A high performance disposable cartridge and reusable holder make the Brownlee MPLC™ Guard Column a unique concept in LC protection.

When placed in front of expensive analytical columns, Brownlee MPLC Guard Columns filter out harmful particles and remove strongly adsorbed compounds from the sample. Result: improved performance and an extended active life for the total LC system.

Prolongs column life
By removing impurities before they reach the analytical column, Brownlee MPLC Guard Columns prolong the useful life of the column.

Lowers cost
The use of Brownlee MPLC Guard Columns can prolong the life of an analytical column about fourfold and save you about 50% in total column costs.

More efficient than pellicular guard columns
Brownlee MPLC Guard Columns employ 5μm and 10μm totally porous slurry-packed cartridges. That means that a Brownlee MPLC Guard Column withstands more injections than a pellicular guard column. A slurry-packed guard column acts like a high efficiency extension of the analytical column and does not degrade total performance.

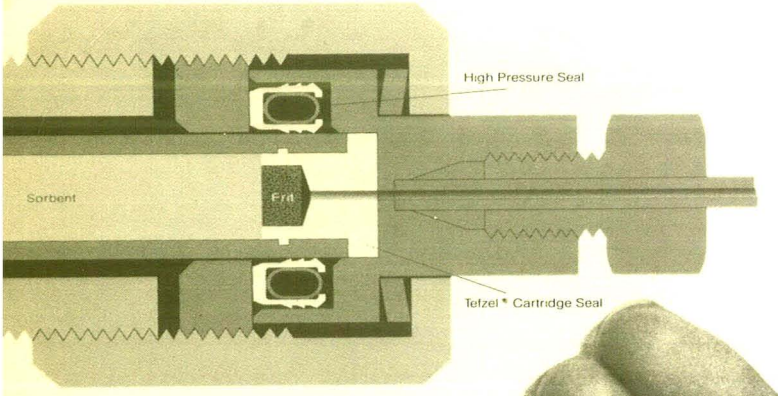
Versatile
You can choose from twenty-two different cartridges for a better match of guard column stationary phase with your analytical column.

Order from a Brownlee distributor, or from Brownlee Labs Inc 2045 Martin Ave., Santa Clara, Ca. 95050, USA. Telex 171 156. Telephone 408/727 1346.

MPLC™ Guard Columns. For economy and protection, no lab should be without them.

Circle reader service number **10**

Indispensable. MPLC Guard Column



Brownlee Labs

JOURNAL OF LIQUID CHROMATOGRAPHY

Editor: DR. JACK CAZES **Editorial Secretary:** ELEANOR CAZES
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

Aims and Scope. This journal publishes papers involving the application of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be a thin-layer chromatography and all modes of liquid chromatography.

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography* are selectively indexed or abstracted in:

- Analytical Abstracts
- Chemical Abstracts
- Current Contents/Life Sciences
- Current Contents/Physical and Chemical Sciences
- Engineering Index
- Excerpta Medica
- Physikalische Berichte
- Science Citation Index

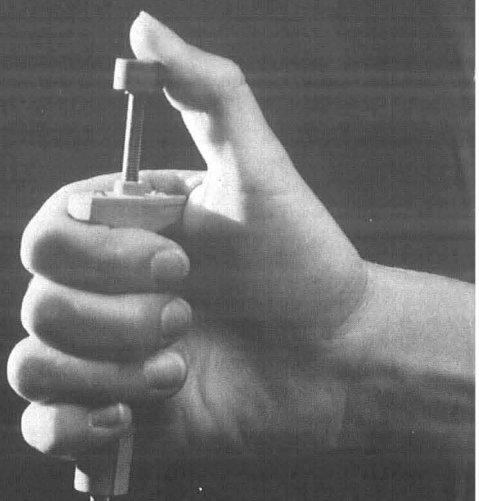
Manuscript Preparation and Submission. See the last page of this issue.

Subscription Information: *Journal of Liquid Chromatography* is published in twelve numbers and two supplements per volume by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 4 (1981), containing twelve numbers and two supplements, is $211.00 per volume (prepaid). The special discounted rate for individual professionals and students is $105.50 per volume. To secure this special rate, your order must be prepaid by personal check. Add $27.30 per volume for surface postage outside the United States. For air mail, add $49.70 per volume.

Mailing address. *Please mail payment with order to:* Marcel Dekker Journals, P.O. Box 11305, Church Street Station, New York, New York 10249.

Contributions to this journal are published free of charge.

Today you need more than technique.



A new approach to sample preparation

BOND ELUT™

BOND ELUT is a family of disposable extraction columns which simplify sample preparation of HPLC, GC, TLC, spectroscopy and RIA, yielding consistent and reproducible results.

Sample materials may be drugs in biological specimens, organic pollutants in water, etc.

The columns function by selective adsorption onto a sorbent modified with a specific chemical moiety. The character of the modificaton and the selection of the particular size/surface make this material particularly effective as a selective adsorbent.

OPERATIONAL SIMPLICI ENSURES ECONOMY AND RELIABILITY

- Typically, 10 samples can be processed in five minutes, ready for instrumental analysis.
- Extraction procedures are flexibl and can accommodate a wide range of sample volumes.
- BOND ELUT eliminates the nee or the use of sophisticated processing instrumentation.
- Because elution solvent volume low, evaporation presents little o no problem.
- There is no preconditioning time problem as in resin columns.
- BOND ELUT offers hig adsorptive surface an capacity utilizing sma diameter, rigid particle without incurring high pressure requirement

Analytichem International
24201 FRAMPTON AVEN
HARBOR CITY, CA 9071
(213) 539-6490

Circle Reader Service Card No. 110

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 4, Number 10, 1981

CONTENTS

Circle Reader Service Card No. 108

SALES AND PURCHASING COORDINATOR

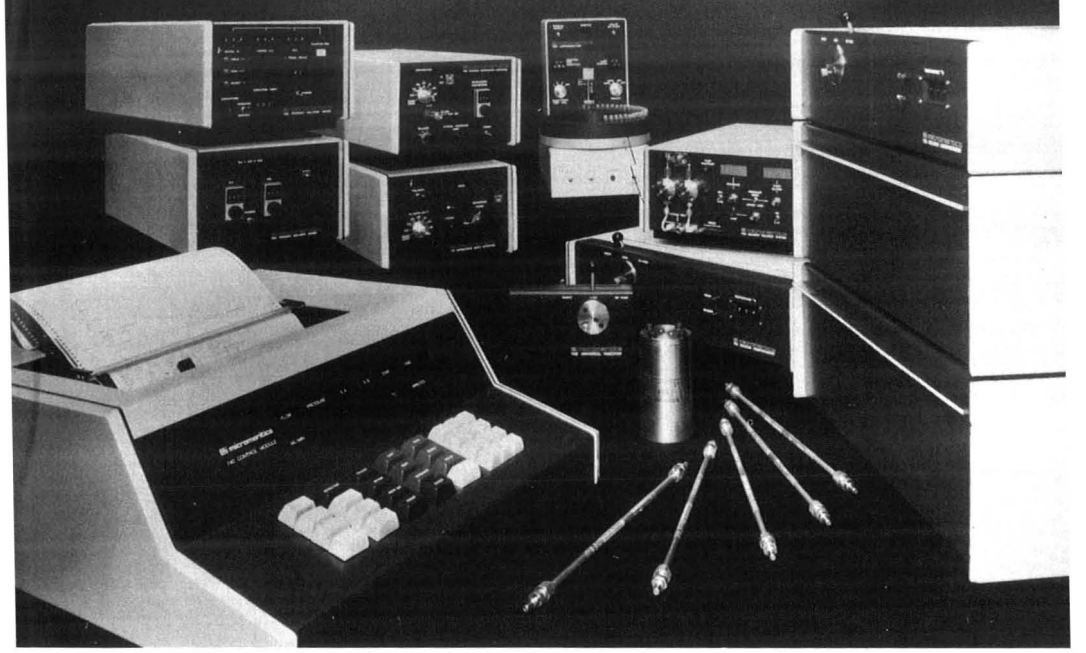
We are Britain's largest supplier of chromatography materials and are expanding our existing U. S. facility in New York.

We need a person with a sound chromatography background, preferably liquid chromatography, with the desire and ability to acquire the commercial skills needed to communicate with customers on a technical and commercial basis and control the purchase and shipment of goods to the United Kingdom for the parent company.

Located in Hauppage, New York, this job offers a challenging opportunity to someone seeking to join a small expanding company.

Reply in confidence to General Manager, Phase Separations, Ltd., 255 Oser Avenue, Hauppage, New York, 11788.

Making Liquid Chromatography Better For You



Whether your separation requirements are for quantitative analysis, purification or identification, Micromeritics can offer you state-of-the-art precision, versatility and sophistication. We offer a complete line of superior HPLC instruments including:

- Pumps and Injectors
- Automatic Injectors
- Ternary Solvent Delivery Systems
- Detectors
- Microcomputer Based Control Modules
- Heated Column Compartment/ Injectors
- Totally Integrated Systems
- Accessories (columns, column packers)

These instruments help make HPLC easier and more precise for you. We have taken the most advanced features available today and packaged them in both individual component and integrated systems. Choose from manual and automatic options. We offer everything from simple isocratic to completely automated systems. We also provide extensive application support including the development of new separation procedures. Fast and professional service is provided worldwide.

For a *free* short form catalog, write:

Micromeritics Instrument Corp.
5680 Goshen Springs Road,
Norcross, Georgia 30093 USA.
Telephone: (404) 448-8282.

micromeritics®

Making Liquid Chromatography Better For You

Circle Reader Service Card No. 104

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1685-1696 (1981)

PROBLEMS IN DETERMINING COMPOSITIONAL HETEROGENEITY OF COPOLYMERS BY SIZE-EXCLUSION CHROMATOGRAPHY AND UV-RI DETECTION SYSTEM

Sadao Mori and Tooru Suzuki
Department of Industrial Chemistry, Faculty of Engineering,
Mie University, Tsu, Mie 514, Japan

ABSTRACT

The compositional heterogeneity of styrene-methylmethacrylate copolymer has been evaluated by a UV-RI dual-detector method and by a RI detector-pyrolysis-gas chromatographic method and problems associated with the determination of the chemical heterogeneity by a UV-RI method are discussed. The quotient of signals H(UV)/H(RI) of both the detectors (UV and RI) for polystyrene increased at the extreme parts of the molecular weight distribution owing to non-linearity of UV response. The correction of the quotient of signals of both detectors for the copolymer was proposed. The UV and RI response factors for the copolymer were supposed to differ from those for homopolymers. Reproducibility for the detector responses influences the values of chemical heterogeneity much more than expected. To overcome these problems, the detection method to obtain each composition independently using different detectors will be preferable.

INTRODUCTION

It is well known that copolymer properties are affected by its composition in addition to molecular weight. Most copolymers may have the molecular weight distribution in addition to the composition distribution. In order to obtain precise values of these distributions, one must determine the molecular weight distribution independently of composition, followed by determination of the relation between composition and molecular weight, or

inversely, the composition distribution must be determined independently of molecular weight, followed by determination of molecular weight at each composition. Several attempts have been made by size-exclusion chromatography (SEC, or GPC) to obtain the relationship between molecular weight and composition. However, in SEC, elution order is dependent on molecular size rather than molecular weight, and molecules of different compositons might have the identical molecular size. Therefore, one can not obtain the precise molecular weight distribution and composition distribution for copolymers by SEC. The measurement of compositional heterogeneity of copolymers by SEC has nevertheless significance under the careful consideration, because SEC is the simplest technique to separate polymers by their molecular sizes.

The use of dual detectors or spectrophotometric detection with dual wave-lengths for analysis of SEC eluents gives information about compositional heterogeneity. A combination of ultraviolet (UV) and differential refractive index (RI) detectors is frequently used, which is applied to the copolymer one component of which has UV absorption, such as styrene-butadiene copolymer (1-3). A combination of RI and infra-red (IR) detectors, where RI is used as a concentration monitor and one component of the copolymer is determined by IR, is applied to the copolymer both components of which have or do not have UV absorption, such as vinyl chloride-vinyl acetate copolymer (4). Selection of two appropriate wave-lengths in IR enables determination of heterogeneity by an IR detector alone (5). Pyrolysis gas chromato - graphy (PGC) has been applied to determine composition of SEC fractions using RI as a concentration monitor (6).

Both UV and RI detectors are estimated to be stable and reliable instruments. The use of a UV-RI dual-detector system, therefore, has been regarded as a precise technique for the determination of compositional heterogeneity of copolymers. However, inconsistency of the compositional heterogeneity at the extreme parts of the molecular weight distribution has been observed in the results obtained by a UV-RI dual-detector method and by a RI

detector-PGC method in our laboratory. In this paper, the compositional heterogeneity of styrene-methylmethacrylate copolymer has been evaluated by a UV-RI dual-detector method and by a RI detector -PGC method, and problems associated with the determination of the chemical heterogeneity by a UV-RI dual-detector method are discussed.

EXPERIMENTAL

Apparatus

A Model LC-08 high-speed preparative liquid chromatograph (Japan Analytical Industry Co., Mizuho-cho, Tokyo 190-12, Japan) equipped with UV(254 nm) and RI detectors and two Shodex A80M HP SEC columns (500 x 8 mm i.d.) packed with polystyrene gels used for polymer fractionation was used for SEC. Chloroform was used as solvent (the mobile phase), The same apparatus was used for PGC as reported previously (6).

Samples

Styrene-methylmethacrylate copolymers (PSM) were prepared in our laboratory by solution polymerization in benzene using α,α'-azo-bis-isobutyronitrile as an initiator. The composition of the copolymers was determined using as ultra-violet spectrophotometer. A PSM having styrene content 48.6 wt% was used as a sample for the determination of compositional heterogeneity by SEC. A polystyrene sample prepared in our laboratory was used as a reference material. Several polystyrenes of known molecular weights and of narrow molecular weight distributions were used for calibration of SEC columns.

Measurement of SEC

Flow rate of the mobile phase was 1.0 ml/min. Concentration of sample solutions was 0.2% for fractionation of PSM and 0.1% for calibration. A 0.5 ml portion of the solutions was injected into the liquid chromatograph. Attenuation of RI was x8 (8×10^{-5} RI unit full scale) and UV x4 (0.04 O.D. full scale). Both chromatograms from UV and RI detectors were used for calculations of compositional heterogeneity and molecular weight distribution.

Twenty-two 0.5-ml fractions were collected over the elution range of the sample PSM.

Distributions of composition and molecular weight

Step 1. Chromatograms from UV and RI detectors were divided into equal parts every 0.25 ml and height (response) at each point i was measured. As UV and RI detectors were connected in series in this order, the dead volume was measured by the elution difference of the peak maximum of several monodisperse samples and it was estimated to be 0.2 ml: the elution volume of a molecule in a RI detector is the elution volume of the same molecule in a UV detector plus 0.2 ml.

Step 2. The ratio, R_j, of responses of UV and RI detectors at elution point j was calculated using

$$R_j = (A_{UV})_j \,/\, (A_{RI})_j \qquad (1)$$

$(A_{UV})_j$ and $(A_{RI})_j$ were calculated using next equation

$$A_j = (H_{i-1} + 2H_i + H_{i+1}) \,/\, 8 \qquad (2)$$

where H_i is the height of the chromatogram (UV or RI) at elution point i. The subscript j corresponds to the elution point i of even number (i.e., j = i (even number)/2). When chart speed of recorder is 1cm/min and the dimension of H is cm, then A_j represents the area (unit:cm^2) of the chromatogram surrounded by the points i-1 and i+1.

Step 3. Styrene weight fraction, W_s, at elution point j was calculated using

$$(W_s)_j = \frac{K_M R_j}{K_s' - (K_s - K_M) R_j} \qquad (3)$$

where K_s, K_s' and K_M are response factors of RI and UV for styrene and RI for methylmethacrylate (MMA) determined from homopolymers or copolymers by using the next equation

$$\Sigma A_j = K \cdot G \qquad (4)$$

where G is weight of polymer injected. Dimension of K is cm^2/mg at attenuations x1 (RI) and x0.01AUFS (UV).

Step 4. The RI response of the SEC chromatogram of the copolymer sample was corrected using

$$(H_{corr.})_j = (H_{uncorr.})_j \ (2.29 - 1.29(W_s)_j) \qquad (5)$$

The RI response of PS is 2.29 times that of PMMA.

Step 5. Molecular weight of the copolymer at the elution point j was calculated using

$$(M_c)_j = 0.806(M_s)_j + 0.194(m_s)_j \ (M_s)_j \qquad (6)$$

where $(M_s)_j$ is the molecular weight of polystyrene obtained from the polystyrene calibration curve at the elution point j and $(m_s)_j$ is the styrene mole fraction at the point j. This equation was obtained as follows. The equation 11 in the literature (4) was converted into the equation

$$M_{copolymer} = m_A M_A + m_B M_B \qquad (7)$$

where subscripts A and B mean components A and B in the copolymer. A ratio of the molecular weight of a PMMA obtained by a classical method to that calculated using the polystyrene calibration curve was 0.806, then

$$(M_{MMA})_j = 0.806 \ (M_s)_j \qquad (8)$$

Replacing A and B with MMA and S and substituting eq. (8) into eq.(7), we obtain the equation (6). Average molecular weights of the copolymer were calculated using $(M_c)_j$ in step 5 and $(H_{corr.})_j$ in step 4.

