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CONTENTS

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(Continued overleaf)

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RECYCLING LIQUID CHROMATOGRAPHY USING MICROBORE COLUMNS

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(Received June 22nd, 1981)

SUMMARY

A study of recycling in high-performance liquid chromatography was carried out using 50 cm × 1 mm I.D. microbore columns. It is demonstrated that, although there are stricter requirements for the recycling microbore column system design than for larger diameter columns, the system can be designed to be highly efficient and can be successfully employed for analytical work. Theoretical aspects of an alternate pumping technique are considered. Equations relating resolution between two peaks of interest and limiting cycle number to various chromatographic parameters are derived. The experimental design of a computerized system operating in an automated mode is described. Several applications demonstrating how closely eluting solutes can be separated are shown and advantages and disadvantages of recycling using scaled down chromatographic systems are discussed.

INTRODUCTION

The technique of recycling liquid chromatography was introduced by Porath and Bennich[1] in 1962 for gel filtration separations. In this method, partially resolved sample components eluted from the column were diverted back onto the column using a specially designed column switching valve. Three basic recycling designs were reported in the literature. An alternate pumping system based on two columns and an eight-port switching valve was designed by Kucera[2] in 1967 and later on studied by Biesenberger and co-workers[3,4] and others[5,6]. A one-column "closed-loop" system was also reported[7–9]. In this case, however, the solute mixture is allowed to pass through the chromatographic pump into the column repeatedly, and since, in most cases, the pump makes a significant contribution to solute band broadening, this system is usually not preferred for analytical work and is only used for preparative scale purposes[10]. An excellent theoretical treatment of "closed-loop" recycling is given by Martin *et al.*[11]. Snyder and co-workers[12,13] recently revived another form of recycling to perform boxcar-recycle chromatography. A novel concept of the recycle combining the advantages of the previous two systems was designed in 1981 by Minarik *et al.*[14].

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It has been suggested in the literature[11] that a relatively strong dependence of recycling resolution on the column diameter makes recycling interesting mainly for the purpose of preparative scale liquid chromatography. If very low dead volume can be achieved, however, favorable analytical use can be made of recycling narrow-bore columns. It has been shown previously[15] that coupling microbore columns together can produce extremely high efficiencies and, generally, efficiency increases linearly with column length[15]. When wider bore columns are joined together, about 40% efficiency is lost during each coupling step, and this has been attributed to interstitial porosity variations across the column and different column heat transfer processes at higher volumetric flow-rates. Thus, all the advantages of the microbore column systems reported previously[16] can be fully utilized and recycling efficiency can be increased. Furthermore, column detector and column switching valve connecting tubes can be completely eliminated because of the 1/16 in. O.D. of the column. A properly designed microbore column recycling system would also be ideally suited for an automated operation using computer data handling.

The purpose of this paper was to investigate an alternate pumping recycling approach using relatively short microbore columns to demonstrate the feasibility of practical analytical operation and to study recycling parameters that affect the desired chromatographic resolution.

THEORETICAL

Chromatographic resolution in recycling

Any analytical chromatographic system can be characterized in terms of three basic attributes: resolution, speed and scope. For the practicing chromatographer, the desire to improve the separation is the main reason to use the recycling technique. However, when using a repeated operation in a microbore loop-type system, such as depicted in Fig. 1, a condition is reached where the retention volume difference between the first and the last eluted peak is greater than the total column dead volume, and any further recycling will involve the loss of part of the sample since this would exit from the loop. Thus, in recycling, we are effectively trading speed and scope or peak capacity for an increase in resolution and the number of theoretical plates. Let us assume that n is the number of passages of the sample through one column; then, at a constant flow-rate of the mobile phase, the retention volumes, $(V_A)_n$ and $(V_B)_n$, of the two solutes A and B after the nth cycle would be proportional to the respective retention volumes in a one-cycle system, V_A, V_B:

$$(V_A)_n = n(V_A + V_S)$$

$$(V_B)_n = n(V_B + V_S)$$

Since microbore columns can be coiled to a relatively small coil diameter[17] without impairing the column efficiency significantly, no connecting tubing between the column and the switching valve is necessary and the only band dispersion would occur in the internal volume of the switching valve, V_S. (V_S represents two internal loop volumes of the switching valve.) Furthermore, using the principle of summation

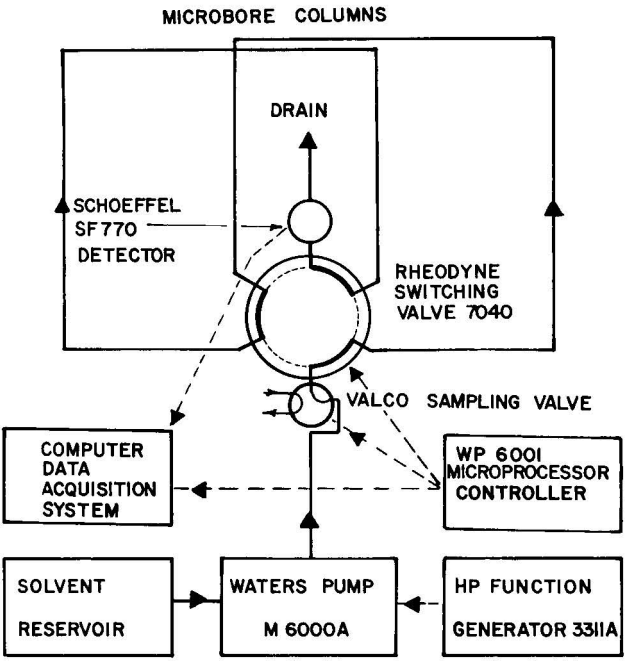

Fig. 1. Block diagram of the recycling microbore column system.

of variances and assuming that both solutes have similar band widths ($\sigma_A = \sigma_B = \sigma$) in a one-cycle system

$$(\sigma)_n = (\sigma_A)_n = (\sigma_B)_n = \sqrt{n(\sigma^2 + \sigma_v^2)}$$

where σ_v^2 is the volume variance resulting from the switching valve, and $(\sigma)_n$ is the solute band width after the nth cycle. Resolution after one cycle, $R(1)$, can now be related to the difference in solute capacity factors, k', on one column

$$R(1) = \frac{(V_B - V_A)}{4\sigma} = \frac{V_0 (k'_B - k'_A)}{4\sigma} \tag{1}$$

where V_0 = column dead volume. For the nth cycle:

$$R(n) = \frac{(V_B)_n - (V_A)_n}{4\sqrt{n(\sigma^2 + \sigma_v^2)}} = \sqrt{\frac{n}{\left[1 + \left(\frac{\sigma_v}{\sigma}\right)^2\right]}} \cdot \frac{(V_B - V_A)}{4\sigma} \tag{2}$$

Thus:

$$R(n) = R(1) \sqrt{\frac{n}{\left[1 + \left(\frac{\sigma_v}{\sigma}\right)^2\right]}} \tag{3}$$

Eqn. 3 is a similar function to that described by Martin *et al.*[11] for closed-loop systems. However, for an alternate pumping system given here, a different function for the relative band spreading is obtained. Assuming now that the solute peak width is increased by $p\%$ during each solute passage through the switching valve

$$\sigma_v = p\sigma$$

and:

$$(\sigma)_n = \sqrt{n(1 + p^2)\sigma^2}$$

We can arbitrarily define efficiency of recycling, η, as:

$$\eta = (1 - p)\,100 \tag{3a}$$

Eqn. 3 can now be written as:

$$R(n) = R(1)\sqrt{\frac{n}{(1 + p^2)}} = R(1)\sqrt{\frac{n}{\left[1 + \left(1 - \frac{\eta}{100}\right)^2\right]}} \tag{4}$$

It can be seen that the resolution after the nth cycle will be a function of the cycle number, n, efficiency of recycling, η, and $R(1)$. η can be experimentally determined from the slope of the $R(n)/R(1)$ *versus* the $\sqrt{n}$ plot. Assuming 100% efficiency of recycling, the relative resolution would be equal to the square root of the cycle number. The volume variance due to the switching valve, σ_v^2, would be proportional to the volume of the switching valve[16], V_S

$$\sigma_v^2 = KV_S^2 \tag{5}$$

where K is a constant.

Substituting for the solute band width, σ, in a one-cycle system

$$p = \left(\frac{\sigma_v}{\sigma}\right) = \frac{4\sqrt{NK}V_S}{\pi D^2 L\varepsilon(1 + k')} \tag{6}$$

where N, L and ε are the column efficiency, column length and the total column porosity, respectively. It can be seen from eqn. 6 that, for columns of larger diameter, D, a much greater internal volume of the switching valve can be tolerated in order to operate the system at the same recycling efficiency. It will be seen later on that, even with a relatively large volume of the switching valve ($V_S = 2\ \mu l$), up to 80% recycling efficiency of the microbore column system can be achieved.

Limiting cycle number

The maximum number of theoretical plates that can be achieved when coupling microbore columns together in series is eventually limited by the pressure rating

of the chromatographic system operated at a given flow-rate. This has been discussed recently[15]. Similarly, in length-programmed systems, the volume of the recycling system, the maximum solute concentration–detector sensitivity relationship and the overall column stability to recycling pressure fluctuations will ultimately limit the number of cycles that can be employed. As the number of cycles increases, the solute peaks broaden and the maximum solute concentration, X, corresponding to the solute peak height decreases

$$(X_A)_1 = m/\sqrt{2\pi} \cdot \sigma \qquad (X_A)_n = m/\sqrt{2\pi} \cdot (\sigma)_n$$

where m is the total solute mass introduced into the system.

$$\frac{(X_A)_n}{(X_A)_1} = \frac{\sigma}{(\sigma)_n} = \frac{1}{\sqrt{n(1 + p^2)}} = \frac{K_1}{S} \tag{7}$$

For the given solute mass, the limiting cycle number, n, will depend on the detector sensitivity, S, expressed as the solute concentration equivalent to twice the noise level, constant K_1 and the efficiency of recycling, or factor p. However, in practice, this condition is usually overridden by the limited volume capacity of the recycling loop system, given by the column dead volume, V_0, and the switching valve internal volume, V_S. Assuming that both peaks will spread to the same extent after the nth cycle

$$(V_B)_n - (V_A)_n + 4(\sigma)_n \leqslant V_0 + V_S \tag{8}$$

and the following second-order equation for the limiting cycle number, n, can be obtained

$$nV_0 \cdot k'_A (\alpha - 1) + 4\sqrt{n} \cdot \frac{V_0 (1 + k'_A)}{\sqrt{N}} \cdot \sqrt{1 + p^2} - V_0 - V_S \leqslant 0 \tag{9}$$

where α is the selectivity coefficient:

$$\alpha = k'_B/k'_A$$

Eqn. 9 leads to the following condition for the maximum cycle number

$$n_{max} \leqslant \left(A - \sqrt{A^2 + B}\right)^2 \tag{10}$$

where

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

and:

$$B = \frac{(V_S + V_0)}{k'_A V_0 (\alpha - 1)} = \frac{V_t}{(V_B - V_A)}$$

Eliminating negligible terms, eqn. 10 can be approximated by:

$$n_{max} \leqslant B \cdot [1 - \sqrt{(16B/N)}] \tag{11}$$

Obviously, a good separation after a given number of cycles requires a resolution factor greater than one. Solving eqn. 2 will yield the relationship for the minimum cycle number:

$$n_{min} \geqslant 4A^2 \tag{12}$$

The maximum theoretical cycle number plotted against the selectivity α for different values of k' of the first eluted peak is shown in Fig. 2 ($\eta = 100\%$). It can be seen from eqns. 10 and 12 and Fig. 2 that the smaller the α, the higher the column efficiency, and the less spreading in the switching valve, the greater the number of cycles possible. Also, the larger the k', the broader the peaks that will be obtained and the total volume capacity, V_t, of the system will be exhausted faster.

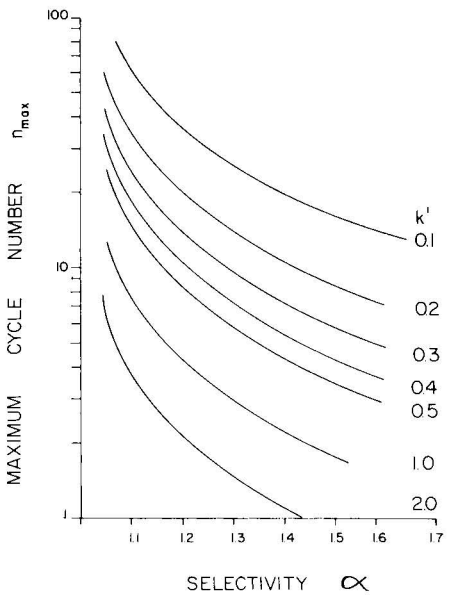

Fig. 2. Graphs of the maximum cycle number *versus* the selectivity for different capacity factors.

It is interesting to note that the constant A is related to the reciprocal of the resolution factor in a one-cycle system where $k'_B \approx k'_A$ and the constant B has the meaning of the relative retention volume difference in a one-cycle system, $V_t/\Delta V$.

Band spreading can be controlled by the ratio V_S/V_0, which should be as small as possible. Thus, recycling should preferably be carried out within the limits given by

$$n_{min} \leqslant n \leqslant n_{max}$$

and the conditions for the special case where $n_{max} = n_{min}$ can be obtained by solving eqns. 10 and 12.

EXPERIMENTAL

Recycling apparatus

Fig. 1 shows a diagrammatic scheme for the recycling system which consists of a Rheodyne six-port, 1-μl internal loop switching valve (Model 7040); two identical 50 cm $\times$ 1 mm I.D. microbore columns packed with DuPont Zorbax ODS 8-μm particles; a Valco sampling valve with a fixed internal loop of 0.5 μl (Model ACV-4UHPa-N60); and a Waters Model 6000A pump driven by a Hewlett-Packard 3311A function generator. A Minarik programmable microprocessor controller (Model WP 6001) was used to initiate sample injection, computer acquisition and column switching. A Schoeffel SF770 UV/VIS spectrophotometer equipped with a 0.5-μl cell was operated at 224, 254 or 350 nm, depending upon the sample application. The detector was connected to the CIS data handling system (Computer Inquiry Systems, Waldwick, NJ, U.S.A.) and to a potentiometric recorder. Assuming port 1 to be the input to the valve and port 4 to be the exit to the detector, the columns are connected to the valve from port 6 to port 3 and from port 2 to port 5, respectively. Column ends are inserted directly into the port openings, butted against 2-μm frits seated within the ports, and sealed with Rheodyne low dead volume fittings. The connection from the sampling to the switching valve is made with 2 cm $\times$ 0.005 in. I.D. tubing, and from the exit port to the 0.5-μl detector cell with a short section of packed microbore column containing the same packing material as the recycling columns.

Ideally, the detector cell should be interposed between each column and the switching valve. This arrangement, however, performed very poorly and, thus, the recycling was carried out in a blind fashion. Only the peaks exiting from the loop system were monitored by the detector. A closeup picture and connections is shown in Fig. 3.

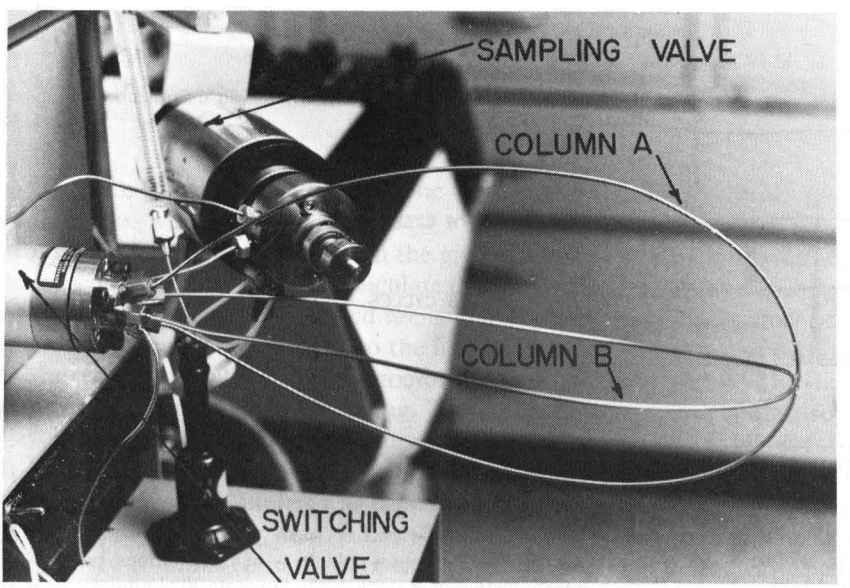

Fig. 3. Detailed picture of the sampling valve/switching valve arrangement.

Determination of switching times

Switching time for a given sample for n number of cycles can be determined by first running the sample with no switching input (two cycles) and then with a single switching input (one cycle). Sample residence time per column, as well as the residence time in the exit line, can then be determined, and programs can be constructed for multiple-cycle operations. However, the increase in the number of theoretical plates and retention times due to the detector connecting tubing was negligible, especially for higher numbers of cycles.

Mobile phases and samples

Glass-distilled solvents (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and deionized water were used to prepare mobile phases. Sample solutes were from commercial sources, except retinoic acids obtained in-house from Hoffmann-La Roche, and were used without further purification. The samples were dissolved in the mobile phase and were placed on the column using the 0.5-μl Valco air-actuated sampling valve.

RESULTS AND DISCUSSION

Since the peak capacity of the recycling system is low, recycling is ideally suited for the separation of closely eluted compounds exhibiting only small differences in their molecular structure. Such would be the case for the separation of fully deuterated benzene and H-6 benzene, which can be seen in Fig. 4. With Zorbax ODS reversed-phase columns and a flow-rate of 30 μl/min, baseline-to-baseline separation between both isotopes was obtained after five cycles; 75,660 theoretical plates for both solutes was achieved, which is exactly five times the column efficiency obtained in a one-cycle system ($k'_{D\text{-}6} = 2.16$; $k'_{H\text{-}6} = 2.25$). The peaks eluted after five cycles

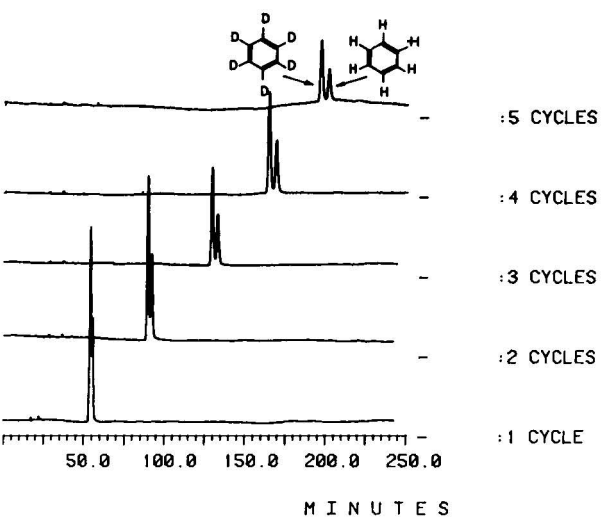

Fig. 4. Separation of benzene isotopes on the recycling microbore column system. Conditions: two Zorbax ODS columns, 50 cm × 1 mm I.D. each; eluent, methanol–water (75:25), flow-rate 30 μl/min; detection at 254 nm.

were extremely symmetrical, and this can be seen in Fig. 5. The asymmetry factor arbitrarily measured at 20% of the peak height was 1.03 for both solutes. Throughout this work, the determination of the number of theoretical plates as well as baselines, asymmetry factors, k's and retention times were done by the CIS computer using special external BASIC programs operating on the digital chromatographic data, and thus all possible errors introduced by the operator were eliminated. Fig. 6 shows another application of the recycling microbore system. Light isomerization of retinoic acid provides within minutes a mixture of 13-*cis*-retinoic acid and all-*trans*-retinoic acid (tretinoin). These solutes could easily be resolved in five cycles with a 5% v/v chloroform–methanol mobile phase modified by the addition of 0.3% ammonium acetate.

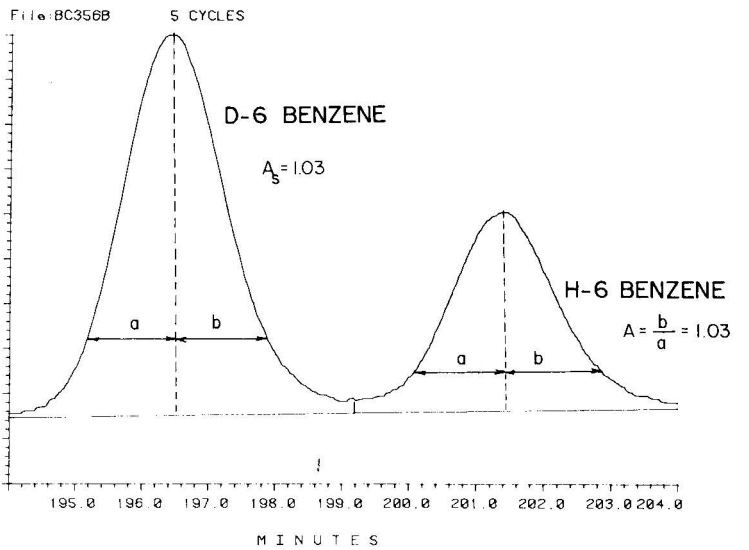

Fig. 5. Demonstration of the symmetry of benzene peaks eluted from the recycling microbore column system.

Since an unmodified Rheodyne switching valve (V_S = 2 μl) was employed during this work, it was of interest to determine the efficiency of recycling, η, as arbitrarily defined by eqn. 3a with the given system. The data from the separation of benzene isotopes were used to calculate the relative resolution of recycling as given by eqn. 3. These values were plotted *versus* the square root of the number of cycles, and the resulting curves were fitted to the linear function. The index of determination of the curve fit was 0.99, and the slope obtained in this way was 0.981. The data from these experiments are plotted in Fig. 7 as a double logarithmic plot; also included is the apparent efficiency of fully deuterated benzene in theoretical plates for five cycles. It can be seen from Fig. 6 that excellent linearity between the number of theoretical plates and the cycle number was obtained. This reflects the excellent symmetry of the peaks eluted from the system. It has previously been observed that, when recycling on larger bore columns (D > 3 mm), the symmetry of eluted peaks was generally worse and, as a consequence, greater departure from efficiency linearity and system ideality

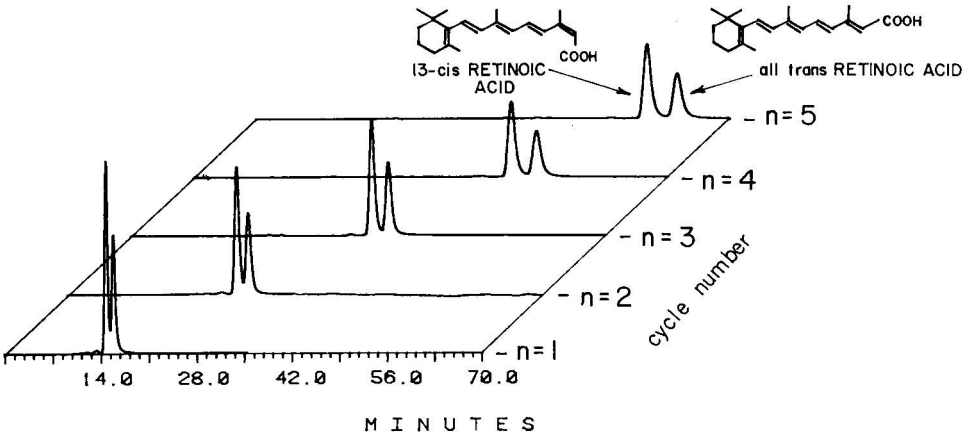

Fig. 6. Separation of *cis/trans* isomers of retinoic acid on the recycling microbore column system. Conditions: two Zorbax ODS columns, 50 cm × 1 mm each; eluent, chloroform–methanol (5:95) containing 0.3% (w/v) ammonium acetate, flow-rate 40 μl/min; detection at 350 nm.

was obtained. This has been attributed to various trans column band spreading processes and will be investigated in greater detail in the future. From the slope of $R(n)/R(1)$ *versus* the square root of the cycle number, the efficiency of recycling was calculated and was found to be 80%. Certainly, the efficiency of recycling could be improved even further if a 0.5-μl internal volume switching valve were used instead of the 1.0-μl valve.

Experimental verification of the maximum cycle number, given by eqn. 10, was

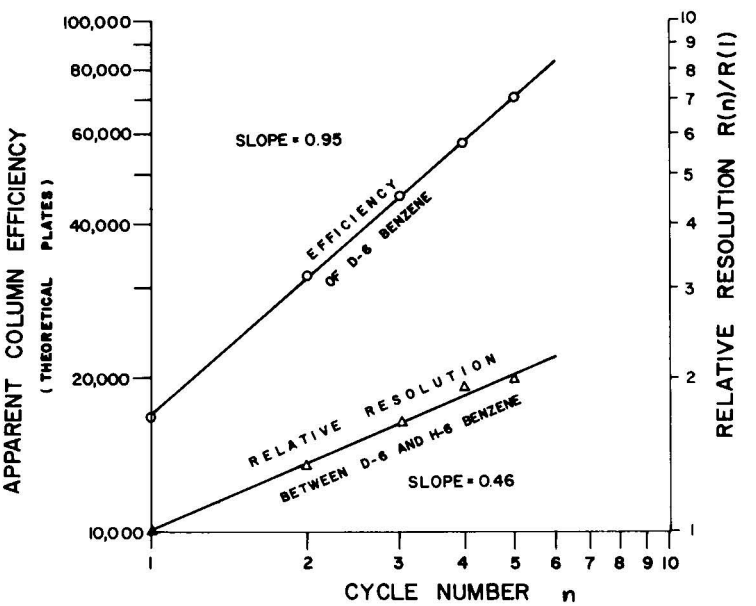

Fig. 7. Graphs of relative resolution and column efficiency against the cycle number.

carried out with a mixture of oligomers present in a non-ionic surfactant, Triton X-100 (isooctylphenoxypolyethoxyethanol). The chromatograms from these experiments can be seen in Fig. 8. The recycling system was operated with a flow-rate of 40 μl/min, 50% acetonitrile in methanol mobile phase and the UV detector was set at 224 nm. The capacity factors of the first and last eluted peaks were 0.52 and 0.78, respectively, which yields $\alpha = 1.5$. Introducing these values into Fig. 2, one can determine that only three cycles should be theoretically feasible. Experimentally, the fourth cycle could not be obtained since part of the solute mass circulating within the recycling loop was always cut off by the switching valve and exited to the detector. Thus, assuming that the peaks are Gaussian in shape, Fig. 2 can be used to predict the maximum cycle number for a given system.

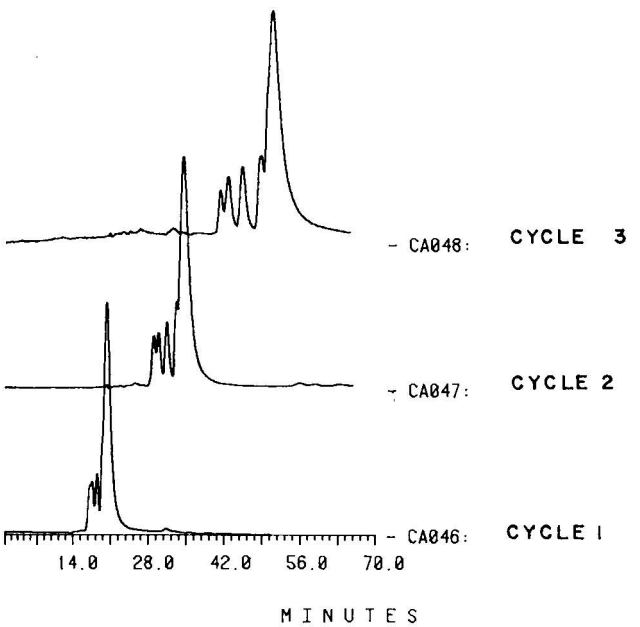

Fig. 8. Experimental verification of the maximum cycle number using Triton X-100. Eluent, acetonitrile–methanol (50:50), 40 μl/min.

CONCLUSIONS

Peaks eluted from microbore columns are, in general, very symmetrical, and, upon connecting microbore columns together in the recycling mode, column efficiencies increase linearly with column length, a result that has been very difficult to demonstrate for larger bore columns. Thus, the recycling microbore column system has distinct advantages over the current conventional recycling systems. Furthermore, the already accepted advantages inherent with the use of microbore columns, such as low solvent consumption of the mobile phase, sample and column material, can be fully utilized. Because the peaks obtained resemble symmetrical Gaussian

curves, experimental data are in good agreement with theory and various recycling parameters can be predicted from experimental data obtained in one cycle run.

Chromatographic resolution in recycling depends on the square root of the cycle number and the recycling efficiency, which in turn is related to the solute band spreading in the switching valve. Factors affecting the maximum cycle number can be determined in a one-cycle run and thus the maximum cycle number which can be employed with a given experimental arrangement can be calculated. The system is easily adaptable to total automation. There is no doubt that, at this stage of miniaturization of a liquid chromatograph, the described recycling system can be successfully employed for analytical work. However, the use of an accurate solvent delivery and a low-volume switching valve is very important. Further decrease in the dimensions of the packed column, switching valve volume and sample size is quite feasible. Quite clearly, the alternate pumping principle is superior over other scaled-down designs. If sufficiently low-cell volume, high-pressure rating, high-sensitivity detectors are developed, there is a possibility that the detector cell can be introduced into the recycling loop and the chromatogram development could be monitored during each sample passage through the respective cycle. Using a detector/computer feedback design would then be a highly desirable system for automation.

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TESTING CAPILLARY GAS CHROMATOGRAPHIC COLUMNS

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SUMMARY

A test procedure for capillary gas chromatographic columns, first published three years ago, is described in further detail, giving practical guidelines based on accumulated experience.

INTRODUCTION

Our "Comprehensive, Standardized Quality Test"[1] has been available for general use for three years, and was recently reviewed[2]. It was developed by one of us (K.G., Jr.) to meet a fundamental need of our (K.G., G.G.) laboratory, which specializes in the development of capillary gas chromatographic columns. The new test has significantly influenced our work, resulting in an obvious saving in time. Furthermore, the quantitative nature of the test has greatly improved our understanding of column quality and has facilitated new developments because of the comparability of the results. It is hard to imagine how we could have developed the persilylation procedure on the basis of our earlier testing methods. Fig. 1 gives an arbitrarily selected example of application of the test method.

While column makers obviously rely on informative and efficient testing, routine users should also apply periodic tests to their capillary columns. Only in this way can they detect alterations and be able to correlate them with their cause, *i.e.*, a given sample, the nature or amount of solvent, a given injection technique or a high temperature which the column is not able to withstand.

The three years of application have not provoked substantial modifications in the original procedure. Thus, for background information we refer the reader to the original paper[1]. For experimental purposes, however, it may be useful to provide a short description, including practical instructions, as well as some recommendations based on long-term experience.

TEST CHARACTERISTICS

Principal merits

The test yields the maximum of information about various objectives from a

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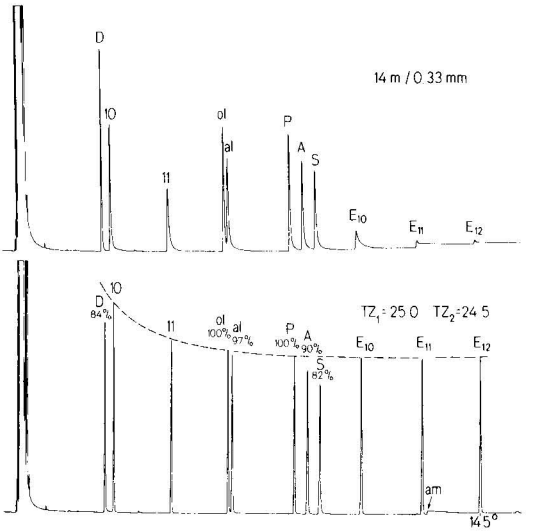

Fig. 1. Column: 14 m × 0.32 mm, coated with experimental, moderately polar silicone phase* suitable for immobilization[3]. Standardized test conditions: methane eluted at 25°C after 28 sec (hydrogen carrier); 1 μl of diluted test mixture I injected at 25° C with splitting ratio 1:30; column heated to 40°C, and temperature program (3.5°C/min) started immediately. Upper chromatogram: freshly coated column conditioned overnight at 240°C with exit connected to the flame ionization detector; flame not lit. Some bleeding was accumulated in the column exit. When the flame was lit (1.5 mm above column end!), the bleeding was carbonized. Typical effect of carbonized contamination: general adsorption, least for most polar substances (very little for D; less for ol than for al), increasing with decreasing polarity and with decreasing volatility (esters!). Lower chromatogram: test after cutting away 3 mm of column exit. Evaluation of the column: column weakly acidic (am strongly adsorbed, A 90%); inertness good (ol 100%), but not excellent (D 84%; S 82% from an acidic column); separation efficiency at maximum; relatively thick film, 0.41 μm (E_{12} eluted 20° above standard elution temperature of 125°C).

minimum of testing materials, analytical manipulation, experimental planning and time. Its chief facilities are as follows:

(1) An automatic optimization of the chromatographic conditions. Thus, no preliminary optimization run is necessary.

(2) Standardized information (from the first run) concerning four basic aspects of column quality, namely, adsorptive activity, acidity/basicity, separation efficiency and film thickness.

(3) In contrast to earlier methods which employed a large number of specific test mixtures, basically one mixture can be used regardless of the polarity of the stationary phase.

(4) Qualitative results.

(5) Full comparability of results.

In the last three years an increasing number of laboratories have utilized the test. However, an examination of the literature reveals that our test mixture is quite often used without the correct composition and without standardization, thus the results obtained are not quantitative or comparable. Typically, the test mixture used under these conditions is called a "polarity mixture". We hope that the information given here will facilitate full utilization of the method.

* Now available as OV-1701.

Temperature programming

The combined merits of this test would not be realized under isothermal conditions. Isothermal testing has to be based on experimental optimization of the conditions, which requires specific mixtures for each different quality aspect (see p. 14, point 2) and different mixtures for different stationary phase polarities. In contrast, temperature programming offers following advantages.

Automatically optimized column temperature. The optimum column temperature is dependent (under optimum flow conditions) on the molecular interactions between the sample and stationary phase and on the film thickness. While the first of these parameters may, to some extent, be theoretically predicted, the second is in many cases unknown, thus requiring repeated test runs at different column temperatures. A further problem is that a given column temperature can be optimum only for one substance within a mixture. Under programmed conditions, the individual test substances begin chromatographic migration at appropriate column temperatures, and with proper standardization, therefore, each substance will migrate automatically under optimum conditions.

Determination of film thickness. Under standard conditions and for a given stationary phase, the elution temperature of a given test substance depends only on film thickness. Consequently, film thickness can be determined in a very simple and non-destructive way.

Analysis of substances of differing volatilities. The applicability of the same test mixture to stationary phases of any polarity requires a set of test substances with a relatively broad range of volatility. Such a mixture cannot readily be analyzed isothermally.

Quantitative results. The quantitative interpretation of chromatograms is based on peak heights (since other criteria, such as peak symmetry or retention indices, are not generally applicable and are less suitable for routine use). Temperature programming facilitates this process.

Reduction of peak broadening. Temperature programming reduces the peak broadening effect due to defects in the equipment or to incorrect sampling technique, as expressed by Kaiser's[4] Q_s value. This is important in evaluation of the column rather than of the equipment or the manipulation.

It is emphasized that with temperature programming no concept of separation' efficiency other than TZ (Trennzahl, separation number) or one basically similar is applicable. An extensive discussion of TZ has appeared recently[5].

Standardization

It is evident that all the advantages of this test hold only when the test is strictly standardized. In a multi-purpose test no standardization can be applied to all functions of the test. Our standardization is set to yield optimum conditions for the determination of separation efficiency. These conditions cannot simultaneously be optimal for the determination of adsorption. The adsorption test, which has to be based on polar test substances, is more stringent for non-polar columns, since the elution temperature of polar substances decreases with decreasing column polarity, thus supporting adsorption effects.

The empirical establishment of the optimal carrier gas flow and temperature program rate was described previously[1]. However, it is often asked why the standard

conditions, in addition to a constant linear gas flow-rate, include a temperature program rate which is inversely proportional to column length. All test substances are, at first, cold trapped in the column inlet. During temperature programming they start to migrate at individual (but not readily observed) temperatures and leave the columns at individual (and easily measured) elution temperatures. The temperature range between the start and elution must be kept constant and independent of column length. A varying temperature range would result in chromatographic conditions which are not comparable on columns of different lengths, and hence in TZ measurements and adsorption/polarity determinations which are not comparable, since both depend on column temperature. If the column length is doubled at constant gas flow-rate, twice the time is required to elute a given substance within the same temperature range. This is countered by simply halving the program rate.

Our standardization produces optimal conditions for TZ only for columns with a medium range of film thicknesses (0.08–0.4 μm) and of internal widths (0.25–0.35 mm). Although TZ values found for a very wide column or for a very thick film may be slightly below the maximum value, we usually prefer to keep the same conditions for all column geometries. If such columns have to be evaluated very stringently, the test conditions are optimized empirically (*i.e.*, as they were more than 3 years ago).

Elution patterns

The order of elution of test compounds is dependent primarily on the polarity of the column. A "fingerprint" is produced which is characteristic for a stationary phase and which may be used to identify an unknown phase. Fingerprints from twenty stationary phases and elution temperatures of individual compounds were reported previously[1].

However, even under the conditions of standardized flow-rate and temperature program, these fingerprints are not absolute, being dependent on two other variables:

The published patterns are valid only for the standard film thickness (0.15 μm). A different film thickness would cause the temperature ranges of migration for all the test compounds to be shifted to lower or higher temperatures. Because of the dependence of polarity on temperature, the test compounds then "see" a column of different polarity from which they are eluted with a different fingerprint.

The published fingerprints were obtained from coatings deposited on barium carbonate surfaces (with Carbowax deactivation for apolar and medium polar coatings). Non-polar phases in particular produce substantially different fingerprints when coated on persilylated columns. These were not available when the original paper was published. Fig. 2 presents new fingerprints together with the slightly different standard elution temperatures.

Experiences with the test mixture

We have found no reason to modify the original mixture, although some of the components give less information than we had originally hoped.

The alkanes are commonly supposed not to suffer from adsorption. This is why we employed them as a basis for the "100% line". However, alkanes are often adsorbed on very inert, apolar columns. The effect is revealed by aldehyde or alcohol peaks which are higher than those of the alkanes. Quantification of adsorption is, of course, not possible in such cases.

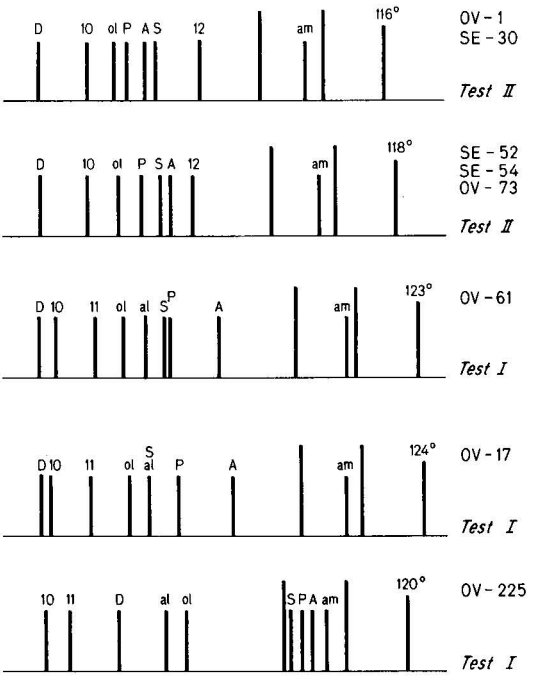

Fig. 2. Elution patterns from persilylated columns. Note the different test mixtures used for different polarities. The unlabelled tall bars represent the esters E_{10}–E_{12}. The given peak sequences and the elution temperatures for E_{12} are correct for the arbitrarily selected standard film thickness of 0.15 μm.

The esters have a dual purpose in this test; they permit determination of TZ, and they serve as a second standard for 100% peak height. Although adsorption of esters is more likely than of alkanes, on columns of reasonable quality it is negligible. It is therefore our policy not to attempt quantification of adsorption on poor columns on which esters are adsorbed.

2-Ethylhexanoic acid is a strongly acidic component which causes problems on apolar columns. A free carboxylic acid is clearly incompatible with an apolar liquid phase; when even less than 1 ng of 2-ethylhexanoic acid has entered the column, the column is already overloaded as can be observed from distorted (leading) peaks. It was this problem which led us to select a branched chain acid. However, there was no appreciable improvement with 2,2-diethylbutyric acid, which has even greater steric hindrance of the carboxyl group. Lacking a strongly acidic and simultaneously sufficiently lipophilic test substance of suitable volatility, we are resigned to interpreting the elution of the free acid from apolar columns on the basis of peak area instead of peak height.

PRACTICAL GUIDELINES

The test mixture

The test mixture is crucial and is the only somewhat demanding part of the method. We have learned that the lack of a suitable mixture or of one of appropriate composition is the major limitation of the test. Therefore, high priority has to be given to this point.

Several test substances, especially acidic and basic ones, are commercially available in various purities and may undergo alteration depending on the storage conditions. A specific problem is 2,3-butanediol which is often available as a mixture of isomers. To circumvent these difficulties, we have encouraged Fluka (Buchs, Switzerland) to distribute the following two test mixtures:

No. 86499, test mixture I (general mixture)

No. 86501, test mixture II (modified mixture for non-polar phases; see under "modified test mixtures")

We have carefully checked the compositions of both mixtures and will repeat the check periodically for this and each new batch. The regular package contains 5 ml of concentrated mixture. A 1-ml volume of the mixture dissolved in 20 ml pure hexane (without volumetric precision) yields a dilute solution ready for use. The durability of the diluted mixture depends on the storage and handling conditions and may vary between 1 month and 2 years. The concentrated mixture has unlimited durability when stored in a freezer. It is supplied with a sheet giving brief information on standardization, and on the interpretation of test chromatograms.

For those who prefer to prepare the test mixture themselves, the individual components and their proportions are given in Table I. If other than Fluka products are used, care must be taken to ensure that only one isomer of butanediol is present. The two alkanes are combined in one 20-ml vial and the three esters in another (nine vials for the twelve substances). To each vial, 20.0 ml hexane are added, except for the vial containing butanediol, the solvent for which is chloroform. After use, the solutions are kept in a freezer. They can be stored for years. The concentrated test mixture is prepared by transferring 1.00 ml of each of the above solutions into a 10-ml vial. The resulting mixture should be stored in a refrigerator. The dilute test mixture is

TABLE I

AMOUNTS OF SUBSTANCES USED FOR THE PREPARATION OF THE CONCENTRATED TEST MIXTURE

Fluka catalogue No.	*Substance*	*Code*	*Amount dissolved in 20.0 ml solvent (mg)*
21479	Methyl decanoate	E_{10}	242
94120	Methyl undecanoate	E_{11}	236
61689	Methyl dodecanoate	E_{12}	230
30560	*n*-Decane	10	172
94000	*n*-Undecane	11	174
44010	*n*-Dodecane*	12	176
74850	1-Octanol	ol	222
76310	Nonanal	al	250
18965	2,3-Butanediol	D	380
39520	2,6-Dimethylaniline	A	205
41350	2,6-Dimethylphenol	P	194
36620	Dicyclohexylamine	am	204
03300	2-Ethylhexanoic acid	S	242

* Only for test mixture II.

prepared by transferring 1 ml of the concentrated mixture to a 20-ml vial and making up to 20 ml with hexane. Fresh solution is made from the concentrated mixture whenever a yellow colour appears in the dilute solution or when any doubt arises as to its composition.

Solutions for identification

Unknown or mixed phases, as well as columns with extreme geometries, may produce fingerprints which lead to doubtful peak identity. In such cases, co-chromatography of the test mixture with solutions of the individual substances should provide the missing information. For this purpose, small amounts of standard solutions (primarily of ol, S, am, A or P, see Table I) diluted 1:50 may be employed.

Modified test mixtures

Our test mixture is designed to give a minimum of peak coincidences on all phases. Incidental overlappings cannot be avoided, however. When a column producing overlapping peaks is of special interest, it may be worthwhile to prepare a specific concentrated test mixture for it.

An example is a modified mixture for non-polar columns, on which a coincidence of *n*-undecane, 2,6-dimethylphenol and nonanal is likely to occur. We omit nonanal, since non-polar, inert columns tend not to adsorb aldehydes, and we replace *n*-undecane by *n*-dodecane, which has no close neighbours on non-polar columns.

Procedure

(1) Cool the oven to room temperature or at least to less than 40°C. Efficient cooling is particularly important with thin films.

(2) Set a suitable carrier gas pressure, and adjust the splitting ratio. Inject a few microlitres of methane (most fuel gases contain enough methane), and measure with a stop-watch the time between injection and the first recorder signal. Adjust the time to the standard time (±5%) of 2 sec/m for hydrogen, 3.5 sec/m for helium. Changing the split flow now may significantly affect the carrier gas pressure!

(3) Set the temperature program as obtained by inter- or extrapolation from the following basic data: column length 10 m, 5.0°C/min with hydrogen, 2.5°C/min with helium; column length 50 m, 1.0°C/min with hydrogen, 0.5°C/min with helium.

(4) Inject the test mixture under conditions that allow *ca.* 2 ng of a single test substance to enter the column (*e.g.*, 1 μl with splitting ratio of 1:20 to 1:50, depending on injector design).

(5) Immediately after the injection, heat the oven to 40°C (for very thin films, to 30°C) and start the temperature programming.

(6) Within the temperature range in which the third ester is eluted (on most columns, 110–140°C), make two marks on the recorder chart noting the actual oven temperature.

(7) At the end of the run, inter- or extrapolate the elution temperature of the third ester.

(8) Draw the "100% line" over the two alkanes and the three esters as shown in Fig. 1.

(9) Express the height of the remaining peaks as a percentage of the distance between the baseline and the 100% line.

(10) Determine TZ as an average of TZ E_{10}/E_{11} and TZ E_{11}/E_{12}.
(11) Determine the film thickness using the nomogram[1].

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CONCEPT OF THE EFFECTIVE MOBILITY OF THE HYDROGEN ION AND ITS USE IN CATIONIC ISOTACHOPHORESIS

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SUMMARY

The concept of the effective mobility of the terminating H^+ ion for cationic isotachophoresis in an acidic medium is introduced and its application to the selection of the electrolyte system for a required separation is discussed. Based on the theoretical description of the migrating reaction boundary, a relationship is derived for the calculation of the effective mobility of the H^+ ion. Further, a rule is defined for the selection of a terminating cation such that the loss of the control of the migration of H^+ ions through the isotachophoretic system is avoided. The method for the calculation and the rule mentioned above have been verified experimentally for model systems.

INTRODUCTION

For isotachophoretic migration, it is necessary to have a state in which each zone contains virtually only one separated substance and a counter-ion system, and in which the contribution of other ions of the same sign as that of the substance being separated to the electrical conductivity in the given zone is negligible. This means that in an aqueous medium either H^+ or OH^- ions, for cationic or anionic isotachophoresis, respectively, must not contribute significantly to the conduction of electrical current.

In anionic isotachophoresis, this requirement can easily be met by performing the separation in the so-called "safe region"[1] at pH < 10. Here the concentration of OH^- ions is less than 10^{-5} M and is therefore negligible in comparison with the concentrations of the substances being separated in their zones (commonly 10^{-3}–10^{-2} M).

Analogously, for cationic analysis the "safe region" is represented by pH > 5. However, here a serious problem occurs in the separation of weak bases. It is necessary to shift the equilibrium $H^+ + B = BH^+$ towards BH^+ and, therefore, a pH range of 2–5 is required. Similarly, an acidic medium is required for suppressing hydrolysis in the electrophoresis of inorganic cations[2,3]. In the pH region mentioned, the concentration of H^+ ions cannot be neglected in comparison with other cations. Moreover, the H^+ ion here possesses the highest ionic mobility and unless the migra-

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tion of H^+ is controlled, these ions penetrate through all zones and, as a result, isotachophoretic migration is disturbed[4–6].

As shown earlier[4], uncontrolled migration of the front of H^+ ions occurs particularly in unbuffered systems, where the anion of a strong acid is used as a counter ion. In such a system the zone of a cation M^+ is always a mixed zone of M^+ and H^+, with more mobile H^+ ions penetrating easily through the boundaries of the M^+ zone. The result is that H^+ ions participate significantly in the flow of electrical current in the migrating zones and, instead of isotachophoretic migration, a moving boundary procedure takes place. This situation occurs particularly when an acidic solution is used as a terminator or when free H^+ ions are generated by electrode reactions on the terminating electrode.

In buffered cationic systems, free migration of H^+ ions is hindered by the buffering counter-ion system, *i.e.*, the anion of a weak acid accompanied by the undissociated free acid. Migrating H^+ ions recombine with the free anion, re-establishing the dissociation equilibrium of the applied acid and in the terminating electrolyte and a zone with an increased concentration of the acid is therefore created, which keeps increasing in the direction of the cationic migration. It can be said that the front of H^+ ions migrates at a certain speed through the given system. If this speed does not exceed the migration velocity of the zones of the separated substances, then H^+ ions do not disturb the separation, and H^+ can be applied successfully as a terminator for the given substances in the given system of electrolytes, which was verified experimentally[5,7,8]. If this speed is greater than the migration velocity of the zones, then H^+ ions penetrate through the zones and the isotachophoretic migration of these substances is disturbed.

As both theoretical and experimental work[9] has already shown, the front of H^+ ions does not interfere with the isotachophoretic analysis provided that the boundary between this front and the last zone of the sample has self-sharpening properties.

This paper presents another approach to the above problem, based on a comparison of effective mobilities, where the migrating front of H^+ ions is considered as a hydrogen-ion constituent[10–12] possessing a certain effective mobility. Based on theoretical considerations, a method is suggested for the calculation of the effective mobility of H^+ ions in cationic systems. The method has been verified experimentally and its application to the selection of suitable electrolyte systems and to the prediction of possible interfering effects in the given cationic system is considered. The theory and its experimental verification are limited to monovalent counter ions, which accords with practice and also makes it possible to express the effective mobility of H^+ ion in a simple explicit form.

THEORETICAL

If a zone λ of the leading electrolyte, containing a weak acid HA and its alkali metal salt L^+A^-, and a zone ϑ of the terminator, containing free weak acid HA, migrate isotachophoretically, then the migration velocity of boundary $\lambda\vartheta$ is equal to that of the leading cation:

$$v_{\lambda\vartheta} = E_\lambda u_L \qquad (1)$$

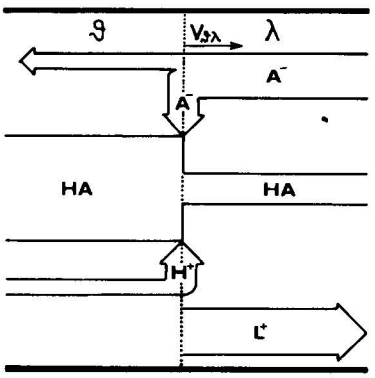

Fig. 1. Schematic diagram of the migrating reaction boundary.

where E_λ is the potential gradient in zone λ and u_L is the electrophoretic mobility of cation L^+. The H^+ ion is present virtually only on one side of the boundary $\lambda\vartheta$ (its concentration in zone λ is neglected) and thus in the sense of the authors'[10,11,13,14] definition of the effective mobility of weak electrolytes it holds that

$$\bar{u}_{H,\vartheta} = \frac{v_{\lambda\vartheta}}{E_\vartheta} \tag{2}$$

where $\bar{u}_{H,\vartheta}$ is the effective mobility of the H^+ ion in zone ϑ. The boundary $\lambda\vartheta$ is a migrating reaction boundary[14], at which the H^+ ion reacts with the counter ion A^- to form the slightly dissociated weak acid HA (see Fig. 1). The mass balance for the H^+ ion can be expressed as follows:

$$v_{\lambda\vartheta}\, c_{H,\vartheta} = E_\vartheta\, u_H\, [H]_\vartheta + v_{\lambda\vartheta}\, [HA]_\lambda \tag{3}$$

where c is the total concentration. By rearranging and combination with eqn. 2, a relationship is obtained for the effective mobility of the H^+ ion:

$$\bar{u}_{H,\vartheta} = u_H \cdot \frac{[H]_\vartheta}{c_{H,\vartheta} - [HA]_\lambda} \tag{4}$$

By expressing the condition of constant current density in the form

$$E_\vartheta\, [H]_\vartheta\, (u_A + u_H) = E_\lambda\, [L]_\lambda\, (u_A + u_L) \tag{5}$$

and by combination with eqns. 1 and 3, the adjusted concentration of acid HA in the terminating zone is given by

$$c_{H,\vartheta} = [L]_\lambda \cdot \frac{u_H}{u_L} \cdot \frac{u_A + u_L}{u_A + u_H} + [HA]_\lambda \tag{6}$$

By expressing the dissociation constant of acid HA, $K_{HA} = [H][A]/[HA]$, and by

employing the condition of electroneutrality, $[H]_\vartheta = [A]_\vartheta$, the concentration $[H]_\vartheta$ can be expressed as

$$[H]_\vartheta = \frac{1}{2}\left[\sqrt{K_{HA}^2 + 4\,K_{HA}\,c_{H,\vartheta}} - K_{HA}\right] \tag{7}$$

By combining eqns. 4, 6 and 7, the final relationship for the effective mobility of the H^+ ion in the terminator zone is obtained in the form

$$\bar{u}_{H,\vartheta} = u_L \cdot \frac{u_A + u_H}{u_A + u_L} \cdot \frac{K_{HA}}{2\,[L]_\lambda} \cdot$$

$$\left[\sqrt{1 + \frac{4}{K_{HA}}\left([L]_\lambda \cdot \frac{u_H}{u_L} \cdot \frac{u_A + u_L}{u_A + u_H} + [HA]_\lambda\right)} - 1\right] \tag{8}$$

Eqn. 8 expresses the value of $\bar{u}_{H,\vartheta}$ explicitly as a function of the mobilities of the participating ions, of the concentrations of the leading ion and the free acid in zone λ, $[L]_\lambda$ and $[HA]_\lambda$, respectively, and of the dissociation constant of the acid, K_{HA}. This equation is suitable for numerical calculations of the value of $\bar{u}_{H,\vartheta}$.

When the leading zone does not contain free acid HA, the quantity $[HA]_\lambda$ disappears from eqns. 4, 6 and 8. Eqn. 4 has then the form identical with the classical Tiselius definition of the effective mobility[15], which is commonly used in isotachophoretic practice[16]. Forms of the other equations are obvious.

EXPERIMENTAL

The isotachophoretic column used, the stabilized-current supply and the potential-gradient detector have been described earlier[17,18]. The effective mobilities were evaluated from the step heights in the potential-gradient record with the aid of the relationship[18] $\bar{u}_2 = \bar{u}_1\, h_1/h_2$, using tabulated (25 °C)[19] values of mobilities of the leading cations. For extrapolating the values obtained to zero heating in the column, the procedure described earlier[20] was adopted. On measuring the model potassium–sodium boundary, the difference between the mobility u_{Na} obtained in the way and the tabulated value[19] was less than 2%.

Acetic acid, formic acid, their potassium salts, tris(hydroxymethyl)aminomethane (Tris) and the other chemicals used were of analytical-reagent grade (Lachema, Brno, Czechoslovakia).

RESULTS AND DISCUSSION

The experimental verification of the method suggested for the calculation of the effective mobilities of the H^+ ion was performed for selected model systems (Table I). A 0.05 *M* solution of formic acid served as the terminator in all instances. Table I and Fig. 2 compare the experimental values of $\bar{u}_{H,\vartheta}$ with theoretical values calculated according to eqn. 8. Tabulated values of mobilities (at 25°C)[19] and dissociation constants[21] (the latter corrected for the ionic strengths of the solutions) were used for the calculation. It can be seen from Table I that, in accord with eqn. 8, $\bar{u}_{H,\vartheta}$

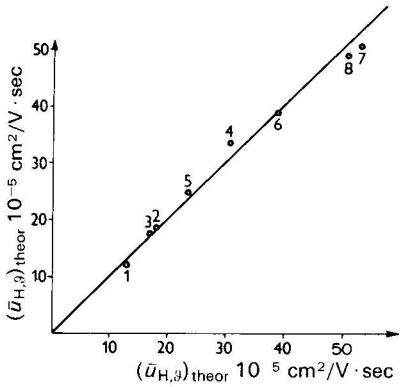

Fig. 2. Comparison of calculated and measured values of $\bar{u}_{H,\vartheta}$ for selected model systems (*cf.*, Table I).

decreases with increasing pK_{HA} of the counter anion [*cf.*, points 1, 5 and 6 in Fig. 2, when for 0.01 *M* solutions the effective mobility decreases in the series formate (pK_{HA} = 3.75), benzoate (pK_{HA} = 4.20) and acetate (pK_{HA} = 4.76)], for a given pK_{HA}, $\bar{u}_{H,\vartheta}$ increases with decreasing concentration of the leading electrolyte, $[L]_\lambda$ (*cf.*, points 1, 2 and 6, 7 in Fig. 2), and addition of free acid to the leading solution causes an increase in $\bar{u}_{H,\vartheta}$ (*cf.* points 1, 3 and 6, 8 in Fig. 2).

