Triketo	–	1.81

* In 5β-cholanoic acid methyl ester unless otherwise indicated. TMS = Trimethylsilyl ether.

** Retention times relative to trimethylsilyl ether of deoxycholic acid methyl ester at 393 and 1308 sec on PEG-HT and OV-1, respectively.

TABLE II

DETECTOR RESPONSE OF TRIMETHYLSILYL ETHERS OF BILE ACID METHYL ESTERS ON A 20-m PEG-HT SUPPORT-COATED OPEN TUBULAR GLASS CAPILLARY COLUMN

The operating conditions were as in Fig. 1. 5α-Cholestane was used as an internal standard. 0.2-nmol amounts of compounds were injected. The results were calculated as mean ± S.D. of the ratios of the area of each bile acid peak to the area of 5α-cholestane, from five standard mixtures. The actual areas of 5α-cholestane in those samples as determined by the integrator were 21,232 ± 1276 (mean ± S.D.). TMS = Trimethylsilyl ether; ME = methyl ester.

Compound	*Detector response (mean ± S.D.)*
5α-Cholestane	1.00 –
Lithocholic acid TMS ME	1.23 ± 0.10
Deoxycholic acid TMS ME	1.43 ± 0.07
Chenodeoxycholic acid TMS ME	1.63 ± 0.08
Ursodeoxycholic acid TMS ME	1.59 ± 0.10
Cholic acid TMS ME	2.00 ± 0.04

unsubstituted parent compounds, but did roughly with the actual injected weight of the substituted compounds. The reproducibility was satisfactory. Linearity of the detector response was demonstrated in the examined range of 0.05–0.8 nmol of the bile acids listed in Table II.

In conclusion, PEG-HT is one of the very few polar liquid phases suitable for bile acid analysis by capillary column gas–liquid chromatography. It offers a short analysis time by its excellent thermal stability, and is particularly useful for most of the hydroxy bile acids in biological samples.

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CHROM. 14,612

SEPARATION OF FLAVONOIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The ability of a reversed-phase high-performance liquid chromatographic system to separate flavonoids [LiChrosorb RP-18 and a combination of an isocratic and a gradient (5% aqueous formic acid and methanol) technique] has been studied. Retention times of some 141 flavonoids ranging from triglycosides to aglycones are reported. The correlations between structure and t_R values are discussed.

INTRODUCTION

For the separation and quantification of non-volatile compounds in complex mixtures, high-performance liquid chromatography (HPLC) is at present perhaps the method of choice. Indeed, in comparison with gas–liquid chromatography (GLC) there is no need for derivatization, and also detection of compounds can be much better adapted to each specific problem, *e.g.*, by variation of wavelength with photometric detection. In addition, quantification is as straightforward as with GLC.

For polar substances, *e.g.*, flavonoids, the reversed-phase (RP) technique is far superior to the normal technique, since there is no danger that some highly polar substance(s) may be retained irreversibly, with the result that the separation characteristics of the column could be gradually changed. The RP column employed throughout this work has been in constant use for about 2 years without any significant change in its separation characteristics.

Most papers published on the HPLC separations of flavonoids have dealt with only a limited number of compounds[1–18]. However, we have successfully separated a whole array of substituted cinnamoyl- and benzoylamino acids and peptides by a reversed-phase HPLC system, consisting of a LiChrosorb RP-18 column and a combination of isocratic and linear gradient elution[19]. We therefore decided to investigate

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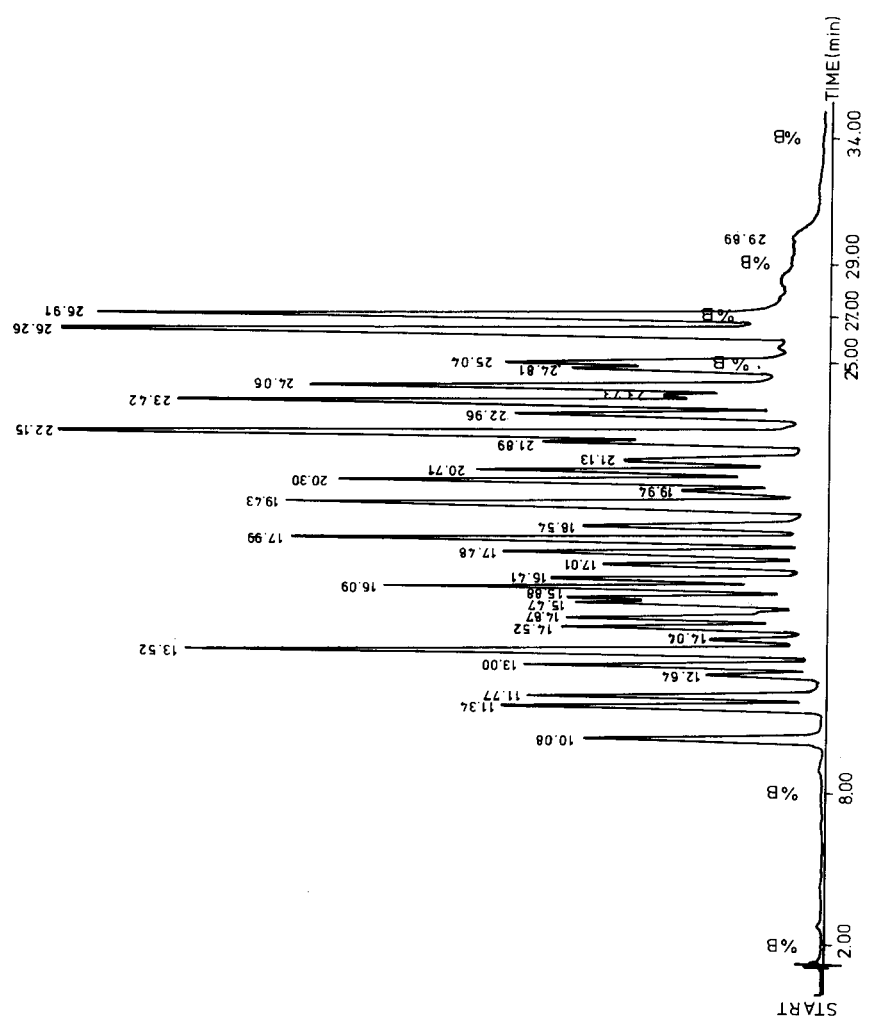


Compound	t_R *(min)*
Kaempferol-3-sophoroside-7-glucoside	10.08
Quercetin-3,7-diglucoside	11.34
Kaempferol-3-sinapoylsophoroside-7-glucoside	11.77
Kaempferol-3,7-diglucoside	12.64
Kaempferol-3-rutinoside-7-glucoside	13.00
Quercetagitrin	13.52
Tricetin-7-glucoside	14.04
Luteolin-3',7-diglucoside	14.52
Myricitrin	14.87
Hyperosid	15.47
Isoquecitrin	15.88
Hesperidin	16.09
Neohesperidin	16.41
Astragalin	17.01
Luteolin-5-methyl ether	17.48
Daidzein	17.99
Quercetin	18.54
Genistein	19.43
Kaempferol-7-rhamnoside	19.94
Kaempferol	20.30
Apigenin	20.71
3',4'-Dimethoxy-7-hydroxyflavone	21.13
7-Hydroxyflavone	21.89
Pinocembrin	22.15
Tricetin-3',4',5'-trimethyl ether	22.96
Chrysin	23.42
5-Methoxyflavone	23.73
Kaempferid	24.06
2'-Methoxyflavone	24.81
4'-Methoxyflavone	25.04
5-Hydroxyflavone	26.26
Tectochrysin	26.91

Fig. 1. The retention time of flavonoids on a Knauer prepacked column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm). For the eluting system see Experimental. The t_R values differ only by 1–2% RTW (retention time identification window) from the values shown in Tables I–V. These differences, which are within the experimental error, are fully acceptable.

the capacity of the foregoing system to separate a relatively large set of flavonoids ranging from triglycosides to permethylated aglycones.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 1084B liquid chromatograph equipped with a variable-wavelength Pye-Unicam LC3 UV detector and a Knauer prepacked analytical column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm) was used throughout this work.

Elution

Two solvents were used: A, formic acid–water (5:95 v/v); B, methanol. The elution profile was: 0–2 min, 7% B in A (isocratic); 2–8 min, 7–15% B in A (linear gradient); 8–25 min, 15–75% B in A (linear gradient); 25–27 min, 75–80% B in A (linear gradient); 27–29 min 80% B in A (isocratic). The temperature of the oven thermostat was set at 35°C. The flow-rate was 2.5 ml/min and the column pressure 80–100 bar.

Detection

The UV detector was set at 280 nm (optical bandwidth 8 nm).

Samples

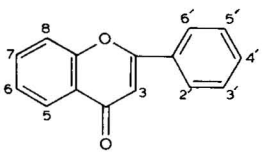
Samples of 0.0025–0.025% solutions in aqueous methanol were applied to the column by means of a 20-μl loop valve. The sources of all the flavonoids examined are given in Tables I–V. Most compounds came from our own laboratories, in which case reference is made either to a paper by one of us, or if the substance has been isolated from a known source, to the name of that plant, and the paper, which describes the first isolation, although the actual isolation might have been performed by present-day methods. Substances marked as synthetic have been synthesized in our laboratories by standard methods, and their identity has been confirmed by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. (Details of these analyses will be published elsewhere.) For substances donated by colleagues, reference is made to the names of those who kindly provided them.

RESULTS AND DISCUSSION

Tables I–V show the retention times of some 141 flavonoids and isoflavonoids and Fig. 1 shows the separation in a single run of a set of 32 different flavonoids. In Table V the t_R values of protogenkwanin (141) and its 4′-glucoside (140) are reported. These two compounds have been included because they must be considered as being very acid labile[24]. However, as they are eluted unchanged it may be concluded that the formic acid-containing solvent system can be safely used with most flavonoids. Further, the t_R values of all compounds are located between 10.05 and 27.39 min; therefore only mixtures of about 40 substances can be separated, because good separations require that the t_R values of the compounds should differ by at least 0.4–0.5. However, 40 flavonoids do not usually occur in a single organism, although in certain

TABLE I

RETENTION TIMES (t_R) VALUES OF FLAVONES AND FLAVONOLS



Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyl and glycosyloxy*		
(a) Flavones					
1 Tricetin-7-glucoside	5,3′,4′,5′	–	7-β-D-Glucopyranosyloxy	13.99	20
2 Vitexin	5,7,4′	–	8-β-D-Glucopyranosyl	14.44	C. Roth, Karlsruhe, G.F.R.
3 Luteolin-3′,7-diglucoside	5,4′	–	7,3′-Di-β-D-glucopyranosyloxy	14.60	20
4 Luteolin-5-glucoside	7,3′,4′	–	5-β-D-Glucopyranosyloxy	15.15	20
5 Luteolin-7-glucoside	5,3′,4′	–	7-β-D-Glucopyranosyloxy	15.52	20
6 Tricetin-3′-methyl ether-7-glucoside	5,4′,5′	3′	7-Glucosyloxy	15.76	21
7 Apigenin-7-neohesperidoside	5,4′	–	7-β-D-(2-O-α-L-Rhamnopyranosido)glucopyranosyloxy	16.77	Synthetic
8 Apigenin-7-glucoside	5,4′	–	7-β-D-Glucosyloxy	16.78	21
9 Diosmin	5,3′	4′	7-β-D-(6-O-α-L-Rhamnopyranosido)glucopyranosyloxy	17.10	G. Hrazdina
10 Isoëtin	5,7,2′,4′,5′	–	–	17.19	Synthetic
11 Luteolin-5-methyl ether	7,3′,4′	5	–	17.43	22
12 Tricetin	5,7,3′,4′,5′	–	–	17.59	23
13 Luteolin-3′-glucoside	5,7,4′	–	3′-β-D-Glucopyranosyloxy	17.95	20
14 7,4′-Dihydroxyflavone	7,4′	–	–	18.74	22
15 Chrysoeriol-5-methyl ether	7,4′	5,3′	–	19.16	22
16 Tricetin-3′-methyl ether	5,7,4′,5′	3′	–	19.38	21
17 Luteolin	5,7,3′,4′	–	–	19.34	23
18 3′,4′-Dihydroxyflavone	3′,4′	–	–	19.80	Synthetic
19 Genkwanin-4′-glucoside	5	7	4′-β-D-Glucosyloxy	20.48	24

20 Tricin	5,7,4′	3′,5′	–	20.87	Synthetic
21 Tricetin-7-methyl ether	5,3′,4′,5′	7	–	20.92	Synthetic
22 Apigenin	5,7,4′	–	–	20.93	23
23 Diosmetin	5,7,3′	4′	–	20.96	Synthetic
24 Chrysoeriol	5,7,4′	3′	–	21.09	22
25 4′-Hydroxyflavone	4′	–	–	21.27	Synthetic
26 3′,4′-Dimethoxy-7-hydroxyflavone	7	3′,4′	–	21.32	Synthetic
27 Primetin	5,8	–	–	21.65	J. Chopin
28 7-Hydroxyflavone	7	–	–	21.68	Synthetic
29 Luteolin-7-methyl ether	5,3′,4′	7	–	22.53	Synthetic
30 2′-Hydroxyflavone	2′	–	–	22.86	Synthetic
31 Tricetin-3′,4′,5′-trimethyl ether	5,7	3′,4′,5′	–	23.24	Synthetic
32 Chrysin	5,7	–	–	23.83	Synthetic
33 Tricetin pentamethyl ether	–	5,7,3′, 4′,5′	–	23.88	Synthetic
34 5-Methoxyflavone	–	5	–	23.95	Synthetic
35 Luteolin-7,4′-dimethyl ether	5,3′	7,4′	–	24.21	Synthetic
36 Flavone	–	–	–	24.29	Synthetic
37 Acacetin	5,7	4′	–	24.29	Synthetic
38 Genkwanin	5,4′	7	–	24.31	24
39 2′-Methoxyflavone	–	2′	–	24.65	Synthetic
40 7-Methoxyflavone	–	7	–	24.77	Synthetic
41 4′-Methoxyflavone	–	4′	–	24.88	Synthetic
42 5-Hydroxyflavone	5	–	–	26.24	Synthetic
43 Tectochrysin	5	7	–	27.39	Synthetic
(b) Flavonoles					
44 Kaempferol-3-sophoroside-7-glucoside	5,4′	–	3-β-D-(6-O-β-D-Glucopyranosido)glucopyranosyloxy-7-β-D-glucopyranosyloxy	10.05	20
45 Quercetine-3,7-diglucoside	5,3′,4′	–	3-β-D-Glucopyranosyloxy,7-β-D-glucopyranosyloxy	11.28	25
46 Kaempferol-3-sinapoylsophoroside-7-glucoside	5,4′	–	3-β-D-2-(2-O-Sinapoyl-β-D-glucopyranosyl)glucopyranosyloxy, 7-β-D-glucopyranosyloxy	11.84	20
47 Kaempferol-3,7-diglucoside	5,4′	–	3-β-D-Glucopyranosyloxy, 7-β-D-glucopyranosyloxy	12.64	20
48 Kaempferol-3-rutinoside-7-glucoside	5,4′	–	3-β-D-(6-O-α-L-Rhamnopyranosyl) glucopyranosyloxy, 7-β-D-glucopyranosyloxy	12.98	20

(Continued on p. 86)

TABLE I *(continued)*

Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyl and glycosyloxy*		
49 Quercetagitrin	3,5,6, 3′,4′	–	7-β-D-Glucopyranosyloxy	13.45	From flowers of *Tagetes erecta*[26]
50 Quercetin-3-sophoroside	5,7,3′,4′	–	3-β-D-(2-O-β-D-Glucopyranosido) glucopyranosyloxy	13.61	25
51 Myricetin-3-galactoside	5,7,3′, 4′,5′	–	3-β-D-Galactopyranosyloxy	13.95	20
52 Gossypitrin	3,5,8,3′,4′	–	7-β-D-Glucopyranosyloxy	14.09	Flowers of *Chrysanthenum segetum*[27]
53 Quercetin-3-(6-O-galloyl) galactoside	5,7,3′,4′	–	3-β-D-(6-O-Galloyl(galactopyran-osyloxy	14.61	20
54 Quercimeritrin	3,5,3′,4′	–	7-β-D-Glucopyranosyloxy	14.61	20
55 Quercetin-3-glucoside-7-rhamnoside	5,3′,4′	–	3-Glucosyloxy, 7-rhamnosyloxy	14.61	28
56 Azaleatin-3-galactoside	7,3′,4′	5	3-Galactosyloxy	14.81	22
57 Kaempferol-3-sophoroside	5,7,4′	–	3-β-D-(2-O-β-D-Glucopyranosido) glucopyranosyloxy	14.91	20
58 Myricitrin	5,7,3′,4′,5′	–	3-α-L-Rhamnopyranosyloxy	14.93	20
59 Robinetin	3,7,3′,4′,5′	–	–	15.01	C. Roth, Karlsruhe
60 Quercetin-7-neohesperidoside	3,5,3′,4′	–	7-β-D-(2-O-α-L-Rhamnopyranosido) glucopyranosyloxy	15.07	G. Hrazdina
61 Patulitrin	3,5,3′,4′	6	7-Glucosyloxy	15.28	Flowers of *Tagetes patula*[29]
62 Hyperosid	5,7,3′,4′	–	3-β-D-Galactopyranosyloxy	15.49	20
63 Azalein	7,3′,4′	5	3-Rhamnosyloxy	15.57	Azalea flowers[30]
64 Gossypetin	3,5,7,8, 3′,4′	–	–	15.64	Leaf of *Ledum palustre*[31]
65 Isoquercitrin	5,7,3′,4′	–	3-β-D-Glucopyranosyloxy	15.69	20
66 Quercetagetin	3,5,6,7, 3′,4′	–	–	15.73	Flowers of *Tagetes erecta*[26]
67 Rutin	5,7,3′,4′	–	3-β-D-(6-O-Rhamnosyl)glucosyloxy	15.76	32

68 Robinin	5,4′	–	3-β-D-(6-O-α-L-Rhamnopyranosido) galactopyranosyloxy, 7-α-L-rhamnopyranosyloxy	15.80	C. Roth, Karlsruhe, G.F.R.
69 Kaempferol-3-glucoside-7-rhamnoside	5,4′	–	3-β-D-Glucopyranosyloxy, 7-α-L-rhamnopyranosyloxy	15.92	20
70 Kaempferol-3-rutinoside-7-glucoside	5,4′	–	3-β-D-(6-O-α-L-Rhamnopyranosido) glucopyranosyloxy, 7-β-D-glucopyranosyloxy	15.92	20
71 Datiscin	5,7,2′	–	3-Rhamnosidoglucosyloxy	15.94	Leaf and stem of *Datisca cannabina*[33]
72 Morin	3,5,7,2′,4′	–	–	16.07	Chromatography of commercial product
73 Guajaverin	5,7,3′,4′	–	3-α-L-Arabopyranosyloxy	16.16	20
74 3′-O-Methylmyricetin-3-rhamnoglucoside	5,7,4′,5′	3′	3-Rhamnosidoglucosyloxy	16.18	34
75 Kaempferol-7-glucoside	3,5,4′	–	7-β-D-Glucopyranosyloxy	16.22	20
76 Myricetin	3,5,7,3′, 4′,5′	–	–	16.54	23
77 Spiraeosid	3,5,7,3′	–	4′-β-D-Glucopyranosyloxy	16.55	20
78 Quercitrin	5,7,3′,4′	–	3-α-L-Rhamnopyranosyloxy	16.55	20
79 Avicularin	5,7,3′,4′	–	3-α-L-Arabofuranosyloxy	16.55	20
80 Quercetin-5,3′-dimethyl ether-3-glucoside	7,4′	5,3′	3-Glucosyloxy	16.63	22
81 Tamarixetin-7-glucoside	3,5,3′	4′	7-Glucosyloxy	16.86	Synthetic
82 Tamarixetin-7-rutinoside	3,5,3′	4′	7-β-D-(6-O-α-L-Rhamnopyranosido) glucopyranosyloxy	16.89	20
83 Fisetin	3,7,3′,4′	–	–	16.99	Chromatography of commercial product
84 Astragalin	5,7,4′	–	3-β-D-Glucopyranosyloxy	17.09	20
85 Isorhamnetin-3-glucoside	5,7,4′	3′	3-Glucosyloxy	17.14	G. Hrazdina
86 Herbacetin	3,5,7,8,4′	–	–	17.21	35
87 Cacticin	5,7,4′	3′	3-β-D-Galactopyranosyloxy	17.23	20
88 Isorhamnetin-3-rutinoside	5,7,4′	3′	3-β-D-(6-O-α-L-Rhamnopyranosido) glucopyranosyloxy	17.40	20
89 Distichin	5,7,4′	3′	3-α-L-Arabopyranosyloxy	17.84	21
90 Kaempferol-3-arabinoside	5,7,4′	–	3-L-Arabinosyloxy	17.94	21
91 Isorhamnetin-3-arabofuranoside	5,7,4′	3′	3-α-L-Arabinofuranosyloxy	18.44	21

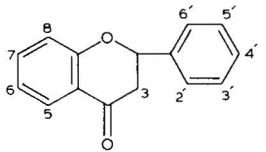
(Continued on p. 88)

TABLE I *(continued)*

Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyl and glycosyloxy*		
92 Galangin-3-rhamnoglucoside	5,7	–	3-Rhamnosidoglucosyloxy	18.51	Leaf and stem of *Datisca cannabina*[33]
93 Quercetin	3,5,7,3′,4′	–	–	18.67	23
94 Patuletin	3,5,7,3′,4′	6	–	18.79	Flowers of *Tagetes patula*[36]
95 3′-O-Methylmyricetin	3,5,7,4′,5′	3′	–	19.04	34
96 Datiscetin	3,5,7,2′	–	–	19.85	Leaf and stem of *Datisca cannabina*[33]
97 Kaempferol-7-rhamnoside	3,5,4′	–	7-α-L-Rhamnosyloxy	20.09	28
98 Kaempferol	3,5,7,4′	–	–	20.48	23
99 Tamarixetin	3,5,7,3′	4′	–	20.86	Synthetic
100 Isorhamnetin	3,5,7,4′	3′	–	21.03	21
101 Rhamnetin	3,5,3′,4′	7	–	22.37	Chromatography of commercial mixture
102 Galangin	3,5,7	–	–	23.97	Rhizoma of *Alpinia officinarum*[37]
103 Rhamnocitrin	3,5,4′	7	–	24.23	Synthetic
104 Galangin-3-methyl ether	5,7	3	–	24.29	Rhizoma of *Alpinia officinarum*[38]
105 Kaempferid	3,5,7	4′	–	24.39	Rhizoma of *Alpinia officinarum*[37]
106 3-Methoxyflavone	–	3	–	24.51	Synthetic
107 Rhamnazin	3,5,4′	7,3′	–	24.53	Chromatography of a commercial mixture with rhamnetine
108 3-Hydroxyflavone	3	–	–	24.88	G. Hrazdina

TABLE II

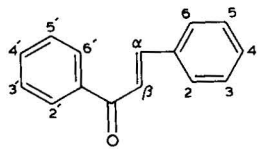
FLAVANONES AND DIHYDROFLAVONOLS



Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyloxy*		
109 Dihydrofisetin	3,7,3′,4′	–	–	11.50	G. Hrazdina
110 Taxifolin	3,5,7,3′,4′	–	–	12.56	W. Steck
111 Eriodictyol-7-glucoside	5,3′,4′	–	7-Glucosyloxy	13.37	Sarsyntex
112 Eriodictyol-7-neohesperidoside	5,3′,4′	–	7-β-D-(2-O-α-L-Rhamnopyranosido)glucopyranosyloxy	14.05	G. Hrazdina
113 Astilbin	5,7,3′,4′	–	3-Rhamnosyloxy	14.72	E. von Rudloff
114 Naringenin-7-glucoside	5,4′	–	7-Glucosyloxy	15.71	Synthetic
115 Naringin	5,4′	–	7-(2-O-Rhamnosido(glucosyloxy	15.71	Sigma
116 Hesperidin	5,3′	4′	7-(6-O-Rhamnosido)glucosyloxy	16.30	Sigma
117 Engelitin	5,7,4′	–	3-Rhamnosyloxy	16.32	E. von Rudloff
118 Neohesperidin	5,3′	4′	7-(2-O-Rhamnosido)glucosyloxy	16.62	W. Steck
119 Eriodictyol	5,7,3′,4′	–	–	16.65	Sarsyntex
120 Naringenin	5,7,4′	–	–	18.71	Sigma
121 Homoeriodictyol	5,7,4′	3′	–	18.72	Leaf of *Eriodictyon californicum*[39]
122 Hesperitin	5,7,3′	4′	–	19.57	Sigma
123 Sakuranitin	5,4′	7	–	21.93	G. Hrazdina
124 Pinocembrin	5,7	–	–	22.41	E. von Rudloff
125 Eriodictyol-7,3′,4′-trimethyl ether	5	7,3′,4′	–	24.30	W. Steck
126 Flavanone	–	–	–	24.53	G. Hrazdina

TABLE III

CHALCONES AND DIHYDROCHALCONES



Substance	*Structure*			*Saturation of α–β double bond*	*t_R (min)*	*Source ref.*
	OH	*OCH_3*	*Glycosyloxy*			
(a) Dihydrochalcones						
127 Phloridzin	4,4′,6′	–	2′-Glucosyloxy	Yes	16.07	BDH
128 Asebotin	4,6′	4′	2′-Glucosyloxy	Yes	18.23	E. von Rudloff
129 Phloretin	4,2′,4′,6′	–	–	Yes	19.28	G. Hrazdina
(b) Chalcones						
130 2′,4′,6′,3,4-Pentahydroxychalcone	3,4,2′,4′,6′	–	–	No	17.76	Sarsyntex
131 Poncirin chalcone	2′,6′	4	4′-β-D-(2-O-α-L-Rhamnopyranosido)glucopyranosyloxy	No	20.47	G. Hrazdina
132 2-Hydroxy-4,6,2′-trimethoxychalcone	2	4,6,2′	–	No	27.39	W. Steck

cases it may be necessary to separate critical pairs of compounds by use of another solvent system or by another suitable method.

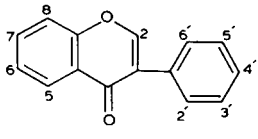
The elution sequence of the individual compounds can best be interpreted by assuming that the compounds are first adsorbed on the hydrophobic stationary phase by "hydrophobic interaction", and that they are subsequently eluted with the mobile phase according to the extent of hydrogen bond formation. Therefore the hydrogen bond donating and/or accepting ability of a given substituent as well as its contribution to the hydrophobic interaction have to be considered. In a methoxyl group, for example, the oxygen is a hydrogen bond acceptor, whereas the methyl group contributes to the hydrophobic interaction. In our system, and with the compounds studied, these two effects balance with the net result that the retention times of tricetin pentamethyl ether (33) and the completely unsubstituted flavone (36) are nearly the same.

The strongest hydrogen bond acceptor in a flavone or isoflavone is the carbonyl group at C-4 which, due to resonance, bears a partial negative charge. If an OH group is present at position 5 a strong internal hydrogen bond is formed between this group and the carbonyl groups, and therefore the latter can no longer interact strongly with the solvent. As a result, the t_R values of the 5-hydroxy-flavones, -isoflavones and -flavonols in Tables I and IV are 1.76–2.29 higher than those of their counterparts not possessing a free 5-OH group. This range applies only to aglycones because for glycosides, which have the ability to form various hydrogen bonds, such generalizations are more difficult (see below).

Hydrogen bonding between the carbonyl group and an OH group in position 3 is much weaker; therefore the t_R value of flavonol (108) is only 0.59 higher than that of flavone (36). If an OH group is present at position 5, introduction of another such group at position 3 usually lowers the t_R values by only 0–1.31. This means that flavones and flavonols, with otherwise identical substitution patterns, are often "critical pairs", which are only poorly separated if at all. On the contrary, introduction

TABLE IV

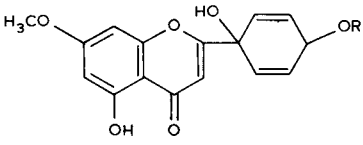
ISOFLAVONES



Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glucosyloxy*		
133 Iridin	5,3′	6,4′,5′	7	16.51	Rhizoma of *Iris germanica*[40]
134 Daidzein	7,4′	–	–	18.15	J. Sachse
135 Genistein	5,7,4′	–	–	19.60	J. Sachse
136 Pratensein	5,7,3′	4′	–	20.31	J. Sachse
137 Irigenin	5,7,3′	6,4′,5′	–	20.43	Rhizoma of *Iris germanica*[40]
138 Formononetin	7	4′	–	21.85	J. Sachse
139 Biochanin A	5,7	4′	–	23.55	J. Sachse

TABLE V

t_R VALUES OF PROTOGENKWANIN AND ITS 4′-GLUCOSIDE



Substance	*Structure*	t_R *(min)*	*Ref.*
140 Protogenkwanin-4′-glucoside	R = β-D-Glucopyranosyl	15.11	24
141 Protogenkwanin	R = H	17.38	24

into a flavanone of an OH group at position 3 (formation of a dihydroflavonol, *e.g.*, 119 and 110) lowers the t_R value considerably (4.09 in the given example).

Hydroxyl groups at positions other than 3 and 5 reduce the t_R values by 1.43–4.59, provided that no OH group is already present at the *ortho* position to the position considered. If an *o*-OH group is already present the decrease in t_R is only 0.86 to 3.27. This means that all flavonoids, which differ in the number of OH groups, at positions other than 3, can easily be separated.

Methylation of OH groups, as already mentioned above, more or less prevents the effect of these groups. This means that, on the one hand, with the exception of flavonol-3-methyl ethers, flavonoids and their partial methyl ethers are easily separated, whereas on the other hand, introduction of additional methoxyl groups has little or no effect on the t_R values. Flavonoids differing only in a methoxyl group are therefore often "critical pairs".

Glycosylation of an OH group means not only introduction of a hydrophilic moiety, but also shielding (be it by hydrogen bonding or just by steric hindrance) of some hydrophilic substituents already present. The latter effect accounts for the striking fact that rutinosides and neohesperidosides show the same t_R values as the corresponding glucosides, although rhamnosylation of a phenolic OH, with no *ortho*-OH, always decrease the t_R value. The same shielding effect also plays a rôle if an OH group located *ortho* to another OH group is glycosylated. For example, if one considers the β-D-glucopyranosides 4, 5, 19, 49, 52, 54, 65, 75 and 84, it can be seen that by comparison with the aglycone, glucosylation of a 7- or 4′-OH, without an adjacent *ortho*-OH, decreases the t_R values by 4.26–3.83, whereas in the presence of an *ortho*-OH the decrease is only 2.28–1.55. Finally, the fact that the t_R value of luteolin-5-β-D-glucopyranoside (4) is only 0.37 smaller than that of the corresponding 7-β-D-glucopyranoside* can also be explained in terms of the shielding effect of the sugar on the carbonyl group. The contributions of various types of sugars to the hydrophilic interaction decrease, as expected, from hexoses through pentoses to methylpentoses. Interestingly enough, arabopyranosides and arabofuranosides are clearly separated. However, glucopyranosides and galactopyranosides, as well as arabofuranosides and rhamnopyranosides, are usually "critical pairs", which are not separated.

* This is rather surprising if one considers the large difference in t_R values of the corresponding methyl ethers.

Saturation of the C-ring, *i.e.*, transformation of a flavone to the corresponding flavanone or of a flavonol to a dihydroflavonol, affects the t_R values in a very complex way. The saturation of the C-ring itself produces only a small effect [*e.g.*, transformation of flavone (36) to flavanone (126) increases the t_R value by only 0.24] but when OH groups are present the t_R values are always decreased. This is because the interruption of the conjugation in the system more or less affects the acidity, and therefore the hydrogen bond accepting and donating abilities of all OH groups. This effect is most pronounced with the 3-OH, which is phenolic in the former (36) and alcoholic in the latter (126) (in our examples dihydroflavonols move 4.41–6.11 and flavanones only 1.06–2.78 min faster than their fully unsaturated counterparts).

In the above discussion attention has been paid not only to the separations which can be achieved by our system, but also to the "critical pairs" of related compounds, which are usually not separated. It is hoped that such a discussion provides a guideline in the search for a suitable complementary system. There remains another type of "critical pairs", *i.e.*, pairs of totally unrelated compounds, and in this case the above discussion cannot be used as a guideline in the search for chromatographic conditions which in a most general way would complement the system described in this paper.

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ROUTINE QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS OF PESTICIDES FOR QUALITY AND PRODUCTION CONTROL USING CAPILLARY COLUMNS AND ON-COLUMN (SYRINGE) SAMPLING*

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(Received December 28th, 1981)

SUMMARY

Quantitative routine analyses of a technical fungicide have been performed and also an inter-laboratory data comparison was carried out using capillary columns and the on-column (syringe) sampling technique. The precision and accuracy of the data obtained with on-column sampling were compared with those obtained by split sampling. The influence of the various sampling parameters on the precision and accuracy, such as solvent volatility, injector and column temperature and adsorptivity of the column support surface, was studied.

INTRODUCTION

Capillary columns allow high-efficiency and therefore also high-resolution separations. In practice, adequate resolution only is necessary for the separation and reliable determination of all significant components of a mixture including those which are present in minor concentrations. A high column separation efficiency may also help to reduce analysis times and to increase signal-to-noise ratios in the analysis for trace components, because of the steeper peak profiles obtainable.

For the routine chromatographic analysis of industrial samples for quality and production control purposes, the following requirements have to be met: (a) the information about the qualitative composition of a certain product has to be as complete as possible (including the information about the trace components that may, for example, have a high toxicity); (b) the quantitative data about the product composition must be of high, if not of ultimate, precision and accuracy for inter-laboratory comparisons, and also for economic and/or environmental reasons; and (c) the overall analysis times should not be too long, in order for results to be obtained rapidly and for optimal utilization of instrument and manpower capacities.

The samples, the quantitative and qualitative gas chromatographic (GC) analysis of which is considered in this paper, preferably contain a major component or their isomers in high concentrations (between 50 and 100%). In some instances such com-

* Dedicated to Professor Dr. mult. Otto Bayer on the occasion of his 80th birthday

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pounds may also be diluted in solvents to give concentrations of about 0.5%. Impurities that may also be present in such products, but in much lower concentrations (*e.g.*, 0.1% and less), must first be separated from the main components and subsequently identified and determined. The relative standard deviations of the repeatability of quantitative data on the major components (which may be present in concentrations between 1 and 99%) should be as low as 0.5–1.0%. With packed columns, such low relative standard deviations are considered common, whereas with capillary columns greater difficulties are expected. Indeed, a good knowledge and some experience are necessary with respect to the various steps of such GC analyses in order to achieve high performance and high resolution. Errors may arise as a result of irreversible adsorption on the support surface within the column and inadequate detection and data processing. The total content of the stationary phase in capillary columns of the commonly used length and inner diameter is as low as a few milligrams, depending on the film thickness, which is usually in the range 0.1–1 μm. Both the advantages and the disadvantages of capillary columns are related to this general feature.

Certain pesticides are highly polar, not very volatile and thermally and catalytically unstable in many instances. With such compounds, the lowest possible column temperatures have to be applied in order to avoid decomposition during sampling or in the column during the chromatographic process. Thermally labile compounds have to be analysed by liquid chromatography (LC), provided that sensitive detection and sufficient resolution can be achieved. Low column temperatures in GC can only be applied, however, if capillary columns with a low content of stationary phase are used, otherwise the retention times would become too long. Moreover, non-polar or weakly polar stationary phases, such as alkylpolysiloxanes, are to be preferred in order to decrease intermolecular interactions of the stationary phase with these polar solutes and thus to avoid long residence times of the solute within the column or too high column temperatures.

Sometimes, however, selectivity and also resolution for certain pairs of solutes have to be sacrificed if a non-polar stationary phase is used. Short retention times can be attained by decreasing the column length at the expense of the separation efficiency, which may become insufficient for the minimum resolution required. Short retention times can be obtained by using thin films of the stationary phase and also by using hydrogen as the carrier gas, which gives separation times about three times shorter than those obtained with nitrogen and argon. Helium can also be used, but the retention times are not as short as those with hydrogen. The application of hydrogen may be considered to be an explosion hazard within an industrial environment, and appropriate precautions should be taken.

Non-polar or weakly polar stationary phases, such as alkylpolysiloxanes, have been used for the more polar types of compounds without difficulty only recently. For such columns complete deactivation of the usual support surfaces in glass capillaries is necessary in order to prevent irreversible adsorption, which causes too high losses of the significant solute at very low column loads, of a few nanograms and less. Complete deactivation of such surfaces before the final coating with temperature-stable alkylpolysiloxanes of the gum type is effected by silanization[1,2], PSD* treat-

* PSD = polysiloxane degradation.

ment[3,4] and related techniques. In addition, the much higher separation efficiency achievable with capillary columns and the effective deactivation of the support surfaces within alkylpolysiloxane columns, low temperatures in the column (and therefore also low sampling temperatures with on-column sampling) can be applied because of the small amount of stationary phase present. This is advantageous for the following reasons: less decomposition of labile sample components, higher selectivity for the resolution of component pairs with similar retentions[5] and less decomposition of the stationary phase, *e.g.*, less bleeding and noise for low detection limits.

Considering the progress described above, we report in this paper on the cooperative work of two laboratories (Bayer and the Max-Planck-Institut), both using capillary columns for routine quantitative analyses of the same technical product at high precision and accuracy. In this connection, different techniques suitable for introducing the sample into the chromatographic system are discussed with special regard to the types of sample to be analysed.

Sampling techniques for quantitative analyses of technical products using capillary columns[6] can be considered as follows. The sample capacity of capillary columns is so low that either the classical split mode or the on-column (direct) mode of introduction[7] of highly diluted samples has to be applied to the type of mixtures specified above. In most instances, in both techniques the original sample has to be diluted with a suitable solvent, either for better manipulation or because the sample is solid or has to be homogenized. With on-column injection, further dilution is necessary in order to achieve a not too high column load in the nanogram range. Volumes lower than 0.2 μl cannot be sampled with high reproducibility when using the usual syringes.

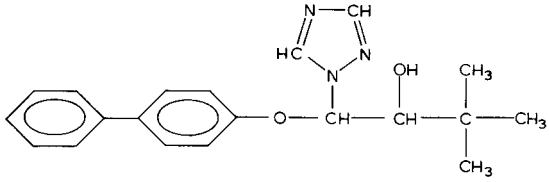
As has been pointed out by Schomburg and co-workers[6,8], Grob and Grob[9,10], Munari and Trestianu[11] and Galli *et al.*[12], various parameters may influence the performance of the sampling procedure with regard to resolution and quantitation. Discrimination of sample components of either low or high volatility and poor precision of relative peak areas are the major problems in quantitation. With split sampling, these parameters are: volatility range of sample components, volatility of solvent or other major components of sample, temperature of injector, temperature of column, splitting ratio and sampling volume, geometry of injector, means of homogenization and type of carrier gas. With on-column sampling, fewer parameters are of importance: temperature of column and the connected inlet valve system, volatility and polarity of solvent or other major components, sampling volume and purity of solvent.

For the comparative assessment of the two sampling techniques, it is also important to realize that many practical samples contain involatile residues. Involatile compounds may also be formed shortly after the injection within the vaporization insert of the splitting device or the column inlet in on-column sampling. With split sampling such deposits remain in the injector insert and do not enter the column. They can be easily removed by exchange or cleaning of the insert after a certain series of injections. Nevertheless, in many instances numerous repeated injections can be executed before cleaning of the vaporization insert becomes necessary. This possibility does not exist with on-column sampling, because the sample enters the column directly. The column inlet could be cleaned by rinsing with solvent if the new cross-linked ("chemically bonded") types of columns are used, but in most instances the

first part of the columns has to be broken off and removed to provide for a fresh, uncontaminated column inlet.

EXPERIMENTAL AND RESULTS

For comparative purposes, a typical sample from the Bayer laboratories in Wuppertal was selected. The active ingredient of the product Baycor® (proposed common name Bitertanol) is a fungicide of the triazole type with the following structure:



Its low volatility requires a column temperature of about 260°C, even with capillary columns. In our experiments, the sample was diluted in three solvents of different volatility and diisooctyl phthalate was added to the solution as an internal standard to calculate the "absolute" content of the two significant (isomeric) species in the technical product and to determine the relative response factors of these components for calibration purposes.

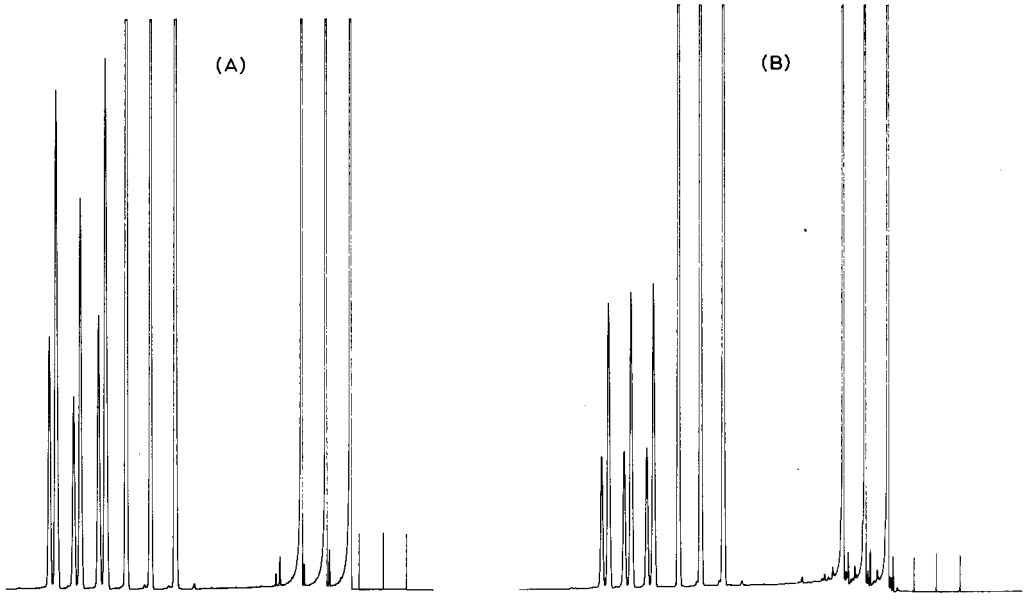
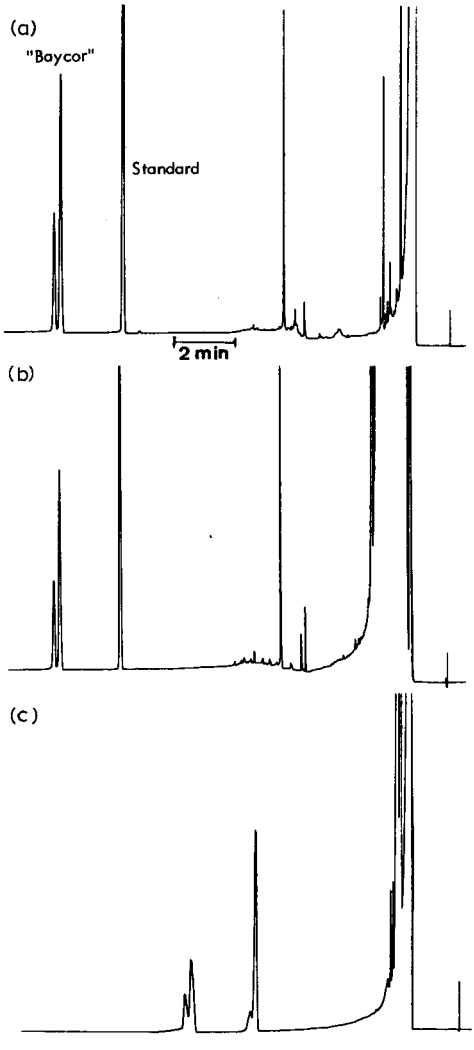


Fig. 1. Isothermal separation of Baycor with split sampling. Solvents: (A), acetone, b.p. 56°C; (B) methyl laurate, b.p. 262°C. Sample: 3 × 0.2 μl each. Column: 30 m OV-101, dealkalinized, PSD treated, soft glass. Temperatures: column, 260°C isothermal; injector, 280°C; detector, 320°C. Carrier gas: 0.78 bar He. Analysis time: 10 min. Solutions: (A) 37.365 mg Baycor, 44.480 mg diisooctyl phthalate, 1058.1 mg acetone; (B) 21.270 mg Baycor, 40.350 mg diisooctyl phthalate, 1304.9 mg methyl laurate.



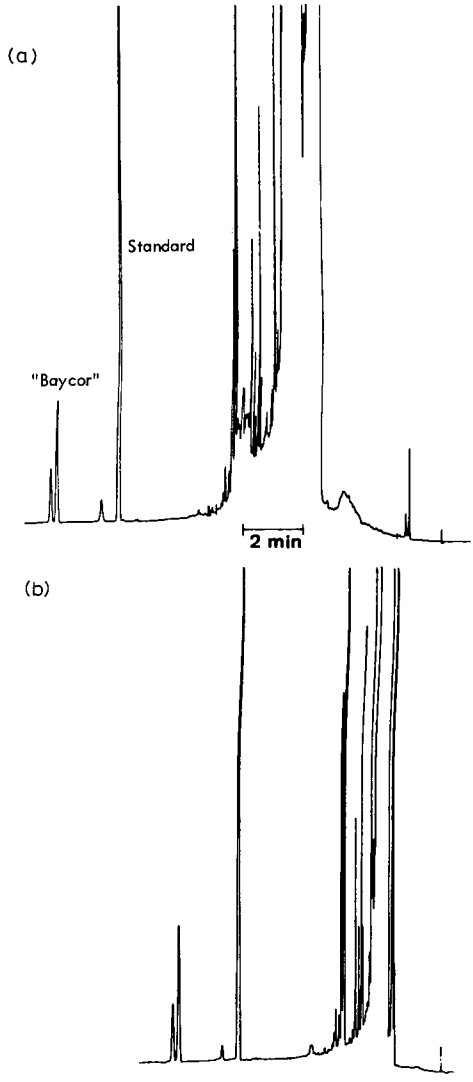
Solvent: 2-propanone, b.p. 56°C.
Sampling temperature: 40°C

Solvent: isobutyl acetate, b.p. 117°C.
Sampling temperature: 40°C

Solvent: isobutyl acetate, b.p. 117°C.
Sampling temperature: 260°C

Fig. 2. Influence of solvent volatility and column temperature on resolution with on-column (syringe) sampling. Sample: 0.2 μl *ca.* 4% Baycor and *ca.* 4% diisooctyl phthalate (diluted 1:400). Column: 30 m × 0.27 mm I.D. OV-1, dealkalinized alkali glass. Carrier gas: 0.78 bar He, flow-rate 27 cm/sec. (a) Temperature, 40–260°C at >40°C/min; solvent, 2-propanone (b.p. 56°C). (b) Temperature, 40–260°C at >40°C/min; solvent, isobutyl acetate (b.p. 117°C). (c) Temperature, 260°C, isothermal; solvent, isobutyl acetate.

Optimization of the chromatographic separation was achieved as follows. With split sampling and using isothermal column operation, an injector temperature slightly higher (280°C) than the column temperature (260°C) was applied. The detector temperature was 320°C and flame-ionization detection was used exclusively. The column dimensions were 25–30 m (length) and 0.27 m (I.D.). A smaller inner diameter could not be used because on-column sampling was also to be applied in these series of experiments. The non-polar stationary phase methylpolysiloxane OV-1 was select-



Solvent: methyl dodecanoate, b.p. 262°C.
Sampling temperature: 40°C

Solvent: methyl dodecanoate, b.p. 262°C.
Sampling temperature: 230°C

Fig. 3. Influence of solvent volatility and column temperature on resolution with on-column (syringe) sampling. Sample: 0.2 μl *ca.* 3% Baycor and *ca.* 4% diisooctyl phthalate (diluted 1:400). Column: 30 m × 0.27 mm I.D. OV-1, dealkalinized alkali glass. Carrier gas: 0.78 bar He, flow-rate 27 cm/sec. (a) Temperature, 40–260°C at 40°C/min; solvent, methyl dodecanoate (b.p. 262°C). (b) Temperature, 230–260°C at 40°C/min; solvent, methyl dodecanoate (b.p. 262°C).

ed for the reasons mentioned above. The splitting ratio for the already diluted sample (4–5% in solvent) was adjusted to 1:280. With on-column sampling, the initial column temperature was 40–50°C (*i.e.* the lowest temperature achievable in the column oven without additional cooling) when the syringe needle was introduced into the column inlet. Such low sampling temperatures are essential, especially when solvents such as the volatile acetone are used. After injecting the sample, the column temperature was increased as quickly as possible (*ca.* 40°C/min) up to the final level of 260°C.

For on-column sampling, the sample had to be further diluted (1:400) in order to achieve the optimal column load because lower volumes, such as 0.1–0.2 μl, cannot be sampled reproducibly enough by the syringe technique, using a Hamilton 701 SN syringe.

The chromatograms obtained with split and on-column sampling are given in Figs. 1–3. Three different solvents were used with both techniques. Fig. 1 shows the chromatograms of two series of three consecutive injections of an acetone and a methyl dodecanoate solution. Complete resolution of the isomers was attained and the solvent peaks showed only slight tailing. The chromatograms in Figs. 2 and 3 illustrate the influence of the solvent volatility and column temperature on the resolution and the peak shape with on-column injection. With isobutyl acetate (b.p. 117°C) as the solvent a column temperature of 260°C proved to be too high, as a lower resolution and irregular peak shapes were observed, whereas with methyl dodecanoate at 230°C (*i.e.*, 30°C below the boiling point of the solvent) the same performance was achieved as at ambient temperature.

The chromatograms obtained with on-column injection also illustrate how many impurities are present in the usually available "pure" solvents. Difficulties with overlapping of the peaks of significant sample components with those of solvent impurities may occur at the very high dilutions applied. Solvents with low boiling points usually have higher purities, and are therefore to be preferred in routine GC analysis, although sampling of mixtures containing large amounts of volatile solvents may become a source of error in quantitation.

Table I gives the operating parameters for the optimized GC separations from which the results in Table II were obtained. Table II gives results for the quantitation of the two Baycor isomers, obtained with both split and on-column injection. The syringe technique of Grob and Grob[9,10] and Galli *et al.*[12] was used. The relative response factors (internal standard: diisooctyl phthalate), the isomer ratio and the corresponding relative standard deviations (calculated from five measurements) were determined.

With split sampling the following conclusions can be drawn from the results. The relative response factors are only slightly dependent on the type (volatility) of solvent, the average value being 1.28 for both techniques. The relative standard deviations of the relative response factors are strongly dependent on the type of solvent, decreasing from a high value of 4.5 % for the volatile acetone to 0.7 % for the less volatile methyl dodecanoate. The relative standard deviations of the isomer ratio, for which no influence of discrimination is to be expected because of their structural similarity, are about 1 % and are independent of the type of solvent used.

With on-column injection using a sampling temperature of 40–50°C, relative standard deviations of the relative response factor always lower than 1 %, independent of solvent volatility, are attained.

Inter-laboratory comparison of quantitative data obtained in analyses of a pure Baycor and a technical product of lower purity

A test mixture containing pure (*ca.* 100 %) Baycor and acetone of standard purity (98.8 %) was analysed at the Max-Planck-Institut für Kohlenforschung in two different non-polar methylpolysiloxane (OV-1) columns, borosilicate glass (Duran) and fused silica (FS) being the capillary (support) material. With both columns, the

TABLE I

QUANTITATIVE GC ANALYSIS WITH HIGH PRECISION AND ACCURACY USING GLASS CAPILLARY COLUMNS; SPLIT *VERSUS* ON-COLUMN* (SYRINGE) SAMPLING

The GC parameters listed gave the results reported in Table II.

Parameter	*Conditions*	
	Split sampling	*On-column sampling*
Column	30 m × 0.27 mm I.D. methylpolysiloxane OV-1, HCl-dealkalinized alkali glass, PSD treated (polysiloxane degradation)	
Temperatures:		
Injector	280°C	
Column	260°C	40°C → 260°C (rapid increase to column temperature after sampling)
Detector	320°C	320°C
Sampling	0.2 μl at a splitting ratio of 1:280	0.2 μl of same sample diluted 1:400
Sample	About 2–4% of each Baycor and internal standard in specified solvent	

* Carlo Erba Model 4160.

TABLE II

INFLUENCE OF SOLVENT VOLATILITY AND SAMPLING TECHNIQUE ON PRECISION AND ACCURACY
Results obtained using the conditions specified in Table I.

Sampling mode	*Solvent*	*Boiling point (°C)*	*RRF**	*RSD (%)***	*Isomer peak-area ratio*	*RSD (%)***	*Sample component weight (mg)*		
							Baycor	*Standard*	*Solvent*
Split	Acetone	56.2	1.248	4.47	2.182	1.18	37.365	44.480	1058.1
	Isobutyl acetate	117	1.296	1.26	2.191	0.67	40.686	39.116	1288.8
	Methyl dodecanoate	262	1.274	0.71	2.167	1.09	21.270	40.350	1304.9
On-column (syringe)***	Acetone	56.2	1.293	0.65	2.180	1.25	37.365	44.480	1058.1
	Isobutyl acetate	117	1.278	0.39	2.186	0.89	40.687	39.116	1288.8

* RRF = relative response factor = $\frac{\text{area of standard} \times \text{weight of substance}}{\text{area of substance} \times \text{weight of standard}}$.