Step 6. The differential molecular weight distribution curve was constructed after converting the values of dW/dV of the normalized SEC chromatogram into those of dW/d log M using the copolymer calibration curve which was constructed using eq. (6).

PGC of the fractions

The stainless-steel column (300 cm x 3 mm i.d.) was packed with Diasolid L coated with 2.5% PEG6000. The procedures for construction of the calibration curve and pyrolysis of the fractions were the same as reported previously (6), except column

temperature being 110 °C and pyrolysis temperature 600 °C. About 10 μg of the copolymer from each fraction were pyrolyzed.

RESULTS AND DISCUSSION

The differential molecular weight distribution curve and the chemical heterogeneity of PSM obtained and calculated from SEC chromatogram through step 1 to step 6 and the chemical heterogeneity of PSM obtained by PGC are shown in Figure 1. Styrene weight fraction obtained by a UV-RI dual-detector method increases at the extreme parts of the molecular weight distribution curve. The situation is quite different for PGC method, where the copolymer seems to have a uniform composition over the whole range of molecular weights.

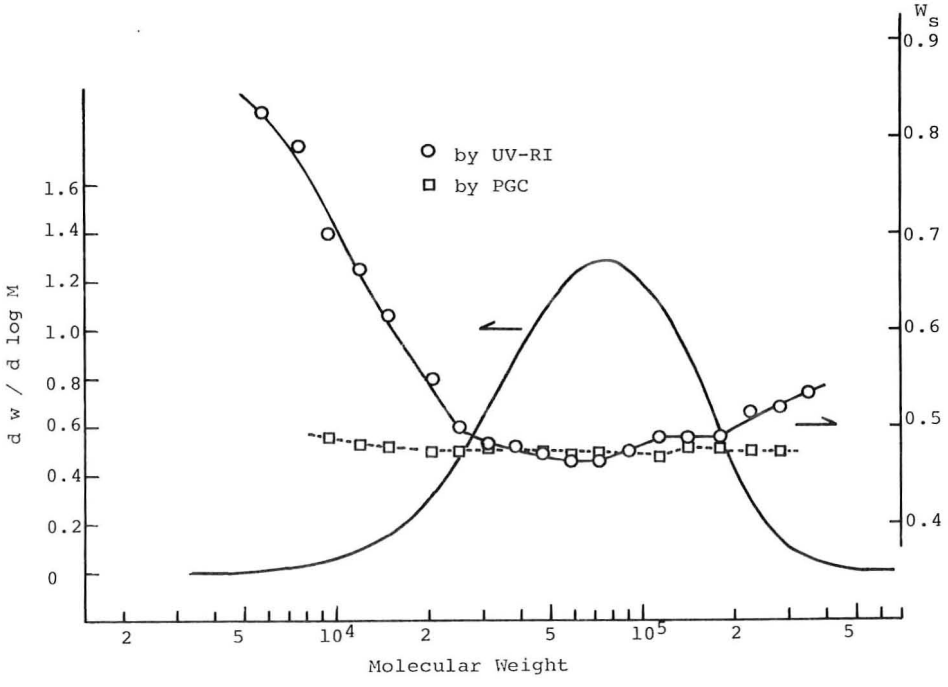


FIGURE 1. The molecular weight distribution and chemical heterogeneity of a styrene-methylmethacrylate copolymer

Response factors K_s, K_M and K_s' are assumed to be constant, whereas the value of R_j will vary with the composition: that is, if R_j is constant for all elution volumes, then, the copolymer will have a uniform composition. Since polystyrene is homopolymer with a constant composition, the quotient of signals A_{UV}/A_{RI} (=R) of both the detectors should be constant for all elution volumes. In practice, however, the result is completely different. Figure 2 shows the SEC chromatograms (RI and UV) of a polystyrene sample ($\bar{M}_w$ = 73000, $\bar{M}_n$ = 36000) and a plot of R_j against the elution volume. A dead volume (0.2 ml) was corrected on the chromatogram measured by an RI detector.

The variation of R_j against the elution volume as in Figure 2 suggests that there is no linear relation between the concentration of a solute and a response of both RI and UV detectors or either of the two. In general, responses of RI and UV detectors are to be proportional to the concentration of a solute in a dilute solution. However, in our laboratory, a next equation was obtained for a polystyrene solution in the range of 0.001 to 0.2 mg solute/ml chloroform and of 0.00075 to 0.085 absorbance at 275 nm of a UV detector

$$\log C = 1.116 \log A + 0.483 \qquad (9)$$

where C is the concentration of a polystyrene and A is absorbance. If the response of an RI detector is proportional to the concentration of a solute (this will be checked hereafter), it can be estimated using eq. (9) that the value R_j increases with decreasing the concentration. Therefore, one of the solution to the problem that the value R_j for polystyrene varies with the concentration will be to use the concentration rather than the height in eq. (2).

The other solution to the problem is to correct the value R_j in eq. (1) for a copolymer multipling by a correction factor which was obtained by dividing the average of R_j (= 0.931 in Figure 2) by the value of R_j at each elution volume for a polystyrene sample. This correction factors are shown in Figure 2. Improvement by

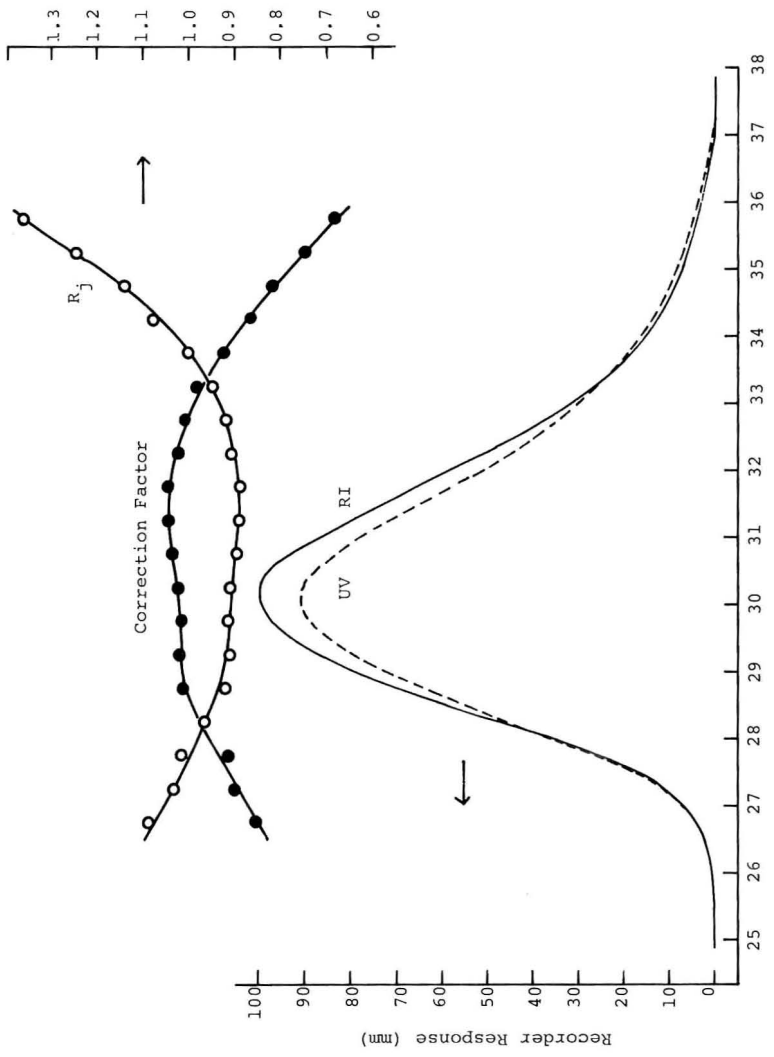


FIGURE 2. SEC chromatograms and plots of R_j and correction factors against the elution volume for a polystyrene sample ($\bar{M}_w$ = 73000, $\bar{M}_n$ = 36000).

correction was observed and the result is shown in Figure 3 with the uncorrected values and the results by PGC for comparison.

One of the reason for the variation of R_j with concentration will be explained by eq. (9). The other reason has been shown by Bressau (7) that the connecting tube disturbs not only by shifting the elution curve, but also by altering its shape, resulting in the deformation of the elution curves and the variation of the quotient curve of signals H_{RI}/H_{UV} of both the detectors. He recommended the use of columns with a large diameter for the multiple detection of narrowly distributed polymer samples. Stojanov and his co-workers (3) have not observed such variation of R_j for polystyrene.

The second problem in the UV-RI method lies down on the determination of response factors in eq. (3). The UV response factor K_s', which is determined using homopolymer, fluctuates slightly with polymers obtained by different synthetic processes and their molecular weights. Table 1 shows UV absorbances of

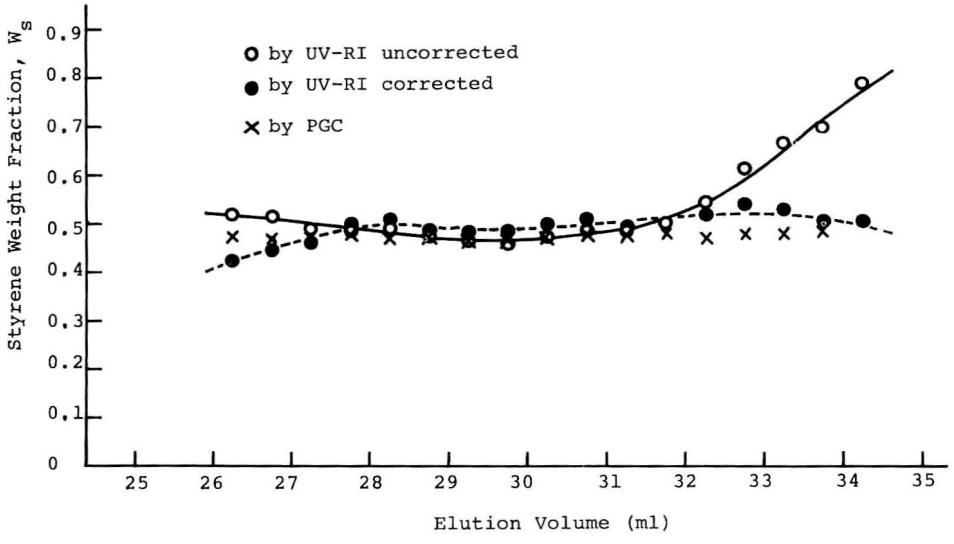


FIGURE 3. Effect of correction of the value R_j to the determination of the chemical heterogeneity

Table 1
Absorbance of Several Polystyrenes in Concentration 1 mg Sample per 10 g Chloroform

Polystyrene	253.7 nm	260 nm
PS 97200	0.260	0.327
PS 20400	0.260	0.326
PS 10000	0.259	0.324
PS 3600	0.259	0.324
PS 2100	0.255	0.319
PS 600	0.246	0.308
S - 5	0.311	0.361
PSt - 1	0.278	0.336
PSt - 2	0.282	0.336

several polystyrenes in solutions in concentration 1 mg polystyrene per 10 g chloroform and in a 1 cm-cell. Polystyrenes from PS97200 to PS600 were synthesized by living polymerization and have narrow molecular weight distributions. Numbers after PS refer to molecular weights. Three polystyrenes from the bottom in Table 1 were synthesized by radical polymerization. Polystyrene S-5 has molecular weight 1×10^4 and the other two have 1×10^5 to 2×10^5. Absorbances in Table 1 correspond to $K_s' = 40$ for PS97200, 42.8 for PS-1, and 47.8 for S-5.

The RI response factor K_c for copolymer can be calculated using eq. (4) and is related to K_s, K_M and W_s as follows

$$K_c = K_M + (K_s - K_M) W_s \qquad (10)$$

Stojanov and his co-workers (8) observed this equation to be valid. However, as shown in Figure 4, some of observed values deviate from the calculated straight line of eq. (10) and the deviation exceeds the standard deviation.

Styrene weight fraction W_s at each elution volume, calculated using eq. (3) and response factors obtained from homopolymers, was about 5% higher than that in Figure 1 and the average styrene weight fraction was 0.540 (the value for unfractionated sample was 0.486). The values in Figure 1 were calculated using response

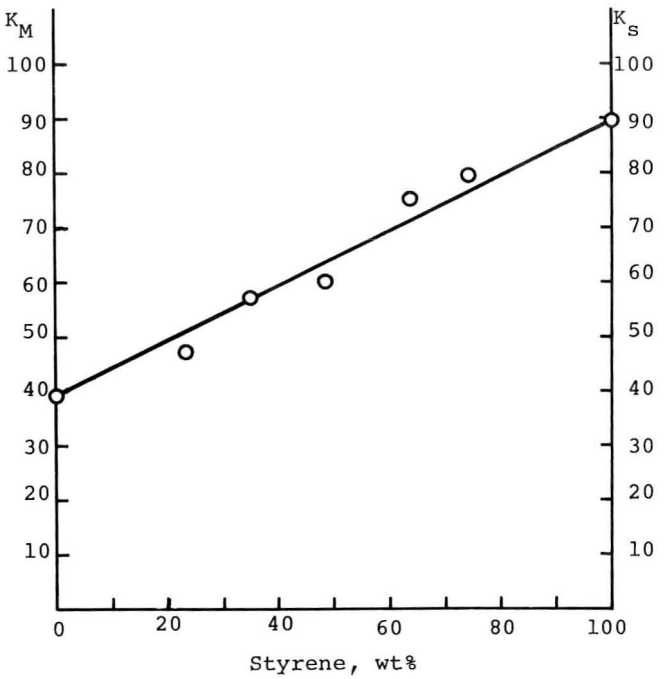


FIGURE 4. Plot of the RI response factor on chemical composition. White circles are observed values.

factors obtained from the copolymer sample by knowing the copolymer composition, the ratio of RI responses for styrene and methyl methacrylate, and UV and RI chromatograms, and the average value of the styrene weight fractions coincideswith the unfractionated value. In case of the sample (W_S = 0.637) in Figure 4, the average value of styrene weight fractions was about 5% lower than that the unfractionated value when response factors from homopolymers were used in eq. (3). Besides these problems, as the equation (3) includes the three response factors and the ration of RI and UV responses, errors from these values are incorporated into the calculated value.

Reproducibility for the detector responses deserves also much attention. Repeatability for the detector responses in the same

day was very good and usually less than 1% as the standard deviation, but reproducibility at the different day went sometimes beyond 5%. Application of eq. (3) will make this problem more serious.

REFERENCES

1. Runyon, J.R., Barnes, D.E., Rudd, J.F., and Tung, L.H., Multiple Detectors for Molecular Weight and Composition Analysis of Copolymers by Gel Permeation Chromatography, J. Appl. Polym. Sci., 13, 2359 (1969).

2. Adams, H.E., Composition of Butadiene-Styrene Copolymers by Gel Permeation Chromatography, Separ. Sci., 6, 259 (1971).

3. Stojanov, Ch., Shirazi, Z.H., and Audu,,T.O.K., Determination of Chemical Heterogeneity of Copolymers by Gel-Permeation Chromatography, Chromatographia, 11, 63 (1978).

4. Mori, S., Determination of the Composition and Molecular-Weight Distribution of a Poly(vinyl chloride-vinyl acetate) Copolymer by Gel-Permeation Chromatography and Infrared Spectroscopy, J. Chromatogr., 157, 75 (1978).

5. Mirabella, F.M., Jr., Barrall, E.M., II, and Johnson, J.F., A Rapid Technique for Measuring Copolymer Composition as a Function of Molecular Weight using GPC and IR, J. Appl. Polym. Sci., 19, 2131 (1975).

6. Mori, S., Determination of the Composition of Copolymers as a Function of Molecular Weight by Pyrolysis Gas Chromatography - Size-Exclusion Chromatography, J. Chromatogr., 194, 163 (1980).

7. Bressau, R., Liquid Chromatography of Polymers and Related Materials II, J. Cazes, ed., Marcel Dekker, New York, 1980, P. 73.

8. Stojanov, Ch., Shirazi, Z.H., and Audu, T.O.K., Contribution to the Determination of Chemical Composition of Copolymers by Gel Permeation Chromatography, Chromatographia, 11, 274 (1978).

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1697-1707 (1981)

SIZE EXCLUSION CHROMATOGRAPHY (SEC) OF COMPLEX POLYMERS - GENERALIZED ANALYTICAL CORRECTIONS FOR IMPERFECT RESOLUTION

A.E. Hamielec,
McMaster University, Hamilton, Canada.
H.J. Ederer and K.H. Ebert,
University of Heidelberg, Heidelberg, Germany.

ABSTRACT

Herein is reported generalized analytical solutions which permit correction for imperfect resolution when the molecular weight calibration curve is nonlinear and the variance of single-species chromatograms changes significantly with molecular size of the polymer solute. Two kinds of generalized analytical solutions have been obtained. One is a solution of Tung's integral equation for the corrected chromatogram or the molecular weight distribution and the other is a solution for the corrected molecular weight averages of the whole polymer. Also discussed is the use of local corrections for imperfect resolution across the chromatogram with detectors such as the low angle laser light scattering spectrophotometer (LALLS) when used with micro and macropackings.