The agreement between the theoretical and experimental data is good and confirms the applicability of eqn. 8 to practical calculations. The method of calculation (*cf.*, eqn. 4) differs from the classical formulation of the effective mobility[15] according to which $\bar{u}_{H,\vartheta} = u_H[H]_\vartheta/c_{H,\vartheta}$. For the systems with a value of $[HA]_\lambda$ different from zero the classical relationship leads to wrong values of $\bar{u}_{H,\vartheta}$ (*e.g.*, for system 3, $\bar{u}_{H,\vartheta} = 10.0 \cdot 10^{-5}$ $cm^2/V \cdot sec$ is obtained, which is almost half the experimental value or the value calculated according to eqn. 4 or 8; *cf.*, Table I).

The calculation of the effective mobility of the terminating H^+ ion is important

TABLE I

COMPARISON OF THE CALCULATED AND MEASURED EFFECTIVE MOBILITIES OF H^+, $(\bar{u}_{H,\vartheta})_{theor}$ AND $(\bar{u}_{H,\vartheta})_{exp}$, RESPECTIVELY, IN VARIOUS MODEL LEADING SYSTEMS

The average relative difference between the theoretical and experimental values, listed in the table, is 4.5%.

No.	*Leading electrolyte*	$(\bar{u}_{H,\vartheta})_{theor}$ $(10^{-5}$ $cm^2/V \cdot sec)$	$(\bar{u}_{H,\vartheta})_{exp}$ $(10^{-5}$ $cm^2/V \cdot sec)$
1	0.01 *M* potassium acetate	13.0	12.3
2	0.005 *M* potassium acetate	18.1	18.6
3	0.01 *M* potassium acetate + + 0.01 *M* acetic acid	17.1	17.5
4	0.003 *M* potassium acetate + + 0.003 *M* acetic acid	30.6	33.7
5	0.01 *M* sodium benzoate	23.5	25.0
6	0.01 *M* potassium formate	38.8	38.8
7	0.005 *M* potassium formate	53.3	50.7
8	0.01 *M* potassium formate + + 0.01 *M* formic acid	51.0	49.1

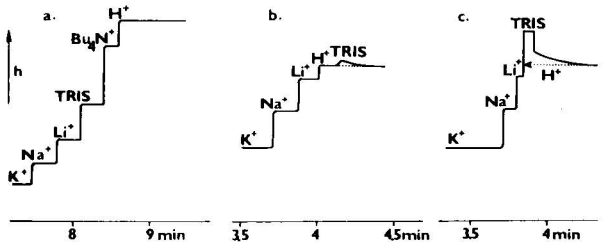

Fig. 3. Experimental results of analyses of a model mixture of cations. Leading electrolyte: (a) system 1, (b, c) system 4 (*cf.*, Table I). Terminator: 0.05 *M* formic acid. Sample: (a) 10 μl of 0.003 *M* $(C_4H_9)_4N^+Br^-$, 2 μl of 0.005 *M* Tris, 0.5 μl of 0.02 *M* LiCl, 0.7 μl of 0.015 *M* NaCl; (b) 1 μl of 0.002 *M* LiCl + 0.002 *M* NaCl + 0.00002 *M* Tris; (c(0.5 μl of LiCl + NaCl + ris (each 0.002 *M*). Driving current: (a) 100 μA, (b, c) 60 μA.

for the correct selection of electrolyte systems for practical separations. Fig. 3a shows the record of a cationic isotachophoretic analysis of a model mixture containing sodium, lithium, Tris and tetra-*n*-butylammonium in system 1 (*cf.*, Table I). Correct isotachophoretic migration of zones of all four components being separated can be seen, as the effective mobility of the terminator, $(\bar{u}_{H,9})_{theor} = 13.0 \cdot 10^{-5}$ cm^2/V · sec, is less than the mobility[19] of the slowest component being separated, $u_{Bu_4N} = 20.2 \cdot 10^{-5}$ cm^2/V · sec. Fig. 3b shows an analysis in system 4 (Table I), where the effective mobility of the terminating H^+ ion lies between the mobilities of Li^+ and Tris. In accord with the theory, isotachophoretic zones are formed by sodium and lithium only, while the slower Tris migrates in the terminator in a zone-electrophoretic manner[22,23]. When a large amount of Tris was injected (Fig. 3c), the H^+ front did not penetrate through the diffusion zone of Tris completely within the time of

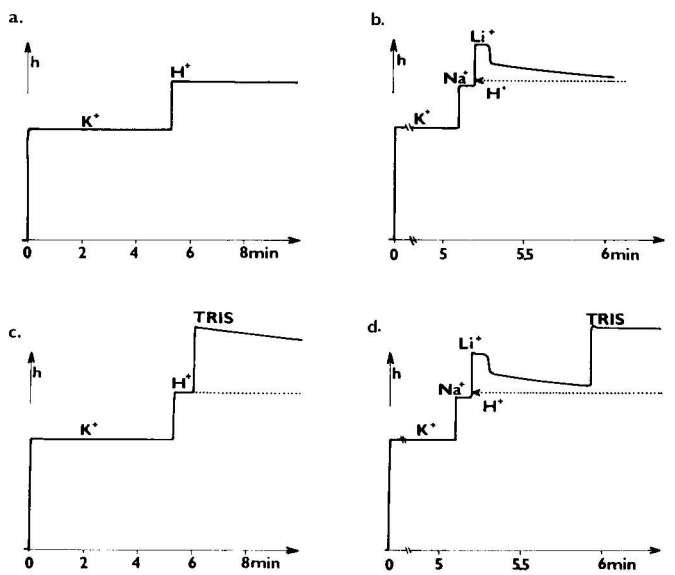

Fig. 4. Experimental results of analyses in system 8 (*cf.*, Table I). Terminator: (a, b) 0.05 *M* formic acid; (c, d) 0.1 *M* Tris. Sample (b, d): 3 μl of 0.002 *M* LiCl + 0.002 *M* NaCl. Driving current: 200 μA.

analysis. It can be seen that the front part of the Tris zone is still homogeneous, whereas its rear shows a diffusion tail. The slope and the length of the diffusion part of the zone depend on u_{Tris} and on the analysis time[22].

A knowledge of the effective mobility of the H^+ ion is important even in "classically" terminated cationic isotachophoresis, because here also the control of the migration behaviour of this ion must be ensured. In system 8 (Table I) the H^+ ion possesses a relatively high effective mobility, $(\bar{u}_{H,9})_{theor}/u_K = 0.669$ (*cf.*, Fig. 4a). When running the same model mixture as in Fig. 3a, it is only sodium that provides an isotachophoretic zone (*cf.*, Fig. 4b, for an analysis of a mixture of Na^+ and Li^+). Here the isotachophoretic zone of sodium is followed by a mixed zone of lithium with H^+ ion, analogous to the migration of Tris in Fig. 3c. If a 0.1 *M* solution of Tris is used as terminator for system 8 (Fig. 4c), with respect to the sequence of the mobilities $\bar{u}_{H,9} > u_{Tris}$, Tris migrates in a frontal manner inside the zone of H^+ ion, which is the real terminator. The analysis of a mixture of Na^+ and Li^+ under the given conditions provides an obscure record of the analysis (Fig. 4d). Based on the above comments on Fig. 4a–c, and with respect to the sequence of mobilities $u_{Na} > \bar{u}_{H,9} > u_{Li} > u_{Tris}$, Fig. 4d can clearly be interpreted. It is only sodium that provides a correct isotachophoretic zone. Lithium and Tris provide mixed zones with H^+ ion and migrate in a zonal or frontal manner, respectively. Thus, H^+ ion serves as background electrolyte for the Li^+ and Tris zones. Analogously, earlier described[4] cases of non-isotachophoretic zone behaviour can be similarly interpreted on the basis of a knowledge of the effective mobility of the H^+ ion.

CONCLUSION

The migration behaviour of the H^+ ion in cationic isotachophoresis in an acidic medium can be described by means of the effective mobility, $\bar{u}_{H,9}$, defined by eqn. 4 or 8. In order to obtain correct isotachophoretic migration it is necessary that the front of the H^+ ion does not pass through the boundary between the terminator and the last zone, *i.e.*, that its effective mobility is lower than the mobility of the slowest cation being separated. If for any cation M^+ $\bar{u}_{H,9} > u_M$, then H^+ ion penetrates through its zone and causes the isotachophoretic character of the migration to deteriorate; M^+ migrates in a zonal or frontal way on the background of the H^+ ion.

According to eqn. 8, $\bar{u}_{H,9}$ is dependent only on the parameters of the leading electrolyte. The method proposed here enables one to predict theoretically $\bar{u}_{H,9}$ for the given leading system and to compare the value thus obtained with the tabulated values of the mobilities of the substances being separated. It is thus possible to evaluate the suitability of a given system for a required separation. As a rule it can be analogously formulated for the selection of the terminator that it is not advisable to terminate a given leading electrolyte (which determines the value of $\bar{u}_{H,9}$) with a cation M^+ for which $u_M < \bar{u}_{H,9}$.

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CHROMATOGRAPHIC STUDY OF OPTICAL RESOLUTION

VIII. THEORETICAL STUDY OF THE CHROMATOGRAPHIC BEHAVIOUR OF THE ENANTIOMERS OF RACEMIC COMPLEX CATIONS ON A CATION-EXCHANGE COLUMN

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SUMMARY

Optical resolution of several mono- and divalent complex cations was achieved with a cation-exchange column using a solution containing various concentrations of a resolving agent as the eluent. The retention volumes of enantiomers, the differences in their retention volumes and the separation factors were determined for various concentrations of the eluent. The trends in these parameters could be expressed by functions derived from the equilibrium expressions of ion association and ion exchange.

INTRODUCTION

In previous papers of this series[1,2] the separation mechanism of enantiomers of metal complexes was discussed mainly from the stereochemical point of view. No consideration was given to the optimal conditions for optical resolution based on the general theory of chromatographic separation. This also applies to most other papers on the chromatographic resolution of racemic mixtures[3]. There have been few papers in which attempts have been made to improve the efficiency of optical resolution by systematically varying the separation conditions. Mikeš *et al.*[4] reported that the separation factor for the enantiomers of helicenes depends on temperature and has an optimum at a certain temperature. Davankov *et al.*[5] reported that in ligand-exchange chromatography using Cu^{2+} complex formation a decrease in temperature or in the concentration of a displacing ligand in an eluent solution leads to an increase in the separation factor of D- and L-prolines. In Part V of this series[6] we reported that *cis*-$[Co(N_3)_2(en)_2]^+$ was resolved into enantiomers on a cation-exchange column and that the separation factor of the enantiomers increases with increasing concentration of the chiral selector anion, antimony *d*-tartrate, $[Sb_2(d\text{-tart})_2]^{2-}$, in the eluent. How-

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ever, no papers appear to have considered the optical resolution from the point of view of chromatographic separation theory. In this paper, we discuss the separation mechanism of enantiomers and the dependence of the separation factor on the concentration of a chiral selector anion in an eluent.

THEORETICAL

Let us consider the case in which a monovalent complex cation M^+ is eluted on a column packed with a cation exchanger. An aqueous solution containing a divalent anion X^{2-}, which potentially forms ion pairs with the complex cation M^+, is used as the eluent. On increasing the concentration of X^{2-}, the concentration of the negatively charged ion pair MX^- increases. This effect overlaps with the effect of the cation exchange and leads to a decrease in the adsorption of the complex cation M^+ Thus, we have to consider two equilibria, ion exchange and ion pair formation.

The ion-exchange equilibrium is

$$RNa + M^+ \rightleftharpoons RM + Na^+$$

where RNa and RM are sodium and complex cations retained by the resin, respectively, and the equilibrium constant K_1 is defined by

$$K_1 = \frac{[RM][Na^+]}{[RNa][M^+]} \tag{1}$$

The ion-pair equilibrium is

$$M^+ + X^{2-} \rightleftharpoons MX^-$$

where the association constant β is defined by

$$\beta = \frac{[MX^-]}{[M^+][X^{2-}]} \tag{2}$$

As the total concentration of the complex M^+ is low in both the resin and eluent phases, *i.e.*, $[Na^+] \gg [M^+]$ and $[RNa] \gg [RM]$, the distribution ratio D_M is described, to a good approximation, by

$$D_M = \frac{[RM]}{[M^+] + [MX^-]} = \frac{K_1}{1 + \beta[X^{2-}]} \cdot \frac{Q}{[Na^+]} \tag{3}$$

where Q is the ion-exchange capacity of the resin (mequiv./ml).

The adjusted retention volume, V_{adj}, for the elution of M^+ can be related to D_M by

$$V_{adj} = D_M V_{resin} \tag{4}$$

where V_{resin} is the volume of the resin in the column.

Hereafter the adjusted retention volume is called simply the retention volume and described by eqn. 5 below. By replacing D_M in eqn. 4 with eqn. 3, we obtain

$$V = \frac{V_{resin} K_1 Q}{1 + \beta [X^{2-}]} \cdot \frac{1}{[Na^+]}$$

$$= \frac{0.5 V_{resin} K_1 Q}{[X^{2-}] + \beta [X^{2-}]^2} = \frac{C_1}{[X^{2-}](1 + \beta [X^{2-}])^2} \quad (5)$$

where $[Na^+] = 2[X^{2-}]$ and $0.5 V_{resin} K_1 Q$ can be regarded as a constant C_1. As the monovalent complex cation M^+ exists in two enantiomeric forms, Δ-M^+ and Λ-M^+, we have to discriminate the quantities concerning them by using the subscripts Δ and Λ. The retention volumes V_Δ and V_Λ are plotted against the concentration of X^{2-} according to eqn. 5 in Fig. 1a, where β_Λ is assumed to be greater than β_Δ. The difference in their retention volumes, $\Delta V = V_\Delta - V_\Lambda$, can be expressed by

$$\Delta V = \frac{C_1 (\beta_\Lambda - \beta_\Delta)}{(1 + \beta_\Delta [X^{2-}])(1 + \beta_\Lambda [X^{2-}])}$$

$$= \frac{C_2}{(1 + \beta_\Delta [X^{2-}])(1 + \beta_\Lambda [X^{2-}])} \quad (6)$$

where $C_1 (\beta_\Lambda - \beta_\Delta)$ is a constant C_2. Fig. 1b shows the plot of eqn. 6.

The separation factor α is given by

$$\alpha = \frac{V_\Delta}{V_\Lambda} = \frac{1 + \beta_\Lambda [X^{2-}]}{1 + \beta_\Delta [X^{2-}]} \quad (7)$$

The separation factor varies with $[X^{2-}]$ and reaches a constant value $\beta_\Lambda/\beta_\Delta$ at a very large value of $[X^{2-}]$ (Fig. 1c). For a particular enantiomeric pair β_Δ and β_Λ have

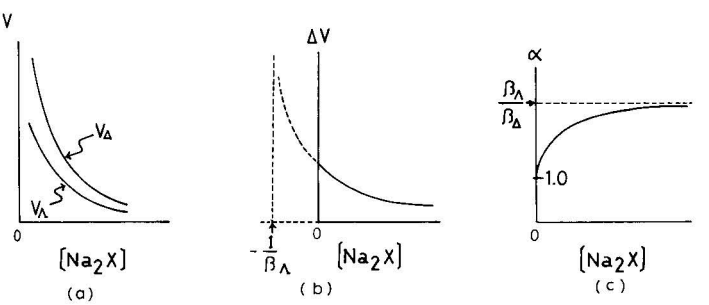

Fig. 1. (a) Dependence of adjusted retention volumes (V) on the concentration of Na_2X. V_Δ and V_Λ are V values for the Δ- and Λ-enantiomers, respectively. (b) Dependence of difference in retention volume (ΔV) on the concentration of Na_2X. (c) Dependence of separation factor (α) for the enantiomers on the concentration of Na_2X.

definite values, so that the separation of two elution peaks (ΔV) and the separation factor α have definite values for a definite value of $[X^{2-}]$. When the separation factor α is fairly large, a good separation (ΔV) is obtained with an appropriate concentration of $[X^{2-}]$. However, when the separation factor is small, we have to use a very low concentration of X^{2-} for a good separation (ΔV) (see Fig. 1b). A very long time would be required for elution. To shorten the elution time, it is necessary to use a concentrated eluent which gives a small ΔV. To resolve this dilemma, we have to use a longer column. If we double the column length, we double the separation (ΔV). Thus, we can rewrite eqns. 5 and 6 as a function of the length of the column:

$$V = \frac{C_1'}{(1 + \beta [X^{2-}])([X^{2-}])} \cdot L \tag{5'}$$

$$\Delta V = \frac{C_2'}{(1 + \beta_\Delta [X^{2-}])(1 + \beta_\Lambda [X^{2-}])} \cdot L \tag{6'}$$

From eqn. 6′ we obtain

$$L = \frac{\Delta V (1 + \beta_\Delta [X^{2-}])(1 + \beta_\Lambda [X^{2-}])}{C_2'} \tag{8}$$

Eqn. 8 is plotted in Fig. 2a.

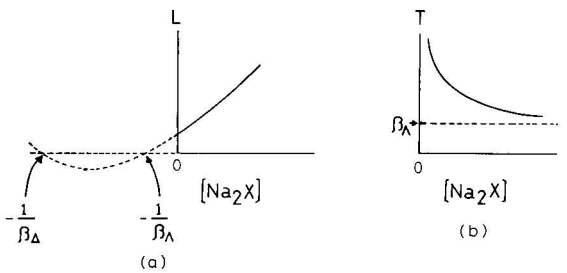

Fig. 2. (a) Column length (L) at various concentrations of Na_2X when the difference in retention volumes for enantiomers is constant. (b) Time (T) required for the second enantiomer (Λ-form) to be eluted at various concentrations of Na_2X, when the difference in the retention volumes of the enantiomers is constant.

We introduce the time (T) which is required for the second enantiomer to be eluted. Let F be a constant flow-rate, then $T = V/F$. From eqns. 5′ and 8

$$T = \frac{C' L}{[X^{2-}] + \beta_\Delta [X^{2-}]^2} \cdot \frac{1}{F}$$

$$= \frac{C_1'}{C_2'} \cdot \frac{\Delta V (1 + \beta_\Delta [X^{2-}])(1 + \beta_\Lambda [X^{2-}])}{[X^{2-}] + \beta_\Delta [X^{2-}]^2} \cdot \frac{1}{F}$$

$$= C_3 \left(\frac{1}{[X^{2-}]} + \beta_\Lambda \right) \tag{9}$$

where $C_3 = C_1'/(C_2' F)$. Eqn. 9 is plotted in Fig. 2b. Thus, when the separation factor is small, higher concentrations of the eluent and longer column lengths are recommended in order to obtain a good separation.

EXPERIMENTAL

Materials

The complexes used were *cis*(O)–*cis*(N)-$[CO(gly)_2en]Cl \cdot H_2O$ (ref. 7), *cis*(O)-*trans*(N)-*cis*(NH_3)-$[Co(gly)_2(NH_3)_2]Cl \cdot H_2O$ (ref. 8), $[Cogly(en)_2]Cl_2 \cdot H_2O$ (ref. 9), $[Cogly(tn)_2]Cl_2 \cdot H_2O$ (ref. 10) and *cis*-α-$[Co(N_3)_2trien]I$. The first four complexes were prepared according to the methods described in the literature, and the last complex was prepared as follows.

A solution containing 3.2 g of *cis*-α-$[CoCl_2trien]Cl$ in 50 ml of water was mixed with a solution of 2 g of sodium azide in 15 ml of water and heated at 60°C for 30 min. After the solution had been allowed to stand at room temperature, dark violet crystals were deposited, which were presumed to be the azide salt of the desired complex and were separated by filtration. The crystals were converted into the iodide by dissolving them in a small amount of water and adding an excess of sodium iodide. The dark violet crystalline powder was filtered, washed with ethanol and dried in air. The β-isomer of this complex was prepared from *cis*-β-$[CoCl_2trien]Cl$ by the same method in order to identify the isomers.

All complexes used were identified by elemental analyses and PMR and UV absorption spectroscopy.

The eluents used were aqueous solutions of various concentrations of sodium antimony *d*-tartrate dihydrate, $Na_2[Sb_2(d\text{-tart})_2] \cdot 2H_2O$, which was prepared from $K_2[Sb_2(d\text{-tart})_2] \cdot 3H_2O$ and sodium perchlorate.

Apparatus

The chromatographic unit was a JASCO LCP-150 pump, PM-150 pressure gauge, PC-150 pump controller, septum injection kit and Shimazu UV-140 double-beam spectrophotometer.

The column was a 20-cm × 3 mm I.D. stainless-steel tube packed with IEX-510 strong-acid cation-exchange resin (Toyo Soda, Japan). A dual-pen strip-chart recorder (Rigakudenki Electronic Recorder Model B-161) was used. The detector, equipped with a 20-μl flow cell, was operated at the first absorption band of each complex. Blue Dextran 2000 (Pharmacia, Sweden) was used as a marker to measure the void volume of the column.

RESULTS AND DISCUSSION

To establish whether the conclusions in the theoretical section are valid in actual chromatographic runs, chromatographic separations of five racemic complexes were performed. The complexes *cis*-α-$[Co(N_3)_2trien]^+$ and $[Co(gly)_2(NH_3)_2]^+$ have already been reported to be separable into enantiomers, but the other three complexes were found to be separable for the first time in this study. Typical elution curves of mono- and divalent complex cations eluted with 0.06 and 0.1 *M* $Na_2[Sb_2(d\text{-tart})_2]$ solution are shown in Figs. 3–5. Fig. 6 shows the dependence of retention volumes,

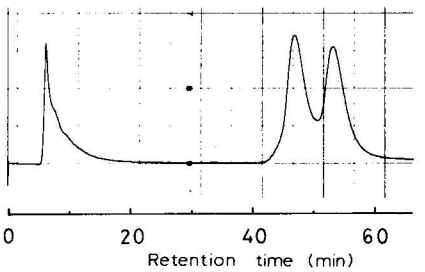

Fig. 3. Elution curve for *cis*-α-$[Co(N_3)_2trien]^+$. Eluent: 0.06 *M* $Na_2[Sb_2(d\text{-tart})_2]$.

differences in retention volumes and separation factors on the eluent concentration for the enantiomers of the complexes.

There is a close resemblance between the curves in Fig. 1 and the corresponding curves in Fig. 6, which indicates the validity of the above theory. As shown in Fig. 6 for the monovalent complexes, the separation factor increases with increasing eluent concentration and reaches a certain limiting value at a high concentration of the eluent. This is just the trend that is expected from eqn. 7. On the other hand, for the divalent complexes the separation factor was found to be almost constant in the experimental concentration range, as shown in Fig. 6. In general, the association constant for the divalent complex cation is presumed to be fairly large so that the term $1 + \beta[X^{2-}]$ in eqn. 7 will come close to $\beta[X^{2-}]$. Thus, the separation factor reaches a definite value even at low concentrations of X^{2-}.

Next, let us compare the trends of the separation factors of two monovalent complexes, *cis*-α-$[Co(N_3)_2trien]^+$ and *cis*(O)-*cis*(N)-*cis*(NH_3)-$[Co(gly)_2(NH_3)_2]^+$. At low concentration of X^{2-} (0.02 *M*), the separation factor is smaller for the diazido complex than for the diammine complex. However, when the concentration of X^{2-} exceeds 0.1 *M*, the separation factor is larger for the diazido complex than for the

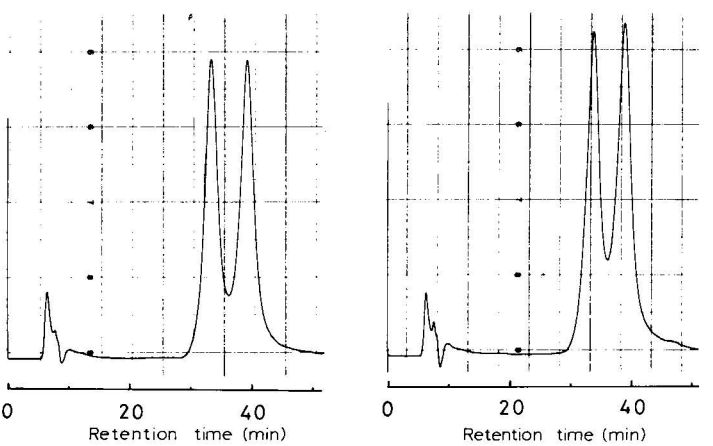

Fig. 4. (a) Elution curve for *cis*(O)-*cis*(N)-$[Co(gly)_2en]^+$. (b) Elution curve for *cis*(O)-*trans*(N)-*cis*(NH_3)-$[Co(gly)_2(NH_3)_2]^+$. Eluent: 0.06 *M* $Na_2[Sb_2(d\text{-tart})_2]$.

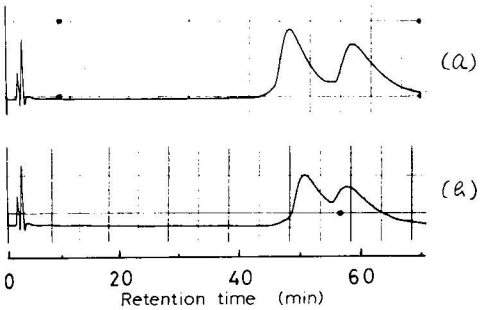

Fig. 5. (a) Elution curve for $[Cogly(en)_2]^{2+}$. (b) Elution curve for $[Cogly(tn)_2]^{2+}$. Eluent: 0.1 *M* $Na_2[Sb_2(d\text{-tart})_2]$.

diammine complex, as shown in Fig. 6. This can be explained by assuming that the association constant with antimony *d*-tartrate is smaller for the azido complex than for the diammine complex but the ratio β_A/β_A is larger for the former complex than for the latter.

Concerning the elution time, let us consider the case of the diazido complex (Fig. 6a). In this study we used a column length of 20 cm. The time (adjusted) required for the elution of the second peak was 21 min with 0.12 *M* eluent and the peak separation (ΔV) was 0.43 ml. With 0.02 *M* eluent, the elution time was 160 min and the peak separation (ΔV) was 1.17 ml. If we want a 1-ml peak separation with 0.02 *M* eluent, we have to use a 17-cm column length, which will require 137 min for elution. With 0.12 *M* eluent we have to use a 46.5-cm column and the elution time (adjusted will be 48.8 min. These relations are illustrated in Fig. 7. Thus, in order to obtain the same separation (ΔV) it is desirable, in order to save time, to use the longer column and more concentrated eluent than to use the shorter column and more dilute eluent.

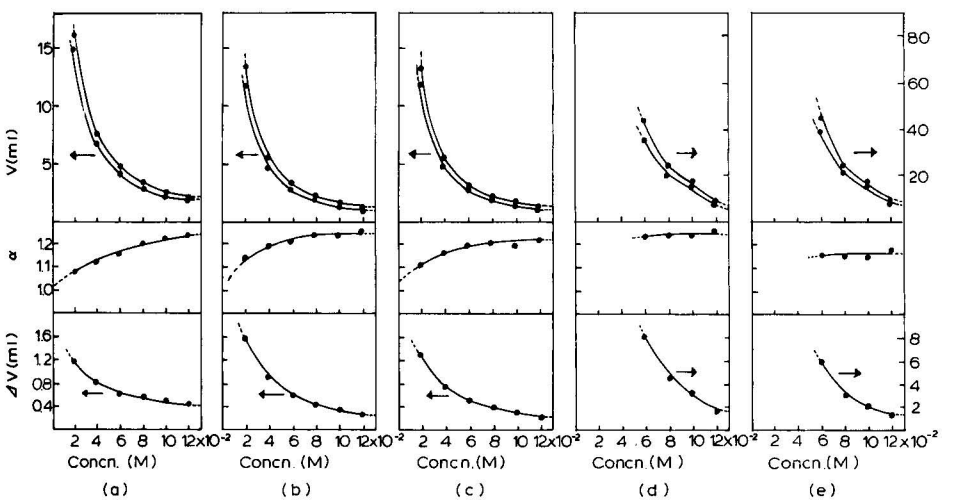

Fig. 6. Dependence of adjusted retention volumes (V), separation factor (α) and difference in retention volume (ΔV) for the enantiomers on the concentration of $Na_2[Sb_2(d\text{-tart})]$. (a) *cis*-α-$[Co(N_3)_2trien]^+$; (b) *cis*(O)-*cis*(N)-$[Co(gly)_2en]^+$; (c) *cis*(O)-*trans*(N)-*cis*(NH_3)-$[Co(gly)_2(NH_3)_2]^+$; (d) $[Cogly(en)_2]^{2+}$; (e) $[Cogly(tn)_2]^{2+}$.

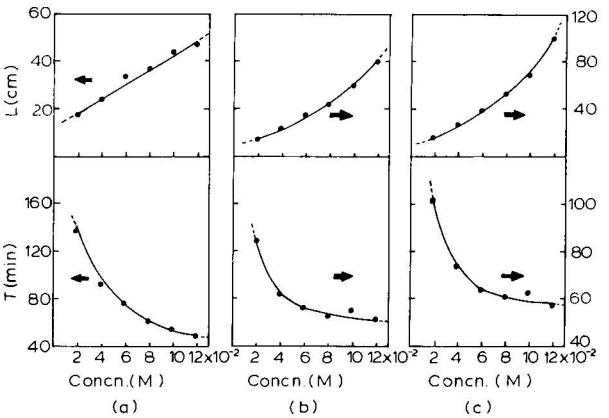

Fig. 7. Column length (L) and elution time (T) for the second enantiomer to be eluted at various concentrations of $Na_2[Sb_2(d\text{-tart})_2]$ when ΔV = 1 ml. (a) *cis*-α-$[Co(N_3)_2trien]^+$; (b) *cis*(O)-*cis*(N)-$[Co(gly)_2en]^+$; (c) *cis*(O)-*trans*(N)-*cis*(NH_3)-$[Co(gly)_2(NH_3)_2]^+$; (d) $[Cogly(en)_2]^{2+}$; (e) $[Cogly(tn)_2]^{2+}$.

CONCLUSIONS

(1) When the complex cation and the chiral selector anion associate only slightly with each other, the separation factor for the enantiomers increases with increasing concentration of the anionic chiral selector, and approaches a certain limiting value.

(2) When strong association takes place between the sample complex cation and the chiral selector anion, the separation factor is expressed as the ratio of the association constants of the enantiomers.

(3) At a constant column length, the lower the concentration of anionic chiral selector in the eluent, the larger is the difference in the retention volumes of the enantiomers.

(4) When the length of the column and the concentration of the chiral selector are both variable, the longer the column and the higher the concentration, the larger is the difference in the retention volume per unit time.

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COMPARISON OF THE DISTRIBUTION CONSTANTS OF ACETONE IN DIFFERENT CHROMATOGRAPHIC SORBENT–GAS SYSTEMS, DETERMINED BY DIRECT MEASUREMENT OF SORPTION EQUILIBRIA AND CALCULATED FROM GAS CHROMATOGRAPHIC RETENTION DATA

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SUMMARY

The distribution constants of acetone in different chromatographic sorbent–nitrogen systems were determined by direct measurement of sorption equilibria at gas-phase concentrations of acetone ranging from tens of ppb (10^9) to hundreds of ppm. Tenax GC, Porapaks P and Q, Apiezon K and silicone oil QF-1 were employed as sorbents. With Tenax GC and Porapak P, the sorption isotherms were almost linear up to concentrations of about 4 ppm of acetone in the gaseous phase, whereas with the other sorbents the isotherms were non-linear over the whole range of concentrations investigated. With the Tenax GC and Porapak P systems, the distribution constants determined at concentrations corresponding to the linear sections of the corresponding isotherms agreed with those calculated from gas chromatographic retention data within about 15% of the values determined.

INTRODUCTION

The concentration of trace components of gaseous and/or liquid materials by trapping them in columns packed with chromatographic sorbents can be carried out in conservation[1] or equilibration[2] modes. In both these modes, it is expedient to know the retention volume of the analyte on the trapping column, from which the so-called breakthrough volume and/or the equilibration (saturation) volume can be calculated. The retention volume, V_R, is given by

$$V_R = V_{M,t} + KW_S$$

$V_{M,t}$, K and W_S being the void volume of the trap, the analyte distribution constant (volume/mass) in the given system and the mass of sorbent in the trap, respectively.

In our previous work[3], the sorption of benzene was studied in different nitrogen–chromatographic sorbent (Tenax GC, Porapaks P and Q, Apiezon K, silicone oil QF-1, Reoplex 400) systems at benzene concentrations in the gaseous phase ranging from tens of ppb (10^9) to hundreds of ppm. The concentration limits of the linearity

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of the sorption isotherms of benzene in the individual systems were determined, and the limiting distribution constants of benzene were compared with those calculated from gas chromatographic (GC) retention data.

In order to assay systems with a polar analyte, the sorption behaviour of acetone on the same sorbents (except Reoplex 400) has now been studied. The concentrations of acetone in the gaseous phase were again varied from tens of ppb to hundreds of ppm.

EXPERIMENTAL

The sorbent materials as well as the instrumentation and procedures employed for the preparation of model gaseous mixtures and the measurements of sorption equilibria and retention data were as described earlier[3,4]. Acetone of analytical-grade purity (Lachema, Brno, Czechoslovakia) was used to prepare the model mixtures.

RESULTS AND DISCUSSION

The distribution constants of acetone, obtained by direct measurement of sorption equilibria in systems with Apiezon K, silicone oil QF-1, Porapaks Q and P and Tenax GC, are plotted against the logarithm of the acetone concentration in the gaseous phase in Figs. 1–5, respectively. Figs. 1 and 2 show that in the systems with liquid sorbents, *i.e.*, Apiezon K and QF-1, the sorption isotherms are non-linear over the entire range of acetone concentrations employed. This is contrary to what has been found for the sorption of benzene on the same sorbents. The non-linearity of the acetone isotherms is probably due to a significant adsorption of acetone at the gas–liquid and/or liquid–solid interfaces which occurs in addition to its dissolution in the bulk liquid[5]. Owing to the small surface areas of these interfaces (about 0.5 m^2/g), the

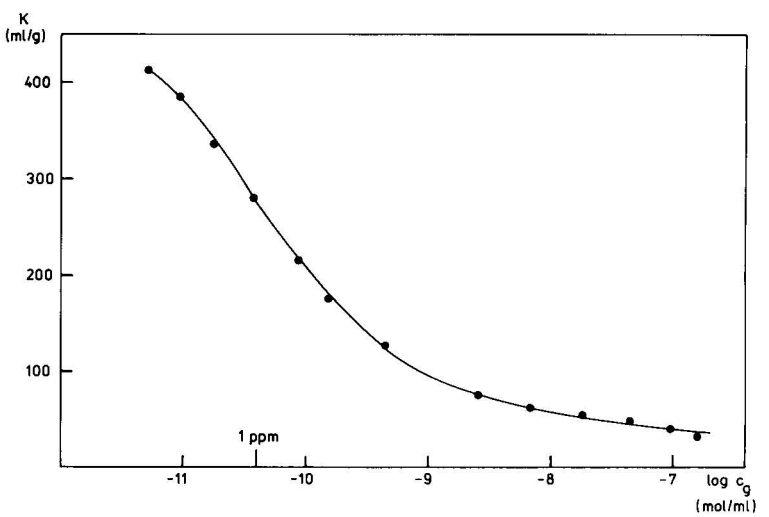

Fig. 1. Dependence of the distribution constant of acetone in the nitrogen–Apiezon K system on the logarithm of the concentration of acetone in the gaseous phase.

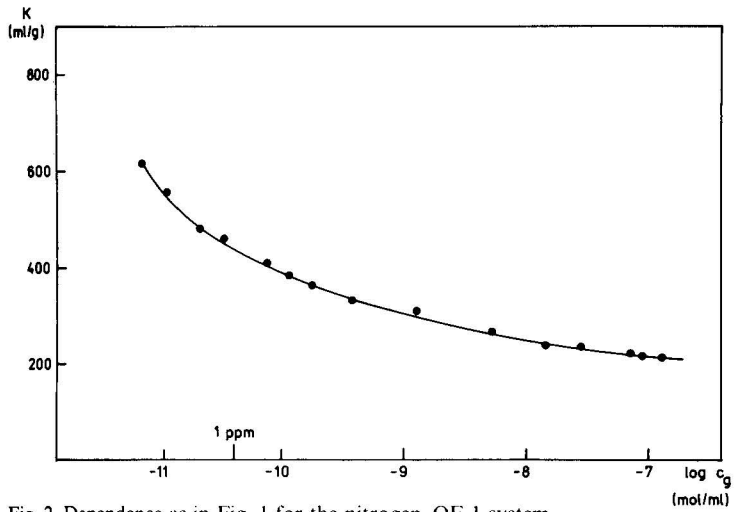

Fig. 2. Dependence as in Fig. 1 for the nitrogen–QF-1 system.

isotherms would probably become linear only at extremely low acetone concentrations.

From Figs. 4 and 5 it is seen that the sorption isotherms of acetone in the systems with Porapak P and Tenax GC are approximately linear up to several ppm of acetone in the gaseous phase. With Porapak Q there is no flat section on the *K versus* log c_g curve within the range of acetone concentrations examined, although for benzene on the same sorbent the corresponding curve was almost flat up to about 1 ppm benzene in the gaseous phase[4]. The cause of this different behaviour of acetone and benzene, on the Porapak Q employed, is not clear.

The acetone distribution constants obtained by direct measurement of sorption equilibria (K_s, K_L) are compared with those calculated from GC retention data (K_r) in

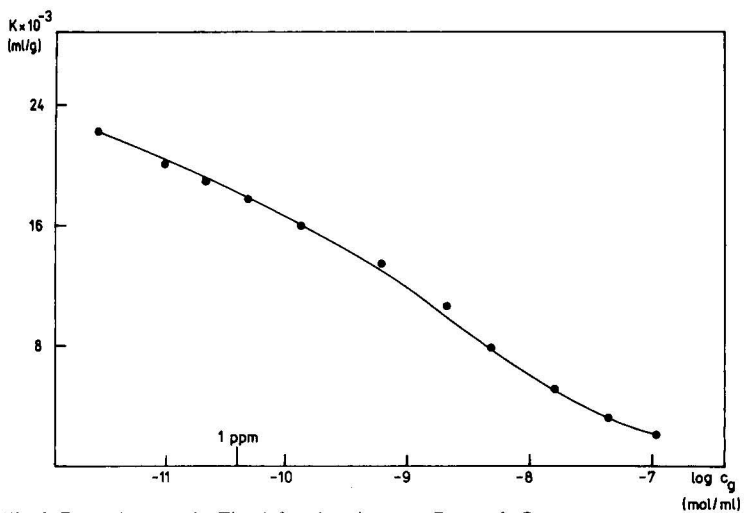

Fig. 3. Dependence as in Fig. 1 for the nitrogen–Porapak Q system.

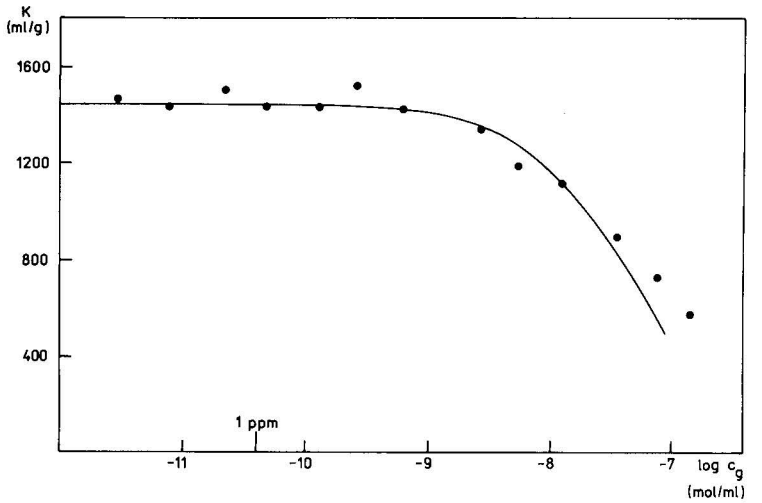

Fig. 4. Dependence as in Fig. 1 for the nitrogen–Porapak P system.

Table I. The K_s values were obtained by simply averaging the K_s values corresponding to the flat sections of the respective K *versus* log c_g curves, and the K_L values were obtained by processing the experimental c_g and c_s data (equilibrium concentrations of acetone in the gaseous phase and in the sorbent, respectively) in terms of Langmuir's isotherm. The K_r values were calculated from the specific retention volumes. The procedures used to calculate K_L and K_r were described in detail in our previous paper[4]. The specific retention volumes, V_g^0, of acetone used to calculate the K_r values were correlated with temperature by:

$$\log V_g^0 = A + B/T$$

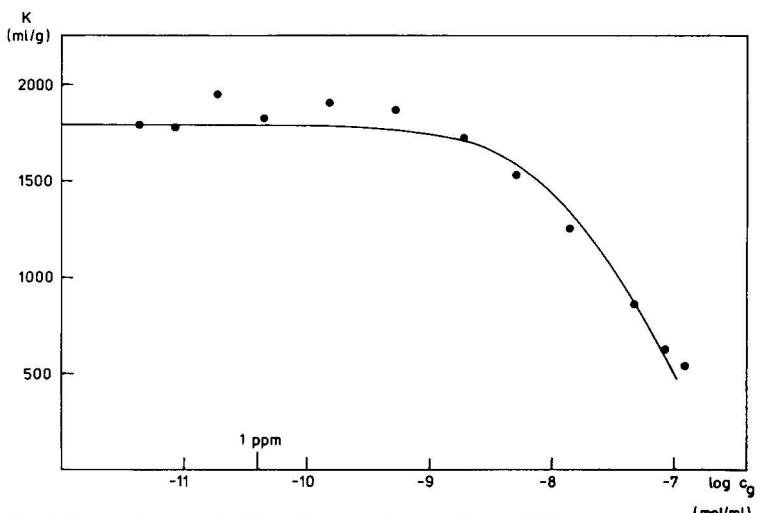

Fig. 5. Dependence as in Fig. 1 for the nitrogen–Tenax GC system.

TABLE I

CHARACTERISTICS OF THE SORPTION ISOTHERMS OF ACETONE

Sorbent	*Linearity limit (ppm)*	K_s *(ml/g)*	K_L *(ml/g)*	K_r *(ml/g)*
Tenax GC	*ca.* 4	1853.0	1787.4	1999.0
Porapak P	*ca.* 4	1462.2	1437.4	1257.9
Porapak Q	<0.1	–	–	13,803
Apiezon K	<0.1	–	–	93*
QF-1	<0.1	–	–	278.8

* The V_g^0 value was dependent on the size of the sample.

The constants A and B, together with the V_g^0 values at 35°C, are summarized in Table II.

With systems in which benzene was used as a model sorbate[4] there was a very good agreement between K_s, K_L and K_r, the maximum difference being less than 2.5%. However, for acetone, differences as large as 15% occur (*cf.*, Table I). It might have been thought that the larger differences between K_s and K_L, relative to benzene, are due to interactions between adsorbed acetone molecules, implying that Langmuir's model is invalid in this case. Upon closer inspection, however, this interpretation is found not to be the case. Thus, Table III shows the coefficients at r^{-6} for the orientation, induction and dispersion contributions to the intermolecular interaction energy[6], calculated for a temperature of 35°C from tabulated data on the polarizabil-

TABLE II

CONSTANTS IN $\log V_g^0 = A + B/T$ AND VALUES OF V_g^0 AT 35°C

Sorbent	A	B	V_g^0 *(35°C) (ml/g)*
Tenax GC	−6.372277	2964.62	1771.9
Porapak P	−5.640364	2677.10	1115.1
Porapak Q	−6.260764	3188.86	12,235.6
Apiezon K	−4.153805	1870.96	82.8
QF-1	−3.942671	1952.33	247.2

TABLE III

COEFFICIENTS AT r^{-6} FOR CONTRIBUTIONS (AT 308.15°K) TO THE INTERMOLECULAR INTERACTION ENERGY OF BENZENE AND ACETONE

Solute	*Coefficient at* r^{-6} $(J \cdot m^6 \times 10^{-79})$		
	Orientation	*Induction*	*Dispersion*
Benzene	0	0	1222.4
Acetone	1056.0	105.8	503.9

ities, ionization potentials and dipole moments of acetone and benzene[7]. It is seen that the sums of the coefficients for benzene are commensurate with those for acetone.

Let us suppose that the surface of the sorbent is homogeneous, that all its parts are equally accessible to the sorbate and that there is no diffusion of the sorbate into the bulk polymer. Then the area of the polymer surface available to each sorbate molecule is

$$A_1 = S/Kc_g N_A$$

where S is the specific surface area of the sorbent, N_A is the Avogadro constant and K and c_g are the distribution constant and the solute concentration in the gaseous phase, respectively. Values of A_1 (mm^2) for acetone and benzene at $c_g = 10^{-10}$ mole/ml and at assumed specific surface areas of Tenax GC, Porapaks P and Q equal to 15, 150 and 550 m^2/g, respectively, are shown in Table IV. The data in Tables III and IV indicate that, for acetone, the processing of c_g and c_s data in terms of Langmuir's model is at least as justifiable as for benzene. The relatively large scatter of the experimental points about the flat parts of the K *versus* log c_g curves for acetone suggests that the larger differences between the K_s and K_L values are probably due to errors incidental to the direct determination of K_s at very low c_g.

TABLE IV

SURFACE AREAS OF SORBENT AVAILABLE TO EACH MOLECULE OF SORBATE

The gas-phase concentration of sorbate was 10^{-10} mole/ml.

Sorbent	A_1 *(nm²)*	
	Benzene	*Acetone*
Tenax GC	12.8	139.2
Porapak P	200.9	1706.1
Porapak Q	200.7	571.0

However, the most important differences are those between the K_s and K_r values. These differences may have several causes, of which the following appear to be most relevant:

(1) The specific retention volumes were measured at concentrations in the migrating chromatographic zone that exceeded the limit of linearity of the corresponding respective sorption isotherm

(2) Slower sorption mechanisms, such as dissolution in bulk polymer, become operative during long contacts of the solute with the sorbent, but are not manifested significantly in the measurement of retention data

(3) Errors due to the extrapolation of retention data at lower temperatures from those measured at higher temperatures.

A result of the non-linearity of the sorption isotherm is illustrated in Table V, for determinations of acetone in model gaseous mixtures by equilibration trapping on Tenax GC; the values were calculated by means of GC retention data. The data for acetone concentrations of up to 3.5 ppm have been taken from a paper by Janák *et*

TABLE V

DETERMINATION OF THE CONCENTRATION OF ACETONE IN MODEL GASEOUS MIXTURES BY EQUILIBRATION TRAPPING ON TENAX GC[8]

Acetone present in model mixture		*Acetone found*		*Relative error*
		g/l	*ppm*	*(%)*
g/l	*ppm*			
4.8	2.0	5.0	2.1	+4
5.8	2.4	5.5	2.3	−5
7.8	3.3	7.2	3.0	−8
8.4	3.5	8.0	3.4	−5
15	6.3	11	4.6	−27
19	8.0	14	5.9	−26
53	22	22	9.2	−58
720	302	260	109	−64
1800	754	470	197	−74

al.[8], and those at higher acetone concentrations are unpublished results from the same work. The concentration limit beyond which the results become grossly inaccurate complies with the concentration limit of the linearity of the corresponding sorption isotherm (*cf.*, Fig. 5).

As discussed earlier[9], the sorption properties of different batches of a given sorbent can differ appreciably. For instance, the following values are available for V_g^0 (ml/g) of acetone on Tenax GC at 20°C: 5505 (this work); 5385 (ref. 10); 4215 (ref. 8) and roughly 4900 (ref. 11, calculated from a value of 3200 at 25°C).

CONCLUSIONS

In the absence of compounds capable of competitive sorption, the retention volumes and, consequently, also the breakthrough volumes of acetone on Porapak P and Tenax GC are practically independent of the acetone concentration in the gaseous phase up to several ppm. With Tenax GC, this conclusion is in accord with the results[8] of the determination of acetone in model gaseous mixtures by a method involving equilibration trapping on Tenax GC and with the findings by Brown and Purnell[10]. The error of the calculation of breakthrough volumes from GC retention data is appreciably larger with acetone (about 15%) than with benzene (about 2%).

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GAS CHROMATOGRAPHY OF HOMOLOGOUS ESTERS

XV. MOLECULAR RETENTION INDICES OF ALIPHATIC ESTERS*

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SUMMARY

Molecular retention indices (ΔMe) of aliphatic esters are examined for use in structure–retention relationships for tentative identification and contrasted with conclusions obtained from retention index plots and increments.

INTRODUCTION

The presentation of retention data as molecular retention indices (ΔMe) as an alternative to the use of retention indices (I) has been proposed by Evans and Smith. While the retention index is defined as the carbon number multiplied by one hundred of a hypothetical *n*-alkane having the same retention as the solute being considered, the molecular retention index considers the molecular weight of the same hypothetical alkane. This value, described as the effective molecular weight, is equivalent to the retention index. The difference between this value and the molecular weight of the compound is the molecular retention index (ΔMe). The relationships are shown in eqns. 1 and 2.

$$Me = 14.026\, I' + 2.016 = 0.14026\, I + 2.016 \quad (1)$$

$$\Delta Me = Me - M \quad (2)$$

where

$I = 100\, I'$

Me = effective molecular weight of the solute or of a hypothetical *n*-alkane with I' carbon atoms;

I' = number of carbon atoms in hypothetical *n*-alkane;

M = molecular weight of solute.

The early papers[1,2] suggested that ΔMe was a useful parameter for the correlation of chemical structure and chromatographic retention, and the the value was

* Part XIV: J. K. Haken, J. R. Chretien and C. Lion, *J. Chromatogr.*, 217 (1981) 125.

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virtually constant within a homologous series. For a proposed structure, M is known and ΔMe for the various structural features could be obtained from correlation charts. Other aspects of the ΔMe scheme have been described by Evans *et al.*[3–5].

Evans and Smith demonstrated these proposals to a limited extent and more recently (6) work with *n*-alkanoates, *n*-alkanols, *n*-alkanones and *n*-alkanals has confirmed the constancy of ΔMe on columns of low (SE-30), medium (OV-25) and reasonably high polarity (SILAR 10C). It was further apparent that the constancy was little influenced by polarity, although the values with all series increased with the phase polarity.

The present work considers ΔMe values of homologous compounds of one functional class as these values have been suggested to be of value in facilitating identification of closely related compounds, such as might be experienced with reaction mixtures. Esters where structural variations occur in both the alcohol or alkyl chain (R′) or in the acid or acyl chain (R) and in the presence of unsaturation have been studied, as considerable information relating retention behaviour and molecular structure has been reported based on retention plots or index increments[7–13].

EXPERIMENTAL

The analyses were carried out using 12 ft. × 1/4 in. O.D. aluminium columns with 10% of the stationary phases on Chromosorb W AW DMCS with isothermal operation at 150 C.

To increase the speed and accuracy of the determination of retention data, a Hewlett-Packard 5750 research gas chromatograph was interfaced with 32 K PDP 11/40 digital computer. The chromatograph employed a splitter and individually operated detectors with injection by a Hewlett-Packard 7670A automatic sampler. Calculations of dead time and retention indices were conducted within the system.

RESULTS AND DISCUSSION

Structure–retention relationships with homologous esters, RCOOR′, based on retention plots and retention index increments, have shown:

(1) The slopes of plots representing homologous esters with the same number of carbon atoms in the acid chain (R) decrease as the value of R is increased; similarly for the same number of carbon atoms in the alcohol chain (R′) the slopes decrease as the value of R′ increases with the phases considered[7–9].

(2) Retention of methyl esters follows boiling point.

(3) On stationary phases containing highly polarizable substituent groups it was evident that a loss of linearity occurs with alcohol ester plots.

(4) The effect of isomerization in the alcohol chain was most apparent with isopropyl esters and to lesser extents with isobutyl and isopentyl esters[13].

(5) It is apparent that the methylene group has a greater effect on retention when in the alcohol chain (R′) than in the acid chain (R), this effect having also been observed with α-alkyl acrylic esters.

(6) Retention increments for the carboxyl group in *n*-alkyl esters indicated that the net retention of the group increased with increasing polar character of the phase.

(7) With unsaturated esters the boiling point was of importance but two fur-

ther effects were experienced. The presence of a double bond in an alkyl group tended to reduce retention on a non-polar phase, the decrease being accentuated where conjugation occurred[10–12].

(8) With increasing polar character of the stationary phase, retention as 2-alkanoates increased with respect to the corresponding branched chain series due to screening of the carboxyl group[10–12].

(9) With increased phase polarity the retention of *cis* isomers of unsaturated esters was affected more than that of *trans* isomers.

Table I shows retention indices, *Me* and *ΔMe* values for homologous esters of varying structure determined on columns of low (SE-30), medium (OV-25) and substantial (SILAR 10C) polar character, together with values for *n*-alkyl acetates extracted from our earlier report[6], the data being considered in terms of the conclusions enumerated above.

With the data determined on a non-polar column (SE-30) it is evident that the approximate degree of constancy previously observed[6] with the homologous acetate esters is maintained with series of increasing carbon number of the acid or acyl chain from propionates to hexanoates. The *ΔMe* values, however, cannot be constant as it would be necessary for esters of the same carbon number to have identical retention.

Table II shows that the *n*-alkyl and isoalkyl alkanoates on all three phases show a decreasing average *ΔMe* value as the chain length R is increased, retention of the series also decreasing; this is in agreement with the earlier reports[7–9]. The effect is less apparent on the non-polar SE-30 column as the separations are greatly enhanced with increased polarity of the phase. The same consideration from a study of R′ variation is not possible.

It has been reported previously that comparison of retention index increments of *n*-alkyl alkanoates has shown that the methylene group has a greater effect on retention when in the alkyl or alcohol chain (R′) than when in the acid or acyl chain (R)[7–9]. Consideration of the data in Table I does not allow such a conclusion to be made, although consideration of the *ΔMe* values of the isoalkyl esters indicates that the reverse effect is apparent, *i.e.* the retention is lower with the isoalkyl hexanoates where the effect of the particularly compact shape of the isopropyl group is minimised[13].

The constancy that exists with the *n*-alkyl alkanoates is not as apparent with the *n*-alkyl iso-esters, although the *ΔMe* values of these series are lower than those of the straight-chain series.

Branching in both the R and R′ chains has similarly been shown to lead to unexpectedly low retention. The *ΔMe* values would, as described above, indicate that reduced retention is experienced as the molecular size is increased. The constancy of the series with branching in both chains is less evident, although the *ΔMe* values are again lower, as would be expected (Table III).

With increasing phase polarity the *ΔMe* values of *n*-alkyl alkanoates increase, as with homologous series of other functional classes[6]. The constancy of the *ΔMe* values however is poorer on both the OV-25 and SILAR 10C phases. With homologous series with branched R′ with R and R and R′ both branched the constancy of the series is poor, although the general *ΔMe* values in all cases increase with phase polarity.