** RSD = Relative standard deviation (> 5 injections).

*** Sample diluted 1:400 in solvent.

TABLE III

INTER-LABORATORY COMPARISON OF RELATIVE RESPONSE FACTORS (RRF) OF THE FUNGICIDE BAYCOR AS A FUNCTION OF COLUMN LOAD AND TYPE OF COLUMN

The weights used were as follows. Calibration: 0.2037 g Baycor (100%), 0.2090 g internal standard, *ca.* 6 g acetone. Analysis: 0.2070 g Baycor (sample), 0.2081 g internal standard, *ca.* 6 g acetone.

Parameter	*MPI**	*Bayer***
Column	A: 25 m × 0.27 mm I.D. methylpolysiloxane OV-1, Duran, HF, PSD treated B: 23 m × 0.32 mm I.D. methylpolysiloxane OV-1, fused silica, PSD treated	C: 30 m × 0.27 mm I.D. methylpolysiloxane SE-30, alkali glass, silanized
Carrier gas	0.52 bar H_2	He
Temperature (°C)	50–260°C at 30°C/min	50–260°C at 30°C/min

Dilution	*Column load (ng)*	*RRF (100% Baycor)*		*RSD (%)****		*Baycor (sample) concentration (%)*		*RSD (%)****	
		MPI (column A)	*Bayer (column C)*	*MPI*	*Bayer*	*MPI (column A)*	*Bayer (column C)*	*MPI*	*Bayer*
1:40	250	1.129	1.148	0.21	0.17	96.62	96.6	0.30	0.34
1:80	125	1.142	1.160	0.12	0.14	96.50	96.9	0.25	0.18
1:160	62.5	1.159	1.140	0.19	0.22	96.42	96.6	0.43	0.25
1:250	40	–	1.159	–	0.31	–	–	–	–
1:480	20	–	–	–	–	98.77	–	0.37	–
1:960	10	1.208	1.178	1.14	1.14	–	–	–	–
		(column B)							
1:2000	5	1.172	–	0.36	–				
1:20,000	0.5	1.251	–	0.67	–				

* Max-Planck-Institut für Kohlenforschung, Chromatographische Laboratorien, Mülheim, G.F.R.

** Bayer AG, Wuppertal, G.F.R.

*** RSD = Relative standard deviation.

column load was decreased from 250 ng of Baycor to 10 ng (Duran column) or from 5 to 0.5 ng (FS column) (see Table III). With the Duran (borosilicate) column, for which (in spite of proper deactivation) a more adsorptive support surface can be presumed, an increase in the relative response factor was already observed with a column load of 10 ng (1.21 compared with 1.15). The relative standard deviation also increased considerably (1.1 % in comparison with 0.2 %) for several reasons, such as increased adsorption and impeded peak area determination because of too low signal-to-noise ratios. With the more inert fused silica column, an appreciable increase in the relative response factor was observed with a column load of only 500 pg, although the relative standard deviation did not increase higher than 0.67 %. The influence of the column adsorptivity on the relative response factors also depends on the difference in the polarities of the solute (Baycor) and the internal standard used. With a less polar internal standard such as an alkane, an even greater increase in the relative response factors will be observed.

A technical Baycor sample of unknown purity was analysed under the same conditions using the previously determined relative response factors. In measurements with three different column loads between 480 and 40 ng, a purity of about 96.50 % was obtained (see Table III). The relative standard deviations for the three series of experiments were about 0.4 % on average. At the Bayer laboratories Baycor contents of about 96.7 % and relative standard deviations of about 0.3 % were found, which can be considered excellent for the analysis of such types of sample.

CONCLUSION

The results show that with the application of modern types of capillary columns, together with on-column (syringe) injection, quantitative data of high precision (with relative standard deviations as low as 0.5 % and less) and accuracy can be achieved. Such low relative standard deviations of the relative peak areas can also be attained with split sampling if all of the parameters are properly optimized. With split sampling it is of advantage that sample constituents of low volatility are deposited in the vaporization insert and do not contaminate the column. Low-boiling solvents cannot be applied if high injector temperatures are necessary owing to the low volatility of the significant sample components itself; increased relative standard deviations are the consequence. The "cold injection" technique described recently by Poy *et al.*[13] and Schomburg[14] may overcome some of these difficulties.

With on-column sampling the column temperature has to be adapted to the volatility of the sample itself and also to the solvent used because otherwise difficulties arise in the period of introduction of the syringe needle into the column. Disadvantages of on-column sampling are the possible contamination of the column inlet by involatile compounds originating from the sample and the necessary high dilution of "concentrated" samples with solvents as pure as possible in order to attain appropriate column loads. Pure solvents with varying volatility are not always available, however. In any other respect, on-column sampling is the superior technique.

It has also been shown again that capillary columns can be used with great advantage for quantitative analyses of high precision and accuracy. High resolution and high signal-to-noise ratios in trace analysis can be easily obtained. Low column temperatures are characteristic of the capillary GC of less volatile samples.

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DETERMINATION OF ACETYL AND FORMYL GROUPS AS PENTAFLUOROBENZYL ESTERS BY MEANS OF GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION*

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SUMMARY

The determination of formate, acetate and propionate as their pentafluorobenzyl esters by glass capillary gas–liquid chromatography has been studied. The separation of pentafluorobenzyl esters of acids is obtained with columns coated with PPSeb stationary phase. Quantitative results are given with samples containing *ca.* 5 nmol of acetyl and formyl groups.

INTRODUCTION

Interest in methods for identification and quantitative determination of acetyl groups in proteins and peptides has grown rapidly since Narita[1] isolated and characterised the N^{α}-acetyl peptide of tobacco mosaic virus protein. Since then many proteins have been found to be N^{α}-acetylated, and a compilation of the sequences of these proteins has recently been published[2]. The presence of N^{α}-formyl groups has also been well established, for instance, N^{α}-formyl methionine in protein chain initiation on the ribosome, N^{α}-formyl valine in Gramicidin A (ref. 3) and N^{α}-formyl glycine in melittin[4].

Acetate has been determined by variety of methods as previously discussed[5,6]. In the previous report, bound acetate and formate were determined as phenacyl esters; the technique required a minimum of 20 nmol for successful derivatisation due to the limited sensitity of the flame ionisation detector (FID)[6]. The availability of the electron-capture detector enabled higher sensitivity to be achieved. Pentafluorobenzyl (PFB) esters of carboxylic acids are known to exhibit high sensitivity to the electron-capture detector and several methods have been proposed to convert carboxylic acids into their respective PFB derivatives[7–16].

* The results described here were taken from the Ph.D. Thesis submitted by J.C. to the University of London.

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A sensitive method was desirable because of the small amount of formyl or acetyl group relative to the weight of the protein. Many proteins being studied are only available in small amounts in a purified form. A method is reported here for the direct determination of acetyl and formyl groups attached to amino acids and proteins. After alkaline hydrolysis of the sample, the sodium salts of acetate and formate are converted into PFB esters prepared by means of crown ether catalysis and determined by gas–liquid chromatography (GLC) with electron-capture detection (ECD).

EXPERIMENTAL

Materials

α-Bromo-2,3,4,5,6-pentafluorotoluene was from Aldrich (Milwaukee, WI, U.S.A.), 15-crown-5 from Fluka (Buchs, Switzerland), acetonitrile (redistilled from P_2O_5), toluene, sodium formate, sodium acetate, propionic acid, *n*-butyric acid, isovaleric acid, nitrobenzene, sodium hydroxide and sodium bicarbonate from BDH (Poole, Great Britain). Other chemicals were obtained as previously reported[6].

Gas chromatography

Gas chromatography (GC) was carried out with the following gas chromatographs: a modified D6 gas chromatograph with gas density balance (GDB) detector as previously reported[17]; Pye series 104, Model 24, fitted with dual flame ionisation detector and Pye Unicam GCD fitted with a 10 mCi ^{63}Ni electron-capture detector. Integration of the peak areas was carried out with Kent Chromalog 2 and Vidar Autolab 6300 digital integrators. The glass capillary column (30 m × 0.27 mm I.D.) was coated with 5% Chromosorb R and 5% PPSeb by a single-step coating method[18]. Direct injection onto the capillary column with sample volumes up to 2.0 μl were made without inlet heater[19].

Hydrolysis of samples and preparation of esters

About 5 nmol of N-acetylated compounds were hydrolysed with 20 μl of 1 *M* sodium hydroxide in 0.5 ml polypropylene microcapped centrifuge tubes in an autoclave at 15 p.s.i. for 3 h. After the hydrolysis, the known amount of internal standards (*n*-butyric acid and isovaleric acid) were added followed by 20 μl of 1 *M* HBr and 2 μl of 1 *M* $NaHCO_3$. Aliquots (5 μl) were transferred to silanised glass tubes (5 cm × 0.3 mm I.D.) and taken to dryness. PFB bromide (50 nmol) and 15-crown-5 (50 nmol) in acetonitrile solution (10 μl) were added. The tube was sealed and incubated at 80°C for 2 h with occasional shaking. A 1-μl aliquot was added to 2 ml of toluene and 1 μl was injected onto the GLC column.

RESULTS AND DISCUSSION

It was necessary to use a mixed stationary phase for the separation of phenacyl esters[6], and mixed stationary phases are always difficult to reproduce. Quantitation of phenacyl formate necessitates a perfectly silylated column. The technique required a minimum of 20–25 nmol for successful derivatisation due to the limited sensitivity of the flame ionisation detector. The availability of ECD enabled higher sensitivity to be achieved, and therefore PFB esters were prepared.

The separation of PFB esters was achieved with PPSeb stationary phase (4%, w/w) coated on 80–100 mesh Chromosorb W-HP, at 110°C and was comparable with that obtained with the phenacyl esters. Fig. 1a–c shows the separation of PFB esters with GDB detection, FID and ECD. In order to determine the yields of esters, standard solutions of PFB formate, acetate and propionate were prepared and run with the internal standards nitrobenzene and *n*-dodecane (C_{12}) on a 4% PPSeb column with a GDB detector. This enabled quantitative yields to be determined in absolute amounts (micrograms). The yields of PFB esters prepared from sodium salts of acids and determined by the GDB detector are given in Table I. Quantitative recoveries for three PFB esters were obtained. The recovery of nitrobenzene against C_{12} as determined by the GDB detector was 98 ± 3.2% (n = 8). The same solution (as for GDB) was diluted and gas chromatographed using the other detectors, from which their relative molar response (RMR) values with FID and ECD were determined (Table II). The separation of PFB esters on PPSeb SCOT column is shown in Fig. 2. A 10-fold increase in amount injected gave the same peak height compared with a packed column, coupled with shorter run time and improved resolution.

PFB esters were formed by using the potassium salts and dicyclohexyl 18-crown-6. Expected recoveries were obtained with a minimum of 25 nmol of starting materials. However, much higher values for formate and acetate were obtained with smaller amounts (in the region of 5 nmol). These higher values were attributed to putative impurities present in KOH. With sodium salts of acids reasonably good recoveries were obtained by using $NaHCO_3$ as a base and 15-crown-5 catalyst (the use of sodium hydroxide and Na_2CO_3 was not satisfactory). Davis[15] derivatised carboxylic acids and phenols with PFB bromide and crown ether catalysis, and has shown that the stronger bases K_2CO_3 and KOH gave good yields of both acids and phenols, whereas the weaker bases $KHCO_3$, CH_3COOK and KCN were suitable for the derivatisation of carboxylic acids, but not phenols. Chan[16] prepared phenacyl valproate by using the sodium salts of valproic acid and dicyclohexyl 18-crown-6. In this investigation, when sodium salts of acids and dicyclohexyl 18-crown-6 were used, no PFB esters were formed. For the sodium salts it was necessary to use 15-crown-5.

It was previously shown for the phenacyl esters[6] that the best results were obtained with phenacyl bromide and dicyclohexyl 18-crown-6 (10:1; mol/mol) in benzene solvent at 80°C for 30 min, but with small amounts much higher concentrations of 15-crown-5 were required. Similarly, for the derivatisation of carboxylic acids (less than 1 μg/ml) higher concentrations of PFB bromide and crown ether were

TABLE I

RECOVERY OF PENTAFLUOROBENZYL ESTERS DETERMINED BY GAS DENSITY BALANCE DETECTOR

Preparation of sample and GLC conditions as in Fig. 1.

Compound	*% Recovery ± S.D. (n = 12)*
PFB Formate	97.0 ± 3.68
PFB Acetate	99.6 ± 1.96
PFB Propionate	98.8 ± 1.50

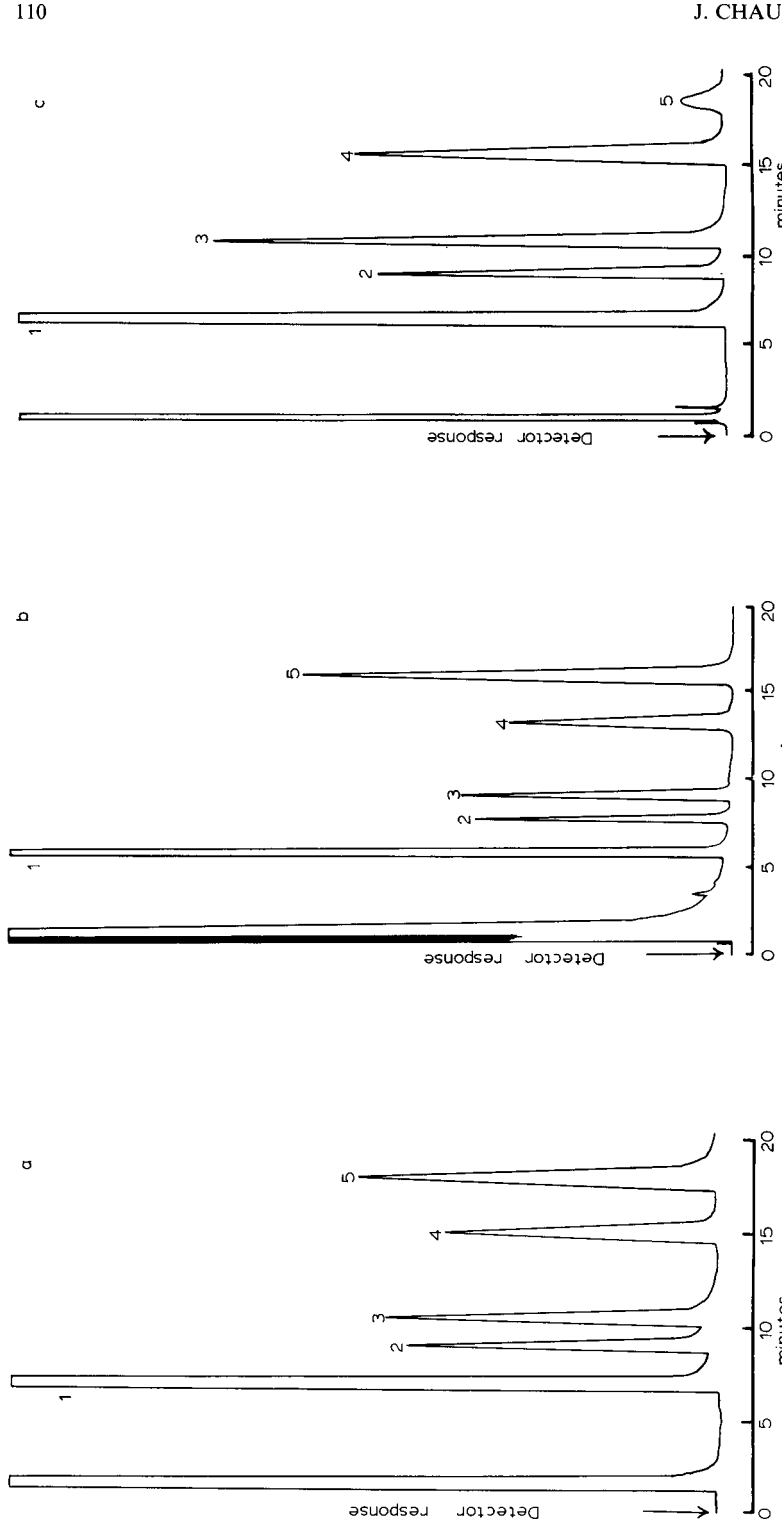

Fig. 1. GLC separation of pentafluorobenzyl esters on a 4% PPSeb column. Preparation of sample: PFB bromide (50 μmol) and 15-crown-5 (5 μmol) were treated with sodium formate (2.89 μmol), sodium acetate (3.01 μmol) and sodium propionate (3.31 μmol) in benzene (400 μl) at 80°C for 30 min in a sealed tube. The tube was cracked open and nitrobenzene (23.08 μmol) in benzene solvent (100 μl) was added. GLC conditions: a glass column (2.25 m × 2.5 mm I.D.) was packed with Chromosorb W-HP 80–100 mesh and coated with 4% (w/w) PPSeb. Carrier gas: nitrogen at a flow-rate of 30 ml/min; oven temperature, 110°C. a, Gas density balance detector: make-up gas, nitrogen at a flow-rate of 30 ml/min; attenuation × 2; sample size, 7 μl. b, Flame ionisation detector: attenuation, 10^{-9} for f.s.d.; to 25 μl of GDB sample 150 μl of benzene was added and 1 μl was injected. c, Electron-capture detector: attenuation, × 32; make-up gas; nitrogen at a flow-rate of 20 ml/min; to 1 μl of FID sample 2 ml of benzene were added and 1 μl was injected. Peaks: 1 = PFB bromide; 2 = PFB formate; 3 = PFB acetate; 4 = PFB propionate; and 5 = nitrobenzene.

TABLE II

RELATIVE MOLAR RESPONSE VALUES OF PENTAFLUOROBENZYL ESTERS DETERMINED AGAINST NITROBENZENE (=1)

Preparation of sample and GLC conditions as in Fig. 1.

Detection	*Relative molar response ± S.D. (n = 12)*		
	PFB Formate	*PFB Acetate*	*PFB Propionate*
FID	1.18 ± 0.06	1.33 ± 0.04	1.59 ± 0.02
ECD	13.81 ± 0.59	22.62 ± 0.75	21.33 ± 0.76

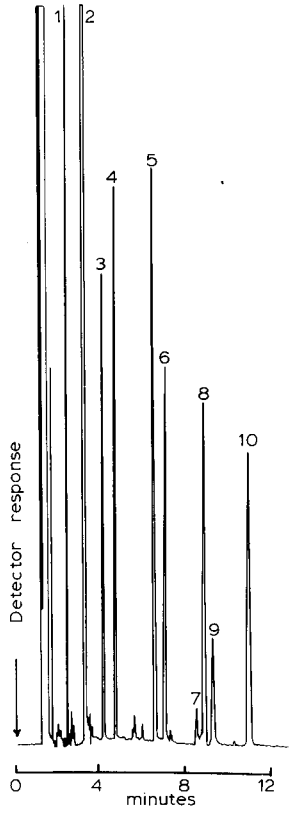

Fig. 2. GLC separation of PFB esters on PPSeb SCOT column. Preparation of sample: a solution containing sodium formate (5.1 nmol), sodium acetate (4.5 nmol), sodium propionate (5.6 nmol), sodium butyrate (5.0 nmol), sodium isovalerate (4.8 nmol) and sodium bicarbonate (250 nmol) was placed in a silanised glass test tube (5 cm × 0.3 mm I.D.) and taken to dryness. PFB bromide (75 nmol) and 15-crown-5 (75 nmol) in acetonitrile solution (20 μl) were added. The tube was sealed and incubated at 80°C for 30 min. A 1-μl aliquot was dissolved in 4 ml of toluene and 1 μl was injected onto the GLC column. GLC conditions: 30 m × 0.27 mm I.D. glass column coated with 5% Chromosorb R and 5% PPSeb; carrier gas, hydrogen at a flow-rate of 1.7 ml/min; make-up gas, nitrogen at a flow-rate of 50 ml/min; detector temperature, 250°C; column temperature, 100°C; attenuation, × 32. Peaks: 1 = PFB chloride; 2 = PFB bromide; 3 = PFB formate; 4 = PFB acetate; 5 = PFB propionate; 6 = nitrobenzene; 7 = artifact; 8 = PFB *n*-butyrate; 9 = artifact; and 10 = PFB isovalerate.

employed (0.3%)[15]. The presence of moisture effects derivatisation, and when samples were not completely dry lower RMR values and larger artifact peaks (Fig. 2, peaks 7 and 9) were obtained. These artifact peaks were attributed to the polymerisation of PFB bromide[15,20]. It was found that pure sodium salts of acids (0.5–5 nmol) were completely derivatised with PFB bromide and 15-crown-5 (1:1; mol/mol) at 80°C in 30 min, but in the presence of 10 μmol NaBr (excess NaBr was always present in the samples due to alkaline hydrolysis conditions) 2 h at 80°C were required.

Fig. 3 shows the response concentration curves for different PFB esters (femtomoles) against the peak area response. The linear dynamic range was *ca.* 100 for each compound under the conditions quoted. The RMR values on a SCOT column are given in Table III; these agree well with packed column RMR values (Table II).

The conditions for the release of bound acetate with alkaline hydrolysis were previously reported[6]. The results for the hydrolysis of N-acetyl and N-formyl amino acids with 1 *M* NaOH are given in Table IV. The recoveries for acetate and formate were close to the those expected.

Fig. 4 shows the GLC trace obtained with chicken egg albumin. Four different proteins were hydrolysed, and all gave peaks which showed the presence of small amounts of formate. Control values were obtained by preparing the PFB esters without previous alkaline hydrolysis. The quantitative results are given in Table V. The controls yielded values of 0.45 and 0.36 mole of acetate per mole of protein, and if these were deducted from the values after alkaline hydrolysis, the results agreed with the expected molar ratios of 4 and 1 for albumin and carbonic anhydrase, respectively.

The unknown protein was isolated from axoplasm of the marine worm (*Myxicola infundibulum*) and was shown to contain two polypeptides with mol.wt. 172,000 and 155,000 (ref. 21). Both polypeptides were blocked with acetate (Table V).

S_{18} (ribosomal protein from *Escherichia coli*) had a high control value of 9.23 moles of acetate per mole of protein, and if this was deducted from the value after alkaline hydrolysis, the molar ratio of 1.46 was obtained (Table V). Owing to the limited solubility of S_{18} in 1 *M* NaOH, the control value was lower then expected and therefore the determined value higher. It is essential with this method that protein should not be grossly contaminated with acetate and formate. However, owing to the limited amount available (S_{18}), it was not possible to dialyse the sample as simple

TABLE III

RELATIVE MOLAR RESPONSE VALUES OF PENTAFLUOROBENZYL ESTERS DETERMINED AGAINST NITROBENZENE (=1) ON A PPSeb SCOT COLUMN

Preparation of sample and GLC conditions as in Fig. 2.

Compound	*Relative molar response ± S.D. (n = 8)*
PFB Formate	13.53 ± 0.75
PFB acetate	22.15 ± 0.87
PFB Propionate	20.47 ± 0.73
PFB *n*-Butyrate	19.45 ± 0.92
PFB Isovalerate	20.83 ± 0.81

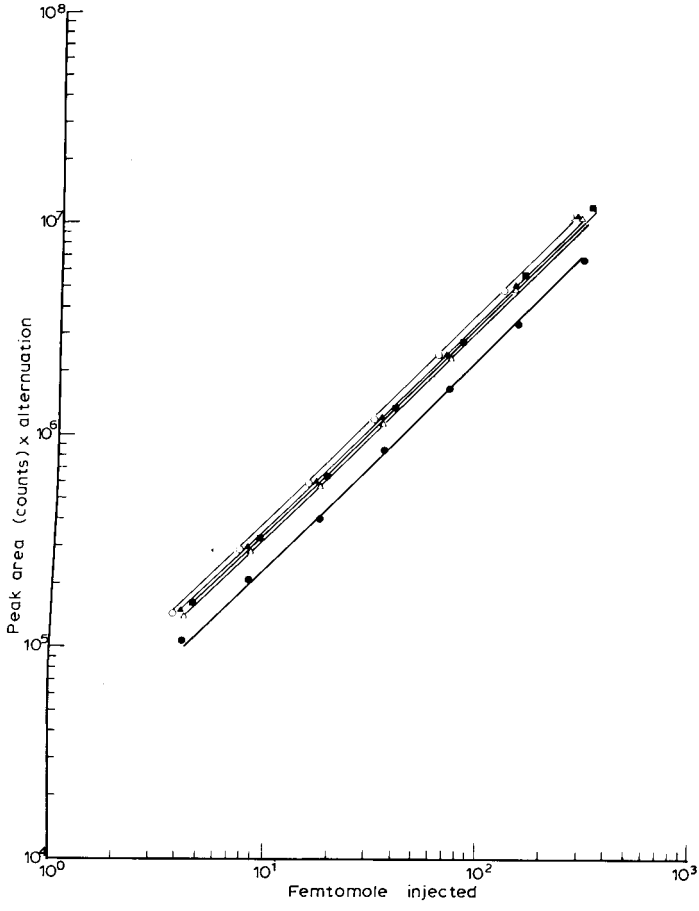

Fig. 3. Response–concentration curves for PFB esters. Preparation of sample and GLC conditions as in Fig. 2. ● = PFB Formate; ○ = PFB acetate; ■ = PFB propionate; △ = PFB *n*-butyrate; and ▲ = PFB isovalerate.

TABLE IV

RECOVERY OF ACETATE AND FORMATE AS PFB ESTERS AFTER ALKALINE HYDROLYSIS OF N-ACETYL AND N-FORMYL AMINO ACIDS

Preparation of sample: see Experimental; GLC conditions as in Fig. 2.

Compound	*Calculated (nmol)*	*% Recovery ± S.D. (n = 4)*
N-Acetyl alanine	4.6	94.2 ± 5.8
N-Acetyl glutamic acid	4.2	96.7 ± 4.3
N-Acetyl phenylalanine	5.6	98.6 ± 6.1
N-Formyl leucine	3.7	95.2 ± 4.6
N-Formyl valine	6.2	97.5 ± 3.8

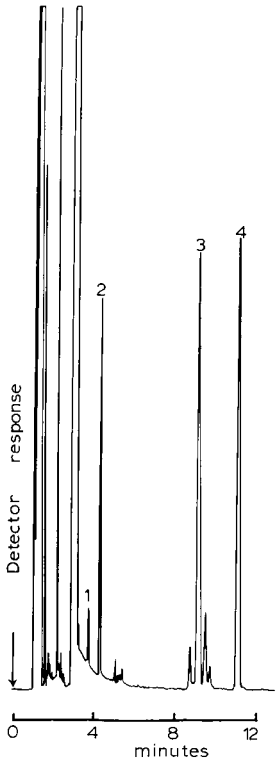

Fig. 4. GLC of PFB acetate and formate obtained from albumin after alkaline hydrolysis. Preparation of sample as in Experimental. GLC conditions as in Fig. 2. Peaks: 1 = PFB formate; 2 = PFB acetate; 3 = PFB *n*-butyrate (internal standard); and 4 = PFB isovalerate (internal standard).

dialysis removes most of the free acetate and formate present. Yaguchi[22] determined the primary structure of S_{18} and showed it to be N-acetylated.

Most of methods reported for the determination of bound acetyl groups either require complicated procedures or large amounts of starting materials. The enzymic methods make use of highly purified enzymes and require 100–900 nmol[23], 17–250 nmol[24], 80 nmol with an average recovery of 90%[25], and 3–12 nmol[26] of acetate.

TABLE V

DETERMINATION OF ACETATE IN PROTEINS

Preparation of sample: see Experimental; GLC conditions as in Fig. 2; (n = 4).

Protein	*Acetate–protein ratio (mol/mol)*		
	Without hydrolysis	*With hydrolysis*	*Difference*
Albumin	0.45 ± 0.05	4.57 ± 0.16	4.12
Carbonic anhydrase	0.36 ± 0.03	1.42 ± 0.09	1.06
Unknown protein	0.62 ± 0.10	2.73 ± 0.13	2.11
S_{18}	9.23 ± 0.75	10.69 ± 1.09	1.46

Protein samples containing 8–17 μmol[27], 0.45–1.13 μmol[28], and 20–100 nmol[6] were analysed by GLC. The method described here allows the determination of *ca.* 5 nmol of covalently bound acetate (also formate and propionate). The limiting factor on this assay's sensitivity is not the detection (ECD can detect 3 fmol of PFB esters) but mainly inherent constraints dependent upon the chemistry of the derivatisation procedure. This method can also be used for the determination of short chain fatty acids in physiological samples (plasma and urine).

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GAS CHROMATOGRAPHIC DETERMINATION OF 1,2-PROPANEDIOL DINITRATE IN BLOOD

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SUMMARY

A method is described for determination of 1,2-propanediol dinitrate in blood at concentrations ranging from 10 ng/ml up to 25,000 ng/ml. It uses double ether extraction with manual shaking in order to complete sample preparation within 5 min. Samples are analyzed via gas chromatography–electron-capture detection using a column of 3% base deactivated SP-2250 on Supelcoport. This column provides excellent separation and little 1,2-propanediol dinitrate tailing.

INTRODUCTION

1,2-Propanediol dinitrate (PGDN) was originally considered as a replacement for 1,2-ethanediol dinitrate in the manufacture of antifreeze dynamite[1] due to the latter's toxicity. It was later found that the effects of vasodilation, hypotension and methemoglobinemia were also present during PGDN intoxication[2,3]. Since PGDN is now used as a principal torpedo fuel (Otto Fuel II) component, further toxicologic investigations were undertaken. These studies required a method of analysis for PGDN in blood that was both rapid and extremely sensitive.

Various analytical techniques have been developed for measuring the organic dinitrate ester compounds. A well established method is alkaline hydrolysis followed by colorimetric determination[4]. This procedure has several drawbacks in that it is time consuming, insensitive with a demonstrated lower detection limit of only 1 μg/ml and relatively non-specific. Polarography is another method which has been applied to PGDN determinations[5]. This equipment is generally not available in most toxi-

cology laboratories. Gas chromatography is by far the most versatile and useful method for PGDN analysis and there are several published methods. Most[6,7] are insensitive or too time consuming and presented technical problems such as peak tailing[8]. In view of our needs and the available methodology, a more sensitive and rapid gas chromatographic technique was developed.

EXPERIMENTAL

Materials

PGDN was obtained as "spirits" in methanol. The neat PGDN was recovered by slowly passing helium gas over the liquid to evaporate the methanol. High-performance liquid chromatography and density measurements were made and compared to published values[9] to ensure adequate PGDN purity for making standard solutions.

The ethenyloxyethene (diethyl ether) used to prepare standards and extract blood samples was purchased as absolute, A.C.S. reagent grade.

Equipment

The gas chromatograph was a Hewlett-Packard Model 5880A, equipped with a modulated-flow thermal conductivity detector (TCD), a ^{63}Ni electron-capture detector (ECD) and a Level Four data integrator. The carrier gas was 30 ml/min helium for the TCD and 30 ml/min methane–argon (5:95) for the ECD.

The column was nickel, 1 m × 2 mm I.D., containing 3 % SP-2250 DB on 100–120 mesh Supelcoport (Supelco). This material is a methyl phenyl silicone, similar to OV-17, which has been deactivated for basic compounds. The column was temperature programmed from 70°C to 120°C at 10°C/min. Following each analysis, the temperature was increased to 200°C for 1 min to elute less volatile residues off the column. The injection port was maintained at 135°C and the detector at 165°C. Although Otto Fuel II decomposition begins above 120°C and becomes rapid above 145°C[10], lowering the injector and detector temperatures to 110°C did not improve the chromatograms.

Sample preparation

A 1-ml sample of freshly drawn whole blood was added to 1 ml distilled water and 5 ml diethyl ether in a capped (PTFE lined) 20 ml glass test tube. The mixture was vigorously shaken manually for 15 sec before centrifuging at 3000 RCF (gravities) for 30 sec to aid separation of aqueous and organic layers. The upper layer was withdrawn and transferred to a capped (PTFE lined) 10-ml graduated centrifuge tube. An additional 5 ml of diethyl ether was used to extract the sample a second time. The extracts were combined and the total volume noted. A 2-μl aliquot of the combined extracts was then injected into the gas chromatograph.

Calculations

The Hewlett-Packard data integrator determined both retention time and area of the generated peaks. Based upon a daily run of nine external standards, the total extraction volume and the PGDN peak area of the sample, a concentration expressed as ng/ml was automatically generated by the integrator.

RESULTS AND DISCUSSION

The use of SP-2250 DB column material permitted excellent separation of PGDN from solvents (Fig. 1). Less volatile constituents which might elute during a following run were removed by using a 1-min post-run column bake-out at 200°C. An additional 2 min were required to reequilibrate the column at 70°C thereby allowing one sample to be analyzed every 10 min. Another advantage of this column was that tailing associated with nitrate esters was greatly reduced. It was also found that recommended daily preconditioning of the column with glycerol trinitrate[4] was not only unnecessary, but undesirable, in that traces of nitrates continued to elute even after many injections.

Since the range of concentration of PGDN in blood was expected to be from 10 ng/ml to 25,000 ng/ml, a nine-point calibration curve was constructed each day. Comparison of the response factors showed a non-linear portion of the calibration curve at the lower concentrations. Use of 1,2,3-propanetriol trinitrate as an internal standard did not prove successful because of significant metabolism in whole blood.

Precision

Several samples were repetitively analyzed in order to demonstrate that the instrument would reliably integrate and report the PGDN values. Since these samples

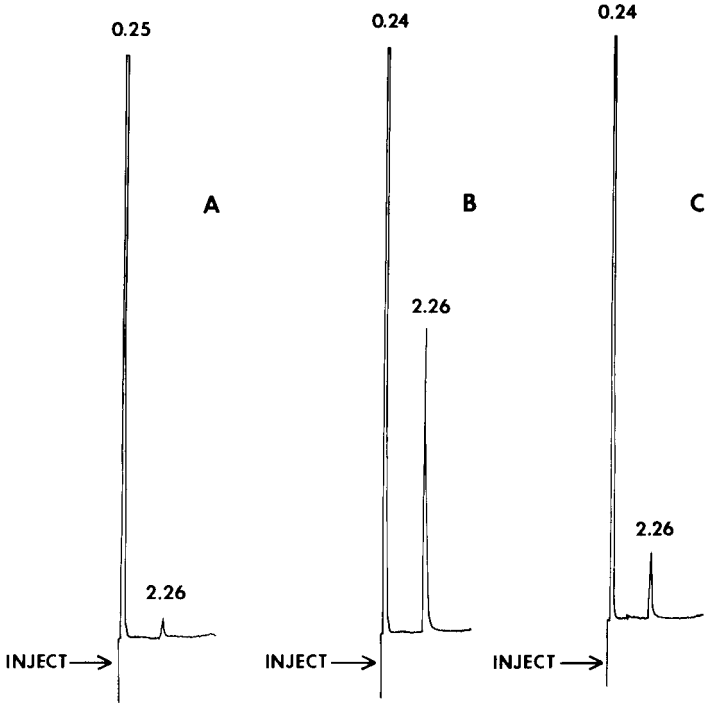

Fig. 1. Chromatograms of diethyl ether standards and sample extract. A, Standard of 10 ng PGDN/ml ether (PGDN retention time of 2.26 min); B, Standard of 100 ng PGDN/ml ether; C, Extract of blood which contained 27 ng PGDN per ml.

TABLE I

PRECISION OF ANALYSIS IN PGDN DETERMINATION

Repetitive determinations of different sample media containing several PGDN concentrations.

Sample type	*PGDN concentration (ng/ml)*	*Number of analyses*	*Mean area counts (± S.D.)*
Blood extract	104	10	8941 (±7%)
Blood extract	7580	10	715753 (±2%)
Ether standard	10	10	1808 (±2%)

were injected manually rather than by mechanical means, the results also reflect operator variability. Table I presents the data for both samples and a standard. Approximately 95% of the samples to be collected during toxicokinetic studies are expected to be within the range of concentrations covered by the two blood extract samples.

Sensitivity

Minimum amounts of PGDN that could be determined were routinely observed for both thermal conductivity and electron capture detectors. The limit of detectability for ECD was 1 pg injected, whereas 75,000 pg was the value on the TCD. These results correspond to concentrations of 5 ng PGDN/ml blood and 150,000 ng PGDN/ml blood, respectively. No interfering peaks were present in extracts of PGDN free blood samples. Concentration of the volume of combined sample extracts to increase sensitivity further was impractical due to variation in recovery.

Reliability

The procedure was tested for two types of reliability. The first was an observation of the number of samples a column could process before PGDN tailing made quantitation difficult. This point was taken to be when the data integrator showed an aborted area count for a 2500 ng/ml standard. Approximately 400 analyses could be

TABLE II

LONG-TERM STABILITY OF A PREPARED EXTRACT

An extract of blood containing PGDN was stored in a capped (PTFE-lined) glass test tube for several days. The GC was calibrated daily before analyses were made.

Time of analysis after sample preparation (h)	*Analyzed concentration (ng PGDN/ml blood)*
0.0	2887
1.0	2964
17.5	2714
20.5	2932
22.5	2972
24.5	3053
42.5	2872
	$\bar{x}$ = 2913 ± 4% (S.D.)

TABLE III

COMPARISON OF TWO SAMPLE PREPARATION METHODS

Blood samples were spiked to contain 4420 ng PGDN/ml and prepared by either rotary or manual shaking extraction. Each succeeding 5-ml extract was analyzed separately, rather than combining extracts as is normally done.

5-ml extraction	*PGDN found (ng/ml)*	
	Rotary shaking for 5 min	*Manual shaking for 15 sec*
1st	2487	3456
2nd	69	134
3rd	7	7
4th	None	None
Composite	2573 (58% recovery)	3597 (81% recovery)

run on this type column before replacement was necessary.

Due to the number of samples taken for analysis in this laboratory, extracts frequently must be stored and run several days following preparation. To test stability of the PGDN in the extract, a sample was prepared and successive analyses made over 42.5 h. These data are presented in Table II and show that no decrease in concentration occurred within the expected limits of variability over that time frame.

Extraction efficiency

Several methods[4,11] for extracting dinitrate ester compounds from biological media incorporate multiple extractions on rotary shakers for periods up to 5 min thereby extending sample preparation to 15 min or more. Since PGDN is rapidly metabolized by blood *in vitro*[2,12] it was desirable to reduce preparation time as much as possible without sacrificing efficiency. Samples of blood containing PGDN were prepared either by shaking the mixture on a rotary shaker for 5 min for each extract or manual shaking for 15 sec. Each extract was analyzed for PGDN. The results, listed in Table III, indicate that all recoverable PGDN has been removed by the third

TABLE IV

ACCURACY OF PGDN DETERMINATION IN SPIKED BLOOD SAMPLES

1-ml aliquots of blood were spiked with known quantities of PGDN before sample preparation and analysis.

PGDN added (ng/ml)	*Number of samples*	*Mean recovery (% ± S.D.)*
10,000	3	83 ± 7
5000	10	89 ± 3
1000	3	72 ± 4
500	3	65 ± 1
100	10	64 ± 5
50	3	47 ± 8
10	3	19 ± 2

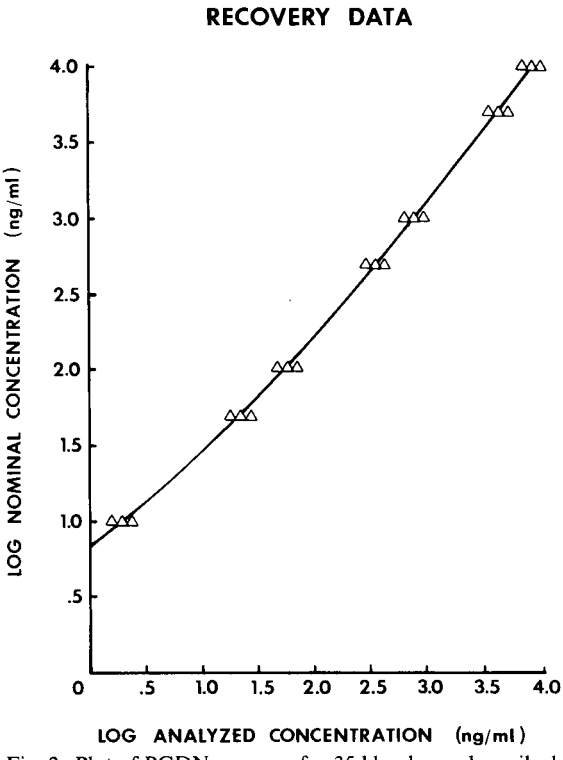

Fig. 2. Plot of PGDN recovery for 35 blood samples spiked to contain various concentrations.

extraction and that the manual technique is more efficient with a 23 % higher recovery of PGDN. Sample preparation time, including centrifugation, was reduced to within 5 min.

Accuracy

The accuracy of the PGDN determination was assessed by analyzing 35 blood samples spiked to contain various PGDN concentrations. The analyses, listed in Table IV, indicate recoveries approach 90 % at higher concentrations but drop below 50 % at levels below 50 ng/ml. Inasmuch as the determined values will be converted to the logarithm of the concentration for toxicokinetic uses, a curve was drawn representing recovery (Fig. 2). A second degree polynomial regression model was fitted to these data. The equation is:

$$y = 0.0664x^2 + 0.54795x + 0.82919$$

This relation has been applied to all raw data before further use.

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HOCHLEISTUNGSFLÜSSIGCHROMATOGRAPHISCHE TRENNUNG DER HERZWIRKSAMEN GLYKOSIDE VON *CONVALLARIA MAJALIS* L. DURCH SERIENSCHALTUNG VERSCHIEDEN POLARER "REVERSED-PHASE" SÄULEN*

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SUMMARY

High-performance liquid chromatographic separation of the cardiac glycosides of Convallaria majalis L. by coupling "reversed-phase" columns of different polarity

A systematic investigation of high-performance liquid chromatographic (HPLC) conditions employing 27 cardenolide glycosides of *Convallaria majalis* showed that LiChrosorb RP-2 possesses the best separation efficiency for the "polar" glycosides whereas LiChrosorb RP-8 can be used for analysis of the "less polar" fraction. For crude extracts from *Convallaria majalis* the best results were obtained by coupling of a LiChrosorb RP-2 precolumn and two analytical columns (LiChrosorb RP-2 and LiChrosorb RP-8) in series in combination with stepwise gradient elution with acetonitrile–water. A comparison of the new method with the paper chromatographic technique currently used showed that the HPLC method gives much more information about the composition of these complex mixtures of cardiac glycosides. It also offers great advantages for the quantitative determination of single cardenolides in *Convallaria majalis* L.

EINLEITUNG

Das Maiglökchen (*Convallaria majalis* L.) wird seit langem wegen seines Gehaltes an herzwirksamen Cardenolidglykosiden[1–14] pharmazeutisch verwendet. Für die Qualitätskontrolle zog man zunächst biologische Verfahren oder die photometrische Bestimmung des Gesamtcardenolidgehaltes heran (vgl. Lit. 15–18). Aussagekräftiger ist die auch heute noch häufig angewendete[19–21] Methode nach Wichtl *et al.*[15], welche die Erfassung der Convallariaglykoside nach papierchromatographischer Trennung in 6 bzw. 7 Zonen vorsieht. Dieses Verfahren kann jedoch in Anbetracht der grossen Anzahl an inzwischen neu gefundenen

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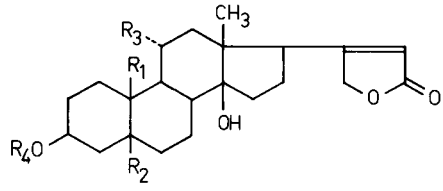
Convallaria-Cardenoliden[13] (vgl. Tabelle I) nicht mehr befriedigen. Da auch die Dünnschichtchromatographie eine zu geringe Trennkapazität aufweist (kurze Laufstrecke, störende Ballaststoffe), können einigermassen sichere Aussagen über den Gehalt an einzelnen Glykosiden nur durch photometrische Messung nach kombinierter Anwendung von Säulen-, Dünnschicht- und/oder Papierchromatographie gemacht werden.

Eine wesentliche Verbesserung versprach der Einsatz der Hochleistungsflüssigchromatographie (HPLC), die in letzter Zeit verschiedentlich für die Analyse von

TABELLE I

STRUKTUR DER IN DER VORLIEGENDEN ARBEIT VERWENDETEN HERZWIRKSAMEN GLYKOSIDE, GEORDNET IN DER AN "REVERSED PHASE" HPLC-SYSTEMEN AUFTRETENDEN ELUTIONSFOLGE

Allose = β-D-Allopyranosyl; AllM = 6-Desoxy-β-D-allopyranosyl; Arab = α-L-Arabinopyranosyl; Glu = β-D-Glucopyranosyl; GuM = 6-Desoxy-β-D-gulopyranosyl; Rh = 6-Desoxy-α-L-mannopyranosyl.



Glykoside	R_1	R_2	R_3	R_4	*Lit.*
Glykosid P_2	CH_3	OH	OH	Allose-	13
Glykosid K	CH_2OH	OH	OH	Rh-	13
Thollosid	CHO	OH	OH	Rh-	13
Glykosid L	CH_2OH	OH	OH	GuM-	13
Glykosid G	CH_2OH	H	OH	Rh-	13
Glykosid P_1	CH_3	OH	OH	Rh-AllM-	13
Lokundjosid	CH_3	OH	OH	Rh-	8
Glykosid E_3	CHO	OH	OH	GuM-	13
Glykosid E_1	CH_3	OH	OH	GuM-	13
Rhodexosid	CH_3	H	OH	Glu-Rh-	10
Rhodexin A	CH_3	H	OH	Rh-	10
Convallatoxolosid	CH_2OH	OH	H	Glu-Rh-	7
Convallosid	CHO	OH	H	Glu-Rh-	3
Convallatoxol	CH_2OH	OH	H	Rh-	4
Desglucocheirotoxol	CH_2OH	OH	H	GuM-	11
Glykosid U	CHO	OH	H	Arab-AllM-	13
Glykosid H_1	CHO	OH	H	Glu-Rh-	13
Convallatoxin	CHO	OH	H	Rh-	1,2
Strophanollosid	CH_2OH	OH	H	AllM-	12
Glykosid F	CHO	OH	H	Rh-AllM-	13
Desglucocheirotoxin	CHO	OH	H	GuM-	5
Glykosid S	CH_2OH	H	H	Glu-Rh-	13
Glykosid Z	CH_2OH	H	H	Rh-AllM-	13
Strophallosid	CHO	OH	H	AllM-	12
Periplorhamnosid	CH_3	OH	H	Rh-	9
Perigulosid	CH_3	OH	H	GuM-	11
Peripallosid	CH_3	OH	H	AllM-	12

Cardenoliden (meist Digitalisglykosiden) und Bufadienoliden herangezogen wurde[22–34]. Der weitaus grösste Teil dieser Arbeiten setzt sich mit der Trennung einer beschränkten Zahl dieser Verbindungen auseinander, wobei neben dem Einsatz verschiedener Sorbentien und mobiler Phasen auch durch Derivatisierung versucht wurde, die Selektivität, Auflösung und Nachweisgrenzen zu optimieren. Mit der Erfassung der herzwirksamen Glykoside in pflanzlichen Drogen beschäftigten sich bisher nur wenige Autoren, wobei neben *Digitalis lanata* kürzlich auch *Urginea maritima*, *Nerium oleander* und *Nerium odorum* untersucht wurden[25,35–39].

Für die HPLC-Bestimmung der Cardenolide in *Convallaria majalis* war das Hauptgewicht auf die Entwicklung eines Systems mit hoher Trennkapazität zu richten, welches weitgehend die Erfassung der einzelnen Cardenolidglykoside (Tabelle I) erlaubt. Im Hinblick auf eine möglichst einfache apparative Anordnung sollte alleine mit UV-Detektion ohne jede Derivatisierung das Auslangen gefunden werden.

EXPERIMENTELLES

Apparaturen

Flüssigkeits-Chromatograph Perkin-Elmer Series 3 + Rheodyne Injektor (175-µl Schleife); Detektor Perkin-Elmer LC 65 T, Wellenlänge 221 nm; Integrator Perkin-Elmer M 2; Schreiber Perkin-Elmer 023.

Säulen (Knauer, Oberursel, B.R.D.) 250 × 4.6 mm I.D.: LiChrosorb RP-2 (10 µm), LiChrosorb RP-8 (7 µm), LiChrosorb RP-18 (7 µm). Vorsäule (40 × 4.6 mm I.D.): LiChrosorb RP-2 (5 µm). Bei Analysen mit Säulenkombinationen wurden die einzelnen Säulen unter Verwendung totvolumenfreier Zwischenstücke jeweils in Serie geschaltet.

Mobile Phase: Acetonitril (HPLC Grade S, Rathburn Chemicals, Walkerburn, Grossbritannien)–Wasser; Zusammensetzung und Durchflussraten vgl. Legenden zu den Abbildungen.

Substanzen: Sämtliche in dieser Arbeit verwendeten Reinglykoside wurden im Institut für Pharmakognosie der Universität Wien aus *Convallaria majalis* L. isoliert (vgl. z.B. Lit. 13).

Testlösungen: Pro Analyse injizierten wir 1–10 µg je Reinglykosid, gelöst in 30 µl 70%igem Äthanol.

Papierchromatographie: Die Aufbereitung der Convallaria-Droge und die papierchromatographische Trennung der Cardenolide führten wir nach der Vorschrift von Wichtl *et al.*[15] durch. Nach Markieren der einzelnen Zonen an Hand des besprühten Leitchromatogrammes schnitten wir die einzelnen Zonen in etwa 1 cm^2 grosse Stücke und eluierten eine Stunde lang unter häufigem Umschwenken mit Methanol. Nach Filtrieren der Lösung wurde unter vermindertem Druck zur Trockene eingedampft und die einzelnen Rückstände in je 1.0 ml 70%igem Äthanol aufgenommen. Von diesen Lösungen wurden 30 µl zur HPLC-Messung verwendet.

Dünnschichtchromatographie: Lokundjosid/Glykosid P_1/Glykosid E_3 bzw. Convallatoxin/Glykosid H_1/Strophanollosid und Desglucocheirotoxin/Glykosid S lassen sich mit folgendem DC-System trennen: Kieselgel 60 F_{254} Fertigplatten (MERCK, Darmstadt, B.R.D.); Mobile Phase: Chloroform–Methanol–Wasser

(70:30:10; Unterphase), hR_F-Werte: Lokundjosid = 47, Glykosid P_1 = 29, Glykosid E_3 = 42; Convallatoxin = 60, Strophanollosid = 53, Glykosid H_1 = 20; Desglucocheirotoxin = 64, Glykosid S = 18; Detektion: Besprühen der auf 103–105°C erhitzten Platte mit Vanillin–Schwefelsäure-Reagens (0.1 g Vanillin + 4 ml Äthanol + 16 ml konz. Schwefelsäure). Die angeführten Cardenolide zeigen —je nach Aglykon— unterschiedliche Färbungen, weshalb auch die Substanzen mit ähnlichem hR_F-Wert eindeutig zugeordnet werden können.

ERGEBNISSE UND DISKUSSION

Nachdem entsprechende Vorversuche gezeigt hatten, dass sich für die HPLC-Trennung der Convallariacardenolide in erster Linie "reversed phase" Systeme mit Acetonitril–Wasser als mobile Phase eignen[30], überprüften wir zunächst die —teilweise differierenden— Literaturangaben[22,23,30,40] bezüglich des Absorptionsmaximums in diesem Lösungsmittel. Sowohl bei "stop flow" Messungen im HPLC-System als auch bei direkter Aufnahme der UV-Spektren lag das durch den ungesättigten Laktonring bedingte Maximum, das in methanolischer Lösung bei 217 nm auftritt, in Acetonitril–Wasser (20:80) bei 221 nm. Wir detektierten daher bei allen folgenden HPLC-Messungen, bei denen ausschliesslich Acetonitril–Wasser als mobile Phase diente, bei 221 nm. Nun wurde mittels einer Lösung von sieben Glykosiden (Lokundjosid, Convallosid, Convallatoxol, Desglucocheirotoxol, Convallatoxin, Desglucocheirotoxin und Periplorhamnosid) versucht, optimale Trennbedingungen zu finden. In erster Linie prüften wir den Einfluss der Polarität der stationären Phase, indem wir das Testgemisch an LiChrosorb RP-18, RP-8 und RP-2 chromatographierten. Dabei variierten wir zunächst im isokratischen Betrieb die Eluenszusammensetzung und -geschwindigkeit, aber auch die Säulentemperatur. Bei Verwendung von LiChrosorb RP-18 liessen sich die meisten Komponenten des Testgemisches gut trennen. Desglucocheirotoxol und Convallatoxin jedoch wiesen unter allen Versuchsbedingungen nahezu gleiche Retentionszeit auf. Da wir uns in diesem Fall von einer Anhebung der Säulentemperatur keine Verbesserung der Trennung versprachen, setzten wir die Untersuchungen an LiChrosorb RP-8 fort: Mit Acetonitril–Wasser (23:77) konnte nunmehr das Paar Desglucocheirotoxol/Convallatoxin bei Durchflussraten von 0.9, 1.0, 1.2 und 1.5 ml/min getrennt erfasst werden, während höhere Flussgeschwindigkeiten auch bei gleichzeitigem Absenken der Acetonitril-Konzentration zu einer Verschlechterung der Auflösung führten; keine Trennung der beiden Substanzen war bei Erhöhung der Säulentemperatur zu erzielen. Auch bei Zusatz weiterer Cardenolide fanden wir unter diesen Bedingungen noch zufriedenstellende Trennleistungen (Fig. 1). Als Nachteil erwiesen sich die niedrigen Kapazitätsverhältnisse der polaren Glykoside: Glykosid G und Lokundjosid konnten nicht einzeln erfasst werden. Im Bereich der weniger polaren Glykoside stellten Desglucocheirotoxin und Glykosid U ein kritisches Paar dar.