INTRODUCTION

To date, analytical solutions for Tung's integral equation for the corrected chromatogram have not been reported. Many numerical techniques have been proposed and evaluated and the better of these include the iterative method of Ishige et al. (1), the method of Chang and Huang (2) and more recently the method proposed by Vozka and Kubin (3). Analytical solutions for Tung's integral equation for the molecular weight averages were first published by Hamielec and Ray (4) for the case of a uniform Gaussian instrumental spreading function.

Provder and Rosen (5) generalized these solutions to include a uniform non-Gaussian instrumental function. Both of these analytical solutions are limited to a SEC operation where the molecular weight calibration curve is linear and the variance of single-species chromatograms is independent of molecular size of the polymer solute. The solutions to be presented herein are analytical and account for non-linear molecular weight calibration curve and non-uniform instrumental spreading function with the variance of single-species chromatograms varying with molecular size of the polymer solute. The limiting factor which remains is that the instrumental spreading function is Gaussian.

THEORY

Tung's integral equation which follows is the starting point for all rigorous methods of correction for imperfect resolution.

$$F(v) = \int_0^\infty W(y)\, G(v,y)\, dy \qquad (1)$$

F(v) is the detector response or chromatogram at retention volume v. The kernel G(v,y) is called the instrumental spreading function and is the normalized detector response for a single species with mean retention volume y. W(y)dy is the area of the detector response for a single species with mean retention volume in the range, y-y+dy. W(y) is the detector response or chromatogram corrected for peak broadening. Species with the same mean retention volume may, in certain circumstances, have different molecular weights and compositions and possibly significantly different instrumental spreading functions as with complex polymers, those having variable long chain branching frequency and copolymers with variable composition. In these instances equation (1) would not be valid and a more complex form involving

multiple integrals would have to be used. Until there is experimental evidence to the contrary, it will be assumed that equation (1) is valid under all circumstances of GPC operation.

We now define a distribution function W(v,y), where W(v,y)dvdy is the area under the detector response in the retention volume range, v-v+dv due to polymer solute species with mean retention volume in the range, y-y+dy. Let us investigate the following properties of W(v,y)

$$W(v,y) = W(y)\, G(v,y) \tag{2a}$$

$$\int_0^\infty W(v,y)dy = F(v) \tag{2b}$$

$$\int_0^\infty W(v,y)dv = W(y) \tag{3}$$

It is clear from equation (3) that a knowledge of W(v,y) permits a simple direct integration to obtain W(y), the chromatogram corrected for peak broadening. At a particular retention volume, v, W(v,y) gives the distribution of polymer molecular sizes in the detector cell. A detector system which could provide a measure of W(v,y), would provide a direct instrumental means of measuring W(y). This possibility will be discussed later. We will now consider the special case where the instrumental spreading function G(v,y) is Gaussian with variance which depends on the size of the polymer solute and where the slope of the molecular weight calibration curve varies with retention volume. For this situation Tung's integral equation takes the form:

$$F(v) = \frac{1}{\sqrt{2\pi\sigma(v)^2}} \int_0^\infty W(y) \exp\left(-(v-y)^2/2\sigma(v)^2 \right) dy \tag{1a}$$

The use of $\sigma(v)^2$ rather than $\sigma(y)^2$ is an approximation which should be valid when peak broadening is not excessive. In this case,

$$W(v,y) = \frac{W(y)}{\sqrt{2\pi\sigma(v)^2}} \exp\left[-(v-y)^2/2\sigma(v)^2 \right] \quad (2c)$$

Let us now derive an expression for $\bar{M}_W(v, uc)$ which is the weight average molecular wieght of the contents of the detector cell. The LALLS detector system would measure this molecular weight average.

$$\bar{M}_W(v,uc) = \int_0^\infty M(y)\, W(v,y)dy \,/\, \int_0^\infty W(v,y)dy \quad (4)$$

where

$$M(y) = D_1(v) \exp(-D_2(v)y)$$

Local linearization is being employed to account for a nonlinear molecular wieght calibration curve. Substituting for W(v,y) and with some algebraic manipulation one obtains

$$\bar{M}_W(v,uc) = \frac{M(v)\exp\{(D_2(v)\sigma(v))^2/2\}}{F(v)\sqrt{2\pi\sigma(v)^2}} \int_0^\infty W(y)\exp -\left\{\frac{(v-D_2(v)\sigma(v)^2-y)^2}{2\sigma(v)^2}\right\}dy \quad (6)$$

Comparing the integrals in equations (1a) and (6) it is clear that

$$\frac{\bar{M}_W(v,uc)}{M(v)} = \frac{F(v-D_2(v)\sigma(v)^2)}{F(v)} \exp\{(D_2(v)\sigma(v))^2/2\} \quad (7)$$

The weight-average molecular weight of the whole polymer corrected for imperfect resolution is given by (7,8)

$$\bar{M}_W(c) = \int_0^\infty F(v)\, \bar{M}_W(v,uc)dv \,/\, \int_0^\infty F(v)dv \quad (8a)$$

$$= \int_0^\infty M(v)F(v-D_2(v)\sigma(v)^2) \exp\{(D_2(v)\sigma(v))^2/2\}dv \,/\, \int_0^\infty F(v)dv \quad (8b)$$

This is an extension of the original analytical solution after Hamielec and Ray (4) for variable variance of the single-species chromatograms

and for a nonlinear molecular weight calibration curve. In a similar manner correction equations for other molecular weight averages and intrinsic viscosity may be derived and these follow (7,8).

$$\frac{\bar{M}_K(v,uc)}{M(v)} = \frac{F\left(v-(K-1)D_2(v)\sigma(v)^2\right)}{F\left(v-(K-2)D_2(v)\sigma(v)^2\right)} \exp\{\frac{(2K-3)}{2}\left(D_2(v)\sigma(v)\right)^2\} \tag{9}$$

$$\frac{[\eta](v,uc)}{[\eta](v)} = \frac{F\left(v-aD_2(v)\sigma(v)^2\right)}{F(v)} \exp\{\left(aD_2(v)\sigma(v)\right)^2/_2\} \tag{10}$$

$$\bar{M}_K(c) = \int_0^\infty F(v)\bar{M}_K^{K-1}(v,uc)dv \;/\; \int_0^\infty F(v)\bar{M}_K^{K-2}(v,uc)dv \tag{11}$$

$$[\eta](c) = \int_0^\infty F(v)[\eta](v,uc)dv \;/\; \int_0^\infty F(v)dv \tag{12}$$

where for $K = 1$, $\bar{M}_1(c) = \bar{M}_N(c)$ and $\bar{M}_1(v,uc) = \bar{M}_N(v,uc)$

$K = 2$, $\bar{M}_2(c) = \bar{M}_W(c)$ and $\bar{M}_2(v,uc) = \bar{M}_W(v,uc)$, and so on.

Equations (9)-(12) may be used to calculate whole polymer molecular weight averages and intrinsic viscosity corrected for imperfect resolution when the variance of single-species chromatograms and the slope of the molecular weight calibration curve vary with retention volume.

We will now derive an analytical solution for W(y), the chromatogram corrected for peak broadening. We approximate W(v,y) with the form

$$W(v,y) = \frac{F(v)}{\sqrt{2\pi\bar{\sigma}(v)^2}} \exp\{-\left(y-\bar{y}(v)\right)^2 / 2\bar{\sigma}(v)^2\} \tag{13}$$

An examination of equation (2a) might suggest that the shape of W(v,y) depends on the shape of W(y). This is true up to a point. However, for a particular retention volume, v, the range of y which is significant

may only include a small portion of W(y). The corrected chromatogram is then given by

$$W(y) = \int_0^\infty \frac{F(v)\ \exp\left[-(y - \bar{y}(v))^2 / 2\bar{\sigma}(v)^2\right]}{\sqrt{2\pi\bar{\sigma}(v)^2}}\, dv \tag{14a}$$

or more directly in this case by

$$W(v) = F(v)\left[\sigma(v)/\bar{\sigma}(v)\right] \exp\left\{-(v-\bar{y}(v))^2/2\bar{\sigma}(v)^2\right\} \tag{14b}$$

A detector system which could say measure $\bar{M}_N(v,uc)$ and $\bar{M}_W(v,uc)$ would also provide a measure of $\bar{y}(v)$ and $\bar{\sigma}(v)^2$. The LALLS detector system which gives a measure of $\bar{M}_W(v,uc)$ with the additional assumption that $\bar{y}(v) = v$ would provide a measure of $\bar{\sigma}(v)^2$. This would in effect with the use of equation (14), provide a direct measure of W(y). For the case of a Gaussian instrumental spreading function it can readily be shown using equations (9) and (13) that

$$\bar{y}(v) = v + \frac{1}{D_2(v)} \ln \frac{F\left(v+D_2(v)\ \sigma(v)^2\right)}{\sqrt{F\left(v-D_2(v)\ \sigma(v)^2\right)\ F\left(v+D_2(v)\ \sigma(v)^2\right)}} \tag{15}$$

$$\bar{\sigma}(v)^2 = \sigma(v)^2 + \frac{1}{D_2^2(v)} \ln \frac{F\left(v-D_2(v)\ \sigma(v)^2\right)\ F\left(v+D_2(v)\ \sigma(v)^2\right)}{F(v)^2} \tag{16}$$

It can readily be shown with a Taylor series expansion accurate to second order that

$$\bar{y}(v) = v + \sigma(v)^2 \frac{F'(v)}{F(v)} \tag{15a}$$

$$\bar{\sigma}(v)^2 = \sigma(v)^2 + \sigma(v)^4 \left(\frac{F''(v)}{F(v)} - \left(\frac{F'(v)}{F(v)}\right)^2\right) \tag{16a}$$

The expansion properly eliminates the dependence on $D_2(v)$. For computational reasons it is easier to employ equations (15) and (16)

with any convenient value for $D_2(v)$. To solve for W(y) one must have values for $\sigma(v)^2$. These may be found by standard calibration techniques. The use of equations derived above to correct the chromatogram and whole polymer molecular weight averages will be illustrated under Results and Discussion.

We will now derive equations which will permit the investigation of the magnitude of corrections for imperfect resolution for the weight average molecular weight of the detector cell contents ($\bar{M}_W(v,uc)/M(v)$) for both micro and macropackings. Micropackings with particles of about 5-15 microns give small peak broadening (small $\sigma(v)^2$) and small peak separation (large $D_2(v)$). Macropackings with particles of about 30-60 microns give large peak broadening (large $\sigma(v)^2$) and large peak separation (small $D_2(v)$). An examination of equation (7) might suggest that the correction for imperfect resolution in the detector cell would be smaller for micropackings that for macropackings due to the $\sigma(v)^2$ term in $F(v-D_2(v)\sigma(v)^2)$. To investigate this further we will begin with a polymer sample which has the most probable distribution and then assume that it is analyzed with an SEC with micropacking and then with macropacking. The MWD of the sample is given

$$W(M) = \frac{M}{\bar{M}_N^2} \exp\{ - M/\bar{M}_N \} \tag{17}$$

with $\bar{M}_N = 2 \times 10^5$ and $\bar{M}_W = 4 \times 10^5$

For convenience we will assume that the molecular weight calibration curve is linear and given by

$$M = D_1 \exp(-D_2 v) \tag{18}$$

The associated chromatogram is given by

TABLE 1

Parameters Employed in Equations (6), (18) and (19) for Micro and Macropackings

Micropacking	Macropacking
$D_2 = 0.357\ ml^{-1}$	$D_2 = 8.62 \times 10^{-2} ml^{-1}$
$\sigma = 0.70$ ml	$\sigma = 2.90$ ml
$D_2\sigma = 0.25$	$D_2\sigma = 0.25$
$D_2\sigma^2 = 0.1750$ ml	$D_2\sigma^2 = 0.725$

$$W(v) = \frac{D_2 D_1^2}{\bar{M}_N^2} \exp(-2D_2 v) \exp\left\{ -\left(\frac{D_1}{\bar{M}_N} \right) \exp(-D_2 v) \right\} \qquad (19)$$

We will consider relatively small corrections for peak broadening and since we have a relatively broad MWD, we will set $F(v) \simeq W(v)$. The parameters in these equations employed for micro and macropackings are given in Table 1. Again for convenience it is assumed that the variance does not vary with retention volume. The results of this comparison will be given under Results and Discussion.

RESULTS AND DISCUSSION

We will begin with a discussion of corrections for imperfect resolution for the LALLS detector system and compare the magnitude of these corrections for micro and macropackings. Table 2 shows the corrections required for typical micro and macropackings. It is clear that the magnitude of the corrections are about the same for both micro and macropackings. Apparently the smaller $D_2(v)\sigma(v)^2$ obtained with micropackings are compensated for my the narrower and steeper chromato-

TABLE 2

Corrections for Imperfect Resolution with the LALLS Detector System

Micropacking			Macropacking		
v(ml)	$F(v)\times10^3$	$\bar{M}_W(v,uc)/M(v)$	v(ml)	$F(v)\times10^3$	$\bar{M}_W(v,uc)/M(v)$
27	16.0	0.745	56	3.6	0.740
28	64.0	0.853	60	14.4	0.846
29	136.3	0.937	64	31.2	0.930
30	186.6	1.002	68	44.1	0.995
31	187.7	1.055	72	46.1	1.041
32	152.1	1.084	76	38.9	1.077
33	105.9	1.109	80	28.2	1.105
34	66.4	1.127	84	18.4	1.122
35	38.6	1.139	88	11.1	1.134
36	21.3	1.153	92	6.3	1.147

gram. It is remarkable that the correction $\bar{M}_W(v,uc)/M(v)$ at the high molecular weight end of the chromatogram is as much as 25% even though the correction to the whole polymer $\bar{M}_W$ is only about 3%. In other words corrections for imperfect resolution are a must when employing the LALLS detector system if quantitative estimates of the molecular weight calibration curve M(v) are to be made.

We will now present an example of the application of equation (14) to solve for the chromatogram W(v) corrected for peak broadening for a situation where the variance of single-species chromatogram varies somewhat with retention volume (or molecular size). We will then use the same chromatograms F(v) and W(v) and use a nonlinear molecular weight calibration curve to solve for the corrected molecular weight averages. One approach is to integrate W(v) directly and the other is to use equations (9) and (11) to generate the molecular weight averages corrected for imperfect resolution.

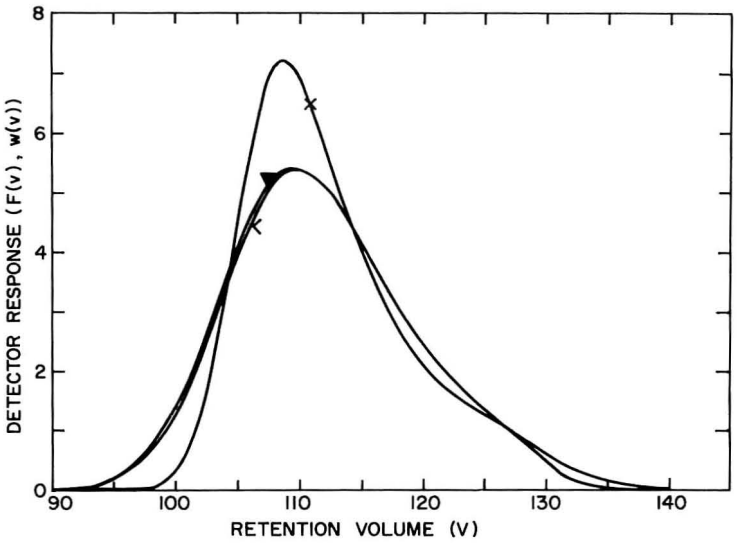


FIGURE 1. Analytical solution of Tung's Integral Equation - A Comparison of the Uncorrected Chromatogram F(v) with the Corrected Chromatogram W(v) and with W(v) Rebroadened.

		Variance (ml^2)	Retention Volume (ml)
X	W(v)		
▼	F(v)		
<	W(v) Rebroadened	16.0	90-100
		17.0	102
		18.0	104-125
		16.7	127.5
		16.2	130
		16.0	132-147

TABLE 3

Molecular Weight Averages Corrected for Imperfect Resolution

Method	$\bar{M}_N(c)$	$\bar{M}_W(c)$	$\bar{M}_Z(c)$
W(v)	1.44×10^6	1.54×10^6	1.65×10^6
Equations (9) and (11)	1.41×10^6	1.57×10^6	1.64×10^6

Figure 1 shows F(v), W(v) and W(v) rebroadened and illustrates the application of equation (14). The variances of single-species chromatograms employed are shown in the caption.

Table 3 shows the corrected molecular weight averages with the molecular weight calibration curve given by equation (20)

$$\ell n\ M = 27.43 - 0.1922v + 6.62 \times 10^{-4}\ v^2 \qquad (20)$$

The agreement between F(v) and rebroadened W(v) shown in Figure 1 is excellent confirming the applicability of equation (14) and the proposed analytical solution of Tung's integral equation. The agreement between corrected molecular weight averages shown in Table 3 also confirms the validity of the proposed method of correcting whole polymer molecular weight averages when the variance and slope of the molecular weight calibration curve vary with retention volume.