Table II shows retention indices and *ΔMe* values of two unsaturated series of

TABLE I

RETENTION INDICES, EFFECTIVE MOLECULAR WEIGHT (*Me*) AND MOLECULAR RETENTION INDICES (*ΔMe*) OF ALIPHATIC ESTERS

	SE-30			*OV-25*			*SILAR 10C*		
	I	*Me*	*ΔMe*	*I*	*Me*	*ΔMe*	*I*	*Me*	*ΔMe*
Acetates									
Propyl	581.69	83.61	−18.52	733.97	104.97	2.85	1143.18	162.37	60.24
Butyl	678.82	97.23	−18.93	831.29	118.62	2.46	1228.09	174.28	58.12
Amyl	785.37	112.18	−18.01	932.48	132.81	2.62	1326.90	188.14	57.95
Hexyl	878.41	125.23	−18.99	1035.25	147.23	3.01	1424.64	201.85	57.63
Proponates									
Methyl							1087.5	154.55	99.43
Ethyl	576.70	82.91	−19.22	727.60	104.08	1.95	1127.07	160.10	57.97
Propyl	678.49	97.19	−18.97	833.35	118.91	2.75	1224.83	173.81	57.65
Butyl	782.16	111.73	−18.46	934.12	133.05	2.85	1327.25	188.19	58.00
Amyl	885.25	126.19	−18.03	1033.59	147.00	2.78	1413.9	200.34	56.12
Hexyl	980.35	139.53	−18.71	1131.84	160.78	2.54			
Butyrates									
Methyl							1196.18	169.79	67.66
Ethyl	671.38	96.19	−19.97	833.06	118.87	2.71	1229.2	174.42	58.26
Propyl	785.37	112.18	−18.01	932.31	132.79	2.60	1309.52	185.69	56.57
Butyl	881.62	125.68	−18.54	1029.89	146.48	2.26	1405.75	199.19	54.97
Amyl	979.0	139.34	−18.90	1126.49	160.03	1.79	1511.8	214.08	55.84
Hexyl	1083.14	153.95	−18.32						
Valerates									
Ethyl	782.28	111.75	−18.44	928.81	132.30	2.11	1309.36	185.68	55.49
Propyl	888.40	126.63	−17.59	1024.13	145.67	1.45	1396.12	197.85	53.63
Butyl	985.20	140.21	−18.03	1124.79	159.79	1.55	1488.85	210.86	52.62
Amyl	1082.47	153.85	−18.42	1223.79	173.68	1.41	1586.47	224.55	52.28
Hexyl	1185.53	168.31	−18.00				1639.2	238.9	52.66
Hexoates									
Ethyl	882.50	125.80	−18.42	1030.26	146.58	2.31	1400.97	198.53	54.31
Propyl	984.75	140.15	−18.09	1127.78	160.21	1.97	1497.14	212.02	53.78
Butyl	1087.67	154.58	−17.69	1228.09	174.28	2.01	1591.25	225.22	52.95
Amyl	1181.99	167.81	−18.49	1327.25	188.19	1.89	1690.70	239.17	52.87
Hexyl	1286.02	182.41	−17.92	1427.63	202.27	1.94	1790.0	253.10	52.77

Isopropyl	649.71	93.15	−23.01	773.4	110.5	−5.66	1130.4	160.58	44.42
Isobutyl	736.04	105.26	−24.93	881.88	125.72	−4.47	1221.9	173.41	43.22
Isoamyl	832.90	118.85	−25.37	985.60	140.27	−3.95	1319.5	187.10	42.88
Butyrates									
Isopropyl	742.50	106.17	−24.02	851.37	121.44	−8.75	1260.55	178.83	48.64
Isobutyl	837.13	119.44	−24.78	959.58	136.61	−7.60	1346.05	190.81	46.59
Isoamyl	931.19	132.63	−25.61	1055.05	150.01	−8.23	1444.79	204.66	46.42
Valerates									
Isopropyl	835.76	119.25	−24.97	951.96	135.55	−8.67			
Isobutyl	932.50	132.81	−25.42	1047.76	148.99	−0.25	1423.3	201.66	43.42
Isoamyl	1030.49	146.56	−25.71	1146.94	162.9	−9.37	1521.41	215.42	43.15
Hexoates									
Isopropyl	938.99	133.73	−24.51	1039.15	147.78	−10.46	1452.51	205.76	47.52
Isobutyl	1042.45	148.24	−24.03	1148.81	163.16	−0.11	1527.23	216.24	43.97
Isoamyl	1142.88	162.33	−23.97	1246.34	176.84	−9.46	1627.53	230.31	44.01
Isobutyrates									
Isopropyl	703.94	100.76	−29.43	773.9	110.57	−19.62	1100.62	156.40	26.21
Isobutyl	801.10	114.39	−29.83	878.38	125.23	−18.99	1251.03	177.50	33.28
Isoamyl	895.49	127.63	−30.61	978.28	139.24	−19.00	1354.09	191.95	33.71
Isovalerates									
Isopropyl	794.1	113.40	−30.82	825.87	117.86	−26.36	1226.63	176.28	32.06
Isobutyl	889.95	126.85	−31.39	968.14	137.82	−20.42	1359.05	195.10	36.86
Isoamyl	990.3	140.93	−31.34	1069.81	152.08	−20.19	1456.73	208.97	36.70
4-Methyl pentanoates									
Isopropyl	887.61	126.52	−31.72	970.96	138.21	−20.03	1344.3	190.58	32.34
Isobutyl	979.4	139.40	−32.87	1108.26	157.47	−14.80	1514.99	214.52	42.25
Isoamyl	1074.3	152.71	−33.59	1212.37	172.08	−14.22	1613.41	228.33	42.03
Isobutyrates									
Butyl	831.47	118.65	−25.57	930.55	132.54	−11.68	1314.59	186.40	42.18
Amyl	929.5	132.40	−25.84	1022.39	145.43	−12.81	1499.72	198.35	40.11
Hexyl	1028.47	146.28	−25.99	1122.16	159.42	−12.85	1501.33	212.61	40.34

(Continued on p. 50)

TABLE I *(continued)*

	SE-30			*OV-25*			*SILAR 10C*		
	I	*Me*	*ΔMe*	*I*	*Me*	*ΔMe*	*I*	*Me*	*ΔMe*
Isovalerates									
Propyl	826.26	117.92	−27.30	937.91	133.58	−10.64	1324.95	190.25	46.03
Butyl	922.98	131.48	−26.76	1031.0	146.63	−11.61	1413.71	202.86	44.62
Amyl	1021.23	145.26	−27.01	1132.09	160.81	−11.46			
Hexyl	1119.79	159.09	−27.21	1231.58	174.77	−11.53			
2-Methyl pentanoates									
Methyl							1262.28	179.08	48.89
Ethyl	836.9	119.41	−24.81	909.89	129.65	−14.57	1291.61	183.19	38.97
Propyl	932.38	132.80	−25.44	1004.73	142.95	−15.29	1378.27	195.35	37.11
Butyl	1029.4	146.41	−25.86	1093.32	155.38	−16.89	1473.78	208.74	36.47
Amyl	1130.71	160.62	−25.68	1195.52	169.71	−16.59	1570.92	222.37	36.07
Hexyl	1228.24	174.30	−26.03				1671.9	236.53	36.20
4-Methyl pentanoates									
Propyl	956.64	136.20	−22.04	1070.20	152.13	−6.11	1448.22	205.16	46.92
Butyl	1051.9	149.57	−22.70	1167.19	165.74	−6.53	1624.95	229.95	57.68
Amyl	1149.3	163.22	−23.07	1267.99	179.88	−6.42	1726.7	244.22	57.92
Acrylates									
Propyl				843.59	120.35	6.21	1274.9	180.85	66.71
Butyl	878	125.17	−3.00	940.76	133.98	5.81	1369.9	194.17	66.00
Amyl	978	139.20	−3.01	1047.11	148.89	6.68	1470.1	208.23	66.02
Hexyl	1068	151.82	−4.43	1146.67	162.86	6.61	1573.2	222.69	66.44
Methacrylates									
Propyl	856	122.09	−6.08	902.51	128.61	0.44	1340.24	190.01	61.84
Butyl	962	136.96	−5.24	1005.04	142.99	0.79	1439.19	203.89	61.69
Amyl	1064	151.26	−4.98	1103.61	156.82	0.58	1537.46	217.68	61.44
Hexyl	1165	164.43	−4.85	1202.88	170.74	0.46	1640.2	232.09	61.81

TABLE II

AVERAGE ΔMe VALUES FOR HOMOLOGOUS ESTERS

	SE-30	*OV-25*	*SILAR 10C*
n-Alkyl acetates	−18.61	2.73	
n-Alkyl propionates	−18.66	2.57	57.43
n-Alkyl butyrates	−18.73	2.34	56.41
n-Alkyl valerates	−18.09	1.63	53.67
n-Alkyl hexanoates	−18.12		
Isoalkyl propionates	−24.44	−4.69	43.40
Isoalkyl butyrates	−24.47	−8.19	47.22
Isoalkyl valerates	−25.40	−9.09	43.28
Isoalkyl hexanoates	−24.17		
Isoalkyl isobutyrates	−29.92	−19.20	31.07
Isoalkyl isovalerates	−31.17	−22.32	32.93
4-Methyl pentanoates	−32.76	−16.34	42.14
n-Alkyl isobutyrates	−25.80	−12.44	40.88
n-Alkyl isovalerates	−26.82	−11.31	45.33
n-Alkyl 2-methyl pentanoates	−25.56	−15.84	36.93
n-Alkyl 4-methyl pentanoates	−22.78	−6.44	57.80
n,Alkyl acrylates	−3.48	6.33	66.29
n-Alkyl methacrylates	−5.28	0.55	61.69

esters, *i.e.* acrylates and methacrylates where, as expected, on a non-polar phase unsaturation leads to a reduction of both retention and ΔMe values. With increased polarity of the phase the effect of unsaturation becomes apparent, and substantial enhancement of both retention and ΔMe values occurs.

CONCLUSIONS

While the actual retention trends measured follow those of earlier reports the conclusions as outlined are largely not apparent from a study of the retention data expressed as molecular retention indices. It would seem that large or obvious reten-

TABLE III

MOLECULAR RETENTION INDICES AND DIFFERENCES OF BUTANOIC ESTERS

	Stationary phase		
	SE-30	*OV-25*	*SILAR 10C*
A *n*-Alkyl butanoates	−18.73	2.34	56.41
B Isoalkyl butanoates	−24.47	−8.19	47.22
C *n*-Alkyl isobutanoates	−25.80	−12.44	40.88
D Isoalkyl isobutanoates	−29.92	−19.20	31.07
Molecular retention index differences			
(A) − (B)	−5.74	10.53	9.19
(A) − (C)	−6.97	12.54	15.43
(A) − (D)	−11.19	21.54	25.34

tion variations are generally apparent, but that ΔMe values are less satisfactory than retention index values for use in the tentative identification of closely related or isomeric compounds.

The use of ΔMe values for assembly into a correlation chart for identification, as suggested by Evans and Smith[2], for use in tentative identification is unlikely, particularly for intra-laboratory studies, as the ΔMe values are particularly susceptible to stationary phase loading as demonstrated recently by Carmi and Evans[5]. This dependence might not be expected as both retention indices and molecular retention indices are dependent on the behaviour of *n*-alkanes, rather than an absolute value of retention.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ALKYLBENZENES ON SILICA

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SUMMARY

Retention data of C_6–C_{16} alkylbenzenes, expressed in the form of capacity factor (k') and logarithms of the retention index (I) were measured on silica. The water content in the recycling mobile phase was controlled. The dependence of log I on the number of carbon atoms in the alkylbenzene molecule are given for different alkyl groups. The length of the alkyl groups, their arrangements (the *ortho*-effect) and numbers and shapes are the main factors affecting the retention of alkylbenzenes.

INTRODUCTION

The higher column efficiencies recently achieved in liquid chromatography allow a better separation of compounds separable previously only as a group.

Liquid chromatography of alkylbenzenes has been realized mostly on alumina[1–3]. The behaviour of some alkylbenzenes has been studied in gel permeation chromatography[4], and a study of the influence of the molecular structure of aromatic hydrocarbons on silica included seven alkylbenzenes[5]. Another study covered the behaviour of alkylbenzenes containing one alkyl group of various lengths (ranging from toluene to amylbenzene, and including also all polymethylbenzenes) on silica with *n*-hexane as the mobile phase[6]. The high-performance liquid chromatography (HPLC) separation of C_1–C_4 alkylbenzenes on three different reversed phases has been compared[7].

The use of preparative HPLC of alkylbenzenes leads to a more detailed separation of these compounds, which may be useful also in the case of some natural mixtures containing alkylbenzenes. When performing preparative separations of C_9 and C_{10} monoaromatic hydrocarbon mixtures on silica gel[8], it was found that the separation takes place according to: (i) the number of substituents; (ii) molecular mass; (iii) the type of hydrocarbon and (iv) the position of substituents in isomers. These criteria have also been verified for the HPLC separation of monoaromatic hydrocarbons in light and middle petroleum fractions (containing C_{10}–C_{17} hydrocarbons)[9]. The fraction of the material obtained by preparative HPLC has now been further analysed by mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy and analytical HPLC. The aim of the present study was to achieve a more detailed understanding of the separation mechanism.

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EXPERIMENTAL

Apparatus

A Varian 8500 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) with a syringe pump was used. Sample injection was performed by the stop flow technique with a 1-μl syringe (Hamilton, Bonaduz, Switzerland) directly into the column, using a septumless injector. A fixed-wavelength (254 nm) UV detector was employed and the chromatograph was operated at ambient temperature. Retention times were measured and printed by a CDS 111 chromatographic data system and recorded simultaneously on a dual-pen strip chart recorder (all Varian).

A stainless-steel (25 cm × 8 mm I.D.) column with tapered inlet (Varian) was filled using the slurry-packing technique with 7.5-μm irregularly shaped silica gel (Silasorb; Lachema, Brno, Czechoslovakia).

Chemicals

A series of alkylbenzene isomers was used. Some of the standards were prepared at the Prague Institute of Chemical Technology (Laboratory of Synthetic Fuels and Department of Petroleum Technology and Petrochemistry), others were commercial products from various manufacturers.

Mobile phase

n-Pentane (analytical grade) was used as the mobile phase. Before use, it was distilled in glass and dried by percolating over a silica gel column (Mikrosil, 100–150 μm; Lachema) which had been dried for 4 h at 200°C. The flow-rate of the mobile phase was 100 ml/h.

Procedure

Two or three injections were carried out for each sample, always together with a test mixture of benzene and naphthalene. As the retention differences between individual alkylbenzenes are rather small, it was necessary to maintain as stable conditions as possible in the course of measurements. Thus, closed-circuit mobile phase recycling was used, in order to eliminate the contact between the mobile phase and air moisture. A column of activated silica gel was incorporated in the circuit (Mikrosil, 80–100 μm; Lachema)[10]. Under these conditions (highly activated silica gel and dry pentane), temperature control of the system was not necessary, contrary to the procedure described in ref. 10.

Retention data are expressed in the form of the logarithm of the retention index x and capacity factor k'. The retention index system was proposed previously for liquid chromatographic retention data of aromatic hydrocarbons in adsorption systems. A retention index of $x = 10$ is assigned to benzene, 100 to naphthalene and 1000 to phenanthrene.

Retention indices for all aromatic hydrocarbons are calculated in accordance with

$$\log x = \log I_n + \frac{\log t_x - \log t_n}{\log t_{n+1} - \log t_n} \qquad (1)$$

where x, n, $n + 1$ represent the measured substance and lower and higher standards, respectively, and the t values are the corresponding adjusted retention times. Eqn. 1 is valid for substances having retention times higher than that of benzene.

For substances with lower retention times, the linear dependence of log t *versus* log I in the interval $10 \leqslant I \leqslant 100$ was extrapolated to the region of $I < 10$. This resulted in

$$\log I = 1 - \frac{\log t_B - \log t_x}{\log t_N - \log t_B} \quad (2)$$

where t_N is the adjusted retention time of naphthalene and t_B the adjusted retention time of benzene.

Capacity factors, k', were calculated using the well-known procedure

$$k' = (t_R - t_0)/t_0$$

where t_R = retention time of the given compound and t_0 = retention time of a compound which is not adsorbed. The values of t_R were obtained by means of a back calculation from log I for the mean value of the retention time of benzene and naphthalene.

RESULTS AND DISCUSSION

The logarithms of the retention indices x and capacity factors k' are given in Table I.

Fig. 1 shows the dependence of log I on the number of carbon atoms in the alkylbenzene molecule. With increasing number of carbon atoms the range of retention data broadens. While maximum retention is limited by the straight line corresponding to the dependence for polymethylbenzenes, with every new methyl group being in the *ortho*-position (curve 1), the lower limit is formed by curve 2, which corresponds to the dependence for alkylbenzenes with the alkyl group extended in a linear manner. The minimum value on this curve corresponds to an alkylbenzene molecule with a sufficiently long alkyl chain. The two curves define the region A for most alkylbenzenes. Some alkylbenzenes have log I values which lie below curve 2 (region B), *e.g.*, compounds with two long alkyl groups (not in *ortho*-position) or with rather long branched alkyl chains.

The main factors affecting the adsorption of alkylbenzenes are as follows:

(i) the length of the alkyl group

(ii) the arrangement of the alkyl groups (the *ortho*-effect)

(iii) the number and shape of the alkyl groups

Alkyl length

Generally, alkylbenzene retention times decrease with increasing alkyl chain length. Fig. 2 demonstrates the dependence of log I on the number of carbon atoms in the molecule. As in the *n*-alkylbenzene series, a decrease in log I can be observed for the following pairs: *o*-xylene–*o*-ethyltoluene; *m*-xylene–*m*-ethyltoluene; *p*-cymene–*p*-isopropylethylbenzene; *p-tert.*-butyltoluene–1-ethyl-4-*tert.*-butylbenzene. Fig. 3

TABLE I

RETENTION DATA

t_R = Retention time (min); k' = capacity factor; I = retention index; $t_x = t_R - t_0$ = adjusted retention time (min).

Compound	t_x	k'	*log I*	*Δlog I*
Benzene	6.90	0.57	1	–
Naphthalene	9.13	1.08	2	–
Toluene	6.93	0.58	1.016	0.016
Ethylbenzene	6.71	0.53	0.901	−0.099
n-Propylbenzene	6.59	0.50	0.838	−0.162
n-Butylbenzene	6.46	0.47	0.764	−0.236
n-Amylbenzene	6.37	0.45	0.712	−0.288
n-Hexylbenzene	6.24	0.42	0.639	−0.361
n-Heptylbenzene	6.18	0.41	0.608	−0.392
n-Octylbenzene	6.14	0.40	0.581	−0.419
n-Decylbenzene	5.98	0.36	0.490	−0.510
o-Xylene	7.33	0.67	1.217	0.217
m-Xylene	6.91	0.57	1.005	0.005
p-Xylene	6.96	0.59	1.031	0.031
1,2,3-Trimethylbenzene	7.58	0.73	1.335	0.335
1,2,4-Trimethylbenzene	7.39	0.68	1.246	
1,3,5-Trimethylbenzene	6.92	0.58	1.009	0.009
o-Ethyltoluene	7.18	0.64	1.144	
m-Ethyltoluene	6.76	0.54	0.928	
Cumene	6.77	0.54	0.932	
1,2,3,4-Tetramethylbenzene	7.87	0.79	1.470	0.470
Isodurene	7.59	0.73	1.339	
Durene	7.51	0.71	1.301	
p-Cymene	7.07	0.61	1.089	
Isobutylbenzene	6.32	0.44	0.688	
tert.-Butylbenzene	6.72	0.53	0.906	
Pentamethylbenzene	8.11	0.85	1.575	0.575
1-Ethyl-2,4,6-trimethylbenzene	7.20	0.64	1.152	
1-Ethyl-4-isopropylbenzene	6.93	0.58	1.017	
p-tert.-Butyltoluene	6.56	0.49	0.818	
2-Methyl-3-phenylbutane	7.12	0.62	1.113	
Isopentylbenzene	6.26	0.43	0.655	
Neopentylbenzene	6.28	0.43	0.662	
Cyclopentylbenzene	6.89	0.57	0.994	
Hexamethylbenzene	8.64	0.97	1.803	0.803
1-Propyl-2,4,6-trimethylbenzene	6.87	0.56	0.984	
1,3,5-Triethylbenzene	6.29	0.43	0.670	
m-Diisopropylbenzene	6.35	0.45	0.705	
p-Diisopropylbenzene	6.19	0.41	0.610	
1-Ethyl-4-*tert.*-butylbenzene	6.43	0.46	0.747	
1-Propyl-2,4,6-triethylbenzene	6.22	0.42	0.631	

shows the separation of an *n*-alkylbenzene mixture. If more than one alkyl chain is extended simultaneously in a molecule, the decrease in the value of the logarithmic retention index is more pronounced, *e.g.*, mesitylene–1,3,5-triethylbenzene; *p*-xylene–*p*-diisopropylbenzene; *m*-xylene–*m*-diisopropylbenzene; 1-propyl-2,4,6-trimethylbenzene–1-propyl-2,4,6-triethylbenzene (Fig. 4). A still greater decrease in the log reten-

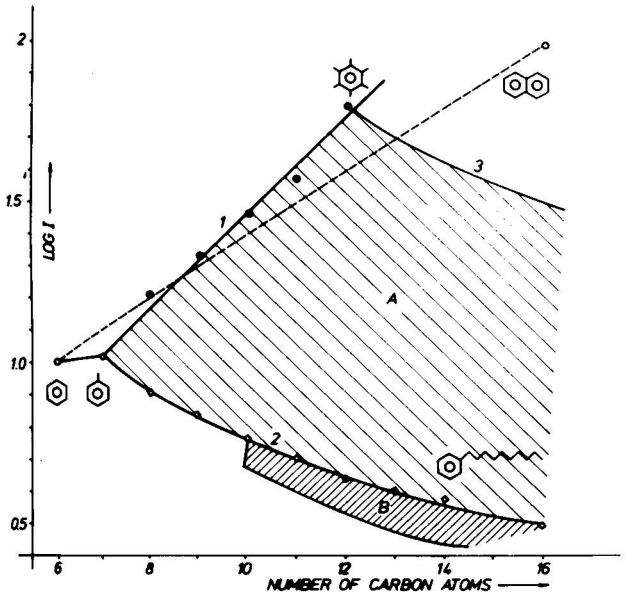

Fig. 1. Dependence of log I of alkylbenzenes on number of carbon atoms. The dashed line represents data for unsubstituted aromatic hydrocarbons, *i.e.*, benzene, naphthalene and phenanthrene.

tion index value is observed on extending an alkyl chain placed between two *ortho*-substituted methyl groups (Fig. 5, curve A).

The largest decrease in log I is observed when *tert.*-butyl replaces isopropyl (*e.g.*, *p*-cymene to *tert.*-butyltoluene, curve B) or isobutyl replaces isopropyl (*e.g.*, cumene to isobutylbenzene, curve C). Further extension of the chain to isoamyl is accompanied by a decrease in the retention index corresponding to the relevant decrease in the basic *n*-alkyl series.

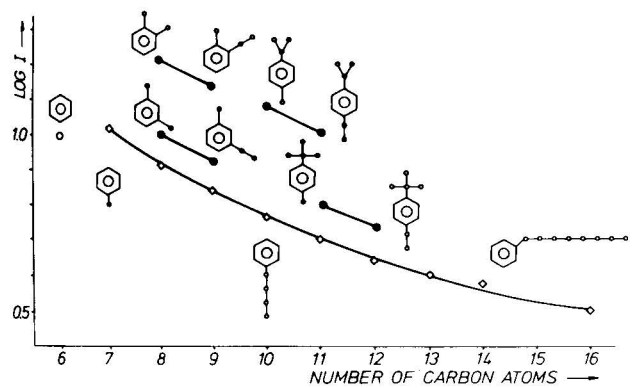

Fig. 2. Dependence of log I on number of carbon atoms for alkylbenzenes with *n*-alkyl chains.

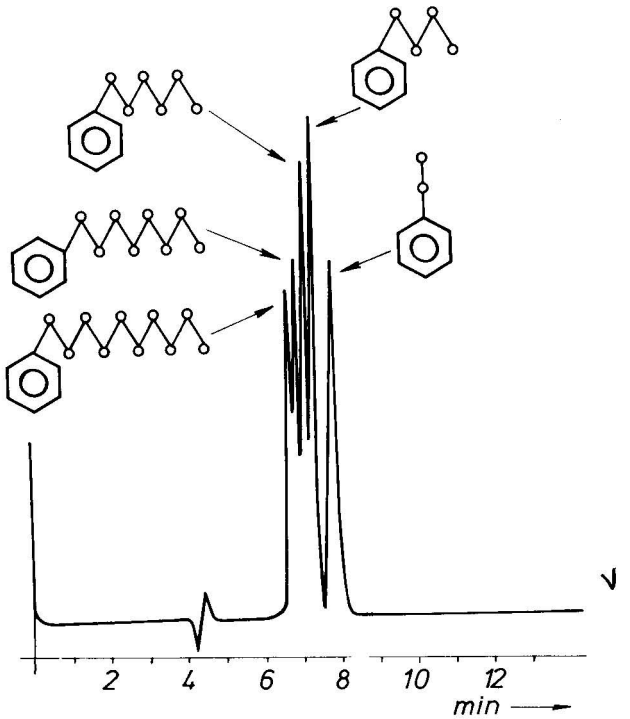

Fig. 3. Chromatogram of some *n*-alkylbenzenes. Column: 25 cm × 8 mm I.D., filled with 7.5-μm silica gel. Mobile phase: moisture controlled *n*-pentane; flow-rate 100 ml/h.

Alkyl arrangement (ortho-effect)

Generally, polymethylbenzenes have the same or longer retention times than benzene. In accordance with previous results[6,8,9], the largest increase in retention time has been found for *ortho*-substituted derivatives (Fig. 1), particularly for the so-called double *ortho*-substitution, *e.g.*, *m*-xylene and 1,3,5-trimethylbenzene or mesitylene and isodurene.

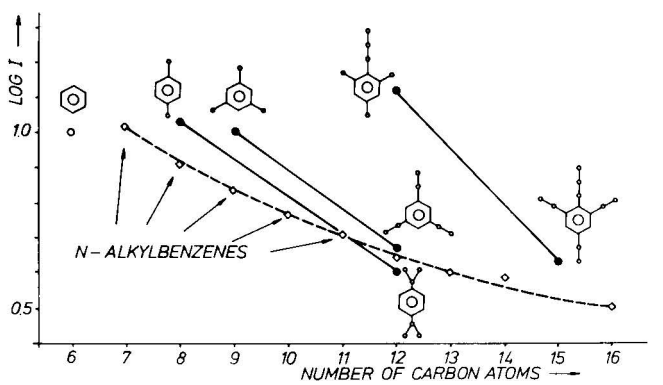

Fig. 4. Dependence of log *I* on number of carbon atoms for alkylbenzenes with extension of more than one alkyl group.

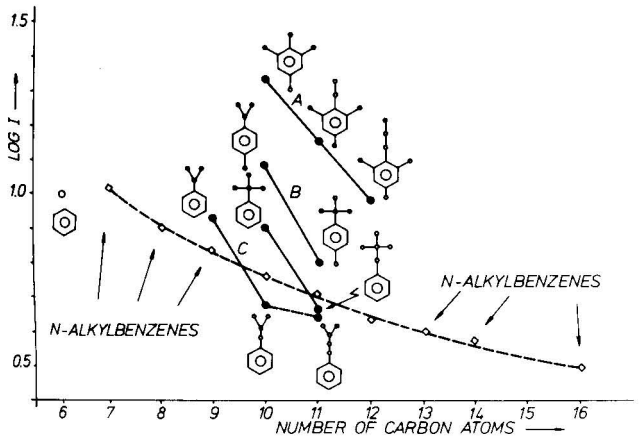

Fig. 5. Dependence of log *I* on number of carbon atoms for alkylbenzenes with extension of the alkyl chain placed between two *ortho*-substituted methyl groups (curve A), for alkylbenzenes when *tert.*-butyl replaces isopropyl (curve B) and isobutyl replaces isopropyl (curve C).

The introduction of another methyl group into a *meta*-position has little effect on the retention data, *e.g.*, in the series toluene–*m*-xylene–1,3,5-trimethylbenzene or for the pairs ethylbenzene–*m*-ethyltoluene, *o*-xylene–1,2,4-trimethylbenzene and 1,2,3-trimethylbenzene–isodurene.

The effects of *ortho*, and *meta*-substitution are illustrated schematically in Fig. 6.

Number and shape of alkyls

Alkylbenzenes with identical molecular masses do not form a group which would be separable as a whole, but on the contrary individual members cover the whole range of retention data.

Generally, higher retention data are found for alkylbenzenes with short alkyl groups (especially methyl) and the *ortho*-effect takes place here. With increasing length of the alkyl group, the effect of the longer chain is more substantial and/or the *ortho*-effect is disturbed, retention times being decreased.

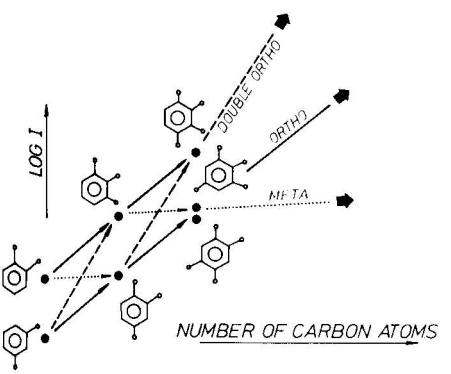

Fig. 6. Effect of *ortho* and *meta* substitution on chromatographic behaviour of alkylbenzenes.

When comparing alkylbenzenes having one alkyl group and the same number of carbon atoms, it is evident that compounds having an alkyl chain branched at the α-carbon atom are more strongly retarded. For example, the retention times of isopropylbenzene and *tert.*-butylbenzene are longer than those of n-C_3 and n-C_4, or even than of ethylbenzene. Cyclopentylbenzene (log I = 0.994) also belongs to this group. On the other hand, if the alkyl chain is branched at the β-carbon atom (or on further carbon atoms), the retention time decreases, *e.g.*, when comparing isobutylbenzene with n-C_4 and neopentylbenzene with n-C_5. In the case of 2-methyl-3-phenylbutane, where the chain is branched at both α and β carbon atoms, there is strong retardation, probably as a result of the mutual arrangement of the alkyl group and the benzene ring.

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ELECTROKINETIC DETECTION AT DIFFERENT POINTS IN A NARROW-BORE GLASS COLUMN IN LIQUID CHROMATOGRAPHY

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SUMMARY

The measurement of the electrokinetic current at different points in a narrow-bore glass column (0.4–0.5 mm I.D.) packed with sorbent is described. The origin of a "derivative" signal and the dependence of its shape on the capacity ratio of the solute and the flow-rate of the mobile phase are explained and verified experimentally. The values of the height equivalent to a theoretical plate, were determined at two places in the column, and a value of $H_{min} = 0.11$ mm (*i.e.*, 3.1 d_p) was calculated.

INTRODUCTION

The electrokinetic principle of detection in liquid chromatography (LC)[1,2] makes it possible to pick up a signal directly from the chromatographic column[3]. Extra-column contributions to peak broadening, originating in the detection cell and in the connecting tubes between the column and the detector, are thus eliminated; this is particularly important when working with narrow-bore and capillary columns. A technique has been described[3] for the direct measurement of the streaming current which results from the transport of the diffusion part of the electric double-layer generated in columns packed with sorbent and in capillary columns.

In the present work, the signal was monitored at different places in the glass column, which allows one to observe the profile of a chromatographic peak during its passage through the column. An advantage is that it is possible to detect solutes having high capacity ratios in the first sections of the column, and which might otherwise be difficult to detect at the column end.

Fig. 1 shows schematically the change in concentration of a solute at the peak maximum, c_{max}, along a column of total length L, and which is expressed by

$$c^i_{max} = \frac{Q_i}{A\,\varepsilon\,\sqrt{2\,\pi\,H_i}\cdot(1 + k_i)} \cdot \frac{1}{\sqrt{L}} \qquad (1)$$

where Q_i is the total amount of solute i introduced into the column, A is the column cross-section, ε is the total column porosity, H_i is the height equivalent to a theoretical

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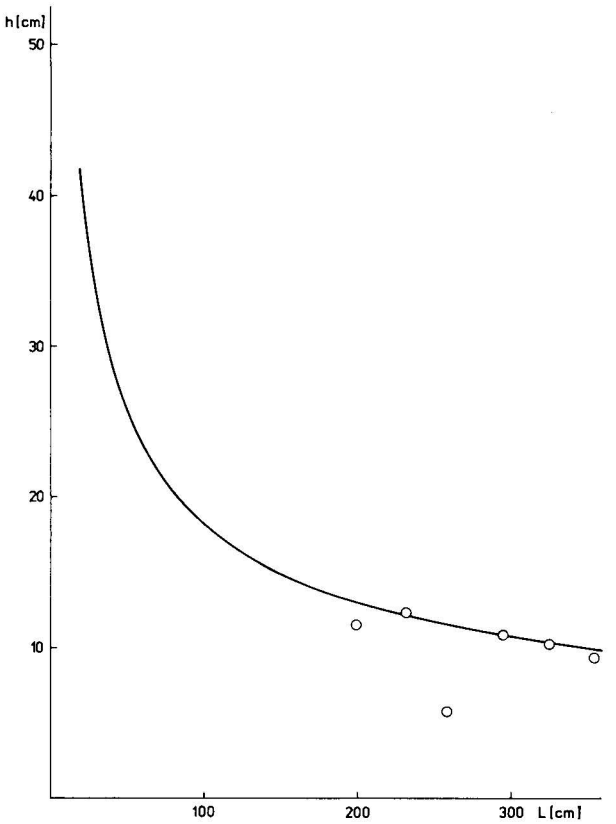

Fig. 1. Change in the concentration of the solute at the peak maximum (expressed in terms of the peak height, h) along the column calculated according to relationship 1 for $h = 10.7$ cm at $L = 295$ cm.

plate of solute i and k_i is the capacity ratio of solute i. For a given column and a given experiment, the first fraction at the right-hand side of eqn. 1 is a constant. It is assumed that the column packing is homogeneous enough so that neither $A\varepsilon$ nor H or k changes along the column length. The nearer to the column inlet the detection element is placed, *i.e.*, the shorter is L, the greater will be the concentration of the analysed component at the peak maximum.

EXPERIMENTAL

Apparatus and columns

Glass columns (2–4 m × 0.4–0.5 mm I.D. × 0.8–1.2 mm O.D.), with thread diameter 100 mm, were used. The columns were packed with Silpearl spherical silica gel (Kavalier, Votice, Czechoslovakia), particle size 30–40 μm. Constantan wire was wound round the column and served as a screening; about 1 cm of the glass surface was left free at the half-length of the column and on the last but one thread. Additionally, a glass column (0.44 mm I.D.) was used which consisted of three segments connected to each other, with two pieces of a stainless-steel tube (length *ca.* 1.5 cm)

into which a stainless-steel capillary (0.25 mm I.D.) was inserted in order to decrease the free volume of the connections. The first two segments of the column consisted of four threads, the third segment of one thread. This column was used for measurements without the wire screening.

The mobile phase contained 7 % *n*-butanol in *n*-hexane with the addition of 10 ppm of perchloric acid. A mixture of aliphatic alcohols, containing methanol, ethanol, *n*-propanol and *n*-butanol, was injected.

The measurements were performed in the arrangement shown in Fig. 2. The column was placed in an earthed metallic box (1) and was connected to a stainless-steel injection port (2) into which the mobile phase was introduced via an inlet (3). A needle valve connected via inlet 4 served to adjust the splitting ratio between the sample entering the column and the sample which was vented. Unscreened segments of the column were connected to electrometers (Vibron 33B; Electronic Instruments, Richmond, Great Britain) (5, 5′); the segments of the column wound with wire were earthed. When the glass column with metallic connections was used, the stainless-steel segments were connected to the electrometers. The mobile phase was pumped by a VCM 300 pump (Development Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) after which a pressure pulse damper according to Locke[4] was inserted. The sample was injected through a septum by means of a Hamilton 5-μl high-pressure syringe.

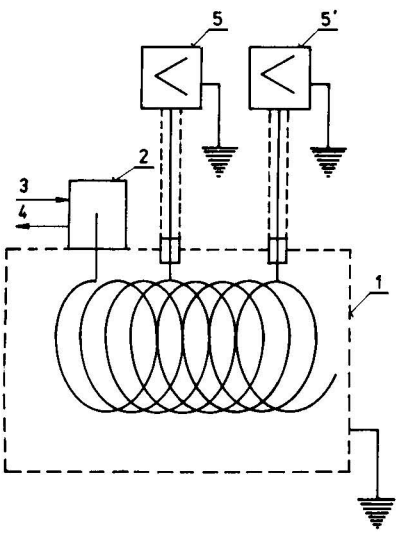

Fig. 2. Diagram of the apparatus for the measurement of the streaming current at two places in the glass column.

RESULTS

Character of the response

An example of a chromatogram obtained by sensing at two places in the glass capillary column with screening is shown in Fig. 3. It is obvious that each solute exhibits a peak that consists of two branches of opposite polarities. This phenomenon is more marked for solutes with lower capacity ratios k. The ratio of the heights of the

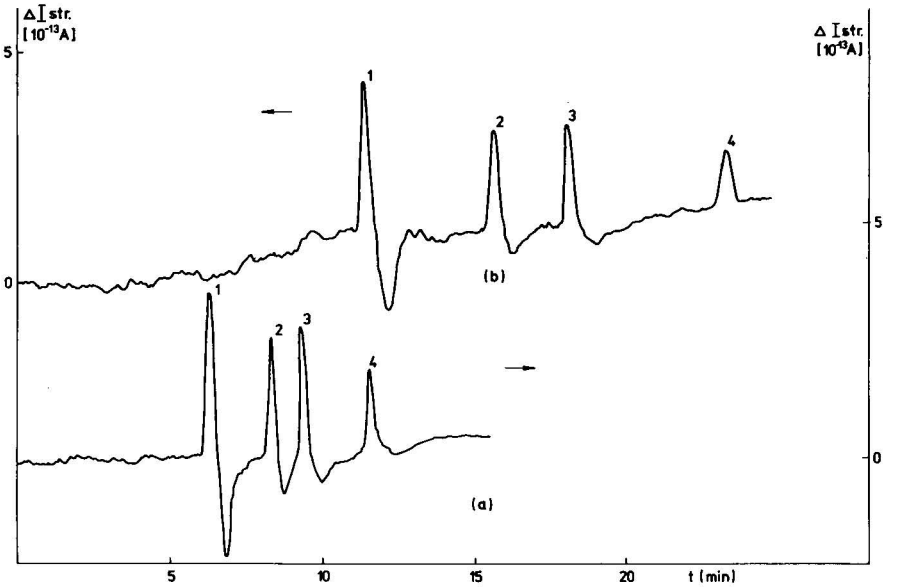

Fig. 3. Chromatogram of a mixture of *n*-butanol (1), *n*-propanol (2), ethanol (3) and methanol (4) obtained at two places in the glass column. L = 185 cm (a) or 310 cm (b).

branches of each peak (h^+/h^-, the plus sign denoting the first branch) does not depend on the mobile phase flow-rate for a given solute (Table I). However, it does depend on the capacity ratio of the solute, and on the position of sensing, increasing with increasing capacity ratio and for sensing at the column end. For methanol, which has a capacity ratio k = 1.4, the height of the second branch of the peak is

TABLE I

DEPENDENCE OF THE RATIO OF HEIGHTS, h^+/h^-, FOR *n*-BUTANOL (k_1 = 0), *n*-PROPANOL (k_2 = 0.35) AND ETHANOL (k_3 = 0.67) ON THE LINEAR VELOCITY OF THE MOBILE PHASE

u (cm/sec)	*L* = 1850 mm			*L* = 3100 mm		
	h_1^+/h_1^-	h_2^+/h_2^-	h_3^+/h_3^-	h_1^+/h_1^-	h_2^+/h_2^-	h_3^+/h_3^-
0.20	1.7	2.0	3.4	1.7	2.4	4.4
0.27	2.2	2.7	3.8	1.8	3.0	4.0
0.39	1.7	2.6	3.6	1.7	2.8	4.3
0.43	1.9	2.0	4.3	1.8	2.3	4.4
0.47	1.8	3.0	3.6	1.4	3.1	5.5
0.53	2.0	2.8	3.9	1.5	5.3	5.4
0.90	1.8	2.5	3.8	1.6	2.7	5.3
1.0	1.7	2.4	3.4	1.3	3.1	5.7
1.1	1.8	2.8	3.6	1.5	3.2	4.0
1.6	1.9	2.6	3.5	1.4	3.5	4.8
2.5	1.9	2.5	3.1	1.8	2.8	4.2
Average	1.8	2.5	3.6	1.6	3.1	4.7

usually too small to be measured with sufficient precision, and the ratio h^+/h^- is therefore not given for this solute.

When a solute enters the unscreened segment of the column, a change occurs in the density of the electric charge of the entering liquid, corresponding to the concentration profile of the solute. The similar change but of opposite polarity takes place when the solute leaves the unscreened segment. Both of the processes can be identified, as shown previously[3]. In the present case, the resulting signal is the sum of the inlet and the outlet signals at the given column segment. Chromatographic peaks with even more complicated shapes for the negative branch were obtained by sensing from the metallic connecting segments of the column. In this case, the situation is made more complicated by the presence of another solid phase, the stainless steel of the connecting capillaries.

The dependence of the concentration of the analysed component at the peak maximum on the distance of the detection element from the column inlet was verified by measurements on the glass column with eleven threads (L = 330 cm). The first five threads were earthed and the signal was picked up from the remaining six threads. *n*-Butanol was injected into the column and the height of the first branch of the chromatographic peak was measured at various sensing points. The measured values of h were plotted in Fig. 1, which also shows the dependence of the chromatographic peak height on the distance of the place of sensing from the column inlet, calculated according to relationship 1. The value h = 10.7 cm for L = 295 cm, as measured on the last but one thread of the column, was taken as the basis for the calculation.

Verification of the mechanism of the response

In order to verify the mechanism of the response picked up from a segment of a narrow-bore glass column, a system described earlier[2,3] was employed. The electrokinetic signal was generated in a packed column (200 × 4 mm I.D.). Metallic capillaries of various dimensions, connected after the column, were applied. The lengths (minimum 120 mm) stainless-steel capillaries (0.3–0.8 mm I.D.) were selected so that the sensing of electrokinetic streaming currents might be performed under the conditions (*e.g.*, flow-rate, conductivity of the mobile phase) providing mass character of the response[2]. The 3200-mm capillary with large volume (2.50 ml) and 1 mm I.D. was made of copper.

The column was packed with Silpearl silica gel. Hexane, purified and dried over activated alumina, with the addition of 5% (v/v) isopropanol was used as the mobile phase. Model solutions of nitroanilines in nitrobenzene, *o*-nitroaniline in nitrobenzene and benzyl cyanide were injected.

The experiments confirmed that measurable electrokinetic currents are generated when solute in the mobile phase flows through empty metallic capillaries. In accord with the theory of the generation of streaming currents[1–3], the magnitudes of these currents depend on the linear velocity of the mobile phase in the capillary. That is why they are greater for a capillary with a given diameter at a higher volumetric flow-rate; at a given flow-rate they increase with decreasing diameter of the capillary (Tables II and III, values with a minus sign). An exception was observed in the case of exclusion response. The "derivative" character of the responses was observed not only with the injected solutes but sometimes also with vacancy of the isopropanol and with the exclusion response[5].

TABLE II

DEPENDENCE OF THE MAGNITUDES OF THE RESPONSES GENERATED IN THE COLUMN AND I THE STAINLESS-STEEL CAPILLARY (0.8 mm I.D.) ON THE MOBILE PHASE FLOW-RATE

The magnitudes of responses are expressed in terms of 10^{-14} A; similarly in Tables III and IV. Responses generated i the sensing capillary possess a negative sign.

Flow-rate (ml/min)	*Exclusion response*		*Vacancy*		*Nitrobenzene*		*o-Nitroaniline*		*m-Nitroaniline*	
	+	−	+	−	+	−	+	−	+	−
3.6	84	20	156	0	144	40	52	8	3	0
0.95	36	12	112	0	112	10	44	0	0	0
0.47	8	0	71	0	78	0	32	0	0	0
0.22	0	0	45	0	47	0	24	0	0	0

The experiments further suggested that mutual shift of the maximum of the signal generated by the solute in the column and of the signal generated in the sensing capillary is associated with the volume of the sensing capillary. The volumes of the applied stainless-steel capillaries were too small to enable a differentiation between the signals. A detection capillary was therefore used with a volume of 2.50 ml, selected so that it might hold not only the zone of the solute (benzyl cyanide) but also the isopropanol vacancy eluted before it. Under these conditions, the responses generated in the column (zones 1–3 in Fig. 4) were perfectly separated from the benzyl cyanide response generated in the sensing capillary (zone 4). The flow-rate dependence of the responses generated in this case in the column and in the sensing capillary is summarised in Table IV. The last column of this table contains the distances between the maxima of the electrokinetic responses generated in the column and in the sensing capillary, expressed in terms of volume. The values measured are in a good accord with the volume of the sensing capillary calculated from its dimensions.

It was thus confirmed that measurable electrokinetic signals are generated not only by the flow of the mobile phase through the chromatographic column but also by its flow along the surface of tubes with diameters sufficiently small that retention can be considered to be negligible. The magnitude of the electrokinetic streaming current generated on the surface of a tube depends (for a given tube material, com-

TABLE III

DEPENDENCE OF THE MAGNITUDES OF THE RESPONSES GENERATED IN THE COLUMN AND I THE STAINLESS-STEEL CAPILLARY (0.3mm I.D.) ON THE MOBILE PHASE FLOW-RATE

Responses generated in the sensing capillary possess a negative sign.

Flow-rate (ml/min)	*Exclusion response*		*Vacancy*		*Nitrobenzene*		*o-Nitroaniline*		*m-Nitroaniline*	
	+	−	+	−	+	−	+	−	+	−
3.6	22	5	4	49	0	80	0	36	2	0
2.2	18	4	4	44	0	70	0	33	2	0
0.96	13	5	8	26	0	33	0	18	0	0
0.47	2	0	7	11	0	13	0	6	0	0

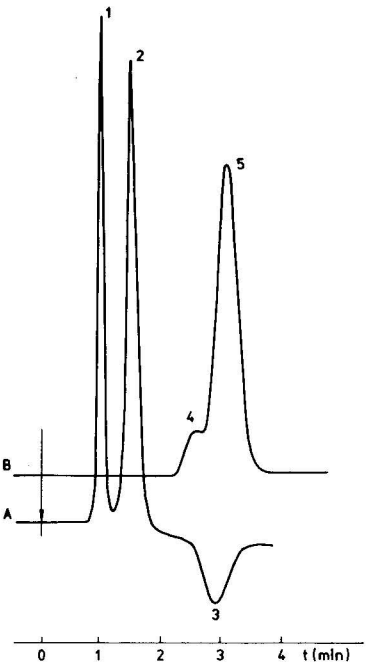

Fig. 4. Chromatogram of benzyl cyanide with sensing of the electrokinetic streaming current by use of a large-volume capillary. A, Record of the electrokinetic response: 1 = response to butanol vacancy generated in the column; 2 = response to the benzyl cyanide zone generated in the column; 3 = response to the benzyl cyanide zone generated in the sensing capillary. B, Comparative record by a refractometric detector connected after the sensing capillary: 4 = response to butanol vacancy; 5 = response to the benzyl cyanide zone. The time of injection is denoted by the arrow.

position and flow-rate of the mobile phase) on the tube diameter. If this tube is applied as a sensing element, its own signal is combined with the signal generated in the column and transported by the mobile phase (*cf.*, Tables II and III with Table IV). At the same time, the mutual shift in time of the maxima of the signals measured in the column and those measured in the sensing tube is determined at a given volumetric flow-rate by the volume of the sensing tube. Composite signals can therefore be expected for sensing elements with sufficiently small volumes (*e.g.*, unscreened seg-

TABLE IV

FLOW-RATE DEPENDENCE OF ELECTROKINETIC RESPONSES OF BENZYL CYANIDE, GENERATED IN THE COLUMN (h) AND IN THE LARGE-VOLUME SENSING CAPILLARY (h')

ΔV is the difference in the elution volumes of the maxima of the responses generated in the column and in the sensing capillary, calculated from the chromatograms.

Flow-rate (ml/min)	h	h'	$\lvert h'/h \rvert$	ΔV *(ml)*
4.56	560	−40	$7.1 \cdot 10^{-2}$	2.50
1.86	220	−16	$7.3 \cdot 10^{-2}$	2.60
0.94	126	9	$7.1 \cdot 10^{-2}$	2.64

ments of a sufficiently non-conducting packed capillary column, a metallic sensing capillary inserted between the two segments of the glass column, a sensing capillary after the packed column). If the polarities of component signals are of opposite sign, the resulting signal can, when the magnitudes of the individual components are comparable, imitate the course of the derivative of the elution peak. If tubes with larger volumes are used for sensing, the parasitic signal of narrow zones can be separated entirely from the column signal, differentiated and quantified. With broader zones, however, it coincides with the column response, in such a way that it distorts the shape of the desorption branch (Fig. 5). Distortion of the time course of the signal sensed from the column, caused by the parasitic signal generated in the sensing capillary, cannot be observed if the secondary signal is small or if it is generated by the zone which, with respect to the volume of the sensing element, can be considered as relatively broad.

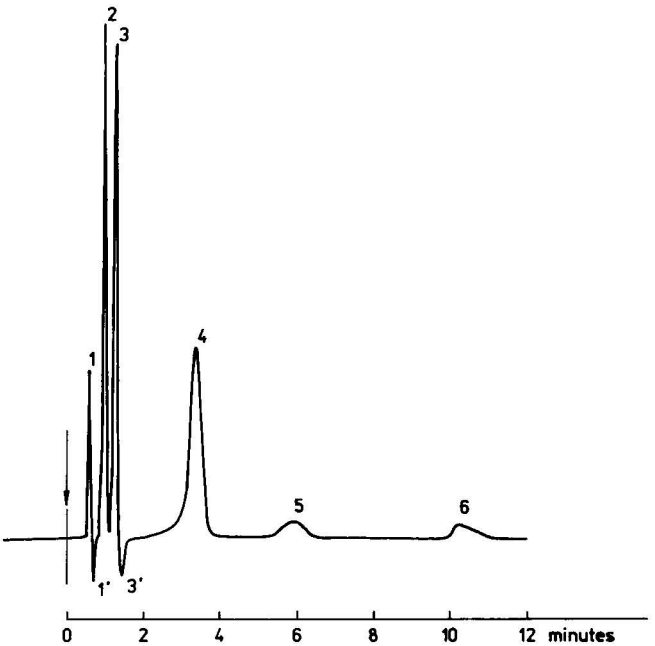

Fig. 5. Chromatogram of a model mixture of nitroanilines. Column: stainless steel, 200 × 4 mm, packed with Silpearl silica gel. Mobile phase: 5% isopropanol in hexane, flow-rate 2.2 ml/min. Sensing capillary: 120 × 0.8 mm. Zones: 1 = exclusion response; 2 = butanol vacancy; 3 = nitrobenzene; 4 = *o*-nitroaniline; 5 = *m*-nitroaniline; 6 = *p*-nitroaniline. The responses generated in the sensing capillary are denoted by primes, the injection time by the arrow.

Measurement of H

In order to calculate H, the peak of methanol was selected, for which it is supposed, with respect to the ratio of the heights of the branches of the peak, that the position of the maximum and the shape of the positive branch will be distorted only negligibly by the negative branch. The calculation was performed on the positive branch. The dependence of the height equivalent to a theoretical plate, H, calculated for both segments of the column, on the linear velocity of the mobile phase, u, is shown in Fig. 6. The values of H measured in both segments agree well for various u.

It was found that $H_{min} = 0.11$ mm, *i.e.*, 3.1 d_p. For a well packed column it was reported[10] that $H_{min} < 3\ d_p$. It is assumed[6,7] that the column can be well packed providing the ratio of the column diameter and the particle diameter $d_c/d_p > 10$. For the present micropacked column $d_c/d_p = 12$ and in this instance the value of H_{min} is considered acceptable. Furthermore, the constant C of Van Deemter's equation was determined from the graph to be $C \approx 0.04$ and was compared with the value calculated according to[8]

$$C = \frac{1 + 6k + 11k^2}{96\,(1 + k)^2} \cdot \frac{d_p^2}{D_m}$$

where D_m is the diffusion coefficient of the solute in the mobile phase. The value $D_m = 3 \cdot 10^{-5}$ cm^2/sec, taken from ref. 9, was used for the calculation. The calculated value of $C = 0.02$ sec is less than the value taken from the graph ($C = 0.04$ sec).

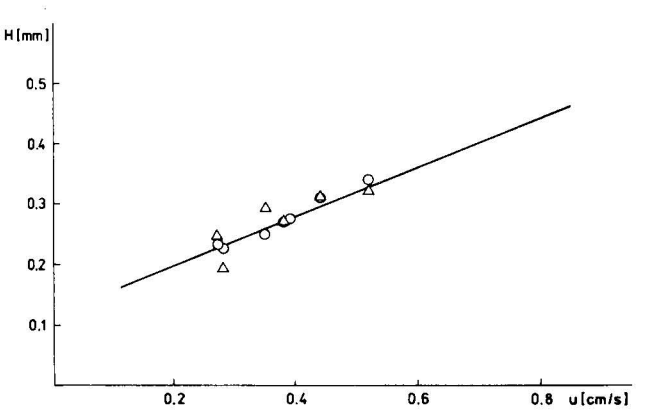

Fig. 6. Dependence of the height equivalent to a theoretical plate, H, on the linear velocity of the mobile phase, u. Column packing: Silpearl (d_p = 30–40 μm). Mobile phase: 7% (v/v) *n*-butanol in hexane, containing 10 ppm perchloric acid. Solute: methanol. L = 185 cm (○) or 310 cm (△).

CONCLUSIONS

The principle of the measurement of electrokinetic streaming currents in chromatographic columns[3] makes it possible to measure the response at different places in the column. The responses obtained for solutes in a single column are comparable with those obtained by measurements in several columns connected in series.

The appearance of a "derivative" signal, the genesis of which was explained and verified experimentally, is a disadvantage of the technique described. However, using this technique, the homogeneity of the packing of narrow-bore columns can be checked by determining the values of the height equivalent to a theoretical plate at different points throughout the column length.

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AFFINITY CHROMATOGRAPHY OF ACETYLCHOLINESTERASE

THE USE OF AMBERLITE CG-120 FOR DISSOCIATING THE ENZYME–INHIBITOR COMPLEX

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SUMMARY

Acetylcholinesterase from rat brain was solubilized with 1% (w/v) Triton X-100 and purified by affinity chromatography. Two different ligands were investigated. The most efficient purification was obtained when the enzyme was eluted from a column containing the acetylcholinesterase inhibitor N-methyl-3-aminopyridinium iodide covalently linked to Sepharose 2B. An initial recovery of 6% of the applied enzyme increased to 70% after treatment with Amberlite CG-120. The partially purified enzyme had a specific activity of 205 μmoles min^{-1} mg^{-1} and a purification of 162-fold with respect to the brain homogenate and 44-fold with respect to the Triton solubilized enzyme.

The effect of metal cations on the stability of the partially purified enzyme during storage at −20°C was also investigated. The addition of $MgCl_2$ to the purified enzyme prevented the rapid loss of enzyme activity.

INTRODUCTION

Acetylcholinesterase (AChE: acetylcholine hydrolase, E.C. 3.1.1.7.) plays a key rôle in the transmission of the nerve impulse at cholinergic neurones and is found in the central nervous system of all vertebrates[1,2]. During the last decade, the enzyme from a number of animal brains has been purified by affinity chromatography using a variety of ligands[3–8]. Once the enzyme has been bound to the affinity material, the AChE has usually been released by running a solution of an irreversible inhibitor through the column. However, the recoveries of the enzyme activity obtained by this method although adequate have not been very high even after extensive dialysis[5–7]. Another problem has been that the partially purified enzyme loses most of its activity after freeze drying[4] or during storage in the deep freeze at −20°C[5].

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In this paper we have examined two affinity columns for purifying AChE from rat brain and the use of ion exchange materials for removing decamethonium from the eluted enzyme–inhibitor complex. The effect of a number of compounds, including several cations, on the stability of this partially purified enzyme has also been investigated.

MATERIALS AND METHODS

Materials

General chemicals were analytical reagent grade from BDH (Poole, Great Britain) or Fisons (Loughborough, Great Britain).

Materials for the two affinity columns were obtained as follows: Sepharose 2B and 4B from Pharmacia (London, Great Britain); 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride from Aldrich (Wembley, Great Britain); anhydrous HBr in glacial acetic acid, isobutyl chloroformate, iodomethane, 9-chloroacridine and 3-aminopyridine from Eastman-Kodak (Rochester, NY, U.S.A.).

The ion-exchange materials used were Amberlite CG-120 (200–400 mesh, Na^+) from BDH, CM-Sephadex C-50 from Pharmacia and Dowex 50W-X4 (50–100 mesh, H^+) from Fluka (Buchs, Switzerland).

Decamethonium bromide, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), acetylthiocholine iodide (ATChI), thyroglobulin, apoferritin, beef liver catalase and lactate dehydrogenase were obtained from Sigma (London, Great Britain). Triton X-100, Folin reagent and bromophenol blue were purchased from BDH, bovine serum albumin (Fraction V) from International Enzymes Ltd. (Windsor, Great Britain). Prepared Gradipore polyacrylamide concave gradient gels (4–26%, w/v), in the form of slabs (70 × 70 × 3 mm) were obtained from Universal Scientific Ltd. (London, Great Britain).

Preparation of the enzyme

Wistar rats of 150 and 250 g body weight were beheaded and the brains removed and stored at −20 C in the deep freeze until required. A 10% (w/v) homogenate of the brains was obtained by grinding the tissue with washed sand in sodium phosphate buffer (30 m*M*, pH 7). The sand was allowed to settle and the supernatant removed by decantation then centrifuged at 100,000 *g* for 1 at 4°C on an MSE SS50 ultracentrifuge. The supernatant from this centrifugation is known as the "naturally soluble" enzyme. The pellet was then resuspended in the same volume of buffer containing Triton X-100 (1% w/v), homogenized and recentrifuged at 100,000 *g* for 1 h. The supernatant is referred to as the "Triton solubilized" enzyme.

Assay of acetylcholinesterase

The enzyme activity was determined by the spectrophotometric method of Ellman *et al.*[9] as described by Reavill and Plummer[8]. One unit of activity represents the hydrolysis of 1 μmole of acetylthiocholine iodide per minute at 30°C.

Estimation of protein

Protein was determined by the method of Lowry *et al.*[10] using crystalline bovine serum albumin as standard. When Triton X-100 was present, a gelatinous

precipitate was formed but this interference was overcome by centrifuging the precipitate (1000 *g* for 5 min) and incorporating Triton X-100 in the reagent blank and standard[11,12].

Affinity column 1 (MAC-agarose)

The ligand, [1-methyl-9-(N$^{\beta}$-aminocaproyl)-β-aminopropylamino]acridinium bromide hydrobromide, was prepared as previously described and coupled to Sepharose 4B activated with cyanogen bromide[8,13].

Affinity column 2 (MAP-agarose)

The affinity material was built up stepwise by a method based on the procedures used by Berman and Young[14] and Goodkin and Howard[15]. A solution of 1,4-diaminobutane was added to the cyanogen bromide activated Sepharose 2B and after washing, succinic anhydride was added. The succinylated resin was treated with the ligand, N-methyl-3-aminopyridinium bromide, in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

Full experimental details for the preparation of the ligands and their coupling to the Sepharose have been presented in a previous report[8].

Conditions for use of the affinity columns

All operations were carried out at 4°C.

Binding. The Triton solubilized enzyme (50–100 ml) was passed through each column which had previously been equilibrated with 30 m*M* sodium phosphate buffer (pH 7) containing 1% (w/v) Triton X-100.