Wir setzten daher LiChrosorb RP-2 ein, wobei wir vor allem in bezug auf die mit kurzer Retentionszeit eluierten Glykoside eine Verbesserung der Selektivität erhofften. Zunächst ergab das an LiChrosorb RP-8 vollständig trennbare Cardenolidgemisch (Fig. 2) bei Verwendung von Acetonitril–Wasser (23:77) nur noch 9 Peaks, allerdings wiesen die Chromatogramme im Bereich der mit kurzer Retentionszeit eluierten Verbindungen eine deutlich verbesserte Auflösung auf. Die vorerst nicht

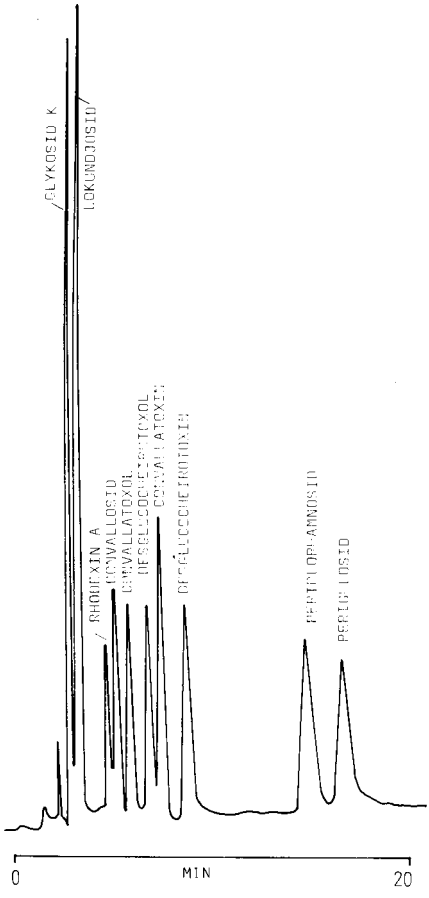


Fig. 1. HPLC-Trennung von 10 Cardenolidglykosiden aus *Convallaria majalis* bei isokratischer Arbeitsweise. Säule: LiChrosorb RP-8 (7 μm), 250 × 4.6 mm I.D. Mobile Phase: Acetonitril–Wasser (23:77), 1.5 ml/min. Temperatur: 22°C.

getrennten Komponenten Convallosid und Rhodexin A konnten nach Senken der Acetonitril-Konzentration auf 18% einzeln erfasst werden, was jedoch mit einer drastischen Verlängerung der Gesamtanalysenzeit verbunden war (Fig. 2). Auch an RP-2 bildeten Lokundjosid/Glykosid G bzw. Desglucocheirotoxin/Glykosid U kritische Paare. Die Lösung dieses Trennproblems versuchten wir durch Einsatz der Gradiententechnik zu erreichen.

Während an LiChrosorb RP-8 weder mit Lineargradienten noch mit Stufengradienten eine Verbesserung der Trennleistung gegenüber isokratischem Betrieb eintrat, fanden wir an LiChrosorb RP-2 bei Anwendung eines Stufengradienten sowohl hinsichtlich der Auftrennung der polaren Glykoside als auch in bezug auf die Gesamtanalysendauer günstigere Bedingungen vor. Die kritischen Paare Lokundjosid/Glykosid G und Desglucocheirotoxol/Glykosid U konnten nunmehr getrennt erfasst werden und auch die Chromatographie eines nach Wichtl *et al.*[15] bereiteten vorgereinigten Drogenauszuges lieferte zufriedenstellende Ergebnisse (Fig. 3). Beim Zuspritzen weiterer Reinglykoside stellten wir aber fest, dass die Glykoside K, P_2 und L_1 ebenso wie

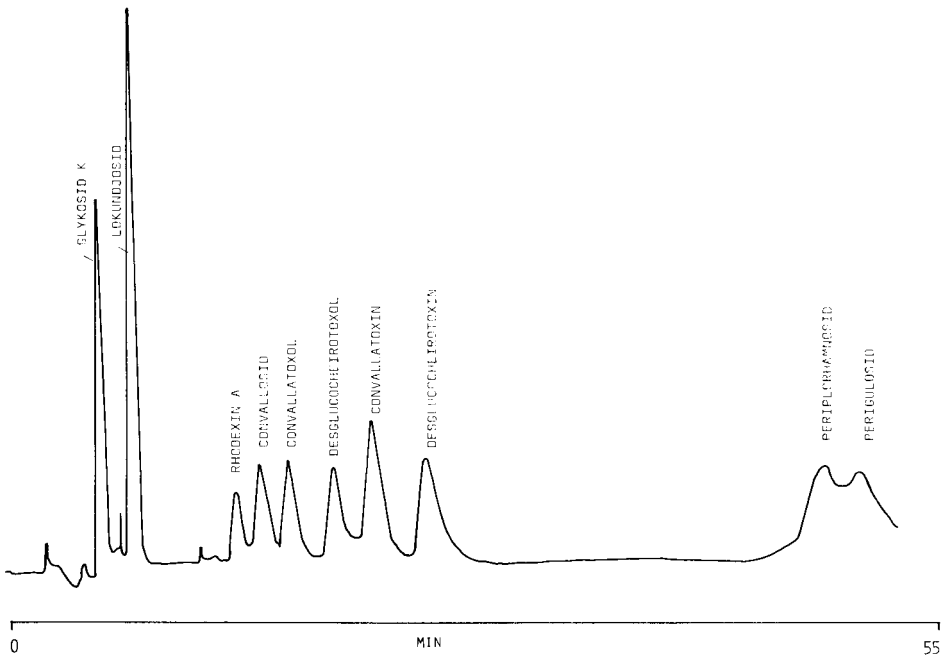


Fig. 2. HPLC-Trennung von 10 Convallariaglykosiden an LiChrosorb RP-2. Säule: LiChrosorb RP-2 (10 μm), 250 × 4.6 mm I.D. Mobile Phase: Acetonitril–Wasser (18:82), 1 ml/min. Temperatur: 22°C.

Thollosid, Glykosid L und K bzw. auch die Glykoside G, E_3 und P_1 mit nahezu gleichen Retentionszeiten eluiert werden.

Obszwar diese einfachen HPLC-Systeme einen wesentlich besseren Einblick in die Zusammensetzung natürlicher Gemische von Convallaria-Cardenoliden erlaubten als andere chromatographische Verfahren, hatten sich auch gewisse Nachteile gezeigt. So mussten wir bei der Messung von mit Bleifällung vorgereinigten Drogenextrakten nach *ca.* 50 Analysenläufen ein drastisches Absinken der Auflösung durch verschmutzte Säulenköpfe feststellen. Der mit Bleiacetat nicht entfernte Begleitstoffanteil wirkte sich hinsichtlich der Auswertbarkeit der Chromatogramme nachteilig aus, da im Bereich der ohnehin schwierig zu trennenden polaren Glykoside störende Peaks auftraten. Zur Lösung dieser Probleme bot sich zunächst der Einbau von Vorsäulen an, die eine rasche Reinigung verschmutzter Säulenköpfe erlauben; die zur Verbesserung der Trennung im ersten Teil des Chromatogrammes notwendige Erhöhung der Trennstufenzahl hofften wir durch Serienschaltung zweier analytischer Trennsäulen zu erreichen.

Während der Einbau einer mit LiChrosorb RP-2 gefüllten Vorsäule keinen signifikanten Einfluss auf die Trennleistung ergab und sich somit für die Analyse verunreinigter Drogenextrakte empfahl, kam es bei Kopplung mit einer weiteren analytischen LiChrosorb RP-2-Säule zu einer starken Peak-verbreiterung und damit drastischen Abnahme der Auflösung. Bessere Ergebnisse erzielten wir bei Anwendung eines Stufengradienten durch Serienschaltung der LiChrosorb RP-2 Vorsäule mit zwei analytischen Säulen, von denen die eine mit LiChrosorb RP-2 und die andere mit LiChrosorb RP-18 gefüllt war (Fig. 4). Die im ersten Abschnitt des Chro-

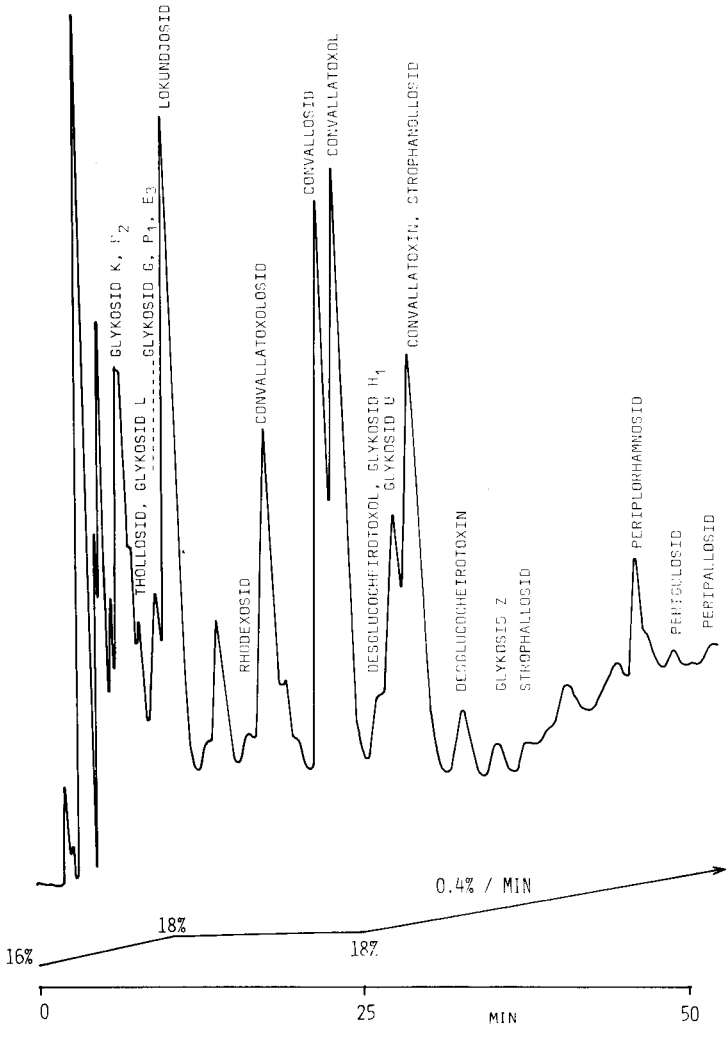


Fig. 3. HPLC eines Drogenauszuges aus *Convallaria majalis*. Säule: LiChrosorb RP-2 (10 μm), 250 × 4.6 mm I.D. Mobile Phase: Acetonitril–Wasser, Gradient 16:84 bis 18:82 mit 0.2%/min, nach 25 min mit 0.4%/min bis Ende der Analyse, Fluss: 1 ml/min. Temperatur: 22°C.

matogramms auftretenden polaren Glykoside lagen nun über einen grösseren Retentionsbereich verteilt vor, Convallatoxol und Convallosid, beides Hauptkomponenten natürlicher Gemische von Convallariaglykosiden, waren fast vollständig getrennt und Desglucocheirotoxin und Glykosid F konnten einzeln nachgewiesen werden.

Eine noch grössere Trennschärfe erzielten wir bei Austausch der LiChrosorb RP-18 gegen eine LiChrosorb RP-8 Säule. Diese Säulenkombination RP-2/RP-8 stellte sowohl hinsichtlich der Trennung der polaren als auch der unpolaren Convallariaglykoside den günstigsten Kompromiss dar. Vor allem Convallatoxol und Convallosid waren fast bis zur Basislinie aufgetrennt und auch zwischen Desglucocheirotoxol, Glykosid U und Convallatoxin waren Minima vorhanden, die eine quantitati-

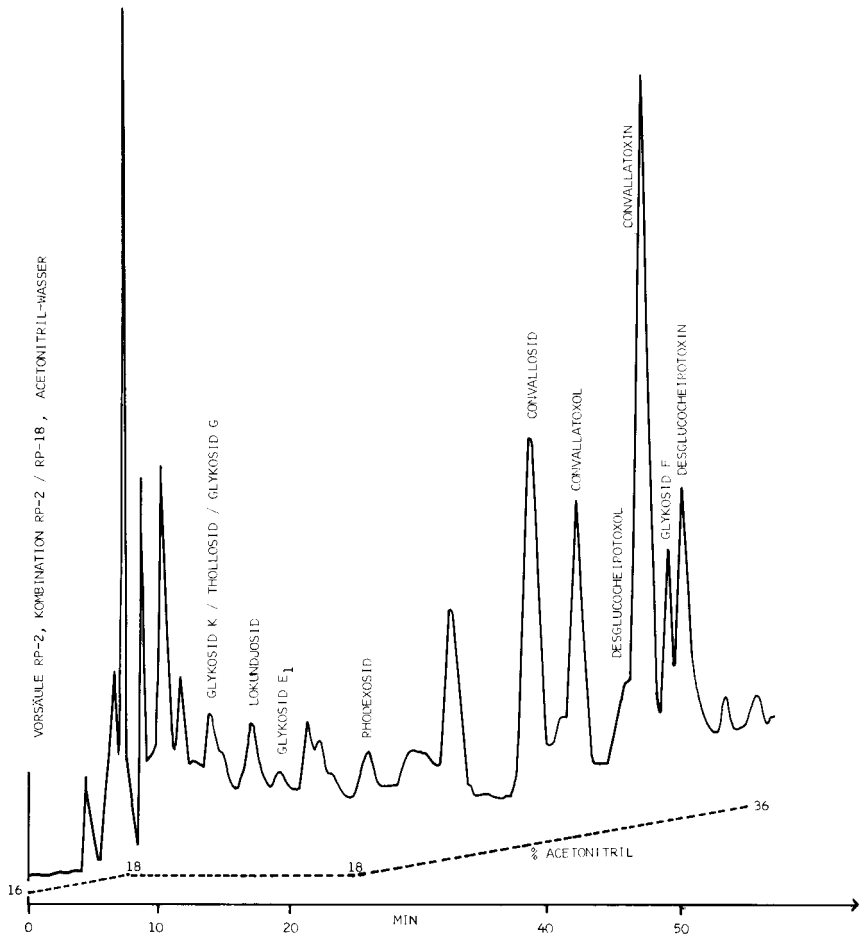


Fig. 4. HPLC eines Drogenauszuges von *Convallaria majalis* unter Verwendung einer RP-2/RP-18 Säulenkombination. Säulen: LiChrosorb RP-2 (5 μm), 40 × 4.6 mm I.D. (Vorsäule), LiChrosorb RP-2 (10 μm), 250 × 4.6 mm I.D. und LiChrosorb RP-18 (7 μm), 250 × 4.6 mm I.D. in Serie geschaltet. Mobile Phase: Acetonitril–Wasser, Gradient 16:84 bis 18:82 mit 0.2%/min, nach 25 min mit 0.4%/min bis Ende der Analyse, Fluss: 1 ml/min. Temperatur: 22°C.

ve Auswertung mittels Integrator erlaubten (Fig. 5). Durch die Verlängerung der Trennstrecke wurde ausserdem eine gute Abtrennung der mit kurzer Retentionszeit eluierten Begleitstoffe erreicht. Das Chromatogramm einer Mischung von Hauptglykosiden und solchen Cardenoliden, die —soweit bisher bekannt— nur in sehr kleinen Mengen in *Convallaria majalis* enthalten sind, zeigt allerdings noch verbleibende Trennprobleme auf (Fig. 5): So besitzt Lokundjosid die gleiche Retentionszeit wie die Nebenglykoside P_1 und E_3, Convallatoxin überdeckt die Nebenglykoside H_1 und Strophanollosid; Desglucocheirotoxin und Glykosid S konnten ebenfalls nicht einzeln erfasst werden. Diese Substanzen lassen sich allerdings dünnschichtchromatographisch trennen, wodurch eine entsprechende Kontrolle des HPLC-Eluates möglich ist.

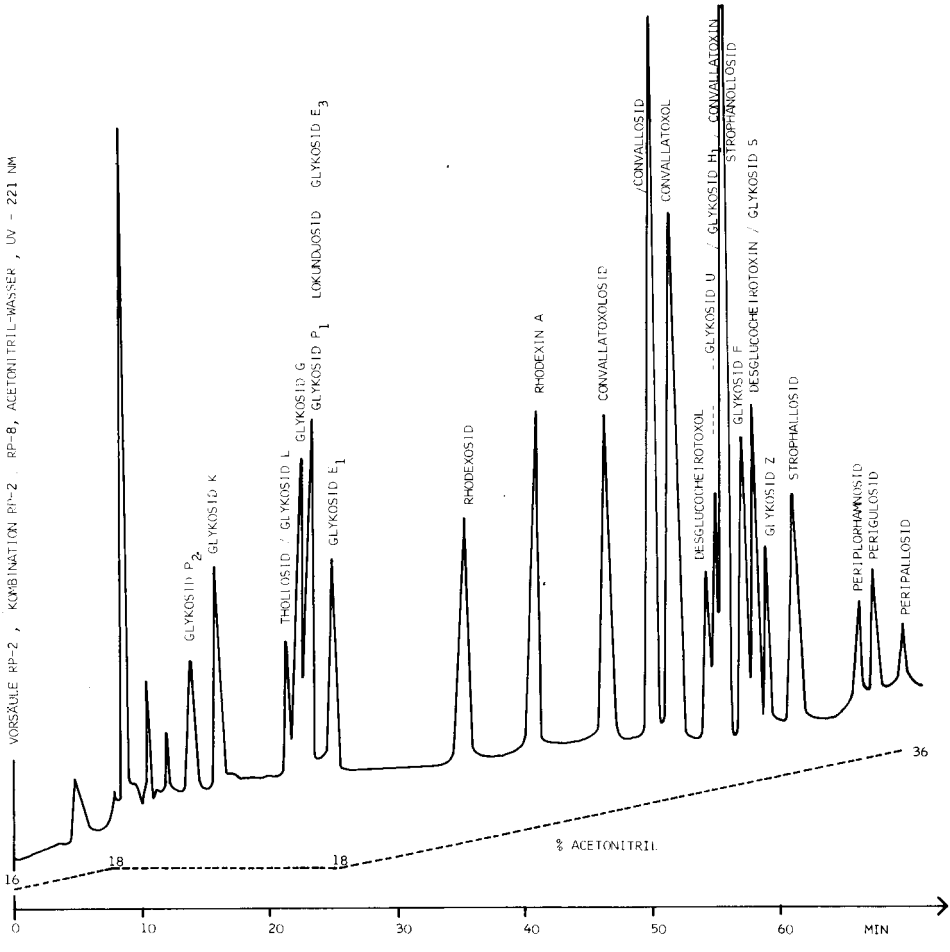


Fig. 5. HPLC-Trennung von 27 Reinglykosiden aus *Convallaria majalis* unter Verwendung einer RP-2/RP-8 Säulenkombination. Säulen: LiChrosorb RP-2 (5 μm), 40 × 4.6 mm I.D. (Vorsäule), LiChrosorb RP-2 (10 μm), 250 × 4.6 mm I.D. und LiChrosorb RP-8 (7 μm), 250 × 4.6 mm I.D. in Serie geschaltet. Mobile Phase: Acetonitril–Wasser, Gradient, Fluss und Temp. vgl. Fig. 4.

Eindrucksvoll lässt sich die Leistungsfähigkeit des neuen HPLC-Verfahrens durch Vergleich mit der bisher für die Einzelglykosidbestimmung eingesetzten Methode[15] demonstrieren. Dazu wurde ein Drogenauszug papierchromatographisch getrennt und die einzelnen Zonen wie für die photometrische Cardenolidbestimmung ausgeschnitten. Die HPLC-Trennung der Cardenolide ergab, dass in den PC-Zonen jeweils bis zu 6 Komponenten vorlagen (Fig. 6). Auffallend war die geringe Trennschärfe der Papierchromatographie: so konnte das in Zone C auftretende Convallatoxin auch in den benachbarten Zonen B und D detektiert werden, ähnliches gilt bezüglich Lokundjosid, Glykosid E_1, Desglucocheirotoxin und Desglucocheirotoxol.

Die durch Serienschaltung unterschiedlich polarer HPLC-Säulen erzielte Trennung der herzwirksamen Glykoside von *Convallaria majalis* stellt somit im Vergleich zum papierchromatographischen Verfahren eine deutliche Verbesserung dar. Wegen

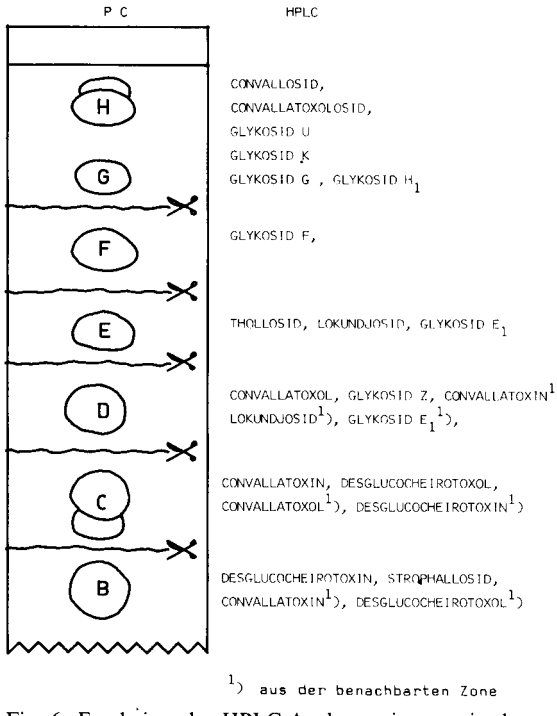


Fig. 6. Ergebnisse der HPLC-Analysen eines papierchromatographisch fraktionierten Convallaria-Extraktes.

der relativ kurzen Analysendauer und der Möglichkeit, auch bei Vorliegen extremer Konzentrationsunterschiede die einzelnen Substanzen zu erfassen, eignet sich das HPLC-Verfahren vorzüglich für die quantitative Erfassung der Herzglykoside in Convallaria-Drogen[41].

ZUSAMMENFASSUNG

Im Laufe einer systematischen Untersuchung zur Trennung der Cardenolide von *Convallaria majalis* L. mittels HPLC wurden 27 Reinglykoside berücksichtigt. Die stationäre Phase LiChrosorb RP-2 zeigte sich für die Trennung der "polaren" Glykosidfraktion besonders geeignet, während sich LiChrosorb RP-8 eher für die Untersuchung der "schwächer polaren" Glykoside bewährte. Da jedoch eine einzige Säule für die Auftrennung des gesamten Komplexes nicht ausreichte, wurde durch Serienschaltung unterschiedlich polarer "reversed phase" Säulen die Trennstrecke verlängert. Am besten geeignet erwies sich das Hintereinanderschalten einer LiChrosorb RP-2 Vorsäule und zweier analytischer Säulen (Lichrosorb RP-2 und RP-8) in Kombination mit einem Stufengradienten von Acetonitril–Wasser. Neben einer Besprechung von Vor- und Nachteilen der untersuchten Systeme wird auch ein Vergleich mit der bisher verwendeten Papierchromatographie beschrieben. Das neue HPLC-Verfahren erlaubt einen wesentlich besseren Einblick in die Zusammensetzung dieser komplexen Substanzgemische; es ermöglicht erstmals die Trennung der Hauptglykoside sowie der Mehrzahl der Nebenkomponenten in einem Arbeitsgang.

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SEPARATION OF DIGOXIN, DIGITOXIN AND THEIR POTENTIAL METABOLITES, IMPURITIES OR DEGRADATION PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and versatile series of high-performance liquid chromatographic systems are described for the resolution of digoxin, digitoxin and their potential metabolites or degradation products and impurities. These systems consist of isocratic, single-step gradient and linear gradient modes that provide resolution of the glycosides in 25, 17 and 14 min respectively. Digoxin, its mono- and bisdigitoxosides, digoxigenin and gitoxin, a potential impurity, may be isocratically separated in 11 min. The two semi-synthetic glycosides α- and β-acetyldigoxin are resolved and separated from digoxin and its metabolites in a chromatographic time of 23 min. Digitoxin and its metabolites or degradation products may be separated in as little as 9 min using an isocratic system.

The solvent systems employ varying proportions of methanol, water, isopropanol and dichloromethane and a conventional 5 μm bonded, octadecyl phase. Detection was accomplished using a variable wavelength detector set at 220 nm.

INTRODUCTION

Digoxin and digitoxin are cardiotonic secondary glycosides obtained from the leaves of *Digitalis lanata* or *Digitalis purpurea*. These purified extracts are the most commonly prescribed medication used in the treatment of congestive heart failure.

Both of the digitalis glycosides are metabolically converted to their respective bis- and monodigitoxosides and finally to the aglycone (genin) steroid by a stepwise elimination of the digitoxose sugars at carbon 3. In addition, the glycosides have been reported to give rise to dihydrodigoxin and dihydrodigitoxin[1–7] and it has also been noted that digitoxin can be converted to digoxin by β-hydroxylation in the liver[1,8,9].

In addition to metabolic cleavage of the digitoxose sugar residues, the acidic hydrolysis of digoxin and digitoxin to their bis-, mono- and aglycone fragments has been observed following *in vitro* tests[10–12]. The digitalis glycosides are also known to contain impurities such as gitoxin in digoxin and digitonin in digitoxin. The United States Pharmacopeia[13] specifies tests and limits to determine these, as well as other unspecified digitoxosides.

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The assessment of plasma levels of drug and metabolites, and the detection of potential degradation products and impurities in drug formulations would therefore require methods that could resolve all of the above glycosides. In the past many investigators have used paper[14,15], thin-layer[16,17], gas–liquid[18,19] and column[20,21] chromatographic methods for the separation of digitalis glycosides. The wide variety of methods that have been reported up to 1974 have been reviewed by Page[22]. High-performance liquid chromatographic (HPLC) procedures have been used to separate various mixtures of cardiac glycosides[23,24]. Separation of digitoxin, digoxin and some of their metabolites by gradient elution in a single chromatogram has been reported by Castle[25] while separations of the high and low polarity groups of digitalis glycosides of the cardenolide series has been reported by Lindner and Frei[23]. HPLC has also been used by two groups of investigators to resolve digoxin and its hydrolysis products[26,27]. A recent paper by Fujii *et al.*[24] has reported a series of solvent systems using a micro-HPLC column for the resolution of various mixtures of digoxin, digitoxin and their respective metabolites or degradation products, as well as lanatosides A and B. Unfortunately the methods employed to resolve the digitalis glycosides thus far have suffered from relatively long elution times, the need for gradient elution, or the need for specialized columns. In addition many methods have been suitable for only certain groups of the cardenolide series of digitalis glycosides. The need for a more robust HPLC method using conventional column technology that would be capable of the isocratic resolution of digoxin, digitoxin and their known metabolites and/or impurities in a single chromatogram is therefore evident. The development of such a method, as well as single-step gradient and linear solvent gradient HPLC modes, is the subject of the present paper. In addition, the isocratic resolution of the semi-synthetic digitalis glycosides, α- and β-acetyldigoxin from digoxin and its metabolites is described.

EXPERIMENTAL

Apparatus

A Beckman High Performance Liquid Chromatograph (Model 322) equipped with dual pumps (Models 100A and 110A) and a Waters Associates injection loop (Model U6K) was used with a Hitachi variable-wavelength detector (Model 100-10) and a Shimadzu Chromatopac (Model C-RIA) electronic data system. The volume of the dynamically stirred mixing chamber and ancillary tubing was 200 μl. The column was a 25 $\times$ 0.46 cm Ultrasphere reversed phase (C_{18}) with 5 μm particle size, obtained from Beckman Instruments.

Materials

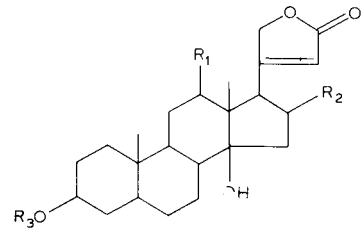
Water, methanol, isopropanol and dichloromethane were HPLC quality (Fisher Scientific, Pittsburgh, PA, U.S.A.). All solvents used were of HPLC grade. The cardiac glycosides and aglycones (Table I) were obtained from Boehringer (Mannheim, G.F.R.) and were used without further purification.

Methods

The glycosides were dissolved in the eluting solvent and injected into the chromatograph immediately after preparation. Samples used for determination of chro-

TABLE I

STRUCTURES OF THE DIGITALIS GLYCOSIDES AND AGLYCONES INVESTIGATED



D = Digitoxose; Ac = acetyl.

Compound	R_1	R_2	R_3
Digitoxigenin	H	H	H
Digitoxigenin monodigitoxoside	H	H	D
Digitoxigenin bisdigitoxoside	H	H	D–D
Digitoxin	H	H	D–D–D
Gitoxin	H	OH	D–D–D
Digoxigenin	OH	H	H
Digoxigenin monodigitoxoside	OH	H	D
Digoxigenin bisdigitoxoside	OH	H	D–D
Digoxin	OH	H	D–D–D
α- and β-acetyldigoxin	OH	H	D–D–D (Ac)

matographic characteristics were freshly prepared. Each data point on the calibration curve is an average of six determinations. The retention time of each compound was determined by separate injections of individual solutions of each sample. Solvent systems were prepared in sufficient quantities before use and degassing was not found to be necessary.

RESULTS AND DISCUSSION

The isocratic separation of digoxin, digitoxin and their metabolites or potential impurities is depicted in Fig. 1. The mobile phase consisted of water–methanol–isopropanol–dichloromethane (47:40:9:4). By a slight alteration in the composition of the mobile phase using the same eluents (43:35:15:7), the total elution time could be reduced from 25 to 13 min, but, the two early peaks due to digoxigenin and digoxigenin monodigitoxoside coalesced. However, the other seven glycosides still maintained baseline resolution. Gitoxin is a known impurity in digoxin formulations[13] and the compendial method specified for its detection is labour intensive. As shown in Fig. 1, this material may be resolved from digoxin in less than 11 min of chromatographic elution time. The chromatogram shown in Fig. 2 is representative of the fastest isocratic separation that could be achieved for the digitoxin series of potential metabolites or degradation products and was obtained with a solvent ratio of 44:34:15:7. In this case digitoxin, its mono- and bisdigitoxosides and digitoxigenin are completely resolved in 9 min.

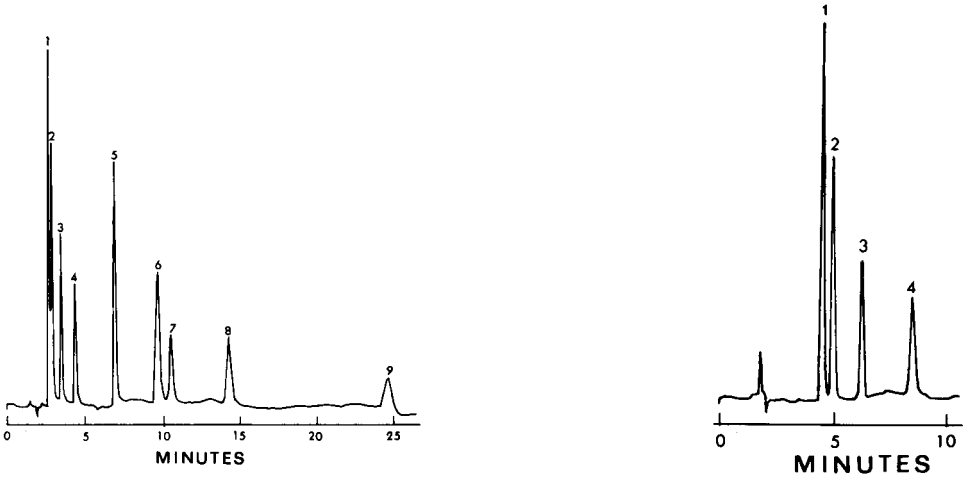


Fig. 1. Isocratic separation of digitalis glycosides. Sequence of elution: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = digitoxigenin; 6 = digitoxigenin monodigitoxoside; 7 = gitoxin; 8 = digitoxigenin bisdigitoxoside; 9 = digitoxin. Solvent system: water–methanol–isopropanol–dichloromethane (47:40:9:4); flow-rate 1.2 ml/min. 160 ng of each glycoside injected in 50 μl.

Fig. 2. Isocratic separation of digitoxin series of glycosides. Sequence of elution: 1 = digitoxigenin; 2 = digitoxigenin monodigitoxoside; 3 = digitoxigenin bisdigitoxoside; 4 = digitoxin. Solvent system: water–methanol–isopropanol–dichloromethane (44:34:15:7); flow-rate 1.2 ml/min. 160 ng of each glycoside injected in 50 μl.

The glycosides, α- and β-acetyldigoxin are prepared by the enzyme hydrolysis of lanatoside C in *Digitalis lanata* leaf and previous attempts[28] to separate these two short acting semi-synthetic glycosides have not been fully successful. The chromatogram depicted in Fig. 3 shows that these two glycosides may be resolved in less than 24 min while maintaining complete separation of digoxin and its digitoxose residues.

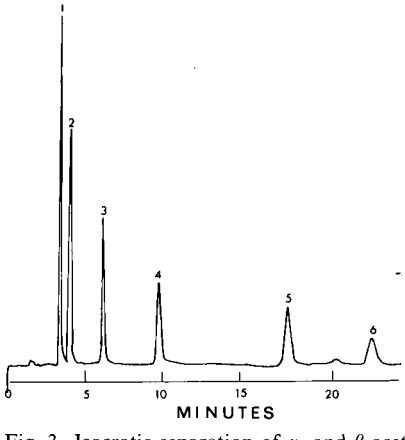


Fig. 3. Isocratic separation of α- and β-acetyldigoxin from the digoxin series of glycosides. Sequence of elution: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = α-acetyldigoxin; 6 = β-acetyldigoxin. Solvent system: water–methanol–isopropanol–dichloromethane (51:42:5:2); flow-rate 1.2 ml/min. 160 ng of each glycoside injected in 50 μl.

Although isocratic solvent systems are generally preferable to linear gradient or step gradient modes as the isocratic mode does not require additional pumps, solvent switching valves or column re-equilibration, the digitalis glycosides were subjected to these techniques to determine if there was any gross benefits in terms of overall resolution or time. Fig. 4 is representative of a single-step gradient that was generated by using two HPLC pumps, although the use of a solvent switching valve would also serve the same purpose. One pump delivered a solvent mixture consisting of water–methanol–isopropanol–dichloromethane (49:41:7:3) for 5 min. At this point the first pump was stopped and the second pump began delivery of a solvent mixture containing the same solvents in a ratio of 41:34:17:8. The total elution time for digitoxin was 17 min but column re-equilibration was found to require 15 min before an identical chromatogram could be generated. A linear gradient starting with water–methanol–isopropanol–dichloromethane (49:41:7:3) that was altered to (38:32:20:10) starting from time 2.5–3 min provided the shortest total elution (Fig. 5) of the eight digitalis glycosides (14 min). Although the single-step gradient and linear gradient modes reported here lead to considerably faster elution times than the 21.5 min reported for an earlier gradient solvent system[29], it is considered that little benefit could be gained over the isocratic solvent mode depicted in Fig. 1 since the gradient modes require column re-equilibration and more sophisticated instrumentation. Quantities of each of the glycosides were increased to accommodate the shift in baseline observed due to the gradient profile.

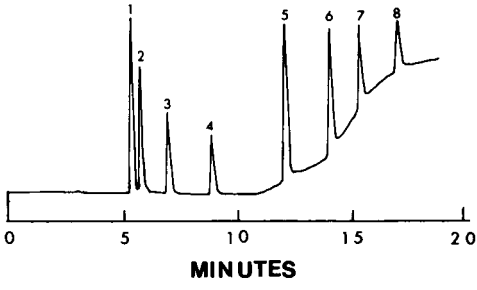


Fig. 4. Single-step gradient separation of digitalis glycosides. Sequence of elution: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = digitoxigenin; 6 = digitoxigenin monodigitoxoside; 7 = digitoxigenin bisdigitoxoside; 8 = digitoxin. Solvent system at time 0: water–methanol–isopropanol–dichloromethane (49:41:7:3). Solvent ratio at 5 min changed to 41:34:17:8. Flow-rate: 1.2 ml/min throughout. 16 μg of each glycoside injected in 50 μl.

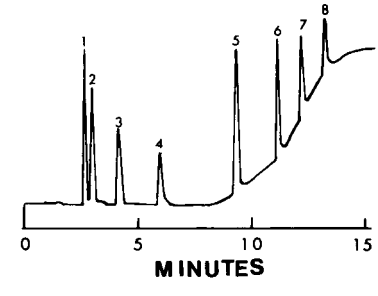


Fig. 5. Linear gradient elution of digitalis glycosides. Refer to Fig. 4 for identification of glycosides. Solvent system at time 0: water–methanol–isopropanol–dichloromethane (49:41:7:3). Solvent was linearly changed from time 2.5–3.0 min to water–methanol–isopropanol–dichloromethane (38:32:20:10) and was maintained at these proportions until completion of the chromatographic elution. Flow-rate: 1.2 ml/min. 16 μg of each glycoside injected in 50 μl.

The retention times of each of the digitalis glycosides were found to be reproducible with a relative standard deviation of 0.5% (n = 6 for each glycoside). A summary of the retention times of the digitalis glycosides evaluated is given in Table II. The capacity factor (k') value for digoxigenin in Fig. 1 is 1.46. This value is below the optimum value of 2 for chromatographic methods but within the minimum value of 1 accepted by most researchers in the field[30].

TABLE II

TOTAL RETENTION TIMES* OF DIGITALIS GLYCOSIDES UNDER THE CONDITIONS SPECIFIED IN THE CORRESPONDING FIGURES**

Compound	*Fig. 1*	*Fig. 2*	*Fig. 3*	*Fig. 4*	*Fig. 5*
Digoxigenin	2.8		3.4	5.5	2.8
Digoxigenin monodigotoxoside	2.9		4.0	5.9	3.2
Digoxigenin bisdigitoxoside	3.6		6.1	7.1	4.4
Digoxin	4.5		9.8	9.0	6.3
Gitoxin	10.6				
Digitoxigenin	7.0	4.5		12.2	9.6
Digitoxigenin monodigitoxoside	9.8	5.0		14.9	11.4
Digitoxigenin bisdigitoxoside	14.4	6.3		15.5	12.4
Digitoxin	24.5	8.5		17.2	13.6
α-Acetyldigoxin			18.0		
β-Acetyldigoxin			23.4		

* Time to the nearest tenth of a minute.
** Times are the mean of six determinations.

The HPLC systems reported in this paper provide good isocratic and solvent program modes for the resolution of the major therapeutic glycosides, digoxin and digitoxin as well as their potential impurities or degradation products. By only subtle changes in the relative composition of four solvents, the glycoside or series of glycosides can be eluted in convenient chromatographic times. In addition, the first reported complete HPLC resolution of α- and β-acetyldigoxin was readily accomplished by the isocratic solvent mode.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH REDUCTIVE ELECTROCHEMICAL DETECTION OF MUTAGENIC NITRO-SUBSTITUTED POLYNUCLEAR AROMATIC HYDROCARBONS IN DIESEL EXHAUSTS

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SUMMARY

A method is described for the measurement of nitro-substituted polynuclear aromatic hydrocarbons (nitro-PAHs) which employs high-performance liquid chromatography with reductive electrochemical detection. A series of reference nitro-PAHs has been separated with a reversed-phase column and quantified over a linear range of 10^3 with a sensitivity of 10–100 pg per compound. Extracts of several samples of diesel-exhaust particulates contained numerous compounds which were reduced at an electrode potential of −0.6 V (*vs.* Ag–AgCl). By comparing retention times and hydrodynamic voltammograms of these peaks with those of reference nitro-PAHs it was possible to confirm the presence of 1-nitropyrene at levels of between 5 and 44 ng/mg in 5 of 6 diesel extracts and to differentiate peaks representing nitro-PAHs from those representing other reducible species (*e.g.*, aldehydes, ketones and quinones).

INTRODUCTION

Recent investigations of emissions from diesel engines have focussed on mutagenic and carcinogenic nitro-substituted polynuclear aromatic hydrocarbons (nitro-PAHs)[1–6]. Of the nitro-PAHs which have been tentatively identified in extracts of diesel-exhaust particulates, several are potent mutagens in Ames' *Salmonella* bioassay[7], a short-term test designed to detect chemicals which may be potential carcinogens. These compounds include 1-nitropyrene (1-NP)[1,4,5], 2,7-dinitrofluorene (2,7-DNF)[1], and 2- and 3-nitro-9-fluorene (2- and 3-NFO)[1]. Other mutagenic nitro-PAHs in diesel exhausts, including 2-nitrofluorene (2-NF)[1,2] and 4-nitrobiphenyl (4-NB)[1], are known to be carcinogenic in laboratory mammals[8]. Numerous other nitro-PAHs whose presence has been indicated in diesel exhausts[1,4] have not been tested

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for mutagenicity or carcinogenicity because of the unavailability of reference compounds.

The quantification of nitro-PAHs in diesel exhausts has been hampered by the small amounts of individual compounds present (pg–ng per mg of extract) and by the extreme complexity of the extracts which contain a myriad of potentially interfering aromatic species including polynuclear aromatic hydrocarbons (PAHs), nitrogen- and sulfur-containing heterocycles and oxidation products of PAHs[5,9]. Thus, methods are required which are not only extremely sensitive but which also provide great selectivity or resolution of individual nitro-PAHs from interfering compounds. Scheutzle *et al.*[4] used tandem mass spectrometry to quantify the prototypical and abundant nitro-PAH 1-NP in extracts of particulates from five diesel engines. Unfortunately, such instrumentation is generally unavailable to most researchers.

We report here the application of high-performance liquid chromatography (HPLC) with reductive electrochemical detection (RED) to measure nitro-PAHs at pg–ng levels and to quantify 1-NP in extracts of diesel exhaust particulates. The use of RED has been proposed by Kissinger *et al.*[10] for several classes of reducible organic compounds, including nitroarenes, which can be conveniently separated by reversed-phase HPLC. This application also illustrates the use of hydrodynamic voltammetry, as proposed by Kissinger *et al.*[10], to provide additional information concerning the identities of compounds whose retention characteristics match those of reference nitro-PAHs.

EXPERIMENTAL

Chemicals and standards

All solvents were distilled in glass (Burdick and Jackson or J. T. Baker). High purity monochloroacetic acid (99.7%) and sodium acetate (99.3%), used for the buffer solution, were obtained from J. T. Baker. Water was purified with a Millipore Q System. Analytical grade 1-nitronaphthalene (1-NN), 2-nitronaphthalene (2-NN), 2-nitrobiphenyl (2-NB), 3-nitrobiphenyl (3-NB), 4-nitrobiphenyl (4-NB), 2-nitrofluorene (2-NF), 9-nitroanthracene (9-NA), 3-nitro-9-fluorenone (3-NFO), 2,7-dinitrofluorene (2,7-DNF), 2-acetamido-3-nitro-9-fluorenone (2-ANFO), 4-nitrofluoranthene (4-NFA), 2,7-dinitro-9-fluorenone (2,7-DNFO), 7-nitrofluorene-1-carboxylic acid (7-NFCA), 1,4-naphthoquinone, anthraquinone, 2-naphthaldehyde, 9-anthraldehyde and 1-pyrenecarboxaldehyde were obtained from Aldrich. 1-Nitropyrene (1-NP) was obtained from Pfaltz and Bauer, 1,3-Dinitropyrene (1,3-DNP) and 1,3,6-trinitropyrene (1,3,6-TNP) were generously supplied by R. Mermelstein of Xerox Corp., Rochester, NY, U.S.A. Pyrene-3,4-dicarboxylic acid anhydride was synthesized as described elsewhere[11]. All reference standards were used without purification.

Collection and extraction of diesel-exhaust particulates

Two types of particulate samples were used in this study. Four of the six samples were obtained from Dr. Thomas Baines of the U.S. Environmental Protection Agency in Ann Arbor, MI, U.S.A. These samples consisted of pleated glass-fiber filters (Dustfoe Type, 13 m^2 collection area per filter, Mine Safety Appliance, Pittsburgh, PA, U.S.A.) containing diesel particulates collected at ambient temperature

from a dilution tunnel where diesel truck engines were tested. Sample No. 1 was collected from a 1979 International Harvester engine, Sample No. 2 from a 1979 Caterpiller engine and Samples No. 3 and 4 from a 1980 Mack engine. All engines were of the 4-stroke, 6-cylinder type with turbochargers and aftercoolers typical of long distance trucks and were designed to meet California emission standards. An electric dynamometer provided the mechanical loading of the engines. The protocol used in the generation and collection of the samples has been described elsewhere[3]. Single sections of each multi-pleated filter were removed from the filter frame for extraction.

The remaining two samples were obtained from Mr. Frank Robben of the Lawrence Berkeley Laboratory of the University of California, Berkeley, CA, U.S.A. These samples consisted of 20.3 × 25.4-cm PTFE-coated glass-fiber filters (Pallflex Products Corp., Putnam, CT, U.S.A.) containing diesel particulates from a medium speed diesel engine manufactured by the Engine and Compressor Division of Transamerica Delaval, Oakland, CA, U.S.A. Designated a DSR-46, this 4-stroke, 6-cylinder engine, with a shaft power of 2700 kW, is a turbocharged and intercooled model of the type used commercially for generating power. Samples were collected by drawing a portion of the diluted exhaust through an in-line filter holder at temperatures of $< 50°C$. Sample No. 5 was obtained when the engine was operated on diesel fuel No. 2. Sample No. 6 was obtained when 5 of the 6 cylinders were operated on diesel fuel No. 2 and the sixth cylinder was operated on a solvent refined coal middle distillate fuel (SRC-II).

Each filter was placed on a Sohxlet extraction apparatus and extracted for 24 h with 300 ml of dichloromethane using a cycling time of about 45 min. Extracts were filtered through PTFE membrane filters of 0.45 μm pore size (Millipore Type FH) and were reduced in volume to a few ml by rotary evaporation. Concentrated extracts were transferred to tared vials with dichloromethane rinsings, dried under nitrogen and weighed. Extracts were stored at $-4°C$ prior to analysis and manipulations were performed under shaded lighting to reduce the possibility of photooxidation.

Preparation of samples

Approximately 20 mg of each extract of diesel-exhaust particulates were dissolved in a minimum volume of dichloromethane (≈ 200 μl) and applied to a small silica gel cartridge (Sep-Pak, Waters Assoc.) with minimal rinsings of dichloromethane. The cartridge was eluted in serial order with 3 ml of hexane, 6 ml of dichloromethane and 3 ml of methanol. Each eluate was collected separately. Eluates were dried under nitrogen at 40°C, weighed in tared vials, dissolved in 200 μl of dichloromethane–*n*-propanol (1:1), and diluted to the desired concentration (0.5–2 mg/ml) with the HPLC mobile phase.

High-performance liquid chromatography

Samples were purged of dissolved oxygen with nitrogen (presaturated with the mobile phase) for about 10 min and aliquots were introduced into the 20-μl loop of a Rheodyne injection valve. The HPLC system consisted of a Beckman/Altex 100A pump, a 25 cm × 4 mm I.D. Beckman Ultrasphere ODS column (5-μm spherical particles) with a short precolumn containing C_{18} Corasil (Waters) both maintained at 50°C in a column oven, and a Bioanalytical Systems electrochemical detector com-

prised of a LC-4A amperometric controller and TL-5 thin-layer flow cell with a glassy carbon working electrode. The electrode was operated at a potential of between −0.1 and −0.8 V *vs.* a Ag–AgCl reference electrode. All PTFE lines in the system were replaced with stainless steel to prevent permeation of oxygen into the mobile phase[10]. The mobile phase, 35% *n*-propanol in 0.05 *M* monochloroacetic acid–sodium acetate buffer at pH 3.8, was continuously heated to 50°C under nitrogen in a flask fitted with a reflux condenser to remove dissolved oxygen as recommended by the manufacturer of the detector (Bioanalytical Systems) for reductive work. The column was purged with the mobile phase at 0.1 ml/min overnight prior to analysis to remove dissolved oxygen. During analysis the flow-rate of the mobile phase was 1.0 ml/min. Some peak areas and retention times were determined manually while others were determined with a Varian Vista 401 data system.

The HPLC system required periodic maintenance to insure optimal performance. After a full day of analysis of diesel-exhaust samples, the system was washed with methanol to remove nonpolar residues. The glassy carbon electrode, when used in the reductive mode, is sensitive to passivation by heavy-metal contamination of the mobile phase. Using the high-purity salts listed, the background current was small and the working electrode required repolishing at roughly 1-week intervals. Initial work, performed with salts of reagent grade obtained from Aldrich, showed substantially higher background current and repolishing of the electrode was required more frequently.

RESULTS AND DISCUSSION

RED of reference nitro-PAHs

Fig. 1 shows a chromatogram of a mixture containing 10 ng each of 16 reference compounds. Although some components in the mixture were not completely resolved, this chromatogram illustrates that most nitro-PAHs containing between 2 and 4 rings, which are the most prevalent such species in diesel-exhaust extracts[1,4], are eluted within about 25 min of injection. Calibration curves of several reference compounds shown in Fig. 2 indicate that the linear range of the RED should typically be about 10^3 and that sensitivity should extend to 10–100 pg for most nitro-PAHs. The detector currents compiled in Fig. 2 were obtained by measuring peaks at −0.6 V, an electrode potential which, as will be shown, is at or above the "plateau" potential of many nitro-PAHs.

RED of diesel extracts

Diesel extracts had been prefractionated prior to HPLC by applying them to silica gel cartridges which were sequentially eluted with hexane, dichloromethane and methanol (see Experimental). Chromatograms of hexane fractions indicated the absence of species reducible at −0.6 V. However, chromatograms of both dichloromethane and methanol fractions contained numerous peaks at −0.6 V indicating the possible presence of nitro-PAHs. Compounds in dichloromethane fractions were eluted predominantly after oxygen (retention time = 6.4 min) in the reversed-phase HPLC system whereas compounds in methanol fractions eluted before oxygen. Because reference standards of nitro-PAHs and nitrosubstituted ketones of PAH eluted primarily after oxygen (Peak No. 2 in Fig. 1) priority was given to the investigation of

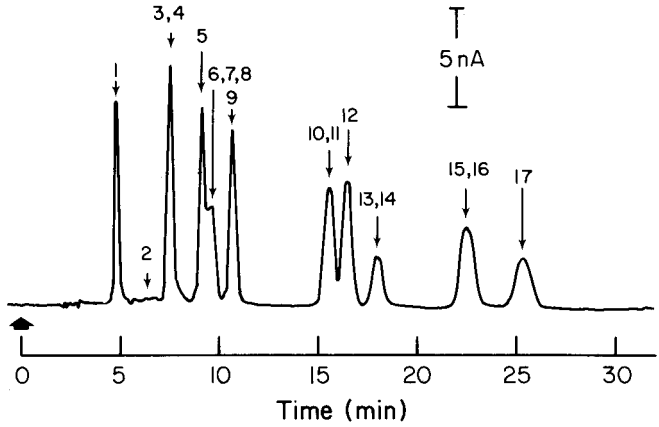

Fig. 1. Chromatogram of 16 reference nitro-PAHs. Chromatographic conditions: Column, 25 cm × 4 mm I.D. Ultrasphere ODS (5 μm spherical particles); Mobile phase, 35% *n*-propanol in 0.05 *M* monochloracetic acid–sodium acetate buffer at pH 3.8, 1.0 ml/min; electrode potential, −0.6 V *vs.* Ag–AgCl reference electrode. Identities of compounds present at 10 ng each: 1 = 2-ANFO; 2 = oxygen; 3 = 7-NFCA; 4 = 2,7-DNFO; 5 = 3-NFO; 6 = 2,7-DNF; 7 = 1-NN; 8 = 2-NB; 9 = 2-NN; 10 = 3-NB; 11 = 4-NB; 12 = 2-NF; 13 = 1,3,6-TNP; 14 = 9-NA; 15 = 1,3-DNP; 16 = 1-NP; 17 = 4-NFA.

dichloromethane fractions and the more polar species present in methanol fractions were not examined further in this study.

Chromatograms of dichloromethane fractions of extracts from various samples of diesel particulates were superficially similar, in that they all contained numer-

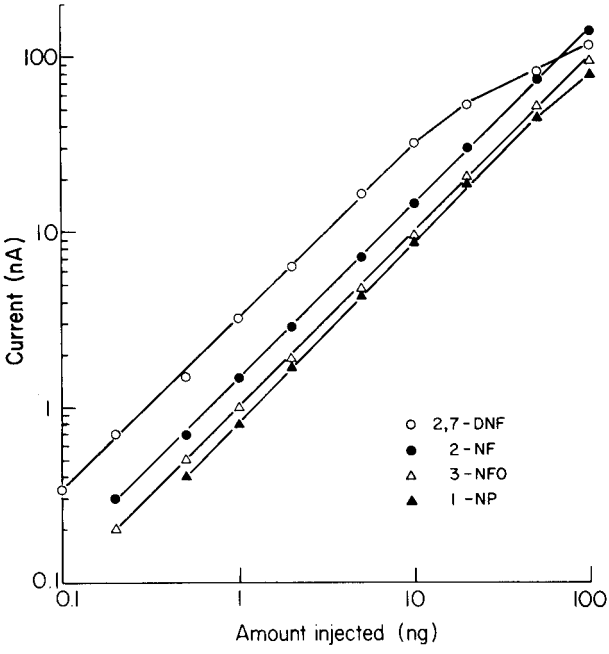

Fig. 2. Calibration curves of 4 nitro-PAHs (3-NFO, 2,7-DNF, 2-NF and 1-NP). Chromatographic conditions as in Fig. 1.

ous peaks in roughly the same range of retention times, but varied considerably in the actual retention times and areas of the peaks. Fig. 3 depicts chromatograms of the dichloromethane fraction from diesel extract No. 1 recorded at −0.6 V (top) and −0.3 V (bottom), respectively. This sample was selected for examination because 3 of the more abundant peaks in the chromatogram, labeled a, b and c, had retention times close to those of several reference nitro-PAHs which have been identified in diesel extracts. [Peak a possibly: 3-NFO, 2,7-DNF, 1-NN; peak b possibly: 2-NF; peak c possibly: 1-NP, 1,3-DNP.]