REFERENCES

1. T. Ishige, S.I. Lee and A.E. Hamielec, J. Applied Polymer Sci., 15, 1607 (1971).

2. K.S. Chang and Y.M. Huang, J. Applied Polymer Sci., 13, 1459 (1969).

3. S. Vozka and M. Kubin, J. Chromatography, 139, 225 (1977).

4. A.E. Hamielec and W.H. Ray, J. Applied Polymer Sci., 13, 1319 (1969).

5. T. Provder and E.M. Rosen, Separation Sci., 5, 437 (1970).

6. A.E. Hamielec and A.C. Ouano, J. Liquid Chromatography, 1, 111 (1978).

7. W.W. Yau, H.J. Stoklosa and D.D. Bly, J. Applied Polymer Sci., 21, 1911 (1977).

8. A.E. Hamielec, J. Liquid Chromatography, 3, 381 (1980).

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1709-1724 (1981)

IONISATION EFFECTS IN THE REVERSED PHASE LIQUID CHROMATOGRAPHIC SEPARATION OF THYROMIMETIC IODOAMINO ACIDS.*

Suew-Jane Su, Boris Grego and Milton T.W. Hearn**,†
Immunopathology Research Unit,
Medical Research Council of New Zealand,
Otago University Medical School,
P.O. Box 913, Dunedin,
New Zealand.

ABSTRACT

The influence of ionisation equilibria on the retention behaviour of iodoamino acids and related compounds on microparticulate octadecylsilica supports has been examined. The chromatographic data for these ionogenic solutes have been discussed in terms of current concepts for reversible solvophobic interactions with the hydrocarbonaceous stationary phase. This treatment permits the conditional effects of the mobile phase composition and pH on solute retention to be assessed and the relationship between the molecular surface area of a solute and its retention to a non polar stationary phase evaluated.

INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) has gained wide popularity over the past few years

---

* High Performance Liquid Chromatography of Amino Acids, Peptides and Proteins, Part XXXIV. For Part XXXIII see ref. [1].

** To whom correspondence should be addressed.

† Present address: St Vincent's School of Medical Research, Victoria Parade, Melbourne, Victoria 3065, Australia.

for the separation of ionic substances, particularly those of biological origin. The large majority of these substances contain ionogenic functional moieties such as carboxyl, amino or phosphate groups. Ion exchange chromatography has traditionally been the method of choice for the separation of such components. Recent developments in RP-HPLC have demonstrated that this technique is eminently suitable for the separation of amino acids, peptides and proteins provided due regard is placed on the role which secondary chemical equilibria can play in the chromatographic distribution process. Attention has been focused on the use of RP-HPLC with eluents containing a variety of anionic or cationic reagents in hydro-organic solvent mixtures. Excellent control over selectivity for amino acids and peptides on chemically bonded microparticulate alkylsilicas can be achieved with these reagents via specific pairing ion interactions. The more lipophilic of these pairing ion reagents are known to act as surfactants and modify the non-polar surface of the sorbent to the equivalent of a dynamic liquid-liquid ion-exchanger. It has also been demonstrated in a variety of studies that the level of solute ionisation plays a very significant role in the retention processes when ionogenic substances are chromatographed on alkylsilicas. Detailed experimental studies with the protein amino acids and small peptides have shown [1-5] that the physico-chemical basis of their chromatographic sorption-desorption processes can be interpreted in terms of solvophobic theory [6,7]. This exacting theoretical treatment allows the parameters affecting solute retention to be individually evaluated. In earlier studies we have described [8-10] methods for the separation of the thyromimetic iodoamino acids by RP-HPLC. Subsequently, these methods have been extended [9,11-13] to the analysis of the iodoamino acids in biological samples. In this paper we examine the effect of protic ionisation on the chromatographic retention of these

solutes and evaluate the chromatographic behaviour in terms of the solvophobic model for solute retention.

MATERIALS

Chromatographic Apparatus.

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which consisted of a M6000A solvent delivery unit, a U6K universal liquid chromatograph injector and a M440 UV absorbance detector coupled to a Rikadenki double channel recorder. The μBondapak C_{18} columns (10μm, 30 x 0.4cm I.D.) were purchased from Waters Assoc. (Aus.) Pty. Ltd. Sample injections were made with Pressure-Lok liquid syringes (0-25μl) Series B110 from Precision Sampling (Baton Rouge, La., U.S.A.). Solvents and dissolved iodoamino acid samples were filtered using AP2500 filters from Millipore Corp. (Bedford, Mass., U.S.A.)

Reagents.

Methanol was supplied by Waters Assoc. (Aus.) Pty. Ltd. Orthophosphoric acid and potassium dihydrogen phosphate were from May and Baker (Dagenham, Great Britain). The iodoamino and thyroacetic acids were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and from Henning (Berlin,G.F.R.). Stock solutions of the iodoamino and thyroacetic acids were prepared by dissolving the compounds in 1% methanolic NH_4OH at a concentration of ca 10mg/ml.

METHODS

A flow rate of 2ml/min was used throughout this study. The solvent reservoirs, precolumn delivery systems and columns were maintained at 18^{o}. All bulk and eluting solvents were prepared and degassed as reported previously[8]. All columns were equilibrated to new elution conditions for at least 30 min. The capacity factors were calculated in the usual way. The retention time of an unretained solute can be readily calibrated

with $NaNO_3$ or D_2O. The analysis of the data by the least squares method was performed on a Burroughs 6700 computer with a modified Biomedical Computing programme (BMD-07R, Univ. of California, Ca. U.S.A.) written in Fortran language. The ionic strengths of the eluents were chosen on the basis of previous experimental studies [3,8,14-16] with simple acids, amines and peptides.

RESULTS AND DISCUSSION

Theoretical Considerations.

In the basic ligand adsorption model for the separation of iodoamino acids and related ionogenic solutes, the chromatographic process is viewed as a reversible association of the solute, S_i, with the hydrocarbonaceous octadecyl ligand L, bound to the surface of the microparticulate, porous silica. The equilibrium association constant K_i, of the solute S_i, is given by

$$K_i = \frac{[S_i L]}{[S_i][L]} \qquad \ldots.(1)$$

If it is assumed that the sorption process does not involve ionic or hydrogen bonding interactions between the solute and the stationary phase, then the capacity factor of the solute S_i can be expressed by

$$k_i' = \psi \cdot K_i \qquad \ldots.(2)$$

where ψ is the phase ratio of the column.

The capacity factor for a diprotic acid, such as diiodothyroacetic acid, as a function of pH can be evaluated[14] from

$$k' = \frac{k_o + k_1 \frac{K_{a_{i,m}}}{[H^+]_m} + k_2 \frac{K_{a_{i,m}} K_{a_{2,m}}}{[H^+]_m^2}}{1 + \frac{K_{a_{1,m}}}{[H^+]_m} + \frac{K_{a_{i,m}} K_{a_{2,m}}}{[H^+]_m^2}} \qquad \ldots.(3)$$

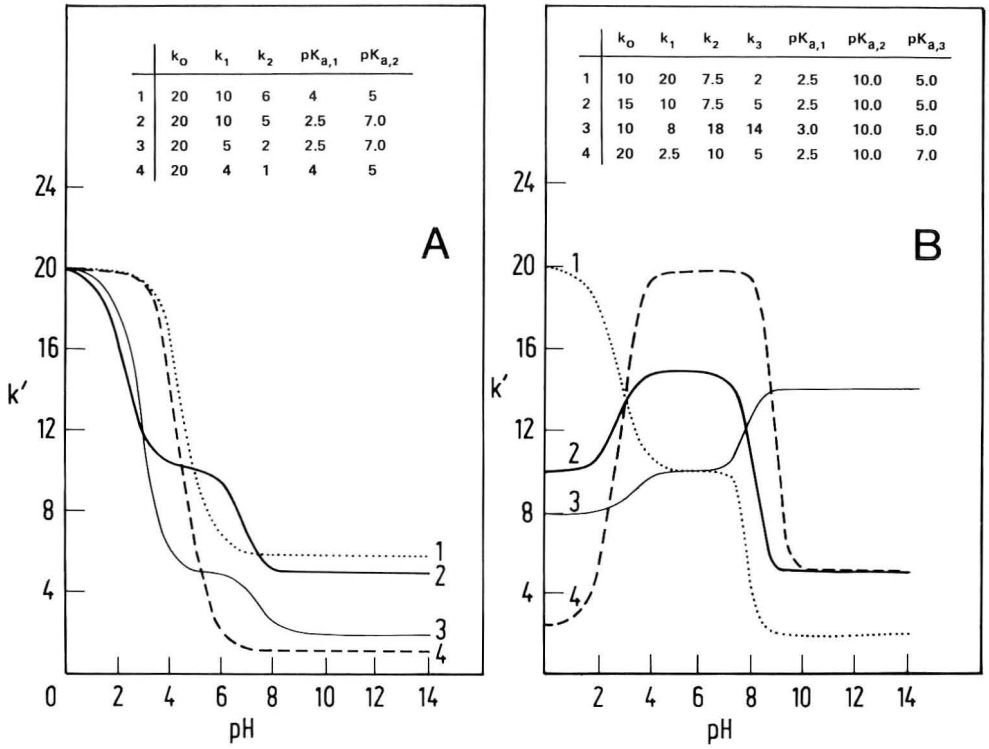
where k_o, k_1 and k_2 are the capacity factors of the undissociated, mono-dissociated and fully dissociated diprotic acid and $K_{a_{1,m}}$ and $K_{a_{2,m}}$ are the corresponding acid dissociation constants.

Similarly, the capacity factor for tyrosine and related ampholyte analogues as a function of pH is given [3,10,14] by

$$k' = \frac{k_o + k_1 \frac{[H^+]_m}{a_{1,m}} + k_2 \frac{K_{a_{1,m}}}{[H^+]_m} + k_3 \frac{K_{a_{1,m}} K_{a_{2,m}}}{[H^+]_m^2}}{1 + \frac{[H^+]_m}{K_{a_{1,m}}} + \frac{K_{a_{2,m}}}{[H^+]_m} + \frac{K_{a_{2,m}} K_{a_{3,m}}}{[H^+]_m^2}} \quad \ldots.(4)$$

where k_o, k_1, k_2 and k_3 are the capacity factors of the zwitterionic, cationic, anionic and doubly anionic charged species, respectively, and $K_{a_{1,m}}$, $K_{a_{2,m}}$ and $K_{a_{3,m}}$ are the three dissociation constants. A given polyprotic ampholyte will in general be characterised by a set of limiting capacity factor values corresponding to the undissociated (zwitterionic, isoelectric) form, k_o, the monodissociated form k_1, the di-dissociated form, k_2, ... fully dissociated from, k_n, with a corresponding set of pK_{a_1}, pK_{a_2} ..... pK_{a_n} values. By choosing appropriate values of the k_o, k_1 ..... k_n and pK_{a_1}, pK_{a_2} .....pK_{a_n} parameters, limiting conditions of the k' versus pH_m dependency for ionisable solutes can be assessed. Figure 1 illustrates the computed curves for the pH dependence of the capacity factor for some typical cases of diprotic acids and ampholytes using this approach.

The above discussion on the dependence of k' on pH does not take into account the effect of an organic solvent on the pH of an aquo-organic solvent mixture or on the pK of an ionogenic solute. It is now well recognised that the pK values of a particular ionogenic solute varies from eluent to eluent by virtue of specific solvation effects, as well as, changes in dielectric properties of the mobile phases [17]. With simple



	k_0	k_1	k_2	$pK_{a,1}$	$pK_{a,2}$
1	20	10	6	4	5
2	20	10	5	2.5	7.0
3	20	5	2	2.5	7.0
4	20	4	1	4	5

	k_0	k_1	k_2	k_3	$pK_{a,1}$	$pK_{a,2}$	$pK_{a,3}$
1	10	20	7.5	2	2.5	10.0	5.0
2	15	10	7.5	5	2.5	10.0	5.0
3	10	8	18	14	3.0	10.0	5.0
4	20	2.5	10	5	2.5	10.0	7.0

Figure 1.

Plots of the capacity factor of diprotic weak acids (A) and triprotic ampholytes (B) versus the pH of the mobile phase. The curves were calculated from eqns. 3 and 4 using the limiting values of the parameters shown on each graph.

monoprotic acids, theory predicts an increase in pK_{a_1} with increasing organic solvent content. This has been observed experimentally although ΔpK_a variations with solvent percentage in general are non-linear. For example, a one unit increase in pK_a for benzoic acid occurs[18] with water-methanol mixtures when the methanol content is increased from 20% to 60%. When the pHs of hydro-organic mobile phases are near a pK value of a solute significant differences in selectivity and retention

behaviour are thus expected for eluents of different organic solvent content.

According to solvophobic theory, solute retention can be expressed in terms of the overall standard unitary free energy changes associated with the transfer of the solute from the mobile to the stationary phase such that

$$\ell n\ k_i' = \ell n\ \psi - \frac{\Delta G_{assoc,i}}{RT} \qquad ....(5)$$

Horvath and his coworkers [6,7] have evaluated $\Delta G_{assoc,i}$ in terms of the association of the solute and the ligand in the gas phase and the transfer of the solute, the ligand and the complex individually into the eluent. The capacity factor of an ionised solute can then be expressed as

$$\ell n\ k_i' = const - \frac{\Delta G_{es,i}}{RT} - \frac{\Delta G_{cav,i}}{RT} \qquad ....(6)$$

where the electrostatic component $\Delta G_{es,i}$, is the energy associated with placing an ionic species into the solvent system and $\Delta G_{cav,i}$ is the free energy required to make a cavity with a surface area identical to that of the solute. The free energy term associated with cavity formation can be evaluated from

$$-\Delta G_{cav,i} = \gamma[N\Delta A_i + 4.836N^{1/3}(\kappa^e - 1)V^{2/3}] \qquad ....(7)$$

where γ is the surface tension of the eluent, N is Avogadro's number, V is the molar volume of the eluent, κ^e the microscopic cavity factor and ΔA_i is the hydrophobic contact area. With ligands of relatively large molecular dimensions and eluents of similar molecular structure, i.e. isocratic phases, ΔA_i is likely to be proportional to the hydrophobic surface area, A, of the solute. It follows that plots of $\ell n\ k'$, corrected for electrostatic effects, versus the hydrophobic surface areas of the various solute molecules should yield straight lines under elution conditions where the mobile phase composition is fixed. Further-

more, the slope of the plots of $\ell n\ k'$ versus the total molecular surface area, A_W, of the solutes will remain constant for eluents of the same composition but different pH only in the absence of pronounced polar interactions in the sorption process or solvation, molecular aggregation and stacking effects specific to the solutes. The slope values will thus respond to changes in the surface tension and dielectric constant of the mobile phase.

Effect of pH on k'.

Because of the chemical instability of octadecylsilica stationary phases to exposure to aqueous solutions at pH values higher than pH 7.5 for extended periods of time, we restricted our investigations to mobile phases have the operational upper limit of pH 7.0. As a consequence, it was not possible to

TABLE 1

Structures of Iodoamino Acids and Related Compounds.

No.	Abbreviation	Structure
1.	Diac	3,5-Diiodothyroacetic acid
2.	Triac	3,3',5-Triiodothyroacetic acid
3.	Tetrac	3,3'5,5'-Tetraiodothyroacetic acid
4.	Tyr	Tyrosine
5.	MIT	3-Iodotyrosine
6.	DIT	3,5-Diiodotyrosine
7.	T_0	Thyronine
8.	T_2	3,5-Diiodothyronine
9.	T_3	3,3',5-Triiodothyronine
10.	T_4	3,3',5,5'-Tetraiodothyronine

examine the retention behaviour of the compounds listed in Table 1 over the pH range where the phenolic groups were completely ionised, i.e. greater than 99.9% in the phenoxide form, or the amino groups were fully deprotonated. Previous studies [14,16] with hydroxylphenylacetic acids and aromatic amino acids have demonstrated, however, that sufficient data can be obtained to allow the retention behaviour of simple ionogenic solutes over this restricted pH range to be analysed in terms of the above theoretical considerations.

The pH dependence of the capacity factors for the thyroacetic acids, (1) - (3), is shown in Fig.2. In common with related studies on mono- and di-protic weak organic acids, sigmoidal shapes for the dependence of the capacity factors of compounds (1) - (3) on the apparent pH of the mobile phase were observed. Least squares fit of the data confirmed that the the experimental data was essentially in agreement with the relationship explicit to eqn. 3. At values below pH 3.0, some divergence from expected behaviour was evident. This effect may be due to the influence that silanophilic interactions have on the retention process under these low pH conditions [5,19]. With diprotic solutes which exhibit widely separated pK_a values, as is the case with the thyroacetic acids, the shape of the k´ versus pH plot can be considered a composite of the respective dissociation curves. However, over the pH range examined, the ionisation of the 4´-hydroxy- group of compounds (1) - (3) does not appear to significantly perturb the basic sigmoidal behaviour characteristic of mono-protic acids. Similar observations have been noted [14] with hydroxyphenylacetic acid analogues. As the pH is increased both the carboxylic and phenolic groups will progressively become more ionised. Since solute retention is dependent on the extent of ionisation, a progressive decrease in the k´ values will ensue until limiting values are reached.