Elution. The standard elution buffer was 30 m*M* sodium phosphate, pH 7.0, containing Triton X-100 (1% w/v) and all solutions were prepared in this medium. The column was first washed with 10–15 column volumes of buffer until the eluate gave a zero reading of protein. The elution was then continued with a volume of the standard buffer containing the competitive inhibitor decamethonium bromide (at various concentrations) and 2–5 ml fractions were collected. Finally, the column was washed with four column volumes of the elution buffer containing 1 *M* NaCl.

Washing. The column was prepared for further use by washing with 6 *M* guanidine hydrochloride followed by 40–60 column volumes of the standard elution buffer.

Removal of the inhibitor from the decamethonium–enzyme complex

Dialysis. The fractions containing the peak of the AChE activity were combined and dialysed for up to 150 h against five changes of 2 l each of the elution buffer.

Ion exchange resins. The ability of three cation-exchange resins (CM-Sephadex, Dowex 50W-X4 and Amberlite CG-120) to remove the decamethonium from the enzyme–inhibitor complex was studied together with their effect on the stability of the Triton solubilized preparation. The resins were washed twice then suspended in cold buffer (20%, w/v) and added to the free AChE or to the enzyme inhibited with 50 m*M* decamethonium bromide. Equal volumes of resin suspension and the enzyme preparation were mixed and after stirring gently, the resin was removed by centrifugation at 1000 *g* for 3 min. The enzyme activity in the supernatant was then determined.

RESULTS

MAC-agarose column

Triton solubilized enzyme (100 ml) was passed through the affinity column: fractions were then collected and assayed for acetylcholinesterase. However, only 8% of the enzyme activity and 9% of protein became bound to the resin. The column was prepared twice but identical results were obtained and so this procedure was abandoned.

Binding of the enzyme to the MAP-agarose

The Triton solubilized enzyme preparation (50 ml) was passed through the column (10 × 1.5 cm) which had been equilibrated with the standard buffer. About 90% of the activity and 80% of protein remained bound to the column. When the eluate gave a zero reading for protein, 25 ml of a solution of the competitive inhibitor decamethonium bromide (10 m*M* in buffer) were applied to the column but only 1% of the total activity was collected after this treatment. A further 25 ml of the elution buffer containing 100 m*M* decamethonium bromide were applied and 10% more of the total activity was eluted from the column. The low yield of acetylcholinesterase activity could be due to the presence of the inhibitor so the active fractions were pooled and extensively dialysed for 72 h and five changes of 2 l of standard buffer in an attempt to remove the decamethonium. However, there was no change in the enzyme activity of the dialysed eluate suggesting that the inhibitor remained firmly bound to the enzyme. Attempts were therefore made to remove the inhibitor by other means.

Removal of the inhibitor with ion exchange materials

The effect of three cation exchange resins on the enzyme was examined to see if they could dissociate the decamethonium–enzyme complex.

The activity of the detergent extract was measured and taken as the 100%

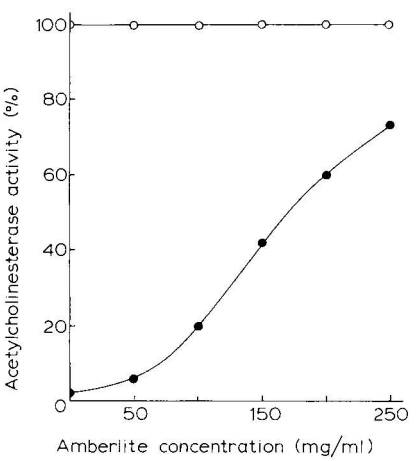

Fig. 1. The effect of Amberlite CG-120 on the activity of acetylcholinesterase in the presence (●) and absence (○) of 50 m*M* decamethonium bromide.

value. A 1-ml volume of this solution was mixed with 1 ml of a solution of 100 m*M* decamethonium bromide and the activity measured again. The activity was reduced to 2.2% of the original activity by this treatment and could not be restored by the addition of Dowex 50W-X4 or CM-Sephadex. In contrast to this, the Amberlite was very effective at removing the inhibitor and more than 70% of the enzyme activity could be recovered after treatment with this resin. The restoration of the activity depended on the concentration of Amberlite present and 250 mg/ml was the highest concentration that could be used in practice (Fig. 1). Amberlite itself had no effect on the uninhibited enzyme. If the enzyme was inhibited by 10 m*M* decamethonium bromide, then addition of Amberlite to 50 mg/ml restored the activity to 98% of its original value but unfortunately a 10 m*M* solution of the inhibitor was unable to remove the enzyme from the column.

The Triton solubilized enzyme preparation was passed through four columns as before and each column was eluted with buffer containing a different concentration of decamethonium bromide (10, 25, 50 and 100 m*M*). The corresponding enzyme recoveries after treatment with Amberlite were 0, 35%, 70% and 75% respectively.

Preparation of the partially purified enzyme

In the light of the above results, 50 m*M* decamethonium bromide was used to remove the enzyme from the MAP-agarose and the eluate treated with washed Amberlite to remove the inhibitor. The fractions were then analysed for protein and enzyme activity and those that contained AChE were pooled and used for the experiments on the storage of the enzyme. A summary of the purification using the above method is shown in Table I. Further elution of the column with 1 *M* NaCl removed a further 9% of the enzyme activity but this fraction was contaminated with other proteins (Fig. 2) and was not used for any other experiments.

TABLE I

PURIFICATION OF ACETYLCHOLINESTERASE BY AFFINITY CHROMATOGRAPHY ON MAP-AGAROSE

	Protein (mg)	*Enzyme activity (μmoles min^{-1})*	*Specific activity (μmoles min^{-1} mg^{-1})*	*Yield (%)*	*Purification (times)*
Brain homogenate	–	–	0.078	100	1
Triton extract					
Total applied	90	25.8	0.287	85	3.7
Affinity column					
Recovered in peak	1.4	17.64	12.6	60	162

Storage of the partially purified enzyme

The preparation of acetylcholinesterase from the affinity column was stored in the deep freeze at −20°C but after 1 week the enzyme had lost virtually all of its activity. Mixing some of the crude Triton extract with the partially purified enzyme increased the stability slightly but most of the activity was gone after 7 days storage (Fig. 3). In contrast to this, the initial extract with detergent retained all its activity on storage.

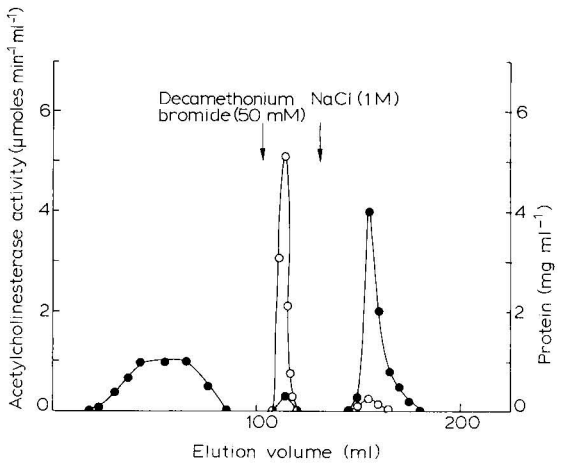

Fig. 2. Purification of acetylcholinesterase by affinity chromatography on column of MAP-agarose. ○, Acetylcholinesterase activity; ●, protein.

The effect of a number of compounds on the stability of acetylcholinesterase was therefore investigated by using the Triton solubilized enzyme that had been inactivated with 50 m*M* decamethonium then reactivated with 250 mg/ml of Amberlite. Storage of this preparation at −20°C resulted in a rapid fall in activity similar to that of the partially purified enzyme (Fig. 4). Storage in the presence of lecithin (5 mg/ml), acetylcholine chloride (150 μM) or sodium phosphate buffer (0.5 *M*, pH 7) had no effect on this rapid decline in activity. However, a number of cations did improve the stability of this preparation and of these, Mg^{2+} was by far the most effective with 80% of the activity retained in the presence of 0.2 *M* $MgCl_2$ (Fig. 4). The protective effect of Mg^{2+} was even more pronounced in the case of the partially

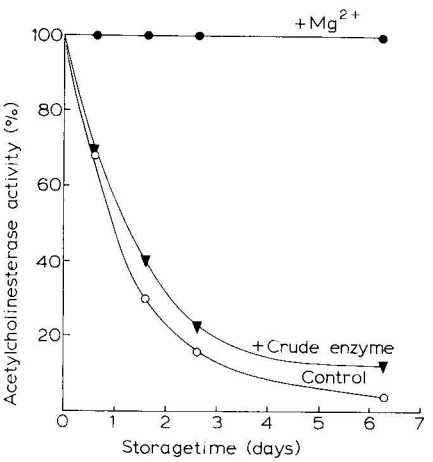

Fig. 3. The stability of partially purified acetylcholinesterase during storage at −20°C. The enzyme was purified by affinity chromatography as described in the text then stored at −20°C by itself (○) and in the presence of the crude Triton extract (▼) or 0.2 *M* $MgCl_2$ (●).

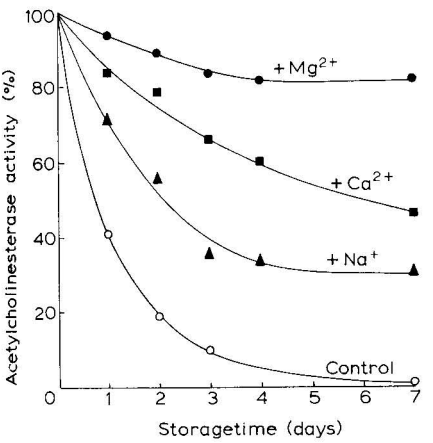

Fig. 4. The stability of Triton solubilized acetylcholinesterase during storage at −20°C. All preparations were inhibited with 50 m*M* decamethonium bromide then reactivated with 250 mg/ml of Amberlite CG-120 as described in the text. Samples were then stored in the presence of 0.2 *M* salt. ○, Control; ▲, NaCl and KCl; ■, $CaCl_2$; ●, $MgCl_2$.

purified enzyme when 0.2 *M* $MgCl_2$ effectively protected the enzyme against loss of activity in the deep freeze (Fig. 3).

DISCUSSION

The first affinity column prepared was MAC-agarose as this had been used by Reavill and Plummer[8] to purify AChE from pig cerebral cortex. Results showed that the column was not effective at binding AChE. In view of this another column was prepared of MAP-agarose which had previously been used by Goodkin and Howard[6] to purify AChE from rat brain synaptosomal membranes. The N-methyl-3-aminopyridinium–agarose was quite efficient in binding rat brain AChE since 92% of the enzyme remained bound to the column. The AChE could be eluted from the column with 50 m*M* decamethonium bromide but the enzyme–inhibitor complex was non-dialysable and so several cation exchange resins were tried with a view to binding the strong positive charge of the inhibitor and thus removing it from the enzyme. Of the resins tested, Amberlite CG-120 proved to be extremely efficient at removing the inhibitor without inactivation of the enzyme (Fig. 1). The effectiveness of this resin is probably due to two main factors, one could be the high binding affinity for the inhibitor and the other the size of the resin particle which allows access of the enzyme–inhibitor complex. The procedure is very rapid and passing the eluate from the affinity column through a second column of Amberlite leads to a partially purified enzyme free of inhibitor which can be used for further studies (Fig. 2). A yield of 70% of the applied enzyme activity was obtained with a specific activity of 12.6 μmoles min^{-1} (mg protein)$^{-1}$ a purification of 162-fold with respect to the crude enzyme. These results are similar to those of Adamson[4] who obtained a preparation from mouse brain with a specific activity of 87 μmoles min^{-1} mg $protein^{-1}$; Adamson used a choline chloride gradient to elute the enzyme and obtained a single peak at a concentration of 40 m*M*. A good recovery was only obtained for rat brain if the concentra-

tion of inhibitor was in the range 40–50 m*M* and so it seems reasonable to postulate that electrostatic and hydrophobic forces are involved in the binding of the enzyme to the affinity column. It was pointed out by Dawson and Crone[5] that the more efficient the purification, the more difficult is the problem of removing the inhibitor from the purified product.

A common characteristic of purified mammalian brain AChE prepared by any procedure is its low stability after storage in the deep freeze[4,5]. In contrast to this, the AChE in the crude Triton extract of rat brain was very stable when stored at −20°C and this stability was unchanged by the addition of Amberlite. However, when the Triton solubilized enzyme was inactivated with decamethonium bromide and reactivated with Amberlite, a dramatic change in the stability of the enzyme took place. Storage of this preparation at −20°C resulted in a rapid loss of enzyme activity (Fig. 4). Decamethonium with its two positive centres probably binds to the active site and also a peripheral anionic site[16,17]. This could result in a distortion of the enzyme into a less stable form which remains even when the inhibitor is removed. The rapid fall in the activity of the AChE was very similar to that of the partially purified enzyme so this inhibited and reactivated preparation was used in screening a number of compounds for their potential stabilizing effect on the enzyme. Another very practical advantage of using this preparation was the much shorter time taken to obtain this preparation compared with the enzyme from the affinity column.

The presence of a substrate often improves the stability of an enzyme but acetylcholine chloride (150 μM) had no effect on the rapid loss of activity experienced at −20°C. Sometimes high ionic strength or a phospholipid detergent stabilises an enzyme but 0.5 *M* sodium phosphate (pH 7) or lysolecithin (5 mg/ml) failed to halt the rapid decline in the activity of the enzyme stored at −20°C. The addition of some of the crude Triton extract also had little effect on the stability of the enzyme (Fig. 3).

Protein–lipid complexes can be stabilized by mono and divalent metal cations[18] and so the effect of several metal ions on the stability of the AChE was examined. In the presence of 0.2 *M* concentration of salt, the stability of the preparation increased as follows $Mg^{2+} > Ca^{2+} > K^{+} > Na^{+}$ > enzyme alone (Fig. 4). The partially purified enzyme was therefore suspended in 0.2 *M* $MgCl_2$ then frozen at −20°C. This treatment protected the AChE during storage at −20°C with very little loss of activity.

MAP-agarose with decamethonium bromide as the eluting inhibitor was very effective at purifying AChE from rat brain. The inhibited enzyme could be reactivated with Amberlite and the purified enzyme stored at −20°C in 0.2 *M* $MgCl_2$ without loss of activity.

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DETERMINATION OF CROSS-CONTAMINATION OF THE DIASTEREOMERS EPHEDRINE AND PSEUDOEPHEDRINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, THIN-LAYER CHROMATOGRAPHY AND CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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SUMMARY

A high-performance liquid chromatographic method for separation of the diastereomers ephedrine and pseudoephedrine is described. The method can be used to determine diastereomeric cross-contamination at the 1 % level and to detect 0.1 % of the contaminant. It employs a phenyl reversed-phase column, mobile phase consisting of 1 % acetonitrile in aqueous phosphate buffer and ultraviolet detection at 210 nm.

A thin-layer chromatographic separation, using reversed-phase separation plates and borate buffer, was also developed. Carbon-13 nuclear magnetic resonance spectroscopy was also examined as a method for determining diastereomeric cross-contamination. The three methods are compared for sensitivity and selectivity.

INTRODUCTION

Ephedrine and pseudoephedrine are diastereomers that are resistant to simple chromatographic separation. Because of their therapeutic importance as adrenergic agents, their analysis has received considerable attention. Many of the published chromatographic methods for ephedrine and pseudoephedrine separate these compounds from other drugs[1–5] but not from each other. Although ephedrine and pseudoephedrine have been separated after derivatization[6,7], some of the reported separations[8,9] are not practical as routine analytical techniques. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy has been used in this laboratory[10] to determine ephedrine in pseudoephedrine after acetylation, but the method is not especially sensitive. The optical rotatory dispersion (ORD) curve of ephedrine passes through zero, but the ORD curve of pseudoephedrine does not; an assay has been reported[11] that takes advantage of this phenomenon. However, racemic samples cannot be analyzed and any racemic pseudoephedrine present would be quantitated as ephedrine. Because of these analytical difficulties, the United States Pharma-

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copeia[12] does not contain a limits test for either ephedrine in pseudoephedrine or *vice versa*.

Commercial formulations labeled to contain either of the diastereomers have a varying risk of cross-contamination, depending on the actual source of the labeled material. (−)-Ephedrine occurs naturally in certain plants of the *Ma Huang* species[13]; the racemic compound is synthesized and resolved[13]. A synthesis has been reported[14] to yield as much as 20% pseudoephedrine, which is then removed by recrystallization as various salts. (+)-Pseudoephedrine is prepared[13] from (−)-ephedrine by the Welsh rearrangement[15] of N-acetylephedrine. Because of the intimate relationship between the two compounds, there may be ample opportunity for low-level cross-contamination.

We report here a simple, rapid high-performance liquid chromatographic (HPLC) method for the complete separation and determination of ephedrine in pseudoephedrine or *vice versa*. We also report a thin-layer chromatographic (TLC) method for separation of the diastereomers and compare these methods to analysis by ^{13}C NMR.

EXPERIMENTAL

High-performance liquid chromatography

The determinations were performed with a Spectra-Physics Model 8000 high-performance liquid chromatograph equipped with an SP 8000 data system, a Spectra-Physics Model 770 UV-visible detector set at 210 nm and a temperature-controlled column compartment. A stainless-steel μBondapak phenyl (particle size 10 μm) (30 cm × 3.9 mm I.D.) column was used. The injector was equipped with a 10-μl sampling loop. The mobile phase was 1% acetonitrile in 0.05 *M* monobasic sodium phosphate which had been recirculated continuously over the column overnight. A flow-rate of 1 ml/min and a column temperature of 22°C were maintained throughout the analysis.

Ephedrine sulfate and pseudoephedrine sulfate were purchased from the United States Pharmacopeia (USP). All solvents (including water) were of suitable grade for HPLC and all solutions were filtered through micropore filters and then degassed before use.

Standard solutions of ephedrine and pseudoephedrine were prepared in water, either separately or in mixtures, over a concentration range of 0.5–500 μg/ml. The standard solutions (10 μl) were chromatographed and calibration curves were obtained by using the data system's peak area measurements.

Single dosage units (tablets or capsules) were dissolved individually in water in small volumetric flasks with the aid of an ultrasonic bath and diluted to volume with water. Samples were filtered and diluted quantitatively to a final concentration of 0.5 mg/ml. The sample solution (10 μl) was chromatographed and peak areas were used for quantitative analysis.

Thin-layer chromatography

Standards used were USP Reference Standards. Ephedrine hydrochloride powder and pseudoephedrine sulfate powder were used (as secondary standards) to prepare the mixed test solutions. Precoated, prescored reversed-phase separation

(RPS) plates (20 × 20 cm, layer thickness 0.25 mm) were purchased from Analtech (Newark, DE, U.S.A.).

Test solutions were prepared to simulate ephedrine (10 mg/ml) cross-contaminated with 1, 5 and 10% pseudoephedrine by dissolving suitable amounts of ephedrine hydrochloride and pseudoephedrine sulfate in methanol–chloroform (1:1). The test solutions (10 μl) were applied to the plate with disposable micropipets as 1-cm streaks about 2 cm from the bottom of the plate. Standard solutions (1 mg/ml) were prepared in methanol–chloroform (1:1) and were applied (10 μl) as spots. Commercial sample solutions (10 mg/ml) were also prepared in the same solvent. An ultrasonic bath was used to facilitate disintegration of the tablet or capsule material. The solution was filtered and 10 μl were applied to the plate as a 1-cm streak about 2 cm from the bottom of the plate.

The developing solvent consisted of 10% acetonitrile, 2% boric acid and 0.5% acetic acid in methanol. Plates were developed at room temperature in a pre-saturated (0.5 h) chamber lined with filter-paper. The solvent was allowed to develop for 15 cm from the spotting line. The plates were removed, air-dried and sprayed with ninhydrin reagent (0.3% in *n*-butanol with 3 ml acetic acid added). Plates were then heated in a 110°C oven for about 15 min to bring out the color.

Carbon-13 nuclear magnetic resonance

^{13}C NMR analyses were performed with a Varian Associates XL-200 Fourier transform NMR spectrometer equipped with a 10-mm broadband probe and deuterium lock.

About 500 mg of the free base (ephedrine, pseudoephedrine or mixtures) were dissolved in 3 ml deuterochloroform in a 10-mm NMR sample tube. The instrumental conditions were: pulse width 5 μsec (about 30°) acquisition time 0.636 sec and delay 0.864 sec (for a total acquisition cycle of 1.5 sec); spectrum width 12,578 Hz (250 ppm) and 3600 transients. Various standard mixtures of the diastereomers were prepared and ^{13}C NMR spectra were determined.

RESULTS AND DISCUSSION

Baseline separations of ephedrine and pseudoephedrine (down to 5% of the minor component) were obtained with the HPLC system reported here. A representative chromatogram is shown in Fig. 1. Linearity curves (concentrations from 0.5 to 0.0005 mg/ml in water) were prepared for each component (Fig. 2). Standard mixtures of ephedrine and pseudoephedrine were prepared and analyzed; concentrations of one component in the other ranged from 1 to 99% (Fig. 3). Chromatograms of mixtures containing 1% of the contaminant are shown in Fig. 4. Other measurements showed that less than 1% could be detected; however, calculation of diastereomers in amounts less than 0.5% were considered to be estimations only. The limit of detection was 0.1%.

Before the determinations, a preliminary system suitability test should be performed. The retention times should be about 16.4 min for ephedrine sulfate and about 19.8 min for pseudoephedrine sulfate. A complete determination will take about 25 min. The resolution factor[16], R, is about 2.6 for equal amounts (25 μg/ml) of a standard mixture of ephedrine and pseudoephedrine. Minor adjustments in the aceto-

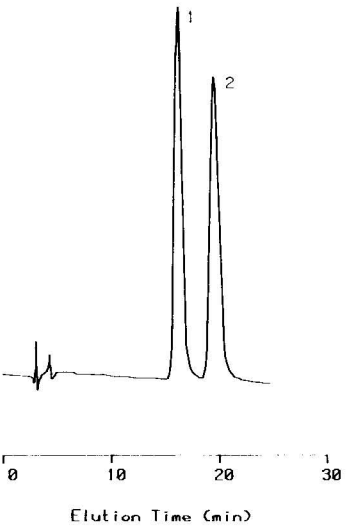

Fig. 1. HPLC separation of ephedrine (1) and pseudoephedrine (2). Column: μBondapak phenyl (10 μm), 30 cm × 3.9 mm I.D. Mobile phase: 1% acetonitrile in 0.05 *M* monobasic sodium phosphate. Flow-rate: 1 ml/min. Injection volume: 10 μl. Detector wavelength: 210 nm.

nitrile concentration and flow-rate may be necessary to attain the optimum resolution factor and retention times.

Because of the sensitivity of the method, extreme care must be exercised in cleaning the injection loop and port between injections of standards and samples, since cross-contamination can be introduced at this point.

Several dosage forms were assayed by using the HPLC method reported here. Single capsule analysis of pseudoephedrine hydrochloride capsules from two manu-

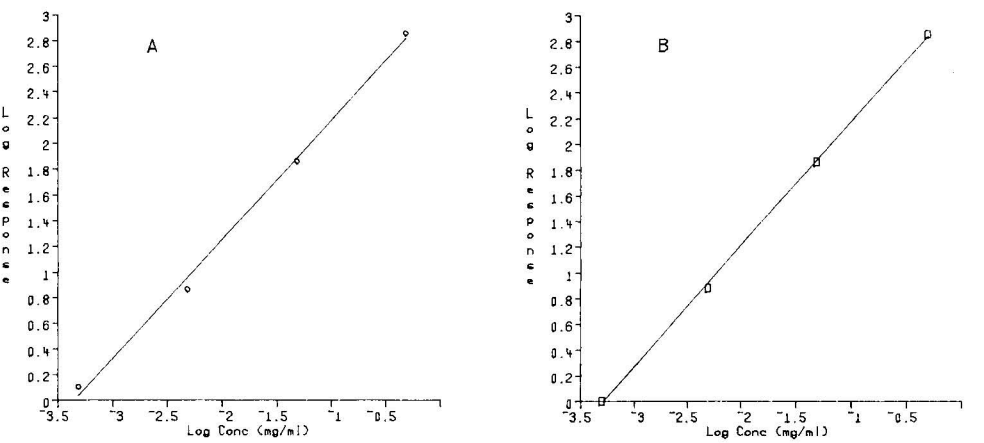

Fig. 2. Linearity curves (log concentration *vs.* log peak response) for ephedrine (A) and pseudoephedrine (B). Concentrations range from 0.5 to 0.0005 mg/ml in water.

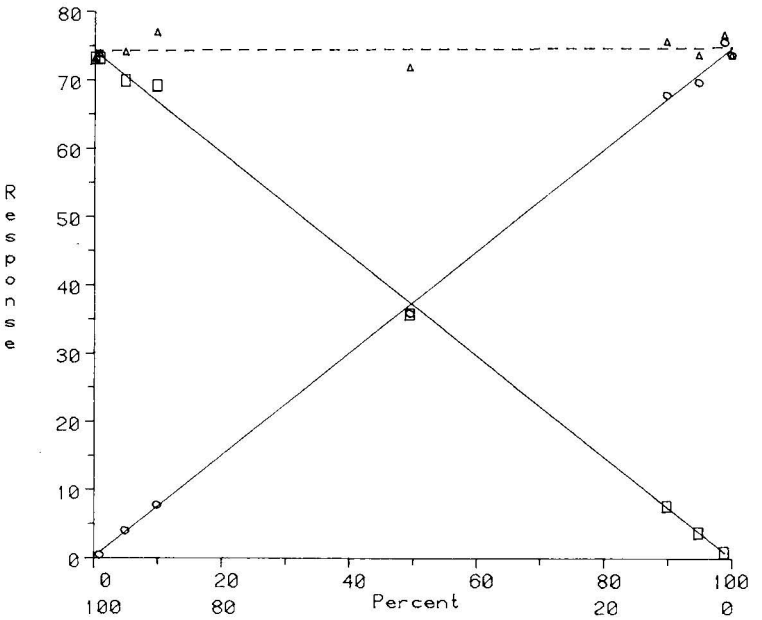

Fig. 3. Plot of peak area response *vs.* composition (in percent) of ephedrine (○), pseudoephedrine (□) and total alkaloid (△).

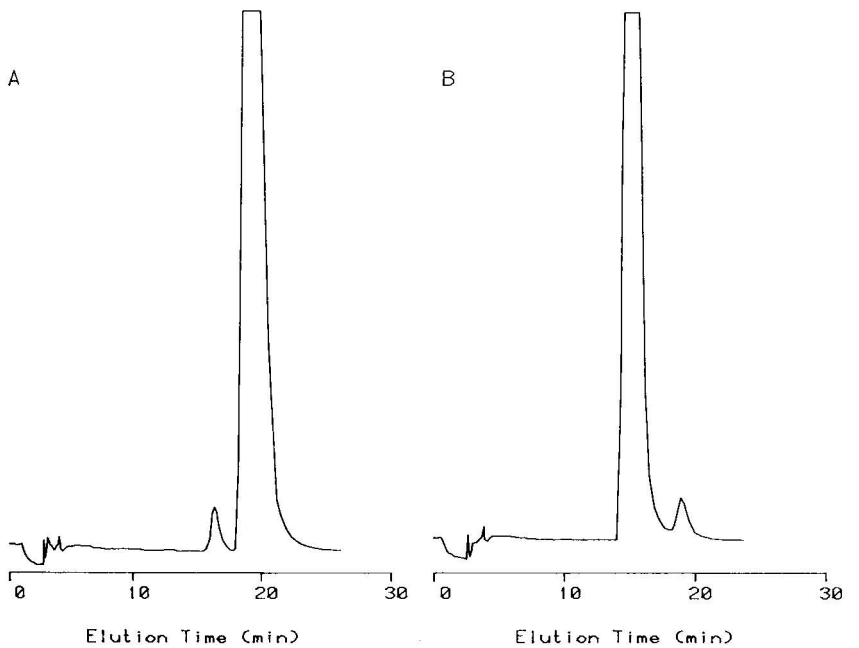

Fig. 4. HPLC chromatograms of 1 % cross-contaminated test solutions: A, 1 % ephedrine in pseudoephedrine; B, 1 % pseudoephedrine in ephedrine.

facturers was performed. One capsule contained 92.7% of label claim and the analysis showed that a small amount of ephedrine was present (about 0.2%). The other capsule contained 102.4% of label claim and the sample had no trace of ephedrine. Ephedrine sulfate syrup was analyzed without any cleanup; the chromatogram is shown in Fig. 5. Despite the potentially interfering presence of sugars, dyes, alcohol, etc., the peak corresponding in retention time to ephedrine sulfate showed that the syrup contained 105.2% of label claim. An ephedrine sulfate capsule contained 92.4% of label claim and no pseudoephedrine was found.

It is important to assay the sample at two different sensitivity levels. The first determination should be performed at the most sensitive attenuation position; this will establish whether or not a small amount of the other diastereomer is present. If there is no indication of the presence of the other diastereomer, the lower (least sensitive) attenuation setting can be used for the remaining sample injections. If the other diastereomer is present, standard mixtures may be prepared containing 1–5% of the contaminant diastereomer in the diastereomer of interest.

The wavelength 210 nm was selected because the extinction coefficients are 40 times greater than the extinction coefficients for the maxima at higher wavelengths, with corresponding increase in sensitivity.

Various HPLC columns and solvent systems were evaluated. A silica column, Zorbax Sil (DuPont), with a normal-phase solvent system successfully separated ephedrine and pseudoephedrine; however, the peak due to pseudoephedrine was unacceptably broad and flat and was therefore unamenable to quantitation. There

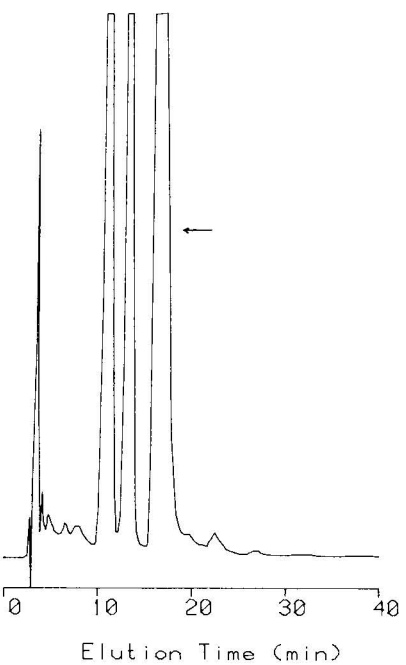

Fig. 5. HPLC chromatogram of an ephedrine sulfate syrup sample diluted with water. The peak corresponding in retention time to ephedrine sulfate is indicated with an arrow.

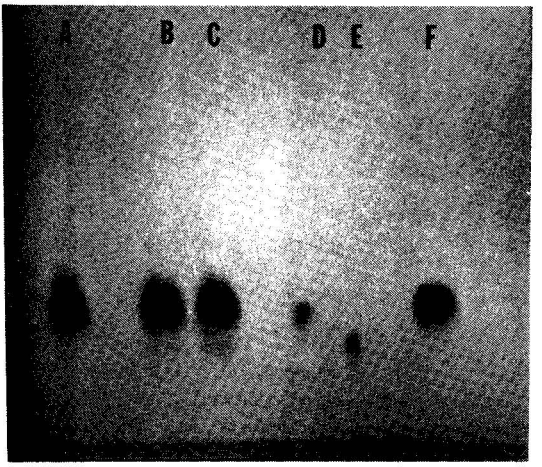

Fig. 6. Photograph of TLC plate: A = 1% pseudoephedrine in ephedrine; B = 5% pseudoephedrine in ephedrine; C = 10% pseudoephedrine in ephedrine; D = ephedrine; E = pseudoephedrine; F = ephedrine sulfate capsule.

was only minimal or no separation of the diastereomers on C_8 columns (E. Merck or DuPont), on Zorbax TMS (DuPont), on μBondapak CN (Waters Assoc.) or on LiChrosorb C_{18} (Altech).

The column of choice was μBondapak phenyl, 10 μm; in fact, separation of ephedrine from pseudoephedrine was observed with three different μBondapak phenyl columns. A 5-μm Spherisorb phenyl column also gave adequate separation of the two diastereomers with 2% acetonitrile. Similar separation was possible if the acetonitrile is replaced by 1% 2-methoxyethanol. In some cases, addition of 2-methoxyethanol sharpened the peaks and gave shorter retention times. A mixture of boric acid, sodium acetate and acetic acid in methanol (pH 4.5) gave the same separation as the phosphate mobile solvent. Other buffers at pH 4.5 gave reasonable separation; we conclude that the identity of the anion is not crucial. The use of phosphate does allow detection at the lower wavelength of 210 nm.

Both silica gel and reversed-phase TLC plates were evaluated for the separation of ephedrine and pseudoephedrine. Good separation was obtained on the reversed-phase plate with the borate system (Fig. 6). The TLC system was initially investigated, but the HPLC assay was found to be more sensitive. Mixtures containing 1, 5 and 10% of pseudoephedrine in ephedrine were prepared and chromatographed by TLC; 5% was the minimum that could be detected with this system.

^{13}C NMR was evaluated because there are usually differences in absorption of the diastereomeric carbons[17]. The proton-decoupled ^{13}C NMR spectrum of ephedrine had absorptions at δ 13.7, 33.7, 60.4, 73.7, 126.2, 126.9, 128.0 and 142.4 ppm; the spectrum of pseudoephedrine had absorptions at δ 15.2, 33.5, 61.1, 77.4, 127.0, 127.4, 128.1 and 1.12.8 ppm with tetramethylsilane as the reference. A problem encountered with the use of ^{13}C NMR as an analytical method was very low sensitivity, which required either very large samples or very long times, in the latter option, for example, more than 8 h per assay. However, the most serious drawback was that 4% of the minor diastereomer was the minimum that could be detected.

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DETERMINATION OF STREPTOMYCIN SULFATE AND DIHYDROSTREPTOMYCIN SULFATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An isocratic, paired-ion reversed-phase high-performance liquid chromatographic method for the determination of streptomycin and dihydrostreptomycin has been developed. The method employs a microparticulate reversed-phase column (μBondapak C_{18}, LiChrosorb RP-18 or Ultrasphere Ion Pair), and a mobile phase composed of 0.02 *M* sodium hexanesulfonate and 0.025 *M* tribasic sodium phosphate in acetonitrile–water (8:92, v/v) at pH 6.0 with detection by ultraviolet absorbance at 195 nm. Resolution and simultaneous quantitation of streptomycin A, dihydrostreptomycin A, streptomycin B, streptidine and process-related substances can be achieved in less than 25 min, with a relative standard deviation of *ca.* 1.1 % for the assay of either streptomycin sulfate or dihydrostreptomycin sulfate. The chromatographic bioequivalency data for streptomycin sulfate and dihydrostreptomycin sulfate were statistically identical with results obtained by the officially recognized microbiological assay. The method is designed for applicability to the analysis of other aminoglycoside antibiotics and antituberculous agents.

INTRODUCTION

Streptomycin and dihydrostreptomycin are clinically useful aminoglycoside antibiotics (Fig. 1) exhibiting comparable antimicrobial activity against a wide range of gram-negative and gram-positive bacteria, as well as mycobacteria, particularly Mycobacterium tuberculosis[1–4]. Streptomycin is produced by the microbial fermentation of *Streptomyces griseus* and was first isolated by Schatz *et al.*[5] in 1944. Dihydrostreptomycin is produced by the catalytic hydrogenation of streptomycin[6]. Numerous chemical and physical methods have been reported for the analysis of streptomycin and dihydrostreptomycin, including paper, thin-layer, and column chromatography, electrophoresis, spectrophotometry and colorimetry, fluorimetry, titrimetry, and polarography[7]. The official methods of analysis for streptomycin sulfate and dihydrostreptomycin sulfate are the USP and EP microbiological turbidimetric procedures and the EP colorimetric method[8,9]. However, none of these methods possesses a desirable combination of speed, specificity, simplicity, sensitivity, and

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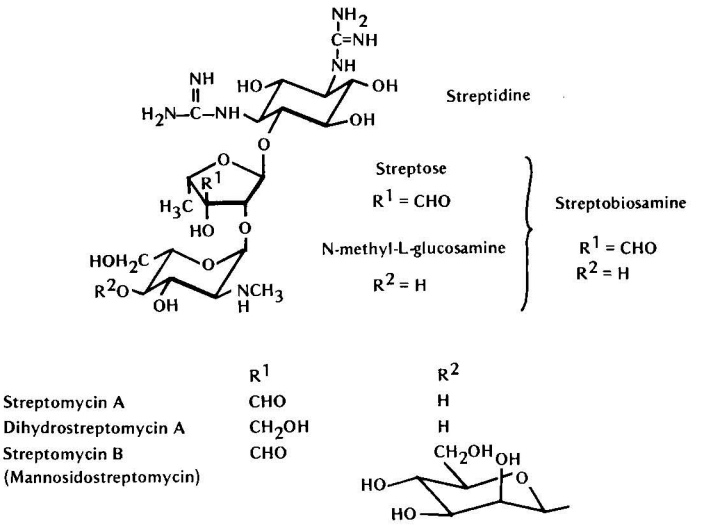


Fig. 1. Structures of streptomycin and process-related substances.

precision. This paper reports the development of a high-performance liquid chromatographic (HPLC) method for separating and quantitating streptomycin A, dihydrostreptomycin A, and their biosynthetic and process-related substances in streptomycin sulfate and in dihydrostreptomycin sulfate. The technique of paired-ion, reversed-phase chromatography is employed, in combination with ultraviolet detection for measurement of the resolved sample components. Using this HPLC methodology, composition profiles of the antibiotics are obtained. Also, the method has potential application to the analysis of the antituberculous agent viomycin sulfate, and the aminoglycoside antibiotics neomycin sulfate and paromomycin sulfate.

EXPERIMENTAL

Apparatus

A Waters M6000A pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a Valco Model CV-6-UHPa-C-20 25-μl loop injection valve (Valco Instruments Co., Houston, TX, U.S.A.) and a Waters 450 variable-wavelength detector attached to a Varian A-25 dual-channel recorder (Varian Instruments Division, Palo Alto, CA, U.S.A.) were employed. The following commercially available prepacked reversed-phase columns were used: (a) LiChrosorb RP-18, 5 μm, 25 cm $\times$ 4.6 mm I.D. (Applied Science Labs., State College, PA, U.S.A., and Altex Scientific, Berkeley, CA, U.S.A.), (b) μBondapak C_{18}, 30 cm $\times$ 3.9 mm I.D. (Waters Assoc.), (c) Ultrasphere Ion Pair, 25 cm $\times$ 4.6 mm I.D. (Altex Scientific). An in-line guard column (25 cm $\times$ 4.6 mm I.D.) packed with either Bondapak C_{18}/Corasil (Waters Assoc.) or Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was connected between the pump and the injection valve. Upon completion of daily analysis, the guard column and analytical column were both washed with a mixture of methanol–water (50:50).

Reagents

Streptomycin sulfate (lot No. 0674-G-5) and dihydrostreptomycin sulfate (lot No. H-1) reference standards were purchased from the United States Pharmacopeial

Convention Inc. (Rockville, MD, U.S.A.). Streptidine sulfate monohydrate (lot No. 3A066-EA) and maltol (lot No. 36326-62EA) were obtained from Pfizer Quality Control Division (Groton, CT, U.S.A.). The reference sample of streptomycin B sulfate was kindly provided by Dr. R. J. Taylor of Pfizer Chemicals Division (Groton, CT, U.S.A.). Dihydrostreptomycin B sulfate was prepared by the sodium borohydride reduction of streptomycin B sulfate in water[10]. Streptobiosamine and dihydrostreptobiosamine were prepared according to the literature[11,12]. Distilled water, acetonitrile, distilled in glass (Burdick & Jackson, Muskegon, MI, U.S.A.), 1-hexanesulfonic acid, sodium salt (Regis, Morton Grove, IL, U.S.A.), sodium phosphate tribasic (Mallinckrodt, Paris, KY, U.S.A.), and 85% phosphoric acid (Mallinckrodt) were used without further purification.

Mobile phase preparation

The mobile phase was prepared by dissolving 3.8 g of sodium 1-hexanesulfonate and 9.5 g of sodium phosphate tribasic in a mixture of 850 ml of distilled water and 80 ml of acetonitrile. The solution was adjusted to pH 6.0 with phosphoric acid, diluted to one liter with distilled water, and filtered through a 5-μm, Type LS, Millipore filter (Millipore, Bedford, MA, U.S.A.) prior to use. The amount of acetonitrile was adjusted to obtain maximum performance of the column. Decreasing the amount of acetonitrile in the mobile phase increased the elution time (*i.e.* improved resolution) of streptidine, streptomycins A and B, and dihydrostreptomycin A, while increasing the amount of acetonitrile decreased the elution time of these substances.

Chromatographic conditions

The column flow-rate was maintained at *ca.* 1 ml/min. The column temperature was maintained at 25.0 ($\pm$0.1)°C. The detector wavelength was set at 195 nm using a 0.2 absorbance units range selection. Dual-channel recorder input of 2 mV and 10 mV and a chart speed of 10 in./h were employed.

Streptomycin sulfate analysis

Streptomycin sulfate standard solution. Approximately 200 mg of dry USP streptomycin sulfate reference standard was accurately weighed into a 50-ml volumetric flask. Distilled water was added to dissolve the substance, then the flask was diluted to volume with additional distilled water. A 3-ml volume of this stock solution was then diluted to 50 ml with mobile phase and analyzed.

Streptidine sulfate monohydrate standard solution. Approximately 125 mg of streptidine sulfate monohydrate reference standard was accurately weighed into a 500-ml volumetric flask and diluted to volume with water. The substance was dissolved with the aid of sonification. A 3-ml aliquot of this stock solution was diluted to 25 ml with water, after which a 2-ml aliquot was removed and diluted to 25 ml with mobile phase and analyzed.

Sample preparation. The streptomycin sulfate sample solution was prepared and analyzed in the same manner as the standard solution.

Calculations

The streptomycin sulfate sample potency and streptidine sulfate monohydrate content are calculated by using the following equations:

$$\text{streptomycin sulfate potency } (\mu g/mg) = \frac{SA_{spl}}{SA_{std}} \times \frac{conc_{std}}{conc_{spl}} \times \text{std. potency } (\mu g/mg)$$

where SA_{spl} and SA_{std} are the streptomycin A peak heights of the sample and standard, respectively; $conc_{std}$ and $conc_{spl}$ are the final concentrations (mg/ml) of the standard and sample solutions, respectively; and std. potency is the potency of streptomycin sulfate standard.

$$\text{streptidine sulfate monohydrate } (\%) = \frac{Sd_{spl}}{Sd_{std}} \times \frac{conc_{std}}{conc_{spl}} \times 100\%$$

where Sd_{spl} and Sd_{std} are the streptidine peak heights of the sample and standard, respectively, and $conc_{std}$ and $conc_{spl}$ are the final concentrations (mg/ml) of the standard and sample solutions, respectively.

Dihydrostreptomycin sulfate analysis

Dihydrostreptomycin sulfate standard solution. Approximately 200 mg of dry USP dihydrostreptomycin sulfate reference standard was accurately weighed into a 50-ml volumetric flask, distilled water was added, the substance dissolved, and the flask was diluted to volume with distilled water. A 3-ml aliquot of this stock solution was then diluted to 50 ml with mobile phase and analyzed.

Streptidine sulfate monohydrate standard solution. The streptidine sulfate monohydrate standard solution was prepared and analyzed as described under *Streptomycin sulfate analysis.*

Trace level streptomycin sulfate standard solution. A 5-ml aliquot of the streptomycin sulfate standard solution was diluted to 50 ml with water, after which a 5-ml aliquot was removed and diluted to 50 ml with mobile phase and analyzed.

Sample preparation. The dihydrostreptomycin sulfate sample solution was prepared and analyzed in the same manner as the standard solution.

Calculations

The dihydrostreptomycin sulfate sample potency, streptidine content, and streptomycin A content are calculated by using the following equations:

$$\text{dihydrostreptomycin sulfate potency } (\mu g/mg) = \frac{DHSA_{spl}}{DHSA_{std}} \times \frac{conc_{std}}{conc_{spl}} \times \times \text{std. potency } (\mu g/mg)$$

where $DHSA_{spl}$ and $DHSA_{std}$ are the dihydrostreptomycin A peak heights of the sample and standard, respectively, and $conc_{std}$ and $conc_{spl}$ are the final concentrations (mg/ml) of the standard and sample solutions, respectively; std. potency is the potency of dihydrostreptomycin sulfate standard.

$$\text{streptidine sulfate monohydrate } (\%) = \frac{Sd_{spl}}{Sd_{std}} \times \frac{conc_{std}}{conc_{spl}} \times 100\%$$

$$\text{streptomycin A base (\%)} = \frac{SA_{spl}}{SA_{std}} \times \frac{conc_{std}}{conc_{spl}} \times 100\%$$

RESULTS AND DISCUSSION

The goal of this investigation was to developed a rapid, sensitive and specific chemical assay for streptomycin, dihydrostreptomycin, and process-related substances. The assay would serve as an alternative to the microbiological method and would be suitable for routine analysis (*i.e.* automation) and facile interlaboratory and compendial adoption. HPLC was selected as the most informative analytical tool in satisfying our assay criteria.

Chromatographic assay and development optimization

Because of the characteristic ionic, water-soluble nature of streptomycin and dihydrostreptomycin, a paired-ion reversed-phase HPLC mode of separation was explored. Recently, this technique has been successfully applied to the analysis of various types of therapeutic agents[13–19]. Initial developmental work using a μBondapak C_{18} column, a heptanesulfonic acid–acetate buffer mobile phase, and refractive index detection resulted in resolution of streptomycin A, dihydrostreptomycin A, and streptidine and thereby demonstrated the feasibility of the paired-ion reversed-phase approach. Further work focused on evaluation of column type and on optimization of mobile phase variables, such as concentration of buffer, organic modifier, type of counter-ion, and pH.

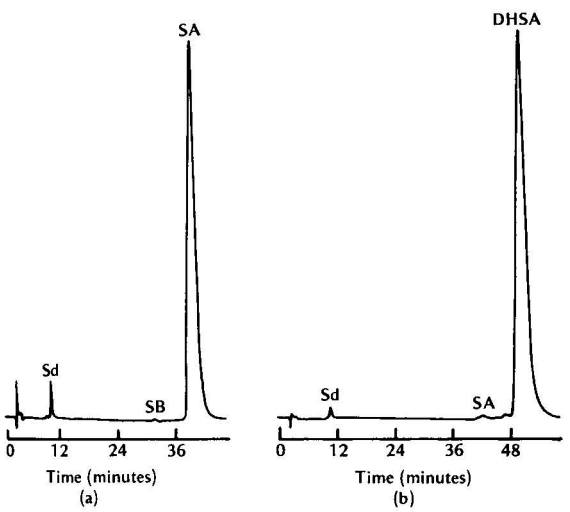


Fig. 2. Paired-ion HPLC chromatograms of (a) USP streptomycin sulfate and (b) USP dihydrostreptomycin sulfate using a LiChrosorb RP-18 (5 μm) column. Mobile phase: 0.02 *M* sodium hexanesulfonate, 0.025 *M* tribasic sodium phosphate in acetonitrile–water (8:92, v/v), pH 6.0. Flow-rate: 0.8 ml/min. Sample size: 10 μg. Detector: UV at 195 nm, and 0.1 a.u.f.s. Peaks: Sd = streptidine; SB = streptomycin B; SA = streptomycin A; DHSA = dihydrostreptomycin A.

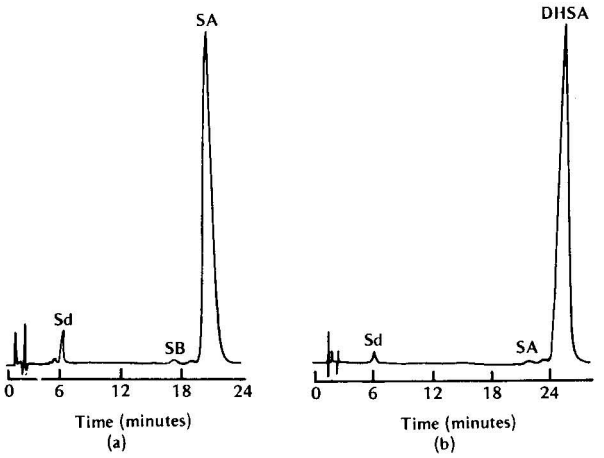


Fig. 3. Paired-ion HPLC chromatograms of (a) USP streptomycin sulfate and (b) USP dihydrostreptomycin sulfate using a μBondapak C_{18} (10 μm) column. Mobile phase: 0.02 *M* sodium hexanesulfonate, 0.025 *M* tribasic sodium phosphate in acetonitrile–water (8:92, v/v), pH 6.0. Flow-rate: 1.3 ml/min. Sample size: 5 μg. Detector: UV at 195 nm, and 0.2 a.u.f.s. Peaks as in Fig. 2.

Column type

Though both Altex LiChrosorb RP-18 and Waters μBondapak C_{18} columns were used and performed satisfactorily, the μBondapak C_{18} column was chosen for the analysis procedure because it was more amenable to routine use. An Altex Ultrasphere Ion Pair column was also been evaluated and was found to be an attractive alternative to the μBondapak C_{18}. Representative HPLC chromatograms of streptomycin sulfate and dihydrostreptomycin sulfate obtained with the LiChrosorb RP-18,

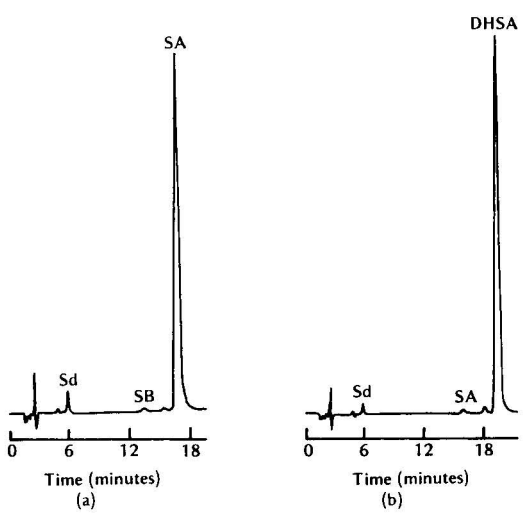


Fig. 4. Paired-ion HPLC chromatograms of (a) USP streptomycin sulfate and (b) USP dihydrostreptomycin sulfate using an Ultrasphere Ion Pair (5 μm) column. Mobile phase: 0.02 *M* sodium hexanesulfonate, 0.025 *M* tribasic sodium phosphate in acetonitrile–water (10:90, v/v), pH 6.0. Flow-rate: 1.3 ml/min. Sample size: 5 μg. Detector: UV at 195 nm, and 0.2 a.u.f.s. Peaks as in Fig. 2.

μBondapak C_{18}, and Ultrasphere Ion Pair columns using the optimal mobile phase described below are presented in Figs. 2, 3 and 4, respectively.

Mobile phase

To achieve maximum assay sensitivity, an acetonitrile–water mobile phase consisting of a phosphate–phosphoric acid buffer in lieu of the aforementioned acetate–acetic acid buffer was chosen to allow for far-ultraviolet (UV) detection. The high UV transparency of the phosphate buffer at *ca.* 195 nm permitted the simultaneous detection of streptomycin A, dihydrostreptomycin A, and several process-related substances. Following these assay improvements, a systematic study was made of the mobile phase variables responsible for assay selectivity and efficiency as characterized by the appropriate capacity factors. The mobile phase variables examined were: concentration of trisodium phosphate and acetonitrile, type and concentration of counter-ion, and pH. The following observations were made.

Effect of mobile phase trisodium phosphate and acetonitrile concentration upon the capacity factor (k'_{SA}) of streptomycin A. Increasing the concentration of either trisodium phosphate or acetonitrile causes a reduction in k'_{SA} and, in general, the capacity factors of all of the process-related substances (*i.e.* streptomycin B, dihydrostreptomycin A, streptidine, etc.). Of these two mobile phase variables, the acetonitrile concentration had a more pronounced effect on the resolution of streptomycin A and the aforementioned substances. Use of a buffer salt in the mobile phase was necessary, as its absence resulted in excessive streptomycin A and dihydrostreptomycin A retention times (*i.e.*, >45 min). Buffer concentration below 0.01 *M* resulted in skewed or tailing component peaks and poor component resolution. A similar phenomenon was observed by Anhalt[16] in his developmental work on a paired-ion HPLC method for assaying gentamycin in serum.

Effect of concentration and type of counter-ion upon k'_{SA}. When the alkylsulfonic acid counter-ion molar concentration is equal to or greater than the trisodium phosphate molar concentration, increasing the counter-ion concentration increases k'_{SA} as well as the capacity factors of streptomycin sulfate process-related substances. When the counter-ion concentration is less than the trisodium phosphate concentration, skewing or tailing of the streptomycin A peak occurs. The type of counter-ion (*i.e.* pentane-, hexane-, or heptanesulfonic acid) used in the mobile phase has a predictable effect upon k'_{SA}, that is, the retention time of streptomycin A increases in the following order: pentane sulfonate, hexane sulfonate, heptane sulfonate. Hexanesulfonic acid was selected in preference to pentanesulfonic acid and heptanesulfonic acid as the preferred mobile phase counter-ion, because it offered optimal resolution of the components of interest within a reasonable chromatographic analysis time (*i.e.*, 30 min or less). A 0.02 *M* counter-ion concentration was selected to obtain assay linearity for both streptomycin sulfate and dihydrostreptomycin sulfate at the sample concentration used for analysis.

Effect of pH on k'_{SA}. Employing the HPLC conditions cited in Fig. 2, the effect of pH on k'_{SA} and capacity factors of streptomycin sulfate process-related substances was examined. As illustrated in Fig. 5, the mobile phase pH has a significant effect upon k'_{SA} and the capacity factors of dihydrostreptomycin A, streptomycin B, and streptidine. A mobile phase pH of 6 was chosen to maximize component resolution and solution stability of the streptomycin and dihydrostreptomycin samples.

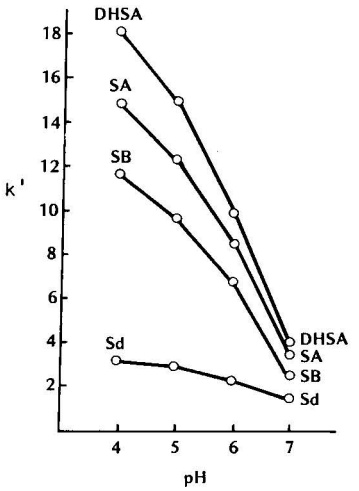


Fig. 5. Influence of pH of the mobile phase on capacity factors of streptomycins and process-related substances.

The information gathered in this systematic study of mobile phase variables proved to be invaluable in arriving at the optimal HPLC conditions for streptomycin sulfate and dihydrostreptomycin sulfate analysis. Based on the above observations, the chosen optimal mobile phase was 0.02 *M* sodium hexanesulfonate and 0.025 *M* tribasic sodium phosphate in acetonitrile–water (8:92, v/v) at pH 6.0. HPLC chromatograms generated with such a mobile phase are presented in Figs. 2–4. Retention data for streptomycin A, dihydrostreptomycin A, and corresponding process-related substances are summarized in Table I.

TABLE I

RELATIVE RETENTIONS OF STREPTOMYCIN A, DIHYDROSTREPTOMYCIN A AND PROCESS-RELATED SUBSTANCES

Compound	*Relative retention*
Streptobiosamine	void*
Dihydrostreptobiosamine	void*
Streptidine	0.30
Maltol**	0.45
Streptomycin B	0.80
Streptomycin A	1.00
Dihydrostreptomycin B	1.05
Dihydrostreptomycin A	1.20

* Under the cited chromatographic conditions, streptobiosamine and dihydrostreptobiosamine exhibited peak UV absorptivities only in the column void volume region of the chromatogram.

** Maltol (3-hydroxy-2-methyl-4H-pyran-4-one) is formed from the streptose portion of both streptomycin A and streptomycin B when either compound is heated in the presence of dilute alkali.

Linearity

Assay linearity for streptomycin A in streptomycin sulfate and dihydrostreptomycin A in dihydrostreptomycin sulfate was established within a concentration range of 0.024–0.24 mg/ml. Trace level assay linearity for streptidine, streptomycin A, and streptomycin B was established over a $1.0–3.0 \cdot 10^{-3}$ mg/ml concentration range.

Precision

The assay precision of the HPLC method was determined for five individual weights of a streptomycin sulfate sample and five individual weights of a dihydrostreptomycin sulfate sample. The relative standard deviation of streptomycin A determination and dihydrostreptomycin A determination was 1.1%. The precision of streptidine determination in both streptomycin sulfate and dihydrostreptomycin sulfate, of streptomycin B determination in streptomycin sulfate, and of streptomycin A determination in dihydrostreptomycin sulfate at approximately the 1% concentration level were 1.9%, 1.7% and 2.0%, respectively. The precision studied over a four day period was comparable.