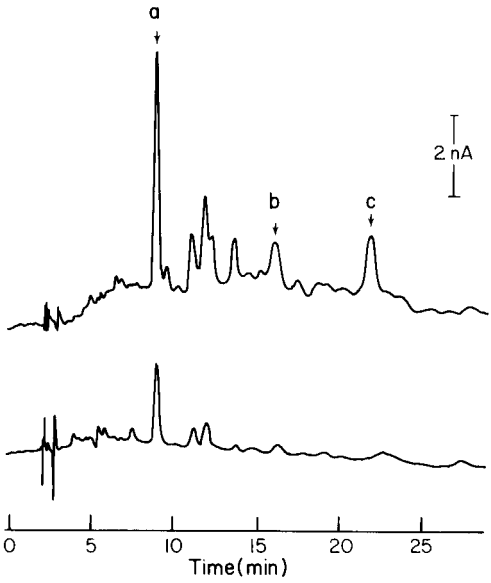

Fig. 3. Chromatograms of 10-μg portions of diesel extract No. 1. Chromatographic conditions given in Fig. 1. Top chromatogram, electrode potential = −0.6 V; bottom chromatogram, electrode potential = −0.3 V.

Hydrodynamic voltammograms

Additional information concerning the identities of peaks a, b and c was obtained by comparing the normalized hydrodynamic voltammograms[10] of these peaks with those of the suspected reference nitro-PAHs as shown in Fig. 4–6. In each case, the relative current ratio Φ, which is the ratio of the current at given potential to the diffusion-limited current, is plotted *vs.* applied potential. The only candidate nitro-PAH whose presence in the sample is entirely consistent with the volammetric data is 1-NP which produces a voltammogram that matches that of peak c within the range of experimental error (Fig. 6). The voltammograms of the other candidate nitro-PAHs are sufficiently different from those of the unknown peaks to preclude the confirmation of identity. However, the data do not eliminate the possibility that these compounds may have coeluted with additional nitro-PAHs or with other reducible species thereby confounding the interpretation of voltammograms.

Quantitation of 1-NP

Five of the six diesel extracts investigated contained peaks whose retention

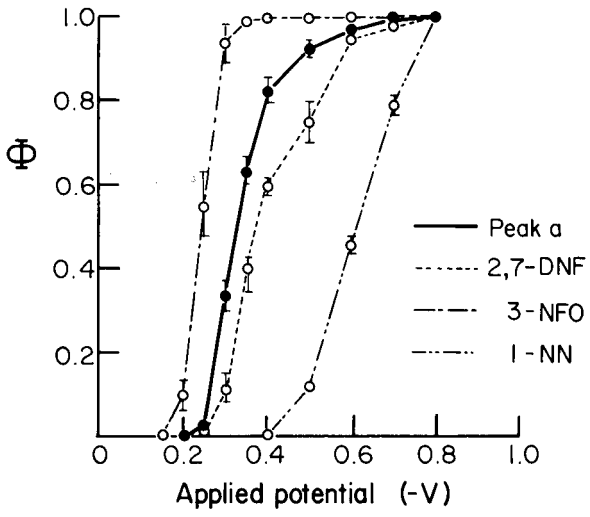

Fig. 4. Hydrodynamic voltammograms of peak a (Fig. 3) and 3 reference nitro-PAHs (2,7-DNF, 3-NFO and 1-NN) with similar retention times. (Φ is the relative current ratio; mean and range are plotted for 3 or more observations.)

times and hydrodynamic voltammograms matched those of 1-NP within the range of experimental error. To determine the concentrations of this compound whose presence is presumed in the extracts, the recovery of 1-NP was determined by spiking 10- to 20-mg portions of diesel extracts with between 0.3 and 0.5 μg of 1-NP, then applying them to silica gel cartridges and performing HPLC as described in Experimental. Based upon 6 trials the recovery was 95.0 $\pm$ 10.4% ($\bar{x}$ $\pm$ S.D.) when corrected for equivalent control samples of the same extracts. The concentrations of 1-NP in the six diesel extracts after correcting for a recovery of 95% are given in

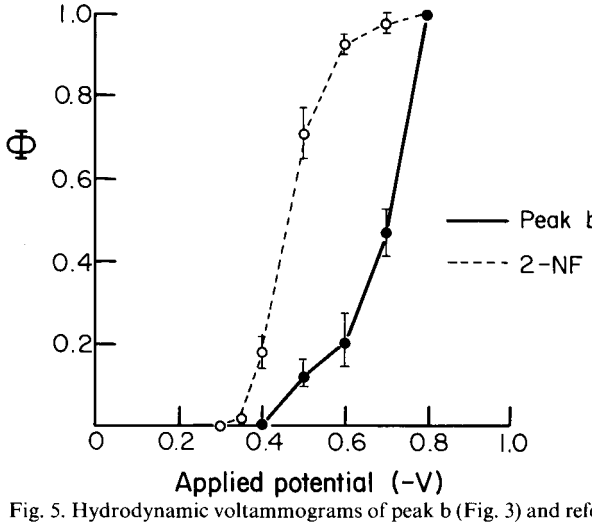

Fig. 5. Hydrodynamic voltammograms of peak b (Fig. 3) and reference 2-NF which has the same retention time. (Φ is the relative current ratio; mean and range are plotted for 3 or more observations.)

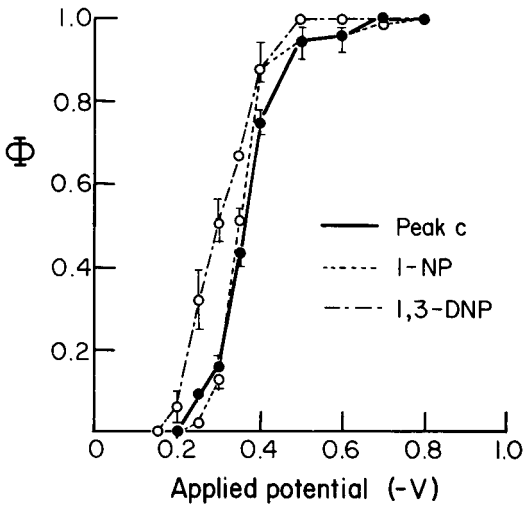

Fig. 6. Hydrodynamic voltammograms of peak c (Fig. 3) and reference 1-NP and 1,3-DNP which have similar retention times. (Φ is the relative current ratio; mean and range are plotted for 3 or more observations.)

Table I. These concentrations, determined by both standard addition and external calibration, ranged from <2 to 44 ng/mg of extract. Three runs with extract No. 1 indicated values of 32, 39 and 36 ng/mg.

Scheutzle *et al.*[4] reported that 1-NP, at levels between 55 and 2285 ng/mg, was one of the more abundant nitro-PAHs in the diesel extracts they investigated. Based upon the relative sizes of peaks of presumed nitro-PAHs in our samples, these results confirm this observation; however, the concentrations of 1-NP we measured were substantially smaller than those reported by Scheutzle *et al.*[4].

Other reducible species

The utility of RED as a tool for measuring nitro-PAHs in engine exhausts and in ambient air rests in part upon its specificity. It is important, therefore, to know whether other classes of reducible compounds might interfere in the interpretation of

TABLE I

CONCENTRATIONS OF 1-NITROPYRENE IN DIESEL EXTRACTS

A recovery of 95% from the prefractionation procedure is assumed.

Sample No.	*Concentration of 1-nitropyrene (ng/mg)*
1 (3 trials)	32, 39, 36
2	44
3	<2
4	8
5	20
6	5

chromatograms. Since the technique is based upon reversed-phase HPLC, only aromatic species with 2 or more rings and little ionic character (at pH 3.8) should be sufficiently retained by the column to elute in the range of interest (*i.e.*, after oxygen in Fig. 1). Of these compounds, the most likely interfering species are oxidation products of PAHs including ketones and quinones, aldehydes and dicarboxylic acid anhydrides[5,9,11].

Compounds representative of these classes of potentially interfering species were tested in the system at an electrode potential of −0.6 V. Of these substances, all three aldehydes (2-naphthaldehyde, 9-anthraldehyde and 1-pyrene carboxaldehyde) and pyrene-3,4-dicarboxylic acid anhydride produced no response. Yet the two quinones, 1,4-naphthoquinone and anthraquinone, were detected with sensitivities comparable to those of the nitro-PAHs. The hydrodynamic voltammograms of these quinones, shown in Fig. 7, indicate that they are more easily reduced than the nitro-PAHs tested. If these reduction potentials are indicative of those of other ketones and quinones of PAH it may be possible to differentiate these compounds from nitro-PAHs by measuring Φ values of the peaks at different electrode potentials. By these criteria, none of the larger peaks shown in Fig. 3 would be attributed to ketones or quinones.

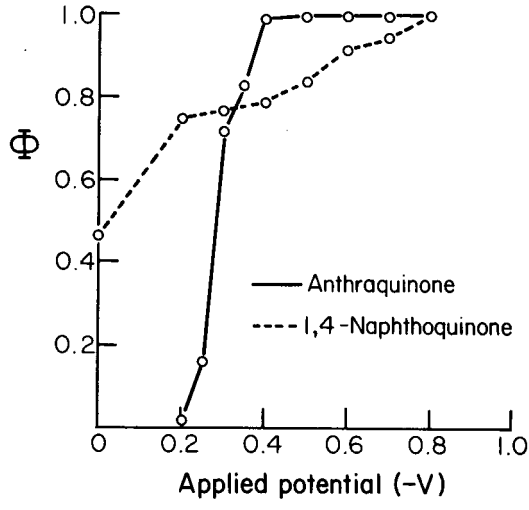

Fig. 7. Hydrodynamic voltammograms of anthraquinone and 1,4-naphthoquinone. (Φ is the relative current ratio.)

The reduction of ketones of PAH which also contain nitro groups is apparently initiated at the carbonyl oxygen (compare the voltammograms of 3-NFO, Fig. 4, and 2-NF, Fig. 5). Thus, it would probably not be possible to differentiate nitro-containing ketones from ketones and quinones which do not contain nitro groups solely on the basis of their Φ values.

CONCLUSIONS

The use of HPLC with RED offers the analyst a relatively straightforward approach to the measurement of nitro-PAHs at pg–ng levels in air samples. Our success in quantifying 1-NP in extracts of diesel-exhaust particulates illustrates the viability of the technique when applied to extremely complex mixtures. A particularly attractive feature of the method involves the use of hydrodynamic voltammetry to assist in the characterization of nitro-PAHs in samples. Preliminary results indicate that some reducible species which might interfere with the analysis (aldehydes and anhydrides) are not measured at the electrode potential used for nitro-PAHs (-0.6 V) while others (ketones and quinones) can probably be differentiated from nitro-PAHs by their hydrodynamic voltammograms.

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RAPID METHOD FOR PURIFICATION OF PLASMID DNA AND DNA FRAGMENTS FROM DNA LINKERS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK-PW GEL

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SUMMARY

High-performance size exclusion chromatography (HPSEC) using TSK-G5000 PW in Tris buffer has been found to be a reliable method for the rapid fractionation of DNA ligation products. Plasmid and fragmented phage DNAs were found to elute in less than 2 min with recoveries greater than 98%. *Escherichia coli* transfection studies, using plasmid DNA that had been subjected to HPSEC column fractionation, showed high transformation efficiencies. $MgCl_2$, a component of the DNA ligation reaction, was found to produce DNA–column support interactions, which resulted in low DNA recoveries. Such interactions were eliminated by chelation with ethylenediaminetetraacetate prior to chromatography.

INTRODUCTION

The evolution of recombinant DNA technology has generated a requirement for a separation technique that will resolve from a DNA ligation reaction, insert DNAs and DNA linkers[1]. It is essential to remove the linker molecules from such a reaction in order to limit the probability of subsequently cloning DNA linkers without insert DNA. There are two approaches to removing linkers from the insert DNA. The first method is to treat the ligation mixture with a restriction endonuclease complementary to the DNA linker site in order to generate a cohesive end that can be joined to vector DNA and to eliminate linker oligomers. This step requires an extremely large and concentrated amount of the appropriate restriction endonuclease, since linkers not incorporated into insert DNA also compete for the endonuclease. After restriction endonuclease treatment the mixture is fractionated by methods with inherently low sample recoveries such as liquid–liquid partition[2,3], partition chroma-

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tography[4,5], gel electrophoresis[6], sedimentation velocity centrifugation[7], or molecular sizing chromatography[8–11]. The second approach is to fractionate first the ligation mixture by one of the methods mentioned above and then to generate cohesive ends by treating the fractionated DNA with the appropriate restriction endonuclease. The fractionation procedures necessary for the two general approaches given above are limited in that they do not meet all of the following requirements: (1) small sample dilution; (2) rapid separation of the ligation products; (3) efficient separation of the DNA–linker complex (1.0 to $5.0 \cdot 10^6$ daltons) from the greatly smaller DNA ligase enzyme, DNA linkers (*ca.* 5000 daltons) and adenosine triphosphate (ATP); and, (4) high recovery of the DNA in a buffer system appropriate for ethanol precipitation of the DNA–linker complex (especially critical when rare eukaryotic genes are being cloned). Recent work with high-performance size exclusion chromatography (HPSEC) has shown that TSK-SW[12] and TSK-PW[13] gels offer significant improvement in speed, resolution and buffer versatility over classical molecular sizing chromatography. However, the effects of high linear flow-rates, as generated in high-performance liquid chromatography (HPLC), on the biological activity of DNAs is largely unknown. These conditions led us to investigate the use of TSK-G5000 PW gel packing for the separation of DNA ligation products.

MATERIALS AND METHODS

Apparatus

For the collection of careful analytical data in this study, a sophisticated solvent delivery system, consisting of a Hewlett-Packard Model 1084B liquid chromatograph interfaced with a Waters Associates Model 450 variable-wavelength monitor, was used. For routine laboratory use, however, a less elaborate system was used. This consisted of an LKB Model 2138 Uvicord UV chromatography column monitor equipped with the HPLC flow cell option and a Model 39-500 low-pressure HPLC pump from Rainin Instruments. The samples were loaded on-column directly with a Rainin model 5020 PTFE rotary injection valve fitted with 0.030 in. I.D. PTFE tubing. A stainless-steel column (200 × 3.2 mm I.D.) was packed by the high-pressure gel filtration slurry method described by Kirkland[14] with 18-μm TSK G5000 PW gel packing (Toyo Soda, Tokyo, Japan). Samples were loaded by suction filling the injection valve loop. After initial calibration of flow-rates with the UV monitor, the system may be monitored using a stopwatch for DNA collection.

Enzymes

The restriction endonuclease *Eco*RI was the gift of Marj Thomas. All other restriction endonucleases were purchased from commercial sources. Bacteriophage T4 DNA ligase was generously provided by S. Scherer and J. Widom. Bacterial alkaline phosphatase (*E. coli*) was from Bethesda Research Labs and bacteriophage T4 polynucleotide kinase was from New England Biolab.

Enzymatic reactions

Restriction endonucleases were used as recommended by the manufacturer. Plasmid and phage DNAs were cleaved at concentrations of 100–200 μg/ml. DNA

ligations were performed in 50 m*M* Tris–HCl (pH 7.4), 10 m*M* $MgCl_2$, 10 m*M* dithiotreitol (DTT), 1 m*M* adenosine triphosphate (ATP) and 100 n*M* T4 DNA ligase. The *Eco*RI cut pBR325 DNA [5.45 kilobase pairs (kb)[15]] was dephosphorylated enzymatically with bacterial alkaline phosphatase and the 5′ end was labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase[16]. *Eco*RI synthetic linker DNA (Collaborative Research) was similarly labeled.

Preparation of bacteriophage T7 DNA

Wild-type T7[17] was grown on *E. coli* B at 37°C in a shaking incubator. Phage particles were purified from the lysate by polyethylene glycol precipitation and CsCl banding[18]. The purified phage were dialyzed against 10 m*M* Tris–HCl, 1 m*M* ethylenediaminetetraacetate (EDTA) (pH 7.4) and extracted with phenol. Residual phenol was removed by ether extraction.

Isolation of plasmid pBR325 DNA

Plasmid pBR325 DNA[15] was prepared by a modification of the procedure of Wensink *et al.*[19]. An overnight culture of *E. coli* containing plasmid pBR325 was used to innoculate 1 l of L-broth. The culture was grown to saturation at 37°C with vigorous aeration. The cells were harvested by centrifugation at 3330 *g* for 10 min in a GSA rotor (Sorvall) at 5°C. The cell pellet was resuspended in 250 ml TE buffer (10 m*M* Tris–HCl, pH 7.4; 1 m*M* EDTA) and collected by centrifugation. The cell pellet was resuspended in 30 ml of 15 (w/v) sucrose in 50 m*M* Tris–HCl (pH 7.4), 50 m*M* Na_2EDTA and 1 mg/ml freshly prepared lysozyme. This mixture was incubated at room temperature for 30 min. Next, 32 ml of Triton solution [0.1 % Triton X-100; 50 m*M* Tris–HCl (pH 8); 50 m*M* Na_2EDTA] was added and lysis was allowed to proceed for 10 min. The mixture was poured into polypropylene tubes and centrifuged at 36,900 *g* for 1 h in an SS34 (Sorvall) rotor at 5°C. The supernatant was poured into a graduated cylinder, the volume was adjusted to 60 ml, and 6 ml of 10 mg/ml ethidium bromide and 57 g of CsCl were added (density = 1.59 g/ml). This solution was centrifuged for 72 h at 112,000 *g* and 20°C in the 50.2 Ti rotor (Beckman). After equilibrium banding the DNA was visualized by fluorescence under long-wavelength UV light, and the lower band (containing the covalently closed plasmid DNA) was removed by side puncture of the polyallomer tube. The DNA-containing fractions were pooled; the density was adjusted to 1.59 g/ml with the CsCl–ethidium bromide solution in a final volume of 39.5 ml, and then re-banded in the 50.2 Ti rotor at 112,000 *g* for 72 h at 20°C. The re-banded DNA was collected and the ethidium bromide was removed by repeated isopropanol extraction. The CsCl was removed by diluting the sample with four volumes of TE buffer followed by ethanol precipitation. The DNA pellet was washed with ethanol, dried in a vacuum, and resuspended in TE buffer.

Plasmid transfection

Plasmid DNAs were used to transform *E. coli* LE392 essentially as described by Mandel and Higa[20]. The efficiency of transformation is about $4 \cdot 10^5$ transformants/μg of Form I DNA. Saturation occurs between 50 and 100 ng of Form I DNA per 10 cm plate ($1 \cdot 10^9$ cells).

Agarose gel electrophoresis

Column fractions of T7 DNA cleaved by restriction endonuclease were electrophoresed from a 6 mm × 15 mm sample well in a 6-mm-thick 1.8% horizontal agarose gel at 4 V/cm in 40 m*M* Tris–acetate (pH 8.1), 2 m*M* EDTA buffer as described by McDonell *et al.*[21]. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed using long-wavelength UV light and Kodak Plus-X film.

Chromatography and scintillation counting of labeled DNA and linkers

The pBR325 DNA (5.45 kb) was purified from *E. coli* and cut with *Eco*RI restriction endonuclease. The DNA and *Eco*RI linkers were labeled independently as described above. The unincorporated [γ-^{32}P]ATP was removed from the labelled DNA and labelled linkers by Sephadex gel filtration prior to HPLC. A mixture of labeled plasmid DNA (0.5 μg) and labeled linkers (0.15 μg) was loaded in a volume of 50 μl. Fractions were collected as drops and were spotted on Whatman DE-52 discs (about 30 μl each). Discs were then washed five times with phosphate buffers, followed by one wash of distilled water, ethanol and diethyl ether. The dried discs were counted in a 2,5-diphenyloxazole–toluene liquid scintillation fluid.

Elution standards and column calibration

Tobacco mosaic virus was a gift of Dr. A. T. Tu. The bovine serum albumin was obtained from Armour and the dinitrophenyl (DNP)-alanine was purchased from Sigma. Column parameters are defined as described by Pharmacia[22]. Here, V_0 (the void volume) refers to the interstitial volume between the beads; V_i and V_s refer to the volume within the beads that is accessible and inaccessible to solvent, respectively. The total column volume, V_t, is the sum of V_0 and V_i. V_e is the elution volume of the sample under study.

Chromatography parameters

Chromatography was performed at 25°C with a mobile phase flow-rate of 0.5 ml/min, a chart speed of 1.0 cm/min and detection at 260 nm. Sample injection volume was 50 μl for both protein and DNA samples. Under these conditions, the chromatography column pressure did not exceed 18 kg/cm^2 (17 bar).

RESULTS

The elution profiles of several standard molecular weight markers are shown in Fig. 1. Tobacco mosaic virus ($40 \cdot 10^6$ daltons)[23], bovine serum albumin ($6.63 \cdot 10^4$ daltons)[24] and DNP-alanine (256 daltons) were found to elute at 1.0 ml, 1.52 ml and 1.75 ml, respectively. These standards were run in 100 m*M* KCl, 10 m*M* $NaPO_4$ (pH 7.0) buffer at a flow-rate of 0.5 ml/min. Although extensive calibration of this column was not made (a procedure we have undertaken on commercially packed TSK columns[12,13]), the approximate values V_0 = 1.0 ml and V_t = 1.75 ml were obtained. The total geometrical column volume, V_g, from column dimensions, was 2.01 ml.

When the first attempts were made at chromatographing the DNA ligation mixture, which contains 10 m*M* $MgCl_2$, 10 m*M* DTT and 1 m*M* ATP, the DNA was greatly retarded on the column and was found to elute at the void volume only after adding EDTA to the mobile phase buffer. It was found that complete recoveries could

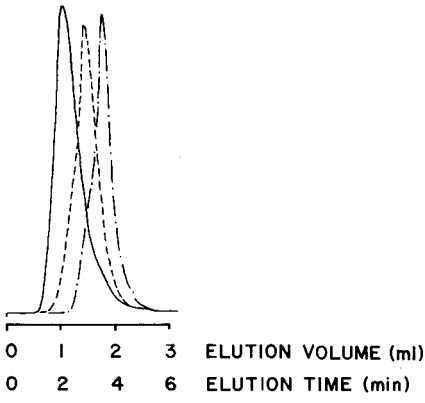

Fig. 1. Chromatography of calibration standards on TSK-G5000 PW. Tobacco mosaic virus (———), bovine serum albumin (------) and DNP-alanine (– · – ·) were used to mark the void volume, intermediate included volume and total column volume, respectively.

be obtained when the ligation buffer was "quenched" with EDTA (final EDTA concentration in the sample $\geqslant$ 15 m*M* before chromatography). When DNA samples had no $MgCl_2$ added, the subsequent chromatography showed no signs of retention and the DNA eluted normally at V_0. These results are summarized in Table I.

A mixture of *Hae*III endonuclease treated T7 DNA[25], and *Eco*RI DNA linkers was chromatographed and collected as drop fractions from G5000 PW. Agarose gel electrophoretic analysis (Fig. 2) showed that DNA fragments from 2.7 to approximately 0.10 kb eluted in the void volume (after 2 min of chromatography). DNA

TABLE I

BUFFER EFFECTS ON DNA ELUTION FROM G5000 PW

Buffer A = 10 m*M* phosphate (pH 7.0) and buffer B = 50 m*M* Tris (pH 7.4).

DNA elution behavior	*Column buffer*	*Injection buffer*
40% recovery —elutes past V_t*	Buffer A + 100 m*M* KCl	Buffer B + 100 m*M* $MgCl_2$ + 10 m*M* DTT**
40% recovery —elutes past V_t	Buffer A	Buffer B + 100 m*M* $MgCl_2$ + 10 m*M* DTT**
20% recovery —elutes past V_t	Buffer B + 10 m*M* $MgCl_2$	Buffer B + 100 m*M* $MgCl_2$ + 10 m*M* DTT**
40% recovery —elutes past V_t	Buffer B	Buffer B + 10 m*M* $MgCl_2$ + 10 m*M* DTT**
98% recovery —elutes at V_0	Buffer B + 15 m*M* EDTA	Buffer B + 10 m*M* $MgCl_2$ + 10 m*M* DTT**
98% recovery —elutes at V_0	Buffer B	Buffer B
98% recovery —elutes at V_0***	Buffer B	Buffer B + 10 m*M* $MgCl_2$ + 10 m*M* DTT + 15 m*M* EDTA

* Recovery estimated by area under elution profile at 260 nm.

** Buffer system used in ligation reaction.

*** Elution at V_0 indicates absence of DNA–column support interactions.

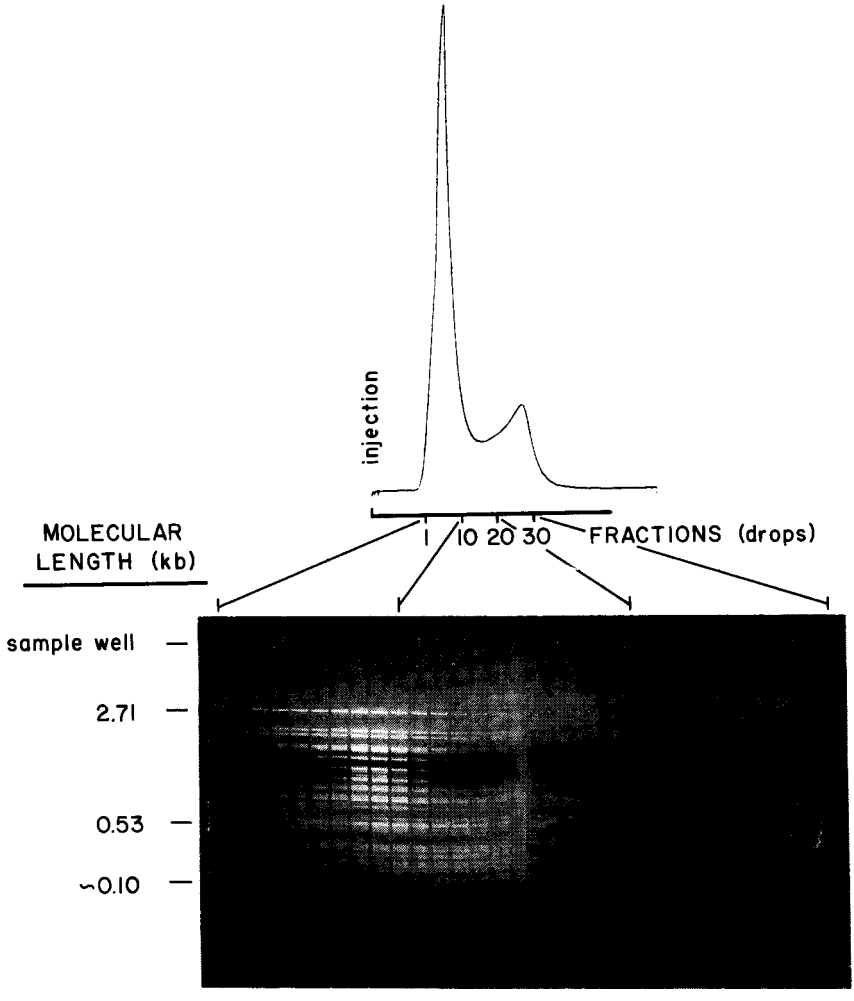

Fig. 2. Composite elution diagram of *Hae*III-treated T7 DNA and *Eco*RI linkers showing correlation between specific elution from G5000 PW and molecular length of DNA fragments. Note that DNA linkers are not visualized by the ethidium bromide stain.

linkers failed to stain with ethidium bromide because they were in single stranded form during electrophoresis and therefore did not bind the stain.

The DNA ligation system was also chromatographed with *Eco*RI cleaved ^{32}P end-labeled pBR325 DNA and ^{32}P end-labeled *Eco*RI DNA linkers. These elution profiles, plotted both as a function of counts per minute and $A_{260\ nm}$ per fraction, are shown in Fig. 3. Here 82,700 total cpm as plasmid DNA and 400,900 total cpm as *Eco*RI linkers were loaded on the column in one injection. Examination of the ^{32}P cpm from peak A on Fig. 3 yielded 81,000 total cpm for an apparent DNA recovery of 98%. The recovery from peak B on Fig. 3 was somewhat lower with 354,000 cpm giving a recovery value of 88% for the DNA linkers.

Studies with *Eco*RI-cleaved pBR325 DNA recovered from the void volume

peak showed that this DNA could be religated and was capable of transforming *E. coli* with an efficiency equivalent to that found for unaltered plasmid DNA.

DISCUSSION

HPSEC of the DNA ligation reaction mixture (containing pBR325 DNA, *Eco*RI DNA linkers, T4 DNA ligase and ATP) on TSK G5000 PW, was found to provide very low column residence times and excellent separation of DNA from oligonucleotide linkers and ATP.

Chromatography of the DNA ligation mixture in this study is effective because the plasmid DNA ($3.6 \cdot 10^6$ daltons) and DNA fragments are large when compared to the DNA linkers (5,300 daltons) and the T4 DNA ligase (65,000 daltons)[26]. Although the small-bore column (3.2 mm I.D.) used in this study provides the gross size fractionation required, it is evident that we are using this column in a "desalting" mode. TSK G5000 PW gel beads have an average pore diameter of about 260 Å[12]; therefore, the DNA is certainly excluded from the gel pores. Partial gel permeation of smaller DNAs may be possible using the noncommercially available gel beads (pore diameter 3500 Å) described by Kirkland[27]. However, we suspect that subjecting medium-sized DNAs to true gel permeation chromatography will generate extensive DNA shearing.

Small-bore columns of the type used in this analysis show peak shape irregularities (as seen with the chromatography standards in Fig. 1) because the injection "bolus" does not approach infinite diameter during chromatography and wall effects occur[28]. However, the inherent high speed and low sample dilution attained in this study are more important to the separation problem discussed here, than peak shape aberrations. The sample dilution is small (relative to column volume) as a 50-μl

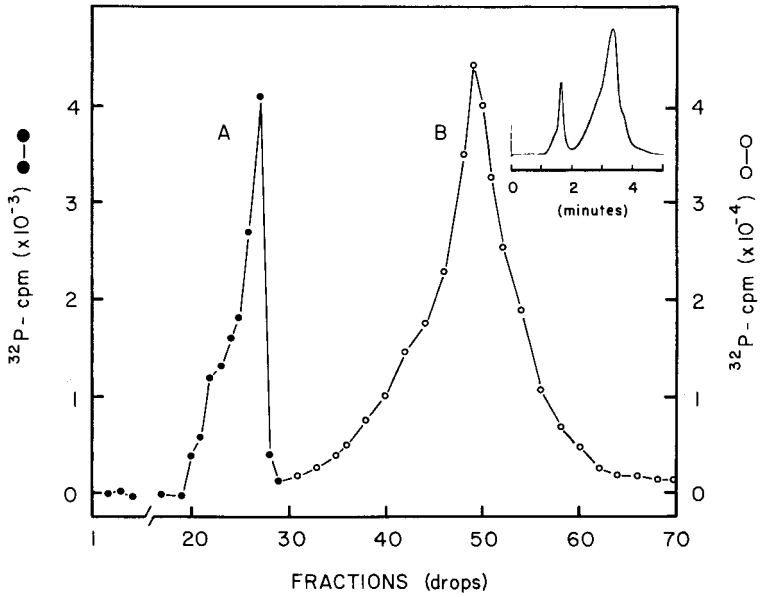

Fig. 3. Elution diagram of ^{32}P-labeled *Eco*RI-cut pBR325 DNA and labeled *Eco*RI DNA linkers on G5000 PW. Insert shows elution time as a function of $A_{260\ nm}$.

sample of DNA may be collected after chromatography in 250 μl; a volume from which it may be easily precipitated with ethanol.

The small-bore column used in this study generates a linear flow-rate of *ca.* 370 $ml \cdot cm^{-2} \cdot h^{-1}$ at 0.5 ml/min. This may be compared to other supports where the maximum linear flow-rates are: Sephadex G-75–G-200 (77–12 $ml \cdot cm^{-2} \cdot h^{-1}$), Sephacryl S-200 and S-300 (30 and 25 $ml \cdot cm^{-2} \cdot h^{-1}$)[22]. The effect of such a high flow-rate on DNA shearing as used in this system is negligible because full-length T7 DNA (40 kb) was unaltered by the chromatography (data not shown). Evidence for the survival of unaltered 5.45 kb linear DNA from possible HPLC shearing effects is shown in this study by the successful transfection of *E. coli* with HPLC treated material. Furthermore, chromatography of other extended macromolecules (7.1×10^6 MW polystyrene) in tetrahydrofuran, showed little evidence of shear sensitivity at linear flow-rates of less than 400 $ml \cdot cm^{-2} \cdot h^{-1}$ (ref. 27).

Electrophoresis of *Hae*III endonuclease treated T7 DNA collected from G5000 PW indicated that all DNA fragments greater than about 150 base pairs were excluded from the gel pores and elute at the void volume. Therefore, elution samples as large as ten linker multimers (*ca.* 100 base pairs) generated during ligation, are separated from DNA fragments as small as 150 base pairs. Similar effects were also observed with plasmid DNAs as large as 5.45 kb. However, in preliminary experiments with *Hind*III digested bacteriophage lambda DNA, a 23.8 kb DNA fragment was found to elute as a third peak at a slightly greater elution volume than the void volume (data not shown). The apparent retention of this larger DNA may be due to a type of physical entrapment within the chromatography packing. We are currently investigating this phenomenon.

The apparent interaction of DNA with the TSK gel support in the presence of Mg^{2+} ions is most intriguing. It is unclear at this time whether the nature of the interaction is purely electrostatic or a complex feature of both DNA charge and shape.

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SEPARATION OF BASIC, HYDROPHILIC PEPTIDES BY REVERSED-PHASE ION-PAIR CHROMATOGRAPHY

I. ANALYTICAL APPLICATIONS WITH PARTICULAR REFERENCE TO A CLASS OF SERINE PEPTIDE SUBSTRATES OF CYCLIC AMP-STIMULATED PROTEIN KINASE

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SUMMARY

Basic, hydrophilic peptides exemplified by Arg-Arg-Ala-Ser-Val, a substrate of cyclic AMP-stimulated protein kinase, could be efficiently retarded by ion-pair formation with *n*-hexanesulphonic acid on a C_{18} column in phosphate buffer systems with ethanol as organic modifier. Reversed-phase ion-pair chromatography appears to be an ideal method for separating peptides of this type. Highly efficient separations under isocratic conditions are reported for peptides composed of 5–9 amino acids, with up to three basic residues, most having sequences derived from the phosphorylatable site of pyruvate kinase.

INTRODUCTION

This paper deals with the problem of separating basic, hydrophilic peptides by high-performance liquid chromatography (HPLC). Among such peptides we were especially interested in serine-containing ones, because they have previously been studied by us and others as substrates of cyclic AMP-stimulated protein kinase[1–5]. The corresponding cyclic GMP-stimulated enzyme has a similar substrate specificity[6,7]. A future paper in this series will deal with the phosphoserine peptides formed in these reactions.

Separation and analysis of peptides is often a difficult and time-consuming step. In the case of arginine- and lysine-containing peptides, the presence of basic groups can generally be exploited for purification purposes by electrophoresis and ion-exchange chromatography. Another interesting approach, pioneered by Schill and co-workers[8], is to generate ion pairs of peptides with hydrophobic anions and

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separate these complexes on straight or reversed-phase columns. Ion-pair HPLC has recently been reviewed[9]. This principle has recently been applied to analysis of the basic peptide somatostatin[10]. To our knowledge, however, it has not yet been used for basic, hydrophilic peptides. The major difficulty with this class of peptides is to retain them from the void volume. It is demonstrated in this paper that, by applying reversed-phase ion-pair HPLC, regulation of retention can be achieved within wide limits.

MATERIALS AND METHODS

Isocratic mobile phases were used consisting of binary aqueous solvents with ethanol as organic modifier. The buffers were prepared from orthophosphoric acid and sodium dihydrogenorthophosphate to an ionic strength of 0.1 *M*. Hexanesulphonate was used as counter ion in the mobile phase. 1-Hexanesulphonic acid was obtained from Eastman-Kodak Co. (Rochester, NY, U.S.A.). All substances and solvents were of analytical or reagent grade.

The peptides used as model substances in this work are summarized in Table I. They were all prepared in this laboratory by the solid-phase method of Merrifield[11,12] as briefly described[2]. Most of them were purified by ion-exchange chromatography on carboxymethyl cellulose[2] and their composition carefully checked by amino acid analysis after acid hydrolysis. Their high purity is confirmed in the present work.

The liquid chromatographic system consisted of a Waters Model 6000A solvent delivery device, a Waters U6K injector, and a Waters variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.). The detection wavelength was 210 nm at high sensitivity, which made possible the detection of peptide quantities at least as small as 0.1 nmol. In a typical experiment 1–2 nmol of each peptide was injected sequentially, dissolved in up to 5 μl of water.

The separation columns, 250 × 4 mm, were packed by the balanced-density slurry technique[13], with Spherisorb C_{18} (10 μm) as chromatographic support for reversed-phase HPLC. These columns were always preceded by a short pre-column. The Spherisorb support was obtained from Phase Separations (Queensferry, Clwyd, Great Britain). The support used in the pre-columns was Bondapak C_{18}/Corasil (Waters).

The separations were run at a nominal flow-rate of 1 ml/min, corresponding to 1.3 mm/sec, at room temperature (22°C) and the pH measurements performed at the same temperature.

RESULTS AND DISCUSSION

The structures of all peptides studied in this paper are given in Table I.

Fig. 1 shows the separation of M 67, M 66 and M 158 which from a structural point of view are reasonably similar and possess one, two and three arginine residues, respectively. In the presence of *n*-hexanesulphonic acid, the hydrophobicity of the ion-pair-containing complexes increases with the number of basic groups in agreement with prediction. The additional leucine residue in M 158 no doubt contributes to an increased hydrophobicity. The differences between the corresponding retention times are, however, considerable and give an indication of the power of this

TABLE I

PEPTIDE STRUCTURES

The peptides are numbered according to a local code. Peptides M 57–M 73 are numbered as in ref. 2. Gva = δ-Guanidinovaleric acid.

M 57	Leu-Arg-Arg-Ala-Ser-Val-Ala
M 66	Arg-Arg-Ala-Ser-Val-Ala
M 67	Arg-Ala-Ser-Val-Ala
M 69	Arg-Arg-Ala-Ser-Val
M 72	Leu-Arg-Ala-Ser-Val
M 73	Arg-Leu-Ala-Ser-Val
M 87	Val-Leu-Arg-Arg-Ala-Ser-Val-Ala
M 97	Gly-Val-Leu-Arg-Arg-Ala-Ser-Val-Ala
M 99	Arg-Lys-Ala-Ser-Val
M 126	Leu-Arg-Arg-Ala-Ser-Val
M 136	Ala-Arg-Thr-Lys-Arg-Ser-Gly-Ser-Val
M 144	Arg-Arg-Ala-Ser-Arg
M 157	Gva-Arg-Ala-Ser-Val
M 158	Leu-Arg-Arg-Ala-Ser-Val-Arg
M 160	Phe-Arg-Arg-Leu-Ser-Ile

technique. This will be further illustrated below with groups of even more closely related peptides associated with the phosphorylatable site of rat liver pyruvate kinase[14].

Fig. 2 shows the separation of three peptides, M 67, M 72 and M 73, all with *one* arginine residue. Components 2 and 3 contain the same amino acids. The only difference is that the two N-terminal amino acids appear in the reversed order, but even in this fast run all the peaks are well resolved. Furthermore, as shown in Fig. 3, four peptides, M 66, M 57, M 87 and M 97, all with *two* arginine residues, have been run and the corresponding peaks are well resolved. Starting from M 66, the four peptides form a series increasing by one neutral amino acid at a time up to the nonapeptide M 97. Although the difference between M 87 and M 97 is only one

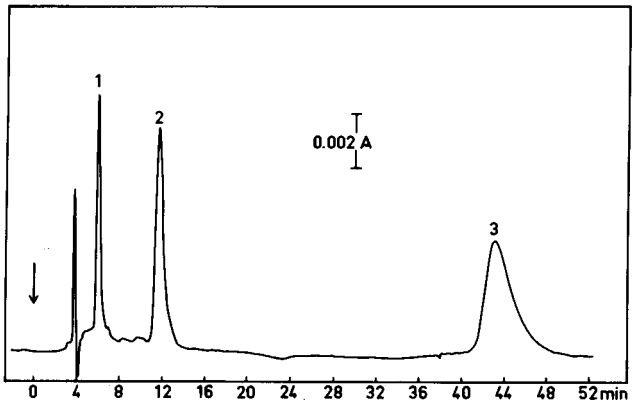


Fig. 1. Separation of three related basic peptides with one, two and three arginine residues, respectively. Mobile phase: phosphate buffer (pH = 3.1)–ethanol (79:21), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0105 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 67; 2 = M 66; 3 = M 158 (for amino acid sequences see Table I).

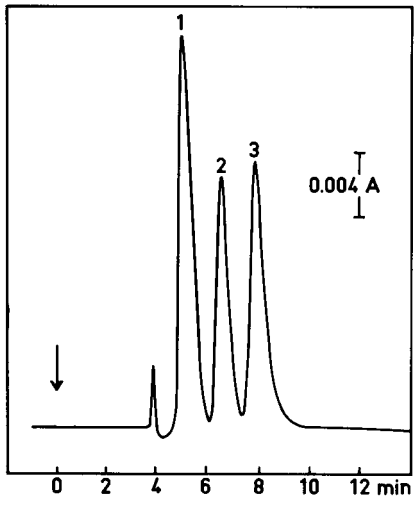
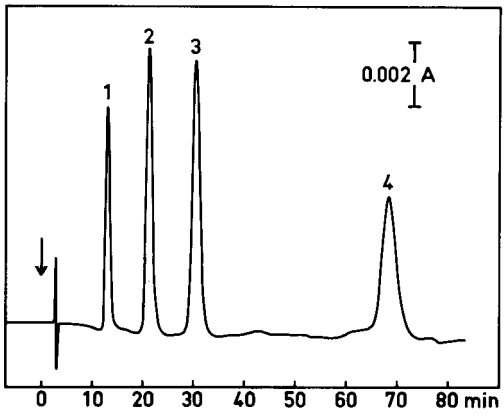


Fig. 2. Separation of closely related basic peptides all with one arginine residue. Mobile phase: phosphate buffer (pH = 4.5)–ethanol (72:28), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0094 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 67; 2 = M 72; 3 = M 73 (for amino acid sequences see Table I).

glycine residue, the separation is considerable. Therefore, we do not anticipate any difficulties in expanding this series by at least a few more residues. In this context, however, we decided to proceed to peptides containing *three* arginine residues and Fig. 4 demonstrates the results obtained with two peptides of this type, M 144 and M



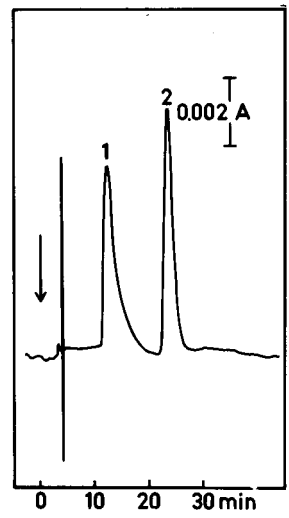


Fig. 3. Separation of four very closely related basic peptides all with two arginine residues. Mobile phase: phosphate buffer (pH = 4.5)–ethanol (79:21), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0105 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 66; 2 = M 57; 3 = M 87; 4 = M 97 (for amino acid sequences see Table I).

Fig. 4. Separation of two very hydrophilic basic peptides with three arginine residues. Conditions as in Fig. 3. Peaks: 1 = M 144; 2 = M 158 (for amino acid sequences see Table I).

158. Even in this case the corresponding peaks are well resolved. Due to lack of material no peptides with four arginines have been run in our new system so far.

The influence of pH was preliminarily studied using the three pentapeptides, M 99, M 69 and M 73 (Fig. 5). The peptide M 99 differs from all previous peptides in that it contains a lysine residue in addition to an arginine. Peptide M 69 is the minimum substrate of cyclic AMP-stimulated protein kinase[2]. The three peptides elute from the column as three well separated peaks. This is particularly the case at pH 3.1.

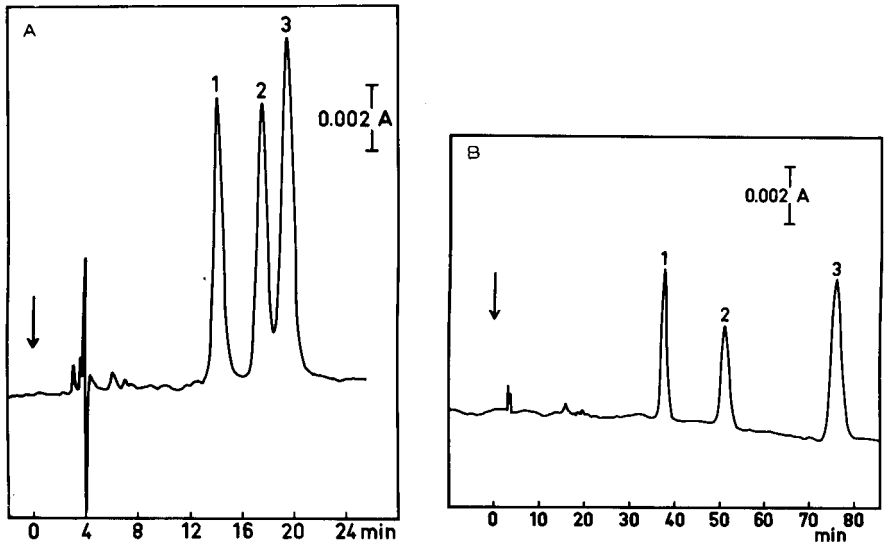
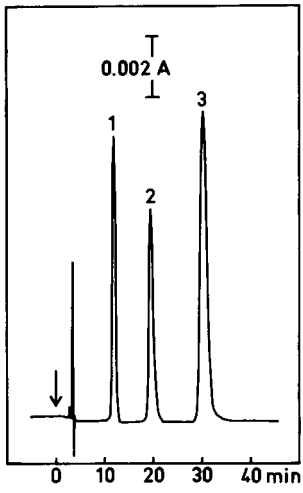


Fig. 5. Separation of three related hydrophilic peptides containing one or two basic amino acid residues. A, Mobile phase: phosphate buffer (pH = 4.5)–ethanol (75:25), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0113 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 99; 2 = M 69; 3 = M 73 (for amino acid sequences see Table I). B, Mobile phase: phosphate buffer (pH = 3.1)–ethanol (75:25). Other conditions and samples as in A.

The influence of the α-amino group and a second hydrophobic amino acid is shown in Fig. 6 for peptides M 157, M 69 and M 126. The lower hydrophobicity of M 157 compared to M 69 is easily understood if we assume that M 157 can only bind two molecules of sulphonic acid[8], *i.e.*, one less than M 69. The addition of leucine to the N-terminus of M 69 has a similar effect upon the retention time of M 126 as seen from the comparison of M 66 and M 57 in Fig. 3.

Fig. 7 shows the separation of two peptides of different origins, M 136 and M 160. Peptide M 136 represents part of the phosphorylatable site of the β-subunit and M 160 that of the α-subunit of phosphorylase kinase[15]. These peptides are also substrates of cyclic AMP-stimulated protein kinase[5]. M 136 is an exceptionally hydrophilic peptide. This is clearly reflected in Fig. 7. On the other hand the occurrence of three hydrophobic amino acids in M 160 compared to only one in many of the previous peptides makes this peptide an interesting model compound for further studies.

As stated above our major interest has so far been to apply ion-pair HPLC to



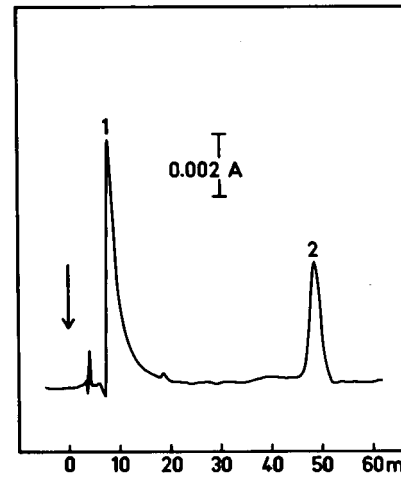


Fig. 6. Separation of closely related basic peptides. Mobile phase: phosphate buffer (pH = 3.2)–ethanol (72:28), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.015 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = M 157; 2 = M 69; 3 = M 126 (for amino acid sequences see Table I).

Fig. 7. Separation of two basic peptides related to the phosphorylatable site of the β-subunit of phosphorylase kinase and the α-subunit of the same enzyme. Mobile phase: phosphate buffer (pH = 3.1)–ethanol (79:21), flow-rate 1.3 mm/sec. Counter ion: hexanesulphonate (0.0105 *M*). Support: Spherisorb C_{18} (10 μm). Peaks: 1 = β-subunit peptide (M 136); 2 = α-subunit peptide (M 160; for amino acid sequences see Table I).

hydrophilic basic peptides. Nevertheless, as this work progressed we have applied the same system to analysis of a few other basic peptides like luteinizing hormone-releasing hormone (LHRH) and analogues of Substance P with promising results. Consequently we now plan a more systematic study of the effect of different separation parameters on resolution in order to improve further the systems described in this paper. For this reason a more detailed discussion of our present results from a theoretical and practical point of view will be postponed pending these experiments.

CONCLUSION

The purpose of this paper is to stress the usefulness of ion-pair HPLC in the analysis of basic hydrophilic peptides. Due to the influence of the hydrophobic counter ion hexanesulphonate, the basic functions are made less polar, and the peptides are more strongly bound to the reversed-phase support[8]. As a result, a number of basic, hydrophilic peptides, which otherwise would have been poorly retarded and resolved, were easily separated. Significant differences in retention time were seen upon variation of content of basic and hydrophobic amino acid residues, and even upon variation of the amino acid sequence.

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PURIFICATION OF HUMAN SERUM HYALURONIDASE USING CHROMATOFOCUSING

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(Received December 30th, 1981)

SUMMARY

A commerical chromatofocusing system was applied to Cohn's fraction III of human serum to purify hyaluronidase (E.C. 3.2.1.35.). The protein that eluted in the pH range 4.7–5.3 was pooled and precipitated by adding ammonium sulphate to 50% saturation. This sequence of fractionation purified hyaluronidase extensively by immunological criteria. It is shown that hyaluronidase is a population of enzymes displaying microheterogeneity. The commercial chromatofocusing system behaved as theoretically expected. The capacity of the gel is 3 mg per ml gel. Any overload will be trapped or precipitated in the gel. The gel is easy to handle and did not deteriorate on repeated use.

INTRODUCTION

Interest is growing in the distribution, organization, metabolism and functional aspects of proteoglycans and glycosaminoglycans. The analysis of these protein polysaccharides is at a quite an advanced stage, and employs chromatographic, enzymatic, spectophotometric and electrophoretic procedures. On the other hand, little has been done on elucidating the human hyaluronidases, which are the central catalytic enzymes in the metabolism of hyaluronic acid, chondroitin-4-sulphate and chondroitin-6-sulphate. There are several reasons for this. First, the catalytic activity is very low with the consequence that long reaction times are needed to obtain sufficient sensitivity in the enzymatic assays. Secondly, there are endogenic inhibitors in plasma and tissue extracts[1–4], which makes interpretation of enzymatic results very difficult. Finally, these difficulties impede exact localization of hyaluronidase in tissues.

The solution to some of these problems is to obtain monospecific antibodies to hyaluronidase. Purified hyaluronidase is required for this purpose. Only a few attempts have been made to obtain purified human serum or lysosomal hyaluronidase[5–7]. This paper describes an efficient procedure for purifying human serum hyaluronidase using alcohol fractionation, chromatofocusing and ammonium sulphate precipitation.

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MATERIALS

Chromatofocusing gel PBE 94, Polybuffers 96 and 74, Sephadex G-25, IEF-agarose, Pharmalyte 2.5–6.0 and isoelectric focusing standards were from Pharmacia (MEDA, Copenhagen, Denmark). Hyaluronic acid (H 1751) was from Sigma (St. Louis, MO, U.S.A.). Rabbit immunoglobulins raised against human serum (100 SF and 100 SG), transferrin (10-061) and immunoglobulin G (IgG) (10-090) were from DAKO-Immunoglobulins (Copenhagen, Denmark). All other reagents were the purest commercial products obtainable.

METHODS

Serum was obtained as a freeze dried product of Cohn's fraction III, method 6 (ref. 8). It was solubilized in 50 m*M* sodium acetate buffer pH 3.7 and dialyzed at 4°C for 2 days against 100 volumes of the same buffer with four equally spaced shifts. It was then centrifuged at 20,000 *g* for 1 h at 4°C and the precipitate discarded. The supernatant (Cohn III) was processed further as described below.

Chromatofocusing

A 10-ml volume of PBE 94 was equilibrated in 25 m*M* histidine hydrochloride buffer pH 6.0. It was packed on a glass column (1.6 × 5 cm) overlaid with 1 cm Sephadex G-25 and washed overnight in the histidine buffer. Cohn III was dialyzed in 10 m*M* histidine buffer pH 6.3 at 4°C for 2 days and centrifuged at 1500 *g* for 15 min. 5 ml (14 mg/ml) were applied to the column followed by 5 ml histidine buffer pH 6.0. The column was eluted with 300 ml Polybuffer 74 diluted 1:8 in degassed deionized water adjusted to pH 4.0. 10-ml fractions were collected.