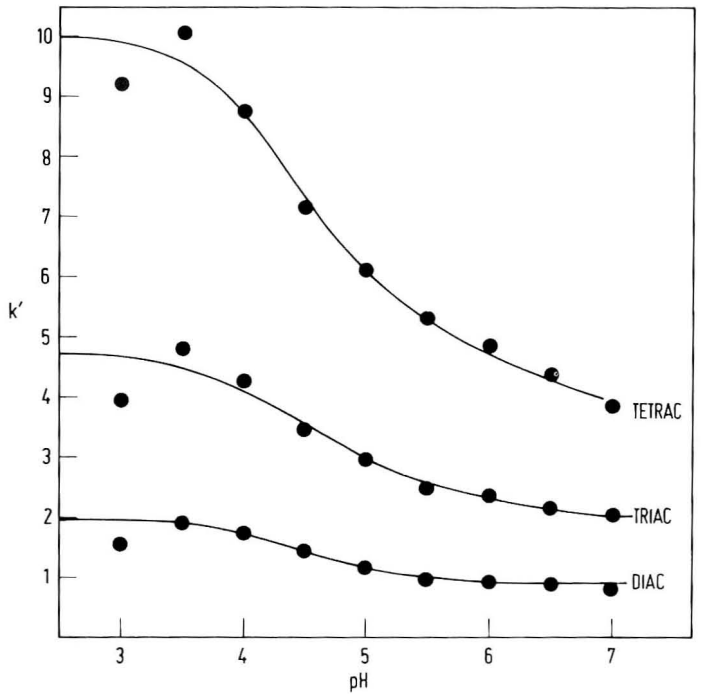


Figure 2.

Capacity factors for several thyroacetic acids as a function of the pH of the eluent. Chromatographic conditions: column, μBondapak C_{18}; flow rate, 2ml/min; temperature, 18°; eluent, 50% methanol-50% water-25mM KH_2PO_4. The legend to the acronyms is given in Table 1.

The data for the capacity factors of the iodoamino acids (4) - (10) are shown in Figs. 3 and 4. Over the pH range studied with these iodoamino acids, the level of amino group protonation will remain essentially constant, i.e. the pK_{NH_2}-pH will generally be larger then 3.0 with the percentage in the protonated form ranging from ca 99.9990 to ca 99.90. Following the procedures outlined earlier, the data were evaluated by least squares fit analysis in terms of eqn. 4.

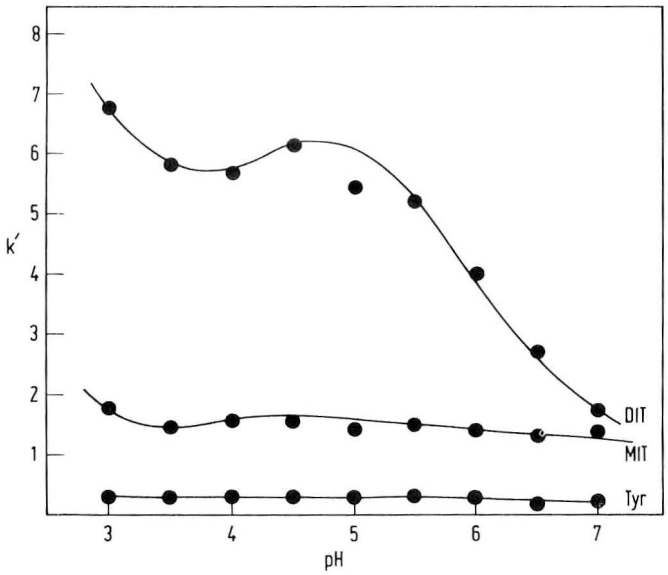


Figure 3.

Plots of the capacity factor versus the pH of the eluent for tyrosine and the iodotyrosines. Chromatographic conditions: column, μBondapak C_{18}; flow rate, 2ml/min; temperature, 18°; eluent 5% methanol-95% water-50mM KH_2PO_4. The legend to the acronyms is given in Table 1.

The chromatographic behaviour of the iodoamino acids under the experimental conditions employed in this study is consistent with the general form of the expression for the k′ of polyprotic ampholytes as a function of the pH of the mobile phase, taking into account solvent effects on ionisation. With ampholytes, theory predicts major differences in the shapes of the k′ versus pH plots even when the relevant limiting parameters of the solutes are similar. By using stationary phases with higher operational upper limits for the mobile phase pH, complete k′ versus pH plots can be achieved [16] and precise pK_a values determined from the chromatographic data.

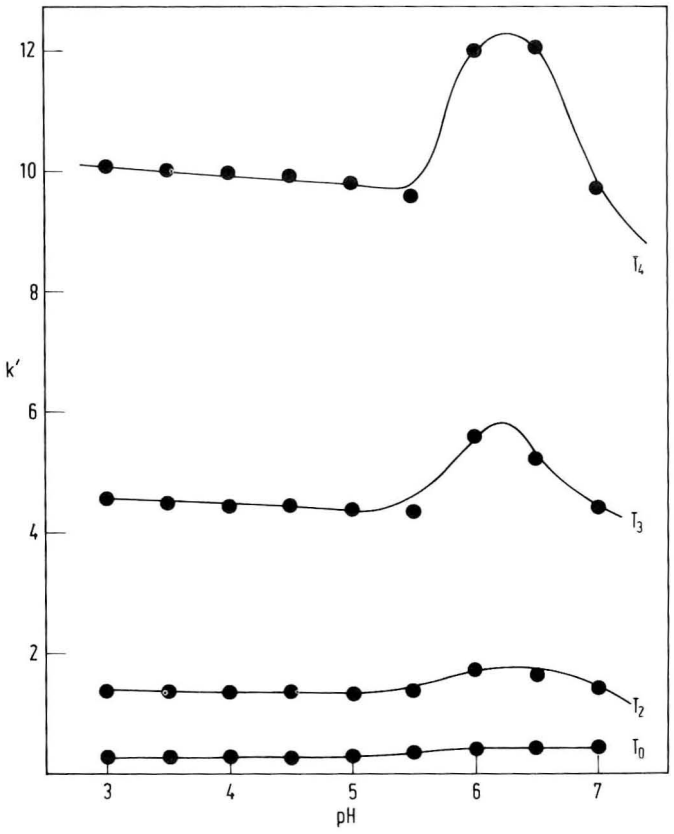


Figure 4.

Capacity factors for several thyronine derivatives as a function of the pH of the eluent. Chromatographic conditions: column, μBondapak C_{18}; flow rate, 2ml/min; temperature, 18°; eluent, 40% methanol-60% water-50mM KH_2PO_4. The legend to the acrynoms is given in Table 1.

Effect of Hydrophobic Surface Area on k´.

There is now general consensus that amino acid and peptide retention in RP-HPLC can be interpreted in terms of hydrophobic interactions between these ionogenic solutes and the hydrocarbonaceous ligand attached to the surface of the silica matrix.

With underivatised amino acids, selectivity depends, inter alia, upon the lipophilicity of the side chains, i.e. their chemical functionality and differences in ionisation state. Solvophobic theory predicts that the k´ values for a series of ionogenic solutes eluted under isocratic conditions should follow changes in the relative interfacial surface area, ΔA_i, of the solute in contact with the stationary phase which can be indicated by the molecular hydrophobic surface area, A, of the solute. If eqn. 7 holds, then linear relationships of ℓn k´ for compounds (1) - (10) as a function of A are anticipated. Shown in Fig. 5 are

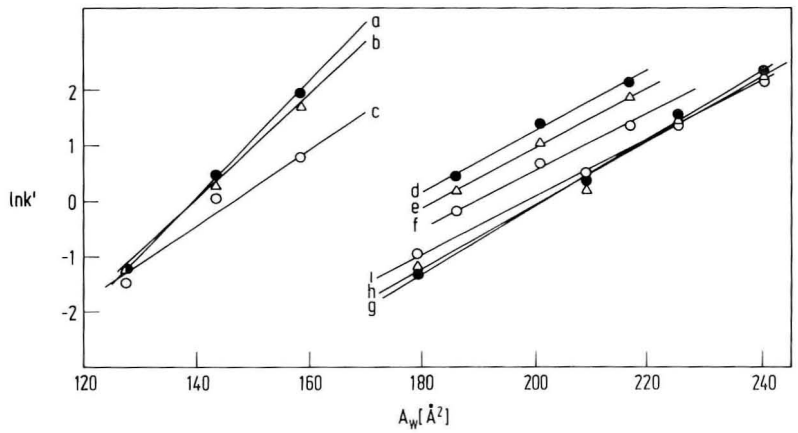


Figure 5.

Plots of the logarithm of the capacity factors versus molecular surface area for the compounds (1)-(10). Data obtained for the tyrosine derivatives at pH 3.0, pH 5.0 and pH 7.0 are shown in plots (a)-(c) with the following r^2 and slope co-ordinates from a least squares analysis, (a), 0.9942, 0.1038; (b) 0.9980, 0.0953; (c) 0.8316, 0.0715. The data for the thyroacetic acids and thyronine derivates obtained under similar pH conditions are shown in plots (d)-(f) and (g)-(i) respectively with the following r^2 and slope values: (d) 0.9930, 0.0534; (e) 0.9900, 0.0537, (f) 0.9853, 0.0507; (g) 0.9956, 0.0602; (h) 0.9946, 0.0591 and (i) 0.9868, 0.0529. Chromatographic conditions are given in Figures 1-3.

the plots of ℓn k′ for compounds (1) - (10) determined at pH 3.0, 5.0 and 7.0, versus the total molecular surface areas, A_W, calculated from the group surface increments of Bondi with the appropriate crowding corrections [20,21]. Each family of compounds - tyrosines, thyronines and thyroacetic acids - yielded linear plots for ℓn k′ versus A_W and similar relationships were evident with plots of ℓn k′ versus hydrophobic surface areas calculated by summation of the area increments for carbon, hydrocarbon and iodo-groups only. The displacement of the lines for the three families of compounds can be accounted for by the differences between the dipole moments of the solutes and the composition of the eluent. According to eqn. 7 the slope of the plots will be predominantly controlled by the surface tension γ, of the eluent. The observation that the slopes of the plots for the tyrosine derivatives (determined at 5% methanol) are noticably different to the corresponding values for the thyronines (determined at 40% methanol) and the thyroacetic acids (determined at 50% methanol) is in accord with the anticipated trend. The changes in slope for the plots of ℓn k′ versus A_W, evident for both iodoamino acid families as the pH of the eluent was varied, are suggestive of the participation of silanophilic and pH dependent specific solvation effects in the retention process. In fact, it is likely that solute-silanol interactions and specific solvation of ionized groups in general play important secondary roles in selectivity modulation for amino acids and peptides on reversed phases. For example, such effects are probably involved in the elution order reversals experienced by some isomeric peptides when the pH of the mobile phase is changed as well as the selectivity deviations seen with polypeptides with mobile phases of high organic solvent content[4,19,21].

Although the three families of solutes are not homologous, it can be seen from the data that the iodo-group effectively has a constant incremental effect on retention for all three groups.

The progressive enhancement of retention, arising from the hydrophobic nature of the iodo-group, has been recognised in previous studies on the separation of iodo-compounds by RP-HPLC. For example, we have demonstrated [8,9] that the plots of ℓn k′ versus number of iodine atoms per aromatic nucleus and ℓn k′ versus partition coefficients also follow linear relationships for these compounds. Collectively, these results can all be ascribed to the common physicochemical phenomenon that governs the retention of amino acids, peptides and proteins to chemically bonded reversed phases. Knowledge of the effects of ionisation, and other secondary equilibrium processes which these ionogenic molecules undergo in solution can be usefully applied in the selection of mobile phase conditions which allow optimal chromatographic resolution. The application of such optimisation strategies has recently been discussed [2,4,5] for the separation of amino acids, peptides and other ionogenic solutes by RP-HPLC.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of New Zealand. Boris Grego is a University Grants Committee Postgraduate Scholar.

REFERENCES

1. M.T.W. Hearn, S.J. Su and B. Grego, J. Liquid Chromatogr. submitted for publication.

2. M.T.W. Hearn and B. Grego, J. Chromatogr., 203, 349, 1981.

3. M.T.W. Hearn, B. Grego and W.S. Hancock, J. Chromatogr., 185, 429, 1979.

4. M.T.W. Hearn, Advan. Chromatogr., 20, in press.

5. M.T.W. Hearn, in HPLC: Advances and Perspectives (Cs. Horvath, ed.) Academic Press, New York, N.Y., vol. 3, in press.

6. Cs. Horvath, W.R. Melander and I. Molnar, J. Chromatogr., 125, 129, 1976.

7. W.R. Melander and Cs. Horvath, in HPLC: Advances and Perspectives, (Cs. Horvath, ed.) Academic Press, New York, N.Y., 1980, vol.2, p.113-319.

8. M.T.W. Hearn, W.S. Hancock and C.A. Bishop, J. Chromatogr., 157, 337, 1978.

9. M.T.W. Hearn and W.S. Hancock, J. Liquid Chromatogr., 2, 217, 1979.

10. M.T.W. Hearn and W.S. Hancock, J. Chromatogr. Sci., 18, 288, 1980.

11. N.M. Alexander and M. Nishimoto, Clin. Chem., 25, 1757, 1979.

12. N.A. Takai, B. Rapoport and M. Yamamoto, Endocrinology, 107, 176, 1980.

13. G.G. Skellern, M. Mahmoudian and B.I. Knight, J. Chromatogr., 179, 213, 1979.

14. Cs. Horvath, W.R. Melander and I. Molnar, Anal. Chem., 49, 142, 1977.

15. S.N. Deming and M.L.H. Turott, Anal. Chem., 50, 547, 1978.

16. E.P. Kroeff and D.J. Pietrzyk, Anal. Chem., 50, 502, 1978.

17. B.L. Karger, J.N. Le Page and N. Tanaka, in HPLC: Advances and Perspectives (Cs. Horvath, ed.), Academic Press, New York, N.Y., 1980, vol. 1, p.117.

18. A.L. Bacarella, E. Grunwald, H.P. Marshall and E.L. Porlee J. Org. Chem., 20, 747, 1955.

19. B. Grego and M.T.W. Hearn, Chromatographia, in press.

20. A. Bondi, J. Phys. Chem., 68, 441, 1964.

21. S.C. Valvani, S.H. Yalkowsky and G.L. Amidon, J. Phys. Chem., 80, 829, 1976.

22. M.T.W. Hearn and B. Grego, J. Chromatogr., submitted for publication.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1725-1744 (1981)

THE SEMI-PREPARATIVE SEPARATION OF PEPTIDES ON REVERSED PHASE SILICA PACKED INTO RADIALLY COMPRESSED FLEXIBLE-WALLED COLUMNS.†

M.T.W. Hearn*, B. Grego,
Immunopathology Research Unit,
Medical Research Council of New Zealand,
University of Otago Medical School,
P.O. Box 913, Dunedin,
New Zealand.

and

C.A. Bishop,
Anac Ltd.,
P.O. Box 5565, Auckland,
New Zealand.

ABSTRACT

The semi-preparative separation of underivatised peptide mixtures from the tryptic digestion of thyroid and pituitary proteins has been accomplished on a 10dp spherical octadecyl-silica stationary phase, Radial Pak A/C18, packed into flexible-walled polyethylene cartridges (10 x 0.8cm). With volatile ionic modifiers, such as ammonium bicarbonate, excellent resolution and peptide recoveries were obtained with this support

---

† High Performance Liquid Chromatography of Amino Acids, Peptides and Proteins XXXV. For the previous paper in the series see ref. [41].

* Author to whom correspondence should be addressed at St Vincent's School of Medical Research, Victoria Parade, Melbourne, Victoria, 3065, Australia.

under gradient elution conditions. Compared to the 'capped' alkylsilicas of high carbon loading per gram packing, this octadecylsilica support exhibits significant differences in selectivity consistent with the greater involvement of silanophilic interactions between the ionised peptides and the stationary phase. With the available flexible-walled cartridges sample loadings equivalent to 10-500nmole peptide(s) per injection can be routinely used. Good recoveries of hydrophobic polypeptides and small proteins were also achieved using shallow gradients of the organic solvent modifier.

INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) has gained wide popularity over the past several years for the structural mapping of polypeptides and proteins and, in particular, for the analysis and micro-preparative separation of enzymatic digests [1-5]. Compared to conventional open column ion exchange and gel permeation chromatographic techniques, RP-HPLC methods exhibits superior peak resolution for complex mixtures of hydrophilic and hydrophobic peptides. These features are also associated with short analysis times and, usually, good recoveries. Most micropreparative procedures have been based on conventional stainless steel analytical columns, e.g. 25 x 0.4cm I.D., packed with 5- or 10-μm dp octyl- or octadecyl-silicas. Depending on the complexity of the separation, the sample capacity of these analytical columns is frequently less than 1mg. In previous studies we have described [6,7] the use of radially compressed polyethylene cartridges (30 x 5.7cm I.D.) containing a 75μm dp octadecyl-silica support in the purification of 1-10gm amounts of unprotected peptides. In the present report a similar approach has been employed for the semi-preparative separation of the tryptic peptides of several proteins, including thyroglobulins and human growth hormone, using a related 10μm dp octadecyl-silica sorbent, packed into flexible-walled polyethylene cartridges of dimensions (10 x 0.8cm, I.D.) and volatile mobile

phases. Application of these procedures to the separation of native and radio-iodinated peptide and polypeptide hormones is also described.