Detection and sensitivity

The mobile phase selection (acetonitrile and phosphate buffer) permitted detection of both streptomycin A and dihydrostreptomycin A and related substances at *ca.* 200 nm or shorter wavelengths. This choice of mobile phase composition simplified the assay development work markedly by eliminating the need to develop a suitable post-column chemical derivatization detector. At a wavelength of 195 nm, the detection limit for streptomycins A and B, and dihydrostreptomycin A in streptomycin

TABLE II

ANALYSIS OF STREPTOMYCIN SULFATE

Sample	*Potency (μg/mg)*		*Streptidine sulfate monohydrate (%)*
	Turbidimetric (microbiological)	*HPLC*	
1	739	743	1.09
2	726	731	1.25
3	732	736	1.32
4	724	730	1.30
5	719	724	1.41
6	709	696	1.58
7	693	691	1.80
8	715	698	1.55
9	719	731	1.15
10	727	718	1.38
11	737	727	1.22
12	737	747	1.61
13	741	741	1.36
14	765	762	1.03
15	762	750	1.06
Average	730	728	

sulfate and dihydrostreptomycin sulfate dry powder products, is *ca.* 2 μg/ml or 50 ng/25 μl injection. Streptidine assay sensitivity of at least 0.5 μg/ml (*i.e.* 12.5 ng/25 μl injection) can be expected in the analysis of similar streptomycin and dihydrostreptomycin samples.

Assay equivalency with microbiological methodology

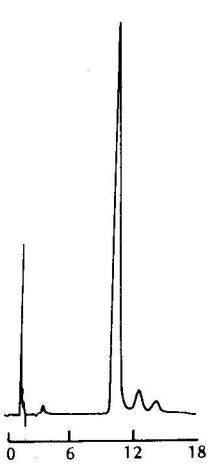
The assay equivalency of the HPLC method was examined by analyzing fifteen samples each of streptomycin sulfate and dihydrostreptomycin sulfate and comparing the calculated HPLC results with those obtained by the turbidimetric microbiological assay methodology (Tables II and III). The results demonstrate that the HPLC assay is equivalent to the bioassay.

TABLE III

ANALYSIS OF DIHYDROSTREPTOMYCIN SULFATE

Sample	*Potency (μg/mg)*		*Streptidine sulfate monohydrate (%)*	*Streptomycin A sulfate (%)*
	Turbidimetric (microbiological)	*HPLC*		
1	680	670	0.81	0.34
2	736	717	0.63	0.69
3	720	722	0.53	0.14
4	706	703	0.44	0.25
5	655	665	0.64	0.12
6	723	734	0.46	0.30
7	721	721	0.49	0.51
8	727	739	0.44	0.19
9	717	713	0.42	0.69
10	728	727	0.75	0.29
11	731	728	0.32	0.42
12	742	737	0.44	0.38
13	753	735	0.42	0.11
14	702	706	1.60	0.39
15	699	703	1.53	0.83
Average	716	715		

The capability of simultaneously determining streptidine, streptomycin A and B, and dihydrostreptomycin A played a decisive role in designing this paired-ion reversed-phase HPLC assay. High assay sensitivity for trace level determination of streptidine and streptomycin B in streptomycin sulfate and of streptidine and streptomycin A in dihydrostreptomycin sulfate is of interest in monitoring studies of streptomycin A biosynthesis and dihydrostreptomycin production (through hydrogenation of streptomycin A) to ascertain optimal conditions for maximum product yield and quality. Use of a dual channel recorder allows the determination of streptidine, streptomycin A, (and streptomycin B) at the 1% or lower concentration level as demonstrated by the data in Tables II and III.



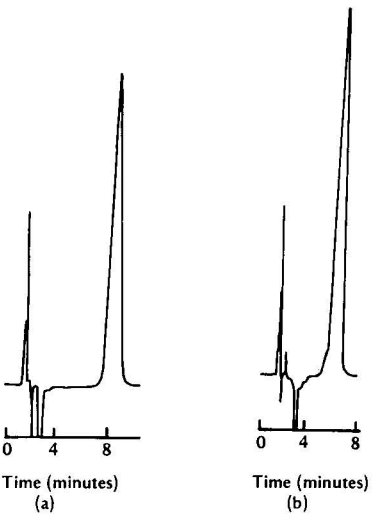


Fig. 6. Paired-ion HPLC chromatogram of viomycin sulfate using chromatographic conditions as described in Fig. 3 except with a two-fold flow-rate.

Fig. 7. Paired-ion HPLC chromatograms of (a) neomycin sulfate (0.25 mg) and (b) paromomycin sulfate (0.25 mg) using a μBondapak C_{18} (10 μm) column. Mobile phase: 0.016 *M* sodium hexanesulfonate, 0.02 *M* tribasic sodium phosphate in acetonitrile–water (17:83, v/v) pH 3.5. Flow-rate: 1.5 ml/min. Detector: refractive index, 8 × range, 10 mV recorder span.

Applicability to the analysis of other aminoglucosides and antituberculous agents

The HPLC conditions described here, without modification, have been applied directly to the analysis of the antituberculous agent, viomycin (Fig. 6). With only a minor mobile phase modification and by employing refractive index detection, the aminoglucosides paromomycin and neomycin can be similarly analyzed by this method (Fig. 7).

CONCLUSION

A rapid, accurate and sensitive paired-ion reversed-phase HPLC assay for separating and quantitating streptomycin A, dihydrostreptomycin A and their biosynthetic and process-related intermediates has been developed. The simplicity of the method should allow facile interlaboratory use. Furthermore, it is suitable for automation and has the potential for compendial adoption. It should be considered as an acceptable alternative to the microbiological method in routine analyses. The method is applicable to the analysis of streptomycin and dihydrostreptomycin finished products, other aminoglycoside antibiotics, and antituberculous agents. Assay sensitivity is adequate for pharmacological studies to determine either streptomycin A or dihydrostreptomycin A in biological matrices.

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Note

Effect of pH on the gas chromatographic behaviour of silica gel

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Silica gel is one of the popular adsorbents[1–4] used in gas chromatography (GC), and belongs to the second type of specific adsorbent in Kiselev's classification[5]. The effects of chemical and geometrical structure[5,6] of the surface of silica gel and the effects of various thermal[7,8] and chemical [9–11] pre-treatments on its GC behaviour have been studied previously. However, unlike organic porous polymers[12,13], the effects of the conditions of synthesis on the GC behaviour of silica gel have not been elucidated.

Recently in our laboratory the effects of gel pH and the conditions of washing on the surface properties of silica gels and their influence on the moisture adsorption capacity have been studied[14]. In this work we have studied these gels in order to establish the effect of gel pH on their GC behaviour.

EXPERIMENTAL

The experiments were performed on a CIC (Chromatography and Instruments Co, Baroda, India) gas chromatograph equipped with a katharometer detector and Omniscribe (Digital Electronics Ltd., Bombay, India) Series 5000 strip-chart recorder complete with an integrator.

Samples of silica gel were prepared by acidification of sodium silicate solutions. The gel was washed with distilled water until it was free from electrolytes. The detailed method of preparation of these gels has been reported earlier[14,15]. The gel was ground and the fraction of 80–100 mesh was evenly packed in copper columns (2 mm I.D.) by a conventional method. Hydrogen was used as the carrier gas. The columns were activated at 200°C for 2 h under a stream of hydrogen before use. The samples were injected with a 2-ml Chirana gas-tight syringe (Laboratory Instruments, Prague, Czechoslovakia).

The specific retention volume (V_g) was calculated from the expression

$$V_g = j\,[F_C(t_R - t_A)/W_L]\,273/T$$

where F_C is the volume flow-rate measured at the column outlet with a soap-bubble flow meter and corrected to the column temperature (T) and outlet pressure, and t_R, t_A, W_L and j are the retention time of the sorbate, the dead retention time, the weight of the sorbent in the column and the pressure correction factor, respectively. The

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peak asymmetry was expressed as the ratio of the distances of the front and the rear of a peak as measured at one tenth of the peak height, from the perpendicular from the peak maximum to the time coordinate[16].

RESULTS AND DISCUSSION

The effect of gel pH on the chromatographic selectivity of silica gel towards permanent gases is depicted in Fig. 1. The chromatographic selectivities of these gels were compared in terms of their ability to separate a mixture of air, carbon monoxide and methane. A decrease in selectivity of the gel as the pH increases from 1.1 to 4.1 is apparent.The best separation was achieved with silica gel prepared at pH 1.1 and this separation is even slightly better than that achieved earlier[1].

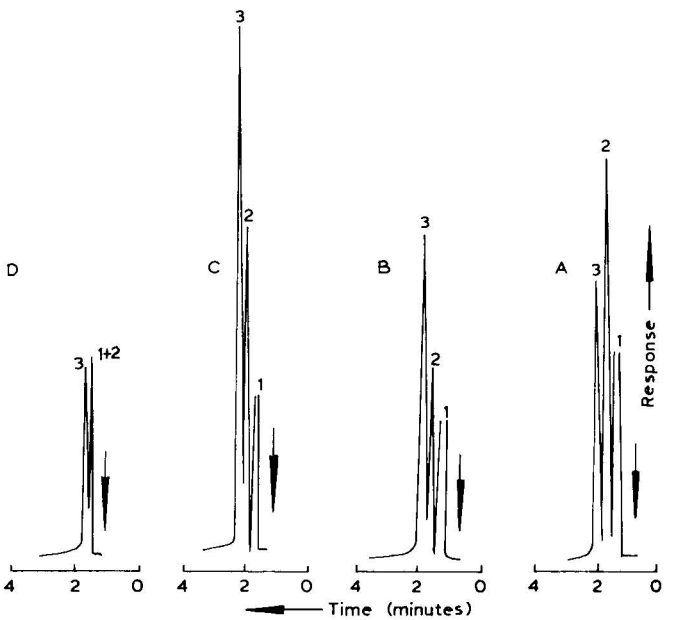

Fig. 1. Effect of gel pH on the GC selectivity of silica gel. Column, 2 m × 2 mm I.D.; temperature, ambient (32–33° C); flow-rate, 30 ml/min. pH: A, 1.1; B, 2.6; C, 4.1; D, 11.0. Peaks 1, air; 2, carbon monoxide; 3, methane.

For practical purposes silica gel of high surface area produced at a low pH is suitable for the separation of common gases and a gel with a low surface area is unsuitable for this separation (Fig. 1D).

A graph of pressure drop (ΔP) *versus* volume flow-rate for columns packed with silica gels produced at different pHs is shown in Fig. 2. The broken line is the average drawn through all the points. Although the lines obtained with individual gel are fairly straight, they vary around the average line within experimental error (*i.e.*, column-to-column reproducibility). Similar results were obtained at ambient temperature. The column permeability is thus independent of the gel pH and is significantly lower than that of Chromosorb P.

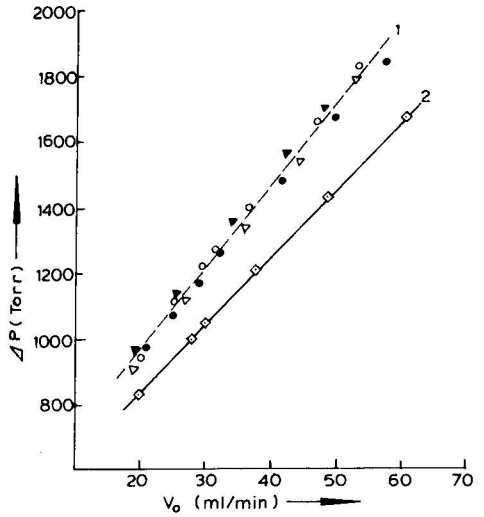

Fig. 2. Plots of pressure drop (ΔP) *versus* volume flow-rate on silica gel. Column, 2 m × 2 mm I.D.; temperature, 100°C. pH: ○, 1.1; ●, 2.6; △, 4.1; ▲, 11.0. □, Chromosorb P.

In Fig. 3, the pressure drop at a flow-rate of 30 ml/min in columns packed with different silica gels and Chromosorb P is plotted against column temperature. The results indicate that the gel pH has no effect on the temperature dependence of the column permeability. The packing structure of the bed seems to be virtually unaffected by the gel pH.

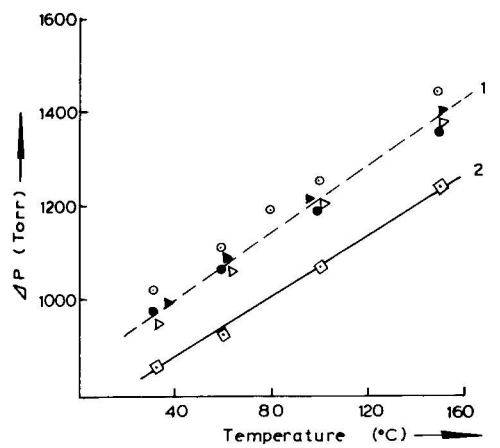

Fig. 3. Plots of ΔP *versus* column temperature on silica gel. Column, 2 m × 2 mm I.D.; flow-rate, 30 ml/min. Symbols as in Fig. 2.

The graphs of log V_g *versus* $1/T$ for these gels are shown in Fig. 4. The parallel lines indicate that the heat of sorption is independent of gel pH. It therefore follows that the chemical nature of the surface is independent of gel pH. With Porasils (porous silica beads), Feltl and Smolková[4] observed an approximately constant concentration

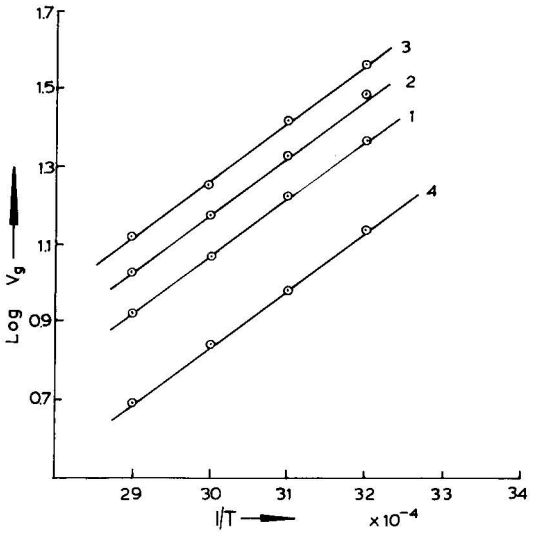

Fig. 4. Plots of log V_g for carbon dioxide *versus* $1/T$ on silica gel. Column, 1.4 m × 2 mm I.D. pH: 1, 1.1; 2, 2.6; 3, 4.1; 4, 11.0.

of hydroxyl groups on the surface and this group is believed generally to be the only specific interaction centre on these surfaces.

Some interesting results of the effect of gel pH on the capacity ratio (k') for carbon dioxide are shown in Table I, together with BET surface area determined from the sorption of nitrogen. Although the surface area decreases with increase in gel pH, k' increases significantly up to pH 4.1 followed by a drastic decrease at pH 11.0.

TABLE I

EFFECT OF GEL pH ON CAPACITY RATIO (k') FOR CARBON DIOXIDE

Column, 1.4 m × 2 mm I.D.; temperature, 70°C; flow-rate, 30 ml/min.

Gel pH	*Capacity ratio* (k')	*Surface area*[14] (m^2/g)
1.1	6.8	858
2.6	10.1	759
4.1	10.3	486
11.0	2.6	190

In order to understand quantitatively the interaction between the sorbate and the sorbent surface, the effective surface area available to the sorbate molecule rather than the true surface area determined by the sorption of nitrogen should be considered. As the gel pH increases, the entire pore spectrum shifts to larger pore diameters[17]. As a result, it is expected that the effective surface area available to the carbon dioxide molecule will increase with increasing gel pH up to a certain stage, and thereafter it may decrease. The increase in k' value with increasing gel pH up to pH 4.1 is thus explained. A similar observation with Porasils was made by Feltl and Smolková[4].

Earlier Guillemin *et al.*[3] found that, anomalies occur in the dependence of the sorption of butene-1 on Spherosils on the surface area, and they explained this phenomenon by a change in the concentration of the so-called reactive hydroxyl groups. The active hydroxylgroups or reactive groups are determined by selective silanization with trimethylchlorosilane. Their conclusion cannot be considered to be authoritative as the results for the reactive hydroxyl groups were related to unit weight of the adsorbent. For direct comparison the results should be related to unit surface area. Further, the values of the specific surface areas determined by the sorption of nitrogen should be replaced with the values obtained by the adsorption of a sorbate of similar structure. Feltl and Smolková[4] treated the data of Guillemin *et al.*[3] in this way and did not find any anomaly.

In adsorption chromatography, peak asymmetry can stem from the surface heterogeneity of the sorbent. The effect of gel pH on peak asymmetry is depicted in Table II. The peak asymmetry increases with increase in gel pH. With gels of pH 4.1 and 11.0 very tailed peaks were found even when the needle volume of the gas was injected. Hence the surface heterogeneity seems to increase with increasing gel pH.

TABLE II

EFFECT OF GEL pH ON THE PEAK ASYMMETRY FOR CARBON DIOXIDE

Column, 1.4 m × 2 mm I.D.; temperature, 70°C; flow-rate, 30 ml/min; sample load, 0.2 ml.

Gel pH	*Peak asymmetry*
1.1	0.75
2.6	0.69
4.1	0.47
11.0	0.31

The structural heterogeneity and the heterogeneity of the adsorption force field of silica gels are known to increase with decreasing average pore size[5]. However, the present results are contrary to this general behaviour. An irregular shape of the curve was also observed earlier[3] on plotting the asymmetry as a function of the specific surface area of Spherosils.

The rate of polymerization of silicic acid varies with pH. The growth of particles of polymers in the pH ranges 9.5–9 and below 2 is due mainly to the polymerization between monosilicic and polysilicic acids, and in the pH range 7–4 mainly to the random condensation of all the polymer species with each other[18]. In acidic solutions, the size distribution of the polymers is symmetrical, whereas a broad and random distribution of particle sizes of the polymers arises from a random condensation of all the polymer species with each other (pH 4–7). A random condensation mechanism may result in a very uneven distribution of the various silanol groups on the surface. The structural heterogeneity (especially with respect to pore shape) and the heterogeneity of the adsorption force field of these gels probably stem from this condensation mechanism. Although we could not include a large number of gel samples in our study, the results on peak asymmetry seem to be related to this pH-dependent condensation mechanism.

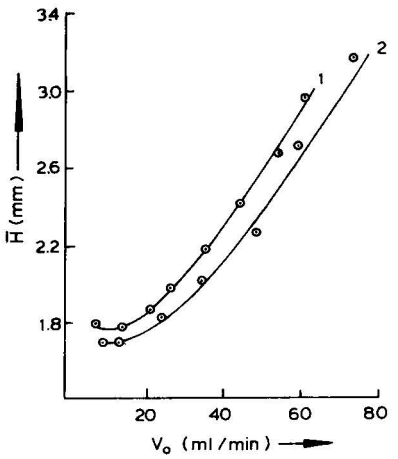

Fig. 5. Effect of carrier gas flow-rate on $\bar{H}$ for carbon dioxide. Column, 1.4 m × 2 mm I.D.; temperature, 70°C. pH: 1, 1.1; 2, 2.6.

The effect of carrier gas flow-rate on the apparent plate height ($\bar{H}$) for the gels produced at pH 1.1 and 2.6 is depicted in Fig. 5. In order to obtain symmetrical peaks the experiments were conducted at a high sensitivity, injecting only the needle volume of gas. Similar plots for the other gels could not be made owing to considerable peak tailing. The slopes of the lines in the mass-transfer predominant flow-rate region are virtually identical. Hence the total non-equilibrium mass transfer coefficients for carbon dioxide are virtually identical for these gels. An increased eddy diffusion contribution to $\bar{H}$ is apparent for the gel produced at pH 1.1 relative to that produced at pH 2.6. However, for practical purposes this difference is not very significant.

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Note

Sorption behaviour of several organic compounds on 6-deoxycyclodextrin polyurethane resins

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(First received May 19th, 1981; revised manuscript received July 6th, 1981)

Many kinds of porous polymer beads have been used as column packings for collecting organic compounds in air or water and/or separating them chromatographically; Amberlite XAD resins are typical examples[1–3]. These and related resins have energetically homogeneous sorption sites and no specific ones. In order to increase the selectivity of resins for organic compounds, we prepared polyurethane resins containing cyclodextrins as specific sorption sites[4]. These cyclodextrin resins exhibited interesting, selective sorption behaviour considered to be based on the formation of inclusion compounds[4–6].

It is of great interest to investigate the sorption behaviour of polyurethane resins containing 6-deoxycyclodextrins whose primary hydroxyl groups are all deoxygenated, because such chemical modification may affect the interaction of the resins with organic sorbates. The sorption behaviour of several model organic compounds on such resins is briefly described here.

EXPERIMENTAL

The materials used were as described previously[4].

Preparation of 6-deoxycyclodextrin polyurethane resins

6-Deoxy-α- or -β-cyclodextrin[7] (10.0 g) was dissolved in 150 ml pyridine in a 500-ml three-necked flask at room temperature. One third of the pyridine was distilled off to remove trace amounts of water. The calculated amount of HDI or H6XDI (see Table I) was added, and the solution was stirred at 115°C for 6 h under nitrogen. The 6-deoxycyclodextrin polyurethane resin was precipitated from a large excess of acetone. It was purified by thorough Soxhlet extraction with acetone and then with water, and dried *in vacuo* at 80°C for 24 h. The resin thus obtained was granulated to particle sizes of 60–80 and 100–200 mesh with an agate mortar and sieved.

Apparatus

A Shimadzu Model GC-3BF gas chromatograph equipped with a flame ionization detector was used. A glass column (80 cm × 3 mm I.D.) was packed with the resin particles (60–80 mesh). The nitrogen flow-rate was 30 ml/min. The detector and column temperatures were maintained at 150°C.

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An Atto Model SF-0396-57 pump and an Mitsumi Model SF-1205 UV monitor (at 254 nm) were used for liquid chromatographic and breakthrough studies. The resin particles (100–200 mesh) were packed in a glass column (5.2 mm I.D.) to form a 40 cm long bed for the former studies and a 10 cm long bed for the latter.

RESULTS AND DISCUSSION

Table I shows the isocyanate/hydroxyl ratio in the monomer feed and the physical properties of 6-deoxycyclodextrin polyurethane resins.

TABLE I

PHYSICAL PROPERTIES OF 6-DEOXYCYCLODEXTRIN POLYURETHANE RESINS

6-D-α = 6-Deoxy-α-cyclodextrin; 6-D-β = 6-deoxy-β-cyclodextrin; HDI = hexamethylene diisocyanate; H6XDI = 1,3-bis(isocyanatomethyl)cyclohexane.

Resin	*NCO/OH in feed*	*Cyclodextrin content* (%, w/w)*	*Temperature limit** (°C)*	*Surface area*** (m^2/g)*
6-D-β-HDI	1.20	53.2	220	7
6-D-α-HDI	1.20	46.8	220	11
6-D-β-H6XDI	0.57	57.7	220	30

* Calculated from elemental analysis.
** Determined thermogravimetrically.
*** Estimated by the BET method.

Fig. 1 is a typical gas chromatogram of a mixture of five organic compounds with similar boiling points. It is apparent that these five compounds can be well separated on the 6-D-β-HDI column more efficiently than on the β-HDI column. This mixture was also chromatographed on four other columns. The peaks of benzene and

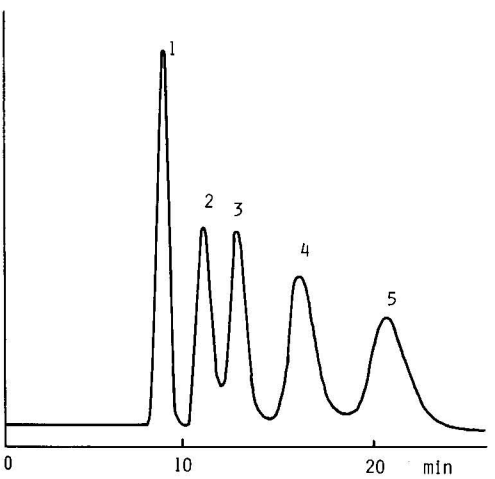

Fig. 1. Gas chromatogram of five organic compounds with similar boiling points on 6-D-β-HDI. Peaks: 1 = cyclohexane; 2 = ethanol; 3 = methyl propionate; 4 = benzene; 5 = methyl ethyl ketone.

cyclohexane overlap on the columns packed with Porapak Q (80 cm at 150°C) or 10% SE-30 (2 m at 45°C). Cyclohexane, methyl ethyl ketone and methyl propionate cannot be separated on 10% dinonyl phthalate (DNP) (1.5 m at 60°C). The PEG HT column exhibits much poorer resolution.

The gas chromatographic retention times of several organic compounds on the polyurethane resins are given in Table II. The retention times of octane and cyclohexane on 6-D-β-HDI are more than ten times larger than those on β-HDI prepared by cross-linking unmodified β-cyclodextrin with hexamethylene diisocyanate. The deoxygenation at the primary hydroxyl groups of the β-cyclodextrin units results in a significant increase in the interaction of the resin with octane and cyclohexane, which may be due to the increase in hydrophobicity at the cyclodextrin torus. Both 6-D-β-HDI and β-HDI exhibit similar retention behaviour for the other compounds in Tabel II. 6-D-β-H6XDI interacts with the organic compounds slightly more strongly, compared with 6-D-β-HDI. All the compounds studied interact with 6-D-α-HDI much more weakly than with 6-D-β-HDI.

TABLE II

RETENTION TIMES (RELATIVE TO BENZENE) ON 6-DEOXYCYCLODEXTRIN POLYURETHANE RESINS

Actual retention times (min) are given in parentheses.

Compound	*6-D-β-HDI*	*6-D-α-HDI*	*6-D-β-H6XDI*	*β-HDI*
Octane	1.03	0.70	2.41	0.07
Ethanol	0.68	1.84	1.25	0.50
Methyl ethyl ketone	1.24	2.04	1.82	1.02
Methyl propionate	0.79	1.31	1.31	0.63
Cyclohexane	0.56	0.12	0.39	0.04
Benzene	1.00	1.00	1.00	1.00
	(16.03)	(4.89)	(19.69)	(22.80)
Pyridine	5.19	9.43	7.82	5.36

The interactions of 6-deoxycyclodextrin polyurethane resins with four disubstituted benzene derivatives in water were estimated from retention volumes measured by liquid chromatography (Table III). A liquid chromatogram of a mixture of *o*-, *m*- and *p*-iodoaniline isomers is shown in Fig. 2 together with that of the three dinitrobenzene isomers on 6-D-β-HDI. A complete baseline separation of the three iodoaniline isomers is obtained, while the *ortho*- and *meta*-isomers of dinitrobenzene or nitroaniline cannot be separated. Compared with *o*-, *m*- and *p*-cresol, the isomers of nitroaniline, dinitrobenzene or iodoaniline are retained more strongly: especially the *para*-isomers have large retention volumes. The three isomers of cresol, however, exhibit nearly equal retentions and cannot be separated. Because 6-D-β-H6XDI interacts with dinitrobenzene and iodoaniline very strongly, 60% aqueous ethanol is used as eluent instead of 40% aqueous ethanol in this case.

Each zone of the solutes placed on columns containing the 6-deoxycyclodextrin polyurethane resins scarcely moves when eluted with pure water. This indicates the possibility of using these resins as sorbents to collect aromatic compounds in water. The breakthrough capacities for the *para*-isomers of nitroaniline, cresol and

TABLE III

RETENTION VOLUMES ON 6-DEOXYCYCLODEXTRIN POLYURETHANE RESINS

Eluent: ethanol–water (40:60); flow-rate 25.5 ml/h.

Solute	*Retention volume (ml)*		
	6-D-β-HDI	*6-D-α-HDI*	*6-D-β-H6XDI*
Nitroaniline			
o-	76.9	63.3	84.3
m-	74.2	62.4	85.7
p-	142.0	75.2	187.0
Dinitrobenzene			
o-	74.3	63.9	27.2*
m-	59.4	65.0	26.5*
p-	108.5	129.2	46.4*
Iodoaniline			
o-	86.7	88.9	30.2*
m-	125.3	108.4	36.6*
p-	171.8	101.1	45.9*
Cresol			
o-	48.4	49.7	62.4
m-	42.6	46.7	56.5
p-	46.4	45.6	60.6

* Eluent: ethanol–water (60:40).

dinitrobenzene on 6-deoxycyclodextrin resins are shown in Table IV, together with those on BDOL-HDI prepared from 1,4-butanediol and hexamethylene diisocyanate. It is clear that the 6-deoxycyclodextrin units in the resins participate in the uptake of the solutes. Both 6-D-β-HDI and 6-D-β-H6XDI give larger breakthrough capacities than 6-D-α-HDI and BDOL-HDI. 6-D-β-HDI is superior to 6-D-β-H6XDI because the breakthrough profiles on the former are well-defined S-shaped curves which rise steeply.

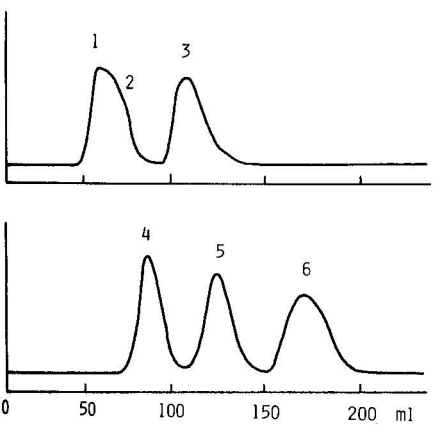

Fig. 2. Liquid chromatograms of dinitrobenzene and iodoaniline isomers on 6-D-β-HDI. Peaks: 1 = *m*-dinitrobenzene; 2 = *o*-dinitrobenzene; 3 = *p*-dinitrobenzene; 4 = *o*-iodoaniline; 5 = *m*-iodoaniline; 6 = *p*-iodoaniline.

TABLE IV

BREAKTHROUGH CAPACITIES ON 6-DEOXYCYCLODEXTRIN POLYURETHANE RESINS

Flow-rate of the sample aqueous solution: 80 ml/h.

Resin	*Breakthrough capacity (mg/g)*		
	p-Nitroaniline, 0.8 mM	*p-Cresol, 0.8 mM*	*p-Dinitrobenzene, 0.4 mM*
6-D-β-HDI	45.3	17.4	18.2
6-D-α-HDI	17.0	6.5	9.9
6-D-β-H6XDI	38.8	18.0	16.0
BDOL-HDI	4.9	2.2	1.5

From the results obtained it is expected that the 6-deoxycyclodextrin polyurethane resins will be used as column packing materials to separate and collect organic compounds.

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Note

Einfluss von intramolekularen Wechselwirkungen auf die gaschromatographische Trennung epimerer Dicyclopentadienderivate*

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(Eingegangen am 21. Juli 1981)

Bei der gaschromatographischen Untersuchung von doppelbindungsisomeren Alkoholen der Dicyclopentadien-Reihe fanden wir, dass das Retentionsverhalten an polaren Trennflüssigkeiten durch intramolekulare Wasserstoffbrückenbindungen erheblich beeinflusst wird. Dadurch wird eine Trennung epimerer Verbindungen, die an unpolaren Säulen nicht trennbar sind, an Polyethylenglykolphasen möglich[1]. Auch bei anderen ungesättigten cyclischen Hydroxyverbindungen, die zur Ausbildung von intramolekularen Wasserstoffbrückenbindungen befähigt sind, konnte dieses Verhalten beobachtet werden[2–6].

In Fortsetzung dieser Untersuchungen studierten wir das Retentionsverhalten von *endo*-Dicyclopentadienderivaten, die am C-Atom 1 eine Hydroxyl- bzw. Acetoxygruppe in *syn*- oder *anti*-Stellung tragen, sowie der entsprechenden Dihydro- und Tetrahydroderivate und einiger strukturell verwandter Ketone bei der Gasverteilungschromatographie an einer unpolaren (OV-1) und einer polaren (Carbowax 20M) Trennflüssigkeit. Die Messungen wurden bei den in Lit. 1 angegeben experimentellen Bedingungen an folgenden Glaskapillarsäulen durchgeführt: (i) OV-1: 50 m × 0.30 mm I.D., vorbehandelt durch HMDS-Gasphasendesaktivierung; (ii) Carbowax 20M: 50 m × 0.23 mm I.D., vorbehandelt durch HCl-Ätzung. (Polaritätskonstanten siehe Lit. 1).

ERGEBNISSE UND DISKUSSION

In Tabelle 1 sind die bei 140°C gemessenen Retentionsindices und die daraus ermittelten Indixdifferenzen $\Delta I = I^{polar} - I^{apolar}$ der untersuchten Substanzen 1–10 zusammengestellt. Zum Vergleich enthält die Tabelle auch die entsprechenden Werte einiger Dicyclopentadiene mit *exo*- bzw. *endo*-ständiger Hydroxylgruppe in Position 9.

Betrachtet man die Elutionsreihenfolge der Alkohole auf der nahezu unpolaren Phase OV-1, so ergibt sich wie bei Kohlenwasserstoffen[1] eine Zunahme der Retention mit steigendem Sättigungsgrad. Die *syn*/*anti*-Epimerenpaare 1a/1b sowie 2a/2b werden nicht aufgetrennt. An der polaren Phase Carbowax 20M werden dage-

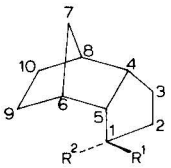
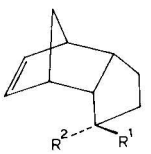
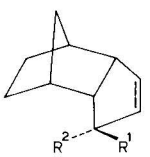
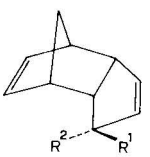
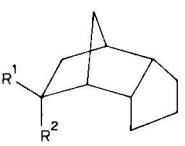
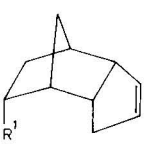
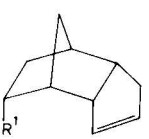
* Molekülstruktur und Retentionsverhalten, XIII. Mitt., XII. Mitt.: W. Engewald, L. Wennrich und E. Ritter, *J. Chromatogr.*, 174 (1979) 315.

0021-9673/81/0000–0000/$02.50

TABELLE I

RETENTIONSINDICES, ΔI-WERTE UND HOMOMORPHIEFAKTOREN VON 1-*anti*-bzw. 1-*syn*-*HYDROXY-, ACETOXY- UND 1.KETODICYCLOPENTADIENEN, SOWIE DEREN DIHYDRO- UND TETRAHYDRODERIVATEN** AN OV-1 (I^{OV}) UND CARBOWAX 20M (I^{C}) bei 140°C

$H^{OV} = I^{OV}$ ungesättigt $- I^{OV}$ gesättigt; $H^{C} = I^{C}$ ungesättigt; $\Delta I = I^{C} - I^{OV}$

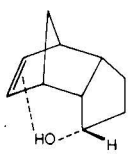
Nr.	*Verbindung*	R^1_{anti}**	R^2_{syn}**	I^{OV}	I^{C}	ΔI	H^{OV}	H^{C}
1a		OH	H	1312	1957	645	–	–
1b		H	OH	1312	1967	655	–	–
5a		CH_3COO	H	1433	1872	439	–	–
5b		H	CH_3COO	1436	1850	414	–	–
8		O		1309	1844	535	–	–
2a		OH	H	1267	1926	659	−45	−31
2b		H	OH	1267	1892	625	−45	−75
6a		CH_3COO	H	1376	1825	449	−57	−47
6b		H	CH_3COO	1393	1870	477	−43	+20
9		O		1258	1810	552	−51	−34
3a		OH	H	1277	1951	674	−35	− 6
4a		OH	H	1233	1943	710	−79	−14
7a		CH_3COO	H	1358	1970	612	−75	+98
10		O		1260	1909	649	−49	+65
13***		H	OH	1321	1967	646	–	–
14***		OH	H	1319	1962	643	–	–
11***		OH	H	1279	1892	613	−42	−75
12***		OH	H	1283	1812	529	−38	−155

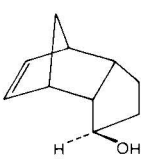
* Entsprechend der IUPAC-Nomenklatur wird Dicyclopentadien als Tricyclo[5.2.1.$0^{2,6}$]deca-3,8-dien bezeichnet mit einer anderen Numerierung der C-Atome als von uns verwendet.

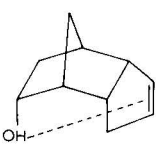
** *syn*- und *anti*- gibt die sterische Anordnung des Substituenten in Bezug zur C_9-C_{10}-Doppelbindung an.

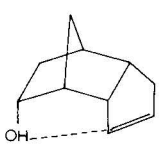
*** Werte aus Lit. 1.

gen die *syn/anti*-Epimerenpaare 1a/1b und 2a/2b jeweils voneinander getrennt. Bemerkenswert ist der um 34 Indexeinheiten (I.E.) geringere Retentionsindex des 1-*syn*-Hydroxy-2,3-dihydro-*endo*-dicyclopentadiens 2b im Vergleich zur epimeren Verbindung 2a, die sich nur durch Stellung der OH-Gruppe in anti-Position am C-Atom 1 unterscheidet. Ursache dieser beträchtlichen Retentionsverringerung ist die infrarotspektroskopisch[7] nachweisbare Ausbildung einer intramolekularen Wasserstoffbrückenbindung zwischen der Hydroxylgruppe in 1-*syn*-Stellung und der olefinischen Doppelbindung zwischen den C-Atomen 9 und 10, wodurch die Verbindung 2b zu geringeren spezifischen Wechselwirkungen mit der stationären Phase in der Lage ist.









	2b	2a	11	12
$\tilde{\nu}$	3592 cm^{-1} (OH_{assoz})	—	3590 cm^{-1}	3566 cm^{-1}
OH	3629 cm^{-1} (OH_{frei})	3625 cm^{-1}	3624 cm^{-1}	—
I^{ov}	1267	1267	1279	1283
I^{c}	1892	1926	1892	1812
ΔI	625	659	613	529

Das gesättigte Epimerenpaar 1a/1b wird mit einem Indexunterschied von 10 I.E. getrennt.

Aus der Beziehung

$$\Delta(\Delta G) = RT \ln \alpha = RT \ln t'_{R(2)}/t'_{R(1)}$$

ergeben sich folgende Unterschiede in den freien molaren Lösungsenthalpien der einzelnen Isomerenpaare an der Trennflüssigkeit Carbowax 20M:

Epimerenpaar	*$\Delta(\Delta G)$*	*Indexunterschied*
11/12	1052 J/grd Mol	80 I.E.
1a/1b	109 J/grd Mol	10 I.E.
2a/2b	375 J/grd Mol	34 I.E.

Bei den Ketonen weist an OV-1 ebenfalls die gesättigte Verbindung die grösste Retention auf. Anders verhält es sich auf der polaren Phase Carbowax 20M. Hier wird zuerst 1-Keto-2,3-dihydro-*endo*-dicyclopentadien 9, dann 1-Keto-tetrahydro-*endo*-dicyclopentadien 8 und zuletzt 1-Keto-*endo*-dicyclopentadien 10 eluiert. Der Retentionsunterschied von nahezu 100 I.E. zwischen den Ketonen 9 und 10, die sich nur durch eine Doppelbindung im Fünfring unterscheiden, ist zwei- bis dreimal grösser als der inkrementelle Beitrag einer Doppelbindung. Dies kommt auch im hohen ΔI-Wert für das Keton 10, der vergleichbar ist mit den für die Alkohole gefundenen Werten, zum Ausdruck. Als Ursache kann der Konjugationseffekt zwi-

schen den π-Elektronen der Carbonylgruppe und der α/β-Doppelbindung im Keton 10 in Betracht gezogen werden, der eine erhöhte Retention zur Folge hat.

Die *syn/anti*-Acetoxyverbindungen 5a und 5b werden sowohl auf der unpolaren Phase OV-1, als auch auf der polaren Phase Carbowax 20M getrennt. Auf Carbowax 20M wird wie bei den Ketonen die 2,3-Dihydroverbindung 6a vor dem Tetrahydroderivat 5a eluiert. 1-*anti*-Acetoxy-*endo*-dicyclopentadien 7a zeigt eine ungewöhnlich hohe Retention an der polaren Trennflüssigkeit Carbowax 20M.

Aus diesen Ergebnissen lässt sich die Schlussfolgerung ziehen, dass intramolekulare Wechselwirkungen zwischen einer Doppelbindung und einer funktionellen Gruppe je nach deren Charakter sich unterschiedlich auf die Retention auswirken können. Zum einen kann eine Verringerung der Retention an polaren Trennflüssigkeiten durch Ausbildung einer intramolekularen Wasserstoffbrückenbindung bewirkt werden; zum anderen können Konjugationseffekte die Retention erhöhen. Diese Effekte lassen sich bewusst zur Epimerentrennung ausnutzen, schränken andererseits aber die Anwendung allgemeiner Retentionsregeln und inkrementeller Methoden zur Vorhersage von Retentionswerten bzw. Retentionsreihenfolgen ein[8–10].

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Note

Determination of carbon chain distribution in alkyl sulfates by *in situ* hydrolysis–gas chromatography

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Sodium dodecyl sulfate (SDS) is routinely used in gel electrophoresis and as a reagent to solubilize proteins. It is now recognized that many commercial samples of SDS are contaminated with homologous alkyl sulfates, mainly tetradecyl sulfate (STS)[1–3]. Dohnal and Garvin[2] reported that STS can alter the affinity of proteins to certain stains and that STS shifts monomer–dimer equilibria of proteins to the monomer form. Thus there is a need for a convenient method for the analysis of SDS. In this paper, we will report a facile method by which the carbon chain distribution in SDS samples may be rapidly determined. SDS samples in water are injected directly into the gas chromatograph with no sample pretreatment and undergo quantitative *in situ* conversion to the corresponding alcohols which are separated and detected.

The determination of the carbon chain distribution in commercial samples of SDS has been accomplished by several methods. All of these methods require sample pretreatment. For example, acid hydrolysis for 1–3 h yields a mixture of homologous fatty alcohols which are extracted into a solvent, *e.g.*, diethyl ether or hexane[1,4]. The solvent final volume is then adjusted prior to analysis by gas chromatography (GC). Pyrolysis–GC of SDS furnishes a mixture of products, primarily 1-olefins and fatty alcohols from which the carbon chain distribution may be derived[5]. Pyrolysis over KOH results in a mixture of 1-olefins with lesser yields of internal olefins and dialkyl ethers which are swept into the gas chromatograph and detected[6]. From pyrolysis–GC over P_2O_5 or H_3PO_4, a mixture of olefins is obtained[7].

EXPERIMENTAL

Materials

Decanol, dodecanol, tetradecanol and 1-dodecene were obtained from Aldrich and were 99% pure by GC. SDS and STS were obtained from Eastman-Kodak. Sodium dodecyl sulfate was obtained from Eastman-Kodak, Fisher Scientific, Pierce, Polysciences, and Sigma. Lithium dodecyl sulfate was obtained from Polysciences and Sigma. Solutions of fatty alcohols were prepared in reagent-grade methanol. Solutions of alkyl sulfates were prepared in distilled, deionized water.

Methods

GC analyses were made with a Varian Aerograph Model 3700 under the fol-

0021-9673/81/0000–0000/$02.50

lowing conditions: injector temperature, 270°C; flame ionization detector, 230°C; attenuation, $128 \cdot 10^{-10}$ A/mV; helium flow-rate, 20 ml/min; column temperature, 200°C. The following 6 ft. × 1/8 in. stainless-steel columns were used: 10% SP-2100 on 80–100 mesh Supelcoport and 5% Carbowax 20M on Anakrom SD. PTFE-coated Microsep septa were utilized. Sample injection volumes of 1, 2 or 3 μl were delivered with a Precision 5-μl syringe. Quantitation of peak areas was accomplished by cutting out the peaks and determination of peak mass with a Mettler H31 AR balance. The elution patterns for aqueous solutions of SDS and methanolic solutions of decanol,

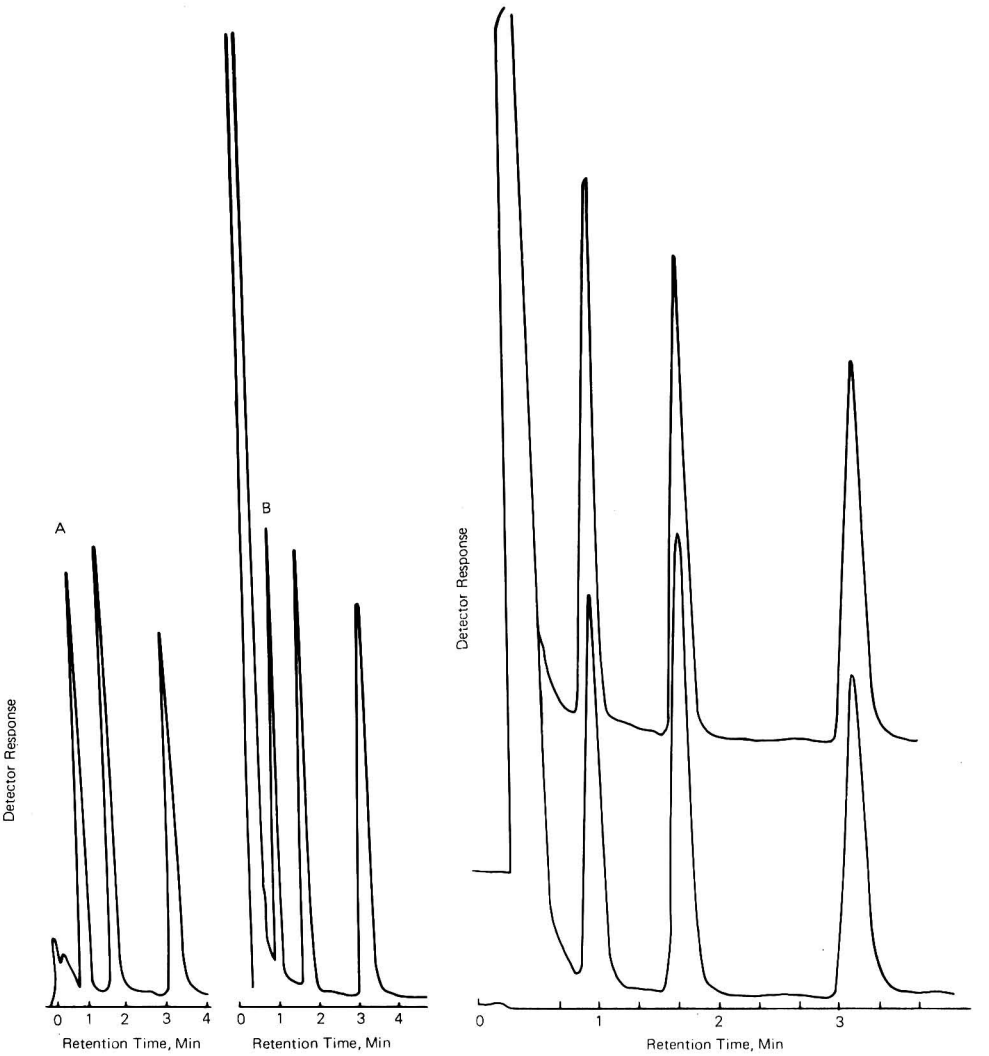


Fig. 1. A, Equimolar 0.026 *M* solution of C_{10}, C_{12}, C_{14} alkyl sulfates in water. B, Equimolar 0.026 *M* solution of C_{10}, C_{12}, C_{14} alcohols in methanol, SP-2100 column. In both series, the order of elution is C_{10}, C_{12}, C_{14}.

Fig. 2. Top, equimolar 0.026 *M* solution of C_{10}, C_{12}, C_{14} alcohols in methanol. Bottom, equimolar 0.026 *M* solution of C_{10}, C_{12}, C_{14} alcohols plus SDS in water, SP-2100 column.

dodecanol and tetradecanol were very reproducible throughout the course of this work. The SP-2100 column was baked at 300°C briefly at the beginning and end of each work day. A glass-lined injector packed with glass wool was used.

RESULTS AND DISCUSSION

It has been found that on an SP-2100 column, the chromatogram of an equimolar mixture of decanol, dodecanol, and tetradecanol in methanol yields three peaks with the identical retention times that are observed for an equimolar mixture of C_{10}, C_{12}, and C_{14} alkyl sulfates in water (see Fig. 1). In Fig. 2, the fatty alcohol mixture has been diluted with an aqueous solution of C_{12} sulfate. The C_{12} peak, at 1.7

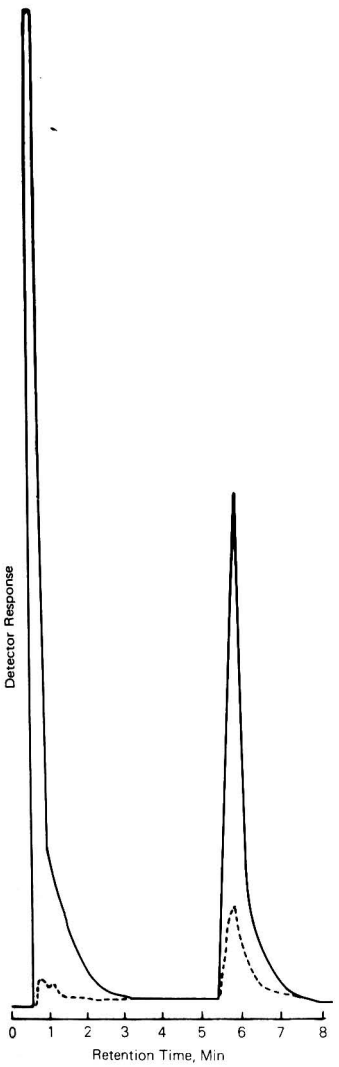


Fig. 3. ———, 0.026 *M* Dodecanol in methanol; ·····, 0.003 *M* SDS in water, Carbowax 20M column.

min, is enhanced and exhibits no shoulders. The basis for chromatographic separation on silicone polymer columns is related to difference in boiling point[8]. Since dodecanol boils at 260°C and SDS melts at 204–207°C, the alkyl sulfate is presumably converted to the corresponding alcohol in the injector. Dodecanol in methanol and SDS in water also yield the same retention time (5.8 min) on a Carbowax 20M column which separates compounds by differences in polarity[8] (see Fig. 3). SDS is not converted to the 1-olefin since 1-dodecene has a retention time of 0.6 min on SP-2100.

Next, the quantitative response (per mole) was determined for dodecanol and SDS and analogously for tetradecanol and STS. Calibration curves for the C_{12} and C_{14} alcohols were prepared by injection of 1-, 2- and 3-μl volumes of an equimolar 0.026 *M* solution in methanol. Peak area determination by cutting and weighing gave superior results to either triangulation or peak height. Significantly, 3 μl of an aqueous equimolar 0.026 *M* solution containing Polysciences SDS and Eastman-Kodak STS yielded the same detector response as the corresponding alcohol (see Fig. 4). This shows that the chromatograms obtained for the alkyl sulfates are a result of a total *in situ* conversion of the sample in the GC injector.

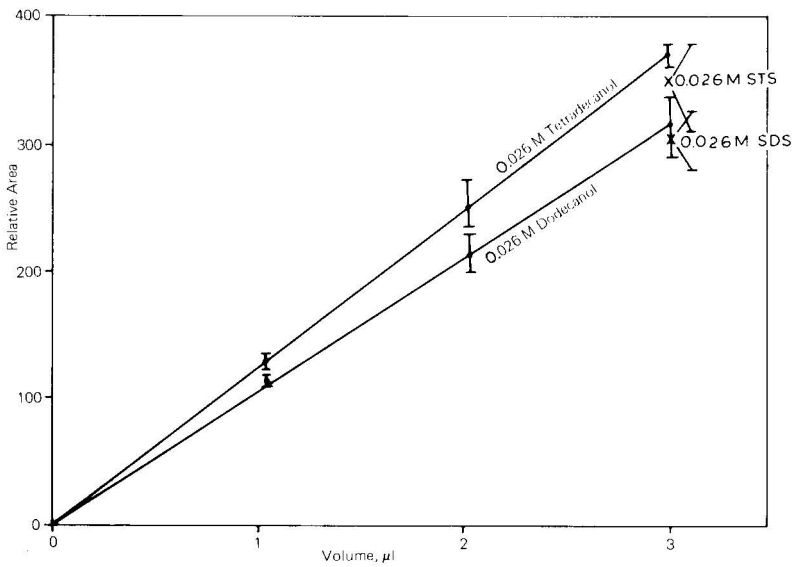


Fig. 4. Quantitative response for tetradecanol and STS (upper line) and dodecanol and SDS (lower line). All data points are the mean of 5 replicates; error bars are ± standard deviation, SP-2100 column.

It has been reported that commercially available samples of SDS are frequently found to contain 25–31 % of STS[1–3]. In Table I are shown the results of carbon chain distribution analysis of commercially available samples of SDS as determined by the direct injection technique. It is apparent that there is a wide range of purity among these samples. The major impurity is STS. It is noted that Pierce SDS yields different results by direct injection *vs.* hydrolysis and extraction. However, the probable cause of this apparent discrepancy is that these SDS samples represent different lots of material. The observed chain distribution for Sigma SDS agrees quite well with the label values.

TABLE I

CARBON CHAIN DISTRIBUTION IN COMMERCIAL ALKYL SULFATES

SDS = Sodium dodecyl sulfate; LDS = lithium dodecyl sulfate; STS = sodium tetradecyl sulfate.

Source	*Alkyl sulfate*	*Percent total area**			
		C_{10} (%)	C_{12} (%)	C_{14} (%)	C_{16} (%)***
Eastman-Kodak, Lot No. A0A	SDS	0.45	99	0.33	–
Fisher Scientific, Lot No. 702789	SDS	2.1	65	26	7.9
Pierce, sequanal grade, Lot No. 021981-9	SDS	0.43	98	1.1	–
Pierce**	SDS	–	99	0.56	–
Polysciences, Lot No. 2-1178	SDS	0.38	98	1.5	–
Polysciences, Lot 04098	LDS	0.85	99	–	–
Sigma, Lot No. F0F-0302	SDS	0.68	67	27	5.6
Sigma, Lot No. F0F-0302, label values	SDS	–	65	27	6
Sigma, Lot No. 11F-0056	LDS	0.80	99	–	–
Eastman-Kodak, Lot No. B9A	STS	–	5.0	95	–

* Percentages computed by cut and weigh procedure; values not corrected for molar response factor. Precision for duplicate injections ±1%. Sample concentration, 2.0% in water; sample volume, 3.0 μl.

** Determined by injection after acid hydrolysis and extraction[4].

*** The data for % C_{16} in SDS samples is based on the presence of peaks with a retention time of 6.0 min which is also observed for hexadecanol in methanol. Hexadecyl sulfate is not commercially available.

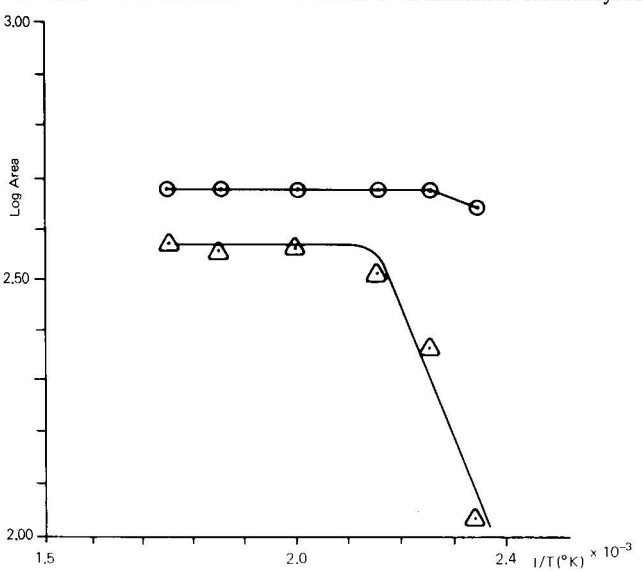
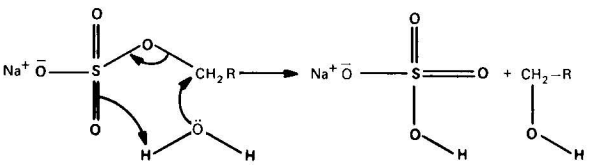


Fig. 5. Effect of injector temperature (T) on peak area for 0.035 M dodecanol in methanol (○) and 0.026 M SDS in water (△), SP-1200 column.

The effect of injector temperature on peak area was compared for 0.035 *M* dodecanol in methanol and 0.026 *M* SDS in water. The effect of temperature from 150–300°C was negligible on dodecanol. In contrast, over the same temperature range, the peak area for SDS markedly diminished as the injection temperature was lowered. The data were transformed and plotted as log area *vs.* $1/T$ (see Fig. 5). From this plot, an activation energy of 11 kcal/mole was calculated.

The gas-phase hydrolysis of the alkyl sulfate must occur within the confines of the injector and therefore must be extremely fast. A concerted mechanism involving a cyclic intermediate is proposed:



ACKNOWLEDGEMENTS

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Note

High-performance liquid chromatography of protein polypeptides on porous silica gel columns (TSK-GEL SW) in the presence of sodium dodecyl sulphate: comparison with SDS-polyacrylamide gel electrophoresis

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(First received May 19th, 1981; revised manuscript received June 15th, 1981)

Although sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE)[1] has a high efficiency, it suffers from disadvantages such as difficulties in the precise determination of mobilities and in the recovery of samples. These shortcomings are inevitable in a separation technique that utilizes a continuous gel matrix. Gel chromatography is free from such shortcomings, but is inferior to gel electrophoresis with respect to resolution and sensitivity. Gel chromatography in the presence of SDS was first reported in advance of SDS–PAGE by Kretscher[2], but found only limited use for the above reasons.

The situation is now being changed by the introduction of porous silica gel suitable for packing in a high-performance liquid chromatographic (HPLC) column. A series of columns pre-packed with such gels has been developed by Toyo Soda, and are available under the name TSK-GEL SW. They have been shown to have high efficiency in protein fractionations[3,4]. Imamura *et al.*[5] have demonstrated that the columns can be used effectively to determine molecular weights of proteins in the presence of SDS. They have not, however, extended their work to a comparison of this promising technique and SDS–PAGE, with which the former must compete in performance. We have therefore studied HPLC (porous silica gel) in the presence of SDS (SDS–HPLC).