On a preparative scale, 100 ml PBE 94 were equilibrated in 25 m*M* Tris–acetic acid pH 7.3, packed on a glass column (2.6 × 19 cm) overlaid with 1 cm Sephadex G-25 and washed overnight. Because the pH dropped in the cold to about 6.5, it was decided to wash the column with two volumes of Polybuffer 96 diluted 1:13 and adjusted to pH 6.0 with acetic acid. Cohn III was dialyzed against 25 m*M* Tris–acetic acid pH 7.3 for 2 days, centrifuged and 28 ml (26 mg/ml) were applied to the column. The sample was followed by 20 ml of Polybuffer 96 and the column was eluted with 1000 ml Polybuffer 74 as described above.

To test the capacity of PBE 94, 2 ml Cohn III (16 mg/ml) were run on a 10-ml column (1.6 × 5 cm) exactly as described in the previous paragraph.

The eluates were pooled and precipitated by 50% or 100% ammonium sulphate (see Results). Precipitates were resuspended in and dialyzed against 50 m*M* sodium acetate buffer pH 3.7.

Electrophoresis

Fused rocket* and crossed immunoelectrophoresis (CIE) were done essentially as described[9].

Isoelectric focusing was done exactly as described in the manual (Pharmacia) using IEF-agarose and Pharmalyte 4–6.5. One lane from the isoelectric focusing was used as a one-dimensional gel in CIE. 1% agarose gel in barbiturate buffer containing

* In the fused rocket procedure the samples from the PBE-columns are placed successively in two slightly displaced parallel rows of sample wells in an agarose gel. The proteins are allowed to diffuse out of the wells for 45 min. Finally the proteins are electrophoresed overnight (2 V/cm) into an antibody-containing gel (see *e.g.* Fig. 2A).

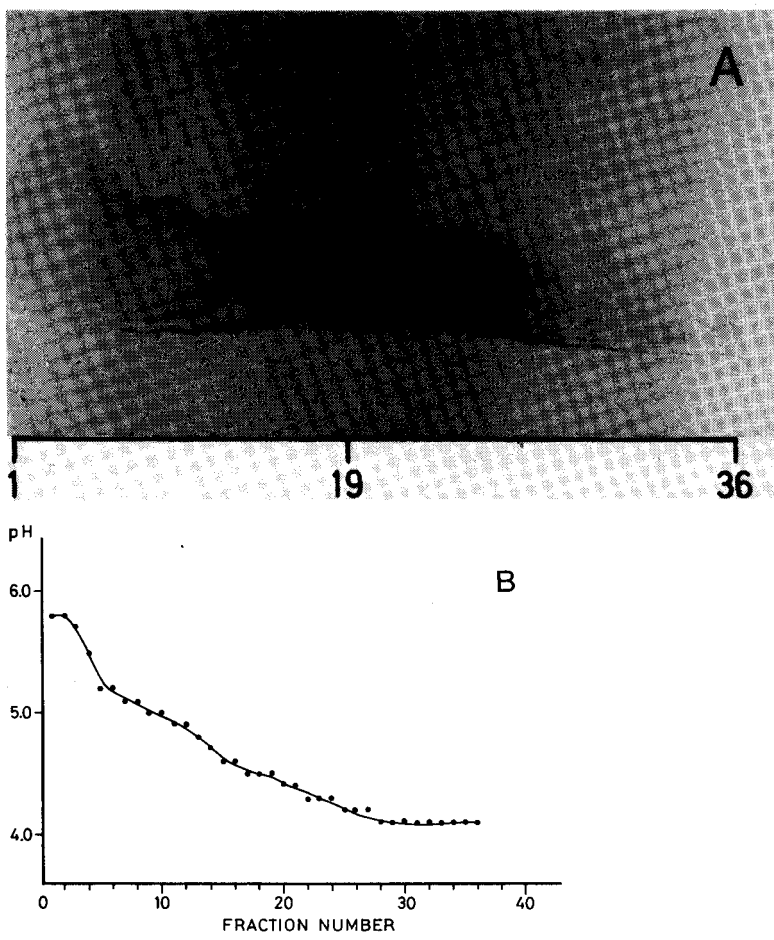

Fig. 1. Fused rocket of the eluates from 10-ml PBE 94 chromatofocusing in the pH range 6.0–4.0 (A). pH-profile from the same chromatofocusing (B). 10-ml Fractions were collected.

polyspecific antiserum (100 SG, DAKO) was cast on a clean glass plate. The IEF lane was transferred and laid parallel to but not in contact with the antibody containing-agarose gel. The sample lane was then moulded in place with 1 % agarose in barbiturate buffer. A potential of 2 V/cm was applied overnight, the cathode being at the IEF lane.

Enzyme assay

The optimum pH for hyaluronidase was found to be 3.7. The final reaction mixture contained 0.2 *M* acetate, 1.5 m*M* saccharolactone, 120 m*M* NaCl, 0.1 % bovine serum albumin, 225 μg hyaluronic acid and enzyme in a total volume of 250 μl. The reaction proceeds at 37°C for 20 h and was stopped by adding 50 μl of 0.86 *M* NaOH. N-Acetylglucosamine end-groups were determined as described previously[10]. One unit of activity was defined as 1 μmol liberated end-group per hour. Polybuffer did not influence the assay or enzymatic activity.

Protein was determined according to Layne[11].

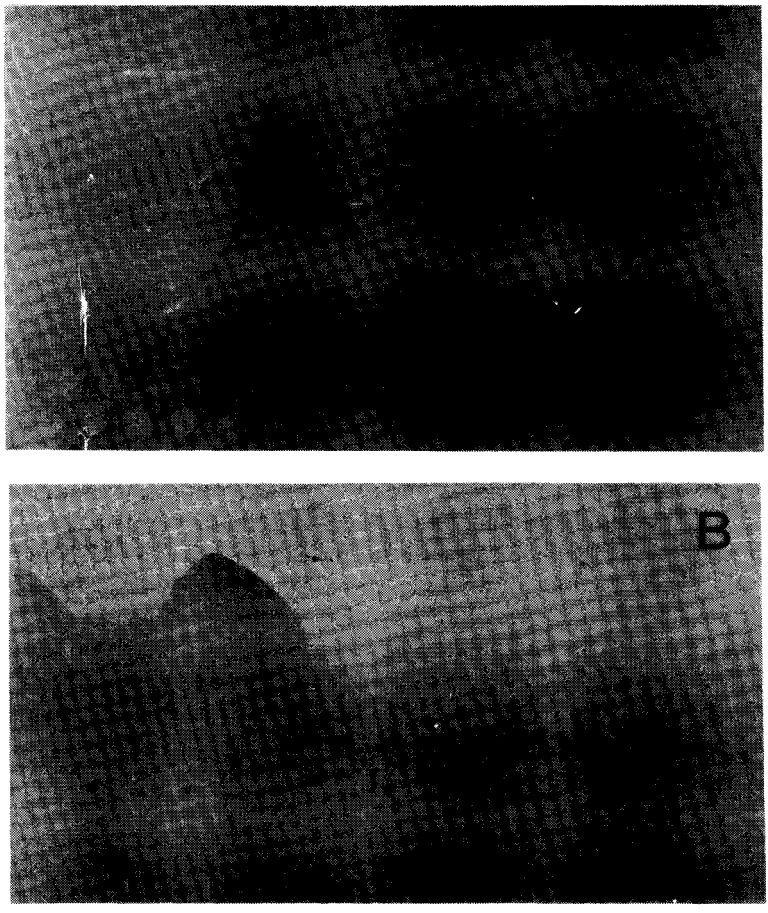

Fig. 2. Fused rocket of the eluates from the 100-ml PBE column. Monospecific antibodies against transferrin (A) and IgG (B) was used. Compare with Fig. 1.

RESULTS

A typical fused rocket immunoelectrophoresis of eluates from a PBE 94 column is shown in Fig. 1A, with the pH-profile in Fig. 1B. Compared with the CIE of isoelectric focusing (not shown), the apparent pH of elution of the proteins, *e.g.*, albumin, is lower than the real p*I* of the proteins. This is in accordance with the theory developed by Sluyterman and co-workers[12,13].

The same pattern emerged when the 100-ml column was used and when the starting buffer was changed from histidine hydrochloride to Tris–HCl. The manufacturers[14] recommend the histidine buffer for this pH range (4.0–6.0) but about 50% of the protein precipitated, without any purification of hyaluronidase. The buffer was therefore changed to Tris–HCl, which preserved almost all the protein.

Fig. 2 shows the fused rocket from a 100-ml PBE column using antibodies against human transferrin and Igb, respectively. These proteins elute in the expected

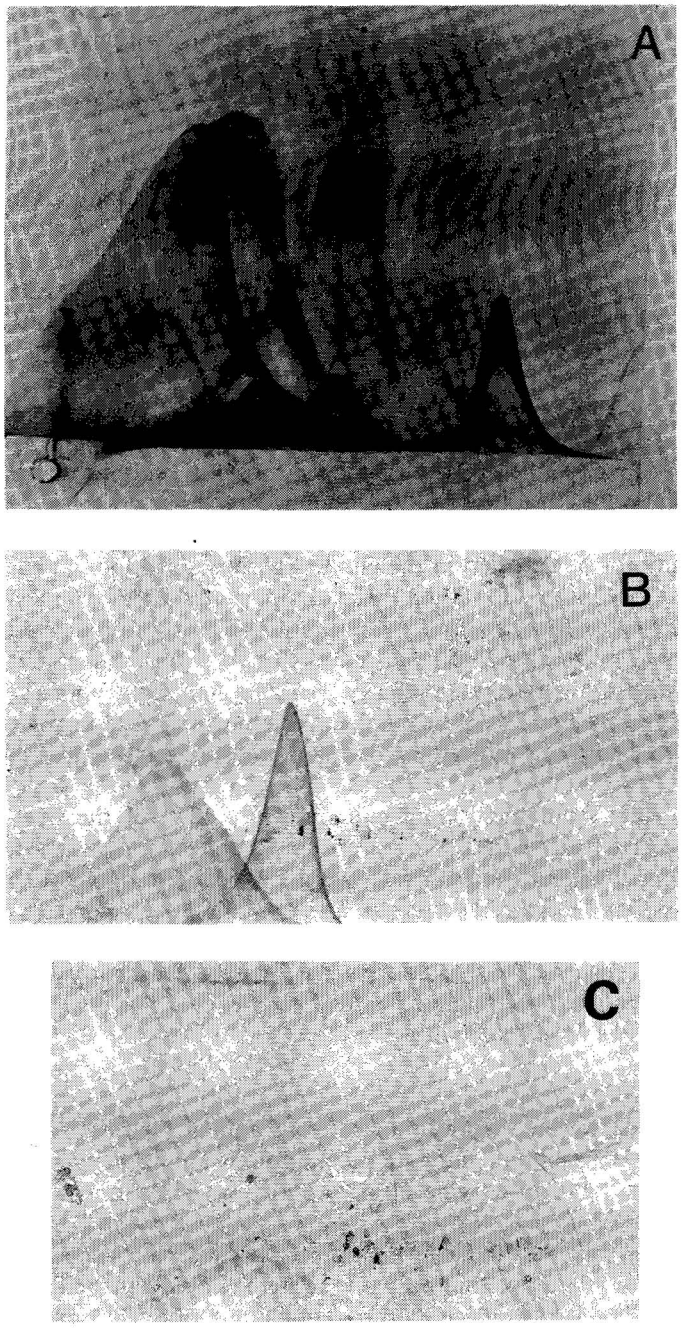

Fig. 3. One-dimensioned CIE using polyspecific antibodies against human serum: A, Cohn III; B, pooled fraction pH 4.7–5.3 from the 100-ml chromatofocusing column; C, the precipitate from 50% saturation with ammonium sulphate of the pool in B. As can be seen in C, only IgG and an unidentified precipitate with β-mobility were left behind.

TABLE I

ELUATES FROM THE 100-ml PBE COLUMN POOLED AND CONCENTRATED WITH AMMONIUM SULPHATE (SEE TEXT)

All fractions were dialyzed before they were analyzed.

Fraction	*Hyaluronidase (mU)*	*Protein (mg)*
Cohn III	1632	728
pH 5.4–6.0	4.5	48
pH 4.7–5.4		
50% Ammonium sulphate		
precipitate	207	44
supernatant	2	13
pH 4.0–4.7	16	8
1 *M* NaCl	456	302

pH ranges. The maximum capacity of PBE 94 was 3 mg per ml gel. If more protein is applied to the column it will partly be trapped in the column. It could be desorbed by 1 *M* NaCl. The absorption is non-specific.

Hyaluronidase could not be detected in the eluates, because of dilution. The fractions in the pH range 4.7–5.3 from the 100-ml PBE column were therefore pooled and solid ammonium sulphate was added to 50% saturation. The precipitate that developed after 16 h at 4°C was centrifuged at 1500 *g* and 4°C for 15 min, re-suspended in 10 ml of 50 m*M* sodium acetate buffer pH 3.7 and dialyzed against the same buffer. Fig. 3B and 3C show the CIE of the pool and the precipitate, respectively. The purification achieved by these procedures is obvious using immunological criteria.

Table I shows the data from the 100-ml column. Only 13% of the hyaluronidase eluted in the expected pH range, 28% were trapped and the rest was lost or denatured. The fractions in the pH ranges 4.0–4.7 and 5.3–6.0 were pooled and saturated with solid ammonium sulphate. The precipitates and the supernatant from 50% ammonium sulphate precipitate described above were dialyzed against 50 m*M* acetate buffer pH 3.7. Practically no activity could be measured in these fractions. If 3 mg protein per ml gel were applied to the column, 40% of activity could be regained, but there was an obligatory loss of 60% activity.

DISCUSSION

Yamada *et al.*[5] partially purified hyaluronidase from human placenta using freezing, thawing, extraction, ammonium sulphate precipitation, ion exchange and gel filtration. They claimed to have an enzyme of 82% purity based on polyacrylamide gel electrophoresis (PAGE).

However, this is not a safe way to determine purity because the unspecific purification procedures cannot guarantee that only one protein, *e.g.*, hyaluronidase, is present in the main band of the PAGE. Besides, the identification of hyaluronidase in PAGE was indirect.

Nevertheless, this is the most pure human serum hyaluronidase which has so far been reported. In the present paper the use of chromatofocusing for purifying human serum hyaluronidase was investigated. A new commercial system from Pharmacia was explored. It is quite clear from Fig. 3A–C that extensive purification of

hyaluronidase based on immunological criteria was achieved. Tripling the antigen amount in the one dimension of CIE did not reveal new antigenic determinants in the preparation; *i.e.*, only three impurities are left behind after this sequence of separation techniques.

The exact pH at which hyaluronidase eluted in the chromatofocusing system could not be assessed because of dilution. However, all the hyaluronidase that eluted in the gradient was eluted in the pH region 4.75–5.34 in agreement with the p*I* of hyaluronidase[5]. No correction for observed p*I* was made according to Sluyterman and co-workers[12,13]. Theoretically, chromatofocusing should have a concentrating effect on a homogeneous protein. The dilution of hyaluronidase indicates fractionation into several hyaluronidases, reflecting the microheterogeneity of human serum hyaluronidase.

The 50% ammonium sulphate precipitate of the pH 4.7–5.3 fraction from chromatofocusing is a potential source for raising antibodies in rabbits and for production of radioactive tracers, to be used in a radioimmunoassay (RIA). The preparation is not pure and the antiserum is not monospecific, but preliminary experiments have shown that a combination of absorption of antibodies and gel chromatography of the iodinated antigen (tracer) will produce suitable tools for a RIA system and will be described in a future paper.

To the author's knowledge, the technique of chromatofocusing has not formerly been used to fractionate human serum proteins. The PBE gel performed well. It is quite easy to handle and no deterioration was observed by repeated use. It is essential for obtaining a smooth gradient and to reach the low pH range that the buffers are degassed. By adjusting the protein load to just below maximum capacity, up to 40% of enzyme activity could be regained in the pH region of the isoelectric point of hyaluronidase.

ACKNOWLEDGEMENTS

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Note

Frit profiles for packed chromatographic column terminations

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(Received December 30th, 1981)

Special arrangements for the uniform distribution of samples near the entrance of a packed column and, likewise, for their uniform collection can serve to reduce band spreading. For this purpose retentionless frits with suitable profiles have been developed. The use of such frits is particularly pertinent in large diameter columns, *e.g.*, $\geqslant 3$ mm I.D.

Figs. 1 and 2 illustrate two column cross-sections with frit profiles calculated by use of the theory outlined below, the first designed for a coarse particle packing and the second for finer particles. The column packing is shown as large squares and the frit as small ones. The parameters are as follows:

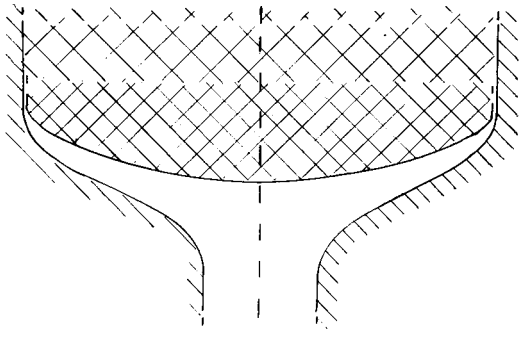

Fig. 1. Frit profile for a coarse particle packing: $s/r_0 = 0.01$.

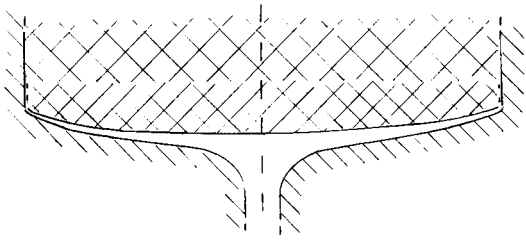

Fig. 2. Frit profile for a fine particle packing: $s/r_0 = 0.001$.

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r_0 = inside column radius
r_1 = entrance and exit pipe radii
r = distance from column axis
t = thickness of the free interspace between the frit and the column wall
t_0 = initial thickness of the interspace at the inner wall of the column exit
V_0 = average fluid velocity within the column
V = average fluid velocity in the interspace
α = angle between the free interspace flow direction and the column axis
p'_0 = vertical pressure gradient within the frit
p'_1 = pressure gradient in the direction of flow in the interspace
s = characteristic dimension of the frit particles
μ = fluid viscosity
x = abscissa normal to the column axis
y = ordinate along the column axis

The characteristic dimension, s, of the frit packing is defined as the quantity which permits the average fluid velocity, v_0, within the column and within the frit to be written as

$$v_0 = p'_0 s/\mu \tag{1}$$

The order of magnitude of s will be one tenth the frit or packing particle diameter.

The fulfillment of four conditions is required to insure the uniform distribution and collection of the fluid, and these conditions can be stated, without loss of generality, in terms of exit conditions. First, the simultaneous arrival of all fluid elements at the collecting point demands that the average fluid velocity within the interspace has a vertical component equal to the average fluid velocity:

$$v \cos \alpha = v_0 \tag{2}$$

Secondly, the average velocity at any distance, r, from the axis, when multiplied by the interspace thickness, t, at that distance and by the circumferencial length at that point should equal the total fluid flow collected between that circumference and the column wall, *i.e.*

$$2\pi rtv = \frac{\pi (r_0^2 - r^2)}{2\,rt} \cdot v_0$$

or

$$v = \frac{r_0^2 - r^2}{2\,rt} \cdot v_0 \tag{3}$$

Thirdly, the condition that isobars within the column packing and within the frit be planes normal to the column axis requires that the pressure drop within the interspace, when multiplied by cos α, be equal to the vertical pressure drop within the column:

$$p'_1 = p'_0 \cos \alpha \tag{4}$$

Fourthly, the pressure drop within the interspace should be such as to produce within the interspace the velocity given by eqn. 3:

$$v = \frac{t^2}{12\,\mu} \cdot p_1' \tag{5}$$

The essential task of obtaining the frit profile is now algebraic. From eqns. 4 and 5, and then with 1, we obtain

$$v = \frac{t^2}{12\,\mu} \cdot p_0' \cos\alpha = \frac{t^2\, v_0}{12\, s^2} \cdot \cos\alpha$$

and, with eqns. 2 and 3:

$$\frac{v}{v_0} = \frac{t^2}{12\, s^2} \cdot \cos\alpha = \frac{1}{\cos\alpha} = \frac{r_0^2 - r^2}{2\, rt} \tag{6}$$

Elimination of cos α from eqn. 6 gives

$$\frac{t^2}{12\, s^2} = \frac{(r_0^2 - r^2)^2}{4\, r^2 t^2}$$

whereby

$$t = \sqrt{\frac{\sqrt{3}\,(r_0^2 - r^2)s}{r}} \tag{7}$$

and again with eqn. 6:

$$\cos\alpha = 2\sqrt{\frac{\sqrt{3}\, rs}{r_0^2 - r^2}} \tag{8}$$

Eqns. 7 and 8 determine the essential portions of the frit profile. When cos α approaches unity near the column inner wall, the behavior of this profile can be determined more conveniently by writing

$$r = r_0 - 2\sqrt{3}\, s - x \tag{9}$$

from which we obtain

$$\cos\alpha \approx \sqrt{1 - \frac{x}{2\sqrt{3}s}} \approx 1 - \frac{\alpha^2}{2} \tag{10}$$

or squaring and writing

$$\alpha \approx \mathrm{d}x/\mathrm{d}y$$

we obtain

$$\frac{dx}{dy} = \sqrt{\frac{x}{2\sqrt{3}s}}$$

$$dy = \sqrt{\frac{x}{2\sqrt{3}s}}\, dx$$

$$y = \sqrt{8\sqrt{3}sx}$$

and finally:

$$x = y^2/8\sqrt{3}s \tag{11}$$

This indicates that the theoretical frit profile ends abruptly at the base of a parabola with the radius of curvature $4\sqrt{3}s$ at that point, which is at a distance $2\sqrt{3}s$ from the inner wall.

Figs. 1 and 2 show the frit profiles for the two cases $s/r_0 = 0.01$ and 0.001, as well as the associated profiles of the steel tube of the column at a distance t from the frit. The part of these two figures which is of interest is the intermediate portion from near the exit (or entrance) tube to near the end of the column straight wall. As shown it represents a physical impossibility near the column exit because supporting ribs must be provided to hold back the frit and the column packing; it is nevertheless an ideal solution which the column designer could strive to approximate.

Near the center, the steel tube profile is shown smoothly joined to an entrance or exit pipe calculated to have that radius, r_1, for which the pipe impedance per unit length is one quarter the column impedance. Since the latter is given by $\mu/\pi\ r_0^2 s^2$, while the pipe impedance is given by $8\mu/\pi\ r_1^4$, for the above condition we have:

$$r_1 = \sqrt{\sqrt{32}\, r_0 s} \tag{12}$$

The frit portion calculated for the immediate proximity of the column wall is also unrealistic. It shows the frit approaching the wall at a distance $t_0 = 2\sqrt{3}s$, which is the exact thickness of free space in which the impedance per unit area would equal the frit impedance. However, even if the frit were to go all the way to the wall there would be a virtually free layer of appreciably greater thickness due to the free passages around the frit particles being much larger than in the frit bulk, as is the case for the column packing itself, where a higher speed layer causes a concentration front distortion which is responsible for a large increase in the basic HETP of the packing.

The elimination of this highly damaging high speed layer at the column inner wall is believed to be one of the most important tasks of column designers, and if and when progress is made in this direction, the adoption of frit designs along the lines discussed here will prove useful.

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Note

Problems encountered during peptide derivatization for gas chromatographic–mass spectrometric analysis

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(First received February 3rd, 1981; revised manuscript received January 12th, 1982)

Vapour-phase analysis of peptides is severely hampered owing to their zwitterionic character, and in order to make them amendable to gas chromatographic–mass spectrometric (GC–MS) analysis they need to be derivatized. Four major types of derivatives are used for peptide analysis: N-acyl peptide esters[1,2], permethylated peptides[3–5], Schiff base derivatives[6,7] and polyamino alcohols[8–13]. The conversion of peptides to polyamino alcohols was introduced by Biemann and co-workers, who have used it extensively for sequencing of proteins[14–16]. In our laboratory we needed to develop a method for protein sequencing and after studying all the options we decided to choose the Biemann approach, but we immediately encountered several difficulties, even with simple dipeptides. In this paper we describe these difficulties and how we finally resolved them. The scheme for reduction of peptides to polyamino alcohols introduced by Biemann and co-workers and modified by Frank and Desiderio[17] is shown in Fig. 1.

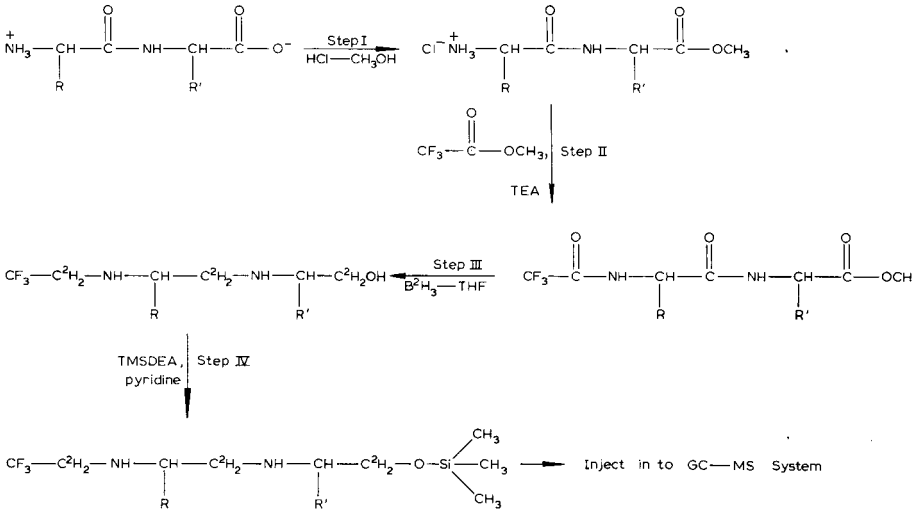

Fig. 1. Derivatization of peptides according to modified Biemann procedure.

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EXPERIMENTAL

A careful investigation of the derivatization procedure revealed that the purity of the reagent played a very important role. Hence we describe in detail below the handling of the glassware and the reagents.

Glassware cleaning and handling

All of the glassware was soaked overnight in a dilute soap solation (approximately half a tablespoon), rinsed with water, distilled water and transferred into a clean glass beaker (1000 ml) containing *ca.* 400–500 ml of 60% nitric acid. The beaker was heated (covered) on a hot-plate for 8 h at 75°C, followed by rinsing with tap water (3–4 times), and was then soaked for 1 h in distilled water and finally dried in an oven for 3–5 h at 110°C. The glassware was allowed to cool to room temperature (covered) and finally stored in zip-fastened polyethylene bags.

All glassware used was of Pyrex-Kimax brand, except for the disposable pipettes (Pasteur), which were made of soda-lime glass.

Reagents

The following reagents were needed for the Biemann procedure.

Step I

3 M hydrochloric acid–methanol. The solution was prepared by bubbling technical hydrogen chloride gas (Matheson) through concentrated sulphuric acid and freshly distilled nanograde methanol. We found it convenient to prepare about 50 ml at a time, divide it into 10-ml portions and store them under refrigeration at all times. We obtained reproducible results with a solution that was 2–3 weeks old. However, we had limited success with commercially purchased solutions.

Step II

(a) Methyl trifluoroacetate (MTFA). Generally about 6 ml were distilled and a middle fraction of about 3 ml was collected.

(b) Triethylamine (TEA). After purchase (P & B Chemicals, CT, U.S.A.), about 100 ml were stirred overnight with 1–2 g of amino acid active ester (N-*tert.*-butyloxycarbonyl-L-alanine pentachlorophenyl ester) to remove trace amounts of primary and secondary amines. The clear solution was distilled and stored in an amber-glass flask.

Step III

(a) Borontrideuteride–tetrahydrofuran. We found that the purity of this reagent was very important. Initially, about 100 ml of 1 *M* solution (Alfa Ventron, Danvers, MA, U.S.A.) were purchased, but according to our experience this must be avoided. We obtained very reproducible results when we purchased a smaller sample (*ca.* 2.5 ml in sealed glass vials) and used up the vial within 2–3 days after opening. The vials were stored under refrigeration at all times.

(b) Reagents needed for work-up of the reduced peptides.

(i) Hydrochloric acid–methanol (1 *M*). The 3 *M* solution prepared for esterification was diluted with distilled methanol to obtain a 1 *M* solution.

(ii) Potassium carbonate. About 100 ml of a 25% solution of potassium carbonate (Fisher) were prepared initially and used throughout the work.

(iii) Chloroform. This was of nanograde quality and was used without further purification.

Step IV

(a) Pyridine. Generally about 5 ml were distilled and about 0.5 ml was collected.

(b) Trimethylsilylydiethyl amine (TMSDEA). We found that it was very important to distill this reagent each time before use, as a large amount of impurities distills (*ca.* 30%) before the correct boiling point. Generally 3 ml were distilled and a middle fraction of about 0.5 ml was collected.

Derivatization

The peptides were derivatized in 100-μg amounts according to the procedure described by Herlihy[16].

Instrumentation

A Varian Model 2700 gas chromatograph interfaced via a 25 cm × 1.15 mm stainless-steel capillary tube to a Nuclide Model 12-90-G mass spectrometer, was used. The GC injection port and the flame-ionization detector (FID) oven were maintained at 280°C. The column (1% or 3% OV-17 on 100–120 mesh Suplecoport) was programmed linearly from 100 to 270°C at 6°C/min. Mass spectra were obtained at 70 eV with a trap current of 50 μA. Data were also obtained on a Varian Model 3700 gas chromatograph and on a GC–MS system at the Massachusetts Institute of Technology (MIT).

RESULTS AND DISCUSSION

The GC retention indices and the mass spectral fragmentation of the polyamino alcohols can be accurately predicted. These unique features provide unambiguous identification of the original peptides[8–16].

We found that the Biemann derivatization procedure could be easily reproduced if necessary precautions are taken. It must be emphasized that it was not necessary to distil all of the reagents each time before use as all the reagents were found to be stable if properly handled.

The importance of the freshness and purity of the reagents can be demonstrated by the following experiments.

Experiment I

The GC (FID) trace of the reduced peptide mixture (standard) shown in Fig. 2 was obtained when all of the reagents were fresh. Four major peaks corresponding to four dipeptides (confirmed by GC–MS) were recorded. The derivatization of this and other more complex mixtures could be reproduced several times.

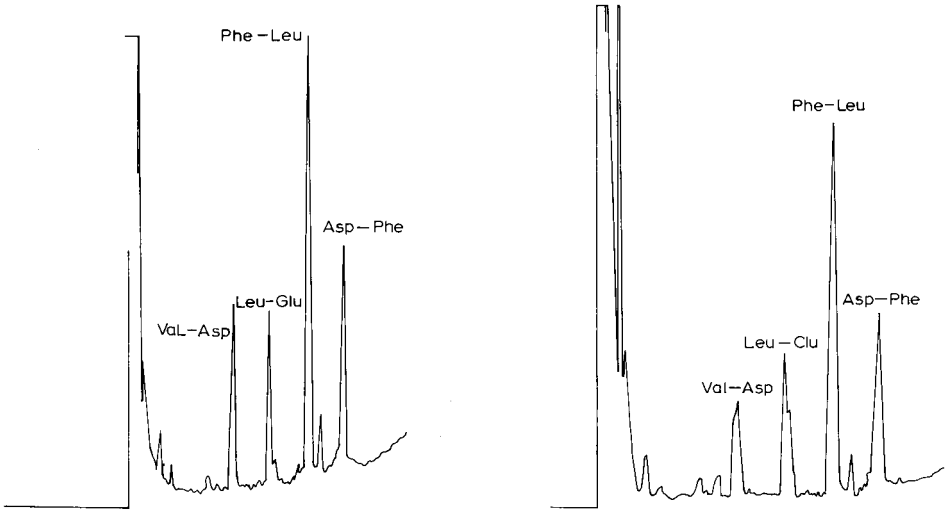

Fig. 2. Gas chromatogram of the reduced peptide mixture (standard) obtained with fresh reagents.

Fig. 3. Gas chromatogram of the reduced peptide mixture (standard) obtained with the reagents in the following states: (a) hydrochloric acid–methanol had a pale yellow colour; (b) MTFA and boron trideuteride–tetrahydrofuran were fresh; (c) TEA, TMSDEA and pyridine were not freshly distilled.

Experiment IIa

The GC (FID) trace of the reduced peptide mixture (standard) shown in Fig. 3 was obtained with the reagents under the following conditions: (1) hydrochloric acid–methanol (3 *M*) was freshly prepared but the solution had a pale yellow colour as the valve on the hydrogen chloride tank was not clean; (2) MTFA was freshly distilled and boron trideuteride–tetrahydrofuran was obtained from an unopened vial; (3) TEA, TMSDEA and pyridine were not freshly distilled.

When the chromatograms in Figs. 2 and 3 are compared, the following major differences can be seen:

(1) The height of all of the major GC peaks was reduced significantly if all the reagents were not fresh. This clearly indicates that the yields of the products are greatly affected by reagent purity.

(2) The height of the shoulder eluting along with the reduced dipeptide Leu–Glu was of much greater intensity than the same shoulder obtained in the experiment in which all the reagents were fresh. This indicates that some undesirable product was formed if impure reagents were used.

Experiment IIb

The GC (FID) trace of the reduced peptide mixture (standard) shown in Fig. 4 was obtained with the reagents under the following conditions: (1) hydrochloric acid–methanol (3 *M*) was about 2 weeks old; (2) MTFA was freshly distilled and boron trideuteride–tetrahydrofuran was obtained from a vial that had been opened 4 months earlier; (3) TEA, TMSDEA and pyriridine were not freshly distilled.

By means of control experiments, it was shown that hydrochloric acid–methanol that was about 2 weeks old gave the same results as a fresh mixture. Hence,

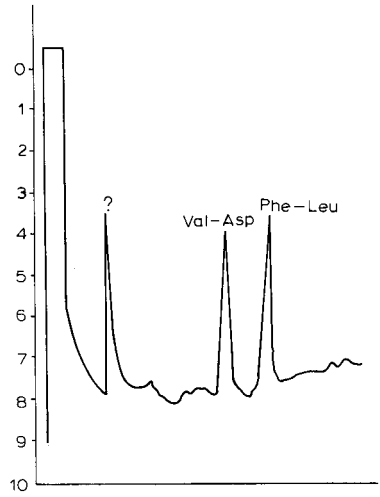

Fig. 4. Gas chromatogram of the reduced peptide mixture (standard) obtained with the reagents in the following states: (a) hydrochloric acid–methanol was about 2 weeks old; (b) MTFA was freshly distilled; (c) boron trideuteride–tetrahydrofuran was obtained from a vial that had been opened about 4 months earlier; (d) TEA, TMSDEA and pyridine were not freshly distilled.

essentially, the difference between experiments IIa and IIb was the freshness of boron trideuteride–tetrahydrofuran. When the chromatograms for experiments IIa and IIb (Figs. 3 and 4, respectively) are compared, the following major differences can be seen: (1) a major unidentifiable peak was recorded; (2) the relative height of the peak corresponding to the dipeptide Phe–Leu was substantially reduced; and (3) dipeptides Leu–Glu and Asp–Phe gave no response.

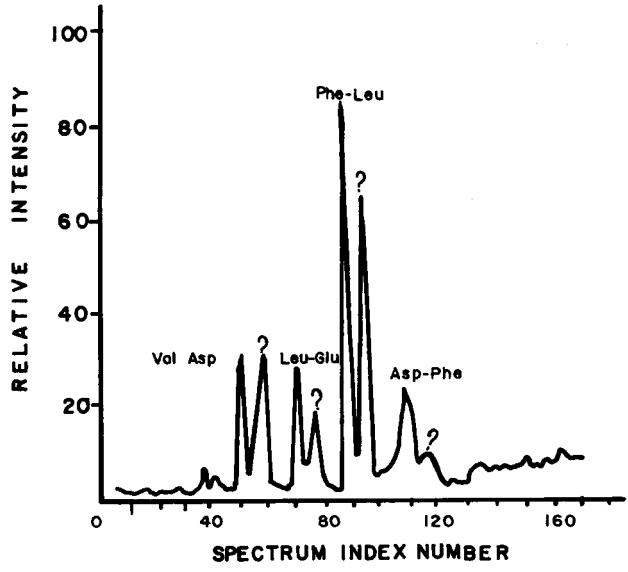

Fig. 5. Total ionization plot of the reduced peptide (standard) obtained with all of the reagents borrowed from the Biemann group but derivatization performed in our laboratory.

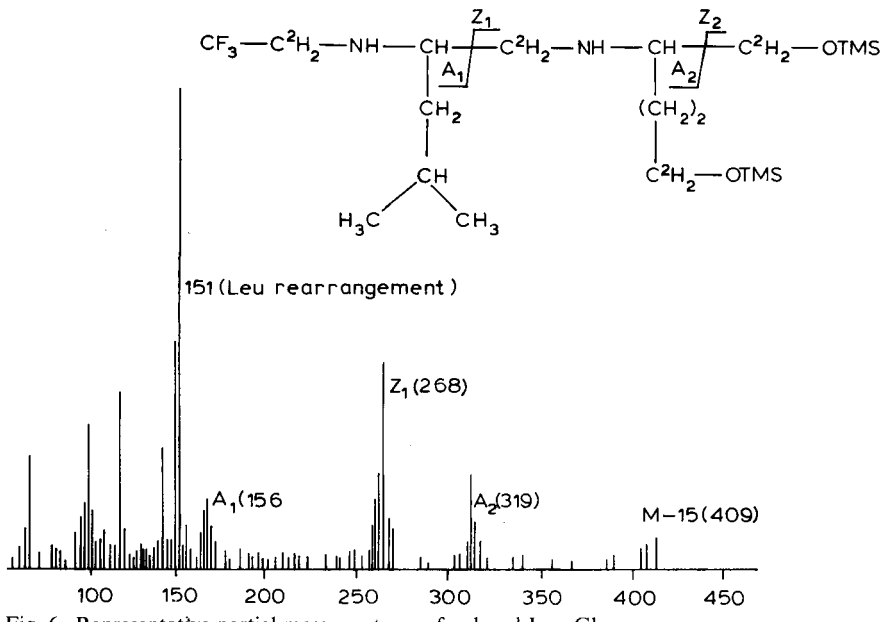

Fig. 6. Representative partial mass spectrum of reduced Leu–Glu.

Experiment IIc

The total ionization plot of the reduced peptide mixture (standard), shown in Fig. 5, was obtained with all of the reagents borrowed from the Biemann group at MIT, but the derivatization was performed in our laboratory. It can be seen that each of the dipeptides gave a major unidentifiable peak in addition to the expected peak.

At this point it became clear that the Biemann sequence was a sensitive scheme and had to be performed carefully. Hence we decided to take precautions with the reagents, and once we had purified them the derivatization could be easily reproduced.

A representative partial mass spectrum of the reduced dipeptide Leu–Glu is shown in Fig. 6.

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Note

Multichannel diode array UV–visible spectrophotometer as detector in screening for unknown butoprozine metabolites in dog bile by high-performance liquid chromatography

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In a recent paper[1] we described the applicability of gradient-elution reversed-phase high-performance liquid chromatography (HPLC) in the screening for unknown drug metabolites in dog bile after administration of radioactive butoprozine, a new anti-anginal drug. The structure of butoprozine and its UV-absorption spectrum are given in Fig. 1. The metabolites were detected by means of conventional UV detection and by counting of the radioactivity in the effluent from the UV detector. UV detection was employed in order to test a detection system in experiments in which only non-radioactive drugs are involved. However, in these investigations it was necessary to differentiate between the UV absorption of the metabolites and that of the background (endogenous bile components, eluent impurities, column bleed, etc.). Thus, we developed a method of recognizing the metabolites based on the impressive reproducibility of the separation system and comparison of continuous gradient chromatograms of dog bile before and after butoprozine administration.

Conventional UV detectors have an important limitation when used to characterize column effluents. In screening processes a single monitoring wavelength must be selected. Although this is usually done by taking into account the absorption spectrum of the parent drug, there is the risk that metabolites with an altered spectrum may go unnoticed at the selected wavelength. Thus, in order to be sure that all

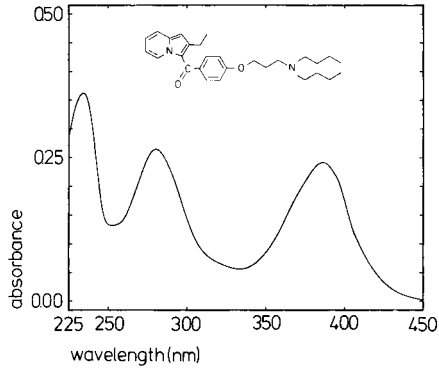

Fig. 1. Structure and UV spectrum of butoprozine.

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metabolites are being detected, a great many consecutive chromatographic runs at various wavelengths have to be carried out. Although stop-flow scanning may be of some help in overcoming this problem, it often appears to be impractical due to losses in resolution and reproducibility. In this area of research there is a need for fast scanning detectors[2,3] or detectors which can record complete spectra during a chromatographic run[4].

We now report the applicability of an universal multichannel diode array UV–visible spectrophotometer in combination with gradient-elution reversed-phase HPLC in screening for unknown butoprozine metabolites in non-radioactive "cold" dog bile.

EXPERIMENTAL

Compounds

Butoprozine was a gift from Labaz (Brussels, Belgium). Methanol and water were HPLC grade obtained from Baker (Deventer, The Netherlands). All other chemicals were analytical grade (pro-analysi) obtained from E. Merck (Darmstadt, G.F.R.).

Bile

Bile was obtained from Beagle dogs after cannulation of the biliary duct and was collected in ice-cooled tubes in the dark 1 h before (blank bile) and 8 h after intravenous administration of butoprozine (5 mg/kg). After the experiment the bile was deep-frozen ($-20°C$) and stored in the dark. Before the bile was used for the HPLC experiments it was subjected to a mild clean-up procedure. Part of the endogenous bile components were precipitated by adding two volumes of methanol to one volume of bile. Then, the supernatant was stored at 5°C in the dark for 25 days. Under these conditions no metabolite losses occurred but UV-absorbing endogenous bile components disappeared, either by precipitation or by decomposition[5].

HPLC

Fig. 2 shows the separation–detection set-up consisting of:

(1) Two eluent reservoirs, containing methanol and water (+0.05 *M* triethylamine, TEA), respectively. Before chromatography the water was led through a reversed-phase column (25 cm × 6.2 mm I.D.) packed with LiChrosorb RP-8,10 μm (Merck) to remove organic impurities. Elution took place at a flow-rate of 1 ml/min, starting with water containing 0.05 *M* TEA and then in a linear gradient mode methanol was added at a rate of 1 % per minute up to 100 min when the final eluent composition was 100 % methanol.

(2) A helium cylinder with a connection to the eluent bottles. To prevent air-bubbles in the low-pressure detector flow cells due to the mixing of the two eluent components[6,7], the water and methanol were saturated with helium before use. To maintain a helium atmosphere during elution, the helium was slowly bubbled through the eluents.

(3) Two M-45 pumps (Waters Assoc., Millford, MA, U.S.A.), as eluent mixing and delivery devices.

(4) A post-pump eluent mixing unit, necessary to reduce extra baseline drift

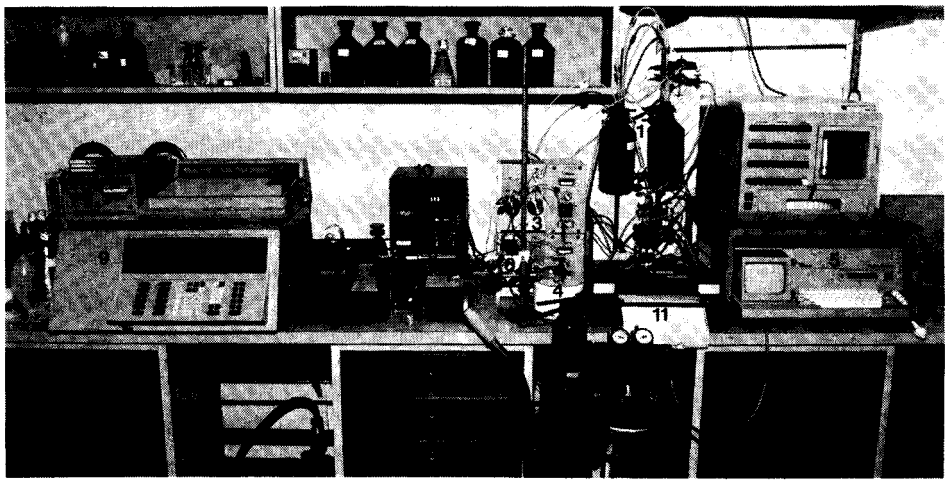

Fig. 2. The HPLC equipment constructed for the detector comparison experiment. The numbers 1 to 11 refer to the components of the set-up and are discussed in the text.

and baseline irregularity because the mixing capacity of the pumps is not optimal.

(5) A M-720 solvent programmer (Waters Assoc.).

(6) A M-7125 variable injection valve (Rheodyne). An 80-μl volume of bile supernatant was injected after the sample preparation.

(7) A WISP-710-B autoinjector (Waters Assoc.).

(8) A stainless-steel column (15 cm $\times$ 4.6 mm I.D.) packed with LiChrosorb RP-8, 5 μm (Merck) by means of a balanced density slurry method.

(9) A HP-8450-A multichannel diode array UV–visible spectrophotometer with a dual tape drive, tapes and plotter (Hewlett-Packard, Palo Alto, CA, U.S.A.). The balance between sample and reference cells, the latter containing methanol–water (50:50), was measured just before the chromatographic run and 30 and 70 min after the start of elution, respectively. This was done because of the special demands placed on this kind of gradient elution. Details will be given in a future paper.

(10) A SF-770 variable-wavelengt UV–visible detector (Schoeffel, NJ, U.S.A.) operated at 380 nm at an attenuation of 0.1 a.u.f.s. and connected in series with array 9.

(11) A BD-40 recorder (Kipp, Delft, The Netherlands) operated at a paper velocity of 0.5 cm/min and connected with detector 10.

All other experimental details were as described earlier[1] or are given, in the case of recording and plotting of the UV–visible spectra, elsewhere[8].

RESULTS AND DISCUSSION

The set-up described in the Experimental was chosen so as to perform a detailed comparison of the two detection systems. On-line measurement could be done without problems when using the helium procedure.

Representative parts of chromatograms of dog bile are shown in Fig. 3. The conventional chromatogram shows the absorption at 380 nm; the three-dimensional

one, which we prefer to call a spectro-chromatogram, depicts absorbance *versus* time over the range 225–450 nm as would seem to be relevant for butoprozine metabolites. For the latter chromatogram, spectra were taken every 8 sec, stored on tape during the run and then recalled for plotting after chromatography had been completed. Comparison of the two chromatograms clearly shows the dramatic gain in selectivity and information provided by the multichannel detector in a single run.

A direct recognition of butoprozine structural analogues against the background can be made immediately, based upon the absorption spectrum of the parent drug (metabolites 1–9). This is facilitated further when a spectro-chromatogram of blank bile is taken into account. The latter has not been reproduced, however, for simplicity. Recent structure elucidation studies have shown that the above metabolites have an intact aromatic system but that hydroxy and/or methoxy groups have been introduced. The peak designated 10 apparently contains a compound with a spectrum different from butoprozine and it thus goes undetected in the conventional chromatogram. Comparisons with chromatograms on blank bile clearly showed that peak 10 had to be a metabolite. Structure elucidation after isolation of the fraction then indicated that in this compound the indolizine moiety has been split off. So, use of the multichannel spectrophotometer represents an important improvement of metabolic screening of excretion liquids. This may be especially important for those metabolic studies in which radio-labelled drug administration cannot be performed, *e.g.*, in human beings.

Besides qualitative information, the spectro-chromatogram also provides greatly enhanced quantitative information, especially with regard to butoprozine-related structures. As is well known, substituents in the parent molecule may cause a shift in absorption maxima and minima with little change the form of the spectrum, therefore one does not know immediately how distant the peaks measured at a fixed wavelength (*e.g.*, 380 nm) are from their absorption maximum. Yet, from the spectro-chromatogram a semi-quantitative picture can readily be obtained. Also, note the changes in peak heights in, *e.g.*, compounds 2, 3, 8 and 9 relative to compound 4 and the parent compound.

A third major advantage of the multichannel diode array detector is that it gives much more information about possible peak overlaps, background interferences, etc., which is of particular importance in the isolation and structure elucidation of potential metabolites. Good illustrations of these phenomena are peak/fraction numbers 1 and 10, respectively. Fraction 1 is contaminated with a component exhibiting short-wavelength UV-absorption, whereas fraction 10 may be contaminated by a compound with a slightly shorter retention time. In these cases it would be necessary to revert to other chromatographic conditions or systems, otherwise interpretation of data from structure elucidation techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy may be rather difficult. We found stepwise gradient elution to be quite useful[9]. The purity of eluting peaks may be checked further by looking at the absorbance ratio at two or more different wavelengths[8].

Finally, background interference in the short-wavelength region between 225 and 300 nm, which is frequently encountered in this kind of gradient elution with conventional detection[10], is not such a problem when using the multichannel detector. This is obviously due to the fact that the latter detector provides significantly enhanced spectral information so that the resulting spectro-chromatogram can more

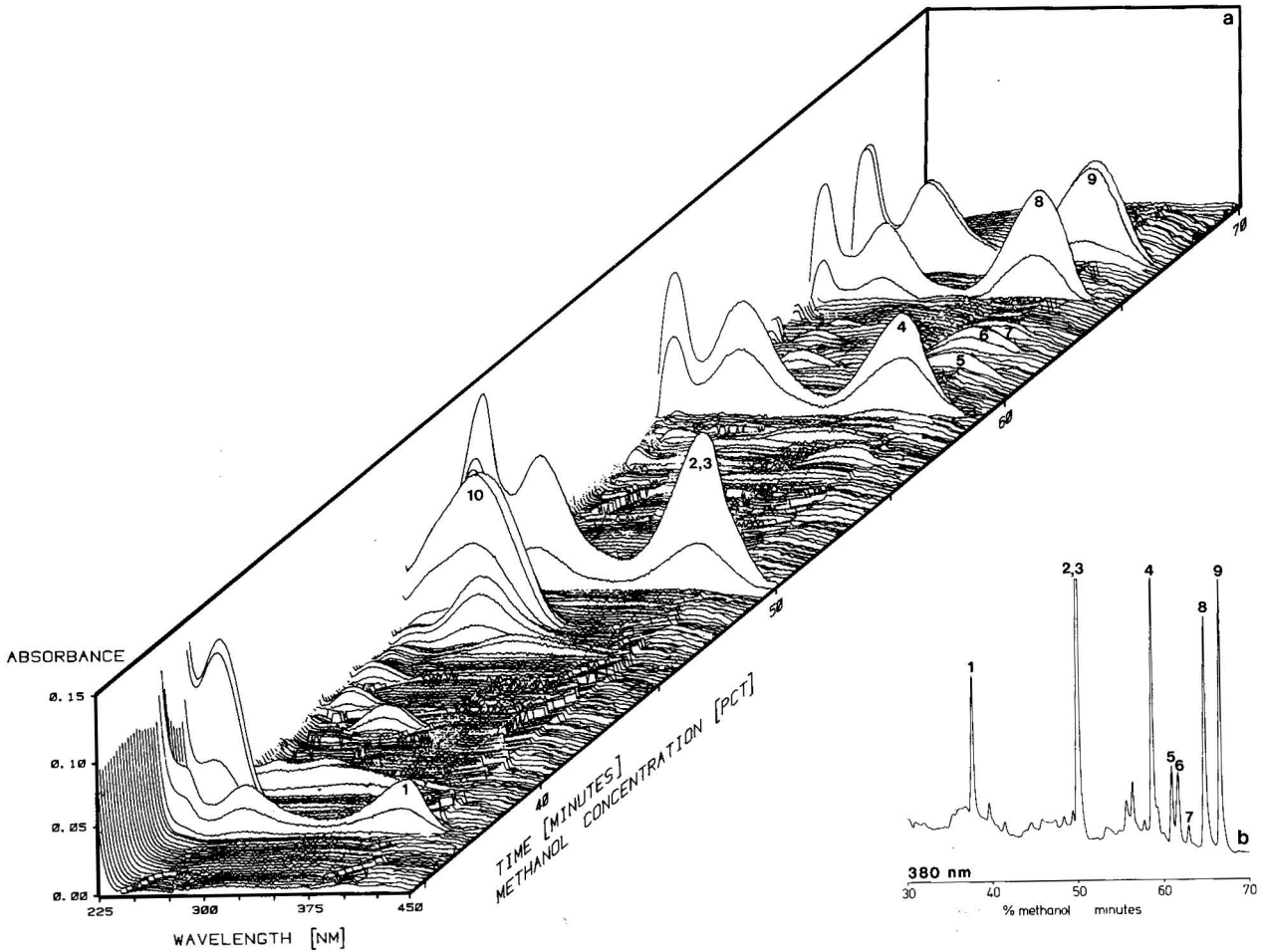

Fig. 3. (a) Spectro-chromatogram of the dog bile fraction obtained from 0 to 8 h after non-radioactive drug administration. (b) Conventional (one-wavelength) chromatogram of the same fraction as in (a), recorded on-line.

readily be interpreted, and is of particular relevance for compounds with absorption maxima only in this wavelength range.

The above results reflect our first experience of applications of the multichannel diode array detector. Other applications are presently being investigated. Although the detector is rather expensive we feel that it will become an important tool in the area of drug metabolic profiling as well as in other areas of bioanalytical research.