MATERIALS AND METHODS

Chemicals and Reagents.

Phosphoric acid, formic acid, ammonium bicarbonate and ammonium sulphate were either ARISTAR or AnalaR grade reagents from B.D.H. (Poole, U.K.). Triethylamine, also from B.D.H., water and acetonitrile were purified using established procedures [8-10].

Human thyroglobulin 19S iodoprotein was extracted from human thyroids by the method of Salvatore et al. [11] and fractionated on Sepharose CL-4B using a 150mM NaCl-10mM Tris-HCl, pH 8.0, buffer followed by affinity chromatography using established procedures [12,13]. Guinea pig thyroglobulin 19S iodoprotein was isolated from an albino inbred strain using the procedure of Haeberli et al. [14]. Human growth hormone was prepared by the method of Chapman et al. [15] based on the procedure of Lumley Jones et al. [16]. The ovine thyrotrophin was isolated from an ovine pituitary extract by salt fractionation, lectin affinity chromatography, ion exchange chromatography and gel permeation HPLC as described elsewhere [17]. Lysozyme (grade 1) was a commercial sample from Sigma Chem. Co. (St Louis, Mo. U.S.A.). The source of some of the peptides and polypeptides has been given previously [18], the remainder were commercial samples, purified in this laboratory, from Sigma (St Louis, Mo., U.S.A.), Bachem (Torrance, Ca., U.S.A.), Calbiochem (La Jolla, Ca., U.S.A.) or Vega Biochem (Tuscon, Ariz., U.S.A.).

Tryptic digestion of the proteins was carried out using TPCK-treated trypsin following the procedure of Chernoff and Liu [19]. Radio-iodination of the proteins was performed using

a modified lactoperoxidase procedure based on the method of Thorell and Johansson [20]. Guinea pig thyroglobulin 19S-iodoprotein was labelled *in vivo* with ^{125}I and isolated by established procedures [14].

Apparatus.

A Waters Assoc. (Milford, Mass., U.S.A.) high performance liquid chromatography system was used which included a M660 solvent programmer, two M6000A solvent delivery systems, a U6K universal injector, coupled via a RCM-module, to a M450 variable wavelength UV detector and a Rikadenki dual channel recorder. The Radial-Pak A (C_{18}) cartridges (10 x 0.8cm and 10 x 0.5cm) were obtained from Waters Assoc. and washed extensively with methanol (*ca* 500ml) prior to use. Aliquots of column effluents containing radio-active peptides were counted in a Packard Tri Carb Gamma Scintillation Counter. Sample injections were made with Pressure Lok liquid syringes, series B110 from Precision Sampling (Baton Rouge, La., U.S.A.). The pH measurements were performed with a Radiometer PHM64 Research pH meter, equipped with a combination glass electrode.

Experimental.

All chromatograms were carried out at ambient temperature (*ca* 18°). Bulk solvents and mobile phases were degassed by sonication. Following a change in mobile phase conditions, all columns were equilibrated to initial or new elution conditions for at least 30min. Flow rates were maintained between 1.0ml/mm and 4.0ml/min. Detection of the peptides was at 210nm or at higher wavelengths depending on the optical transparency of the mobile phase. The triethylammonium phosphate and formate buffers were prepared at different concentrations over the range 15-150mM by titrating the appropriate acid with freshly distilled triethylamine until pH 3.5 was obtained. All peptides and tryptic digests were

made up in the mobile phase corresponding to initial equilibration conditions.

RESULTS AND DISCUSSION

The composite effects due to non-polar and polar group selectivities in peptide separation on alkylsilicas have been much discussed of late [2,4,21-23]. Although the predominant mechanism of retention under these RP-HPLC conditions is the hydrophobic expulsion of the peptidic solutes from the polar mobile phase to the less polar sorbent, polar effects due to unreacted and accessible silanol groups can also influence retention behaviour. For example, several studies [21-24] have demonstrated that both solvophobic and silanophilic processes are involved in the retention of peptides with most commercially available types of reversed phase silicas, even supports of high surface coverage and carbon loading. Under inappropriate elution conditions, these competing distribution processes can result in low solute recoveries and poor resolution due, in part, to undesirable peak broadening. Other column and extra-column effects are also known to influence chromatographic efficiencies. Even in well packed columns, 'wall' and associated non-uniform flow phenomena can lead to significant zone dispersion effects for peptidic solutes. Theory predicts [25-28] that radial compression of flexible walled cartridges can create an efficient and homogeneous chromatographic bed in which these dispersion effects are largely circumvented. Due to their favourable column characteristics, e.g. wide diameter, short columns, relatively high flow rates and sample loadings are also possible with these cartridges without a significant loss of resolution. In recent studies [6,7], we have employed this approach in the purification of multi-gram amounts of synthetic peptides using preparative reversed phase columns with sample capacities greater than

1gm/injection. As is evident from the investigations reported in the present and other recent studies[29], the corresponding analytical system, now available commercially in the form of Radial Pak A/C_{18} columns, is suited to high efficiency semi-preparative separations of complex mixtures of peptides.

Acidic amine phosphate buffers have recently gained popularity in analytical RP-HPLC of peptides and proteins. Low concentrations, usually in the region of 5-20mM, of these buffers have been added to mobile phases of high-intermediate water content in order to mask polar group interactions at the surface of the chemically bonded alkylsilica stationary phase. Compared to the corresponding eluents which lack these buffer components, mobile phases which contain amine phosphates, formates or trifluoroacetates generally give rise [2,4,21-23,30] to reduced retention times and improved peak shapes for small peptides when chromatographed on such conventional reversed phase silica supports as μBondapak C_{18} or LiChrosorb RP-18. With the Radial Pak A/C_{18} support, it was found necessary to use considerably higher concentrations of these buffer combinations to avoid excessive band spreading. Although satisfactory column efficiencies could be achieved with, for example, 150mM triethylammonium phosphate incorporated into the mobile phase (Fig. 1a), mobile phases containing lower concentrations of the acidic triethylamine buffers resulted in inferior chromatographic performance. The decreased resolution observed with eluents containing low concentrations of the acidic amine buffers was most noticable with basic peptides and particularly with peptides containing a N-terminal arginine residue. These observations are consistent with the reported [21] surface characteristics of this totally porous, spherical, reversed phase support and parallel earlier experiences [2,21,22] on convenient procedures to mask silanophilic interactions. In order to obtain optimal chromatographic reproducibility, it was

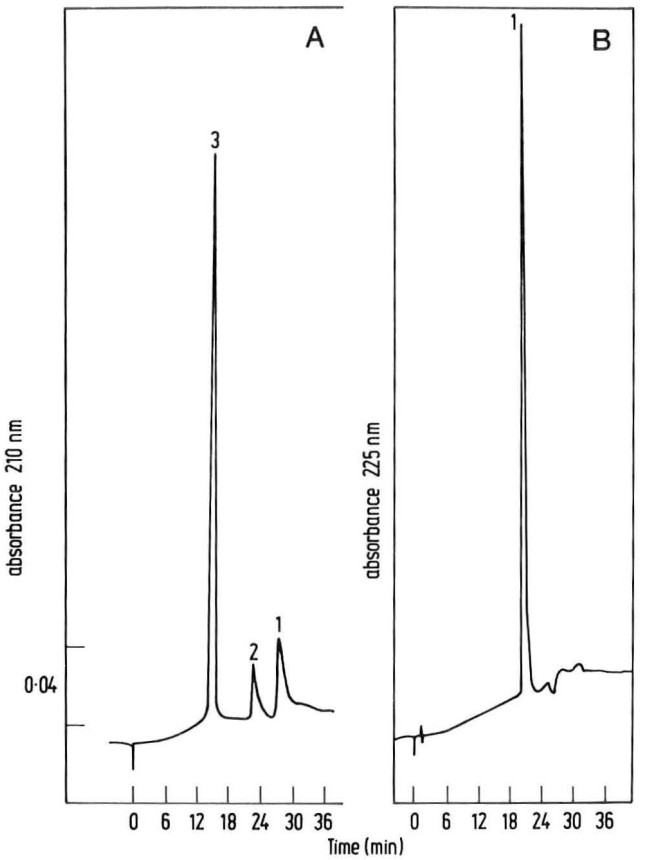


Figure 1. (A) Gradient elution profile of a mixture of bovine insulin (1), angiotensin I (2) and trityrosine (3) (= ca 20µg each). Chromatographic conditions: column, Radial Pak A/C_{18}; flow rate, 2ml/min; gradient elution beginning with 150mM triethylammonium phosphate, pH 2.6 with a linear 30min. increase of the modifying mobile phase 50% acetonitrile-50%-water-150mM triethylammonium phosphate. (B) Chromatogram of bovine insulin (1) obtained under 30min. linear gradient elution conditions on the same Radial Pak A/C_{18} column. Flow rate, 2ml/min; mobile phase, aqueous 100mM ammonium sulphate (pH3.0) tp 50% acetonitrile-50% water-100mM ammonium sulphate (pH3.0).

also found necessary to condition the sorbent by means of an extensive wash with either methanol or another alcohol followed by elution with the appropriate mobile phase containing the acidic amine buffer for ca 200 column volumes. As most commercial organic amines are either of low purity or low volatility, triethylamine has proved to be the reagent of choice. However, even with triethylamine it is essential for semipreparative and analytical separations to ensure that associated contaminants, which interfere with high sensitivity UV or fluorometric detection, are removed by distillation procedures, followed by percolation of the freshly prepared mobile phase containing the appropriate concentration of the acidic triethylammonium buffer through an octadecylsilica support prior to use.

Ammonium sulphate, at concentrations up to 100mM, was also found effective as an ionic modifier with the Radial Pak A/C_{18} support. With mobile phases of similar pH and organic solvent content, peptides show different selectivities with this reagent compared to triethylammonium phosphate buffer systems. Typical of these selectivity differences are the elution profiles for bovine insulin compared in Figs. 1a and 1b.

The major limitation of acidic amine phosphate or sulphate buffers in preparative RP-HPLC is the necessity to subsequently remove the buffer components. Although this desalting step can be achieved by classical open column gel permeation, high performance gel permeation and RP-HPLC techniques using volatile eluents, these subsequent steps may be undesirable and could well lead to lower recoveries. Volatile amine formate, acetate and triflouroacetic buffers have been employed [1,2,6,7,30] in preparative and semi-preparative RP-HPLC separations of peptides. Prior purification of the buffer reagents is invariably a prudent precaution. Ammonium bicarbonate offers a convenient alternative despite its limitations as an ionic modifier of peptide selectivity due to the restriction this reagent places

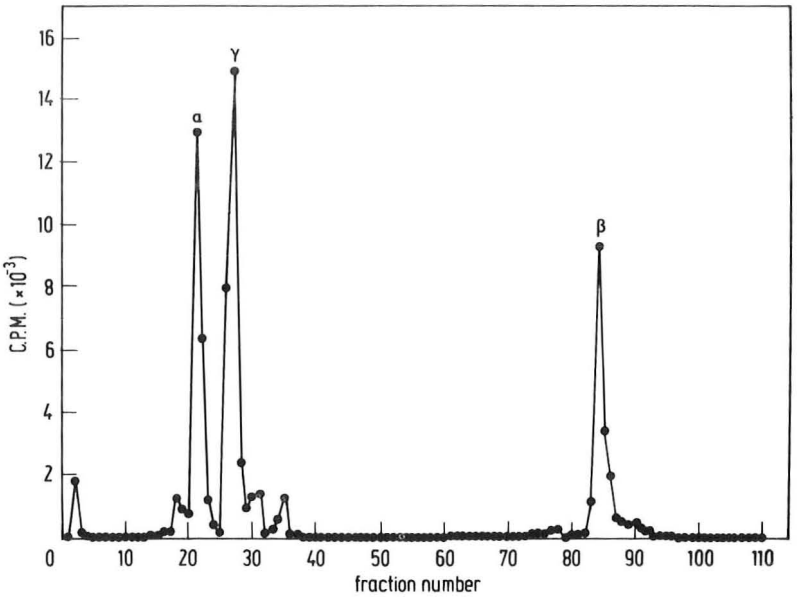


Figure 2. Separation of [^{125}I]-labelled α-, β- and γ-endorphin on a Radial Pak A/C_{18} column under gradient elution conditions. Chromatographic conditions: flow rate, 4ml/min; mobile phase A, aqueous 100mM ammonium bicarbonate; mobile phase B, 65% acetonitrile-35% water-100mM ammonium bicarbonate. The gradient elution commenced with 20% B with a 30min. linear increase up to 100% B.

on the operational pH range of the mobile phase. Desirably, peptides can be recovered directly by lyophilisation from hydro-organic solvent eluents containing this reagent. Micropreparative separations of peptides on conventional octadecylsilica supports have been described [1,2,4,32] using ammonium bicarbonate-based mobile phases. As is evident from the studies illustrated in Figs.2-7, similar eluents can be employed for the separation of peptides, including radio-iodinated polypeptides and hydrophobic tryptic peptides, on the Radial Pak A/C_{18} support.

Figure 2 shows the separation of [^{125}I]-labelled α-, β- and γ-endorphin. The recovery of radioactivity for each of

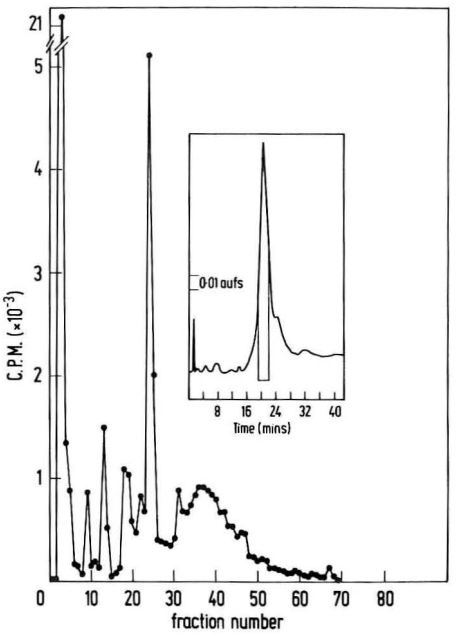


Figure 3. Gradient elution profile of a partially purified [^{125}I]-labelled ovine thyrotrophin preparation chromatographed on a Radial Pak A/C_{18} column. Chromatographic conditions: flow rate, 2ml/min; a 30min. linear gradient was generated from aqueous 100mM ammonium bicarbonate to 50% acetonitrile-50% water-100mM ammonium bicarbonate. Also shown is the elution profile and recovery of concanavalin A-purified ovine thyrotrophin (500µg) chromatographed under the same conditions.

these labelled peptides was greater than 97% using a linear 30min acetonitrile gradient at a flow rate of 4ml/min. Similarly, high recoveries were obtained for a crude [^{125}I]-labelled ovine thyrotrophin preparation (Fig.3) (99% recovery) and for an *in vivo* labelled guinea pig thyroglobulin 19S iodoprotein complete tryptic digest (85% recovery). Also shown in Fig. 3 is the elution profile and recovery of a 500µg loading of Concanavalin A purified ovine thyrotrophin (*ca* 4µU/µg bioactivity based on a U.S.P. bovine thyrotrophin standard) chromatographed under the same elution conditions.

The high recovery of this glycoprotein hormone from the Radial Pak A/C_{18} column parallels similar results [17] obtained with a 500Å pore diameter octadecylsilica support and suggest that relatively hydrophobic proteins with molecular weights at least up to 30,000 may be successful separated on a semi-preparative basis using these radially compressed columns and mobile phases of suitable elutropicity. Because of their favourable solvent characteristics and peptide solubility parameter dependencies [2,23], acetonitrile and 2-propanol appear to be the two common organic modifiers best suited to polypeptide/protein separation on Radial Pak A/C_{18} supports.

The application of analytical RP-HPLC to peptide mapping of proteins has generated much valuable information on the primary structure of a large variety of proteins of current interest in molecular biology. Following our earlier reports on the development of elution strategies [1,2,18,33,34] for the micropreparative RP-HPLC separations of enzymatic digests of proteins, we have extended this micromethodology to haemoglobin variants [35], protein hormones [1,36] and other proteins with intrinsic biological activity [37,38] using in several cases as little as 100pmole of protein digest. In several instances where the parent protein was available on the nanomole scale, semi-preparative RP-HPLC would have been most useful.