EXPERIMENTAL

Sodium phosphate buffer (0.10 *M*) of pH 7.0 was used throughout, and contained 0.1 % of SDS (special grade for protein analysis from Nakarai Chemicals) and 0.02 % of sodium azide. The buffer was supplied to two TSK-GEL G3000SW columns (each 60 cm × 7.5 mm I.D.) connected in series by a Milton-Roy Model SF Minipump, after filtration through a glass and a stainless-steel sintered-type filter. A Rheodyne injector with a 100-μl sample loop was used. Elution was monitored at 280 nm by the use of a flow cell with an internal volume of 8 μl. All of the above components comprised a Model HLC-803 liquid chromatograph (Toyo Soda). All operations were carried out at 20 ± 2°C.

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SDS–PAGE was carried out essentially according to the method of Weber and Osborn[1]. A frequently used sample was a lyophilized mixture of six kinds of purified proteins (Electrophoretic Calibration Kit; Pharmacia). The other proteins used were preparations of the best grade available.

RESULTS AND DISCUSSION

Fig. 1 shows a typical example of the elution patterns for the Pharmacia Kit obtained with the two G3000SW columns. About one third of a vial of the Kit, nominally containing 60–150 μg of each protein, was applied to the columns. Four of the six kinds of proteins could be separated but the first and second did not show a baseline region between their peaks. When only one of the columns was used all of the peaks were resolved fairly well, but there were no baseline regions between the neighbouring peaks. The anomaly in the elution curve at a retention time of about 130 min is an optical artifact due to the elution of the micelles of SDS added in excess in the sample solution. The elution of the micelles often gave a positive peak, contrary to the case shown in Fig. 1. The shape of the elution pattern of the micelles is dependent not only on the amount of SDS added to a sample but also on the extent of incorporation of materials absorbing at 280 nm into the micelles. Care should therefore be taken not to mistake it for a protein peak when it gives a positive deflection. Other materials of low molecular weight in the sample mixture were eluted far behind the micelle band (not shown).

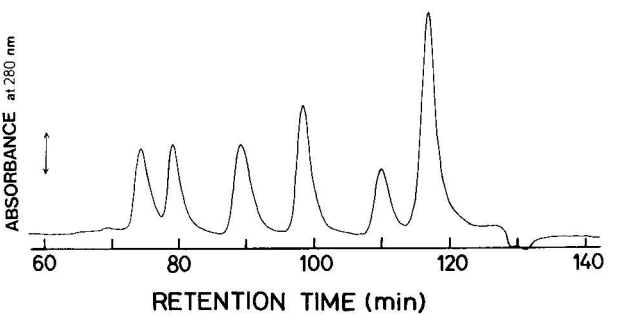

Fig. 1. Typical elution pattern from the two columns of TSK-GEL G3000SW connected in series. Flow-rate, 0.30 ml/min. The bar in the left indicates 0.01 absorbance unit. The contents of a sealed vial from the Pharmacia Kit were dissolved by injection of 200 μl of 0.10 *M* sodium phosphate buffer (pH 7.0) containing 30 mg of SDS and 10 mg of dithiothreitol. The mixture was heated in a water-bath at 100°C for 5 min. One third of the mixture was applied to the SDS–HPLC system. The vial nominally contains 64, 83, 147, 83, 80 and 112 μg of rabbit phosphorylase *b* (mol.wt. 94,000), bovine serum albumin (mol.wt. 67,000), ovalbumin (mol.wt. 43,000), bovine carbonic anhydrase (mol.wt. 30,000), soybean trypsin inhibitor (mol.wt. 20,100) and bovine α-lactalbumin (mol.wt. 14,400).

Fig. 2 shows the elution patterns obtained when the weights of protein sample applied were successively halved. Retention times were also measured with more than ten other proteins. The retention time was reproducible within 1 % provided that the run was continued without resetting the pumping rate.

Fig. 3 shows a plot of the molecular weights of the proteins *versus* retention times relative to that of bovine serum albumin. The retention time of bovine serum

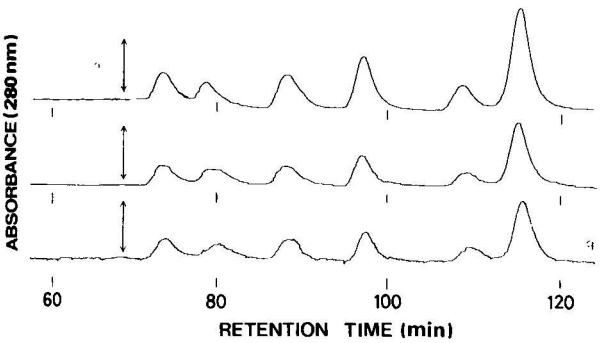

Fig. 2. Elution patterns obtained with the Pharmacia Kit in experiments in which the weights of the sample proteins were successively halved in comparison with Fig. 1 from the top to the bottom. The bars on the left indicate 0.01, 0.01 and 0.005 absorbance unit from top to bottom.

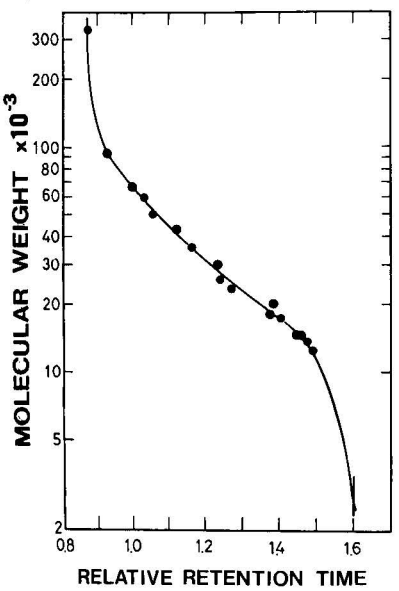

Fig. 3. Plots of molecular weights of proteins *versus* their retention times relative to that of bovine serum albumin in SDS–HPLC. Proteins from left to right: thyroglobulin, phosphorylase *b*, bovine serum albumin, catalase, immunoglobulin G heavy chain, ovalbumin, lactic dehydrogenase, carbonic anhydrase, chymotrypsinogen A, immunoglobulin G light chain, β-lactoglobulin, trypsin inhibitor, myoglobin, lysozyme, α-lactalbumin, ribonuclease, cytochrome *c* and insulin A and B chains. The insulin chains failed to separate, and their elution position is denoted by the bar on the right. Relative retention times of blue dextran (frontal edge) and DNP-alanine were 0.885 and 2.41, respectively.

albumin was measured for every series of experiments in order to obviate the effect of fluctuation of the pumping rate. The correlation between the two parameters is good enough for the SDS–HPLC technique to be used to determine molecular weights of proteins between 10,000 and 100,000.

Fig. 4 shows a typical electrophoretic pattern obtained with the Pharmacia Kit using SDS–PAGE with the standard procedure[1]. Comparison of Fig. 4 with Figs. 1

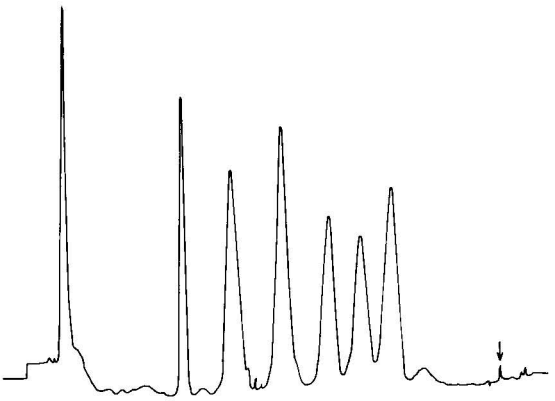

Fig. 4. Typical SDS–PAGE profile obtained with the Pharmacia Kit. The contents of a vial from the Kit were dissolved by injection of 1 ml of 0.1 *M* sodium phosphate buffer (pH 7.0) containing 25 mg of SDS, 50 mg of mercaptoethanol and a small amount of malachite green. The mixture was heated in a water-bath at 100°C for 5 min, then 10 μl of the mixture were applied to the top of the gel (5%). Electrophoresis (left to right): 6 mA per tube, 140 min, 25°C. The peak on the left is an artifact due to the gel top. Other major peaks correspond to those in Fig. 1 in the same sequence. The arrow on the right indicates the position of the marker dye.

and 2 indicates that the SDS–PAGE and the SDS–HPLC techniques give similar resolutions except for proteins of higher molecular weight, for which the former technique performed better. The use of TSK-GEL SW of other grades (G4000 and G2000) in addition to G3000 can expand the range of molecular weights covered by the SDS–HPLC technique. Clearly the SDS–HPLC technique can act as an alternative to SDS–PAGE. The former exceeds the latter in ease of operation, sample recovery, reproducibility, time required and variability of solvent composition. The latter technique, on the other hand, exceeds the former in the amount of sample required and the simultaneous operation of many runs at low cost.

The bottom SDS–HPLC elution curve in Fig. 2 was obtained by application of a protein sample only five times larger than that used to obtain the SDS–PAGE electrophoretic pattern in Fig. 4. The detector used to monitor the eluate in the present study was of a conventional type. If a more sensitive detector were to be used, there might be no significant difference in the amounts of sample required in the two techniques.

The SDS–HPLC technique separates complexes between SDS and various polypeptides derived from proteins predominantly on the basis of molecular sieving. The SDS–PAGE technique, on the other hand, separates them on the basis of both molecular sieving and electrophoresis. The determination of protein molecular weights by either of these techniques is therefore empirical in nature. Such empirical methods do not always give a correct estimate of molecular weight, but the development of the SDS–HPLC technique has made available two techniques with comparable performances.

A particular protein is expected to behave differently in the two techniques. Thus, the SDS–HPLC technique is promising not only as a substitute for the SDS–PAGE technique but also as a means for examining any abnormal behaviour of a protein in the latter. The SDS–HPLC technique is free from the restrictions imposed

by the electrophoresis conditions in the SDS–PAGE technique. In principle, various aqueous solutions can be used as solvents in SDS–HPLC, thus making modifications of the technique possible, unless the solvents attack the chromatographic system or have a high viscosity.

The resolution in SDS–HPLC is reported to be affected markedly by the concentration of salt added to the buffers[5–7]. A study on the parameters that affect the elution behaviour of proteins in SDS–HPLC is needed, as a rapid expansion of the use of this technique is expected in the near future.

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Note

Isoelectric focusing, gel chromatography and electrophoresis of pyridoxalated and polymerized stroma-free haemoglobin

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New variants of oxygen transporting blood substitutes are based on irreversibly pyridoxalated and polymerized stroma-free human haemoglobin (SFH)[1–9]. Little is known about the heterogeneity of the above complex preparations. Electrophoresis on cellulose acetate strips makes it possible to separate the pyridoxalated and native haemoglobin into two fractions[3]. We have observed previously[9] one zone of "reversibly" pyridoxalated oxyhaemoglobin of p*I* 6.7 among about eight subfractions of native haemoglobin during flat bed electrofocusing in isoelectric focusing (IEF) agarose gels.

In the present model experiments we used isoelectric focusing together with gel filtration and gel electrophoresis to investigate the heterogeneity of protein molecules in SFH derivatives prepared by covalent binding of pyridoxal 5-phosphate to deoxyhaemoglobin in the presence of borohydride and by subsequent polymerization with glutaraldehyde[1–4,10].

MATERIALS AND METHODS

SFH (90 g/l of haemoglobin) was prepared from outdated human erythrocytes by a standard technique[11]. The reaction of deoxyhaemoglobin with pyridoxal 5-phosphate (Fluka, Buchs, Switzerland) in a molar ratio 1:4 was performed in the presence of potassium borohydride (Lachema, Brno, Czechoslovakia) in 0.1 *M* Tris–HCl buffer, pH 7.4, under nitrogen gas at 5–10°C as described by Sehgal *et al.*[3], with minor modifications. The intermediate product (SFH-P) was treated with 10–100 mg glutaraldehyde (E. Merck, Darmstadt, G.F.R.) per 1 g of haemoglobin for 3 h under the above conditions and the reaction was then stopped by the addition of 20–200 mg of lysine per 1 g of haemoglobin to form the final product (SFH-P-G)[4]. Comparative deoxyhaemoglobin samples were treated with pyridoxal 5-phosphate in the absence of borohydride (SFH···P)[6]. When necessary, the low-molecular-weight reagents were removed by gel chromatography on Sephadex G-25.

Analytical thin-layer isoelectric focusing was performed in the usual manner[8] on IEF agarose with Pharmalyte 3-10 (Pharmacia, Uppsala, Sweden) or with Am-

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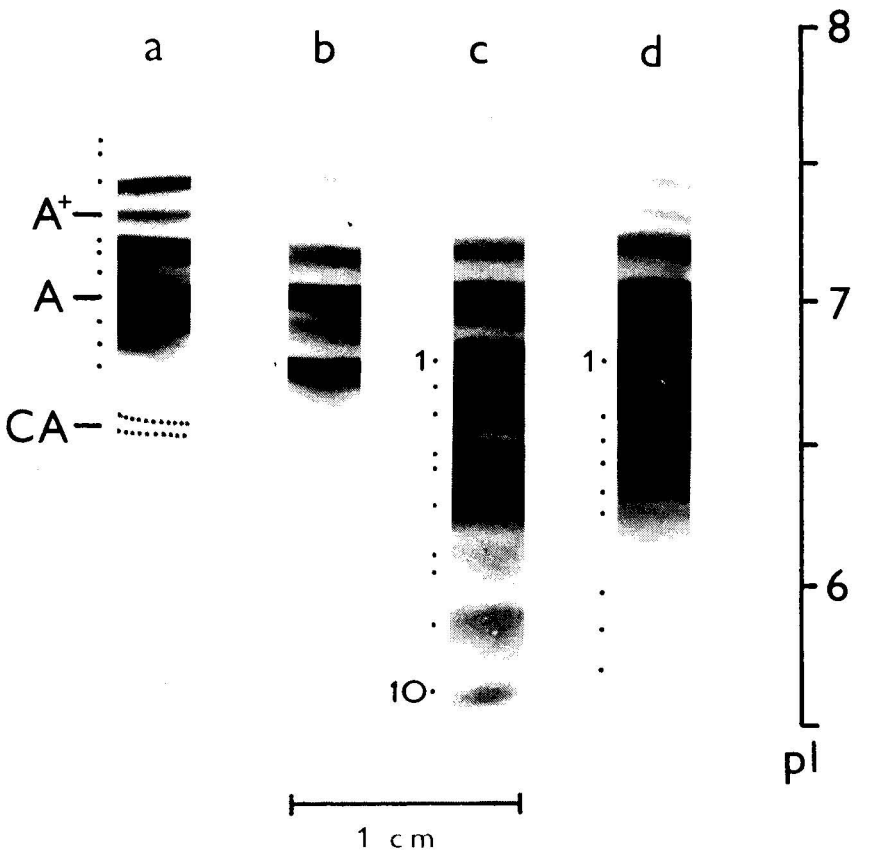

Fig. 1. Electrofocusing of stroma-free haemoglobin samples in IEF agarose gels. a, Stroma-free haemoglobin (SFH); b, SFH + pyridoxal 5-phosphate (SFH···P); c, SFH + pyridoxal 5-phosphate + borohydride (SFH-P); d, SFH-P + 10 mg glutaraldehyde per 1 g of haemoglobin (SFH-P-G/10). A = Haemoglobin A; A^+ = methaemoglobin A; CA = carbonanhydrase. The main pyridoxalated fractions in the patterns b, c and d are indicated by arabic numerals. Pharmalyte pH range 3–10. Staining with Coomassie blue.

pholine 3-10 (LKB, Bromma, Sweden). Both ampholytes led to similar results. Pharmacia as well as Serva kits of protein standards for electrofocusing were used together with previous data[9] to establish an approximate p*I* scale.

The relative molecular mass, M_r, distribution of proteins in SFH derivatives was estimated by gel chromatography on a calibrated Sepharose 6B column as before[9]. Lyophilization of SFH in the presence of 5% sucrose as lyoprotector was performed[12]. The formation of pentyl cross-links was detected by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS)[9]. Haemoglobin and methaemoglobin were estimated as their cyano derivatives[9].

The oxygen dissociation curves were estimated automatically by biotonometry[13,14] at 37°C, pH 7.40, in an isotonic phosphate buffer. The values of P_{50} and the Hill's coefficient of sigmoidity, n, were deduced from the oxygenation curves by means of simple graphical constructions[13,14].

RESULTS AND DISCUSSION

Fig. 1 shows the marked differences between native SFH, reversibly pyridoxalated SFH · · · P (where the binding is predominantly a Schiff-base like), irreversibly pyridoxalated SFH-P (where the Schiff base was reduced by borohydride to form stable imino bridges) and glutaraldehyde-treated SFH-P-G. Visual evaluation of the patterns before staining with Coomassie blue yielded three or four brown-red zones of various methaemoglobins with p*I* above 7.1 and three more red zones in SFH, four in SFH · · · P, eight in SFH-P and three in SFH-P-G. After staining, we usually found eleven zones in SFH, seven in SFH · · · P, fifteen in SFH-P and twelve in SFH-P-G (treated with 10 mg glutaraldehyde). Three to six faint non-haemoglobin zones were present, mostly between p*I* 4.5 and 5.5. About 60–70 % of the total haemoglobin was modified during the above reactions. The unreacted haemoglobin subfractions remained at their original positions. The acid shift of the pyridoxalated fractions is evidently due to the induction of phosphate groups. Glutaraldehyde invoked a stepwise smearing of the patterns and disappearance of certain zones in the p*I* region below 6.7. However, these changes were less profound in comparison with the marked changes in the IEF pattern of native SFH after reaction with 10 mg glutaraldehyde alone[7,9]. Evidently, pyridoxalation of SFH has lowered the amount of determinant groups available for the reaction with glutaraldehyde[15] in our samples. The question whether the large number of zones in pyridoxalated SFH might be artefacts due to interaction of SFH-P with the components of the ampholyte remains open. Anyway, the patterns are reproducible and typical for the given SFH derivative.

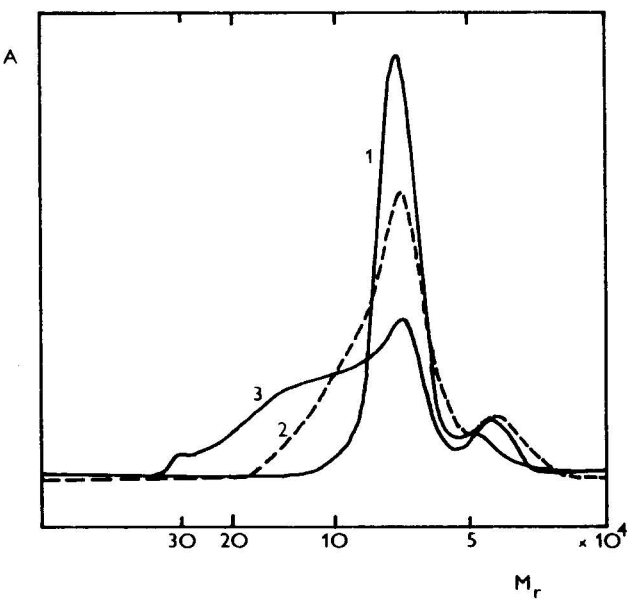

Fig. 2. Gel chromatography of pyridoxalated haemoglobin (SFH-P) treated with different amounts of glutaraldehyde. Sepharose 6B column (27 × 1.5 cm); isotonic phosphate buffer, pH 7.4, saturated with CO; absorbance measured at 254 nm; flow-rate 4 ml/cm² · h. The samples were treated with CO before chromatography. Curves: 1, SFH-P and SFH-P-G/10; 2, SFH-P-G/33; 3, SFH-P-G/66 (the index indicates mg of glutaraldehyde per 1 g of haemoglobin).

Fig. 2 is an example of the dependence of particle size distribution in SFH-P-G on the amount of glutaraldehyde used during polymerization. SFH-P and SFH-P-G samples treated with 10 mg glutaraldehyde yield a relatively uniform fraction having M_r = 64,000 (about 90%) and a smaller fraction corresponding to M_r = 44,000–50,000. At higher amounts of glutaraldehyde, *e.g.*, 30 mg and 60 mg, diverse populations of particles were found in a broad interval of M_r between about 40,000 and 330,000. The use of 100 mg glutaraldehyde caused gel formation.

Similar conclusions can be made from the results of polyacrylamide gel electrophoresis with sodium dodecyl sulphate, Fig. 3. In untreated SFH and SFH-P, haemoglobin is split into monomers and (SFH) into dimers. After treatment with glutaraldehyde, higher fragments corresponding to tri- and tetramers are also present.

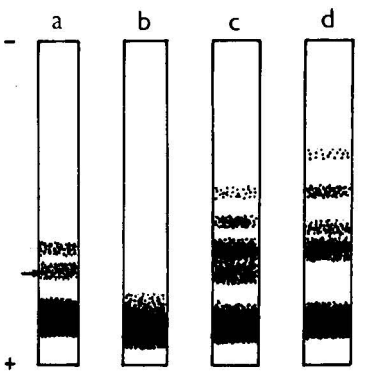

Fig. 3. Gel electrophoresis of fragments of modified SFH after splitting with sodium dodecyl sulphate. Polyacrylamide gel (50 g/l); phosphate buffer, pH 7.2, with SDS (1 g/l). Haemoglobin samples were split for 2 h at 37°C in SDS (20 g/l). Staining with Coomassie blue. a, SFH; b, SFH-P; c. SFH-P-G/10; d, SFH-P-G/50. The position of bromophenol blue is indicated by the arrow.

Table I summarizes the oxygen transporting and offloading ability of the given haemoglobin derivatives. Only SFH-P and to a lesser extent also SFH-P-G samples with covalently bound pyridoxal phosphate had a permanently lowered mean affinity to oxygen (as compared to unmodified SFH), even after removal of the unbound reagents by Sephadex G-25. Freshly prepared samples with methaemoglobin contents below 7% gave sigmoidal dissociation curves and the Hill's coefficients, n, indicated a fair cooperation between the subunits within the SFH-P and SFH-P-G tetramers. However, elevated concentrations of glutaraldehyde and of methaemoglobin resulted in lower P_{50} and n values.

The above parameters decreased slightly (by about 10%) in lyophilized samples (with 5% sucrose)[1,2] when stored dry at 20°C for 1 week and redissolved in distilled water.

Certain differences in the oxygen affinity and mean molecular mass of our SFH-P-G model samples as compared to those of Sehgal *et al.*[4], Rozenberg *et al.*[1] and Bogomolova *et al.*[2] seem to be due to the different protein and glutaraldehyde concentrations used.

The present results show that thin-layer isoelectric focusing in IEF agarose gels reveals several new details and yields substantially more information on the hetero-

TABLE I

COMPARISON OF OXYGENATION AND SUBUNIT COOPERATION CHARACTERISTICS OF DIFFERENT HAEMOGLOBIN SAMPLES

Standard deviation, S.D. $\leqslant 8\%$. SFH = Stroma-free haemoglobin; SFH···P = deoxyhaemoglobin (1 mole) + pyridoxal 5-phosphate (4 moles); SFH-P = as above, pyridoxal phosphate bound covalently in the presence of borohydride; SFH-P-G/*a* = the above sample treated with *a* mg of glutaraldehyde per 1 g of haemoglobin (see text) (*a* = 10, 20, 33 and 66).

Sample	P_{50} *(kPa)*	*Hill's coeff. n*
Fresh human blood	3.6	2.7
Banked blood after 24 days	2.3	2.6
SFH	1.8	2.6
SFH···P	4.0	2.5
SFH···P purified by Sephadex G-25	2.2	2.5
SFH-P purified by Sephadex G-25	2.9	2.4
SFH-P-G/10	2.8	2.2
SFH-P-G/20	2.7	2.0
SFH-P-G/33	1.9	1.7
SFH-P-G/66	1.4	1.5

geneity of pyridoxalated and polymerized haemoglobins than, *e.g.*, zone electrophoresis[3]. In combination with other techniques it seems to be most suitable for further investigation and routine quality tests of the above blood substitutes, especially of the pyridoxalated ones.

The complexity of the above samples has recently been confirmed by chromatofocusing which will be described elsewhere.

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Note

Improved method of resolving nucleotides by reversed-phase high-performance liquid chromatography

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Recent work in our laboratory has been directed to the relationship between the purine salvage enzymes and *de novo* purine biosynthesis in both eukaryotic and prokaryotic cells. We have recently demonstrated that in *E. coli* there is a selection for purR$^-$ (repressor negative) mutants in an hpt$^-$ gpt$^-$ background[1]. It has also been demonstrated that hgprt$^-$ cells may have altered regulation of the *de novo* purine pathway[2], and in man, individuals suffering from hgprt deficiency (Lesch-Nyhan syndrome) have elevated levels of uric acid[3]. Many other human diseases have been described that involve defects in purine salvage enzymes[4–6].

We have attempted to develop a rapid and sensitive method of quantitating changes that might occur in nucleotide pools in cells grown under different conditions using high-performance liquid chromatography (HPLC). Our aim has been to avoid the use of complex gradients that might require a programmer. Using simple isocratic elution and a C_{18} reversed-phase column we have obtained excellent and reproducible separation of nucleotides and bases in 25 min. Since we are using an isocratic elution system the column need not be equilibrated between runs of similar materials, and the baseline absorbance remains constant. The effect of varying ionic strength and pH on the elution profile of nucleotides was examined. Our results demonstrate that by choosing a specific combination of pH and ionic strength various types of separations can be achieved.

Thus depending on the interests of the investigator different conditions may be used for different types of separation.

MATERIALS AND METHODS

Apparatus

For HPLC, a Waters Assoc. (Milford, MA, U.S.A.) M45 solvent delivery system, Model U6K sample injector, and Model 440 wavelength detector were used. The column was a pre-packed reversed-phase column (30 × 0.4 cm I.D.), utilizing an octadecyl (C_{18}) chemically bonded stationary phase (Waters Assoc.). A pre-column consisting of a short stainless-steel column (5 cm × 4.6 mm I.D.) packed with C_{18} reversed-phase material was used to protect the main column.

Chemicals

Methanol (spectral quality) was from Fisher Scientific. All liquids were pre-

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filtered through a 0.2-μm Millipore filter. Nucleotide standards were obtained from Sigma (St. Louis, MO, U.S.A.), and were prepared as 1 m*M* stock solutions in double-distilled water, and diluted before use. Stock solutions were maintained at −20°C.

Preparation of eluents

A stock solution (1 *M*) of ammonium dihydrogen phosphate (Baker) was prepared, filtered, degassed, and diluted to the required ionic strength with double-distilled water. The pH was adjusted with NH_4OH or H_3PO_4. All eluents were pre-filtered through a 0.2-μm Millipore filter before use.

Chromatographic conditions

A Bondapak C_{18} column was used in all studies. Between use, the column was stored in methanol–water (30:70). The column was cleaned at the end of the day with the methanol–water mixture. The column was routinely run at 1–2 ml/min (1000–2000 p.s.i.).

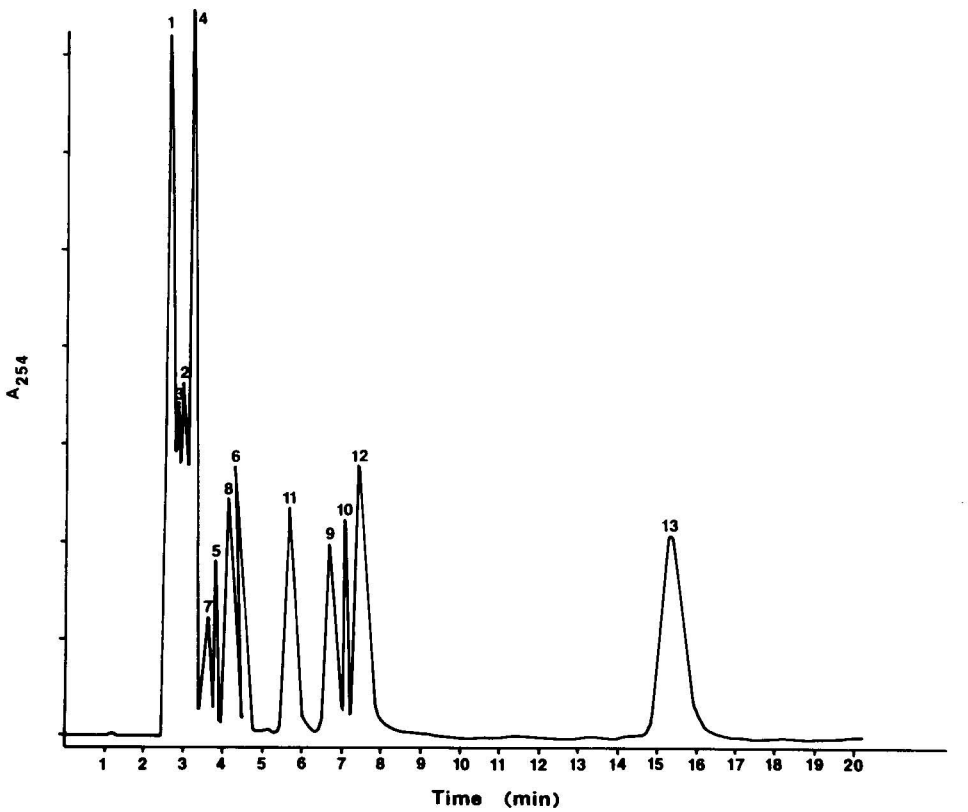

Fig. 1. Chromatogram of nucleotide standards on a Bondapak C_{18} column, using UV (254 nm) detection. Eluent 0.02 *M* $(NH_4)H_2PO_4$, pH 5.1. Flow-rate 1 ml/min. All peaks identified individually: 1 = CTP; 2 = CDP; 3 = UTP; 4 = UDP; 5 = CMP; 6 = UMP; 7 = GTP; 8 = GDP; 9 = GMP; 10 = IMP; 11 = ATP; 12 = ADP; 13 = AMP.

Cell extracts

Acid-soluble nucleotides and bases were extracted from tissue culture cells by a modification of the method of Jensen *et al.*[7]. Tissue culture cells were removed from flasks by trypsinization with 0.25% trypsin, washed in phosphate-buffered saline in the cold to remove the trypsin, and resuspended in 0.33 *M* formic acid. After 30 min on ice, the extract was centrifuged and the supernatant applied directly to the column. Under these conditions there is little breakdown of triphosphates to mono- or di-phosphates. Soluble nucleotides were extracted from *E. coli* in a similar fashion.

RESULTS

Effects of ion concentration

Standard nucleotides (AMP, CMP, GMP, UMP, ADP, CDP, UDP, GDP, GTP, CTP, UTP, ATP) were eluted from the C_{18} column at 1.0 ml/min with $(NH_4)H_2PO_4$ (pH 5.1, 0.01 *M*) containing 6% methanol according to the method of Davis *et al.*[8] for the separation of nucleotides. However, in our hands this method did not give good resolution of nucleotides, all of them eluting very early. Resolution of the different nucleotides could be improved, by removing methanol and chromatographing with 0.02 *M* $(NH_4)H_2PO_4$ (pH 5.1) (Fig. 1). By increasing the ionic strength, better resolution could be achieved. The retention times of mono-, di- and triphosphates at pH 5.1 with 0.02, 0.05, 0.1, 0.2, and 0.5 *M* $(NH_4)H_2PO_4$ are compared in Fig. 2. At higher salt concentrations there was a greater retention time of adenine

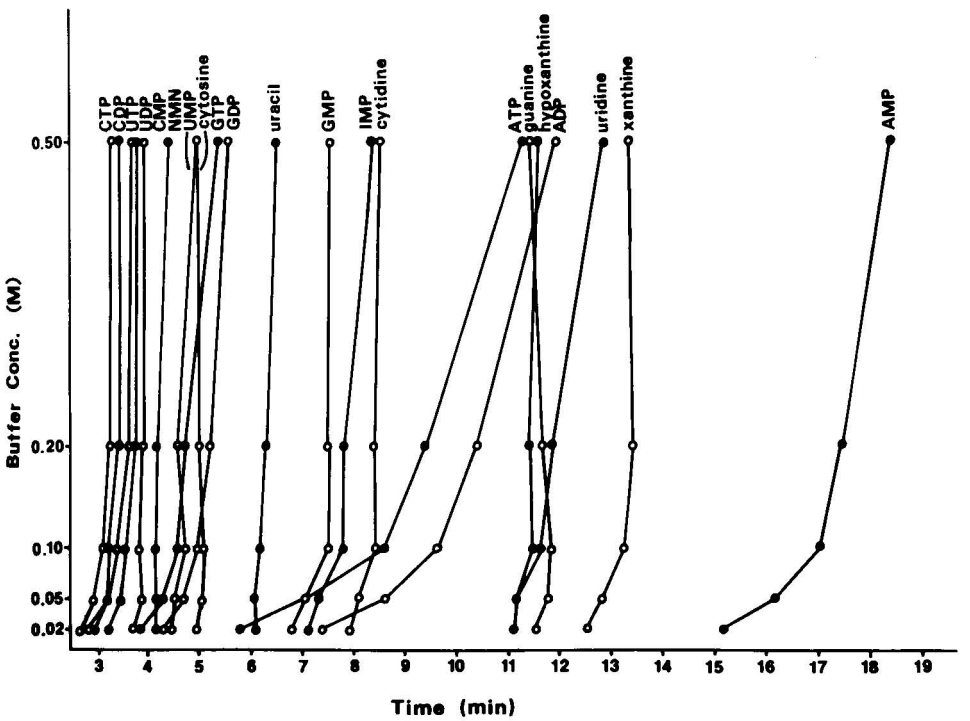

Fig. 2. Composite of retention time of nucleotide standards as a function of salt concentration, from a Bondapak C_{18} column (30 × 0.4 cm I.D.) using UV (254 nm) detection. Isocratic elution with 0.02 *M* $(NH_4)H_2PO_4$–0.5 *M* $(NH_4)H_2PO_4$, pH 5.1. Flow-rate 1 ml/min.

nucleotides, with ATP, ADP and AMP eluting later at higher salt concentration. Increasing salt concentration had very little effect on time of elution of other nucleotides.

Effect of pH

Nucleotides were eluted from the C_{18} column using 0.2 *M* $(NH_4)H_2PO_4$, and 0.5 *M* $(NH_4)H_2PO_4$ at pH 3.4–6.6 in 0.5 pH unit differences. As expected[9,10], pH had a much greater effect on separation than salt concentration. The elution of cytidine and uridine nucleotides was not influenced by changes in pH as markedly as the purine nucleotides. In particular the retention time of ATP, ADP and AMP was greatly altered at different pH values. The monophosphates were most affected and AMP retention was particularly sensitive to pH alteration (13 min at pH 6.6, 20 min at pH 5.5, and 11.5 min at pH 3.65). These data are summarized in Fig. 3. The pK_a of the primary phosphate group (*ca.* 6 for all the nucleotides) probably accounts for these results, with the polarity of the base and the ratio of base to phosphate groups accounting for the greater shifts in monophosphates and adenine nucleotides. The pK_a values of the bases appear to have only a minor effect.

Chromatography of cell extracts

In order to ascertain that similar results were obtained with biological samples, total nucleotide pools of Chinese hamster cells (CHO), L-cells, and *E. coli* were

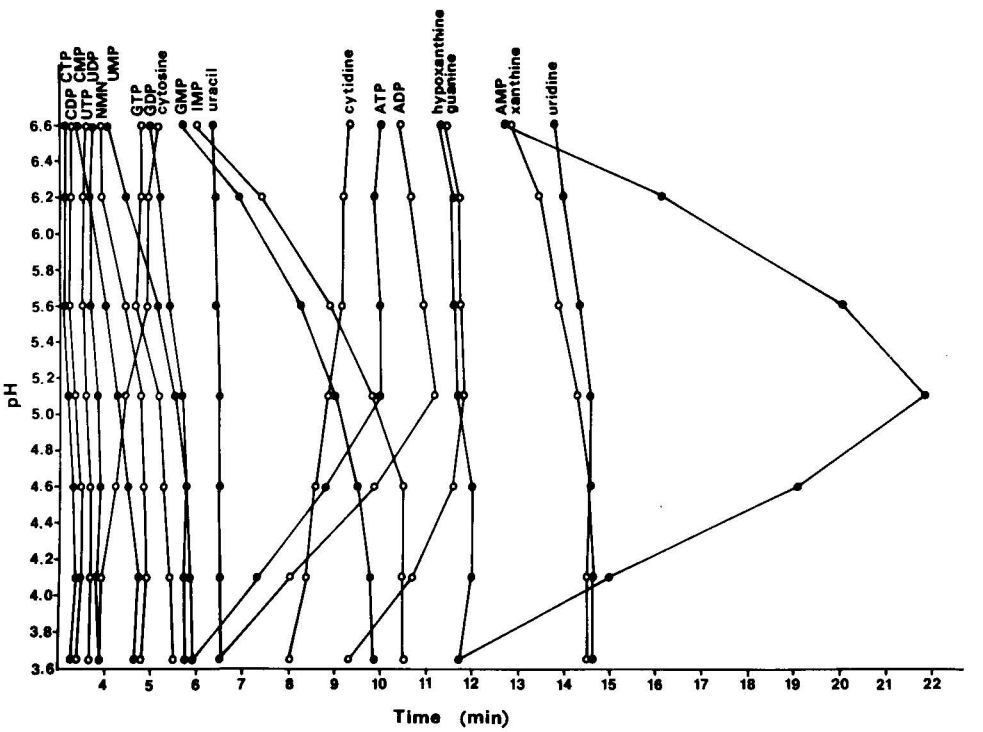

Fig. 3. Composite of retention time of nucleotide standards as a function of pH. Conditions as in Fig. 1, except 0.2 *M* $(NH_4)H_2PO_4$ used at different pH values.

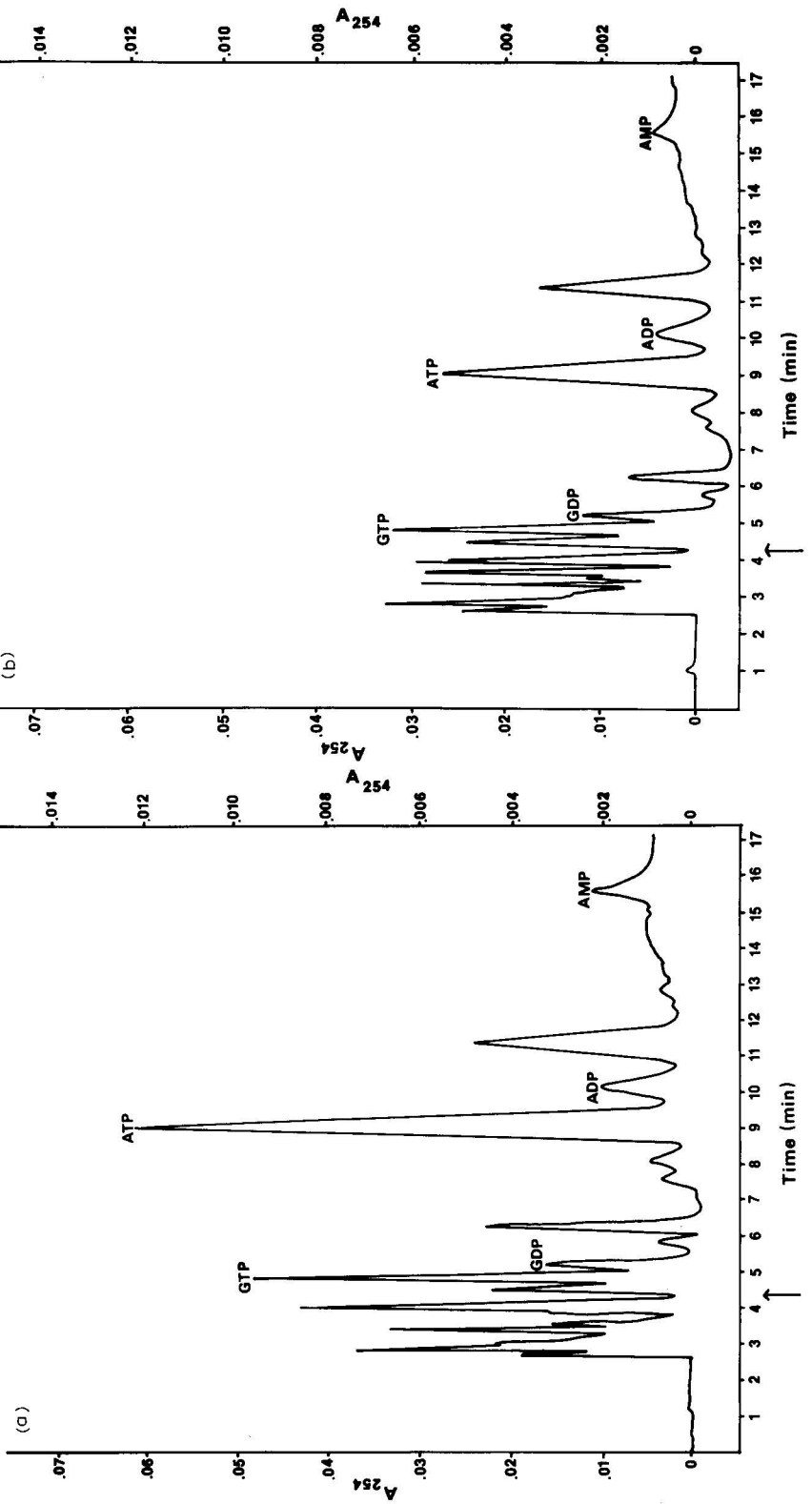

Fig. 4. Chromatogram of total acid-soluble pool of *E. coli* grown in the presence (a) and absence (b) of azaserine ($1 \cdot 10^{-4}$ *M*). Chromatographic conditions: 25 μl injected; flow-rate 1 ml/min; 0.2 *M* $(NH_4)H_2PO_4$, pH 5.1.

measured. To test whether nucleotide pool changes could be detected using our analytical method, an exponential culture (5 ml) of *E. coli* strain TL525 (ara, Δleu, V169, StrA purE::lac) was treated with azaserine ($1 \cdot 10^{-4}$ M) for 15 min. The glutamine analogue azaserine has been shown to inhibit *de novo* purine biosynthesis[11]. After this period samples were removed and the nucleotide pools extracted with 0.33 *M* formic acid. Cultures that had not been exposed to azaserine were processed identically and served as controls.

As can be seen in Fig. 4a and b, addition of azaserine specifically affects the pools of purine nucleotides. There is approximately a two-fold difference in ATP and GTP concentrations. Thus the separation of nucleotides in a cell extract is identical to that received using standards.

Chinese hamster ovary cells and L-cells, grown as monolayers, were extracted as described in materials and methods. A 100-μl volume of the cell extract (from 10^6 cells/ml) was sufficient to give a good profile of nucleotides. All the nucleotides and bases could be identified from standards. To identify peaks, radioactive and non-labelled standards were injected and co-eluted with the sample. Fig. 5 compares the total nucleotide pools of CHO cells and L-cells grown in culture. The differences in nucleotide pools, ATP/AMP and GTP/GMP ratios, as well as the amounts of nicotinamide mononucleotide (NMN) are quite striking. The resolution in this case has been enhanced (two-fold) by using two columns in tandem.

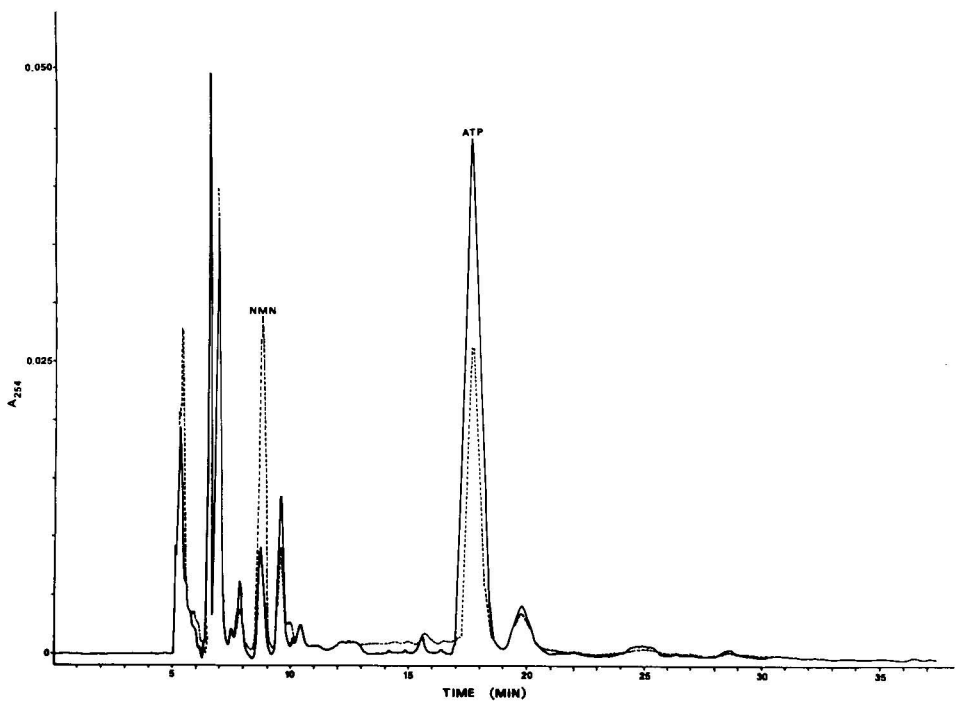

Fig. 5. Comparison of total nucleotide pools of CHO (———) and L (ATCC) (– – –) cells. Chromatographic conditions: 50 μl cell extract injected; 0.05 A_{254} total absorbance; flow-rate 1 ml/min; 0.2 *M* $(NH_4)H_2PO_4$, pH 5.1

The analysis of total nucleotides using reversed-phase column HPLC has numerous advantages over other methods. The method is rapid, highly reproducible, and does not require equilibration between samples. Cyclic nucleotides and nucleosides are only eluted if methanol is added to the salt solution, or after a lengthy period of time (over 60 min) without methanol (unpublished results).

The normal procedure used in examining nucleotide contents of biological samples relies on radioactive labelling procedures. However it is difficult in such procedures to calculate the specific activity of each nucleotide pool, or to measure the turn-over rate of various nucleotides. The method described in this paper does not rely on such measurements, and can also be used in conjunction with labelling to directly measure the fate of any specific radio-labelled compound added to the medium. The specific activity of each nucleotide can thus be calculated.

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Note

Use of routine preparative high-performance liquid chromatography in the separation of isomers

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Recent advances in techniques[1–10] and in the efficiency of the apparatus available for preparative high-performance liquid chromatography (HPLC)[11,12] has made the separation of multigram quantities possible. A major problem encountered in the use of these machines, however, is the correct choice of eluent. Although some authors[13–15] recommend direct scale-up from thin-layer chromatography (TLC), in our experience this is not always a good guide and often leads to either a failed separation or waste of time and solvent with mid-course adjustments to eluent composition and flow rate.

Many reactions performed in the synthetic laboratory yield one or more products which can be readily isolated and purified by gravity column chromatography or by non-chromatographic techniques such as distillation or crystallisation. However, it frequently occurs that a reaction will give a mixture of two components with very similar chemical, physical and chromatographic properties, this usually being the case when the products are isomeric. In this case the laboratory chromatography techniques are inadequate and our work has been concerned with the development of an HPLC system using commercially available equipment for the routine separation of such isomeric mixtures.

To simplify the procedure for choosing the eluent system, a mixture of two solvents was used as eluent. In practice either ethyl acetate–pentane or methanol–dichloromethane in various ratios have been found to cover most situations adequately. Water–methanol mixtures were used for reversed-phase separations. Initially several thin-layer chromatograms of the sample mixure are run and the results used as a guide for the choice of absorbent and eluent. The system which results in R_F values in the range 0.2 to 0.5 is considered appropriate. A glass column (250 × 15 mm) is then packed using the chosen absorbent and eluent and a 50-mg sample of the mixture is run repeatedly, the eluent being adjusted until an acceptable separation is obtained. The pure components separated by this means are usually sufficient for structural determination by physical methods. If it is then desired to carry out a large-scale separation, a large column may be set up in the Jobin-Yvon apparatus using the same absorbent and eluent system.

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EXPERIMENTAL

Materials

The mixtures of isomers for separation were submitted by chemists in our research laboratories and were the products of chemical synthesis. Large-scale separations and some small-scale separations were carried out using Merck silica gel K60 (40–63 μm). Other small-scale separations were carried out using Merck LiChroprep Si 60 (15–25 μm). The reversed-phase material was prepared in our own laboratories from Merck silica gel K60 (40–63 μm) and octadecyl trichlorosilane supplied by Magnus. The material was capped with trimethyl silyl chloride.

Equipment

Large-scale separations were carried out using a Jobin-Yvon Chromatospac Prep 100. Small-scale separations were carried out using a Kontron pump (10 ml/min maximum), Jobling glass columns, an LDC sample injector, an Altex single-wavelength UV detector and an LDC refractive index detector. A Gilson CPR was used for fraction collection.

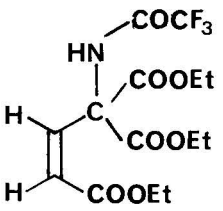
RESULTS AND DISCUSSION

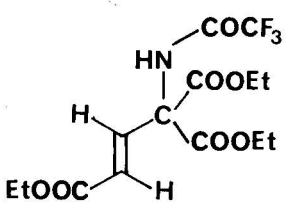
The criteria governing the use of HPLC in the organic synthesis laboratory include cost, versatility, safety and efficiency relative to other forms of chromatographic and classical methods of compound isolation. One variable factor which relates directly to the efficiency of this technique is the choice of the eluent and absorbent used for a particular separation. We have described here a systematic procedure which enables the correct absorbent and eluent for a large-scale separation to be determined quickly and efficiently using a small glass column. This enables larger-scale chromatography to be carried out efficiently with minimum cost in time and money.

The basis of the system we chose was the Jobin-Yvon Chromatospac Prep 100, descriptions of which can be found elsewhere[10,11]. Initial results suggested that progression directly from TLC to the Jobin-Yvon was feasible. In practice, however, this was only possible in the case of relatively simple separations and this led us to experiment with an intermediate system.

The system chosen for an intermediate separation was based on the 250 × 15 mm and 450 × 15 mm Jobling glass columns. These columns are easy to dry-pack with a simple 10 ml/min flow-rate pump and are inexpensive. The 450 × 15 mm column holds about 30 g of packing which will separate 50–100 mg of a two-component mixture. This gives enough sample for nuclear magnetic resonance and infrared spectra. Thus, components may be identified at an early stage and a decision can then be taken on whether a large-scale separation is required.

Figs. 1–5 are given to illustrate the utilisation of this technique. In Fig. 1 a mixture of *cis*- and *trans*-triesters was produced by the addition of diethyl N-trifluoroacetylaminomalonate to ethyl propiolate[16]. Clean separation of the two isomers initially resulted from the use of pentane–ethyl acetate (9:1), but it was found that the ratio could be reduced to 4:1 with no loss of resolution, but significant decrease in time. Accordingly, these conditions were used on a large scale and pure samples of both isomers readily isolated.





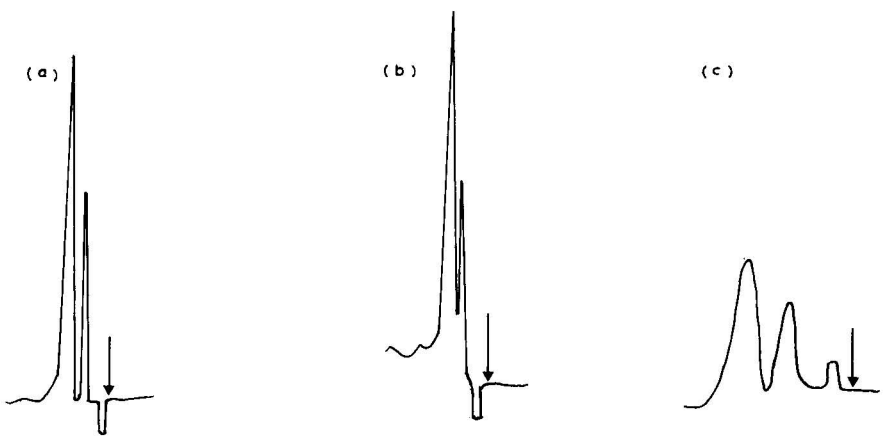


Fig. 1. Separation of *cis*- (left structure, less polar) and *trans*- (right structure, more polar) triesters on silica using refractive index detection. Et = Ethyl.

Separation	*Eluent: n-pentane–ethyl acetate*	*Flow-rate (ml/min)*	*Packing (g)*	*Injected*	*Retention time (min)*		*Yield (g)*	
					cis	*trans*	*cis*	*trans*
(a) Small scale 1	9:1	4	12	50 mg	5	8	–	–
(b) Small scale 2	4:1	4	12	50 mg	2	4.5	–	–
(c) Large scale	4:1	30	1500	6.5 g	120	190	1.2	2.7

Note: Although the 9:1 solvent ratio gives a better separation than 4:1 on the small scale, the 4:1 ratio was chosen for the large-scale separation because this is just enough to give a complete separation. Also, the relatively lower flow-rate used on the preparative scale tends to improve the separation of the two components provided that diffusion does not increase.

The separation in Fig. 2 was more complicated in that a four-component mixture was involved but again it was found that the most polar solvent mixture which was capable of separating the components on the small scale gave a good separation on a large scale.

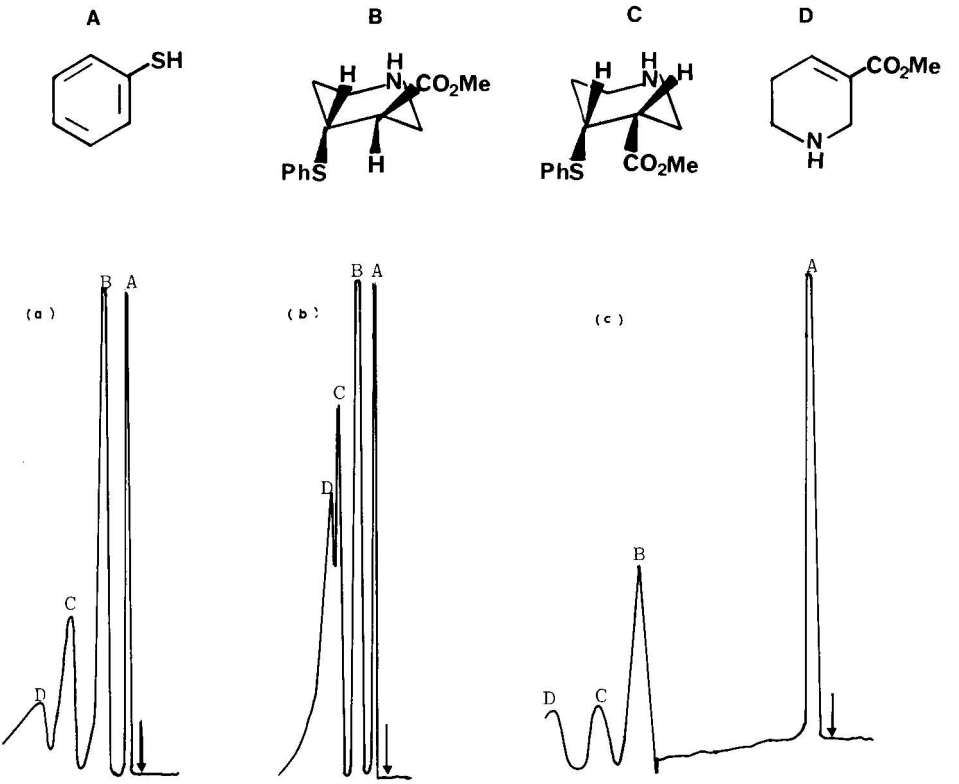


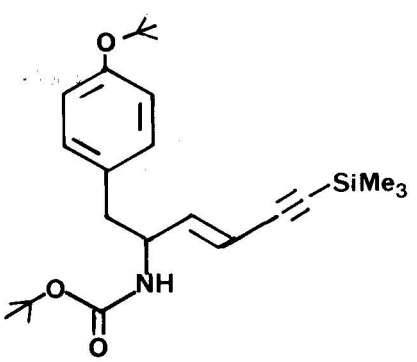
Fig. 2. Separation of four components [structures A–D, where B (*trans*) and C (*cis*) are isomers] on silica using UV detection. Me = Methyl; Ph = phenyl.

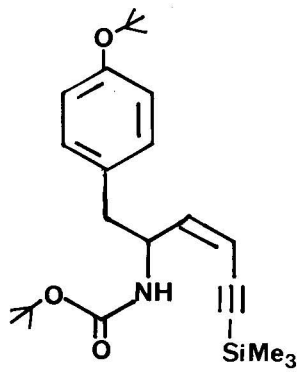
Separation	*Eluent: dichloro-methane–methanol*	*Flow-rate (ml/min)*	*Packing (g)*	*Amount injected*	*Retention time (min)*		*Yield (g)*	
					trans	*cis*	*trans*	*cis*
(a) Small scale 1	99.5:0.5	1	12	50 mg	11	22.5	–	–
(b) Small scale 2	99:1	1	12	50 mg	6.5	12.5	–	–
(c) Large scale	99:1	30	500	0.9 g	400	480	0.2	0.04

The conditions which were just enough to give adequate separation of the components were chosen. The relatively lower flow-rate and the smaller injection mixture-to-packing ratio tends to improve the large-scale separation.