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Note

Liquid chromatographic determination of clorazepate decomposition rates

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Clorazepate is a benzodiazepine-type tranquilizer available in oral dosage forms as either the monopotassium or dipotassium salt. Product information[1] indicates that aqueous solutions of clorazepate are unstable and undergo rapid decomposition. The decomposition occurs by loss of the carboxyl group at the 3-position of the benzodiazepine nucleus. This decarboxylation process has been shown to be acid catalyzed and the rate of the reaction increases as solution pH decreases[2]. Clorazepate is stable only as the salt form and the decomposition occurs following protonation of the carboxylate anion. Clorazepate salts are highly water soluble with little or no organic solvent solubility. The rapid decarboxylation of clorazepate under acidic conditions results in therapeutic plasma levels of the decomposition product, N-desmethyldiazepam, and only trace levels of the parent drug[3]. Thus, the pharmacological profile of this product is essentially that of N-desmethyldiazepam which has been shown to be a very potent benzodiazepine[4]. N-Desmethyldiazepam is also the major metabolite of diazepam in humans.

In previous studies[5], administration of clorazepate with sodium bicarbonate and other antacid preparations reduced the rate and extent of appearance of N-desmethyldiazepam in blood. Chun *et al.*[6] have shown a trend toward slower absorption for clorazepate when administered with antacids, however, no significant effect on extent of absorption as measured by the area under the plasma level-time curves was observed. In a similar study[5] the bioavailability of clorazepate determined by evaluating its conversion to and absorption as N-desmethyldiazepam, was significantly reduced in the presence of a higher pH, and the peak metabolite plasma level and the time of occurrence of this peak was reduced and delayed respectively.

The direct analysis of clorazepate by some analytical techniques[7] including gas chromatography is very difficult due to the ease of the decarboxylation reaction. Previous reports[2,3] concerning the rates of clorazepate decomposition have required extractions and the measurement of N-desmethyldiazepam levels. This report describes the result of direct liquid chromatographic measurement of clorazepate levels during decomposition studies.

EXPERIMENTAL

Equipment

The liquid chromatograph was a modular system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, Model U6K injector and a Model 440 ultraviolet detector operated at 254 nm and 0.02 a.u.f.s. The column (15 cm × 4.6 mm I.D.) was packed with C_{18} chemically bonded spherical silica (5 μm), Ultrasphere ODS (Altex, Berkeley, CA, U.S.A.). All pH measurements were made using a Beckman (Fullerton, CA, U.S.A.) model 3500 digital pH meter with a combination electrode.

Reagents and chemicals

All reagents were of ACS reagent-grade quality and were used without further purification. HPLC grade methanol, mono- and dipotassium phosphate and phosphoric acid were purchased from Fisher Scientific Company (Atlanta, GA, U.S.A.). Three stock solutions of 0.2 *M* phosphate were prepared: dipotassium phosphate, 34.8 g/l; monopotassium phosphate, 27.2 g/l; and phosphoric acid, 23.1 g of 85%/l. Five 0.1 *M* phosphate buffer solutions ranging in pH from 2 to 6 at approximately one pH unit increments were prepared by mixing varying amounts of the stock solutions to obtain the desired pH and diluting with an equal volume of water.

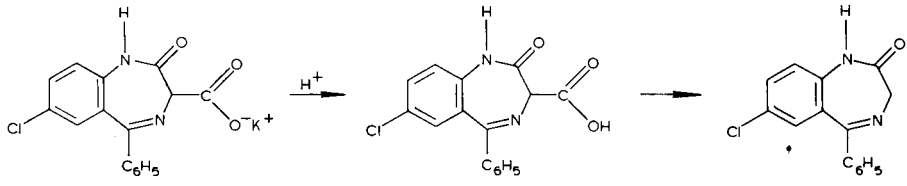
The chromatographic mobile phase was a mixture of 0.1 *M* phosphate buffer (pH 7.46) and methanol (3:7). All aqueous solutions were prepared in double-distilled water.

Decomposition studies

Samples of clorazepate (4-mg range) were accurately weighed and transferred to 10-ml volumetric flasks. The flasks were filled to volume with 0.1 *M* phosphate buffer at the desired pH. Triplicate samples were prepared and analyzed at pH 2.00, 2.99, 4.01, 4.99 and 6.05. The addition of buffer to the clorazepate sample was recorded as time zero and the times of injection recorded until the clorazepate peak had virtually disappeared. Liquid chromatographic analysis of the solutions was accomplished by injecting 5-μl samples with a mobile phase flow-rate of 1.5 ml/min.

RESULTS AND DISCUSSION

The acid-catalyzed decomposition of clorazepate to yield N-desmethyldiazepam is illustrated in Fig. 1. The decomposition reaction is known to result in the loss of the carboxyl-group from the 3-position of the benzodiazepine ring. The reversed-phase liquid chromatographic separation of clorazepate and N-desmethyldiazepam is shown in Fig. 2. The chromatograms in Fig. 2 illustrate the results obtained for clorazepate decomposition at pH 2.99. The separation was achieved using a mobile phase of 70% methanol in 0.1 *M* phosphate buffer pH 7.46. The high mobile phase pH is required to insure that no clorazepate decomposition occurs during the analysis. The decarboxylation reaction has been shown[7] to occur at a rate rapid enough to preclude the liquid chromatographic observation of the parent molecule in an aqueous mobile phase of pH 4.6 or less and substantial on-column decarboxylation was observed at pH 5.6. The high mobile-phase pH used in this study



1 2

Fig. 1. Decomposition pathway for clorazepate. Structures: 1, clorazepate monopotassium; 2, N-desmethyldiazepam.

keeps the carboxyl-group in the ionic form preventing the decarboxylation. Thus, the clorazepate is being chromatographed as an ionic species and this accounts for its relatively low capacity factor (k'). Previous work[7] has confirmed the identity of peak 1 in Fig. 2 as clorazepate. The k' value for clorazepate can be increased by increasing the aqueous component of the mobile phase indicating that the ionic solute is undergoing the typical reversed-phase retention process. A similar liquid chromatographic procedure[8] for the analysis of clorazepate in pharmaceutical products made use of hydrophobic ion-pairs by adding tetrabutylammonium ion to the mobile phase. Sufficient retention was obtained in this study with the more hydrophilic phosphate counterion. Both the monopotassium and dipotassium salts of clorazepate have been shown to produce species in solution of identical chromatographic characteristics[7].

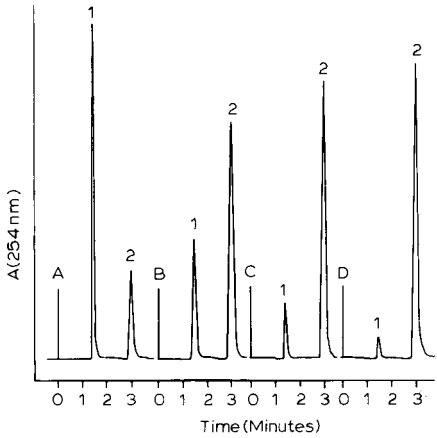
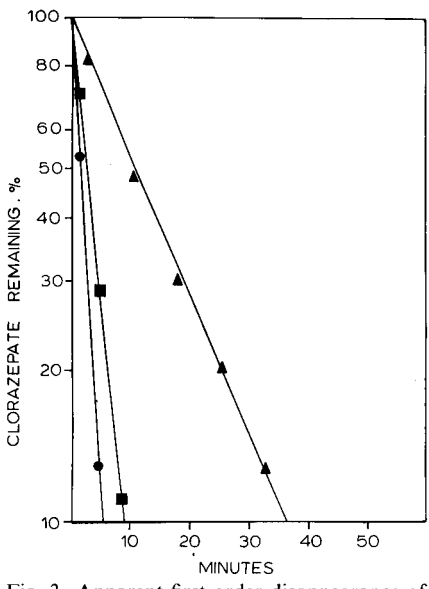


Fig. 2. Liquid chromatographic analysis of a clorazepate solution of pH 2.99. Reaction times: A, 1.43 min; B, 5.56 min; C, 9.07 min; D, 12.64 min. Peaks: 1 = clorazepate; 2 = N-desmethyldiazepam.

The rate of clorazepate decomposition was studied as a function of solution pH with the concentration of unchanged clorazepate measured by liquid chromatography. The pH and buffer capacity of the mobile phase were sufficient to inhibit the decomposition reaction upon injection. The decomposition experiments were conducted by preparing solutions of clorazepate in phosphate buffer at pH 2.00, 2.99, 4.01, 4.99 and 6.05. At least three trials were conducted at each pH level. The insolubility of N-desmethyldiazepam in water results in precipitation as the decomposition progresses and prevents the quantitation of its accumulation. Thus, no attempts were made to measure the corresponding formation rates for N-desmethyldiazepam.



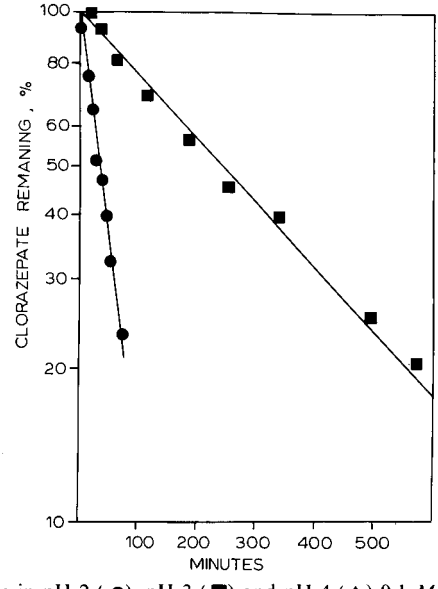


Fig. 3. Apparent first order disappearance of clorazepate in pH 2 (●), pH 3 (■) and pH 4 (▲) 0.1 *M* phosphate buffers at 22.0°C.

Fig. 4. Apparent first order disappearance of clorazepate in pH 5 (●) and pH 6 (■) 0.1 *M* phosphate buffers at 22.0°C.

Figs. 3 and 4 show the semilogarithmic plots for the percentage of clorazepate remaining as a function of time for the pH buffer systems studied. At constant pH and temperature, the degradation followed an apparent first order process. The linear relationship describing an apparent first order degradation mechanism is shown in eqn. 1.

$$\log C = \log C_{\text{initial}} - K_d t/2.303 \quad (1)$$

The terms C and C_{initial} are the concentrations of clorazepate in the buffer at time t and time zero, respectively and K_d is the apparent or observed first order degradation rate constant. Degradation kinetics were followed through at least three half-lives for all

TABLE I

CLORAZEPATE DEGRADATION RATE CONSTANTS AS A FUNCTION OF pH

Conducted at 22°C.

pH	K_d (h^{-1}), *mean ± S.D.*	*Correlation coefficient, r, mean ± S.D.*
2.00	24.94 ± (1.30)	0.993 ± (0.007)
2.99	14.95 ± (0.40)	0.982 ± (0.002)
4.01	4.02 ± (0.40)	0.990 ± (0.005)
4.99	1.29 ± (0.34)	0.987 ± (0.014)
6.05	0.17 ± (0.02)	0.994 ± (0.003)

pH systems to assure the reaction order. Final estimates of K_d were obtained by fitting the data to Eq. 2 by means of a non-linear squares method[9].

$$C = C_{\text{inital}}\ e^{-K_d t} \tag{2}$$

The mean degradation rate constants at each of the pH conditions studied are presented in Table I. Degradation half-lives for pH conditions 2 to 6 were 1.7, 2.8, 10.3, 32.3, and 241.7 min, respectively. Previous studies[2,10] employing extraction methods and analysis reported half-lives of 1.8 and 28 min at pH values of 2 and 5 for studies at 27.5°C. While it would be of interest to evaluate the decarboxylation reaction via appearance of N-desmethyldiazepam, the observed precipitation of degradation product prevented including this kinetic approach.

A profile of log K_d as a function of buffer pH for the decarboxylation of clorazepate is illustrated in Fig. 5. Over the pH range of 2 to 6, the apparent first order rate constant varied by 150-fold (24.9 to 0.17 h^{-1}). The non-linearity of the profile between pH 2 and 4 may be a consequence of protonation of the nitrogen at the 4-position of the benzodiazepine ring. Other benzodiazepines have reported pK_a values for the nitrogen at the 4-position ranging from 1.8 to 3.4 (refs. 11 and 12). Either slower or the absence of decarboxylation of the N-4 protonated form of clorazepate relative to that of the unprotonated form (pH 4–6) may offer some explanation for the noted curvature. Similar curvature in pH profiles has been noted at various temperatures[2] for this drug. While the relationship of log K_d *versus* pH appears linear from pH 4 to 6 as expected for an acid catalyzed reaction involving one drug species, the limited pH range examined does not permit its complete evaluation.

The results of this study show that degradation rates determined by direct measurement of clorazepate using liquid chromatography are very similar to those

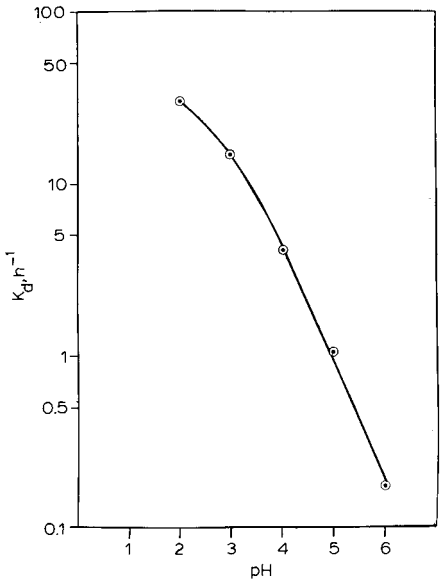


Fig. 5. The pH–rate profile of clorazepate at 22.0°C.

determined by other methods. Clorazepate, the prodrug form of N-desmethyldiazepam, has a degradation half-life of 1.7 min at pH 2.0 which increases to 241.7 min at pH 6.0.

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Note

High-performance liquid chromatography of 34 selected flavonoids

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The analysis of flavonoids by high-performance liquid chromatography (HPLC) offers an accurate, sensitive technique which yields results in minutes compared to classical procedures requiring large amounts of material, and days, if not weeks, for analysis. In 1974, Ward and Pelter[1] published the first application of HPLC to flavonoid analysis. Wulf and Nagel[2] later demonstrated, with a dozen flavonoids, the theory and practicality of separating flavone type compounds for identification purposes. Their solvent system of methanol–acetic acid–water (30:5:65) on a C_{18} column was considered adequate for the resolution of a mixture of aglycones containing the same sugar.

In the last five years, researchers have applied HPLC analysis to flavonoids in citrus fruit[3–7], tobacco leaf[8], soybeans[9,10], celery and tomatoes[7], poinsetta[11,12], and species of Larix[13–15] and Cedrus[16]. Attention has also been given to particular flavones such as polymethoxylated flavones[5,7], biflavones[1], and acetylated flavones[7].

The customary column packing was C_{18} but C_8[4,5,7,17] and alkylphenyl columns[10] have been employed. There has been only one report of columns (C_{18}) in series[18]. Most of the work has been reversed-phase and, in addition to methanol, acetonitrile[9,16], ethanol[10,13], and tetrahydrofuran[4,5] have been used.

This note reports the results of HPLC analysis of 34 flavonoids using a methanol–acetic acid–water eluting system on a C_{18} column. Also the mechanism of the HPLC analyses of certain types of flavonoids is interpreted as being related to structure or adsorption qualities.

EXPERIMENTAL

A Waters Assoc. liquid chromatograph equipped with a Model 440 absorbance detector, 254 nm wavelength; two 6000 A pumps and a Model 660 solvent programmer were used. The column was 30 cm × 3.9 mm. I.D. packed with μBondapak C_{18}, 10 μm. Solvents were filtered using a glass Millipore system with a 0.45-μm filter and degassed at room temperature under vacuum with magnetic stirring. Samples were obtained from commercial sources (C) or isolated at Southern Regional Research Center (S) and were used as received. Working solutions contained 1 mg of sample per 2 ml of methanol filtered through a Swinny stainless unit with a 0.45-μm filter. The elution solvent was water–acetic acid (495:5) from pump A and methanol

from pump B. Flow-rate was 2 ml/min with pump A providing 70% and pump B 30% of the solvent mixture for 28 of the compounds studied. The other six used the same solvent system and flow-rate but with each pump providing 50% of the solvent mixture.

The retention times were measured to calculate two chromatographic parameters: the capacity factor, k', and the relative retention, α. These parameters were calculated by the equations[19]:

$$k' = \frac{t_r - t_0}{t_0} \text{ and } \alpha = \frac{k'_2}{k'_1}$$

where t_r = retention time of compound, t_0 = time of the non-retained solvent peak; k'_2 = capacity factor of component 2, and k'_1 = capacity factor of component 1.

RESULTS AND DISCUSSION

Table I lists the common name, descriptive name, capacity factor, and relative retention times of the 34 flavonoids selected for the study.

These data confirmed and extended the findings of Wulf and Nagel[2]. The solvent system used here allowed for greater separation, *e.g.*, quercetin–quercetrin α = 1.90 (Wulf and Nagel α = 1.67).

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF FLAVONOIDS

Column, μBondapak C_{18}; solvent system, methanol–(acetic acid–water, 5:495) (30:70 for compounds 1–28; 50:50 for compounds 29–34); flow-rate 2 ml/min.

	Common name	*IUPAC name*	t_r *(min:sec)*	k'
1	D-Catechin	3,3′,4′,5,7-Flavanpentol (C)	2:14	0.43
2	Epeatechin	3,3′,4′,5,7-Flavanpentol (C)	2:49	0.80
3	Fustin	3,3′,4′,7-Tetrahydroxyflavanone (C)	3:50	1.45
4	Dihydroquercetin	3,3′,4′,5,7-Pentahydroxyflavanone (C)	4:59	2.18
5	Eriodictyol-7-glucoside	3,4′,5,7-Tetrahydroxyflavanone-7-D-glucoside (S)	5:09	2.29
6	Galongin	3,5,7-Trihydroxyflavone (C)	5:17	2.37
7	Isohesperidin	3′,5,7-Trihydroxy-4[1]-methoxyflavanone-3′--rhamnoglucoside (C)	9:38	5.15
8	Phloridzin	4,6-Dihydroxy-2(β-D-glucosido)-β-(*p*-hydroxyphenyl)-propeophenone (C)	10:25	5.65
9	Myricitrin	3′,4′,5,5′,7-Hexahydroxyflavone-3-rhamnioside (C)	10:25	5.65
10	Quercetin-3-neohesperidoside	3,3′,4′,5,7-Pentahydroxyflavone-3-(2-O-α-L-rhamnosyl-D-glucoside) (S)	10:39	5.80
11	Hesperidin	3,5,7-Trihydroxy-4′-methoxyflavanone-7-(6-O-α-L-rhamnosyl-D-glucoside) (C)	10:44	5.85
12	Quercetin-3-glucoglucoside	3′,4′,5,7-Tetrahydroxyflavone-3-O-glucoglucoside (S)	11:00	6.02
13	Isoquercitrin	3,3′,4′,5,7-Pentahydroxyflavone-3-glucoside (C)	13:00	7.30
14	Rutin	3,3′,4′,5,7-Pentahydroxyflavone-3-(6-O-α-L-rhaminosyl-D-glucoside) (C)	13:14	7.45
15	Quercetin-3-robinoside	3,3′,4′,5,7-Pentahydroxyflavone-3-O-D-galacatose-1-rhamnoside (S)	13:20	7.51

(Continued on p. 204)

TABLE I *(continued)*

	Common name	*IUPAC name*	t_r *(min:sec)*	k'
16	Kaemferol-3-neohesperidoside	3,4′,5,7-Tetrahydroxyflavone-3-(2-O-α-L-rhamnosyl-D-glucoside (C)	15:32	8.91
17	Kaemferol-3-glucoglucoside	3,4′,5,7-Tetrahydroxyflavone-3-glucoglucoside (S)	15:49	9.10
18	Myricetin	3,3′,4′,5,5′,-7-Hexahydroxyflavone (C)	16:30	9.53
19	Robinin	3,4′,5,7-Tetrahydroxyflavone-3-O-D-galactose-1-rhamnoside-7-O-rhamnoside (C)	16:31	9.54
20	Apigetrin	4,5,7-Trihydroxyflavone-7-D-glucoside (C)	16:48	10.04
21	Morin	2′,2,4′,5,7-Pentahydroxyflavone	19:20	11.34
22	Apiin	4′,5,7-Trihydroxyflavone-7-apoiosylglucoside (C)	19:21	11.35
23	Quercitrin	3,3′,4′,5,7-Pentahydroxyflavone-3-L-rhamnoside (C)	19:46	11.61
24	Naringenin	4′,5,7-Trihydroxyflavanone (C)	23:23	13.93
25	Hespertin	3′,5,7-Trihydroxy-4-methoxyflavanone (C)	32:36	19.81
26	Phloretin	2′,4′,6′-Trihydroxy-3-(*p*-hydroxyphenyl)-propcophenme (C)	34:45	21.18
27	Isorhamnetin	3,4′,5,7-Tetrahydroxy-3′-methoxyflavone (S)	35:36	21.72
28	Quercetin	3,3′,4′,5,7-Pentahydroxyflavone (C)	36:06	22.04
29	Kaemferol	3,4′,5,7-Tetrahydroxyflavone (C)	6:49	–
30	Apigenin	4′,5,7-Trihydroxyflavone (C)	7:33	–
31	Rhamnetin	3,3′,4′,5-Tetrahydroxy-7-methoxyflavone (S)	8:36	–
32	Flavone		12:22	–
33	Acacetin	5,7-Dihydroxy-4′-methoxyflavone	18:46	–
34	Techtochrysin	5-Hydroxy-7-methoxyflavone	36:25	–

The separation of kaemferol (3,4′,5,7 tetrahydroxyflavone) and luteolin (3′,4′,5,7 tetrahydroxyflavone) was attributed to the lesser polarity of the 3-hydroxyl group compared to that group at position 3′. The position of the 3-hydroxyl group in hydrogen bonding with the 4-keto group was given as the reason[2]. Examination of the α-values of several pairs show the hydrogen bonding between 3′ and 4′ hydroxyl groups to be more important for separation purposes than the hydrogen bonding between the 3-hydroxyl and the 4-keto group. The α-value for kaemferol–luteolin is only 1.15 compared to the quercetin–morin pair's 1.45. Although the methylation of a hydroxyl group should reduce a compound's polarity and increase its retention time, the elimination of hydrogen bonding between 3′ and 4′ has a greater effect. Isorhamnetin and quercetin have essentially the same retention times. Rhamnetin, methylated on the 7 hydroxyl group, however, did not even elute in a reasonable time with the methanol–acetic acid–water (30:1:69) system.

This work and Rouseff's[4] with polymethoxylated flavones show that another quality, adsorption, and not size, is operative. Methoxy groups on the B ring reduce an aglycone's retention time, *e.g.*, sinensetin (5,6,7,3′,4′-pentamethoxy flavone) elutes faster than tetramethoxyscutellarein (5,6,7,4′-tetramethoxyflavone). In contrast, a methoxy group on the A ring dramatically increases an aglycone's retention time (rhamnetin *versus* quercetin).

It is possible that the theories of Wulf and Nagel and their extension in this report will enable researchers to predict a known compound's retention time and/or assign a tentative structure to an unknown based on its retention time.

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Note

Improved N-chlorination procedure for detecting amides, amines, and related compounds on thin-layer chromatograms

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(First received November 12th, 1981; revised manuscript received January 21st, 1982)

In connection with a study on the isolation of drugs containing an amide group from the tissue of farm animals, we have developed and describe below a simple, sensitive procedure for detecting these and some other biologically important classes of nitrogenous compounds on thin-layer chromatograms. The method involves the N-chlorination of a primary or secondary amide or amino group with chlorine vapor evolving from the slow, spontaneous decomposition of calcium hypochlorite, followed by selective reduction of the excess chlorine with formaldehyde vapor. The N-chloro compounds are then detected as purplish-blue spots by spraying with potassium iodide–starch solution containing a wetting agent. Although there are several N-chlorinating procedures available[1–6], the proposed procedure is faster and more convenient.

EXPERMENTAL*

All chemicals were from J. T. Baker (Phillipsburg, NJ, U.S.A.). Thin-layer plates were products of Analtech (Newark, DE, U.S.A.); Whatman (Clifton, NJ, U.S.A.); and E. Merck (Darmstadt, G.F.R.). Silica gel plates 2.5 × 10 cm and 20 × 20 cm, with and without fluorescent indicator, and coated with a 250-μm-thick layer were employed.

The plates were spotted with nanogram amounts of the compounds and developed with a solvent which moved the spots above the origin but behind the solvent front. They were dried at 100°C for 5–10 min unless there were compounds on the plate that volatilized at this temperature, in which case they were dried at room temperature for 15–60 min, or overnight. The plate (cooled to room temperature, if heated) was placed for 2 min in a tank containing an approximate 0.5-cm layer of calcium hypochlorite (renewed every 6–7 days) spread over the bottom. It was then transferred to a tank containing a beaker of formalin (renewed every 6–7 days) (20 ml for a tank 30 × 27.5 × 8.7 cm) for 30–45 sec, removed, and sprayed with a solution containing 1% soluble potato starch (dissolved by heating), 1% potassium iodide, and 0.05% Triton X-100.

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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RESULTS AND DISCUSSION

The compounds studied, developing solvents, R_F values, and the minimum amount detectable by the procedure after chromatographic development are given in Table I. Animal drugs, lipids, nucleotide bases, amino acids, peptides, and a few miscellaneous compounds are represented in Table I. Several compounds did not give a positive response at relatively high concentration. Diphenylamine, 2,4-dinitrobenzamide (nitromide) and methionine sulfoxide gave no response at the 1–2-μg level.

Reproduction of two chromatoplates showing the expected contrast between spots and background is shown in Fig. 1.

TABLE I

LOWER LIMIT OF DETECTION OF COMPOUNDS BY N-CHLORINATION PROCEDURE

Solvent systems: a = Ethyl acetate; b = ethyl acetate–methanol–water (68.5:26.5:5); c = methanol; d = water–methanol (2:1); e = methylene chloride. R_F values were determined on Analtech plates. Minimum detectable amounts were determined on Analtech plates, 2.5 × 10 cm.

Compound	*Solvent system*	R_F	*Minimum detectable amount (ng)*
Chloramphenicol	a	0.61	60
Aklomide	a	0.64	50
Zoalene	a	0.79	57
Sulfanitran	a	0.78	46
Nitrofurazone	a	0.28	136
Anthranilic acid	a	0.22	43
Methyl anthranilate	e	0.67	37
p-Aminobenzoic acid	a	0.30	38
Sulfanilic acid	b	0.29	32
Creatine	c	0.26	28
Cytosine	c	0.52	13
Inosine	c	0.61	77
5-Methylcytosine	c	0.52	10
Adenine	c	0.64	10
Guanine	c	0.65	11
Xanthine	c	0.71	20
Thymine	c	0.81	23
Uracil	c	0.81	23
Hypoxanthine	c	0.70	10
Histidine · HCl	d	0.47	17
DL-Phenylalanine	c	0.09	66
Glycylglycine	d	0.82	33
Glycylglycylglycine	d	0.82	33
O-Phosphoethanolamine	d	0.54	29
Sphingosine	a	0.72	50
DL-Phosphatidylethanolamine dipalmitoyl glycerol	b	0.20	29
Octadecylamine	b	0.14	31
α,α-Dimethylphenethylamine	b	0.12	30
Dicyclohexylamine	b	0.14	33

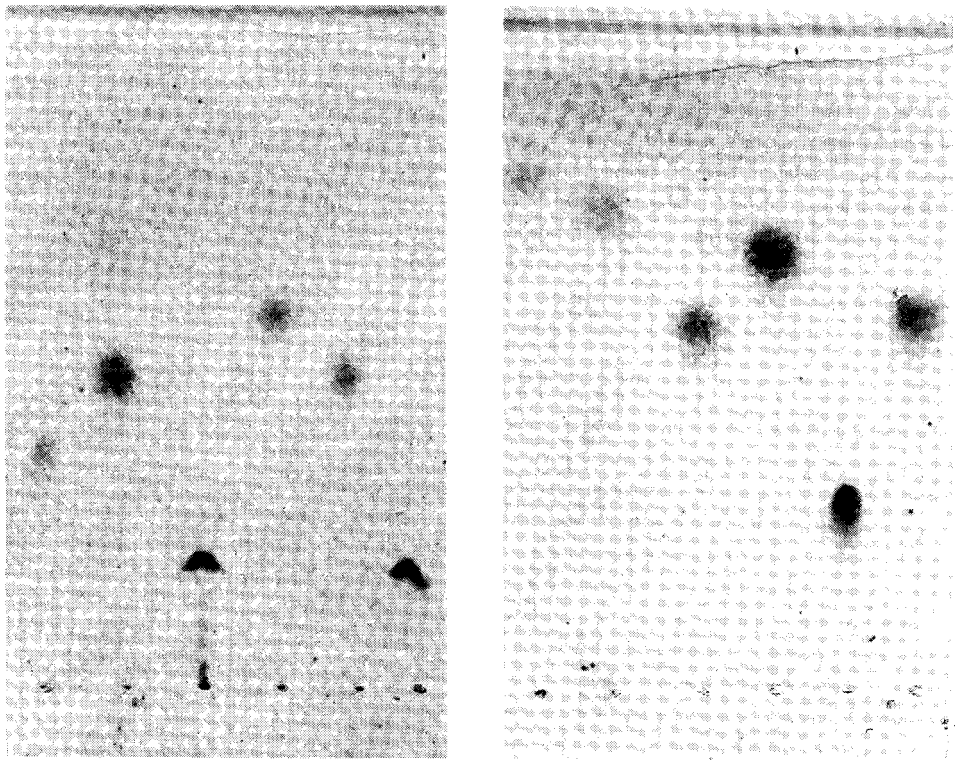

Fig. 1. Showing spots after N-chlorination procedure. Right chromatoplate: compounds from left to right are thymine (46 ng), xanthine (40 ng), 5-methylcytosine (20 ng), guanine (22 ng), creatine (56 ng), and cytosine (26 ng). Analtech silica gel G 2.5 × 10 cm; developing solvent, methanol. Left chromatoplate: compounds from left to right are chloramphenicol (120 ng), sulfanitran (92 ng), anthranilic acid (86 ng), zoalene (114 ng), aklomide (100 ng), and *p*-aminobenzoic acid (76 ng). Analtech silica gel G 2.5 × 10 cm; developing solvent, ethyl acetate.

Developing systems containing ammonia or nitrogen-containing organic solvents can give high background color and should be avoided unless sufficient time is allotted for their complete volatilization from the plate prior to N-chlorination.

Silica gel layers incorporating a fluorescent indicator were less amenable to the detection of the compounds studied than were normal plates, requiring about twice the minimum detectable levels listed in Table I.

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Note

Pyrolysis–gas chromatography of separated zones on thin-layer chromatograms

III. Application to the determination of some amino acids

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Amino acids occur naturally along with peptides and proteins in vegetaion, fruit, milk and meat and are also found in preparations derived from these products. They may be produced from hydrolysis of peptides and proteins, as for example in studies of amino acid sequences in such polymeric substances. Determination of one or more amino acids in a commercial product can sometimes provide useful information about quality as well as basic composition. Chromatographic methods, particularly based on ion-exchange as in commerical amino acid analysers, are widely used for their separation and determination. Thin-layer chromatography (TLC) can provide a versatile, inexpensive alternative for the separation but the technique is somewhat restricted in utility by problems associated with determination of the separated constituents.

In this paper, a new approach to the determination of amino acids separated by TLC is offered. It makes use of pyrolysis–gas chromatography (Py–GC) as set out in previous papers[1,2]. Five amino acids, selected for structural and compositional variation are separated by TLC and conditions established for the reliable determination of each by Py–GC. They are L-cystine, L-glutamic acid, L-methionine, L-proline and D,L-leucine. A gas chromatograph coupled to a mass spectrometer was used as an aid to identification of some of the pyrolysis products on which the determinations are based.

EXPERIMENTAL

Reagents

Amino acids were taken from an Amino Acid Reference Collection obtained from B.D.H., Poole, Great Britain. Standard solutions of each amino acid in water or 0.1 *M* NaOH were prepared at concentrations of 0.100 and 1.00 g l^{-1}. Solutions containing all 5 amino acids were also prepared so that each was at a concentration in the range 0.100 to 1.00 g l^{-1}.

Analytical Reagent grade solvents were used as supplied by Fisons Scientific Apparatus, Loughborough, Great Britain.

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Apparatus

Plates for TLC, the apparatus for Py–GC and for Py–GC–mass spectrometry (MS) are described in Part II[2]. The carrier gas for Py–GC was nitrogen at a flow-rate of 50 ml min^{-1} and for Py–GC–MS it was helium at the same flow-rate.

Thin-layer chromatography

The solvent system for separation of the amino acids was *n*-butanol–acetic acid–water (4:1:1) as described by Opienska-Blauth *et al.*[3]. The chromatogram was developed in the ascending mode for 10 cm in a closed tank saturated with solvent vapour at 25°C. Iodine vapour in a closed system at room temperature was used as revealing agent.

Pyrolysis–gas chromatography

Experiments similar to those described for the vitamins in Part II[2] were conducted with the amino acids either in solution or adsorbed on thin-layer substrate.

Mass spectrometry of pyrolysis products

Each amino acid was treated in the way described in Part II[2] and under the same experimental conditions except for the pyrolysis temperature which was chosen to be optimum for the particular test substance. In addition glutamic acid, proline and leucine were each mixed with water-saturated silica gel and the experiments repeated.

RESULTS AND DISCUSSION

The experiments in which each amino acid was introduced into the pyrolysis chamber in solution was used to establish the furnace temperature for optimum production of the decomposition product on which the determination was to be based. A selection of the pyrograms taken from the series conducted with methionine is reproduced in Fig. 1. It is seen that the pyrograms obtained at the lower end of the

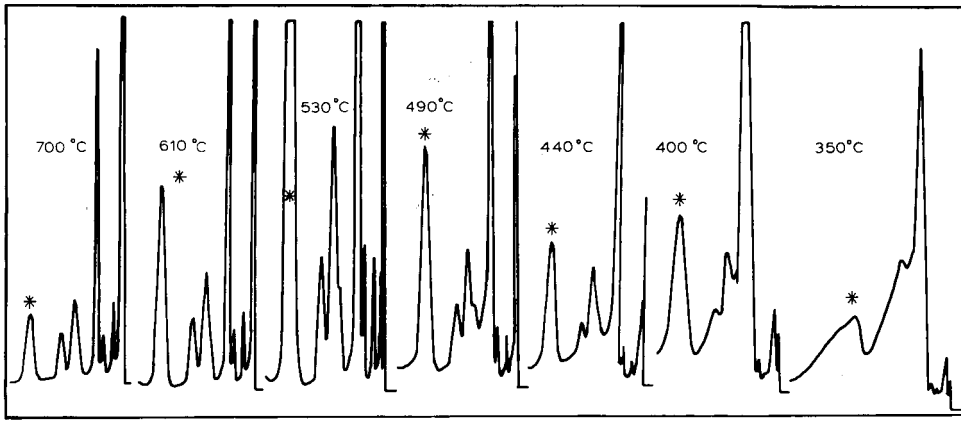

Fig. 1. Effect of changing temperature of pyrolysis on the pyrograms produced from L-methionine introduced into the furnace in aqueous solution. The pyrolysis product chosen for quantitative measurement is marked by an asterisk. The GC oven temperature was 170°C.

temperature range are not well developed. The peak, on which the measurement is based, grows in height and area as the temperature is increased. However, above temperatures of around 530°C there is a decline in yield of this particular product with increasing furnace temperature. The decline arises from the production of increasing yields of lower molecular weight products as the temperature rises. The behaviour of methionine is typical of that observed for the other amino acids studied and the vitamins reported earlier[2].

The results of the experiments with solutions of each amino acid are summarised in Table I. The table sets out the optimum pyrolysis temperatures for each amino acid and the oven temperature found to be most satisfactory for optimum resolution and sensitivity. A linear relation exists between the amount of each amino acid and the height of the peak produced by the GC detector for the chosen product of pyrolysis over the sample ranges recorded in column 3 of the table. Statistical tests indicated the reproducibility was good, relative errors never exceeding 3% for 3 to 5 replicate measurements. The temperature settings presented in Table I were confirmed to be optimum for pyrolysis of each amino acid on the thin-layer substrate.

TABLE I

SUMMARY OF DATA DERIVED FROM THE Py–GC OF SOLUTIONS OF THE AMINO ACIDS

The GC retention times are given for the pyrolysis products used for quantitative determination.

Amino acid	*Solvent*	*Sample range (μg)*	*Pyrolysis temp. (°C)*	*GC oven temp. (°C)*	*GC retention time (min)*
L-Cystine	Water	0.4–2.5	530	170	2.1
L-Glutamic acid	0.1 *M* NaOH	0.6–4.0	530	180	2.1
L-Methionine	Water	0.2–2.0	530	170	4.5
L-Proline	0.1 *M* NaOH	0.2–2.0	450	180	2.0
D,L-Leucine	Water	0.2–2.0	410	180	7.5

However, yields of the selected products were different. The ratios of yields from amino acid on the solid substrate relative to that from aqueous solution for the chosen pyrolysis products were 1.15, 3.20, 0.71, 0.74 and 0.61 for cystine, glutamic acid, methionine, proline and leucine in that order. Thus it is seen that yields are in some instances enhanced and in other suppressed by pyrolysis on thin-layer substrate. This is as might be expected from the general comments made in the previous paper[2]. Typical pyrograms for each amino acid pyrolysed on thin-layer substrate are set out in Fig. 2.

For each of the amino acids, experiments were conducted to identify the pyrolysis product on which the determination is based. The results of this work are summarised in Table II, column 5. The Py–GC–MS experiments and GC retention time data point to the products being thiirane (ethylene sulphide) and acetaldehyde for cystine and glutamic acid, respectively. With methionine, the product is ethenyl methyl sulphide as deduced from the Py–GC–MS measurements only. (The substance was not available for confirmation by retention time studies.) In the Py–GC–MS studies with proline and leucine pyrolysis products with GC retention times greater than about 1 min did not appear to reach the mass spectrometer. (Changing the

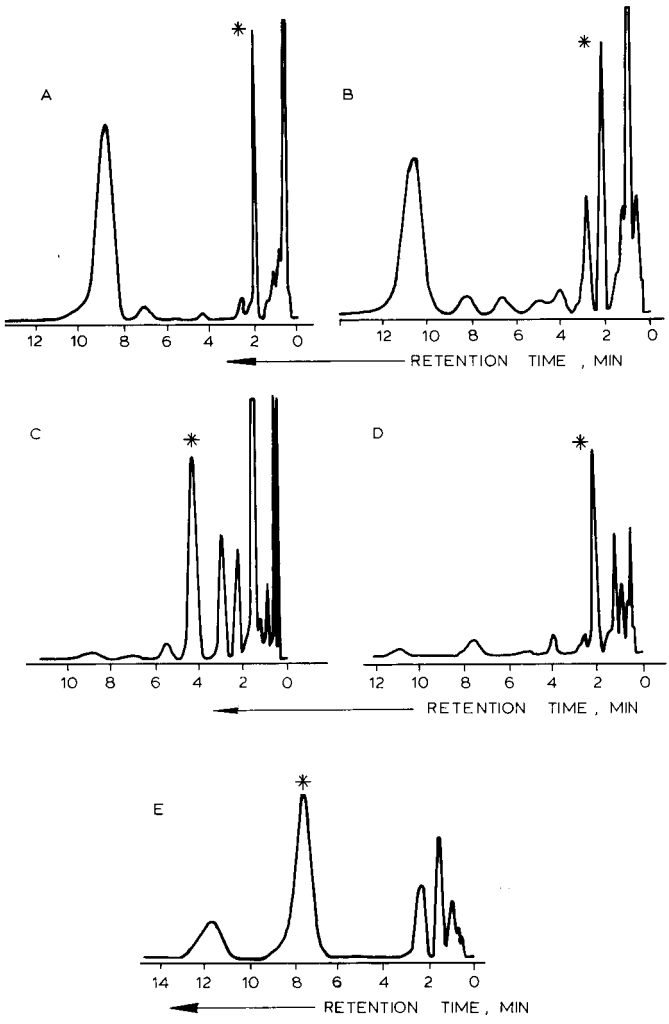

Fig. 2. Pyrograms obtained under optimum conditions on thin-layer substrate. Peaks chosen for quantitative measurement are marked by asterisks. A, proline; B, glutamic acid; C, methionine; D, cystine; E, leucine.

physical conditions of the sample to be pyrolysed as described in the experimental section did not lead to any useful mass pyrogram.) This result would suggest that the high concentration of fragments in these experiments, relative to that in the Py–GC studies leads to polymerisation or other secondary reactions which efficiently remove volatile components before the detector is reached. Retention time data suggest that the product measured following pyrolysis of proline may be one or both isomers of *n*-butene. In the case of leucine, the product determined remains unknown.

When amino acid mixtures were subjected to TLC, separated zones were located using iodine vapour as revealing agent. Mean R_F values for solvent travel of 10 cm on the chromatogram were 0.07, 0.32, 0.43, 0.24 and 0.53 for cystine, glutamic acid, methionine, proline and leucine, respectively. Neither chromatographic devel-

TABLE II

DATA RELEVANT TO THE DETERMINATION OF EACH AMINO ACID PYROLYSED ON THIN-LAYER SUBSTRATE

Amino acid	*Sample range (μg)*	*Limit of detection (μg)*	*Maximum sampling rate (hour^{-1})*	*Product determined*
L-Cystine	0.2–3.0	0.1	5.0	Thiirane
L-Glutamic acid	0.4–2.0	0.4	4.6	Acetaldehyde
L-Methionine	0.2–2.7	0.1	6.0	Ethenylmethyl sulphide
L-Proline	0.4–3.0	0.3	5.0	*n*-Butene?
D,L-Leucine	0.2–3.0	0.1	4.3	?

opment nor use of the revealing agent had an effect on the yield of pyrolysis product chosen for the determination of each amino acid. The lower limit of determination of a particular amino acid is set by the limit of detection (Table II) of the zone on the thin-layer chromatogram as found in the work with the vitamins[2]. The rate at which determinations can be made depends on the retention times of the least volatile pyrolysis product; sampling rates, estimated on this basis are set out in Table II. Sample ranges over which each of the five amino acids are known to give a response directly proportional to the amount of sample are also set out in Table II. For each amino acid in these ranges, reproducibility was good; relative errors of 3% or less were attained in the middle of each range with careful working and attention to experimental conditions as discussed in Part II[2].

For the determination of these amino acids separated by TLC from each other or from other substances, the technique presented here possesses some useful advantages. Because of the good reproducibility it can provide reliable results without the need for several replicate determinations as may be the case in applying the elution technique or densitometry when accurate results are required[4]. Errors arising from losses by elution and subsequent separation from thin-layer substrate are avoided as is the problem of finding a solvent compatible with the method of determination. Under conditions strictly specified the pyrogram of an amino acid can be regarded as a fingerprint. This can be compared with pyrograms from separated components and thus provide information about the character and quality of the substance in the thin-layer zone. The amino acids examined in this work have been separated from each other and from many substances by thin-layer methods[3,5]. Where such separations are possible on silica gel and probably other as yet untested stationary phases, it follows that the method of determination described here can be used. However, where clean-up of sample and derivatisation are readily accomplished the technique offered here is, of course, not competitive as it stands with GC, GC–MS or liquid chromatographic analysis, particularly where many amino acids are to be determined in a single sample, *e.g.* protein hydrolysate. It can be expected to be of more use where TLC would offer a convenient separation of one or a few named amino acids from a complex mixture of substances without resort to elaborate sample pre-treatment or if an independent method was required for comparative non-routine purposes.

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Note

Glass capillary gas chromatography of C_2–C_5 2-nitro-1-alkanols

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The separation of C_2–C_5 2-nitro-1-alkanols on a Carbowax 20M packed column was reported earlier[1]. The analysis time was 40 min and the resolution, R_s, was not always greater than 1. We have therefore studied the separation of these compounds on Carbowax 20M wall-coated open tubular (WCOT) glass capillary columns. The influence of the analysis temperature, gas hold-up determination method (with methane or according to Peterson and Hirsch[2] with different groups of *n*-alkanes) and the nature of the standards used (*n*-alkanes and *n*-alkanols) was investigated in order to obtain retention data that would permit an accurate identification.

EXPERIMENTAL

Preparation of the glass capillary column

A SIMAX borosilicate glass tube, 7–8 mm O.D. and 2–3 mm I.D., was used for drawing a capillary of 0.25–0.30 mm I.D. The inside surface of the capillary was then coated with isopropyl 1,2-difluoro-2-chloroethyl ether (TEE), which was first passed through one end of the column for 3 h in one direction and then for an additional 3 h through the other end in the opposite direction. Both ends of the capillary were sealed and the column was heated at 623°K for 24 h. After purging the capillary with nitrogen it was coated dynamically with Carbowax 20M using 2.5–3 ml of a 4% solution in chloroform at a velocity of 0.3–0.5 cm/sec. The column was purged again with nitrogen overnight and then conditioned using temperature programing from 313 to 513°K at a rate of 1°K/min and finally heated for 3 h to the maximum operating temperature.

GC conditions

A Carlo Erba Fractovap 2407T gas chromatograph equipped with a flame-ionization detector (FID) was used. Retention time and peak area measurements were carried out with an Autolab Model 6300 integrator.

Optimal results were obtained when using 24-m capillary column at 413°K. Nitrogen was used as the carrier gas at a flow-rate of 0.6 cm^3/min; the splitting ratio was 1:150, the injection port temperature 523°K and the sample size 0.1 μl.

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RESULTS AND DISCUSSION

The separation of C_2–C_5 2-nitro-1-alkanols is shown in Fig. 1. Elution from the column required only 12 min. The separation of all peaks is very good and the resolution, R_s, is always greater than 1.

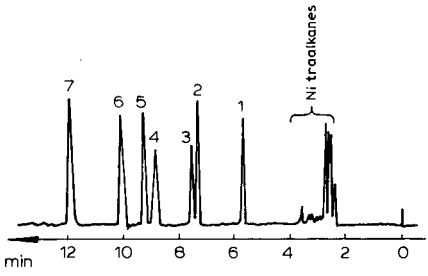

Fig. 1. Chromatogram of C_2–C_5 2-nitro-1-alkanols an a Carbowax 20M WCOT glass capillary column at 413°K. Peaks: 1 = 2-nitro-2-methyl-1-propanol; 2 = 2-nitro-1-propanol; 3 = 2-nitro-2-methyl-1-butanol-1; 4 = nitroethanol; 5 = 2-nitro-1-butanol; 6 = 2-nitro-3-methyl-1-butanol; 7 = 2-nitro-1-pentanol.

The retention indices were calculated at different temperatures. The gas hold-up time was determined with methane and according to Peterson and Hirsh with C_{15}, C_{16}, C_{17} and C_{18} *n*-alkanes. The retention indices were calculated using different standards (*n*-alkanes and *n*-alkanols). The dispersions between the experimental retention indices were compared by the Fisher and Gohren[3] criteria. It was established that the dispersions were always homogeneous but with *n*-alkanols as standards they were smaller. On this basis the mean values of the retention indices were calculated and the results are given in Table I.

TABLE I

RETENTION INDICES OF C_2–C_5 2-NITRO-1-ALKANOLS ON A CARBOWAX 20M GLASS CAPILLARY COLUMN

Compound	*Standard*	
	n-Alkanes (403–423°K)	*n-Alkanols (413–423°K)*
2-Nitro-2-methyl-1-propanol	1898 ± 5	1095 ± 1
2-Nitro-1-propanol	1990 ± 5	1184 ± 1
2-Nitro-2-methyl-1-butanol	2000 ± 5	1193 ± 1
Nitroethanol	2045 ± 5	1244 ± 1
2-Nitro-1-butanol	2067 ± 5	1256 ± 1
2-Nitro-3-methyl-1-butanol	2093 ± 5	1281 ± 1
2-Nitro-1-pentanol	2140 ± 5	1334 ± 1

The data in Table I show that better reproducibility of the retention indices is obtained when *n*-alkanols are used as standards.

CONCLUSIONS

The separation of C_2–C_5 2-nitro-1-alkanols on a Carbowax 20M W COT glass capillary column is better than that on a Carbowax 20M packed column; he resolution is greater than 1 for all of the peaks and the analysis time is shorter. Retention indices calculated using *n*-alkanols as standards have better reproducibility.

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Note

Determination of epichlorohydrin in blood by gas chromatography and selected ion monitoring

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Epichlorohydrin (3-chloro-1,2-epoxypropane) is a monomer widely used in the manufacture of epoxy resins[1]. It has been shown to be mutagenic *in vitro* and *in vivo*[2–4], and the possibility of it having carcinogenic effects has been raised[5,6].

Epichlorohydrin has been determined by a series of different techniques such as spectrophotometry[7,8], infrared spectroscopy[9,10], gas chromatography[11–13] and mass fragmentography[14]. The methods so far described, however, lack specificity or sensitivity, or both, and are not applicable to *in vivo* determinations.

Interest in the biochemical mechanisms of the toxicity of compounds used in the plastics and rubber industry led us to develop a procedure involving gas chromatographic separation and selected ion monitoring for the determination of nanogram concentrations of epichlorohydrin in complex matrices such as biological materials.

EXPERIMENTAL

Chemicals

Epichlorohydrin was kindly supplied by the Division of Occupational Health, Montedison (Milan, Italy). Epibromohydrin (3-bromo-1,2-epoxypropane), used as internal standard for quantitation, was obtained from Merck (Darmstad, G.F.R.). All solvents were of analytical-reagent grade.

Animals

Male CD_2F_1 mice (body weight 20–22 g) were obtained from Charles River Italy (Calco, Como, Italy). Animals were given a single intraperitoneal injection of epichlorohydrin (200 mg/kg dissolved in corn oil). Groups of ten mice were killed by decapitation 1, 3, 5, 7, 10, 15, 20 and 30 min after this treatment and blood samples were collected and immediately processed for epichlorohydrin determination.

Micro-extraction procedure

To 1 ml of blood, 2 ml of 1.15% potassium chloride solution and 200 μl of methylene chloride containing epibromohydrin were added (10 μg/ml, depending on

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the expected concentration range for quantitation and the detector utilized). The appropriate amount of epibromohydrin to be added to the 3-ml biological sample was determined from a preliminary experiment to establish a suitable ratio between the peak areas. The tubes were capped, shaken on a rotary shaking system for 15 min and centrifuged at 1000 *g* for 2 min. The methylene chloride phase was transferred with a Pasteur pipette into capillary-ended glass tubes, which were further centrifuged at 4000 *g* for 10 min. Any traces of biological material and water were removed by aspiration and 2–5 μl of the methylene chloride phase were injected on to the gas chromatographic column. Addition of epichlorohydrin to epichlorohydrin-free blood at concentrations from 50 ng/ml to 100 μg/ml resulted in an extraction recovery of 65.0 $\pm$ 3.9%.

Gas chromatography

Gas chromatography was carried out on a Carlo Erba Model G1 instrument with a flame-ionization detector. The column was a glass tube, 2 m $\times$ 4 mm I.D., packed with 100–120-mesh Gas-Chrom Q coated with 3% OV-17 (Applied Science Labs., State College, PA, U.S.A.). All newly prepared columns were conditioned at 280°C for 1 h without carrier gas and then for 12 h with a carrier gas flow-rate of 15 ml/min. During analysis, nitrogen was used as the carrier gas at a flow-rate of 35 ml/min; the air and hydrogen flow-rates were adjusted to give maximal detector

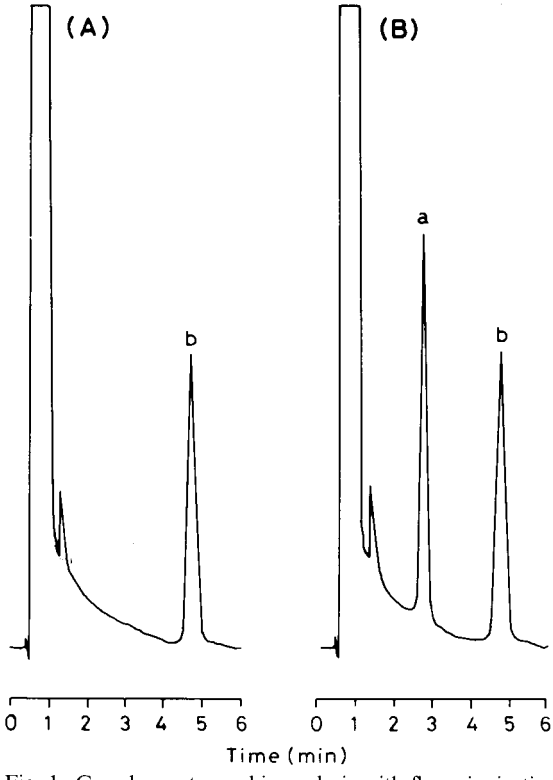

Fig. 1. Gas chromatographic analysis with flame-ionization detection of the blood of (A) an untreated mouse and (B) an epichlorohydrin treated mouse. Peaks: a = epichlorohydrin; b = epibromohydrin.

response. The column oven temperature was 60°C, injection port heater temperature 150°C and flame-ionization detector temperature 250°C.

Mass spectrometry

An LKB 2091 mass spectrometer was used, equipped with a Model 2130 computer system for data acquisition and calculation. The gas chromatographic conditions were as above, except that helium was used as the carrier gas. Selected ion monitoring was performed at 70 eV, focusing the instrument on the ions at *m/e* 62 and 64 for epichlorohydrin, *m/e* 106 and 108 for epibromohydrin and on the ion at *m/e* 57 which is common to both compounds.

RESULTS AND DISCUSSION

Fig. 1 shows typical gas chromatograms, recorded with flame-ionization detection, for the determination of epichlorohydrin in the blood of control mice (A) and treated mice (B). Epibromohydrin was chosen as the internal standard for quantitative purposes because of its similarity in structure, its suitable retention time and its pattern of fragmentation under electron impact. The chemical natures of peaks a and b were checked by mass spectrometry and the resulting mass spectra are shown in Fig. 2.