Fig. 4 shows the elution profiles obtained after application of the 18h and 20h tryptic digest of chick lysozyme to a Radial Pak A/C_{18} column and elution with linear water-acetonitrile-100mM ammonium bicarbonate gradient. Under these chromatographic conditions excellent resolution and peptide recoveries were obtained. Consistent with the earlier results, the corresponding low pH phosphate based eluent gave significantly inferior resolution. As can be seen from Fig. 5, a 0-50% 2-propanol gradient with 100mM ammonium bicarbonate

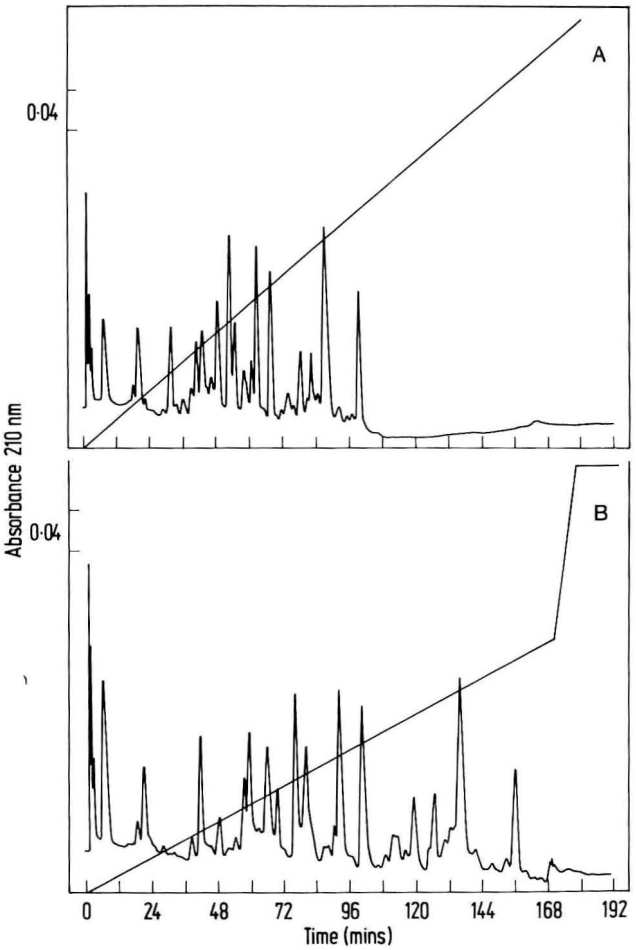


Figure 4. Separation of the tryptic peptides of chick lysozyme on a Radial Pak A/C_{18} column. In (A) is shown the chromatogram for the 18h. digest eluted at a flow rate of 1.5ml/min with a linear 3h. gradient generated from aqueous-100mM ammonium bicarbonate to 50% acetonitrile-50% water-100mM ammonium bicarbonate, sample size, 1400μg. In (B) is shown the chromatogram for the 20h. digest (sample size 2100μg) eluted under similar conditions except that a biphasic 5h. gradient was employed.

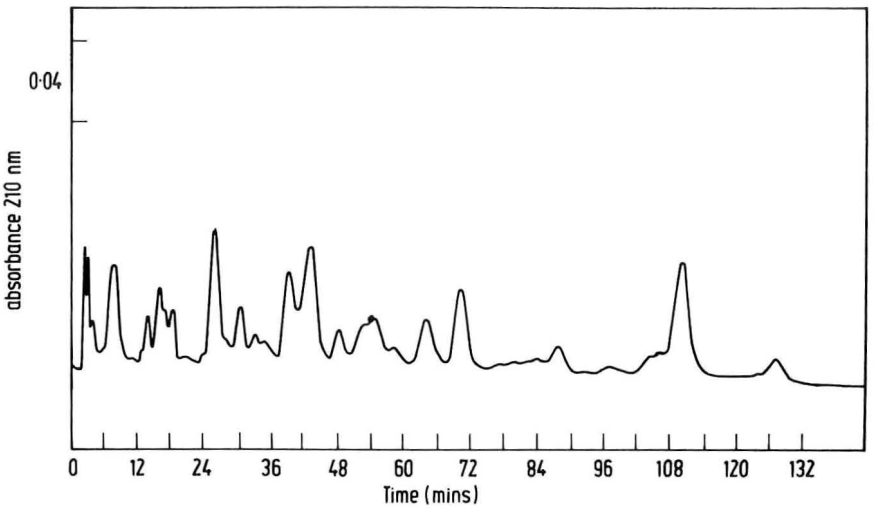


Figure 5. Gradient elution profile for the tryptic peptides of chick lysozyme chromatographed on a Radial Pak A/C_{18} column using a 5h. linear gradient generated from aqueous 100mM ammonium bicarbonate to 50% propan-2-ol-50% water-100mM ammonium bicarbonate at a flow rate of 1.5ml/min. Sample size 2100µg in 150µl.

present resulted in more rapid elution of the less polar lysozyme tryptic peptides but reduced efficiencies compared to acetonitrile gradients. These results are typical for these two solvent modifiers and reflect relative differences in their elutropicities and solute diffusion rates [23].

Fig. 6a illustrates the elution profile obtained for the 6h tryptic digest of human growth hormone eluted under ammonium bicarbonate-acetonitrile gradient conditions. In Fig. 6b is shown the corresponding chromatogram obtained for the same tryptic digest on an analytical µBondapak C_{18} column eluted with a low pH phosphate-based mobile phase. As anticipated the Radial Pak A/C_{18} column gave better column performances with larger sampling loadings for the human growth hormone enzymatic digests. Application of these methods has

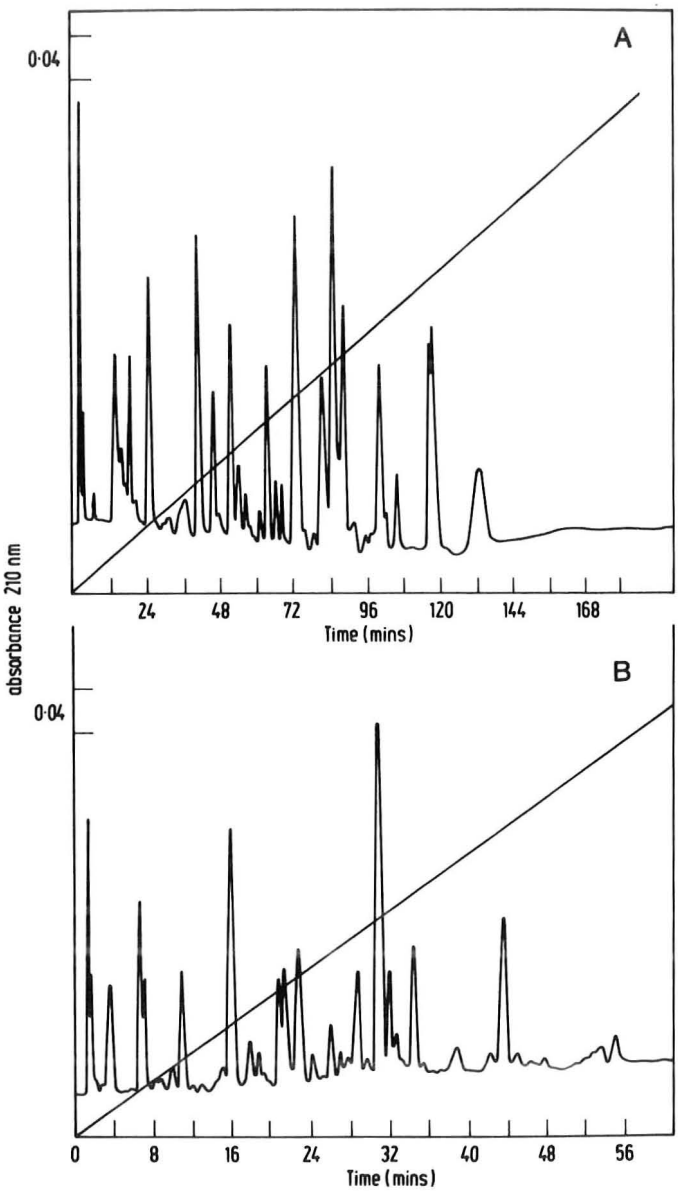


Figure 6. Separation of the tryptic peptides of human growth hormone on (A) the Radial Pak A/C_{18} column with the elution conditions given in the legend to Fig. 4A, and (B) on the μBondapak C_{18} analytical column with a 60min. linear gradient generated from water-15mM orthophosphoric acid to 50% acetonitrile-50% water-15mM orthophosphoric acid, flow rate, 2ml/min.

permitted the amino acid sequence of the 20K human growth hormone variant to be confirmed in this laboratory [36].

Fig. 7 demonstrates further the general applicability of the Radial Pak A/C_{18} column to resolve particularly complex peptide mixtures on a semi-preparative scale. In this figure, the elution profiles of the 20h tryptic digests of human (A) and guinea pig (B) thyroglobulin 19S iodoproteins are shown. Compared to previous peptide mapping techniques used in the study of these two macroglobulins [39] these rapid, high resolution RP-HPLC separations are clearly superior. Using similar chromatographic techniques, we have been able to resolve [38] milligram quantities of [^{125}I]-labelled peptides from *in vivo* or *in vitro* iodinated thyroglobulins from several species. Despite the reported [14,40] similarities in amino acid composition for human and guinea pig thyroglobulin 19S iodoproteins, significant sequence and cleavage site differences are evident from a comparison of the chromatograms shown in Fig.7. At this stage, little information is available on the amino acid sequence of the subunits of these microheterogeneous macroglobulins due, in part, to the poor resolution of the enzymatic or the CNBr-fragments of these proteins obtained with classical open column gel permeation or ion-exchange techniques. For example, on Sephadex G75 columns, the same tryptic digest as used for the RP-HPLC separation shown in Fig. 7A is resolved into only nine discrete fractions [38].

In summary, high efficiency separations of complex peptide and polypeptide mixtures can be achieved using the Radial Pak A/C_{18} support packed into flexible-walled polyethylene cartridges. From the available chromatographic data, this non-polar sorbent exhibits different selectivities for peptides including the angiotensins, insulins and endorphins when compared to conventional octadecylsilica supports such as μBondapak C_{18}, LiChrosorb RP-18 or Hypersil ODS. A further

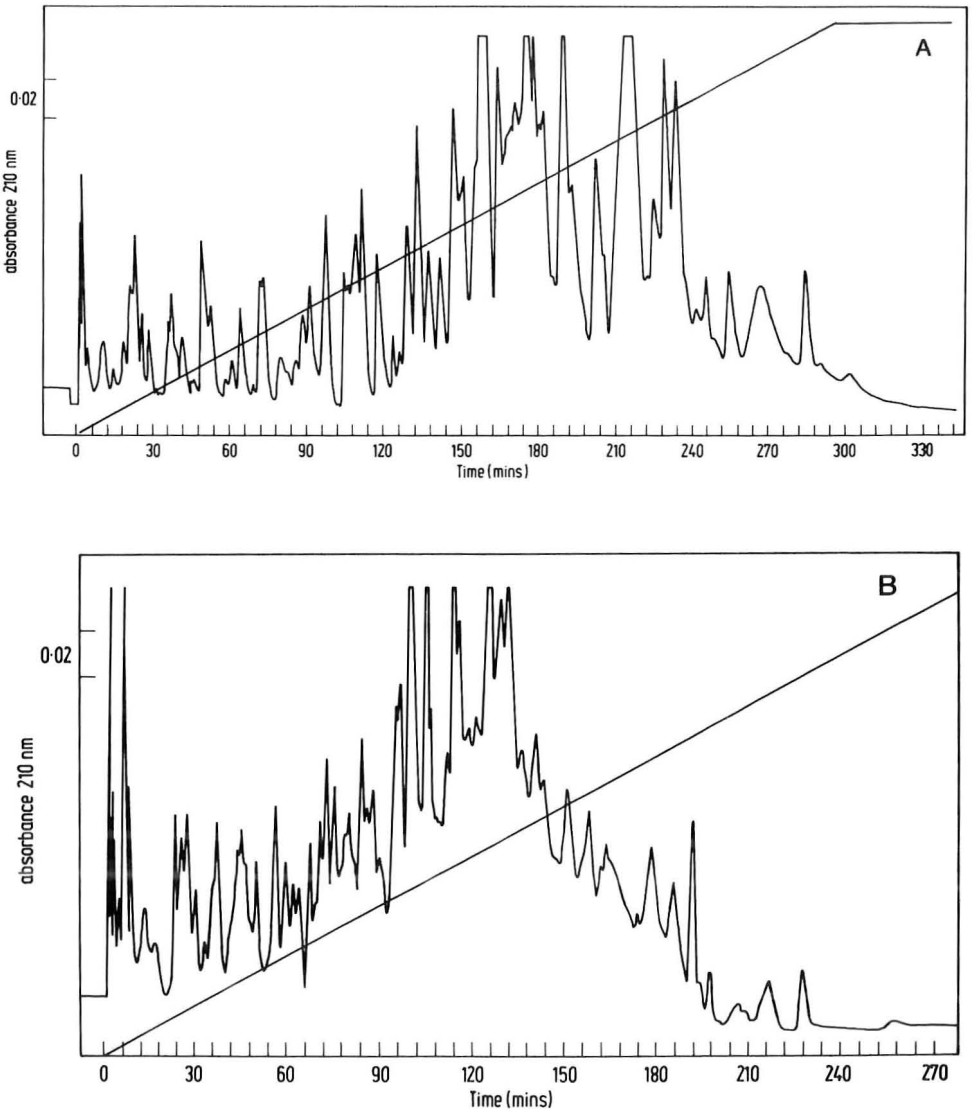


Figure 7. Gradient elution profiles for the tryptic peptides of human thyroglobulin 19S iodoprotein (A) and guinea pig thyroglobulin 19S iodoprotein (B) chromatographed on Radial Pak A/C_{18} columns at a flow rate of 1.5ml/min. A linear 5h. gradient was employed in each case using the mobile phase conditions given in the legend to Fig. 4.

distinction can be made with these other types of bonded reversed phase silicas in so far that hydrophobic peptides tend to be eluted from the Radial Pak A/C_{18} support with mobile phases of lower organic solvent content. With mobile phases containing volatile buffers such as triethylammonium formate or ammonium bicarbonate, good selectivity and recoveries were obtained. Shallow gradients were preferred for the separation of larger polypeptides or complex mixtures derived from the enzymatic digestion of proteins. Because higher flow rates can be used with these flexible-walled columns than is the case with stainless steel columns, re-equilibration times were significantly shorter. No significant loss of resolution was noted with the Radial Pak A/C_{18} support, packed into 10 x 0.8cm cartridges, for sample loadings between one and two orders of magnitude greater than those typical for standard (25 x 0.4cm) stainless steel HPLC columns. For example, the semi-preparative capability of the radially-compressed chromatographic system permitted 10-100nmoles per injection of the thyroglobulin tryptic digests to be separated with excellent resolution and recovery in ca 6h.

ACKNOWLEDGEMENTS

This investigation was supported in part by the Medical Research Council of New Zealand. B. Grego is a recipient of a University Grants Committee Postgraduate Scholarship. We also wish to acknowledge grant assistance from Waters Assoc. (Australia) to M.T.W. Hearn.

REFERENCES

1. M.T.W. Hearn, J. Liquid Chromatogr., 3, 1255, 1980.

2. M.T.W. Hearn in 'Advances in Chromatography' (J.C. Giddings, P.R. Brown, E. Grushka and J. Cazes, eds.) Marcel Dekker, New York, N.Y., vol. 20, in press.

3. S. Stein in 'The Peptides' (E. Gross and J. Meienhofer, eds.) Academic Press, New York, N.Y., in press.

4. M.T.W. Hearn, in 'HPLC, Advances and Perspectives' (Cs. Horvath, ed.) Academic Press, New York, N.Y., vol. 3, in press.