In Fig. 3 another *cis–trans* mixture[17] was readily separated. In this case the same eluent mixture was used on a large scale but the flow-rate could be increased tenfold with no loss of resolution.

The use of the intermediate column to give sufficient material for spectroscopic analysis is shown in Fig. 4. Despite a very difficult separation, sufficient material was obtained of each isomer for their structures to be defined.





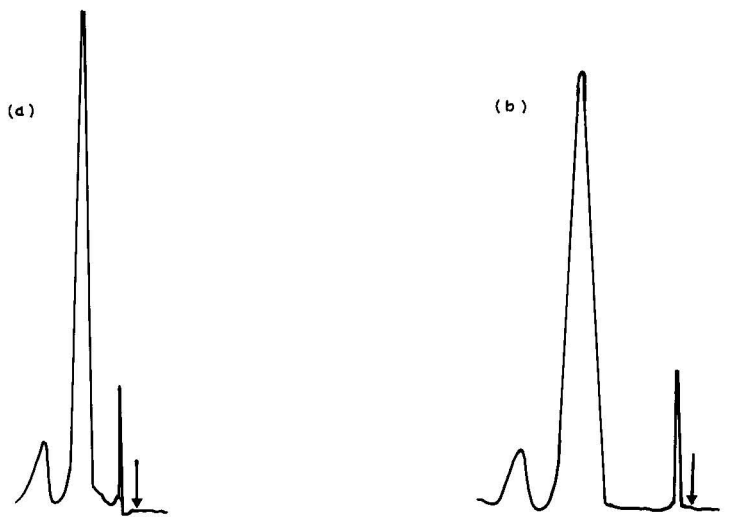
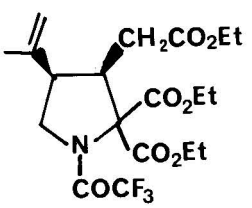
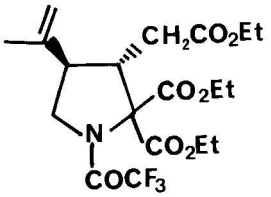


Fig. 3. Separation of *trans* (left structure, less polar) and *cis* (right structure, more polar) isomers on silica using UV detection.

Separation	*Eluent: n-pentane-ethyl acetate*	*Flow-rate (ml/min)*	*Packing (g)*	*Amount injected*	*Retention time (min)*		*Yield (g)*	
					cis	*trans*	*cis*	*trans*
(a) Small scale	95:5	4	32	100 mg	16	33	–	–
(b) Large scale	95:5	40	1000	9.0 g	400	640	5.7	1.1

In all the previous separations silica was used as the support and the final example shows that the method could be extended to the reversed-phase technique. No separation occurred on ordinary phase under the conditions used. Accordingly, a





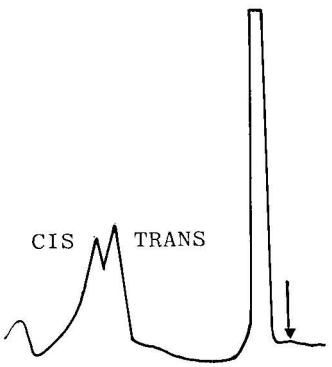


Fig. 4. Small-scale separation of *cis* (left structure, more polar) and *trans* (right structure, less polar) isomers on silica using refractive index detection. Conditions: Eluent, *n*-pentane–ethyl acetate (9:1); flow-rate, 3 ml/min; packing, 30 g; amount injected, 50 mg. Retention times: *cis*, 30 min; *trans*, 33 min.

column was set up which was capable of separating the test mixture shown and then was injected with successive 50-mg samples of the mixture. Fractions were collected as shown (Fig. 5) and pooled. Analytical HPLC confirmed that one isomer was recovered in fraction 1 (f1), the other in fractions 3 (f3) and 4 (f4) and the purity of the fractions can be clearly seen.

It should be emphasised that in most of these cases the components were either inseparable by TLC and were discovered to be mixtures only by anomalies in the nuclear magnetic resonance spectra or showed incomplete separation.

We consider that the system developed allows the routine separation of difficult-to-separate mixtures, as illustrated here in the case of isomeric mixtures, efficiently and routinely by preparative HPLC.

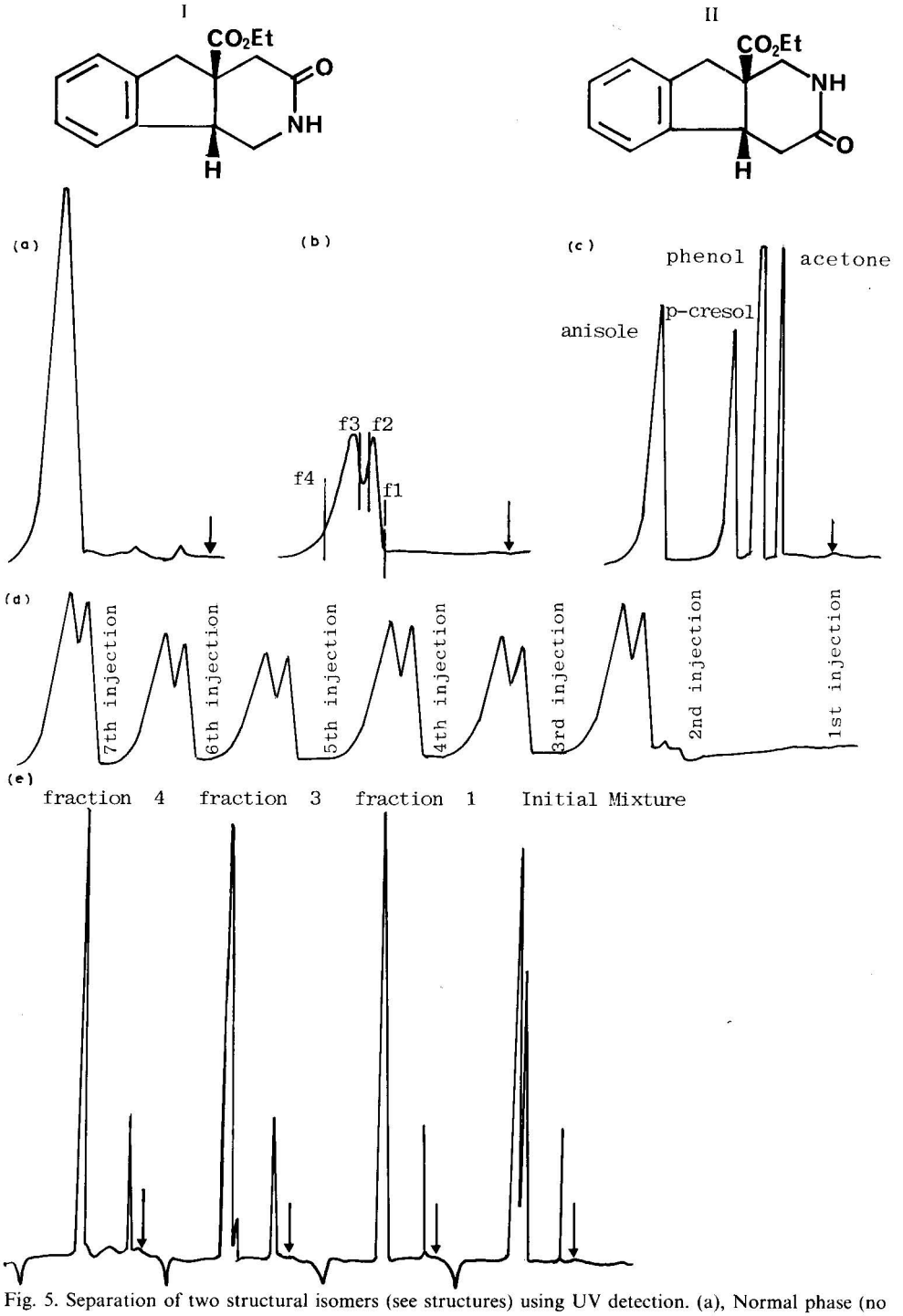


Fig. 5. Separation of two structural isomers (see structures) using UV detection. (a), Normal phase (no separation); conditions: eluent, dichloromethane–methanol (98:2); flow-rate, 3.5 ml/min; packing, 31 g; amount injected, 25 mg. (b) and (c), Reversed phase (b: research mixture; c: test mixture); condition eluent, methanol–water (40:60); flow-rate, 5 ml/min; packing, 30 g; amount injected, 50 mg; retention times: I, 94 min; II, 106 min. (d), Separation of 800 mg of mixture by repeated injections of 50 mg on a small column; fractions 1–4 (f1–f4) were taken as indicated in (b); yields (total injected 800 mg): f1, 230 mg; f2, 150 mg; f3 + f4, 350 mg; total, 730 mg; useful recovery: I, 29%; II, 44%. (e), Comparison of fractions by analytical HPLC; conditions: column, Spherisorb ODS (5 μm); eluent, methanol–water (65:35); pump, LDC constametric; detector, LDC spectromonitor.

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Note

Interaction of heme proteins and thyroid hormone

II. Localization of the site on thyroid hormone that binds to hemoglobin

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(Received July 14th, 1981)

The binding of iodothyronines by hemoglobin[1] and myoglobin[2] has recently been described by this laboratory. Interaction of thyroid hormone and these heme proteins is poorly dissociable[1–3], suggestive of the apparently covalent complexes of iodothyronine and tissue proteins described by Surks and Oppenheimer[4]. The site or sites on thyroxine (T_4) and triiodothyronine (T_3) involved in binding to heme proteins have not been previously identified and are the subject of this report.

MATERIALS AND METHODS

Isotopic hormones

[^{125}I]T_4 and [^{125}I]T_3 were obtained from Amersham (Arlington Park, IL, U.S.A.) and their purity verified by thin-layer chromatography (TLC)[5]. [^{125}I]Tetraiodothyroacetic acid (TETRAC) was custom-synthesized from unlabeled triiodothyroacetic acid (Sigma, St. Louis, MO, U.S.A.) by New England Nuclear Corporation (Boston, MA, U.S.A.) and was re-chromatographed to purity in our laboratory. Labeled 3,3′,5′-triiodothyronine (reverse T_3, rT_3) was obtained from Serono (Braintree, MA, U.S.A.) as [^{125}I]rT_3 and was shown by TLC to contain a small amount of contaminating radioiodide. The rT_3 was used in binding studies without further purification.

Reagents

Purified human hemoglobin was purchased from Sigma; polyacrylamide gel electrophoresis at pH 9.0 (ref. 6) showed this preparation to be homogeneous.

Gel filtration

Sephadex G-100 (Pharmacia, Piscataway, N.J., U.S.A.) chromatography of solutions of hemoglobin and labeled iodothyronines was carried out as previously described[1], using 0.02 *M* phosphate buffer, pH 7.6 as the eluting buffer. Fractions were monitored for absorbance at 555 nm (deoxyhemoglobin) and radioactivity.

Bound/free partition of labeled T_4 and T_3 in solutions of hemoglobin

In order to determine the possible effects of phenolic hydroxyl group ionization

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of iodothyronines on the binding of the latter to hemoglobin, we measured hemoglobin binding of [^{125}I]T_3 and [^{125}I]T_4 in phosphate-buffered solutions of hemoglobin at various pH values. Bound/free partition of hormones was quantitated by the addition of dextran-coated charcoal[1]. "Control binding" at each pH was defined as the amount of hormone remaining in the supernatant of buffer without hemoglobin after the addition of charcoal and was subtracted from the bound hormone which was measured in the presence of hemoglobin.

RESULTS

Gel filtration of purified hemoglobin previously reacted with radioactive TETRAC, rT_3, T_4 and T_3

Fig. 1A indicates that TETRAC is not bound by hemoglobin, in contrast to T_4 (Fig. 1C), T_3 (Fig. 1D) and rT_3 (Fig. 1B). Thus, the alanyl amino group is required for the iodothyronine–hemoglobin interaction.

Effect of pH on hemoglobin–thyroid hormone interaction

The pH of hemoglobin solutions was varied over the range of 6.0–10.0 and hemoglobin-binding of labeled T_3 and T_4 was measured (Table I). Gemmill[7] has shown and Handwerger *et al.*[8] have confirmed that the p*K* of the phenolic hydroxyl group of T_3 is *ca.* 8.3, whereas that of T_4 is *ca.* 6.6. If ionization of the hydroxyl site were important to hormone-binding by hemoglobin, we would expect pH changes in the 6.0–8.0 range to affect T_3- and T_4-binding differentially. It is clear from Table I

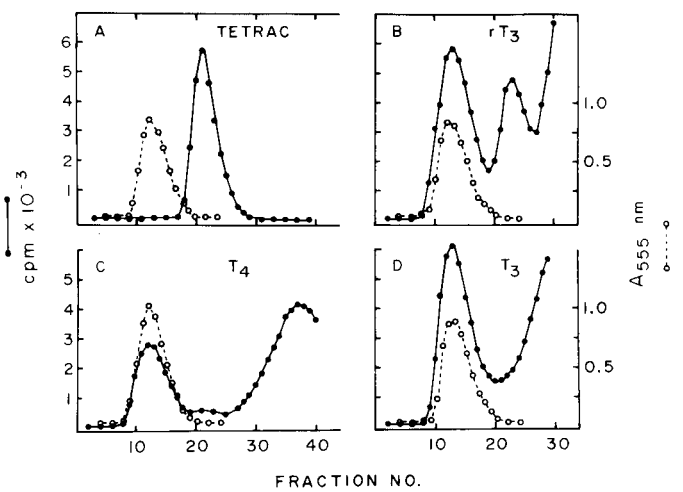

Fig. 1. Gel filtration (Sephadex G-100) of human hemoglobin, 1.0 g/dl 0.02 *M* phosphate buffer, pH 7.6, incubated for 24 h at 4°C with labeled thyroid hormone analogues: A, [^{125}I]-tetraiodothyroacetic acid (TETRAC); B, [^{125}I]-reverse T_3 (rT_3); C, [^{125}I]-thyroxine (T_4) and D, [^{125}I]-triiodothyronine (T_3). Emergence of hemoglobin from columns was monitored spectrophotometrically at 555 nm. Co-elution of hemoglobin fraction and labeled rT_3, T_4 and T_3 is demonstrated; TETRAC does not elute with hemoglobin. Column dimensions were 30 × 1.5 cm, fraction volume was 1 ml, void volume included tubes 1–9. Panel B shows elution of contaminating radioiodide (tubes 21–25) and of unbound rT_3 (tubes 26–30). Panel C shows elution of unbound T_4 (tubes 27–40) and panel D reveals emergence of unbound T_3 in tubes 21–30.

TABLE I

EFFECT OF pH ON BINDING OF IODOTHYRONINES BY HUMAN HEMOGLOBIN (Hb)
t = Calculated by paired *t*-test; NS = not significant.

	Fractional binding of hormone by Hb		
	pH 6.0	*pH 8.0*	*pH 10.0*
$[^{125}I]T_4$	0.066 ± 0.006*	0.142 ± 0.018	0.249 ± 0.160
$[^{125}I]T_3$	0.018 ± 0.004	0.130 ± 0.008	0.226 ± 0.038
t	6.76	0.62	0.32
P T_4 *vs.* T_3	<0.01	NS	NS

that pH influences hemoglobin-binding of these iodothyronines similarly, rather than differentially. T_3- and T_4-binding increase comparably at pH 8.0 and 10.0, while at pH 6.0 T_3 and T_4 at pH 6.0, however, is small (<7%). We conclude that the contribution of the thyroid hormone phenolic hydroxyl to the heme protein binding of iodothyronines is small or negligible.

DISCUSSION

The interaction of thyroid hormone and hemoglobin is a recently recognized phenomenon[1] that we have found to occur progressively with time in the intact human erythrocyte[3]. Once bound to hemoglobin, T_4 and T_3 are no longer detectable by radioimmunoassay[1] and the hemoglobin–thyroid hormone complex is one from which labeled iodothyronine is minimally displaceable by unlabeled thyroid hormone[1]. The qualities of non-displaceability of heme-bound hormone and lack of detectability of unlabeled hormone bound to hemoglobin precluded conventional ligand–protein analysis (such as determination of affinity constants) and mandated that the current studies of hemoglobin interactions be conducted only with labeled thyroid hormone and iodothyronine analogs.

The data presented here suggest that the amino group of the alanine side chain on iodothyronines is the principal site of interaction with hemoglobin. The alanyl carboxyl group cannot be primarily involved in the interaction. The fact that hemoglobin binds T_4, T_3 and rT_3 indicates that the 5- and 5′-iodines are not essential to binding. It remains to be established whether the thyronine structure is required for binding; conformational rigidity of the diphenyl ether structure, conferred by the 5-iodine[9], is obviously not needed. Because the 5′-iodine was also found to be unimportant to the interaction, we anticipated that the phenolic hydroxyl group of the iodothyronines would not relate to binding; the 5′-iodine is known to influence the p*K* of the hydroxyl group[7,10]. Studies of hormone-binding in the pH range of 6.0–10.0 confirmed that T_3- and T_4-binding were not differentially affected, indicating that ionization of the hydroxyl was unimportant to binding. The nature of the thyroid hormone amino group bonding to hemoglobin is unknown, but the fact that this interaction appears to involve the heme prosthetic group of hemoglobin[1] and that thyroid hormone may form apparently covalent complexes non-enzymatically with proteins[11] raises the possibility that an amide bond may develop between the amino group of hydrophobic thyroid hormone and an accessible carboxyl group (*e.g.*, propionyl carboxyl) of hydrophobic heme.

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Note

High-performance liquid chromatography of progesterone and its metabolites

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The estimation of progesterone and its three principal metabolites in testis, ovary, adrenal gland and placenta is required for the determination of the activity of the enzymes involved in the biosynthesis of testosterone from this precursor (Fig. 1). Steroid 17α-monooxygenase (E.C. 1.14.99.9) converts progesterone to 17α-hydroxyprogesterone the substrate for 17α-hydroxyprogesterone aldolase (E.C. 4.1.2.30). The product of this stage, androst-4-ene-3,17-dione, is reduced by testosterone 17β-dehydrogenase (NADP, E.C. 1.1.1.64) to testosterone.

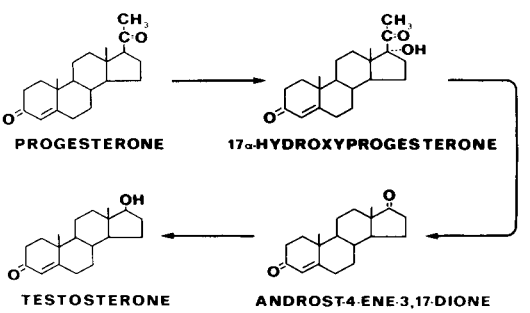

Fig. 1. Microsomal metabolism of progesterone to testosterone.

We were concerned to develop a suitable procedure to measure rapidly and reproducibly the stages in this metabolic process in rat testis as a replacement for the time consuming and somewhat variable thin-layer chromatographic (TLC) methods[1,2] previously in use in our laboratory.

Although high-performance liquid chromatography (HPLC) has been widely applied to the analysis of steroid hormones in general[3] and to the separation[4,5] and quantification[6,7] of this type of hormone, a robust and rapid reversed-phase method of adequate resolution was not available. This paper describes a satisfactory technique for determining these four steroids in radiolabelled form in incubations of interstitial and germinal cells of the testis or in sub-cellular fractions.

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EXPERIMENTAL

Materials

[4-^{14}C]Progesterone, [4-^{14}C]androst-4-ene-3,17-dione (The Radiochemical Centre, Amersham, Great Britain), [4-^{14}C]-17α-hydroxprogesterone (NEN Chemicals, Dreieich, G.F.R.), testosterone, progesterone, 17α-hydroxyprogesterone and androstenedione (Sigma London, Poole, Great Britain) were used as supplied. Identity and radiochemical purity was confirmed as >98% by TLC using silica gel plates with benzene–acetone (4:1, v/v) as solvent. HPLC grade methanol and tetrahydrofuran were obtained from Rathburn Chemicals, Walkerburn, Great Britain. Prepared solvent mixtures were sparged with helium and maintained under a helium atmosphere during use.

Equipment

An Applied Chromatography Systems (Luton, Great Britain) LC750 high-performance liquid chromatograph fitted with 250 × 4.6 mm I.D. columns packed with Partisil PXS 10 ODS, Partisil PXS 10 ODS-2, or Partisil PAC was used for all separations. A pre-column of CO:PELL:ODS (Whatman Labsales, Maidstone, Great Britain) was fitted for biological samples. Detection was either by UV absorbance at 240 nm using a Cecil Instruments CE2012 variable-wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain) or by scintillation counting[8] of collected effluent fractions[9] in Tritosol[10] scintillant.

RESULTS AND DISCUSSION

Initial attempts to achieve an adequate separation within a practicable analysis time using water–methanol mixtures and gradients and ODS, ODS-2 or PAC stationary phases proved unsuccessful. With tetrahydrofuran–water mixtures and ODS-2 as stationary phase more promising results were obtained. Complete resolution of the 4 steroids was possible in tetrahydrofuran–water (25:75) at 25°C but the analysis time of 50 min was unacceptably long. It was not found to be possible to shorten the analysis time and retain adequate resolution under isocratic conditions. The introduction of a linear gradient following an isocratic phase produced the desired result in that satisfactory resolution was achieved with a total analysis turnaround time of 20 min (Fig. 2).

Biological application

Sample preparation methods of known efficiency were already available from TLC studies of biological matrices[1,2]. In order to avoid problems arising from incompatibility of the extraction solvent (chloroform) with the eluent, the samples were dried under a stream of nitrogen and redissolved in a methanol solution containing carrier steroids. Recoveries were checked with radiolabelled materials. Fig. 3 shows the separation of radiolabelled metabolites following incubation of [4-^{14}C]progesterone with testis microsomes. A comparison of the TLC and HPLC systems for replicate incubations of testes microsomes showed correlation coefficients of 0.98 for 17α-hydroxyprogesterone (n = 10), 0.95 for androstenedione (n = 12 and 0.99 for testosterone (n = 11) in enzyme assays using the appropriate radiolabeled

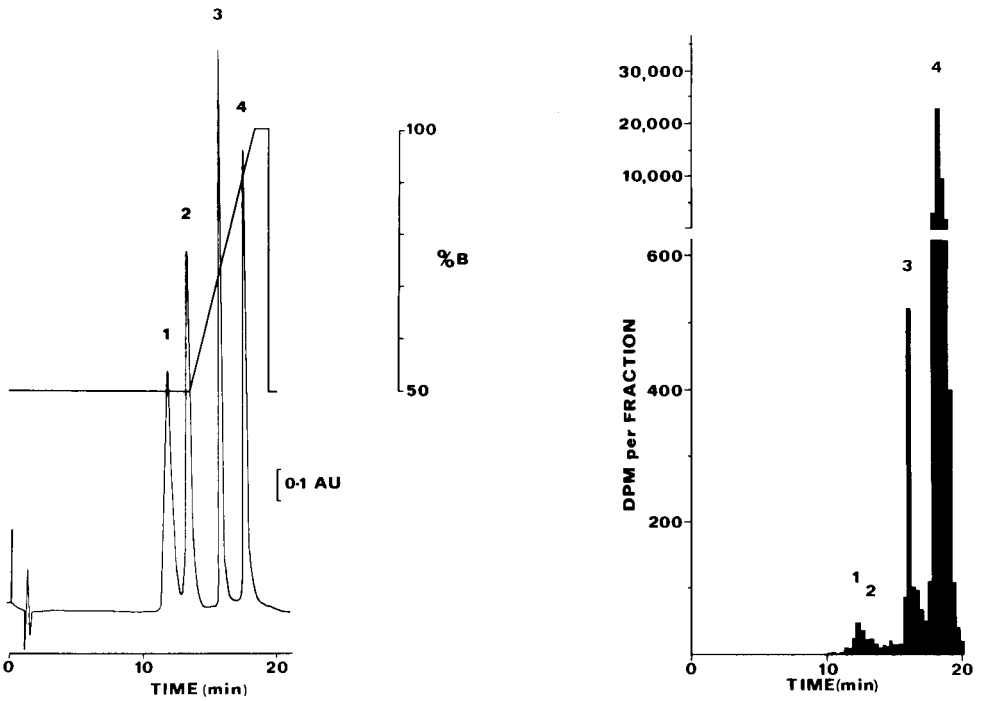

Fig. 2. Chromatogram obtained with Partisil PXS 10 ODS-2 eluted with a linear gradient of tetrahydrofuran–water; flow-rate 2 ml/min. Injection (6 μl) contained 24 μg of each steroid: androstenedione (peak 1), testosterone (peak 2), 17α-hydroxyprogesterone (peak 3), progesterone (peak 4). Reservoir A contained 1% tetrahydrofuran. Reservoir B contained 50% tetrahydrofuran. Elution conditions were: 50% B for 9 min followed by an increase of 10% B per min for 5 min then held at 100% B for 1 min. 3 min to reset starting condition (50% B).

Fig. 3. Separation of metabolites of progesterone from an incubation of [4-^{14}C]progesterone with rat testis microsomes in the presence of an NADPH generating system. Peaks identified and chromatographic conditions correspond to Fig. 2. Injection 80 μl, contained carrier steroids at 0.2 mg/ml of each.

precursors. The reproducibility of the determination of progresterone was assessed by replicate incubations with rat testis microsomes which yielded an overall coefficient of variance of 7.5% on subsequent HPLC analysis.

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Note

Rapid determination of aflatoxin M_1 in dairy products by reversed-phase high-performance liquid chromatography

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It has been shown that aflatoxin M_1 (AfM_1) is the aflatoxin residue excreted in milk. The amount of excreted AfM_1 in milk corresponds to 0.25–1.5% of the amount of ingested AfB_1[1–4].

The thin-layer chromatographic (TLC) methods available before 1976 could detect AfM_1 levels in dairy products down to 1.0 ppb*. Since then, several methods have been reported which permit lower detection limits. Stubblefield[5] used a silica gel mini-column clean-up procedure, but this procedure has the disadvantage that an important emulsion frequently occurs at the extraction step and that automation for routine control is impossible. Winterlin *et al.*[6] and Beebe and Takahashi[7] reported high-performance liquid chromatographic (HPLC) methods that are interesting for routine control; however, they cannot be used for dried milk and dairy baby food. These HPLC methods[6,7] have detection limits (0.6 and 0.2 ppb) that are higher than that of the TLC method[5] (0.1 ppb).

We now describe a method that involves a rapid clean-up of several dairy products: liquid and dried milk, liquid and powdered whey, caseinate, fresh and ripened cheeses. The detection limit is comparable to that of TLC: 0.1 ppb. The identification of AfM_1 is rapidly confirmed by a chemical reaction with trifluoroacetic acid (TFA), according to Beebe[8].

EXPERIMENTAL

Equipment

The following equipment was used: an oscillatory shaker (Laboral; Prolabo, Paris, France); a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph, equipped with a M6000A pump and a U6K septumless injector; a Schoeffel (Westwood, NJ, U.S.A.) Model 970 fluorescence detector with variable-wavelength excitation, excitation at 360 nm and cut-off filter at 389 nm; and a stainless-steel column (25 cm × 4 mm I.D.), packed with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.; article No. 9334).

* Throughout this article, the American billion (10^9) is meant.

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Reagents

All solvents were distilled-in-glass solvents for HPLC; the mobile phases were filtered on Millipore (Bedford, MA, U.S.A.) FH (0.5 μm) and HA (0.45 μm) filters.

Sep-Pak silica gel cartridges (Waters Assoc.) and LiChrosorb RP-18 (E. Merck) were used as stationary phases. For Sep-Pak, chloroform–methanol (9:1) was used as the mobile phase, for LiChrosorb water–acetonitrile (72:28).

An AfM_1 standard solution was prepared by dissolving 10 μg of commercially available AfM_1 (Makor, Jerusalem, Israel) in 2 ml methanol; the solution was diluted to 0.5 μg AfM_1/ml.

Extraction

A 50-ml volume of liquid milk was transferred into a 250-ml glass-stoppered erlenmeyer flask to which 125 ml chloroform and 5 g of diatomaceous earth were added.

For powdered milk, a 10-g sample was weighed, and 15 ml water, 125 ml chloroform and 5 g of diatomaceous earth were added.

For cheese, a 10-g sample was weighed, cut in small pieces, and 15 ml water, 125 ml chloroform and 5 g of diatomaceous earth were added.

All samples were shaken slowly for 30 min, using an automatic shaker. The samples were then filtered through filter-paper the filtrate (the volume of which should be recorded) was transferred into a 250-ml round-bottomed flask and evaporated to near dryness, using a 50°C water-bath and a rotary evaporator.

Clean-up

A Sep-Pak cartridge was connected to a 10-ml glass syringe. The residue was dissolved in 2 ml chloroform and transferred to the syringe. The round-bottomed flask was washed twice with 1-ml portions of chloroform and the washings were quantitatively transferred to the syringe. The resulting solution was injected into the Sep-Pak. Then the Sep-Pak cartridge was washed with 2 ml hexane and with 2 ml diethyl ether. The eluates from the Sep-Pak were discarded. A 3-ml volume of chloroform–methanol (9:1) was added to the syringe. The first 1-ml fraction eluted from the Sep-Pak was discarded, and the following 2 ml (containing the AfM_1 fraction) were transferred to a 5-ml vial. The solvent was evaporated to dryness over a 50°C water-bath with a gentle stream of nitrogen. The residue was dissolved in 100 μl of methanol for HPLC analysis.

Standard curve and determination

The following conditions were used to obtain a standard curve: AfM_1 standard solution, 0.5 μg/ml in methanol; flow-rate, 2 ml/min; pressure, 1200 p.s.i.; fluorescence detector, excitation wavelength 350 nm, emission cut-off filter 389 nm, sensitivity 6.30, range 0.01 μA, time constant 5 sec. The calibration curve was obtained by plotting several amounts of AfM_1 (0.5–10 ng) against the observed peak areas.

For determination, the sample extracts was injected under the same conditions; the injection volume was either 20 or 40 μl, depending on the concentration of AfM_1 in the sample.

Identification

The methanolic solution of the residue employed in the HPLC analysis was evaporated to dryness over a 50°C water-bath under a gentle stream of nitrogen. The residue was dissolved in 100 μl hexane. A 25-μl volume of TFA was added, and the compounds are mixed. After 10 min in the dark at room temperature, the reaction mixture was evaporated to dryness over a 50°C water-bath under a stream of nitrogen. The TFA-treated sample residue was dissolved in methanol; samples of this solution should be injected on the same day as they are derivatized.

Simultaneously, 20- and 40-μl volumes of the AfM_1 standard solution were transferred to a 4-ml vial and evaporated to dryness with minimum heating under a stream of nitrogen. The derivatization solvents were added and the standard treated as the dairy sample.

RESULTS AND DISCUSSION

The Sep-Pak silica cartridge removes much of the lipids and pigments from the sample.

AfM_1 is completely eluted by the elution solvent used. This is a quick and easy clean-up step. The AfM_1 levels in the sample can be estimated easily when they are in the range of the calibration curve (linear response from 0.5 to 15 ng). Table I shows the recoveries of AfM_1 added to dairy product samples in the range 0.1–5.0 ppb. The recoveries were between 80 and 120%, the variation being due mainly to the extraction step (see Table I).

TABLE I

PERCENTAGE RECOVERIES AND DETECTION LIMITS

NM = Detected but recovery not measured precisely ($\approx 110 \pm 25\%$); NR = not recovered, not detected.

Dairy product	*Sample*	*AfM_1 added (ng)*	*Level (ppb)*	*Recovery (%)*	*No. of determinations*
Liquid raw milk	50 ml	25	0.5	71 (±10)	4
		5	0.1	85 (±15)	4
		1	0.05	NM	4
		0.5	0.01	NR	2
Dried milk	10 g	10	1	110 (±10)	3
		5	0.5	90 (±10)	4
		1	0.1	NM	4
		0.5	0.05	NR	3
Cheese	10 g	10	1	60 (± 8)	4
		5	0.5	70 (±15)	3
		1	0.1	NM	4
		0.5	0.05	NR	2

Fig. 1 illustrates a typical chromatogram of 4 ng AfM_1 standard and of 4 ng AfM_1 standard derivatized by the TFA reaction. The retention times of AfM_1 and derivatized AfM_1 are 8.0 and 4 min, respectively.

Fig. 2 shows a chromatogram of a sample of powdered milk in which no AfM_1

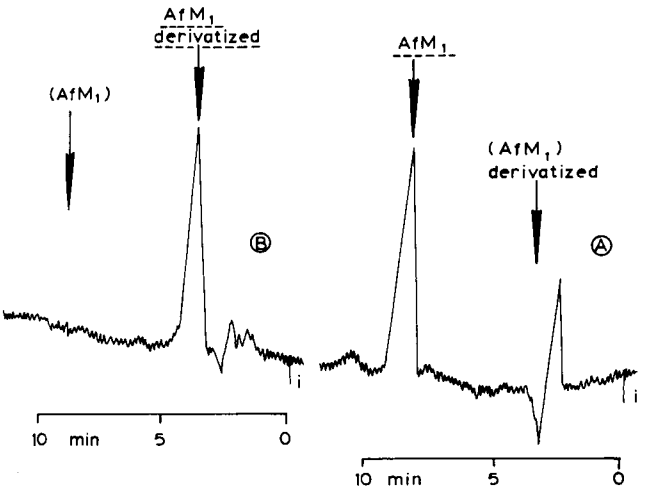

Fig. 1. Chromatograms of AfM_1 standard (4 ng) (A) and of TFA-derivatized AfM_1 standard (4 ng) (B).

was detected, and a chromatogram of the same sample spiked with 0.1 ppb of AfM_1.

Fig. 3 shows chromatograms of a liquid milk sample and a cheese sample.

Fig. 4 illustrates the TFA reaction to confirm the AfM_1 identity in a powdered milk sample. From the fact that the AfM_1 peak disappears, it can be concluded that the TFA reaction is complete.

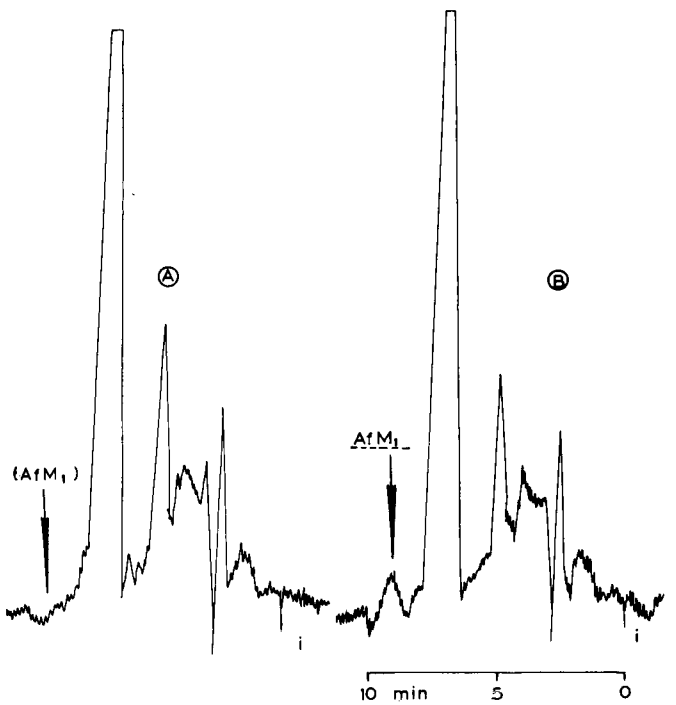

Fig. 2. Chromatograms of a sample of powdered milk (A) and of the same sample spiked with 0.1 ppb AfM_1 (B).

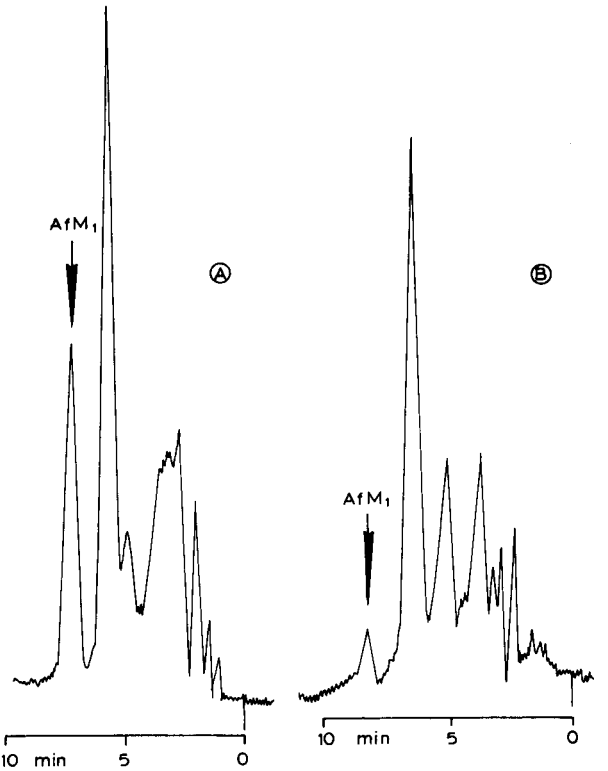

Fig. 3. Chromatograms of liquid raw milk containing 0.5 μg/l AfM_1 (A), and of Camembert cheese containing 0.2 μg/kg AfM_1 (B).

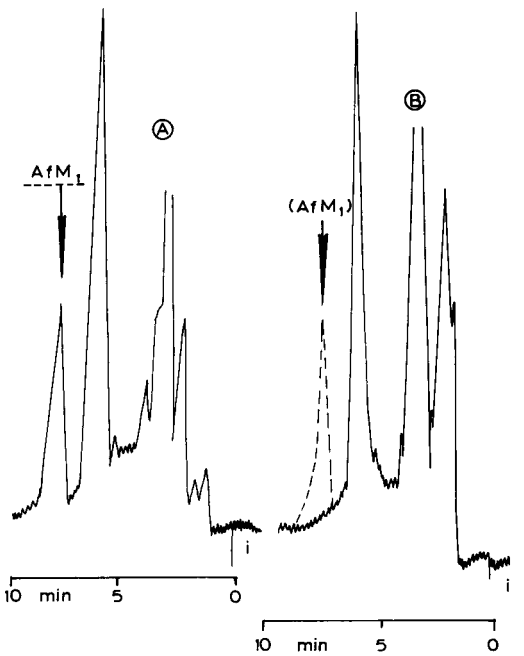

Fig. 4. Chromatograms illustrating the confirmation test of the AfM_1 identity. A, Naturally contaminated powdered milk (1.2 ppb); B, as A, but after TFA derivatization.

In conclusion, the method described is suitable for the rapid determination of AfM_1 in dairy products. The complete analysis, including the confirmation of the AfM_1 identity, can be performed in 3 h. The method is both simple and cheap. AfM_1 can be detected at 0.1 ppb levels in cheese, dried whey and dried milk (results can be given in μg/l: 0.01 μg/l reconstituted 10% liquid milk) and at 0.05 ppb levels in liquid raw milk.

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Note

Assay method for aflatoxin in milk

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During the last three years we have been interested in developing a sensitive, reliable method to detect and quantitate aflatoxins in milk. The method has now been successfully tested and used to study the effects of some parameters on the appearance of aflatoxins in milk and the analysis of commercial dairy samples in France obtained during a period of importation of groundnut cakes heavily contaminated by aflatoxins. For these purposes the previously reported techniques were not very satisfactory. For example, the method of Pons *et al.*[1], modified by Stubblefield and Shannon[2], did not permit a sufficiently clean extract for the sensitivity desired; this observation was equally true for the technique of Jacobson *et al.*[3] applied to powdered milk. The major inconvenience of Tuinstra and Bronsgeest's method[4], which offers low detection limits, is the utilization of two-dimensional thin-layer chromatography (TLC). Finally, in our hands, the method elaborated by Gauch *et al.*[5] using an Extrelut column did not produce the high quality clean-up claimed by the authors.

Our method was designed to: (1) use a powerful extraction agent in order to break the anticipated bonds of aflatoxin M1 which is more polar than aflatoxin B1; (2) avoid the employment of a completely water immiscible organic phase for the extraction of aflatoxin from milk, which is probably incomplete and depends on several factors; (3) prevent the formation of a colloidal protein precipitate by contact with certain organic solvents; (4) avoid the adsorption of the mycotoxin on the precipitate of proteins caused by heavy metals; (5) obtain a detection limit of 5 ng/kg, at least for the liquid milk, and 50 ng/kg for powdered milk, with good repeatability.

MATERIALS AND METHODS

Reagents

The solvents were isopropanol, methanol, chloroform, toluene, diethyl ether (0.01% ethanol) and acetone (Merck "pro analysis", or Carlo Erba RPE or equivalent). Reagent grade epichlorohydrin and glass-distilled water were also used. Celite 545 (Johns-Manville, Denver, CO, U.S.A.) was employed as filter aid. The chromatographic adsorbents were Aluminoxid 90 (aktiv-basisch, Merck, 70–230 mesh) and Kieselgel H 60 (Merck). Crystallized aflatoxins M1 and M2 were obtained from Serva-Feinbiochem (Heidelberg, G.F.R.).

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Apparatus

The following were employed: 500-ml separatory funnels; rotary evaporator with cold-trap; micro-syringes or Microcaps (Drummond, Broomall, PA, U.S.A.); chromatographic columns, fitted with glass valves; thin-layer plates, Merck Si 60-5721; filter-paper, Whatman No. 1; UV lamp, Philips HP WI25; photodensitometer (Model PHI 5; Vernon, Paris, France).

Procedure

Extraction of milk powder. A 10-g amount of milk powder were mixed with 30 ml water, then added to a separatory funnel containing 60 ml isopropanol. The mixture was shaken vigorously for 1 min. Then 50 ml methanol, 10 g Celite and 250 ml chloroform were added successively. After each addition the funnel was shaken and the contents allowed to settle. The clear lower layer was filtered through paper containing 10 g of anhydrous Na_2SO_4, 300 ml of the filtrate being collected. This solution was evaporated *in vacuo* to about 5 ml on a rotary evaporator at 50°C. The remaining solvent consisting mainly of isopropanol was evaporated as an azeotrope by the addition of 30 ml chloroform. The mixture was evaporated to near dryness and the residue dissolved in 30 ml toluene. After concentration to 0.5 ml, another 30 ml toluene were added and then concentrated to 5 ml.

Extraction of liquid milk. A 50-ml sample of milk was heated in a water-bath at 100°C for 5 min. After cooling to 20°C, the mixture was extracted as above.

Alternatively 100 ml of heated milk were concentrated *in vacuo* to about 25 ml in a rotary evaporator. The volume was adjusted to 30 ml by adding water. The concentrate was then treated as described previously.

Column chromatography. The column was prepared by dry packing 10 g of anhydrous sodium sulphate and 1 g alumina in the ascending order. The column was partially filled with toluene and a slurry of 2 g silica gel in the same solvent was poured into it and firmly packed under pressure. Anhydrous sodium sulphate (2 g) was added to the toluene above the settled silica gel. All the interfaces of the packed materials were separated by pieces fo filter-paper.

The milk extract in toluene was passed through the column. The fats and other substances which interfere with TLC were eluted first with 50 ml toluene and then with 50 ml chloroform. The milk aflatoxins were eluted with 100 ml chloroform–acetone (4:1 v/v). The eluate was collected in a 250-ml round-bottomed flask and concentrated *in vacuo*. Before evaporation was complete, the residue was quantitatively transferred with chloroform to a small vial (*ca.* 1 dram) fitted with a tight cap, dried under a stream of nitrogen and redissolved in 100 μl of chloroform ready for TLC.

Thin-layer chromatography. TLC was performed on silica gel plates heated at 110°C for 20 min. 20-μl aliquots of the extract, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ng of aflatoxin M1 were spotted. A further 10 μl of the extract were spotted on a 0.3 ng of M1 which serves as an internal standard. (The disposition of this standard helps to discriminate aflatoxin M1 from other blue fluorescent spots given by extracts of some milks.) The plate was developed for 12 cm in an unlined tank containing diethyl ether–epichlorohydrin–methanol–water (90:6:3:1 v/v) which had been equilibrated for 2 h.

If aflatoxin M1 was judged to be present in a sample without internal standard,

the mycotoxin was quantitated by matching its fluorescence with a standard spot. If the fluorescence intensity of the sample spot exceeded that of the highest standard, TLC was repeated after dilution of the extract, taking into consideration the 30% of extract already used. If no aflatoxin was detected in the 20-μl sample spot the experiment was repeated with a 50-μl aliquot.

Quantitative densitometry was performed as described by Gauch *et al.*[5].

Confirmation. To confirm the nature of the fluorescent material the test elaborated by Przybylski[6] or the modification proposed by Van Egmond *et al.*[7] was used.

Calculation of the aflatoxin M1 concentration. The value given by the chromatogram corresponds to the amount of the aflatoxin contained in 300 ml of organic extract. Since the total volume of the recoverable organic phase is 360 ml for dry milk and 345 ml for liquid milk, it is necessary to introduce factors of 1.2 and 1.15 respectively for calculation of the aflatoxin level in the analysed material.

RESULTS AND DISCUSSION

The described method has been used to analyze about 1000 samples of dry or fluid milks. Several other experiments have been conducted with artificially contaminated products.

Recovery of aflatoxin M1

Samples (0.5 g) of milk powder without aflatoxin were contaminated by adding titrated solutions of aflatoxin M1. After evaporation of solvent, each of these samples was incorporated either in solutions of powdered milk (9.5 g in 30 ml distilled water) or in liquid milks without any aflatoxin.

The results in Table I show that for aflatoxin levels of 20–800 ng/l (natural or reconstituted milk) the proposed method gives average recoveries of 98.2%.

TABLE I

RECOVERY (%) OF AFLATOXIN M1 ADDED TO MILK

Aflatoxin M1 added (ng/l)*	*Dried milk*		*Liquid milk*	
	No. of tests	*Average recovery***	*No. of tests*	*Average recovery*
20	7	96	6	105
50	7	98	6	96
100	6	95	6	95
250	2	103	2	97
800	6	98	5	99

* Natural or reconstituted milk.

** Calculated (see text).

Repeatability

The present results using the described method were obtained by only one group of analysts. On the other hand, these results all relate to naturally contaminated milks. Table II shows the slight scattering of the values even when the contamination level is low.

TABLE II

PRECISION FOR NATURALLY CONTAMINATED MILKS

m = Average value; s = standard deviation.

Sample	*No. of tests*	*m (ng/kg)*	*s*	*s/m (%)*
Dried milk				
a	6	11,033	312	2.8
b	8	4975	134	2.7
c	5	2210	72	3.3
d	6	104	11	10.7
e	8	75	9	12.2
Liquid milk				
1	6	510	15.6	3.0
2	8	372	9.3	2.5
3	6	122	6.4	5.3
4	8	71	4.8	6.8
5	8	44	6.0	13.3

Sensitivity

The detection limits for aflatoxin M1 are 5 ng/l for liquid milk and 25 ng/kg for powdered milk. It should be noted that the values for aflatoxin M are the totals for aflatoxins M1 and M2, which are not separated by the TLC phase employed.

On several occasions we have verified that M2 represents only a small fraction of the total aflatoxin M and therefore this method of expressing the results does not overestimate the (potential) health risk.

TABLE III

DETERMINATION OF AFLATOXIN M1 (ng/kg) IN NATURALLY CONTAMINATED MILKS BY THE METHOD OF STUBBLEFIELD (A) AND BY THE PROPOSED METHOD (B)

The values are means from two assays for each sample. T = Trace; ND = not detected.

Liquid milks			*Dried milks*		
Sample	*A*	*B*	*Sample*	*A*	*B*
I	250	510	A	9100	11,650
II	200	362	B	8500	11,125
III	100	235	C	4650	5875
IV	150	210	D	2950	4950
V	100	175	E	2050	2475
VI	100	160	F	400	450
VII	ND	105	G	200	275
VIII	T	80	H	100	225
IX	ND	42	I	T	160
X	ND	20	J	ND	125
XI	ND	15	K	ND	75
			L	ND	62
			M	ND	50
			N	ND	47

Comparison with the Stubblefield method

During the development of our method, an assay for aflatoxin M1 was described by Stubblefield[8]. It was therefore of interest to compare results obtained by the two methods using the same samples. Experiments were performed with naturally contaminated liquid and dried milks. The results in Table III show, chiefly for samples of liquid milk, some of the difficulties of applying the Stubblefield method.

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Note

High-performance liquid chromatographic analysis of permethylated cytokinins*

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Cytokinins exist in most plant tissues in minute amounts. The isolation of material of sufficient purity for structural determination by physical techniques requires extensive use of chromatographic methods. The polar nature of most of the naturally occuring cytokinins places limits on the types of HPLC systems that can be utilized for their isolation and analysis. The latter necessitates formation of volatile derivatives for GLC and GLC–MS. The most widely used derivatives for cytokinins have been TMS[1,2]. With the advent of permethylation[3] more recent use of these cytokinin derivatives have been made[4,5]. Advantages of permethyl over TMS derivatives include their chemical stability and lower molecular weight. In addition, the permethylated cytokinin is non-polar which expands the chromatographic potential for alternate purification procedures.

The purpose of this work was to modify the permethylation procedure and to devise HPLC purification techniques for permethylated cytokinins.

EXPERIMENTAL

Reagents

Potassium *tert.*-butoxide from Aldrich and reagent-grade DMSO from Pierce were stored in a drying cabinet. After redistillation methyl iodide was dispersed into small vials over molecular sieve and a drop of mercury, and the vials held in a plastic bottle containing silica gel and stored at 4°C.

The DMSO anion was prepared by mixing DMSO plus potassium *tert.*-butoxide for 1 h at 45°C with nitrogen flowing through the system at 30 l/h. The DMSO anion at a final molarity of 0.1 to 0.2 was the easiest to handle with respect to viscosity

* Abbreviations: TMS = Trimethylsilate; Z = zeatin; ZR = zeatin riboside; ZROG = zeatin riboside-O-glucoside; Z9G = zeatin-9-glucoside; Me = methyl; GA = gibberellins; DMSO = dimethylsulphoxide; GLC = gas–liquid chromatography; HPLC = high-performance liquid chromatography; MS = mass spectrometry; 2iP = N^6-isopentenyl adenine; 2iPA = N^6-isopentenyl adenosine; diHZR = dihydrozeatin riboside; diHZROG = dihydrozeatin riboside-O-glucoside; diHZ = dihydrozeatin; diHZOG = dihydrozeatin-O-glucoside.

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and can be achieved by mixing 6 ml DMSO with about 180 mg potassium *tert.*-butoxide. After preparation the DMSO anion was dispensed into small vials and stored over silica gel in a plastic container at −20°C.

Permethylation procedure

Cytokinins were placed in Reactivials (Pierce) and dried with a stream of nitrogen. Experiments with and without vacuum drying indicated the vacuum step was not essential. Reactivials were positioned on a warming plate at 40°C; 50 μl DMSO anion were added (*ca.* 10 × excess of the cytokinin) and the vial shaken; 10 μl methyl iodide then added and shaken; allowed to stand; 50 μl water added, and finally partitioned with 100 μl chloroform to remove the Me cytokinin from the reagents. *Note:* the relatively non-polar cytokinins 2iP and 2iPA will partition into chloroform even when not permethylated.

HPLC

Two separate purification systems for methylated cytokinins were developed. One using an adsorption column of 150 × 4.5 mm I.D. Spherisorb eluted with hexane–isopropanol–triethylamine (85:15:0.1) at 2 ml/min and the other a polar column of 250 × 4.5 mm I.D. Partisil 10PAC eluted with hexane–ethanol–triethylamine (90:10:0.1) at 2 ml/min.

UV spectrometry

UV spectra were obtained using a Beckman DU-8 spectrophotometer. Samples of Me cytokinins were run in methanol.

Mass spectrometry

MS spectra of Me cytokinins were obtained with a Pye 104 GC connected to a Kratos MS 30. The GLC conditions were: column, 3% OV-1 on Gas-Chrom Q (100–120 mesh); helium flow-rate, 35 ml/min; column temperature, for Z 220–280°C at 8°C/min and for ZR isothermal at 300°C. For Z and ZR the MS 30 was set at 70 eV, 300 μA, 3 sec/decade scan speed, source temperature at 190°C and 200°C, respectively, and jet temperature of 280°C.

Scintillation counting

[^{14}C]Cytokinins were permethylated and assayed in 5 ml of a solution containing 1.5 *M* toluene, 6 g PPO and 750 ml Triton X-100. Counting was conducted in a Phillips PW 4540 liquid scintillation analyzer.

RESULTS AND DISCUSSION

The commonly used procedure for methylation of cytokinins has many steps and is time consuming[6]. Our modifications of the procedure result in favorable changes. The use of potassium *tert.*-butoxide in place of sodium hydride for the preparation of the DMSO anion has several advantages. The rate of reaction is rapid and no hydrogen is produced. The starting material does not need to be washed free of mineral oil, thus contamination of small samples prior to MS analysis is reduced. The DMSO anion solution is free of particulate matter which can cause syringe

blockage as when NaH is used. The time in DMSO anion prior to adding methyl iodide was checked at intervals of zero to 60 min using [^{14}C]ZOG. No greater than 3% difference of recovered activity could be found and the zero time period was chosen for all subsequent experiments. In practice this meant adding the DMSO anion to each vial, vigorously shaking each vial and then adding the methyl iodide. Similar results were found for the time in methyl iodide, however, in this instance 30 min was chosen for convenience.

The efficiency of derivatisation was estimated by the use of ^{14}C-labelled cytokinins together with HPLC and MS analysis of the products. In all cases the procedure outlined above gave greater than 90% permethylation with S.E. less than 2. An advantage of this procedure is that samples do not need to be exhaustively dried as they do for the production of TMS derivatives. Though samples were routinely dried over phosphorus pentoxide under vacuum overnight it was found that the same efficiency was obtained for samples dried by an stream of nitrogen immediately prior to derivatisation. Once permethylated the cytokinins are stable for long periods and can be used as laboratory standards.

An aspect worth emphasizing is the fresh preparation of DMSO anion on a regular basis. If prepared each 2 months, a point stressed by others[4], one can expect good permethylation results. We have had numerous permethylation failures traced to DMSO anion stored beyond 2 months at $-20°C$.

Hormone analysis from plant organs involves organic solvent extraction of large samples and subsequent separation of materials via solvent partitioning and chromatography. While these procedures are useful and important the putative compound must be further separated from contaminants before reliable identification and estimation of amounts present are possible. When purifying a putative cytokinin from plant extracts it is useful to have a wide range of separation procedures throughout the scheme. In the last stage of extract preparation for MS analysis it is important to attempt separation of the methylated putative cytokinin from remaining contam-

TABLE I

HPLC OF METHYLATED CYTOKININS ON SPHERISORB OR PARTISIL 10 PAC

Compound	*Retention time (min:sec)*	
	*Spherisorb**	*Partisil 10 PAC***
Me 2iPA	2:15	2:45
Me diHZR	2:45	3:15
Me ZR	3:00	3:30
Me diHZROG	3:30	4:00
Me Z9G	3:30	3:30
Me ZROG	4:00	4:15
Me 2iP	4:45	5:30
Me diHZ	6:45	6:30
Me Z	8:00	7:45
Me diHZOG	12:30	8:15
Me ZOG	13:45	9:30

* Isocratic. Hexane–isopropanol–triethylamine (85:15:0.1), 2 ml/min.

** Isocratic. Hexane–ethanol–triethylamine (90:10:0.1), 2 ml/min.

inates. Toward that end we investigated HPLC systems for permethylated cytokinins. Since permethylation reduces cytokinin polarity we chose adsorption columns and polar columns eluting both with non-polar solvents. The most useful results were obtained with adsorptive Spherisorb and polar Partisil 10 PAC (Table I). Each contrasted system allows opportunity for further purification of the permethylated cytokinin from extract contaminants prior to MS analysis. It is even possible on Spherisorb to separate mixtures of cytokinins (Fig. 1). While one does not usually examine multiples of cytokinins the added flexibility is useful. In each HPLC system it is necessary to use a basic solvent system to suppress ionization of the amino group. This was achieved by adding triethylamine to the eluant. Attempts to achieve good separation under neutral or acidic conditions were unsuccessful.

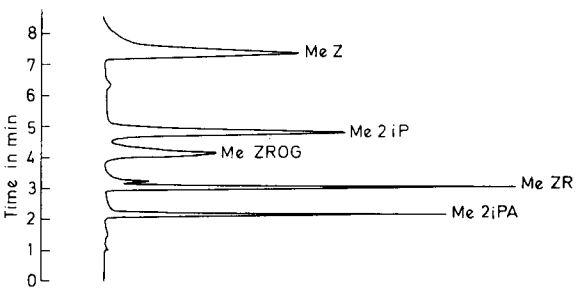

Fig. 1. Isocratic development of Me 2iPA, Me ZR, Me ZROG, Me 2iP and Me Z on Spherisorb with hexane–isopropanol–triethylamine (85:15:0.1) at 2 ml/min.

The Me cytokinins were collected after HPLC and UV spectrum obtained. Of the 11 analyzed all provided peaks at 275 nm. From each HPLC column one cytokinin was obtained for an MS spectrum. From Spherisorb, Me Z showed M^+ at *m/e* 261 with a base peak at *m/e* 230 (ref. 7), while Me ZR from Partisol 10 PAC showed M^+ at *m/e* 421 with a base peak at *m/e* 216 (ref. 7).