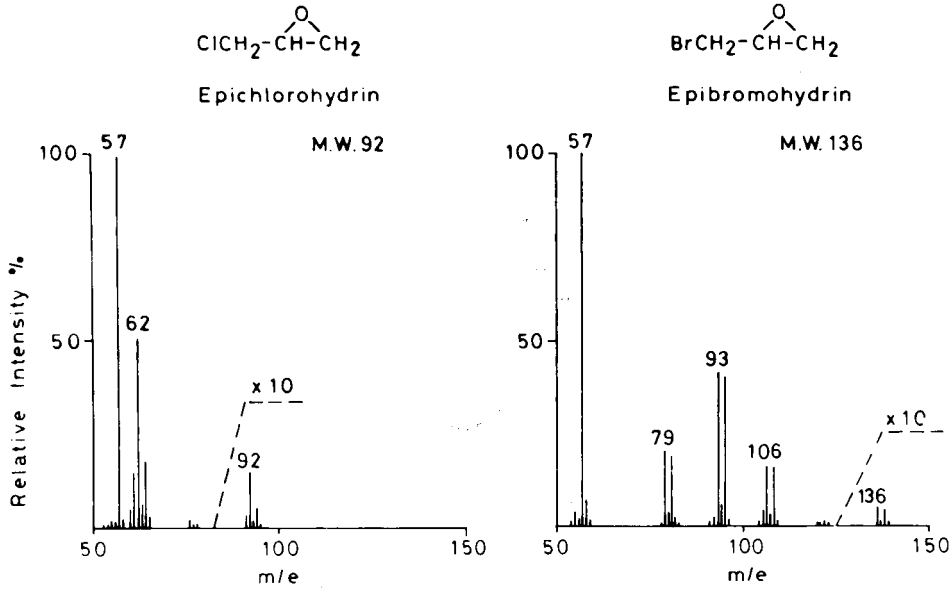

Fig. 2. Mass spectra of epichlorohydrin and epibromohydrin obtained at 70 eV.

For analysis at the nanogram level a selected ion monitoring procedure was applied, and Fig. 3 shows a typical recording for mouse blood. The mass spectrometer was focused on the ions at *m/e* 106 (isotopic 108) and 62 (isotopic 64), arising from the loss of formaldehyde from the molecular ions of epibromohydrin and epichlorohydrin, respectively, as shown in Fig. 4, and on the ion at *m/e* 57 (CH_3–CH_2–C≡O^+), the base peak in the spectra of both compounds. During the *in vivo* determinations

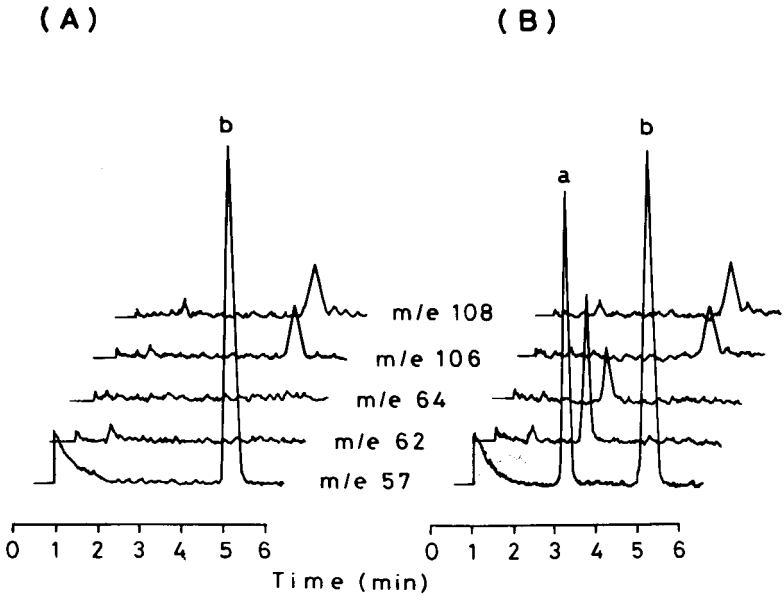

Fig. 3. Selected ion monitoring of (A) analysis of the blood of an untreated mouse and (B) analysis of the blood of an epichlorohydrin-treated mouse. Peaks: a = epichlorohydrin; b = epibromohydrin.

interferences from endogenous compounds were never observed and the detector response (flame-ionization or selected ion monitoring) was linear over a range of epichlorohydrin concentrations from 50 ng/ml to 100 μg/ml in blood.

The precision of this procedure, expressed as the coefficient of variation (C.V.), was also determined. Table I reports the results with both flame-ionization and selected ion monitoring. The concentrations selected ranged from values near the sensitivity limit of the procedure up to 100 μg/ml in blood. The method can be considered precise, as shown by the low coefficients of variation.

A kinetic study of epichlorohydrin in mouse blood was also performed to check the validity of the method for determining this compound in biological material. Fig. 5 shows a semi-logarithmic plot of the time course of epichlorohydrin concentrations in mouse blood after i.p. administration of 200 mg/kg. The monomer

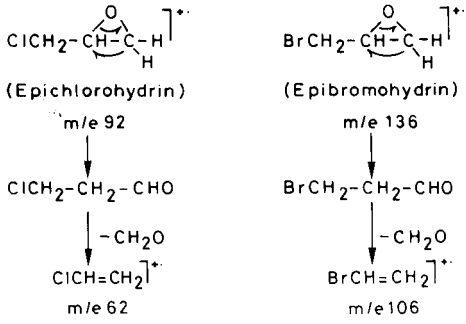

Fig. 4. Rationale for the loss of formaldehyde from the molecular ions of epichlorohydrin and epibromohydrin.

TABLE I

PRECISION OF THE METHOD FOR THE DETERMINATION OF EPICHLOROHYDRIN IN BLOOD

Parameter	*Concentration (μg/ml)*					
	Selected ion monitoring			*Flame-ionization detection*		
	0.05	*0.25*	*1*	*5*	*25*	*100*
$\bar{x}$	0.048	0.261	1.034	4.91	23.4	98.9
S.D.	0.006	0.011	0.091	0.57	1.2	3.6
C.V. (%)	12.5	4.2	8.8	11.6	5.1	3.6
n	5	5	5	5	5	5

is rapidly absorbed, reaching a peak concentration of 3.7 μg/ml within the first few minutes, after which it disappears so fast that after 15 min it is only just detectable.

The parameters describing the kinetics of epichlorohydrin in mouse blood are reported in the legend to Fig. 5 and were calculated by the method of residuals[15]. The extremely short *in vivo* half-life of epichlorohydrin provides evidence of its biological lability. Apart from its chemical reactivity towards cellular molecules and/or macromolecules, epichlorohydrin may also be a good substrate for epoxide hydrolase and glutathione-S-epoxide transferase, two ubiquitous enzymatic systems.

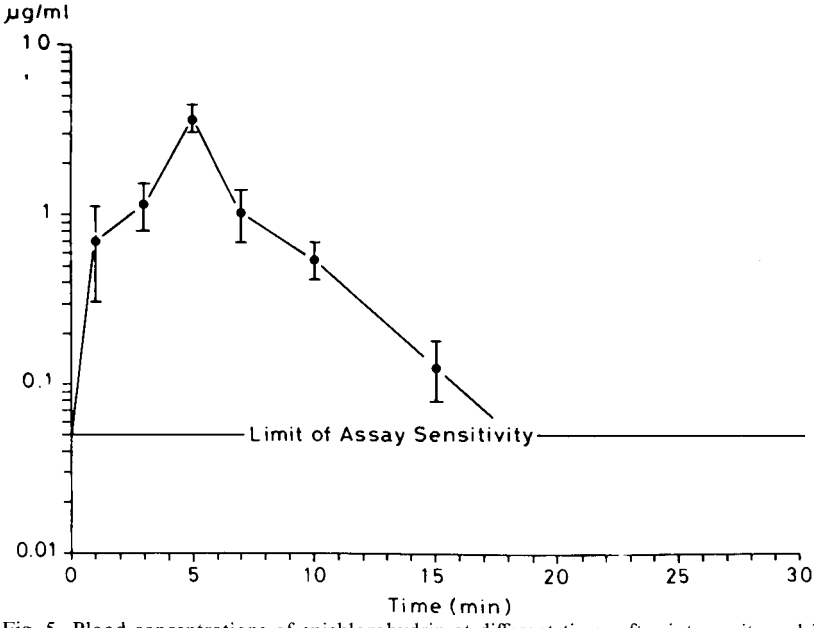

Fig. 5. Blood concentrations of epichlorohydrin at different times after intraperitoneal injection of 200 mg/kg. Each point is the mean value ± standard deviation of ten determinations. Kinetic parameters: K_a, rate constant of absorption = 0.405 min^{-1}; K_{el}, rate constant of elimination = 0.518 min^{-1}; $T_{\frac{1}{2}}$, half-life = 1.3 min; C_0, concentration extrapolated at time zero = 13.6 μg/ml; V_d, apparent distribution volume = 68.4 l/kg; AUC, area under the curve = 9.2 μg/ml · min.

In conclusion, the simple and rapid method described here offers better specificity and sensitivity than the procedures previously published, and should lend itself to application in experimental toxicology to help clarify the molecular mechanisms of epichlorohydrin toxicity.

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Note

Dns derivatization of anabolic agents with high-performance liquid chromatographic separation and fluorescence detection

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(Received October 19th, 1981)

Anabolic agents are natural or synthetic chemical compounds which have properties of the sex hormones. A major and often controversial use of such agents is in meat production, because of the rapid weight gain produced in treated animals[1]. Samples of some of the more important anabolic agents were supplied to our laboratory by the Institute for Research on Animal Diseases with the goal of developing a sensitive, general method for their detection[2]. Although these were quite different chemical groups of compounds all of them, except, trenbolone, have one or more phenolic OH groups present. Therefore all of these phenolic compounds should react with Dns chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride or Dns-Cl) to form fluorescent derivatives[3].

Various thin-layer chromatographic (TLC) methods have been published for the analysis of steroids and other anabolics by sulphuric acid-induced fluorescence[4,5] or the fluorescence of Dns derivatives[6,7]. High-performance liquid chromatography (HPLC) has also been used for the separation of selected anabolics with UV detection[8] and for the specific analysis of zenone based on its natural fluorescence[9,10]. Dns derivatives have been prepared of phenols[11] and of hydroxybiphenyls[12], separated by HPLC and TLC, respectively, and detected by their fluorescence.

The versatility and selectivity of HPLC separation, coupled with the great sensitivity of fluorescence detection, provides the analyst with an analytical system that is difficult to rival for speed, simplicity, reproducibility and sensitivity[13]. Pre-column Dns derivatization, followed by HPLC separation of the crude reaction mixture with fluorescence detection, was used to determine low nanogram (ppb, 10^9) levels of phenolic estrogenic steroids (estrone, estriol, and estradiol) by Schmidt and co-workers[13,14].

In this paper, we describe our successful modification and extension of the work of Schmidt *et al.*[14] for the detection and quantitation of nanogram levels of anabolic agents of the resorcylic acid lactones (RAL) and stilbene groups by a general procedure of pre-column Dns derivatization, HPLC separation, and fluorescence detection. Total reaction and chromatography time is less than 1 h. Partly successful attempts at analysing these compounds in samples of body fluids (serum, plasma, urine and bile of

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cows and sheep) are described. Finally, unsuccessful attempts to use the same chromatographic system for the detection of Dns hydrazone derivatives of anabolics with a keto group are reported.

EXPERIMENTAL

Solvents and reagents

Water was distilled and deionized and the pH adjusted to 3.5 with phosphoric acid. It was filtered through a Millipore 0.45-μm filter. Acetonitrile (Fisons HPLC grade) was used without further purification. HPLC solvents were thoroughly degassed before use. Dns-Cl was from BDH biochemicals. A 1.5 mg/ml-standard solution of Dns-Cl in acetone was used[14]. This solution was stored in a regrigerator. A buffer solution of $NaHCO_3$ in water (4 g/l) adjusted to pH 10.5 with 5 *N* sodium hydroxide was used[14]. Samples of the anabolic agents were donated by Dr. S. Dixon of the Institute for Research on Animal Diseases[2] and were determined to be pure by melting point determinations. Standard solutions of these anabolic agents were made up by dissolving 2 mg of each in 100 ml of ethanol (BDH, AnalaR, 99.7–100%).

Dns derivatization procedure

The appropriate amount (usually 100 μl or less) of the anabolic agent solution was transferred to a Pierce 1-ml Reacti-Vial. The solvent was evaporated with a stream of dry nitrogen at room temperature. To the residue in the vial was then added 40 μl of buffer solution and 100 μl of the Dns-Cl solution. The Dns-Cl is therefore present in large excess. The vial was vigourously shaken for 30 sec and then placed in a Pierce Reacti-Therm Heating Module at 100°C for 5 min. The vial contents changed from a pale yellow before reaction to colourless after reaction. The yield of Dns derivatized product was not changed by longer reaction times of 10 and 20 min. The vial was stored in a refrigerator after reaction.

Separation and detection procedures

The crude Dns derivatization product mixtures were injected via a 20-μl Rheodyne injector directly into a Perkin-Elmer Series 3-B liquid chromatograph. The column used was a 25 × 0.26 cm PAH-10 (reversed-phase C_{18}) LC column, Perkin-Elmer 258-0082, Serial No. 1155. Gradient elution was performed from acetonitrile–water (60:40) to acetonitrile–water (95:5) over 15 minutes (curve 1) at a flow of 1 ml/min. The solvent compensation was held at acetonitrile–water (95:5) over a 10-min period. An overnight flush with pure acetonitrile (0.1 ml/min) was employed. The column effluent was allowed to pass through a 20-μl flowcell in a Perkin-Elmer Model LS-3 fluorescence spectrometer. Excitation and emission wavelengths of 335 nm and 540 nm, respectively, (both slits 10 nm) were chosen after an examination of the spectra of several of the Dns derivatized anabolics using the stop-flow feature of the Series 3-B chromatograph. Chromatograms were recorded on a Perkin-Elmer Model 56 recorder.

Animal samples

Samples of serum, plasma, urine, and bile from cows and sheep were supplied to our laboratory[2]. After a hydrolysis with acid or with β-glucuronidase and sul-

phatase, samples were extracted with diethyl ether. The ether layer was dried and then evaporated to dryness in a Reacti-Vial. Dns derivatization was carried out in the manner described above. Body fluid samples were spiked with the anabolic agent when appropriate.

RESULTS AND DISCUSSION

Dns derivatization and chromatography

Fig. 1 is the chromatogram of a blank of the buffer plus Dns-Cl solution. Fig. 2 is the chromatogram which results from the Dns derivatization of a mixture of estriol,

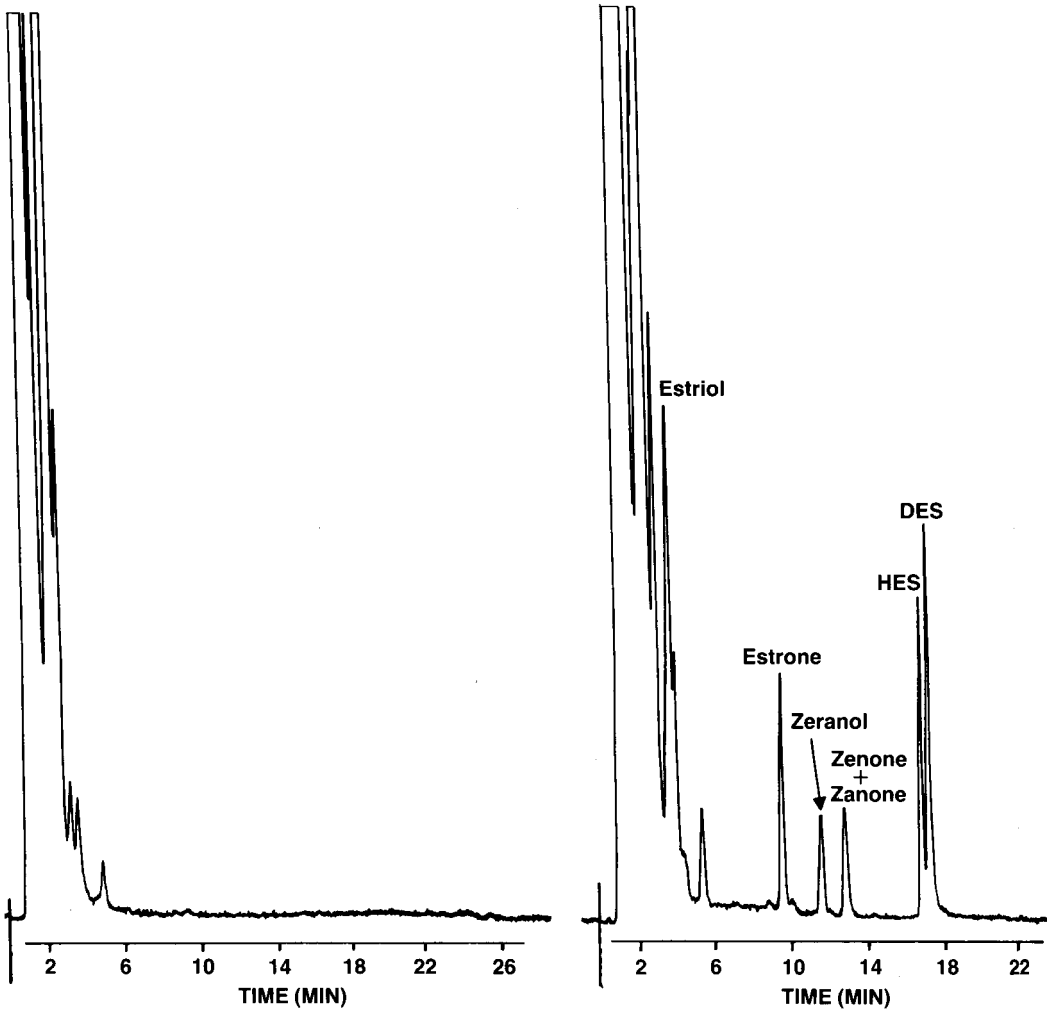

Fig. 1. Chromatogram of a blank of the buffer plus Dns-Cl solution.

Fig. 2. Chromatogram after Dns derivatization of estriol, estrone, zenanol, zanone, zenone, HES and DES.

estrone, zenanol, zenone, zanone, hexestrol (HES) and diethylstilbestrol (DES). The amounts of each compound were not the same, as will be discussed later. The presence or absence of a double bond is a sufficient enough structural difference to allow HES and DES to be separated. The double bond is a less significant feature of the zenone and zanone structure and these two compounds are not separated from each other, although they do separate from zeranol. Retention times for each compound were confirmed by separate individual Dns derivatization and chromatography. Dns derivatives of both the RAL and stilbene compounds were shown to be stable for at least four days in the dark at 4°C (refrigerator).

Sensitivity

Separate experiments showed that the Dns derivatization of identical amounts of anabolic agent gave a peak height for the RALs which was only about 1/10 of that for the steroids and 1/15 of that for the stilbene. Therefore, the peaks in Fig. 2 are those which result from the Dns derivatization of several μg of the RALs but several hundred μg of the other anabolic. By separate experiments, the lower limit of detection of Dns derivatized DES was determined to be 5 ng, whereas that for zeranol was found to be 80 ng. Time of reaction, product stability, wavelength of excitation and emission, and air oxidation were all experimentally excluded as possible reasons for the smaller peak size for the RALs. The cleavage of the lactone ring under the basic buffer conditions is a possibility which requires further exploration. The basic conditions required for the Dns derivatization of phenols may reduce yields when there is a lactone ring present in the same structure.

Quantitation of Dns derivatization

Triplicate samples of DES ranging from 50 to 150 ng were Dns derivatized and the resulting peak heights averaged to construct a calibration curve. Each separate Dns derivatization gave a peak height within $\pm 15\%$ of the average value shown. Dns derivatization is a chemical reaction whose yield would not be expected to be exactly the same each time it is performed. Given that the amounts derivatized were very small and that the reactions and analyses were not all done on the same day, we were pleased with the stability of the analytical system and with the reproducibility of the data.

Dns-hydrazone experiments

Trenbolone does not have a phenolic OH group but it does have a keto group as do two of the RALs, zenone and zanone. DNS-hydrazones of these compounds were prepared[3] and subjected to the chromatographic conditions given above for the Dns derivatization of phenolic OH groups. Despite the similarity of structures of these derivatives (an –N=N– group replaces an –O–), no peaks, other than those in a blank, were observed.

Animal samples

Dns derivatization of cow and sheep serum and plasma gave very few peaks which might interfere with the identification of the anabolic agents. Unfortunately, these agents do not appear at any appreciable levels in serum and plasma but are found in much higher levels in urine and bile[2]. Fig. 3 shows the chromatogram that

results from the dansylation of the extract from 0.5 ml of sheep urine to which 100 ng (200 ppb) had been added. Therefore, only the anabolics with long retention times, HES and DES, can be detected and the lower limit of detection of these is rather high because of the very large number and high concentration of materials in urine which are extracted and Dns derivatized.

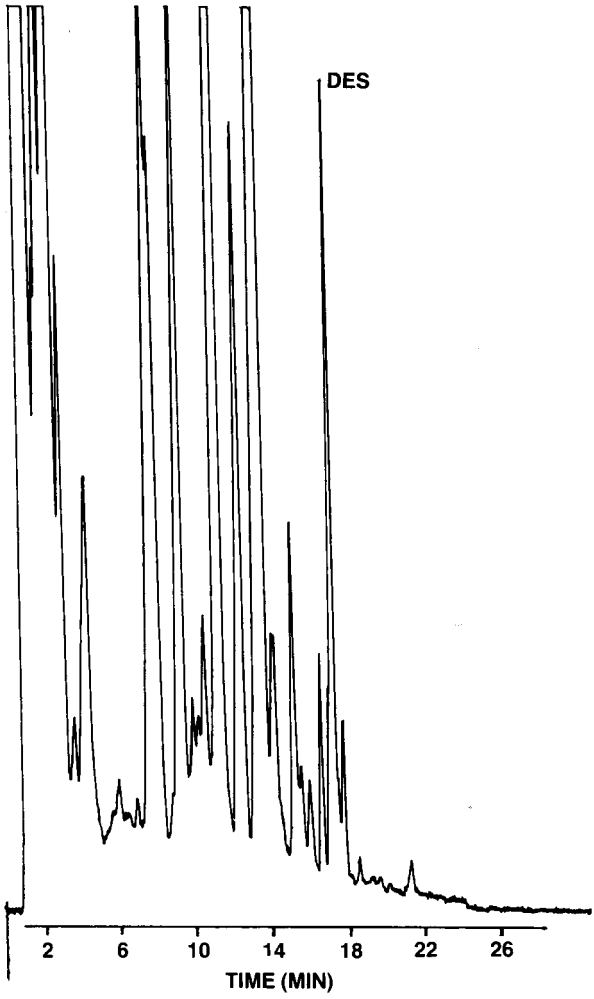

Fig. 3. Chromatogram after Dns derivatization of sheep urine plus DES (200 ppb).

Hydrolysis and ether extraction of cow bile gave large amounts of residual material. Unsuccessful attempts at Dns derivatizing spiked (DES) samples of cow bile led us to conclude that unacceptably high concentrations or amounts of Dns-Cl would be required to detect anabolic agents in bile by this method.

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Dr. S. Dixon of the Institute for Research on Animal Diseases, Longston, Newbury, Great Britain, provided us with samples of anabolic agents and with animal samples of plasma, serum, urine, and bile.

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Note

Determination of furazolidone in swine plasma using liquid chromatography

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Furazolidone is used as a therapeutic antibacterial agent in swine, cattle, and poultry and is also used as a growth promotant in swine. In the latter use the drug is added to feeds in subtherapeutic doses for most of the life of the animal. The Food and Drug Administration recently proposed to list furazolidone as a category III drug for medicated feeds[1]. This action would continue the present "zero" tolerance level (no residue) restriction for this drug in edible tissue because of the concern over the potential carcinogenicity of this compound.

In connection with other studies, we required a sensitive method for the quantitative determination of furazolidone in swine plasma. Plasma levels of furazolidone have been determined by a colorimetric method[2]. In this method furazolidone is hydrolyzed to 5-nitro-2-furaldehyde. This compound is subsequently reacted with phenylhydrazine to yield the corresponding phenylhydrazone which is determined by measurement of the absorbance at 430 nm. This method is nonspecific and is also relatively insensitive. The lowest level of furazolidone reported was 1 μg/ml.

Methods have recently been reported which used liquid chromatography (LC) for the determination of furazolidone in turkey[3,4] and chicken[5] tissue and in animal feeds[6–10] but not in plasma. We report a sensitive method for the determination of furazolidone in swine plasma using LC in which both ultraviolet (UV) and electrochemical detection were examined.

EXPERIMENTAL

Reagents and materials

Furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidin-2-one] was obtained from Norwich-Eaton (Norwich, NY, U.S.A.). Glass-distilled methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and doubly-deionized, glass-distilled water were used for the LC analysis. Culture tubes (100 × 16 mm) with screw caps and tapered centrifuge tubes (15 ml) were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.) and were amberized with Amber-stain® No. 29-346 (Drakenfelt Colors Division of Ciba-Geigy, Washington, PA, U.S.A.) because furazolidone is light sensitive[10].

Heparinized swine blood was obtained from a local slaughterhouse at the time of slaughter. Plasma was spiked via syringe with the appropriate amount of a solution of furazolidone in water–methanol (70:30).

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Sample preparation

To an amber-colored culture tube were added 100 mg of sodium chloride (to prevent emulsion formation) and 2 ml of swine plasma. The plasma was extracted four times with 5 ml of ethyl acetate by shaking on a wrist-action shaker for 10 min. The ethyl acetate extracts were transferred with a Pasteur pipet to a 15-ml, amber-colored centrifuge tube, and the ethyl acetate was removed under a stream of nitrogen. LC mobile phase (200 μl) was added via a 250-μl syringe, and the tube was agitated for 1 min on a Vortex mixer to wash the surface with the solvent. The tube was centrifuged for about 3 min on a bench-top centrifuge to remove the small amount of insoluble material which was often present. The LC injector loop was overfilled with *ca.* 70 μl of the supernatant.

Chromatography

An Altex 100A pump (Altex, Berkeley, CA, U.S.A.), an Altex 210 injection valve fitted with a 50-μl loop, and a Whatman (Clifton, NJ, U.S.A.) Partisil PXS 10/25 ODS column were used. The pump was fitted with high-pressure inlet check valves (Altex) so that all plastic tubing could be replaced with 1/8-in. stainless steel. The mobile phase consisted of methanol–water (30:70) which was buffered to pH 4.0 with dibasic sodium phosphate (16.5 m*M*) and sodium citrate (13.1 m*M*). A flow-rate of 1.1 ml/min was used. The mobile phase was degassed by heating to 55°C under an atmosphere of helium. A Schoeffel SF770 Spectroflow variable-wavelength UV detector set at 362 nm and an electrochemical detector comprised of a TL5 glassy-carbon electrode and a LC4 amperometric controller (both from Bioanalytical Systems, West Lafayette, IN, U.S.A.) set at a potential of −0.75V relative to Ag/AgCl were employed.

RESULTS AND DISCUSSION

Since we could find no reports in the literature concerned with the distribution of furazolidone between plasma and red cells, we performed experiments to determine the distribution. In these experiments 10-ml samples of fresh, heparinized swine blood were spiked with the desired amount of furazolidone. The samples were immediately centrifuged, and 2 ml of the resulting plasma were analyzed. The amounts of furazolidone recovered were 96 and 193 ng/ml, respectively, when blood was spiked with 100 and 200 ng/ml. If one assumed that the amounts recovered were identical to those obtained with spiked plasma (see Table I and below), the actual amounts of furazolidone present in the plasma were 103 and 212 ng/ml, respectively. Since the volumes of plasma and red cells in swine plasma are about equal, these results suggest that the drug is distributed about equally between the plasma and the red cells.

Other experiments showed that the amount of furazolidone recovered from refrigerated whole blood decreased with time. Several 10-ml samples of heparinized blood were spiked with 100 ng/ml of the drug. Plasma from three samples was analyzed immediately and the remaining samples of whole blood were refrigerated. Plasma from these samples were analyzed in triplicate at various times. The average amounts of furazolidone were 92, 82, 74, and 44 ng/ml at 0, 24, 48, and 120 h, respectively. However, plasma containing furazolidone showed less than 10% de-

TABLE I

RECOVERY OF FURAZOLIDONE FROM PLASMA

Amount added (ng/ml)	*Recovery ± S.D. (ng/ml)*	*Recovery (%)*
200	182 ± 6.0	91.1
100	93.4 ± 4.1	93.4
60	55.6 ± 2.7	92.6
30	27.8 ± 1.1	92.4
10	9.47 ± 1.0	94.7

crease per day when refrigerated. The reason for this difference between whole blood and plasma is not known at this time, but is under study. The point is that whole blood containing furazolidone should be separated immediately and not stored prior to analysis. In this study plasma was spiked immediately before analysis.

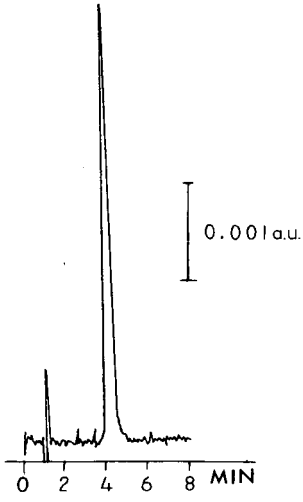
A chromatogram from a plasma extract containing furazolidone and detected at 362 nm is shown in Fig. 1. Plasma from more than ten animals was examined; the chromatogram shown is typical. The response factor was $9.2 \cdot 10^{-5}$ a.u./ng, and the minimum detectable level (signal-to-noise ratio 2:1) was 0.5 ng which corresponded to 1 ng/ml of plasma. Detection was carried out at 362 nm because this is the wavelength of maximum absorption of furazolidone in the mobile phase and, more importantly, because of the absence of interfering peaks in the chromatograms. Detection at lower wavelengths, *e.g.* 254 nm, was unsatisfactory because of a large amount of background absorption. Similar observations have been made for nitrofurantoin in human plasma[11,12].

Recovery data for plasma spiked with furazolidone at levels which ranged from 10 to 200 ng/ml are shown in Table I. Each result shown represents the average of at least six determinations. The percent recoveries were similar for all levels examined. The standard deviation ranged from approximately 3% at 200 ng/ml to 10% at 10 ng/ml. For plasma which contained less than 100 ng/ml of furazolidone, identical recoveries were obtained with one less ethyl acetate extraction. Amounts of furazolidone were determined by comparing the sample peak with the average peak height from a standard solution which contained a similar amount of the drug. Alternatively, a standard curve could have been used since the detector response showed excellent linearity over the range examined.

In some experiments serum was substituted for plasma. Amounts of furazolidone added were 60, 100, and 200 ng/ml. Recoveries were identical, within experimental error, to those obtained with plasma.

In an effort to increase the sensitivity of the method, the use of a commercially available electrochemical detector was investigated[13]. It has been known for years that nitrofurans can be reduced electrochemically[14,15]. Polarography has, in fact, been used to determine furazolidone in feeds[16–18]. The polarographic behavior of a structurally similar nitrofuran, nitrofurantoin, has recently been investigated[19].

Because of the facile reduction of oxygen, efforts were made to exclude oxygen from the chromatographic system and from the sample when this detector was used. Oxygen dissolved in the mobile phase will increase the background current and thus



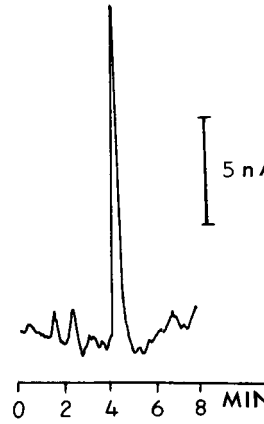


Fig. 1. Chromatogram obtained from plasma containing 100 μg/ml furazolidone and detected at 362 nm. Peak corresponds to 47 ng of furazolidone injected.

Fig. 2. Chromatogram obtained from injection of 50 ng of furazolidone dissolved in mobile phase and detected at a glassy-carbon electrode at a potential of −0.75 V *vs.* Ag/AgCl.

decrease sensitivity; oxygen dissolved in the sample will appear as a peak in the chromatogram. In addition to degassing the mobile phase, all plastic tubing was replaced with stainless steel to prevent permeation of air. Samples were degassed prior to injection by bubbling mobile phase-saturated nitrogen through the sample for 10 min.

We found that a glassy-carbon electrode at a potential of −0.75 V relative to Ag/AgCl gave the best signal-to-noise ratio for furazolidone with the chromatographic conditions used. Changing the pH of the mobile phase, the buffer-salt concentration, or the buffer composition, *e.g.* acetic acid–sodium acetate, did not increase the signal-to-noise ratio. Injection of standard solutions showed that the detector response was linear over the range examined (15–500 ng); the correlation coefficient from linear regression calculations was 0.999. As with UV detection at 362 nm, no peaks were present in the region where furazolidone eluted when plasma blanks were analyzed. The signal-to-noise ratio observed with this detector was similar to that found with 362 nm detection (Fig. 2). Similar detection limits for the two types of detectors used in this study is not surprising since Lund *et al.*[20] reported identical detection limits (3 ng) for a nitrobenzene derivative (nitrazepam) when detected at 254 nm and at a glassy-carbon electrode. Thus, electrochemical detection is a viable alternative to UV detection for furazolidone. Dual detection using both types of detectors would yield additional evidence to confirm the presence of furazolidone in plasma.

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Note

Bestimmung des Einzel- und Gesamtcardenolidgehaltes in *Convallaria majalis* L. mittels Hochleistungsflüssigchromatographie*

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(Eingegangen am 30. Dezember 1981)

Zur quantitativen Erfassung der Cardenolidglykoside in *Convallaria majalis L.* bediente man sich bisher eines Verfahrens, das nach papierchromatographischer Trennung des Glykosidkomplexes in sieben Zonen und Umsetzung mit Baljet-Reagens eine photometrische Bestimmung zuliess[1]. In Anbetracht der grossen Anzahl inzwischen bekannt gewordener Convallariaglykoside[2–4] genügt diese Methode jedoch keineswegs mehr den heutigen Anforderungen.

Nachdem kürzlich die Trennung von über 20 Convallaria-Cardenoliden mittels Hochleistungsflüssigchromatographie (HPLC) gelang[5], war es naheliegend, diese Methode auch für quantitative Zwecke nutzbar zu machen. Im Vordergrund stand dabei die Frage nach einer möglichst einfachen Probenaufbereitung und die Suche nach einem geeigneten inneren Standard, da neben der Bestimmung der Zusammensetzung des Glykosidkomplexes auch die Erfassung des Gesamtgehaltes an herzwirksamen Glykosiden in Herba Convallariae angestrebt wurde.

EXPERIMENTELLES

Apparaturen: Flüssigkeitschromatograph Perkin-Elmer Series 3 + Rheodyne Injektor (175-μl Schleife); Detektor Perkin-Elmer LC 65 T. Wellenlänge 221 nm; Integrator Perkin-Elmer M 2; Schreiber Perkin-Elmer 023.

Säulenkombination: Vorsäule (40 × 4.6 mm I.D.) LiChrosorb RP-2 (5 μm) und 2 Trennsäulen (250 × 4.6 mm I.D.) LiChrosorb RP-2 (10 μm) und LiChrosorb RP-8 (7 μm) mittels totvolumenfreier Zwischenstücke in Serie geschaltet. Alle Säulen von Knauer (Oberursel, B.R.D.). Mobile Phase: Acetonitril (HPLC Grade S, Rathburn Chemicals, Walkerburn, Grossbritannien)–Wasser, Gradient 16:84 bis 18:82 mit 0.2%/min, nach 25 min mit 0.4%/min bis Ende der Analyse, Fluss: 1 ml/min. Temperatur: 22°C.

Vergleichssubstanzen: Sämtliche in dieser Arbeit verwendeten Convallariaglykoside wurden im Institut für Pharmakognosie der Universität Wien isoliert (vgl. Lit. 5). Als innerer Standard diente Helveticosid reinst (Serva, Heidelberg, B.R.D.). Die einzelnen Cardenolide wurden an Hand der Retentionszeiten und durch Zuspritzen der Vergleichsubstanzen identifiziert.

* Teil der Diplomarbeit E. Bamberg-Kubelka, Universität Wien, 1980.

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Probenaufbereitung: 1.5 g Folium (Herba, Flos) Convallariae (Sieb V, ÖAB 9) werden in einem 100-ml fassenden Rundkolben mit 15.00 ml Standardlösung (10.00 mg Helveticosid in 100.0 ml 70 %igem Äthanol) übergossen und 15 min auf dem siedenden Wasserbad unter Rückflusskühlung erhitzt; nach dem Abkühlen auf Raumtemperatur fügt man 35 ml Wasser und zur Fällung der Ballaststoffe 10.0 ml Bleiessig (DAB. 6) zu und mischt gut durch. Der Bleiüberschuss wird durch Zugabe von 12.5 ml einer 10 %igem wässerigen Lösung von Dinatriumhydrogenphosphat ausgefällt. Der entstandene Niederschlag wird durch Zentrifugieren abgetrennt, die klare Lösung abegegossen und ein aliquoter Teil von 50 ml mit 30 ml und viermal mit je 20 ml Chloroform–*n*-Butanol (2:1) ausgeschüttelt. Die in einem Rundkolben gesammelten Fraktionen bringt man unter vermindertem Druck bei max. 60°C zur Trockene und löst den verbleibenden Rückstand in 10 ml 70 %igem Äthanol. Nach Filtrieren durch ein Millipore-Filter (Type FA; Porenweite 1 μm) verwendet man 30 μl der klaren, hellgelb gefärbten Lösung für die HPLC-Messung. Berechnung: Die Korrekturfaktoren und die Einzelglykosidanteile bzw. der Gesamtcardenolidgehalt werden in der üblichen Weise berechnet[6]. Es empfiehlt sich eine tägliche Kontrolle der Faktoren.

Untersuchungsmaterial: Probe 1: Herba Convallariae ÖAB. 9, Kottas, Wien; Probe 2: Herba Convallariae, Wolkersdorf, Niederösterreich; Probe 3: Flos Convallariae, Wolkersdorf, Niederösterreich; Probe 4: Herba Convallariae ÖAB. 9, Kottas, Wien; Probe 5: Folium Convallariae russ. Typmuster 24536, Paul Müggenburg, Hamburg, B.R.D.

ERGEBNISSE UND DISKUSSION

Bei der direkten Einspritzung alkoholischer Drogenauszüge erschwerten intensive, durch Ballaststoffe verursachte Peaks die Zuordnung der Cardenolide erheblich. Eine Reinigung über Sep-Pak Silika bzw. C_{18} Kartuschen (Waters Assoc., Milford, MA, U.S.A.) verlief unbefriedigend, da die Begleitstoffe nur zum Teil zurückgehalten wurden. Wir verwendeten daher im wesentlichen das schon von Wichtl *et al.*[1] vorgeschlagene Verfahren (Fällung der Ballaststoffe mit Bleiacetat, Ausschütteln der Cardenolide mit organischem Lösungsmittel) mit geringfügiger Modifikation. Vor der HPLC-Messung wurden die Proben durch Millipore-Filter gepresst, um eine Verlegung der Säulen zu vermeiden. Für die Aufbereitung der Proben war somit eine Reihe von Arbeitsschritten erforderlich, die eine gewisse Fehleranfälligkeit befürchten liessen. Um derartige Fehlerquellen auszuschliessen, versuchten wir einen inneren Standard zu finden, der schon bei der Drogenextraktion zugesetzt werden konnte. Die Digitalisglykoside Digoxin, α-Acetyldigoxin, Digitoxin und Lanatosid C erwiesen sich als ungeeignet, weil sie erst bei einer Steigerung des Acetonitrilanteiles in der mobilen Phase auf 60 % eluiert wurden, was die Analysenzeit nahezu verdoppelte. Cymarol, Cymarin, k-Strophanthin β, Erysimosol, α-Antiarin, Acovenosid A, Cheirosid A und Helveticosol überlappten mit einzelnen Convallariaglykosiden. Von allen geprüften Substanzen bildete nur Helveticosid eine Ausnahme: diese Verbindung war nahezu vollständig (4σ-Trennung) von Periplorhamnosid getrennt und bewirkte durch ihre günstige Lage im Chromatogramm auch keine Analysenverlängerung (Fig. 1).

Für die Eichung des Messsystems zogen wir die Hauptglykoside Lokundjosid, Convallatoxol, Convallatoxin, Desglucocheirotoxin und Convallosid heran, von wel-

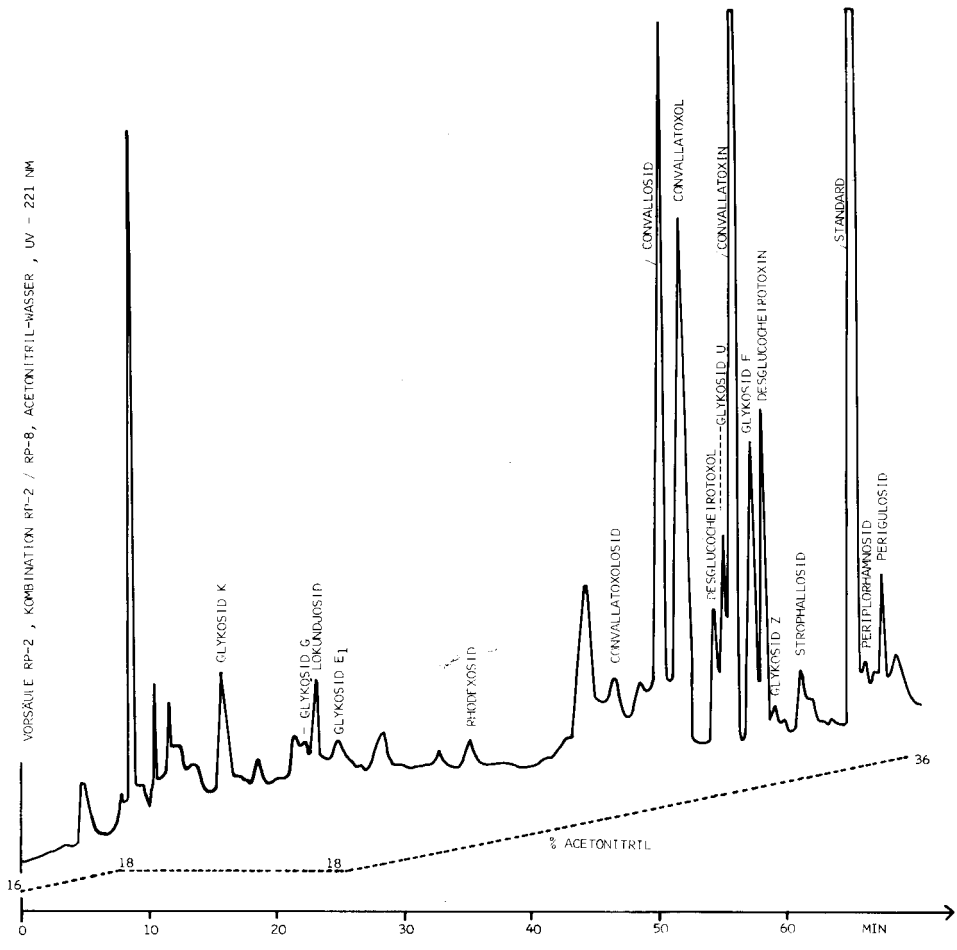

Fig. 1. HPLC eines vorgereinigten Drogenauszuges von *Convallaria majalis*. Säulen: LiChrosorb RP-2 (5 μm), 40 × 4.6 mm I.D. (Vorsäule), LiChrosorb RP-2 (10 μm), 250 × 4.6 mm I.D. und LiChrosorb RP-8 (7 μm), 250 × 4.6 mm I.D. in Serie geschaltet. Mobile Phase: Acetonitril–Wasser, Gradient 16:84 bis 18:82 mit 0.2%/min, nach 25 min mit 0.4%/min bis Ende der Analyse, Fluss: 1 ml/min. Temperatur: 22°C.

chen letzteres ein Diglykosid darstellt. Es ergaben sich in allen Fällen Eichgerade, die durch den Nullpunkt verliefen, womit die Korrekturfaktoren in der üblichen Weise[6] berechnet werden konnten. Als Beispiel ist in Fig. 2 die Eichgerade von Convallatoxin dargestellt.

Da die Korrekturfaktoren (F) der Monoglykoside (Lokundjosid $F = 1.08$; Convallatoxol $F = 1.12$; Convallatoxin $F = 1.16$; Desglucocheirotoxin $F = 1.10$) relativ geringe Unterschiede aufwiesen, schien es gerechtfertigt, bei den späteren Gehaltsbestimmungen von Drogen für die bei der Eichung nicht berücksichtigten Monoglykoside —diese liegen ja nur in geringer Menge vor— einen aus den oben angegebenen Faktoren gemittelten Wert ($F = 1.12$) heranzuziehen. Für die Ermittlung der Diglykoside verwendeten wir den Faktor von Convallosid ($F = 1.41$). Der gemittelte Korrekturfaktor der Monoglykoside verhält sich zum Faktor von Convallosid annä-

TABELLE I

QUANTITATIVE AUSWERTUNG VON PROBE 2

Anteil am Gesamtglykosidgehalt in %. Mw = Mittelwert.

Cardenolide	*1. Extraktion*			*2. Extraktion*			*3. Extraktion*			*Gesamt-mittelwert*
	*1**	*2**	*Mw*	*1**	*2**	*Mw*	*1**	*2**	*Mw*	
Glykosid K	1.85	1.87	1.86	2.44	2.46	2.45	2.17	2.18	2.17	2.16
Lokundjosid	11.53	11.10	11.32	10.72	11.57	11.15	8.88	10.22	9.55	10.67
Glykosid E_1	1.16	1.22	1.19	0.99	1.25	1.12	1.22	1.29	1.26	1.19
Rhodexin A	2.17	2.79	2.48	3.28	1.34	2.31	1.45	1.69	1.57	2.12
Convallatoxolosid	1.26	1.60	1.43	1.68	0.58	1.13	2.43	1.52	1.98	1.51
Convallosid	11.75	11.65	11.70	12.62	12.29	12.45	12.44	13.53	12.99	12.38
Convallatoxol	16.87	15.77	16.32	15.26	15.57	15.41	13.45	14.39	13.92	15.22
Desglucocheirotoxol	3.82	3.28	3.55	3.49	3.17	3.33	3.41	3.50	3.46	3.45
Convallatoxin	28.34	29.06	28.70	27.83	27.79	27.81	26.91	27.08	26.99	27.83
Glykosid F	4.72	4.99	4.85	5.05	5.57	5.31	6.36	6.24	6.30	5.49
Desglucocheirotoxin	14.37	14.66	14.52	13.67	15.19	14.43	14.90	13.80	14.35	14.43
Strophallosid	0.48	1.01	0.75	1.33	2.00	1.67	2.89	1.96	2.38	1.60
Periplorhamnosid	0.98	0.53	0.75	0.27	0.45	0.36	0.23	0.67	0.45	0.52
Perigulosid	0.71	0.45	0.58	0.18	0.29	0.24	1.43	0.40	0.92	0.58
Peripallosid	–	–	–	1.20	0.48	0.84	1.74	1.53	1.64	1.24
% Gesamt-glykosidgehalt	0.273	0.267		0.266	0.272		0.306	0.306		0.282

* Getrennte HPLC-Messung.

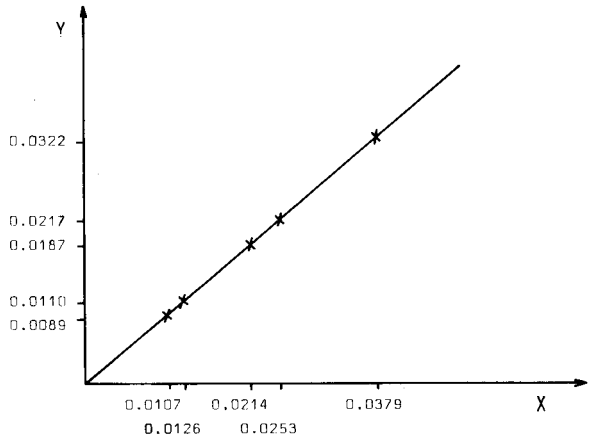

Fig. 2. Eichgerade von Convallatoxin. x = Einwaage Convallatoxin in mg; y = [Einwaage (mg Standard) × Peakfläche (Convallatoxin)]/Peakfläche (Standard).

hernd umgekehrt proportional wie die entsprechenden spezifischen Extinktionskoeffizienten, was die Richtigkeit der näherungsweisen Ermittlung der unbekannten Faktoren bestätigt.

Zur Überprüfung der Reproduzierbarkeit und Richtigkeit des neuen HPLC-Verfahrens wählten wir Convallaria-Proben mit unterschiedlichem Begleitstoffanteil, aber bekanntem Cardenolidgehalt (photometrische Messung nach Baljet-Reaktion[1]), um Auskunft über die Anwendbarkeit des Verfahrens bzw. über die Effizienz der Probenvorbereitung zu erhalten. Zunächst führten wir von den Convallaria-Mustern 2 und 4, deren Gesamtcardenolidgehalt im üblichen Bereich[1] lag, jeweils drei getrennte Extraktionen durch und chromatographierten jeden Auszug zweimal (Tabelle I und II).

Aus den Daten ermittelten wir sodann die absolute Standardabweichung[7] für die Einzelglykosidzusammensetzung (Tabelle III) und den Gesamtcardenolidgehalt. Letztere lag mit ± 0.024 in einem sehr günstigen Bereich, da sich die Werte durch Summierung einer grossen Anzahl von Einzeldaten ergeben.

Auch die in Tabelle III gezeigten absoluten Standardabweichungen der Einzelglykosidanteile liegen in einem für biologisches Material günstigen Bereich, wenn man berücksichtigt, dass es durch die relativ grosse Peakanzahl zu Flächenüberlappungen kommt. Die für die Hauptkomponenten gefundene maximale Abweichung von ± 1.08 spricht ebenfalls für die gute Reproduzierbarkeit des Verfahrens.

Um die allgemeine Anwendbarkeit des Verfahrens zu gewährleisten, wurden im weiteren auch die Proben 1, 3 und 5 analysiert, wobei sich ebenfalls keine Störungen durch Ballaststoffe ergaben. Die Ergebnisse sind in Tabelle IV zusammengestellt, die zusätzlich auch einen Vergleich mit den photometrisch ermittelten Gesamtcardenolidgehalten erlaubt. Unter Berücksichtigung der doch sehr unterschiedlichen Messtechniken stimmen die Daten ausgezeichnet überein, was als Beweis für die Präzision des neuen HPLC-Verfahrens gewertet werden kann, das sich hinsichtlich Zeitaufwand und Informationsgehalt den bisherigen Methoden überlegen zeigt.

Das neue HPLC-Verfahren scheint auch für die Standardisierung von Herba Convallariae geeignet. Wegen der in Tabelle IV aufgezeigten Variabilität der Einzel-

TABELLE II

QUANTITATIVE AUSWERTUNG VON PROBE 4

Anteil am Gesamtglykosidgehalt in %. Mw = Mittelwert.

Cardenolide	*1. Extraktion*			*2. Extraktion*			*3. Extraktion*			*Gesamt-mitelwert*
	*1**	*2**	*Mw*	*1**	*2**	*Mw*	*1**	*2**	*Mw*	
Lokundjosid	0.46	0.22	0.34	1.02	0.70	0.86	0.95	0.69	0.82	0.67
Rhodexosid	1.50	1.25	1.37	1.50	1.50	1.50	1.40	1.25	1.32	1.0
Convallatoxolosid	0.31	–	0.31	0.31	0.26	0.28	0.58	0.50	0.54	0.38
Convallosid	22.80	23.31	23.05	20.98	22.09	21.53	26.47	25.67	26.07	23.55
Convallatoxol	14.68	14.28	14.48	15.25	15.02	15.13	16.69	15.91	16.30	15.30
Glykosid ?	3.33	4.0	3.66	3.34	2.38	2.86	–	–	–	3.26
Desglucoheirotoxol	5.40	3.85	4.62	4.24	3.28	3.76	3.24	3.31	3.27	3.88
Convallatoxin	31.38	31.73	31.55	32.46	33.14	32.80	31.60	33.60	32.60	32.32
Glykosid F	7.56	7.35	7.45	7.85	7.75	7.80	7.15	7.46	7.30	7.52
Desglucocheirotoxin	9.11	9.20	9.15	9.94	9.83	9.88	8.85	8.94	8.89	9.31
Strophallosid	1.25	1.13	1.19	1.06	1.59	1.32	1.27	1.15	1.21	1.30
Periplorhamnosid	0.70	0.62	0.66	0.48	0.97	0.72	0.59	0.51	0.55	0.64
Perigulosid	1.39	2.86	2.12	1.55	1.35	1.45	1.20	1.0	1.10	1.56
Peripallosid	0.12	0.18	0.15	0.02	0.12	0.07	–	–	–	0.11
% Gesamt-glykosidgehalt	0.269	0.265		0.253	0.234		0.226	0.239		0.248

* Getrennte HPLC-Messung.