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Note

Determination of aprophen in biological samples by normal-phase high-performance liquid chromatography

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Aprophen, a diethylaminoethyl analog of 3-quinuclidines, possesses spasmolytic and cholinolytic activities similar to that of benactyzine which is used in nerve agent antidotal formulations[1]. The increased anticholinergic potency and decreased toxicity of aprophen as compared to benactyzine make it attractive as a potential nerve agent antidote[2].

Current analytical methodology employed for the analysis of aprophen hydrochloride and its structural congeners utilizes reversed-phase high-performance liquid chromatography (HPLC). This methodology effectively separates and quantitates the quinuclidine salts and their hydrolytic by-products in pharmaceutical formulations[3]. Aprophen, however, is recovered as the free base from biological samples after simple solvent extraction and as such has limited solubility in the mobile phases of reversed-phase systems. Therefore the present method describes a simple and sensitive normal-phase HPLC chromatographic system for the separation and quantitation of aprophen in biological samples.

EXPERIMENTAL*

Apparatus

A liquid chromatographic system (Waters, Milford, MA, U.S.A.) consisting of two Model 6000A solvent delivery systems, a Model 660 solvent programmer, a Model U6K sample injector and a Model 440 absorbance detector set at 254 nm was employed. Chromatograms were recorded on a Houston recorder and integrated with a Columbia Scientific Supergrator-3.

Reagents

Spectroquality or reagent-grade solvents were employed. Triethylamine (TEA) was obtained from Eastman (Rochester, NY, U.S.A.), and was redistilled before use. 2,2-Diphenylpropionic acid was purchased from Aldrich (Milwaukee, WI, U.S.A.).

* The manufacturer's names and products are given as scientific information and do not constitute an endorsement by the United States Government.

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Aprophen was obtained by in house synthesis as previously described[3]. Benactyzine was USP grade obtained from Millmaster (New York, NY, U.S.A.).

Procedure

Samples were separated at ambient temperature on a Whatman Partisil 5 column (25.0 cm × 4.6 mm I.D.). The mobile phase consisted of methanol–acetonitrile (30:70) with 0.01 % TEA. The composition of the mobile phase was controlled by the solvent programmer at a flow-rate of 1.0 ml/min. All separations were monitored at 254 nm at sensitivities of 0.02 to 0.005 absorbance units.

*Sample preparation**

Rat serum was extracted directly with diethyl ether. Five volumes of reagent-grade ether were used for each volume of aqueous phase extracted. The ether was evaporated under nitrogen and the residue resuspended in 1 ml of absolute methanol. Tissues were washed in cold saline, blotted dry, weighed and homogenized in 0.067 *M* Na_2HPO_4 (pH 8.5). Aliquots of the homogenate (0.5–1.0 ml) were extracted with 10 ml of diethyl ether, centrifuged and the clear supernatent removed, measured and evaporated to dryness as described above.

RESULTS AND DISCUSSION

Although it is possible to quantitate accurately and reproducibly low concentrations of aprophen hydrochloride in neat solutions by reversed-phase HPLC, quantitative and reproducible elution of the free base is not possible due to the limited solubility of aprophen in these aqueous–organic mobile phases. A normal-phase HPLC system was therefore required to determine aprophen in the biological fluids and tissue samples after simple solvent extraction. The chromatographic conditions for aprophen were chosen by comparing the resolution of standards of aprophen and benactyzine on a Partisil-5 silica column while varying the mobile phase composition as shown in Fig. 1. Baseline resolution was not achieved with more than 50 % methanol. With less than 30 % methanol, the compounds were not quantitatively eluted from the column. A solvent composition of methanol–acetonitrile (30:70) provided the best compromise for resolution and reproducibility.

The method was found to be linear for column concentrations of aprophen from 12 to 1000 ng (Fig. 2). The lowest limit of detection of a standard solution was determined to be 10 ng. The coefficient of variation for retention times was 2.5 %.

Aprophen was extracted from serum and tissue samples prior to injection on the column. An extraction step was necessary to concentrate the aprophen for greater sensitivity and to remove material which may interfere with the assay technique. The recovery of aprophen from spiked serum samples ranged from 75–77 % and the extraction efficiency from spiked tissues was 89–95 %.

A representative chromatogram showing aprophen recovered from a serum sample is shown in Fig. 3. Concentrations of aprophen as low as 25 ng can be determined by this procedure.

* In conducting the research described in this report, the investigator(s) adhered to the "Guide for the care and use of laboratory animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

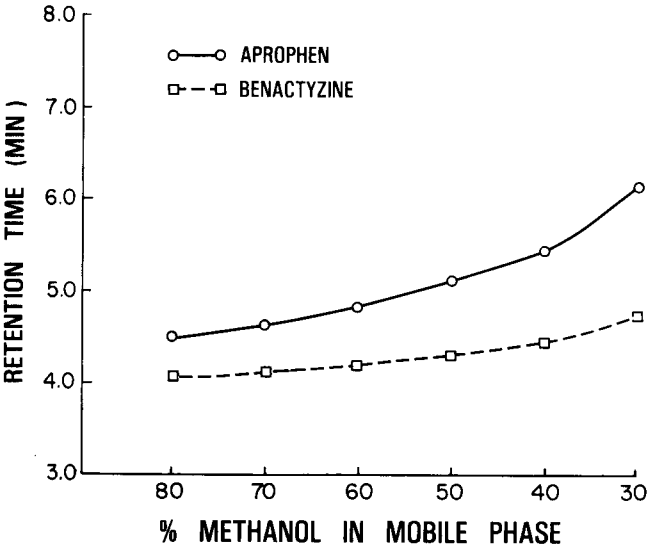

Fig. 1. Variation of the retention time with solvent composition of aprophen and benactyzine.

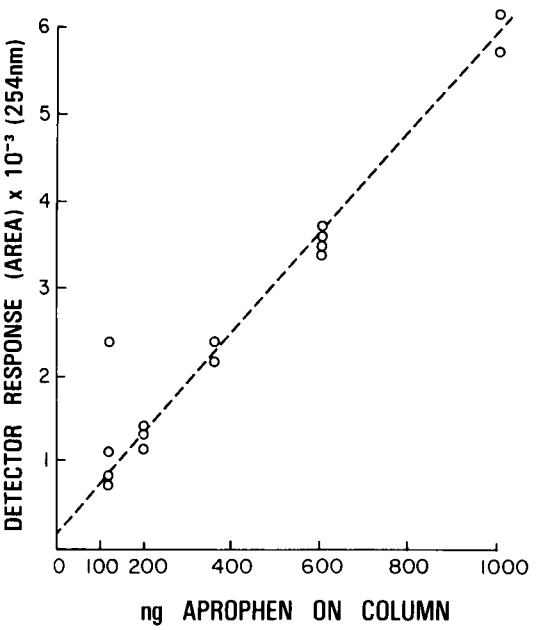

Fig. 2. Calibration curve of aprophen detected at 254 nm. Column: 25.0 cm × 4.6 mm I.D. Partisil-5. Mobile phase: methanol–acetonitrile (30:70) with 0.01% triethylamine.

Fig. 3. Chromatogram showing the separation of aprophen (1) in spiked rat serum. Column: 25.0 cm × 4.6 mm I.D. Partisil-5. Mobile phase: methanol–acetonitrile (30:70) with 0.01% triethylamine. Flow-rate: 1 ml/min. U = Unidentified sustances present in the serum extract. Time in minutes.

The present chromatographic method clearly demonstrates the usefulness of normal-phase chromatography for the analysis of compounds that in reversed-phase HPLC present problems because of limited solubility in the aqueous–organic mobile phases. The system employs a deactivated silica column and an alkylamine to reduce tailing and enhance resolution. This separation is very reproducible and the chromatographic system is simple to maintain. It can be easily employed for the determination of aprophen levels in serum and tissues for pharmacological and clinical studies.

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Note

High-performance liquid chromatographic determination of acetaldehyde in wine as its lutidine derivative

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Suzuki and Maruyama[1] reported on the high-performance liquid chromatography (HPLC) of acetaldehyde (Ac) in wine as its 2,4-dinitrophenylhydrazone derivative. This method was not successful in the determination Ac, because the high temperatures and high concentrations of acid required to transform Ac into hydrazone with the reaction system equipped with the specific apparatus, also caused corrosion of the stainless-steel columns in the HPLC system. In a previous paper[2], we considered the HPLC retention behaviour of low molecular weight aldehydes as their lutidine derivatives on 3-aminopropyltriethoxysilane (3APTS) treated silica gel. Therefore, we studied the determination by HPLC of Ac in wine as its lutidine derivative on the 3APTS-treated silica gel or the commercial NH_2-chemically bonded stationary phase.

EXPERIMENTAL

Reagents

Ac, propionaldehyde (PA) and acetylacetone (AA) were obtained from Wako (Osaka, Japan). 3APTS was purchased from Aldrich (Milwaukee, WI, U.S.A.). Nucleosil 5 NH_2 was purchased from Macherey, Nagel & Co. (Düren, G.F.R.). A highly microporous spherical silica gel (mean pore diameter 95 Å, surface area (BET) 380 m^2/g, particle size distribution 5.5 μm) was obtained from Fuji-Davison (Nagoya, Aichi, Japan). Hexane and ethanol were used after distillation. The other reagents and organic solvents were reagent grade.

Apparatus

Three liquid chromatographs were employed: a Hitachi 635 T equipped with a visible spectrophotometer and Hitachi 204 S fluorescence spectrophotometer; a Jasco Twincle equipped with a Jasco Uvidec 100-III visible spectrophotometer and a Jasco FP 110 fluorescence spectrophotometer; and a Type KHU 16 of Kyowa Seimitsu Mini Micro Pump equipped with a Type KLC 200 of Kyowa Seimitsu variable-wavelength detector.

Determination of the number of accessible NH_2 surface groups per gram (or 100 $Å^2$) of homemade gel

Dried silica gel (5 g) was added to 50 ml of a 2.5% benzene solution of 3APTS.

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After stirring for 24 h at room temperature, the silica gel was filtered with a glass filter (1 μm), washed several times with benzene and methanol, and then dried *in vacuo* at 70°C for 2 days. According to the assumption made in a previous paper[2,3], the number of accessible NH_2 surface groups per gram of silica gel was calculated to be $0.45 \cdot 10^{21}$, and the number of accessible NH_2 surface groups per 100 $Å^2$ of silica gel was calculated to be 1.76.

Preparation of Ac-AA and internal standard

According to a previous paper[2], the lutidine derivatives of Ac and PA (internal standard) used in this report were Ac-AA (m.p. 157–159°C; analysis for $C_{12}H_{17}NO_2$ (calculated): C, 69.53; H, 8.27; N, 6.76; (found): C, 69.47; H, 8.51; N, 6.73) and PA-AA (m.p. 162–164°C; analysis for $C_{13}H_{19}NO_2$ (calculated): C, 70.54; H, 8.66; N, 6.33; (found): C, 70.42; H, 8.82; N, 6.37).

Column preparation

Silica gel treated with 3APTS or Nucleosil $5NH_2$ was packed into stainless-steel columns (250 mm × 4 mm I.D.) using a balance density method and a 10-ml stainless-steel packer at a rate of 500 kg/cm^2 (Kyowa Seimitsu Type KHW-20 ultra-high-pressure pump).

Procedure

To a 15-ml aliquot of wine, 5 ml of a mixture[6] of 2 *M* ammonium acetate, 0.05 *M* acetic acid and 0.02 *M* AA was added. The reaction mixture was warmed in a water-bath at 60°C for 30 min as described by Suzuki and Tani[4]. After cooling, 5 ml of internal standard solution (15 μg of the lutidine derivative of PA in 1 ml of chloroform) were added. The mixture was shaken well and allowed to stand for some minutes. The aqueous phase was then discarded, and the organic phase dried over anhydrous sodium sulphate. A 50-μl volume of the resulting solution was subjected to HPLC. The operating conditions are given in the legend to Fig. 2.

When PA was omitted from the mixture, it was not detected as present in the wine. Therefore PA was selected as the internal standard.

RESULTS AND DISCUSSION

To determine the best extracting solvent for Ac-AA in wine, the variation of the peak shape to peak area ratio with Ac-AA/PA-AA was measured on the 3APTS-treated silica gel column and on the Nucleosil $5NH_2$ column with methylene chloride, ethyl acetate or chloroform. Of these, chloroform was chosen as the most suitable for extraction, as it clearly separated from the aqueous phase as a lower layer, which was convenient for the separation procedure.

Hexane was used as a component of the mobile phase, and the effect of an alcohol on the retention behaviour of Ac-AA was studied. According to the polarities of alcohols[5] methanol is the most suitable, but it has a very low solubility in hexane. Therefore, ethanol seems to be best from the viewpoint of the separation. The dependence of capacity factor on the alcohol concentration was studied with ethanol. The optimal separation for Ac and internal standard was attained for a hexane:ethanol ratio of 25:1 (Fig. 1). Elution with this mobile phase on the 3APTS-treated silica gel

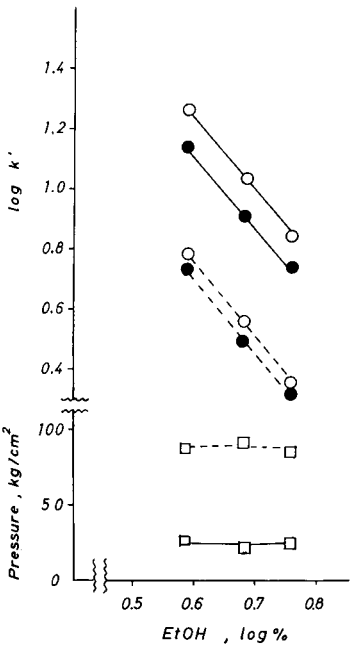

Fig. 1. Relationship of capacity factor, k', and column pressure to the concentration of ethanol (EtOH) in the mobile phase. – · · –, 25% 3APTS-treated silica gel column; – – – –, Nucleosil $5NH_2$ column; ○, acetaldehyde, ●, propionaldehyde (i.s.); flow-rate, 1.2 ml/min.

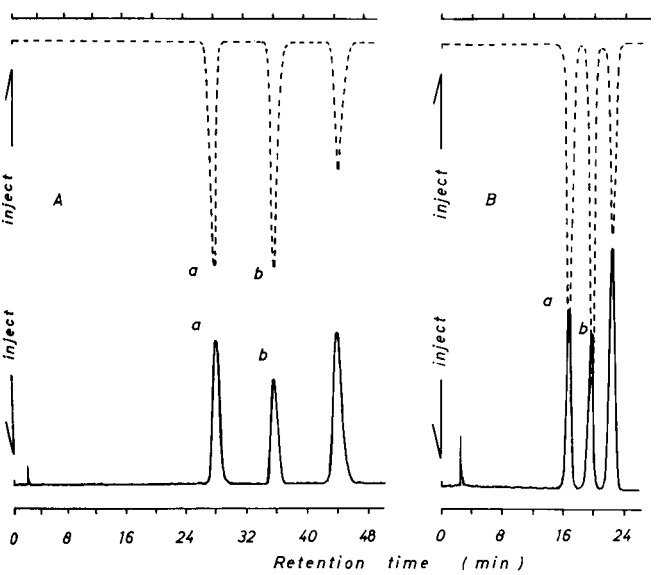

Fig. 2. Comparative liquid chromatograms of acetaldehyde in wine and propionaldehyde (internal standard). Stationary phase: A, 25% 3APTS-treated silica gel column; B, Nucleosil $5NH_2$ column. Mobile phase, hexane–ethanol (25:1); flow-rate, 1.2 ml/min. Detection: ———, 375 nm; – – – –, 395 nm (Ex) 460 nm (Em). Peaks: a = propionaldehyde; b = acetaldehyde.

or the Nucleosil $5NH_2$, resulted in a resolution between the Ac and PA peaks of 4.00 or 1.88, respectively.

Fig. 2 shows the comparative chromatograms obtained from Ac in wine and PA as internal standard on the 3APTS-treated silica gel column or the Nucleosil $5NH_2$ column. No interfering peaks were observed at the retention times of the compounds of interest.

A calibration graph constructed by plotting the ratio of the peak area of Ac to that of the internal standard was linear and passed though the origin, in the ranges 0.01–0.5 and 0.1–6 μg using fluorescence and visible spectrophotometers, respectively. Nine replicate determinations on a test solution containing Ac (0.2 μg) gave a standard deviation of 3.38% and 1.98%, using fluorescence and visible spectrophotometers, respectively; the limit of detection was 0.01 μg.

Table I shows the acetaldehyde concentration in the samples commercial wines tested.

TABLE I

ACETALDEHYDE CONCENTRATION IN WINE

Wine sample	*Acetaldehyde (μg/ml)*
A	1.2
B	0.7
C	0.4
D	1.0
E	0.2

ACKNOWLEDGEMENT

The authors are indebted to Dr. Hiroshi Kishimoto for technical assistance.

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Note

Separation of diamantane-3-oneoxime stereoisomers by preparative high-performance liquid chromatography

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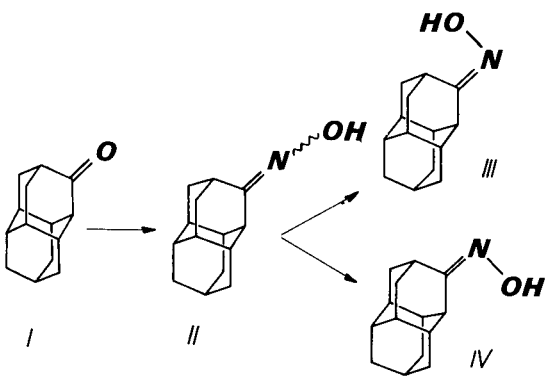
During the preparation of diamantane-3-oneoxime (II) by the reaction of diamantanone (I) with hydroxylamonium chloride and sodium hydroxide, a mixture of two stereoisomers[1] is formed, *viz.* the *E*-form (III) and the Z-form (IV).



As the oximes are thermally unstable and therefore cannot be subjected to gas chromatography, a method has been elaborated for their analysis using liquid chromatography. This paper describes a method for the separation of the above two stereoisomers by preparative high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Separation of stereoisomers

The mixture of diamantane-3-oximes (II) was separated into individual compounds on a Chromatospac Prep 100 preparative chromatograph (Jobin Yvon, Longjumeau, France). A 200-g amount of silica gel of irregular shape (particle size 10–20 μm) was packed into a column of 40 mm I.D.; the height of the bed was 270 mm. The adsorbent was obtained from silica gel L-40 (Lachema, Brno, Czechoslovakia) by screening on a Zig-Zag classifier (Alpine, Augsburg, G.F.R.), washing out with methanol and drying at 200°C for 5 h before use.

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n-Pentane (Reakhim, U.S.S.R.) containing 4% (w/w) of 2-propanol was used as the mobile phase. The mobile phase was degassed before use by connecting its reservoir in the preparative chromatograph to a vacuum for 10 min. The flow-rate of the mobile phase was 18 ml/min at a pressure of 150 kPa. A 10% (w/w) solution of the sample in *n*-heptane–2-propanol (96:4, w/w) was injected using a sample injection reservoir. The amount of sample solution injected varied from 1 to 15 ml. Detection was effected with a refractive index (RI) (Varian, Palo Alto, CA, U.S.A.). Individual fractions were collected according to the shape of the chromatographic curve.

Analysis of fractions

HPLC analysis of fractions obtained by preparative separation was carried out on a Varian 8500 instrument with an RI detector. The column (250 × 3 mm I.D.) was packed with 10-μm spherical silica gel (Pragosil). The flow-rate of the mobile phase [*n*-heptane–2-propanol (98:2, w/w)] was 1 ml/min.

The crystal structure of the *Z*- and *E*-forms of diamantane-3-oneoxime was determined by three-dimensional X-ray diffraction analysis[2].

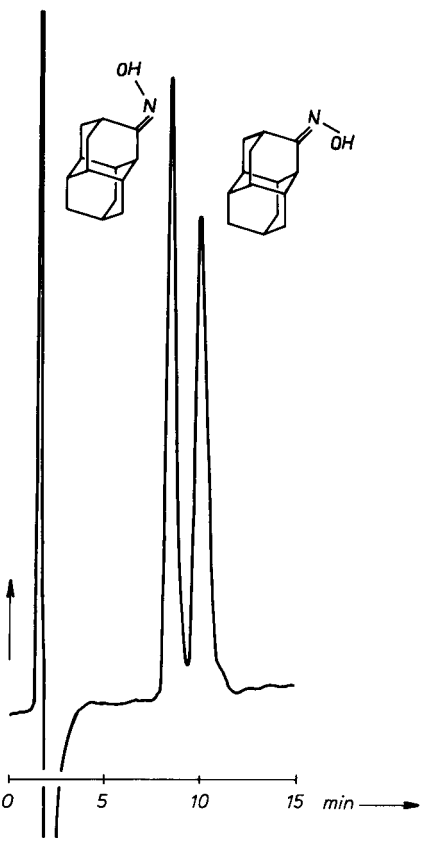

Fig. 1. Analytical separation of diamantane-3-oneoxime stereoisomers. Column: 250 × 3 mm I.D. packed with spherical silica (10 μm). Mobile phase: *n*-heptane–2-propanol (98:2, w/w), flow-rate 1 ml/min. Detection: RI detector.

RESULTS AND DISCUSSION

An analytical column of relatively high efficiency was used; the number of theoretical plates for naphthalene (capacity ratio, $k' = 1.6$) was 9000 (determined using *n*-pentane as the mobile phase) and 6000 for phenanthrene ($k' = 3.6$).

A chromatogram of the two diamantanone oximes is presented in Fig. 1. As the separation of these stereoisomers is difficult (separation factor, $\alpha = 1.09$), the preparative parameters were chosen so as to achieve maximal efficiency for sufficiently large amounts of sample. Silica gel particles of size 10–20 μm were used, *i.e.*, the minimal size fraction that was obtainable from the original material in a sufficiently large amount. The use of 200 g of the silica gel resulted in a column of nearly the maximal length (275 mm), allowing the whole amount of stationary phase to be introduced into the column at once.

n-Heptane–2-propanol (98:2, w/w) was used as the mobile phase for the analytical separation, but for preparative purposes *n*-pentane was used instead of *n*-heptane as it is more readily available (particularly UV-grade material), is easily regener-

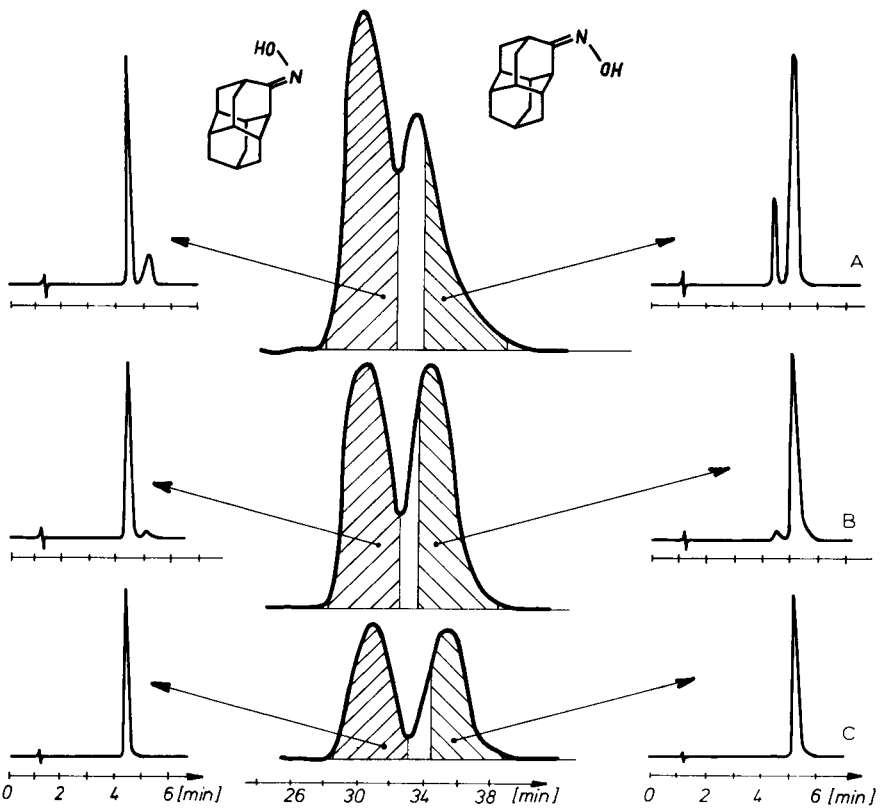

Fig. 2. Preparative chromatograms for various injection volumes, together with analytical chromatograms for the fractions obtained. Preparative run: column, 270 × 40 mm I.D. packed with irregular silica (10–20 μm); mobile phase, *n*-heptane–2-propanol (96:4, w/w), flow-rate 18 ml/min. Analytical run: column, 250 × 3 mm I.D. packed with spherical silica (10 μm); mobile phase, *n*-heptane–2-propanol (98:2, w/w), flow-rate 1 ml/min. Detection: RI detector. Amounts injected: A, 1.5 g; B, 0.9; C, 0.5 g.

ated, has a lower viscosity and is cheaper. Also, the separation of *n*-pentane from the fractions obtained is easy, owing to its low boiling point. It was also found that a much better separation during the preparation is possible when *n*-pentane was used instead of higher hydrocarbons with similar properties such as *n*-heptane and iso-octane. In contrast to the analytical column separation, the contents of 2-propanol in the mobile phase had to be increased to 4%.

The flow-rate of the mobile phase was chosen on the basis of test experiments; the testing was carried out on a preparative column using dry *n*-pentane as the mobile phase. The number of theoretical plates for a column packed with an identical amount of the same silica gel (flow-rate 20 ml/min) was determined to be 5800 for naphthalene ($k' = 2.0$) and 6100 for phenanthrene ($k' = 4.1$).

When injecting the optimal amount of 0.5 g of the compound, it was possible to obtain both isomers sufficiently pure without further preparation. However, it is obvious that with increasing amount injected the separation becomes poorer and thus it is necessary either to collect only parts of the relevant peaks, or to repeat the preparation. Fig. 2 shows some preparative chromatograms for various the injected volumes, together with chromatograms of prepared fractions for comparison.

Of the total amount of 17.0 g of a mixture containing 52% of the *E*-form and 48% of the Z-form of diamantane-3-oneoxime, the preparation resulted in 6.0 g of the *E*-form and 4.5 g of the *Z*-form, respectively, in addition 3.5 g of a mixture of both compounds.

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Note

Analytical and preparative separation of the *cis*- and *trans*-isomers of 4-(4′-*tert.*-butylcyclohexyl)-4-methylpentan-2-one by reversed-phase high-performance liquid chromatography

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Our studies of the relationships between chemical structure and odour has yielded individual *cis*- and *trans*-isomers of 4-(4′-*tert.*-butylcyclohexyl)-4-methylpentan-2-one (I and II). Synthesis of this compound was first described by Beets and Van Essen[1]. Later, Theimer *et al.*[2] reported that only the liquid *cis*-isomer I had a specific "urinous" odour, similar to the odour of 5α-androst-16-en-3-one[3], and that crystalline *trans*-isomer II was odourless. The authors did not specify how these substances had been obtained. They mentioned that solutions of the *trans*-isomer had a weak "urinous" odour, presumably because of the presence of *cis*-I as an impurity.

Here we describe the synthesis of a mixture of I and II, the separation of the isomers by preparative reversed-phase high-performance liquid chromatography (HPLC), structural evidence, spectral data and odour evaluation.

EXPERIMENTAL

Apparatus

HPLC was performed with a DuPont 830 preparative HPLC system equipped with a Rheodyne 7120 injector (loops 50 μl and 2 ml), a UV-photometer (254 nm), a DuPont refractive index (RI) detector and a Hewlett-Packard 3380 integrator. Stainless-steel (25 cm × 4.6 mm) columns packed with DuPont Zorbax C_8 (Ser. No. L947; test efficiency 13,890 theoretical plates) and Zorbax ODS (F3823; 15,130) were used for the analytical experiments. Adsorption and reversed-phase HPLC were performed with DuPont preparative columns (25 cm × 22.7 mm) packed with LiChrosorb SI 60 (particle diameter, d_p, 7 μm; test efficiency 11,320 theoretical plates) and LiChrosorb RP-8 (d_p 10 μm; 7490).

Gas–liquid chromatography (GLC) was performed with a Pye Unicam 104 gas chromatograph equipped with a flame ionization detector (FID) on a glass column (2 m × 4 mm) packed with 3% SE-30 or 10% free fatty acid phase (FFAP) on Chromosorb W at 180°C.

Combined GLC–mass spectrometry (MS) was carried out using an LKB 2091 mass spectrometer (70 eV), equipped with a capillary column (SE-30, 30 m × 0.2 mm) programmed from 100°C (5 min) to 260°C at 5°C/min.

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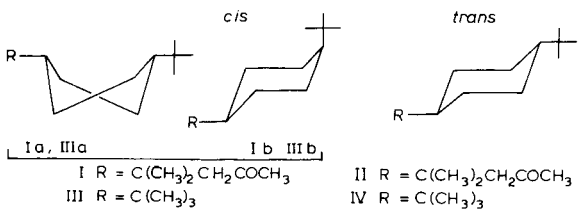
Infrared (IR) spectra (5% carbon tetrachloride solutions) were obtained using a Beckman Acculab VI spectrometer. ^{13}C Nuclear magnetic resonance (NMR) spectra were measured on a 20-MHz CFT-20 Varian spectrometer with tetramethylsilane internal standard.

Chromatographic conditions

The mobile phases were: adsorption preparative HPLC, hexane–chloroform (80:20), flow-rate 13 ml/min, pressure 840 p.s.i.; reversed-phase HPLC, water–acetonitrile (1:3), analytical columns, flow-rate 2 ml/min, pressure 1300 p.s.i., preparative column, flow-rate 19 ml/min, pressure 800 p.s.i. Fractions obtained in preparative reversed-phase HPLC were evaporated until a colourless emulsion was formed, then NaCl and hexane were added. The organic layer was separated and concentrated *in vacuo*.

RESULTS AND DISCUSSION

In previous papers[2,3] the synthesis of a mixture of *cis*- and *trans*-isomers of 4-(4′-*tert*.-butylcyclohexyl)-4-methylpentan-2-one (I and II) and their odour characteristics were described. The authors did not indicate how the individual isomers had been obtained. There was no spectral evidence in favour of the existence of any definite conformations for I and II.



We obtained a mixture of I and II by successive benzene alkylation with mesityl oxide and *tert*.-butanol according to ref. 1, hydrogenation of the resulting 4-(4′-*tert*.-butylphenyl)-4-methylpentan-2-one over Raney nickel at 180°C and 160 atm H_2, oxidation by Corey reagent[4] and preparative HPLC of the reaction mixture (LiChrosorb SI 60).

The fraction isolated was not only homogeneous to adsorption HPLC but also to packed and capillary GC, and its IR, mass and ^1H NMR spectra corresponded to 4-(4′-*tert*.-butylcyclohexyl)-4-methylpentan-2-one. Only reversed-phase HPLC revealed the presence of the two peaks in this sample in a ratio of about 85:15, which could be separated on Zorbax ODS and C_8 analytical columns with α values of 1.27 and 1.065 respectively (Fig. 1a, b).

The separation strategy for preparative HPLC was selected according to Snyder and Kirkland[5]. During the first run (Fig. 2a) a homogeneous fraction A (oil) was isolated and after rechromatography of fraction B (Fig. 2b) we obtained peak I (the same substance as in fraction A) and peak II (colourless crystals, m.p. 46.8–47.8°C). Analytical HPLC confirmed that the compounds from peaks I and II were homogeneous (purity >99.9%, RI detector, integrator).

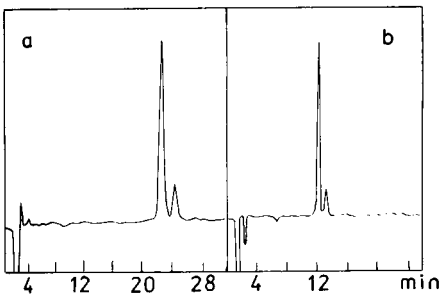


Fig. 1. Separation of *cis*- and *trans*-isomers of 4-(4′-*tert*.-butylcyclohexyl)-4-methylpentan-2-one by reversed-phase HPLC. Columns: 25 × 0.46 cm. Mobile phase: water–acetonitrile (1:3); flow-rate 2 ml/min. Pressure: 1300 p.s.i. Detector: RI, $2 \cdot 10^{-4}$ RI.u.f.s. a, Zorbax ODS; b, Zorbax C_8. Peaks: first = *cis*; second = *trans*.

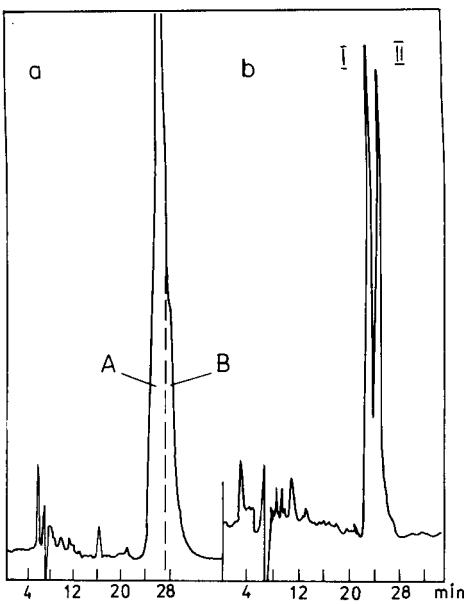


Fig. 2. a, Preparative HPLC separation of a mixture of I and II. Column: 25 × 2.24 cm, LiChrosorb 10 RP-8. Mobile phase: water–acetonitrile (1:3); flow-rate 19 ml/min. Pressure: 800 p.s.i. Detector: UV, 254 nm, 0.04 a.u.f.s. Sample: 493 mg. Fractions: A, 370 mg; B, 58 mg. b, Rechromatography of fraction B. Same conditions, except detector 0.01 a.u.f.s. Peaks: I = *cis*, 30 mg; II = *trans*, 25.4 mg; balance 94%.

Capillary GLC–MS showed that the retention time of these substances was identical but that their mass spectra differed slightly (Figs. 3 and 4). Based on these observations we presumed that the compounds from peaks I and II (Fig. 2b) were *cis*- and *trans*-isomers of 4-(4′-*tert*.-butylcyclohexyl)-4-methylpentan-2-one. These results are in agreement with the findings of Van Bekkum *et al.*[6], to the effect that catalytic hydrogenation of *p*-di-*tert*.-butylbenzene at 190°C leads to a mixture of *cis*- and *trans*-isomers of 1,4-di-*tert*.-butylcyclohexane (III and IV) in a ratio of about 8:2. The two sets of isomers have similar differences in melting points (20° and 94–96°C for III and IV and −5° and 46.8–47.8°C for I and II) and in IR spectra (see Fig. 5).

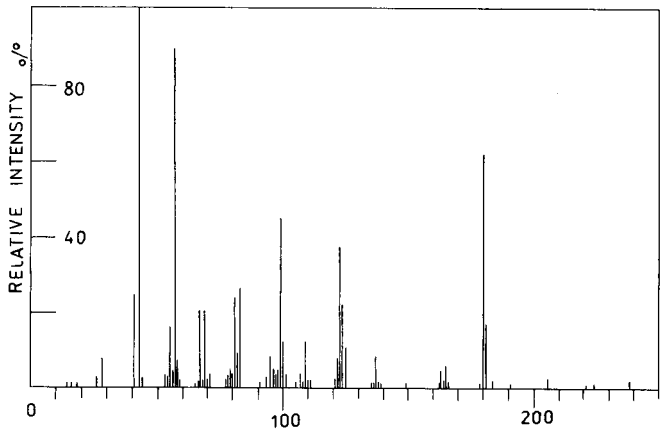


Fig. 3. Mass spectrum of isomer I: *m/e* (relative intensity in %) = 238 (0.6, M^+), 180 (62), 124 (22), 123 (38), 99 (45), 83 (28), 81 (24), 67 (20), 57 (90) and 43 (100).

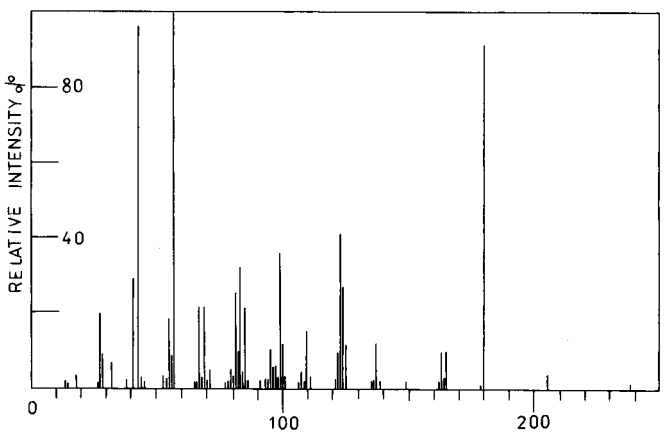


Fig. 4. Mass spectrum of isomer II: *m/e* (relative intensity in %) = 238 (1.1, M^+), 180 (90), 124 (27), 123 (41), 99 (36), 83 (32), 81 (27), 69 (23), 57 (100), 43 (96) and 41 (30).

Theoretically, the diastereomers I and II (as III and IV) may exist in various stereoisomeric forms, which differ in the conformations of the cyclohexane ring and in the axial and equatorial positions of the substituents. It was found earlier that stereoisomers with the bulky *tert.*-butyl group in the axial position were less stable[7]. From a X-ray structure analysis and a number of other methods, Van Bekkum *et al.*[6] showed that the *cis*- and *trans*-isomers of 1,4-di-*tert.*-butylcyclohexane existed in forms having equatorial substituents (IIIa and IV). However, it was found later that *cis*-isomer III should be considered as a mixture of the conformers IIIa and IIIb, with the former predominating ($\approx 60\%$)[8–12].

In the ^{13}C NMR spectrum of *cis*-isomer I the signals of atoms C_5 and C_8 have lower chemical shifts compared to *trans*-isomer II (Table I). This is in agreement with Looms and Robinson's data[13] on the chemical shifts of atom $C_{1(4)}$ in the spectra of III and IV (δ_{TMS} = 42.8 and 48.4 ppm respectively), supporting their conclusion that

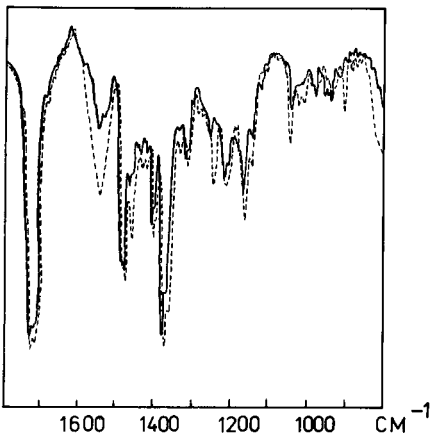


Fig. 5. Infrared spectra of *cis*- and *trans*-isomers I (———) and II (– – –) (5% carbon tetrachloride solutions).

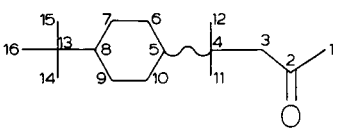
"... carbon atoms on twist rings are on average considerably more shielded to those in diastereoisomers in chair conformers with equatorial substituents". Similar results were obtained by Roberts *et al.*[14].

We conclude that the *trans*-isomer of 4-(4′-*tert.*-butylcyclohexyl)-4-methylpentan-2-one exists in form II, whereas its *cis*-isomer is a conformational mixture of forms Ia and Ib with the twist-form Ia predominating.

TABLE I

^{13}C NMR SPECTRAL DATA OF *cis*- AND *trans*-ISOMERS OF 4(4′-*tert.*-BUTYLCYCLOHEXYL)-4-METHYLPENTAN-2-ONE (I AND II)

s = Singlet; d = doublet; t = triplet; q = quartet; m = multiplet.



cis-I			*trans-II*		
δ_{TMS} *(ppm)*	*Multiplicity, "off-resonance"*	*No. of C atoms*	δ_{TMS} *(ppm)*	*Multiplicity, "off-resonance"*	*No. of C atoms*
52.59	t	3	52.61	t	3
42.07	d	5, 8	48.24	d	5, 8
41.31	d		46.67	d	
36.41	s	4, 13	35.87	s	4, 13
33.00	s		32.70	q	1
32.75	q	1	32.37	s	4, 13
27.68	m	11, 12,	27.58	m	11, 12, 14–16
24.76	m	14–16	24.94	m	6, 7, 9, 10
24.44	t	6, 7, 9, 10	209.50	s	2
23.06	t				
218.46	s	2			

In the odour evaluation experiments (ten panelists, sensitive to the odour of 5α-androst-16-en-3-one) we found that *cis*- and *trans*-isomers I and II both had "urinous" odour, but the odour perception thresholds were about 10^3 and 10^5-fold greater respectively than that for 5α-androst-16-en-3-one. Also, *trans*-isomer II, twice purified by preparative reversed-phase HPLC to a constant odour perception threshold, had a "urinous" odour, although its threshold was about 10^2-fold greater than that of *cis*-isomer I.

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CHROM. 14,113

Note

Application of the combined redox–complexation reaction to the detection of antipyrine and its derivatives in thin-layer chromatography

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(First received February 5th, 1981; revised manuscript received June 11th, 1981)

The aim of this work was to obtain lower detection limits for phenylpyrazolone derivatives by using the following systems as detection agents: (I) Ce(IV), arsenazo III; (II) Fe(III), 1,10-phenanthroline or 2,2′-dipyridyl; (III) Cu(II), 2,2′-diquinolyl or disodium 2,2′-dicinchonate.

The reaction path is as follows:

$$n\mathrm{M}^{(m+1)+} + \mathrm{R_R} \overset{ne}{\rightleftarrows} n\mathrm{M}^{m+} + \mathrm{R_0} \quad (1)$$

$$\mathrm{M}^{m+} + qL \rightleftarrows [\mathrm{M(L)}_g]^{m+} \quad (2)$$

where $\mathrm{M}^{(m+1)+}$ and M^{m+} are the metal ion in oxidation states $(m + 1)+$ and $m+$, L is the complex ligand, q is the number of coordinated ligands, $\mathrm{R_0}$ and $\mathrm{R_R}$ are the organic redox reagent in its oxidized and reduced forms, respectively, n is the number of exchanged electrons and $[\mathrm{M(L)}_q]^{m+}$ is the coloured complex.

This combined redox–complexation reaction has been applied previously in the absorption spectrophotometry of the antypyrine compounds[1,2].

EXPERIMENTAL AND RESULTS

Reagents and materials

Aqueous solutions of 1-phenyl-2,3-dimethylpyrazolone-5 (antipyrine), 1-phenyl-2,3-dimethyl-4-dimethylaminopyrazolone-5 (pyramidon), and sodium 1-phenyl-2,3-dimethyl-5-pyrazolone-4-methylaminomethanesulphonate (novalgin, dipyrone) were prepared at concentrations of 1, 0.5, 0.25, 0.01, 0.0025 and 0.0005 mg/cm^3.

A 0.05 *M* solution of 1,10-phenanthroline in 0.15 *N* sulphuric acid, a 0.05 *M* solution of 2,2′-dipyridyl in 0.2 *N* hydrochloric acid, a saturated ethanolic solution of 2,2′-diquinolyl, a 1% aqueous solution of disodium 2,2′-dicinchonate and a 0.05% aqueous solution of arsenazo III were prepared.

A Ce(IV) solution containing 1 mg/cm^3 of cerium, an Fe(III) solution containing 5 mg/cm^3 of iron and a Cu(II) solution containing 12.5 mg/cm^3 of copper were used.

0021-9673/81/0000–0000/$02.50

Other reagents and organic solvents were of analytical-reagent grade.

Glass thin-layer chromatographic (TLC) plates covered with silica gel 60 (without a fluorescent agent) were obtained from Merck (Darmstadt, G.F.R.).

Detection of antipyrine and its derivatives after chromatographic separation

Our aim was to obtain the best detection effects with the above group of compounds in terms of the highest colour contrast between the chromatographic spots and their background. The best results were obtained with the following concentrations of detection agents: (1) Ce(IV) solution, 1 mg/cm^3; arsenazo III solution, 0.05%; (2) Fe(III) solution, 1 mg/cm^3; 1,10-phenanthroline and 2,2′-dipyridyl solutions, 0.05 *M*; and (3) Cu(II) solution, 12.5 mg/cm^3; saturated ethanolic solution of 2,2′-diquinolyl and 1% solution of disodium 2,2′-dicinchonate.

It was established that drying of the chromatograms in a thermostatic chamber (80°C) after each spraying with a detection agent accelerated the colour reaction and increased the colour intensity of the spots.

Detection limits for antipyrine, pyramidone and novalgin

The efficiency of the applied systems as detection agents for antipyrine and its

TABLE I

"DETECTION INDICES" FOR ANTIPYRINE AND ITS DERIVATIVES

Detection agent	*Sample compound*	*(μg) Detection limit*	*Mean surface area of chromatographic spot (n = 6) (mm²)*	*"Detection index"* (μg/mm²)*
Fe(III)–1,10-phenanthroline	Antipyrine	–	–	–
	Pyramidone	0.025	3.7	0.007
	Novalgin	0.05	4.2	0.012
Fe(III)–2,2′-dipyridyl	Antipyrine	–	–	–
	Pyramidone	0.005	4.1	0.001
	Novalgin	0.05	4.0	0.012
Cu(II)–2,2-diquinolyl	Antipyrine	–	–	–
	Pyramidone	0.005	4.2	0.001
	Novalgin	0.005	4.0	0.001
Cu(II)–Na_2DC**	Antipyrine	–	–	–
	Pyramidone	0.05	3.9	0.013
	Novalgin	0.005	3.8	0.001
Ce(IV)–arsenazo III	Antipyrine	1.25	4.0	3.1
	Pyramidone	0.05	4.6	0.011
	Novalgin	0.05	4.1	0.012
Dragendorff reagent	Antipyrine	12.5	4.7	2.66
	Pyramidone	5.0	4.2	1.2
	Novalgin	50.0	4.0	12.5
UV light	Antipyrine	1.25	6.2	0.20
	Pyramidone	0.5	5.3	0.09
	Novalgin	5.0	5.8	0.86

* "Detection index" is the ratio of the smallest amount detected in micrograms with a given reagent to the area of the chromatographic spot[3].

** Na_2DC = disodium 2,2′-dicinchonate.

derivatives was established by comparing the detection limits with those obtained using Dragendorff's reagent and examination under UV light. Volumes of 5 μl of the sample solutions were applied to the TLC plates, which were placed in chromatographic chambers and developed by means of the ascending technique to a distance of 10 cm with chloroform–acetone (1:1) as the mobile phase[4]. After drying, the chromatograms were treated with the detection reagents. The results obtained are shown in Table I.

CONCLUSION

The detection agents examined produced high permanence and good colour contrast with antipyrine and its derivatives. The most efficient were Cu(II), 2,2′-diquinolyl; Fe(III), 2,2′-dipyridyl; and Cu(II), disodium 2,2-dicinchonate. "Detection indices" for all the pyrine derivatives examined obtained using Dragendorff's reagent were greater than 1.0 $\mu g/mm^2$. Application of UV light gave "detection indices" in the range 0.1–0.9 $\mu g/mm^2$. The use Cu(II)–2,2′-diquinolyl as the detection agent reduced the "detection indices" to 0.001 $\mu g/mm^2$.

Hence it can be concluded that the detection agents examined give significantly lower TLC detection limits for antipyrine and its derivatives.

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CHROM. 14,127

Book Review

Theory and mathematics of chromatography, by Abdel Salam Said, Dr. Alfred Hüthig Verlag, New York, Heidelberg, 1981, 210 pp., 44 figs., price US$ 28.00, DM 65.00, ISBN 3-7785-0616-1.

This book starts with very elementary material (the solution of linear equations with determinants, logarithms) then progresses to simple differential calculus, a side excursion into optimum and definite values and subsequently to integrals, Hermitian polynomials, etc. It covers in a few pages that which would generally require a student two or three years of study. Altogether, the impression gained is of a hodge-podge obtained from existing text-books, sometimes without full comprehension. Thus, the important Poisson distribution function, φ_n^*, is introduced followed by the Poisson summation distribution, P_n^*, without a statement that the latter is simply the integral of the former. Another puzzle is the equation between (270) and (271) on p. 90: I could not understand how the term $(\varphi_n^*)^2$ arises. Further on, the concept of the negative binomial distribution is introduced, but its relevance to chromatography is not demonstrated and is doubtful.

I was curious to see how my basic capillary chromatography equation (543) was treated. It appears in the section on rate theory, where it does not particularly fit, but no mention of its derivation and meaning are given, and it contains an error, leaving the reader completely at sea.

This book is far from being a text-book. The only part which I found of interest was the solution to the differential equation in (117), with which I was not familiar.

Lausanne (Switzerland) MARCEL GOLAY

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Book Review

Introduction to analytical gas chromatography: History, principles and practice (*Chromatographic Science Series*, Vol. 14), by J. A. Perry, Marcel Dekker, New York, Basel, 1981, XX + 426 pp., price SFr. 74.00, ISBN 0-8247-1537-3.

Based on his long-term experience of giving an evening course in gas chromatography to working chemists having no previous training in this field, the author attempts to present the uninitiated audience ("readers thoroughly ill at ease with mathematics and electrical circuitry") with a tool that facilitates a quick orientation and subsequently provides a deeper insight into the practice and principles of chromatography. Clarity and easy readability are prominent features throughout the text. After having read the first five chapters, the reader should already be equipped for a reasonably efficient start in practical work. It is somewhat unusual that column performance is the first of the topics dealt with; the factors causing peak broadening are discussed before the mechanisms of solute retention. Also, neither the plate nor the rate theory of the chromatographic process is presented; a price which must inevitably be paid for keeping the mathematics involved on an elementary level.

Several highlights of the book should be mentioned. The derivation of and comments on the Van Deemter equation which point out its practical consequences are very useful as is the convincing demonstration of the prevalent importance of column efficiency over solvent selectivity for complex mixtures, presented in the chapter on stationary phase characterization and selection. (There is, however, a slip here: in Table 5.2 squalane is incorrectly classified as a cycloparaffin.) An excellent review of chromatographic supports is completed with lovely micrographs of various Chromosorb types. Advanced techniques in qualitative and quantitative analysis are also presented; gas chromatography–infrared spectroscopy and gas chromatography–mass spectrometry coupling are discussed in some detail as are the problems of data processing with computers.

Each chapter is concluded by a judiciously selected list of references which the reader will find to be a very useful guide to chromatographic literature.

As a whole, then, this book should prove to be a valuable aid not only for beginners but for experienced chromatographers as well.

Lausanne (Switzerland) D. FRITZ

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Book Review

Analytical chemistry of polycyclic aromatic compounds, by M. L. Lee, M. V. Novotny and K. D. Bartle, Academic Press, New York, London, Toronto, Sydney, San Francisco, 1981, XII + 462 pp., price US$ 60.00, ISBN 0-12-440840-0.

This is an excellent book which surveys an important field of environmental analytical chemistry in a thorough fashion. However, reading it made me aware of the following problem, which the book (unfortunately) does not deal with.

Polycyclic aromatic compounds (PAC) are being determined because they cause cancer. On p. 68 we are told, "Unlike many other carcinogens that are polar and can bind directly to biological molecules, PAC need metabolic activation". We are then told that the "ultimate carcinogens" are probably hydroxylated compounds, *e.g.*, in the case of benzo[*a*]pyrene especially dihydrols. Now, everybody who has worked with benzo[*a*]pyrene (like myself) will have noticed that the compound is unstable in solution even at room temperature and hence presumably also in smoked meat, atmospheric dust, etc., and yields precisely these dihydrols. Why, then, do chemists determine only benzo[*a*]pyrene and not the various hydroxylated derivatives? If one looks through the book one finds numerous data for benzo[*a*]pyrene (gas chromatographic retention values, etc.) but not a single value or method for the hydroxylated derivatives. I would not be surprised if in some environmental samples all of the benzo[*a*]pyrene has already decomposed and the "ultimate carcinogens" are still present.

A minor comment: on p. 134 we are told, "Disadvantages of paper chromatography include long separation times, irreproducible papers, often inadequate resolution and poor quantification". This is true for some paper chromatographic methods, but is also true for some column and gas chromatographic methods. It is certainly not true for all paper chromatographic methods. If the authors had worked with Tarbell's system (their ref. 79) they would not have made this statement. It is fast, I found it highly reproducible and it gives excellent resolution.

Lausanne (Switzerland) MICHAEL LEDERER

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CHROM. 14,187

Book Review

Fats and oils: Chemistry and technology, edited by R. J. Hamilton and A. Bhati, Applied Science Publ., London, 1981, XII + 255 pp., 53 figs., price £24.00, ISBN 0-85334-915-0.

This book is based on the symposium *Recent Advances in Chemistry and Technology of Fats and Oils*, held in December, 1979. There are eleven papers on subjects ranging from synthesis of glycerides to protected lipids in animal feeds. The chapters vary widely in their treatment: some are detailed and comprehensive, others simply introduce the topic. Nevertheless, taken together, they represent useful, up-to-date statements on a number of aspects of oils and fats which are currently of importance.

The chapter on analytical techniques gives a brief but useful over-view in a rapidly changing area which will encourage the reader to seek more detail elsewhere. A chapter on the application of wide-line nuclear magnetic resonance to the determination of solids content of fats gives a detailed and authoritative account of the basis and application of a technique which is of growing importance. The section on processing gives one of the best presented and illustrated brief accounts of the subject I have seen. Glyceride synthesis is dealt with in considerable detail with copious references. It deals with the effect of acyl migration in a clear manner. This chapter is essential reading for those who need to prepare pure glycerides or those concerned with the purity of these materials in their work.

The remainder of the book provides short but authoritative reviews of prostaglandins and naturally occurring oxyacids. There are also contributions on some selected applications of fats, particularly protected feeds for ruminants and confectionery fats. The latter serves to point to the need for a more extended view of this important subject.

Overall the book contains a lot of useful material for the oils and fats scientist and technologist. It is in no way comprehensive. The quality and depth of the various aspects is variable. The title of the book is, however, misleading and one's expectations differ from the content and arrangement one finds. Few people will find all the contents useful but it is a very useful addition to the bookshelf and in certain cases it is strongly recommended reading.

Sharnbrook (Great Britain) A. CROSSLEY

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CHROM. 14,218

Book Review

Connective tissue research: Chemistry, biology and physiology (Proc. VII Eur. Connective Tissue Club Meeting, Prague, September 8–11, 1980), edited by Z. Deyl and M. Adam, Alan R. Liss, New York (Heyden & Son), 1981, X + 251 pp., price £ 16.40, DM 72.00, ISBN 0-8451-0054-8.

This volume contains the plenary talks and introductions to workshops of the meeting directly reproduced from the manuscripts. The main field of interest in these twenty papers is of course that of collagen structure and properties. However, the chapter on chromatographic and electrophoretic methods for collagen separation may be of general interest to the chromatographer.

The production of the volume is good; all the papers are legible and the figures clear and what is also rather surprising is that it appeared quickly.

Lausanne (Switzerland) M. JANOVSKY

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CHROM. 14,211

Book Review

Katalytische Methoden in der Spurenanalyse, by H. Müller, M. Otto and G. Werner, Akademische Verlagsgesellschaft Geest & Portig K.-G., Leipzig, 1980, 168 pp., 50 figs., 28 tables, price M 45.00.

Catalytic methods have been used in flat-bed chromatography for some time, the most frequent method being the detection of iodoamino acids using the Ce(IV)–As(III) reaction.

Chromatographers will thus be interested in a survey of catalytic methods in general as the need for very sensitive reactions arises frequently these days. This small volume can be warmly recommended as a reference work and as a first orientation of the possibilities of catalytic methods for a specific problem. The book contains 338 references, many of which cite Russian journals; most of these references concern recent work.

Lausanne (Switzerland) M. LEDERER

PUBLICATION SCHEDULE FOR 1981

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

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Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	212/3 213/1 213/2	213/3 214/1 214/2	214/3 215 216	217 218 219/1	219/2 219/3
Chromatographic Reviews							220/1					220/2		220/3
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2	224/3	225/1	225/2	226/1	226/2

INFORMATION FOR AUTHORS

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STATISTICAL TREATMENT OF EXPERIMENTA DATA

By J.R. GREEN, *Lecturer in Computational and Statis Science, University of Liverpool, U.K. and* D. MARGERIS *Senior Lecturer in Inorganic, Physical and Industrial Chem University of Liverpool, U.K.*

PHYSICAL SCIENCES DATA 2

This book first appeared in 1977. In 1978 a revised reprin published and in response to demand, further reprints appe in 1979 and 1980. Intended for researchers wishing to an experimental data, this work will also be useful to studer statistics. Statistical methods and concepts are explained the ideas and reasoning behind statistical methodology cla Noteworthy features of the text are numerical worked exan to illustrate formal results, and the treatment of many pra topics which are often omitted from standard texts, for exa testing for outliers, stabilization of variances and polyn regression.

What the reviewers had to say:

"The index is detailed; the format is good; the presentation is clear; and no mathematics beyond calculus is assumed".
–CHOICE

"A lot of thought has gone into this book and I like it very much. It deserves a place on every laboratory bookshelf".
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1977. Reprinted 1978, 1979, 198 xiv + 382 pages US $39.25/Dfl.9 ISBN: 0-444-417

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