TABELLE III

ABSOLUTE STANDARDABWEICHUNGEN DER EINZELGLYKOSIDANTEILE DER PROBEN 2 UND 4, BERECHNET NACH LIT. 7

Glykosid K	±0.29	Convallatoxin	±0.77
Lokundjosid	±0.72	Glykosid F	±0.55
Glykosid E_1	±0.07	Desglucocheirotoxin	±0.37
Rhodexin A	±0.48	Strophallosid	±0.59
Rhodexosid	±0.29	Periplorhamnosid	±0.16
Convallatoxolosid	±0.32	Perigulosid	±0.53
Convallatoxol	±1.08	Peripallosid	±0.40
Desglucocheirotoxol	±0.33		

TABELLE IV

MITTELWERTE DER EINZEL- UND GESAMTCARDENOLIDGEHALTE DER UNTERSUCHTEN CONVALLARIA-PROBEN IM VERGLEICH ZUM PHOTOMETRISCH BESTIMMTEN GESAMTCARDENOLIDGEHALT

Anteil am Gesamtglykosidgehalt in %.

Droge	*1*	*2*	*3*	*4*	*5*
Glykosid K	2.92	2.16	1.23	–	3.15
Lokundjosid	11.73	10.67	6.51	0.67	9.78
Glykosid E_1	2.08	1.19	1.26	–	1.36
Rhodexosid	1.70	–	–	1.0	3.09
Rhodexin A	1.80	2.12	–	–	3.23
Convallatoxolosid	1.53	1.51	0.93	0.38	2.19
Convallosid	19.90	12.38	7.37	23.55	11.71
Convallatoxol	16.81	15.22	17.95	15.30	11.66
Desglucocheirotoxol	2.31	3.45	4.08	3.88	–
Convallatoxin	24.18	27.83	42.10	32.32	21.03
Glykosid F	1.95	5.49	–	7.52	6.45
Desglucocheirotoxin	7.56	14.43	15.81	9.31	15.13
Strophallosid	0.74	1.60	1.73	1.30	1.94
Periplorhamnosid	2.82	0.52	0.48	0.64	3.02
Perigulosid	0.74	0.58	0.29	1.56	3.72
Peripallosid	0.62	1.24	0.27	0.11	0.71
% Gesamtglykosidgehalt (HPLC)	0.215	0.282	0.407	0.248	0.191
% Gesamtglykosidgehalt (photom. Lit. 1)	0.244	0.324	0.461	0.235	0.216

glykosidanteile von Haupt- und Nebenkomponenten müssten zu diesem Zweck die Wirkwerte aller erfassbaren Herzglykoside bekannt sein, weshalb es notwendig erscheint, die Ergebnisse der pharmakologischen Prüfung der in geringerer Menge vorkommenden Convallaria-Cardenolide abzuwarten.

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CHROM. 14,706

Note

Reversed-phase high-performance liquid chromatography of some irritants

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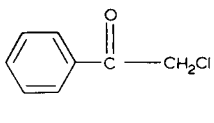
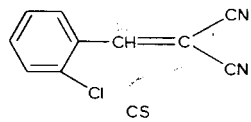
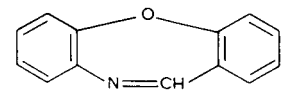
We report here the reversed-phase liquid chromatography of three commonly used irritants, *viz.*, ω-chloroacetophenone (CN), *o*-chlorobenzylidene malononitrile (CS) and dibenz[*b*,*f*]-1:4-oxazepine (CR).



CN



CS



CR

CN and CS have been used by military and police establishments for over two decades; CR is reported to be better than CN and CS in view of its mode of delivery and sustained irritant and lachrymatory action associated with low toxicity[1]. Sass *et al.*[2] reported a gas–liquid chromatographic (GLC) method of analysis of CN and CS. Blood levels of CS in animals exposed to aerosol sprays were determined by GLC by Leadbeater[3]. The same group[4] reported the detection and measurement in blood of CS H_2, a metabolite of CS, formed by the reduction of the olefinic bond in CS. No method for the determination of CR has previously been reported, and a high-performance liquid chromatographic (HPLC) procedure is described here.

EXPERIMENTAL

Apparatus

A Waters Model ALC-GPC-244 high-performance liquid chromatograph consisting of two Model 6000A pumps, a U6K injector, a Model 660 solvent programmer and a Model 440 absorbance detector was used. The separations were carried out with a μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.). Detection was carried out at 254, 280 and 313 nm.

Chemicals

Methanol and acetonitrile were of analytical reagent grade. Water, triply distilled in an all-glass distillation apparatus, was degassed by refluxing for 1 h and filtered over a 0.45-μm Millipore filter before use. CN was recrystallized from methanol. CS was synthesized by Knoevenagel condensation of *o*-chlorobenzaldehyde and

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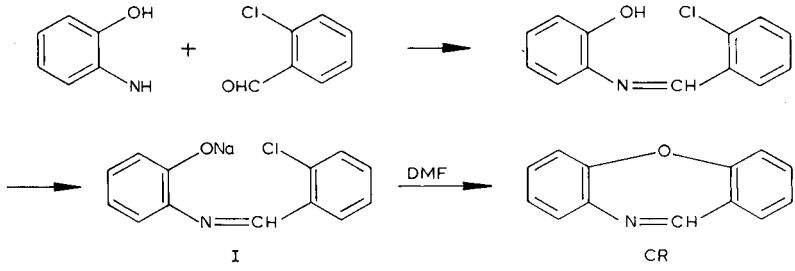
malononitrile with alcoholic potassium hydroxide as catalyst and recrystallized from cyclohexane. CR was synthesized by a modification of the Higginsbottom and Suschitzky procedure[5] by condensing *o*-aminophenol with *o*-chlorobenzaldehyde. The sodium salt of the Schiff's base (I) so formed was cyclized to CR by refluxing in dimethylformamide (DMF):



I CR

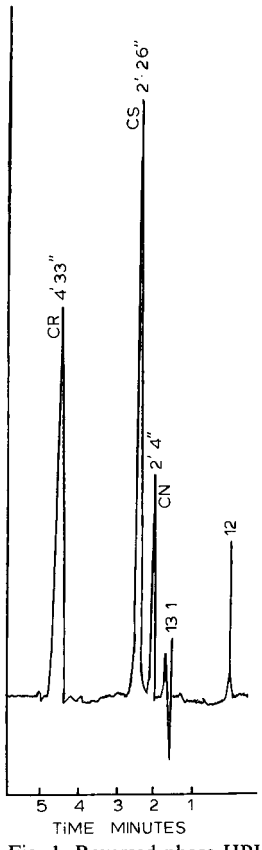


Fig. 1. Reversed-phase HPLC of CN, CS and CR. Column, μBondapak C_{18}; mobile phase, methanol–water (7:3); flow-rate, 2 ml/min; detection, 280 nm; 0.01 a.u.f.s.; chart speed, 1 cm/min; amounts, CN 40 ng, CS 24 ng, CR 30 ng.

The purities of the three compounds were established by spectroscopic methods, thin-layer chromatography and GLC.

Procedure

The mobile phase was methanol–water (7:3) mixed with a solvent programmer. Each solvent was pre-mixed with 10% of the other before use, as CR appeared to be unstable in methanol and water mixed as such, without pre-mixing. Acetonitrile was used for preparing solutions of all three compounds as CR tends to decompose in methanol. The flow-rate was maintained at 2 ml/min.

RESULTS

CN, CS and CR absorb at 254, 280 and 313 nm to different extents. Baseline separation of these compounds at 280 nm in the 10–100-ng range is shown in Fig. 1. Trace-level detection in the range 1–10 ng was carried out at 254 nm for CN and 313 nm for CS and CR. The latter two compounds absorb at 280 nm also, with the peaks of CR being larger than those at 313 nm for the same concentration and at the same sensitivity, *viz.*, 0.005 a.u.f.s. In spite of this, a calibration plot for CR shows better linearity at 313 than at 280 nm (see Fig. 2.). Calibration graphs for the three compounds are shown in Fig. 2 and are linear in the concentration range referred to.

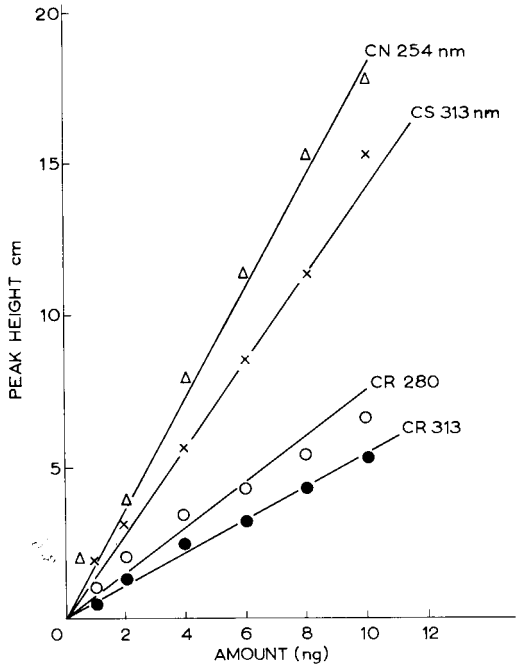


Fig. 2. Calibration graphs for CN (△), CS (×) and CR (○) at 280 nm and CR (●) at 313 nm.

The HPLC method reported here can serve as an adjunct to the GC electron-capture methods of Sass *et al.* and Leadbeater owing to its linearity in the 1–10-ng range, which is essential for quantitative determinations.

ACKNOWLEDGEMENTS

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Note

Separation of atrazine and some of its degradation products by high-performance liquid chromatography

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Environmental studies on the fate of herbicides in soils have raised much interest in recent years[1–3]. Atrazine is one of the *s*-triazine group of herbicides which are currently being used world-wide[4]. Uncontrolled factors such as rainfall and temperature affect the atrazine degradation rate in certain soils[1], necessitating the measurement of residual levels before rotational crops can be planted. High-performance liquid chromatography (HPLC) is eminently suitable for monitoring the fate of small amounts of such herbicides and their degradation products, especially in complex mixtures such as soil extracts[5,6]. Often no elaborate pretreatment[7] or derivatization is necessary as for gas chromatography (GC)[1,8]. The HPLC behaviour of atrazine has been studied on amino[9], cyano[10] and reversed-phase (C_{18})[11] columns. Atrazine and its derivatized degradation products have also been studied by GC[1]. This paper describes the determination of atrazine and its underivatized degradation products by reversed-phase (C_8) HPLC.

MATERIALS AND METHODS

Atrazine and its degradation products were kindly donated by Ciba Geigy (Johannesburg, South Africa). Methanol, acetic acid and ammonium acetate of the highest quality were obtained from Merck (South Africa), while water was obtained from a Millipore Milli-Q system. The compounds investigated are listed in Table I and were injected individually as 100 ppm solutions, or as a mixture containing 10 ppm of each, with a 20-μl sample loop.

An HPLC system comprising a Beckman Model 322 Gradient Liquid Chromatograph with a fixed (254 nm) wavelength detector was used. Ultrasphere octyl (25 × 0.4 cm) columns were employed at ambient temperature and a constant flow-rate of 1 ml/min. Investigations at 220 nm were performed on a Spectra Physics SP 8000 B liquid chromatograph equipped with a Model SP 8400 UV–visible variable-wavelength detector using the same operating conditions as above. The water and metha-

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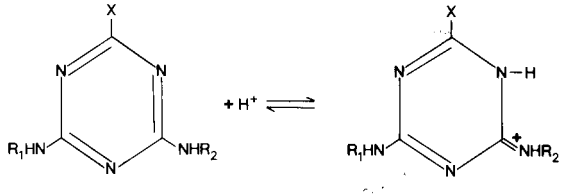
nol solvents were either neat or fortified with 1 % acetic acid or 50 m*M* of ammonium acetate. When ammonium acetate was used the pH was adjusted to 7.4; for the methanol phase, acetic acid, and for the water phase, ammonium hydroxide, was used to adjust the pH.

The pK_a values of the *s*-triazine compounds were determined spectrophotometrically[12] on a Beckman Spectrophotometer Acta Model MVI and are listed in Table I. The absorption spectra of the atrazine derivatives were determined in methanol at a concentration of 6.25 μg/ml and are shown in Fig. 5.

A sandy loamy soil obtained from the University of Pretoria's experimental farm was spiked with 5 ppm of atrazine and each of its degradation products. The spiked soil samples were extracted with a 10 % water–acetonitrile solution[5,7] and the extract filtered through a Whatman No. 1 filter-paper. Three ml of the filtrate were evaporated in a stream of nitrogen. The residue was dissolved in 3 ml methanol, filtered through a 0.45-μm Millipore filter and injected (20 μl) as such without further purification.

RESULTS AND DISCUSSION

Initial studies on the separation of atrazine and its degradation products by reversed-phase HPLC indicated that a column with a C_8 bonded stationary phase should be used in preference to a C_{18} bonded stationary phase, since some of the derivatives were too polar to be retained on the latter column. Furthermore, these initial studies also revealed that tailing of the hydroxyatrazines could be improved by addition of 1 % acetic acid to both solvents. This problem could also be completely eliminated by addition of 50 mmol ammonium acetate per litre of each solvent and adjusting the pH to 7.4. This could be expected since *s*-triazines are weakly basic polar compounds which dissociate in aqueous solutions according to eqn. 1:



(1)

if X = Cl pKa ≃ 2 $R_1 = -CH_2-CH_3$

if X = OH $pKa_1 \simeq 5$ $R_2 = -CH(CH_3)_2$

Thus the separation of atrazine and its derivatives should be influenced by a change in pH. The principle of suppressing or enhancing ionization to improve tailing of peaks is well established[13,14]. From the dissociation constant (pK_a) values in Table I it can be seen that these compounds can be divided into two groups, namely the chloro- and hydroxyatrazines, with pK_a about 2 and 5, respectively.

The dissociation constant for the hydroxy group on the hydroxyatrazines is about 11. This group would therefore remain undissociated in the pH range of 2–8, wherein silica-based columns can be used. The dependence of k' of these derivatives on solvent strength of the mobile phase, containing water–methanol, both of which

TABLE I

STRUCTURES OF THE DEGRADATION PRODUCTS OF ATRAZINE USED

Name	*Abbreviation*	pK_a	R_1	R_2	*X*
Atrazine	ATRZ	1.71*	C_2H_5	$CH(CH_3)_2$	Cl
Deethylatrazine	DEA	1.65	H	$CH(CH_3)_2$	Cl
Deisopropylatrazine	DIA	1.58	C_2H_5	H	Cl
Hydroxyatrazine	HA	5.15**	C_2H_5	$CH(CH_3)_2$	OH
Deethylhydroxyatrazine	DEHA	4.57	H	$CH(CH_3)_2$	OH
Deisopropylhydroxyatrazine	DIHA	4.65	C_2H_5	H	OH

* Reported as 1.85 and 1.68 in ref. 12 and 16, respectively.

** A pK_a of 5.20 has been reported for 2-hydroxy-4,6-bis(isopropylamino)-*s*-triazine in ref. 12.

contain 1% acetic acid, is shown in Fig. 1. The pH of these mixtures varies from 3.0 to 3.6 increasing with methanol concentration, as expected from the dependence of pK_a of weak acids on the dielectric constant of solvents. At these pH values the chloroatrazines should be neutral while the hydroxyatrazines should be protonated. The retention times of the neutral chloroatrazines decrease with increasing solvent strength. However, the retention times of the hydroxyatrazines increased with increasing solvent strength. These anomalous results can possibly be explained in terms of solubility effects. It is known that hydroxy-*s*-triazines are relatively more soluble in aqueous solutions of pH 3 rather than neutral pH as they

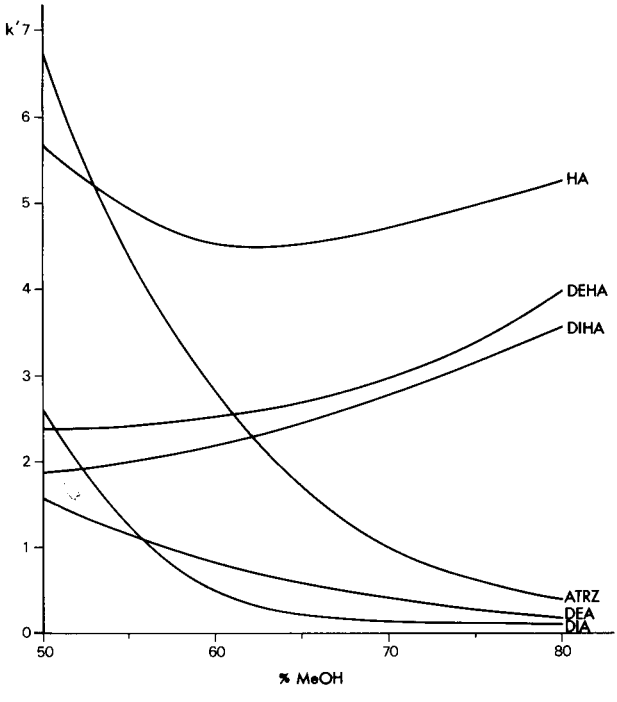


Fig. 1. Dependence of k' on the % methanol (MeOH) in the mobile phase. Both the methanol and water reservoirs contained 1% acetic acid, and the pH was 3.0–3.6.

are protonated at pH 3 (ref. 15). Reduction of the polarity of the solvent (increasing methanol concentration) should decrease their solubility.

Notwithstanding the fact that these molecules are positively charged, they are still retained on the column, as shown in Fig. 1. The differences in retention times between the hydroxyatrazine derivatives indicate that typical reversed-phase forces are probably operative, through the difference in their alkyl groups. That is, the retention times increase in the order of DIHA < DEHA < HA with two, three and five alkyl carbons respectively.

It was then decided to develop a solvent system where all the atrazine derivatives were present in the unprotonated form. This was achieved by adding 50 mmol of ammonium acetate to each litre of the two solvents and adjusting the pH to 7.4. The results of these investigations are shown in Fig. 2. Satisfactory separation of all these derivatives can be obtained with methanol–water (40:60), with ammonium acetate at pH 7.4, as shown in Fig. 3. The separation can be increased if necessary by decreasing the percentage of methanol.

The sensitivity of this method depends on the molar absorptivity of the compounds at a particular wavelength. The detection limits for atrazine at 220 and 254 nm were about 0.1 and 1 ppm respectively. From the spectra shown in Fig. 5, it can be seen that the detection limit of the other compounds will be of the same order. We were able to extract and determine atrazine and its degradation products from a soil sample which had been spiked with 5 ppm of each of these compounds, as shown in Fig. 4.

The linearity of the detector had been confirmed in the 1–10 ppm range for all

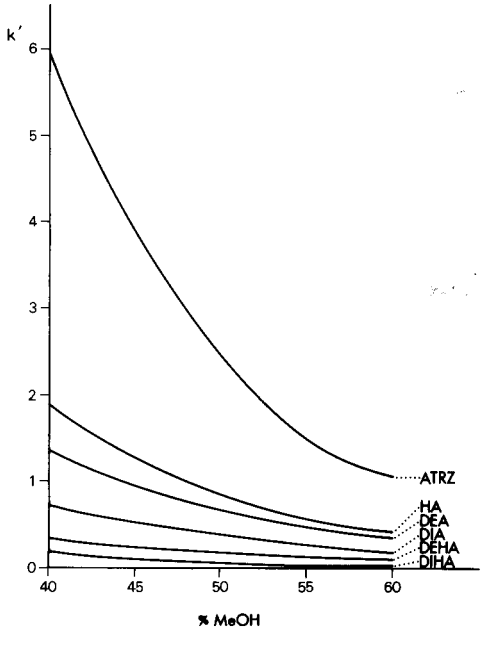
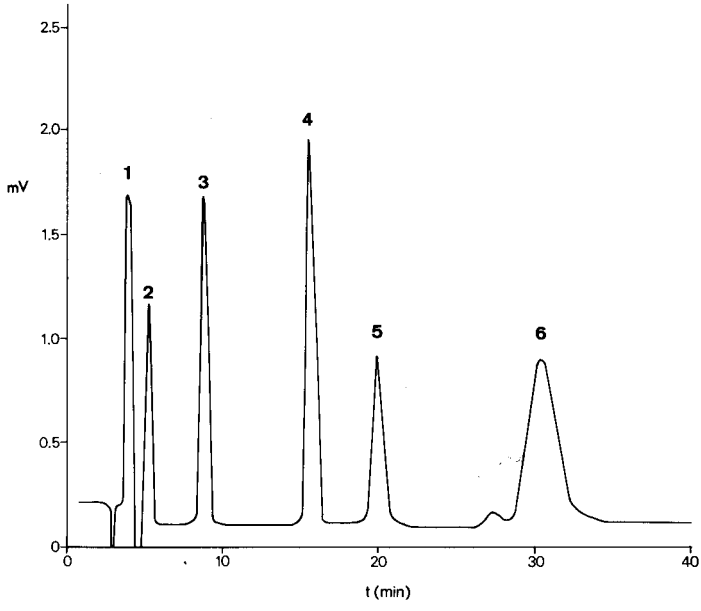


Fig. 2. Dependence of k' on the % methanol in the mobile phase. Both the methanol and water reservoirs contained 50 mM ammonium acetate and the pH was adjusted to 7.4.



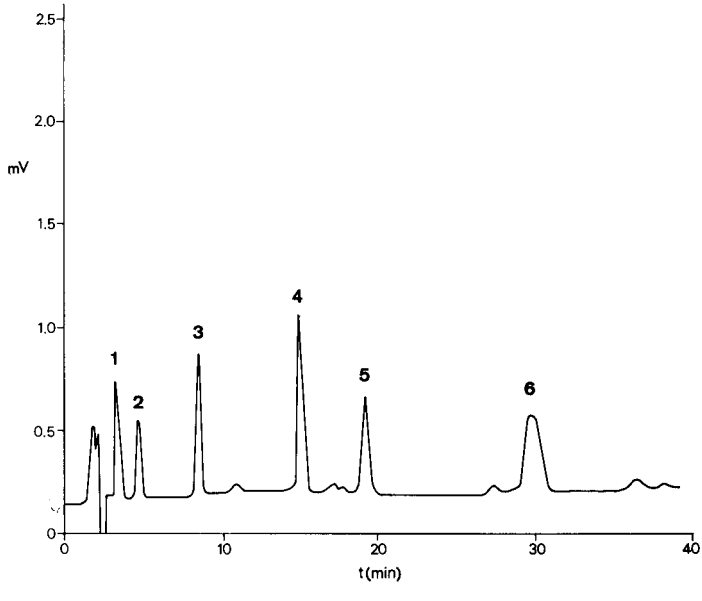


Fig. 3. Separation of atrazine and its degradation products on Ultrasphere octyl (C_8) stationary phase. Mobile phase: methanol–water (40:60) both solvents with 50 m*M* ammonium acetate at pH 7.4. Flow-rate: 1.0 ml/min. Pressure: 2000 p.s.i. Detection at 220 nm, each compound 10 ppm. Peaks: 1 = DIHA; 2 = DEHA; 3 = DIA; 4 = DEA; 5 = DHA; 6 = ATRZ.

Fig. 4. Separation of atrazine and its degradation products extracted from a soil sample which had been spiked with 5 ppm of each. Chromatographic conditions as in Fig. 3.

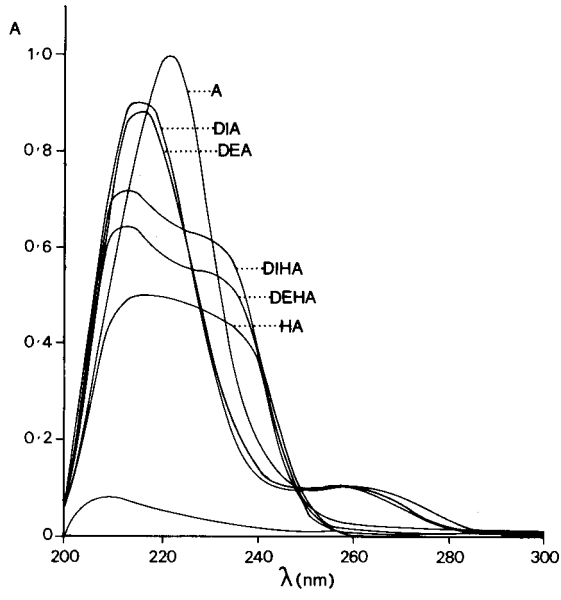


Fig. 5. Absorption spectra of atrazine (A) and its degradation products, 62.5 μg derivative per 10 ml methanol.

the derivatives. The recoveries of the spiked soil samples were 78.4 ± 4.2% for the atrazines and 72.5 ± 4.8% for the hydroxyatrazines. However, we have found that these recoveries vary with soil type.

CONCLUSION

This method can be used to measure residual amounts of atrazine and its degradation products in soil samples. It should be possible to measure most *s*-triazines and their degradation products in soil samples, as well as in plant and animal tissues, with slight modifications.

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Note

Analysis of ethylenethiourea in beer by high-performance liquid chromatography

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Ethylenebis(dithiocarbamates) including Maneb, Zineb and Mancozeb form an important class of fungicides for controlling crop diseases including those of hops. Ethylenethiourea (ETU) can be produced as a degradation product of these fungicides and in view of its toxicity there is a need for analytical methods to check residues which may arise in treated crops. Current methods include thin-layer chromatography (TLC)[1] and gas–liquid chromatography (GLC)[1,2,3] but these require derivatization and also extensive clean-up procedures particularly for samples of plant origin. High-performance liquid chromatography (HPLC) has considerable potential in trace organic analysis of biological samples and has very recently been used in the ETU analysis of rat plasma[4] and urine[5]. This note illustrates the use of HPLC in the analysis of ETU in beer and also the application of column-switching to resolve the analyte from coeluting matrix components.

EXPERIMENTAL

High-performance liquid chromatography

Two independent isocratic HPLC systems linked by a 4-way switching valve were used for analysis. System 1 comprised a Waters 6000A solvent delivery pump linked in series to a Rheodyne loop injector (20-μl loop), two Spherisorb CN 5 μm columns (25 cm × 5 mm I.D.), a Rheodyne 4-way switching valve and a waste reservoir. System 2 comprised a second Waters 6000A pump linked in series to the 4-way switching valve, a Spherisorb NH_2 5-μm column (25 cm × 5 mm I.D.) and a Pye LC3 UV detector operating at 240 nm. A mobile phase of hexane–ethanol (2:1), flow-rate 1.0 ml/min, was used for both systems.

The beer extract (20 μl) was injected onto the first Spherisorb CN column of system 1 and the eluent passed to waste via the switching valve. After 15.0 min the eluent from system 1 was switched to the Spherisorb NH_2 column of system 2 for 1 min and then switched back to waste again. Under these conditions ETU eluted from the Spherisorb NH_2 column 25.3 min after the initial injection.

Beer extraction procedure

Ethanol (1 ml) was added to 5.0 g beer and the sample quantitatively transfer-

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red to the top of a sintered glass column (25 × 2.5 cm I.D.) containing 50 g anhydrous sodium sulphate. After absorption of the sample (*ca.* 1 min) the column was eluted with 3 × 20 ml dichloromethane–methanol (99:1) and the extract concentrated to 2 ml on a water bath at 60°C using a Kuderna-Danish concentrator. The concentrate was diluted with 8 ml dichloromethane and passed through a silica gel Sep-Pak and the eluent discarded. ETU was eluted from the Sep-Pak with 10 ml dichloromethane–methanol (98:2) and the eluent concentrated to 300 μl at 60°C.

RESULTS AND DISCUSSION

A typical HPLC chromatogram of a beer extract using two 5-μm bonded-cyano columns linked in series to a UV detector operating at 240 nm is shown in Fig. 1. Under these conditions a peak corresponding to 200 μg/kg ETU eluted after 15.6 min. However, reanalysis of the sample at 250 nm showed the absorbance ratio of this peak (250 nm:240 nm = 1:0.5) to be quite dissimilar to that of standard ETU (250 nm:240 nm = 1:1.3) indicating the presence of coeluting beer components. When the fraction containing this peak was switched to a bonded-amino column (as described in the Experimental section) no peaks eluting with the same overall retention time as ETU (25.3 min) were observed subject to a detection limit of 10 μg/kg. The column switching HPLC chromatogram of a different beer sample at 240 nm is shown in Fig. 2. The three major components were well resolved by the bonded-amino column with

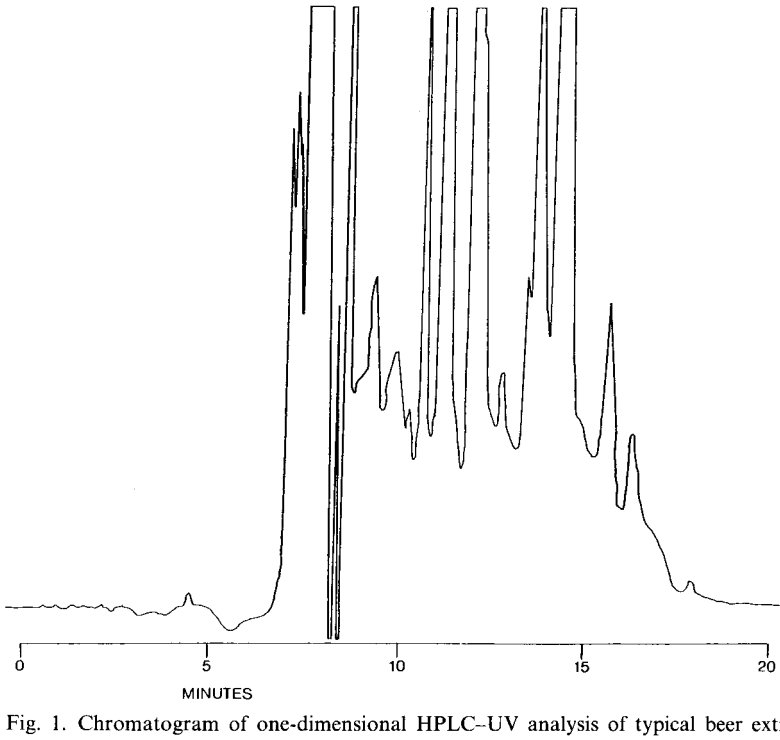

Fig. 1. Chromatogram of one-dimensional HPLC–UV analysis of typical beer extract; two 5-μm CN columns in series; detection at 240 nm, 0.08 a.u.f.s. (retention time of standard ETU 15.6 min).

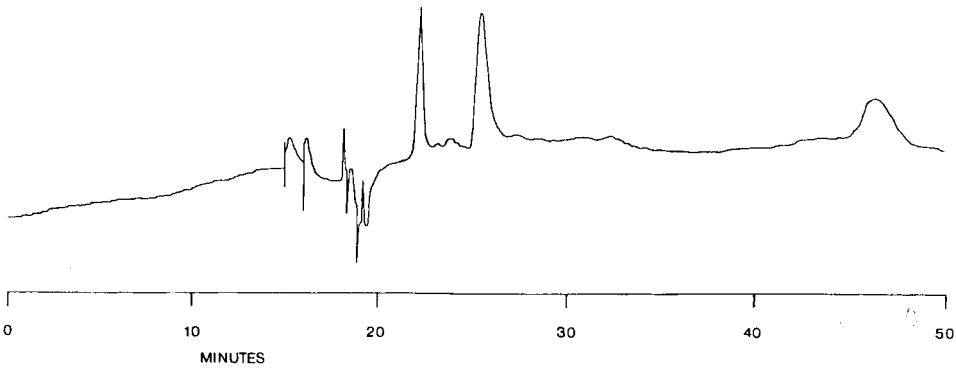

Fig. 2. Chromatogram of column switching HPLC–UV analysis of beer extract; two 5-μm CN columns and one 5-μm NH_2 column; detection at 240 nm, 0.01 a.u.f.s. (retention time of standard ETU 25.3 min).

the peak at 25.3 min corresponding to 370 μg/kg ETU. Reanalysis of the sample at 250 nm showed this peak to have the same absorbance ratio as standard ETU.

Using the extraction procedure described in the experimental section the recovery of ETU added to beer at 20 μg/kg and 600 μg/kg averaged 62.4% (standard deviation 4.9, 5 determinations) and 75% (standard deviation 1.9, 5 determinations) respectively with a detection limit of 10 μg/kg.

Conventional one-dimensional HPLC has been found to give spuriously high results in the analysis of ETU in beer due to the presence of coeluting matrix components. However, the more powerful resolving ability of column switching HPLC using polar-bonded columns of differing selectivities has proved highly effective in separating ETU from these coeluting materials. Additionally the procedure of monitoring the eluent at two different wavelengths has been found to be a valuable confirmatory technique.

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Note

Rapid determination of sugars in cantaloupe melon juice by high-performance liquid chromatography

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(Received January 15th, 1982)

Recent developments promise to make high-performance liquid chromatography (HPLC) a convenient method for analysis of sugars in crude extracts of plant tissues. One such development is the use of silica columns with an aqueous acetonitrile mobile phase to which an amine modifier has been added[1]. This technique avoids the use of chemically bonded amine columns, which are expensive and deteriorate with use, especially when crude extracts containing non-sugars are analysed[1,2]. The use of tetraethylenepentamine (TEPA) as an amine modifier was proposed by Wheals and White after an extensive survey[2], and a recent study on the use of TEPA with radially compressed silica shows that high resolution and good column life can be readily obtained with this amine[3].

Another development is the use of water as a mobile phase for sugar separations, in conjunction with cation-exchange[4] or reversed-phase C_{18} columns[5]. The risk of components in a crude aqueous sample matrix precipitating upon introduction to the mobile phase is avoided where water is the eluent.

In the course of a study on the accumulation of sugars in the developing cantaloupe melon fruit (*Cucumis melo* L. var *reticulatus* Naud.), we have used two HPLC methods for sugar determination. The first method uses a proprietary stationary phase with water as eluent, and the second method uses radially compressed silica as the stationary phase, and an aqueous acetonitrile mobile phase containing the amine modifier TEPA. This paper describes these methods, which require minimal sample preparation, and permit rapid analysis of each sugar constituent.

EXPERIMENTAL

Sample preparation

Cantaloupe melons were harvested at several stages of maturity and juice recovered from the edible flesh with a domestic juice extractor. Sodium azide (0.1 %, w/v) was added as a preservative, and suspended solids were removed by centrifu-

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gation (2000 *g*, 5 min). The supernatant was filtered prior to analysis by drawing it through a 2-μm porosity filter (Supelco, Bellefonte, PA, U.S.A.), fitted onto the polypropylene tip of an automatic pipettor. In one experiment, juice was extracted in the presence of boiling 80% (v/v) ethanol, followed by centrifugation and removal of ethanol at 40°C *in vacuo*.

Reagents

Water for elution was distilled, and then passed through a Millipore Milli-Q purifier (Bedford, MA, U.S.A.). Acetonitrile and methanol were of HPLC grade (Waters Assoc., Milford, MA, U.S.A.). TEPA (technical grade) was supplied by Waters Assoc., Chippendale, Australia. Eluents were degassed before use by vacuum filtration through a 0.5-μm membrane filter. Glucose, fructose, sucrose, ethanol, and sodium azide were of analytical reagent grade.

Apparatus

A Waters liquid chromatograph was used, which consisted of an M45 pump, U6K injector, RCM-100 radial compression module, and R401 differential refractometer connected to a 10-mV potentiometric recorder. The columns were Waters Radial-Pak cartridges of Dextropak and silica (both 100 × 8 mm I.D.).

Operating conditions

The Dextropak column was washed with 30 ml of methanol before each use, and then equilibrated with water. This took about 45 min at a flow-rate of 2 ml/min.

The silica column was initially pretreated with 500 ml of a solution of 75% (v/v) aqueous acetonitrile containing 0.1% TEPA. A mobile phase of 75% (v/v) aqueous acetonitrile containing 0.01% (v/v) TEPA was prepared, and 250 ml of this solution was pumped through the column to waste before each use. The effluent was then placed in the eluent supply reservoir, which was stirred continuously, and this solvent was recirculated overnight at 2 ml/min. Recirculation was maintained during the subsequent analysis. This procedure improved resolution and baseline stability, and conserved solvent.

RESULTS AND DISCUSSION

The separation of melon juice sugars on the Dextropak column with water as eluent is shown in Fig. 1. Salts and acids eluted first as a single peak (a). Glucose and fructose eluted next as a single peak (b), with a retention time of 1.5 min, followed by sucrose (c), with a retention time of 1.9 min. Identification of sugars was based on their retention times relative to standards. Juice from immature melons contained mostly monosaccharides (Fig. 1A), whereas sucrose was the major component in juice from mature melons (Fig. 1B).

When another sample of juice from a mature melon was fractionated on the silica column with aqueous acetonitrile containing TEPA as eluent, fructose (b), glucose (c), and sucrose (d) were resolved with retention times of 2.2, 2.7 and 4.4 min, respectively (Fig. 2). Two unknown peaks (e, f) were found in this separation. Baseline stability using 0.01% (v/v) TEPA was excellent (Fig. 2). Hendrix *et al.*[3] concluded, however, that the baseline could be improved by increasing the concentration of TEPA to 0.02% (v/v).

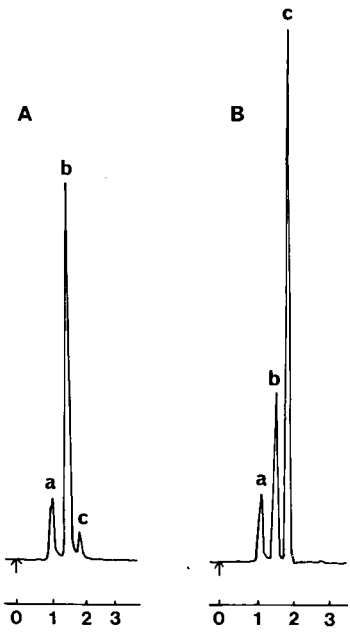

Fig. 1. Separation of juice extracts from (A) immature cantaloupes and (B) mature cantaloupes. Salts and acids (a), total monosaccharides (b) and sucrose (c). Column: Dextropak; eluent: water. Sample size, 10 μl. Flow-rate, 2 ml/min. Detector attenuation, $\times 32$.

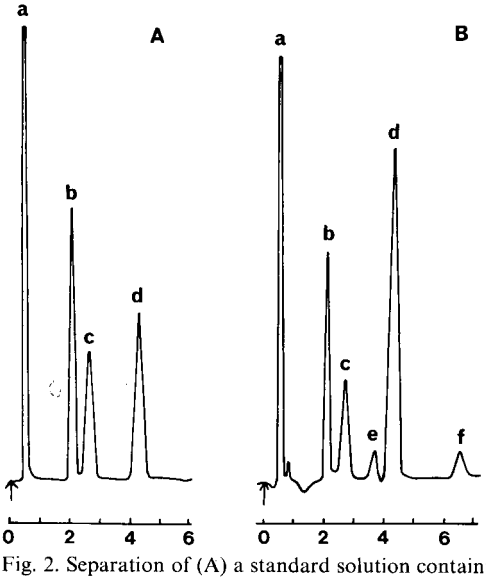

Fig. 2. Separation of (A) a standard solution containing fructose, glucose, and sucrose (each at 2%, w/v) and (B) a juice extract from a mature cantaloupe. Solvent peak (a), fructose (b), glucose (c), and sucrose (d). Peaks (e) and (f) are unknowns. Column: Radial-Pak silica; eluent: acetonitrile–water (75:25), 0.01% (v/v) TEPA. Sample size, 15 μl. Flow-rate, 3 ml/min. Detector attenuation, $\times 16$.

Carbohydrates in plant tissue extracts are subject to enzymic degradation[6]. Sucrose in particular is liable to undergo hydrolysis, so the stability of sucrose in melon juice was examined. The recovery of sucrose from freshly prepared juice was 102 ± 1% relative to the recovery from a boiling 80% (v/v) ethanol extract. Since 80% ethanol inactivates sugar-degrading enzymes[6], this result is evidence that sucrose was not being lost during juice preparation.

In a second test, freshly prepared juice was incubated for 24 h with and without added sucrose and added azide. Added sucrose was recovered quantitatively immediately after addition (Table I). After incubation for 24 h, only about 95% of the sucrose originally present could be recovered from the samples without azide. Addition of sodium azide (0.1%, w/v), which is an antimicrobial preservative, prevented this loss of sucrose, and did not interfere with the separation. The analysis of crude melon juice samples is, therefore, valid.

TABLE I

EFFECT OF AZIDE ON STABILITY OF SUCROSE IN JUICE

Juice with or without added azide or sucrose was analysed by the HPLC method of Fig. 1 immediately after expression, or after incubation at 30°C for 24 h.

Additives		*Sucrose recovered (%, w/v)*	
Azide (0.1%, w/v)	*Sucrose (0.5%, w/v)*	*0 h*	*24 h*
−	−	3.6 ± 0.05	3.4 ± 0.03
+	−	3.6 ± 0.06	3.6 ± 0.06
−	+	4.1 ± 0.06	3.9 ± 0.05
+	+	4.1 ± 0.04	4.1 ± 0.05

Using the methods described, we have been able to make several hundred injections onto each column. Frequent cleaning of pre-column filters, and back-flushing of the columns is, however, necessary, and the columns ultimately fail from particulate blockage. Where maximum column life is sought, samples should be freed of high-molecular-weight material by methods such as ethanol precipitation or ultrafiltration, and a guard column should be placed in the system.

A feature of both methods is the speed of analysis, which compares very favourably with other published procedures. The need for minimal sample preparation is a further advantage. The Dextropak column gives the same analytical data as two colorimetric reducing sugar assays performed before and after inversion (*i.e.* total reducing sugars and sucrose). The amine-treated silica column gives additional data which, if obtained by colorimetry or enzyme assay, would require specific assays for both glucose and fructose. Near-baseline resolution of glucose and fructose can be obtained routinely within 3 min. Both columns are robust and relatively economical to use. The use of water or of recirculated aqueous acetonitrile as mobile phases also reduces the cost of each assay.

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Note

Selective and highly sensitive spray reagent for detection of nanomolar quantities of carbodiimides on thin-layer plates

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In the course of our investigations we required several peptides, which had to be absolutely uncontaminated by the carbodiimides employed during synthesis. In order to follow the complete disappearance of carbodiimides during the reaction, a selective and highly sensitive spray reagent had to be developed, since no suitable assay for carbodiimides on thin-layer chromatographic (TLC) plates had previously been reported. Such an assay could be of general interest since, upon charring of silica plates, carbodiimides leave no traces, and remain undetected when reaction mixtures are assayed.

Recently the reaction between carbodiimides, pyridine and barbituric acid or N,N′-dimethylbarbituric acid was employed for the determination of carbodiimides in solution[1]. We found that the same reaction, which results in the formation of an intensely coloured purple dye ($\varepsilon \approx 150{,}000$ l mol^{-1} cm^{-1}), can also be used for the detection of carbodiimides on TLC plates. Screening for interfering substances showed that urea forms derivatives, like dicyclohexylurea, imidocarbonates, carbamates, isourea derivatives and α-amino acids, which are totally unreactive. Carbodiimides[1], cyanates (R–OCN)[2], triazine derivatives[2] and cyanogen halides[3,4] seem to be the only compounds which react with the pyridine–barbituric acid reagent. Of these compounds only the carbodiimides are commonly used in peptide synthesis. On TLC silica gel sheets the reaction is highly sensitive. Using dicyclohexylcarbodiimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as models for water-insoluble and water-soluble carbodiimides respectively, the formation of clearly visible purple spots allowed the detection of as little as 1 nmol of carbodiimide (≈ 0.2 μg). These spots exhibited a strong cherry-red fluorescence, and remained visible for several hours up to several weeks. Unfortunately they are unstable for longer periods. In terms of sensitivity, the colour reaction is comparable to assay methods based on radioactively labelled carbodiimides.

EXPERIMENTAL

Spray reagent was prepared by dissolving 500 mg, N,N′-dimethylbarbituric acid in 10 ml pyridine–water (9:1). The TLC plate was spayed intensively until transparency or dipped briefly into the reagent. Without drying or heating, the moistened plate was kept in a closed petri dish for 5–10 min. The appearance of blue or purple

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spots indicates the presence of as little as 1 nmol of carbodiimide. The background remains absolutely colourless.

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Note

Steroids and related studies

LVI. Thin-layer chromatography of some azasteroids

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Previously we have carried out thin-layer chromatographic (TLC) studies with some steroidal ketones[1,2], oximes[1,2], amides[2], lactams[1–3], tetrazoles[2,3] and basic and quaternary azasteroids[3]. In this paper we report the results of chromatographic studies on different azasteroids, which include secondary and tertiary bases, hydrochloride salts of certain tertiary bases and mono- and bisquaternary iodides.

EXPERIMENTAL

Azasteroids

Several of the basic and quaternary azasteroids were prepared in our laboratory. Appropriate references to the reported methods of preparation are given in the tables.

Adsorbent and TLC plates

Silica gel G (E. Merck, Darmstadt, G.F.R.) was mixed with distilled water (30 g in 60 ml of water) and coated on 20 × 20 cm plates to a thickness of 0.25 mm. The plates were air-dried for 15 min, heated at 110°C for 1 h and then stored in a cabinet over calcium chloride.

The running distance was 16 cm at a temperature of 25–30°C, and the amount of an azasteroid applied was 50–100 μg.

Detection

Cerium(IV) sulphate solution (2 g in 100 ml of 10% sulphuric acid) was used as the spray reagent, followed by heating at 150°C for 30 min, which gave permanent black spots. Exposure to iodine vapour was also used and gave brown spots in 2–4 min.

Solvents

All of the solvents employed were of analytical reagent grade and were used without further treatment; the strong ammonia solution used was 30% (w/w). The following solvent systems were tried:

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(1) methanol–strong ammonia solution (9:1)
(2) ethanol–strong ammonia solution (9:1)
(3) ethyl acetate–1-propanol–strong ammonia solution (40:30:3)
(4) 1-butanol–ethanol–strong ammonia solution (6:3:1)
(5) ethanol–chloroform–ethyl acetate–water (4:2:2:1)
(6) methanol–strong ammonia solution (1:1)
(7) 1-butanol–acetic acid–water (5:4:3)
(8) ethanol–ethyl acetate–chloroform–water–concentrated hydrochloric acid (120:48:32:20:1)
(9) ethanol–concentrated hydrochloric acid–chloroform–ethyl acetate–water (20:12:10:10:5)

TABLE I

THIN-LAYER CHROMATOGRAPHY OF SOME BASIC AZASTEROIDS IN SOLVENT SYSTEMS 1-4

Compound	*R_F value*			
	1	*2*	*3*	*4*
4-(2-Hydroxyethyl)-4-aza-5α-cholestane[4]	0.76	0.72	0.75	0.74
3-Pyrrolidino-17a-aza-D-homo-5α-androst-2-en-17-one[5]	0.77	0.69	0.54	0.67
3β-Pyrrolidino-17a-aza-D-homo-5α-androstan-17-one[5]	0.61	0.64	0.44	0.64
3β-Pyrrolidino-17a-aza-D-homo-5α-androstane[5]	0.22	0.28	0.10	0.37
17a-Ethyl-3-pyrrolidino-17a-aza-D-homoandrosta-3,5-diene[5]	0.63	0.65	0.44	0.58
17a-Ethyl-3β-pyrrolidino-17a-aza-D-homoandrost-5-ene[5]	0.55	0.63	0.39	0.65
17a-Methyl-3β-pyrrolidino-17a-aza-D-homo-5α-androstane[5]	0.43	0.53	0.19	0.57
17a-(2-Hydroxyethyl)-3-pyrrolidino-17a-aza-D-homoandrosta-3,5-diene[4]	0.65	0.69	0.42	0.57
17a-(2-Hydroxyethyl)-3β-pyrrolidino-17a-aza-D-homoandrost-5-ene[4]	0.50	0.60	0.41	0.66
4,17a-Diethyl-4,17a-diaza-D-homo-5α-androstane[6]	0.46	0.58	0.45	0.66
4-(2-Hydroxyethyl)-4-aza-5α-androstan-17β-ol[4]	0.76	0.72	0.69	0.70
4,17a-Di(2-hydroxyethyl)-4,17a-diaza-D-homo-5α-androstane[7]	0.61	0.65	0.38	0.60
4-(2-Chloroethyl)-4-aza-5α-androstan-17β-ol[7]	0.57	0.63	–	0.60
17a-Aza-D-homoandrost-4-en-3-one[8]	0.31	0.30	0.12	0.34
17a-Ethyl-17a-aza-D-homoandrost-5-en-3β-ol[5]	0.61	0.65	0.54	0.69
17a-Ethyl-17a-aza-D-homoandrost-4-en-3-one[5]	0.59	0.70	0.52	0.64
17a-(2-Hydroxyethyl)-17a-aza-D-homoandrost-5-en-3β-ol[4]	0.69	0.69	0.56	0.65
17a-(2-Hydroxyethyl)-17a-aza-D-homoandrost-4-en-3-one[4]	0.70	0.68	0.52	0.64
17a-(2-Acetoxyethyl)-17a-aza-D-homoandrost-5-en-3β-yl acetate[4]	0.71	0.69	0.62	0.67

TABLE II

THIN-LAYER CHROMATOGRAPHY OF HYDROCHLORIDES OF CERTAIN TERTIARY AZASTEROIDS IN SOLVENT SYSTEMS 5, 7–9

Compound	R_F *value*			
	5	*7*	*8*	*9*
4-(2-Acetoxyethyl)-4-aza-5α-cholestane hydrochloride[4]	0.65	0.61	0.75	–
4-(2-Chloroethyl)-4-aza-5α-cholestane hydrochloride[7]	0.69	0.66	0.70	–
4-(2-Chloroethyl)-4-aza-5α-androstan-17β-ol hydrochloride[7]	–	–	–	0.67
3β-Chloro-17a-(2-chloroethyl)-17a-aza-D-homoandrost-5-ene hydrochloride[7]	0.53	0.58	0.68	–

TABLE III

THIN-LAYER CHROMATOGRAPHY OF SOME MONO- AND BISQUATERNARY AZASTEROIDS IN SOLVENT SYSTEMS 5–9

Compound	R_F *value*				
	5	*6*	*7*	*8*	*9*
4-(2-Hydroxyethyl)-4-aza-5α-cholestane methiodide[4]	0.48	–	0.63	0.65	–
4-(2-Acetoxyethyl)-4-aza-5α-cholestane methiodide[4]	0.67	–	0.60	0.68	–
4-(2-Hydroxyethyl)-4-aza-5α-androstan-17β-ol methiodide[4]	–	0.59	–	0.61	–
4-(2-Acetoxyethyl)-4-aza-5α-androstan-17-β-yl acetate methiodide[4]	–	0.61	0.46	0.47	–
17a-(2-Hydroxyethyl)-17a-aza-D-homoandrost-5-en-3β-ol methiodide[4]	–	0.58	0.40	0.42	–
17a-(2-Acetoxyethyl)-17a-aza-D-homoandrost-5-en-3β-yl acetate methiodide[4]	0.21	0.58	0.42	0.52	–
17a-Ethyl-3β-pyrrolidino-17a-aza-D-homoandrost-5-ene diethiodide[5]	–	–	–	–	0.47
17a-(2-Hydroxyethyl)-3β-pyrrolidino-17a-aza-D-homoandrost-5-ene dimethiodide[4]	–	–	–	–	0.39
17a-(2-Acetoxyethyl)-3β-pyrrolidino-17a-aza-D-homoandrost-5-ene dimethiodide[4]	–	–	–	–	0.39
17a-Methyl-3β-pyrrolidino-17a-aza-D-homoandrost-5-ene diethoiodide[5]	–	–	–	–	0.47
17a-Methyl-3β-pyrrolidino-17a-aza-D-homo-5α-androstane diethiodide[5]	–	–	–	–	0.31
17a-Methyl-3β-pyrrolidino-17a-aza-D-homo-5α-androstane dimethiodide[5]	–	–	–	–	0.32

RESULTS

In Table I are listed the R_F values of basic azasteroids, which were secondary and tertiary amines. Solvent systems 1–4 gave consistent results; systems 2 and 4 are best since the R_F values are relatively higher.

The R_F values of the hydrochlorides of some tertiary azasteroids are given in Table II. Of the solvent systems useful for the hydrochloride salts (5, 7–9), system 8 was best, except for one salt which was more mobile in system 9.

Table III lists the R_F values of some mono- and bisquaternary azasteroids in solvent systems 5–9. For monoquaternary iodides, systems 7 and 8 were suitable and 9 worked well with bisquaternary iodides.

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Book Review

Chemical criminalistics, by A. Maehly and L. Strömberg, Springer, Berlin, Heidelberg, New York, 1981, VII + 322 pp., 70 figs., 65 tables, price DM 162.00, *ca.* US$ 75.50, ISBN 3-540-10723-1.

This volume is first of all an excellent book on forensic science. The introductory chapters survey the structure of forensic services in various countries and the development of these services. Then follow a number of chapters on the various materials that a forensic scientist may have to examine, and here the authors make extensive use of chromatographic methods. However, what is even more important, and not only for forensic scientists, they point out clearly the limitations of chemical evidence.

For example, in the chapter on soils, they compare two soils by numerous methods, all of which give identical results, but the conclusion is worded as follows: "Based on the results of the investigation, it cannot be excluded that sample 1 (from the hotel room) has come from the field in question". This is clearly all that can be said under the circumstances, but this would not have prevented many people from jumping to conclusions and affirming that the soil sample "must have come from the field in question". In this sense the book can be recommended to chromatographers in general, as I can list numerous instances in the literature where authors claimed "identification" or even "unambiguous identification" simply on the evidence of identical retention values and spectra.

The final section of the book deals with the organisation of a forensic science laboratory. Page 303 and Table C.17 discuss a project in which 240 forensic laboratories participated in 21 tasks. The number of "correct answers" is listed. Many a defence attorney, facing "unrefutable forensic evidence" may be grateful!

Lausanne (Switzerland) M. LEDERER

PUBLICATION SCHEDULE FOR 1982

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

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	234/1 234/2 235/1 235/2	236/1 236/2	237/1 237/2 237/3	238/1 238/2 239	240/1 240/2 241/1	The publication schedule for further issues will be published later.						
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