5. M. Rubinstein, S.C. Kiang, S. Stein and S. Udenfriend, Anal. Biochem., 95, 117, 1979.

6. C.A. Bishop, D.R.K. Harding, L.J. Meyer, W.S. Hancock and M.T.W. Hearn, J. Chromatogr., 192, 222, 1980.

7. C.A. Bishop, L.J. Meyer, D.R.K. Harding, W.S. Hancock and M.T.W. Hearn, J. Liquid Chromatogr., 4, in press.

8. M.T.W. Hearn, C.A. Bishop and W.S. Hancock, J. Chromatogr., 157, 337, 1978.

9. J.C. Sauer, Org. Synth. Coll. Vol., 4, 560, 1963.

10. L.J. Durham, D.J. McLeod and J. Carson, Org. Synth. Coll. Vol., 4, 555, 1963.

11. G. Salvatore, M. Salvatore, H.J. Cahnman and J. Robbins, J. Biol. Chem., 239, 3267, 1964.

12. A.J. Paterson and M.T.W. Hearn, Aust. J. Exp. Biol. Med. Sci. 57, 641, 1979.

13. M.T.W. Hearn, G.S. Bethell, J.S. Ayers and W.S. Hancock, J. Chromatogr., 185, 463, 1979.

14. A. Haeberli, J. Bilstad, H. Edelhoch and J.E. Rall, J. Biol. Chem., 250, 7294, 1975.

15. G.E. Chapman, A.G.C. Renwick and J. H. Livesey, personal communication.

16. R. Lumley Jones, G. Benker, P.R. Salacinski, T.J. Lloyd and P.J. Lowry, J. Endocr., 82, 77, 1979.

17. P.G. Stanton, B. Grego and M.T.W. Hearn, manuscript in preparation.

18. M.T.W. Hearn and B. Grego, J. Chromatogr., 203, 349, 1981.

19. A.I. Chernoff and J.C. Liu, Blood, 17, 54, 1961.

20. J.I. Thorell and B.G. Johansson, Biochim. Biophys. Acta, 251, 363, 1971.

21. B. Grego and M.T.W. Hearn, Chromatographia, in press.

22. K.E. Bij, Cs. Horvath, W.R. Melander and A. Nahum, J. Chromatogr., 203, 65, 1981.

23. M.T.W. Hearn and B. Grego, J. Chromatogr., in press.

24. C. Hunter, K. Sugden and J.G. Lloyd-Jones, J. Liquid Chromatogr., 3, 1335, 1980.

25. J.H. Knox and J.F. Parcher, Anal. Chem., 41, 1599, 1969.

26. J.H. Knox, G.R. Laird and P.A. Raven, J. Chromatogr., 122, 129, 1976.

27. C.H. Eon, J. Chromatogr., 149, 20, 1978.

28. C.H. Eon and P. Sharrock, J. Liquid Chromatogr., 2, 485, 1979.

29. W.S. Hancock, J.D. Capra, W.A. Bradley and J.T. Sparrow, J. Chromatogr., in press.

30. J. Rivier, J. Liquid Chromatogr., 1, 347, 1978.

31. S.P. Assenza and P.R. Brown, J. Liquid Chromatogr., 3, 41, 1980.

32. C. Schwabe, J.K. McDonald and B.G. Steinetz, Biochem. Biophys. Res. Commun., 75, 503, 1977.

33. W.S. Hancock, C.A. Bishop, R.L. Prestidge and M.T.W. Hearn, Anal. Biochem., 89, 203, 1978.

34. M.T.W. Hearn, B. Grego and W.S. Hancock, J. Chromatogr., 185, 429, 1979.

35. C.A. Bishop, W.S. Hancock, S.O. Brennan, R.W. Carrell and M.T.W. Hearn, J. Liquid Chromatogr., 4, 4, 1981.

36. M.T.W. Hearn, B. Grego and G.E. Chapman, manuscript in preparation.

37. A.A. Hobbs, B. Grego, M.G. Smith and M.T.W. Hearn, J. Liquid Chromatogr., 4, 513, 1981.

38. A.J. Paterson, B. Grego and M.T.W. Hearn, submitted for publication.

39. P. de Nayer and G. Vassart in 'The Thyroid Gland' (M. de Visscher, ed.) Raven Press, New York, N.Y., 1980, p.21-37.

40. M.J. Spiro, Biochem. Biophys. Res. Comm., 77, 874, 1977.

41. S.J. Sue, B. Grego and M.T.W. Hearn, J. Liquid Chromatogr., 4, in press.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1745-1764 (1981)

ANALYSIS OF GROUP RETENTION CONTRIBUTIONS FOR PEPTIDES SEPARATED BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

Suew-Jane Su, Boris Grego, Brian Niven
and Milton T.W. Hearn†
Immunopathology Research Unit and
Biometrics Unit,
University of Otago Medical School,
P.O. Box 913, Dunedin,
New Zealand.

ABSTRACT

Within the framework provided by solvophobic theory, selectivities for unprotected peptides separated on fully porous, microparticulate, chemically bonded alkylsilicas can be ascribed to differences between the effective hydrophobic contact areas of the solutes. Furthermore, this theoretical treatment predicts that retention behaviour differences can be evaluated from topological parameters which accomodate the influence of amino acid side chain and end group contributions in the retention process. With data obtained for 57 peptides, including a variety of peptide hormones, eluted under the same conditions from a μBondapak C_{18} column, these predictions have been rigorously tested using two methods of numerical analysis. The results provide further evidence that the hydrophobic group retention contributions of the amino acid residues in small peptides have an essentially additive effect on peptide retention with alkylsilicas. Divergences in retention

---

* High Performance Liquid Chromatography of Amino Acids, Peptides and Proteins, XXXVII. For the previous paper in the series see ref. [1].

† Author to whom reprint requests should be sent at St Vincent's School of Medical Research, Victoria Parade, Melbourne, Victoria, 3065, Australia.

behaviour are interpreted in terms of specific silanophilic and solvation interactions.

INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) has become firmly established as a powerful technique for the analysis and isolation of underivatised peptides [2,3]. This technique predominantly depends upon the hydrophobic expulsion of ionised peptidic solutes from polar mobile phases with the concommitant adsorption onto the surface of a non-polar stationary phase. Under these chromatographic conditions, peptides are retarded to different extents depending on their intrinsic hydrophobicities, the elutropicity of the mobile phase and the nature of the hydrocarbonaceous stationary phase. Because of their favourable mechanical and chemical characteristics, the fully porous, microparticulate chemically bonded alkylsilica supports have attracted most attention as chromatographic packings in RP-HPLC separations of peptides. In order to accomodate the great structural diversity which peptides can exhibit, a large variety of mobile phase combinations have been developed. By suitable manipulation of the mobile phase conditions, precise control over the chromatographic distribution processes can be achieved. The effect of organic solvent modifiers and the participation of secondary chemical equilibria including ionisation, pairing ion and solvation effects, on peptide retention to alkylsilica supports have received much detailed attention [2,3]. It has become apparent from these studies that the nature and relationship of the amino acid side chains to ionised centres have dominant influences on the retention behaviour of peptides with alkylsilicas under elution conditions which involve aquo-organic solvent mobile phases of high to intermediate water content covering the range pH 2.0-7.0.

Considerable success has been achieved with the prediction of retention behaviour of neutral and polar solutes, such as benzene derivatives and weak organic acids and bases, on reversed phases from quantitative estimates of solute hydrophobicities. In many cases, these estimates have been based on such topological indices as the Hansch π constants derived from classical n-octanol/water partition coefficients and related parameters. With homologous peptides it has been noted [2,4-6] that the retention behaviour appears to follow that predicted on the basis of the summated hydrophobic contribution from each amino acid side chain. For example, Molnar and Horvath have demonstrated [4] a linear dependency exists between $\ell n\ k'$ and the number of residues for alanine oligomers. With most other peptides, such approaches have met with more limited successes presumably due to the participation of secondary conditional effects in the retention process. It is apparent from data presented [2,4-11] from several laboratories that with some structurally unrelated peptides the retention order can predominantly be equated with the amino acid composition. However with peptide positional isomers and analogues, subtle selectivity deviations have been described which cannot simply be accomodated in terms of the summated hydrophobic contribution of each amino acid residue. Anomalies of this type have been attributed to polar interactions between the peptide and the stationary phase, competing protic or pairing ion dissociation equilibria, hydrogen bonding interactions and conformation effects. Despite their obvious limitations, tables of retention coefficients and group contributions have recently been used [5,7,9] to predict peptide retention, in some cases with surprisingly high correlation between the actual and the predicted retention orders for peptides up to ca 20 residues. In most earlier studies, the procedures used to derive individual amino acid group retention contribution values have been based [6,12,13]

on repetitive regression analysis associated with forcing routines. The basis of these calculations assumes that peptide retention can be described solely in terms of ideal reversed phase behaviour. The purpose of this paper was to examine more closely some of the assumptions used in the compilation of amino acid retention coefficients from RP-HPLC data for peptides. To this end, we have applied two methods of numerical analysis, using chromatographic data accumulated for various peptides, to assess the reliability of such approaches in the prediction of retention behaviour of peptides on silica-bonded non-polar stationary phases.

MATERIALS AND METHODS

The HPLC system was assembled from modular components and consisted of two Model 600A solvent delivery pumps, a M660 solvent programmer, a U6K universal chromatographic injector and a Model 450 variable wavelength UV detector, all from Waters Assoc., (Milford,Mass, U.S.A.) and a Rikadenki dual channel recorder. Sample injections were made with Microliter #810 Syringes from Hamilton Co., (Reno, Nev., U.S.A). The sources and characterisation of the peptides used in this study have been given previously [5]. All amino acids except glycine were of the L-configuration. All solvents and chemicals were AnalaR grade, water was de-ionised by reverse osmosis (Milli-Q) and double distilled. All chromatograms were carried out at ambient temperature (ca 18^{o}). The peptides were chromatographed with linear gradinets of acetonitrile (0.83% per min.) commencing with 50mM sodium dihydrogen phosphate-15mM orthophosphoric acid (pH 2.65) at 0 min. after injection. The final elution condition was 50% acetonitrile-50% water-50mM sodium dihydrogen phosphate-15mM orthophosphoric acid. The μBondapak C_{18} column was equilibrated to initial conditions for at least 30min. following a gradient elution experiment. The flow rate was

1.0ml/min. Sample sizes varied between 5 and 10μg peptide material injected in volumes of 5-10μl. The relative capacity factors for gradient elution experiments were calculated in the usual way using $NaNO_3$ to calibrate the column void time.

A Burroughs 6700 computer was used to analyse retention coefficients of amino acids. Programme 1, written in Pascal language, was used to perform repetitive regression analysis via a forcing routine. Programme 3, written in Algol language, was used to perform a mathematical analysis by solving linear equations. A subroutine (S/LINEANIMPRV, Burroughs 6700 numerical analysis programme library) written in Fortran was included in Programme 3 to solve linear equations by Gaussian elimination with partial pivoting. Programme 2, written in Pascal was used to convert the input file of programme 1 to the input file for programme 3. Therefore only one input file is needed for these two different methods of analysis.

RESULTS AND DISCUSSION

Theoretical Considerations.

In most previous RP-HPLC studies, the chromatographic process has been viewed as a series of reversible associations between the solute molecules, S_1, S_2 ... S_n and the hydrocarbonaceous ligand, L. In the absence of electrostatic or hydrogen bonding effects, the solute-stationary phase interactions will thus be characteristed by a set of equilibrium association constants, K_1, K_2, ... K_n with solute retention determined solely be the nature of the solvophobic solute-ligand associations. Solute retention in RP-HPLC is usually expressed in terms of the capacity factor, k', which is proportional to the equilibrium association constant, such that in the general case the capacity factor for the solute, S_i, is given by

$$k_i' = \psi \cdot K_i \qquad \ldots.(1)$$

$$\text{where} \quad K_i = \frac{[S_i L]}{[S_i][L]} \qquad \ldots.(2)$$

and ψ is the phase ratio, (volume stationary phase)/(volume mobile phase).

Under conditions of ideal linear liquid-liquid chromatography, column selectivity between two peptides, S_i and S_j, can be expressed as

$$\ell n\ \alpha_{i,j} = \ell n\ k'_i/k'_j \qquad \ldots.(3)$$

Since the separation of peptides by classical liquid-liquid partition chromatography and RP-HPLC has a similar physico-chemical basis, it is thus possible to relate selectivities to both the partition and the association coefficients, i.e.

$$\ell n\ \alpha_{i,j} = \ell n\ P_i/P_j = \ell n\ K_i/K_j \qquad \ldots.(4)$$

where P_i and P_j are the partition coefficients and K_i and K_j the association coefficients of the peptides, S_i and S_j, respectively for a particular mobile phase-stationary phase combination. The liquid-liquid partition model presupposes that the bonded hydrocarbonaceous ligand acts as a bulk liquid. As has been pointed out in several studies [14-16], bonded monolayers of octyl- or octadecyl-phases differ from ideal liquids due to the relatively ordered nature of the alkyl chains. However, determination of the Langmuir adsorption isotherms for a number of organic solvent-water systems in contact with hydrocarbonaceous phases has shown [17-19] that the organic solvent is distributed between the mobile and stationary phases. The non-polar phase thus takes on the characteristics of a dynamically coated support which bears a close surface similarity to nonbonded, physically coated classical liquid-liquid partition chromatographic systems.

If we consider two peptides of similar sequence differing only by one amino acid residue, then the group retention contribution τ due to the different amino acid can be defined as

$$\tau = \ln \alpha_{i,j} \qquad \ldots.(5)$$

where α is the selectivity coefficient of the two peptides, S_i and S_j. The τ contribution is thus a function of the differences in the overall standard unitary free energy changes associated with the transfer of the peptide solutes from the mobile to the stationary phase. Solvophobic theory, as elaborated by Sinanoglu and coworkers [20], has been successfully adapted by Horvath et al. [21] to permit an evaluation of these free energy terms. According to this approach, the surface area, ΔA_s, of the solute molecule in contact with the non-polar stationary phase plays a significant role in determining the magnitude of the hydrophobic interactions. Since linear free energy relationships are also anticipated between bulk phase partition parameters and functional group contributions, linear relationships should exist between retention behaviour, as expressed by $\ln k'$ values, and the hydrocarbonaceous surface areas of the solutes. Under a defined set of chromatographic conditions selectivity can be evaluated from an analysis of the differences in effective hydrophobic contact areas, i.e. from an analysis of the $\Delta(\Delta A_s)$ terms. Experimental studies with non-polar solutes, weak acids and weak bases have generally been in good agreement with these theoretical considerations. For example, RP-HPLC studies with benzoic acids have revealed [22] a linear dependence of selectivity parameters on topological indices, the general form of this relationship being

$$\ln \alpha_{i,j} = a\rho_{i,j} + b \qquad \ldots.(6)$$

where $\rho_{i,j}$ is an appropriate functional group contribution such as the Hansch π terms, the Rekker hydrophobicity fragmental constants or analogous terms. The retention of amino acids and small peptides appears [23] to follow similar dependencies when eluted isocratically from alkylsilicas with low pH mobile phases.

In many cases, the separation of complex mixtures of peptides by RP-HPLC requires gradient elution conditions. Over the range 0-100% organic solvent modifier, binodal dependencies of $\ell n\ k'$ values for peptides and polypeptides on the volume percentage of the organic solvent have been observed [24-26]. Over the operational limits commonly employed in gradient elution of peptides from alkylsilicas with such solvent modifiers as acetonitrile or 2-propanol, i.e. up to ca 50% organic solvent content, the dependence of $\ell n\ k'$ on the volume fraction, ψ, can however be approximated to a linear relationship [2,5,9]. As a consequence, under linear solvent strength gradient elution conditions encompassing this restricted range of mobile phase compositions, the solvophobic model anticipates linear increases in k' (apparent) as amino acid residues are added in an ordered manner to a homologous peptide series, i.e. the k' (apparent) value of a peptide can be expressed in terms of summated group contributions such that

$$k'_{i,app} = \Sigma^{n} c_n \chi_n + d \qquad \ldots(7)$$

where c_n is a numerical factor indicating the incidence of a given fragment in the structure and χ_n represents the group retention contribution due to amino acid, n. In the ideal circumstance when only solvophobic interactions mediate the retention process the intercept term, d, should have the value zero. When the sorption process involves competing polar and non polar equilibrium interactions, the intercept term, d, may

diverge from zero. Curvilinear relationships between k'_{app} (actual) and k'_{app} (predicted) will also result in these circumstances. Using data obtained with 72 different solutes (Table 1), chromatographed on the same μBondapak C_{18} column under identical elution conditions, the assumptions explicit to the solvophobic model have been examined using two methods of numerical analysis, namely multiple regression via an iterative forcing procedure and by a mathematical routine for solving linear equations. Both these methods of numerical analysis can be expanded to include memory parameters which accomodate amino acid residue triplet orders and hence recognise sequence features. Since these expanded subroutines would require for the common protein amino acids at least 9261 data points, we have limited the present analysis to non-isomeric peptides.

Forcing Approach.

The statistical framework of multiple regression analysis with forcing, i.e. the introduction of a few arbitarily chosen data points such that the intercept term, d, has a negligible value and the predicted and actual values are equal, has previously been applied [12,13,22] for the evaluation of sets of equations of the type of eqn. 7 to obtain the desired set of hydrophobic group contribution coefficients for polar solutes. Meek has used [6] this approach to derive retention coefficient values for peptides chromatographed on a BioRad ODS support. In the present study, the starting values for the group retention contribution, χ_n, for the 26 amino acids and end groups were obtained by plotting the k' (apparent) values of oligomeric peptides versus the number of residues, n. The slope of these plots equals the amino acid group retention contribution per residue, the intercept at n=0 represents the end group contribution. In other cases, starting values of the group retention contribution were computed from data reported

TABLE I.

Linear Sequences of Peptides used in the Present Study*

1. LW
2. LWMRF
3. LWMR
4. LWM
5. RF
6. RFA
7. MRF
8. MRFA
9. AY
10. PY
11. LY
12. VY
13. YYY
14. AK
15. YTPKA
16. KG
17. AG
18. GV
19. GF
20. VL
21. FA
22. GLY
23. VV
24. DRVYIHPFHL
25. DRVYIHPF
26. GIVEQCCASVCSLYQLENYCN
27. FVNQHLCGSHLVEALYLVCGERGFFYTPKA
28. FVQWLMNT
29. GLA
30. HSQGTFTSDYSKYLDSRRAQDFVQYLMNT
31. FPTIPLSR
32. LFDNAMLR
33. AHR
34. LHQLAFDTYEEFEEAYIPK
35. EQK
36. YSFLQDPETSLCFSESIPTPSNRNYGLLYCFR
37. EETQK
38. SNLQLLR
39. ISLLLIQSWLEPVEFLR
40. SVFANSLVYGASNSDVYDLLK
41. DLEEGIETLMGR
42. LEDGSPR
43. TGQIFK
44. QTYSK
45. ETYSK
46. FDTNSHNDDALLK
47. DMDKVETFLR
48. IVQCRSVEGSCGF
49. LHQLAFDTYEEFDPETSLCFSESIPTPSNRNYGLLYCFR
50. DRVYIHP
51. FF
52. FFF
53. FFFF
54. FFFFF
55. YGGFLTSEKSQTPLVTLFKNAIIKNAHKKGQ
56. YGGFMTSEKSQTPLVTLFKNAIIKNAHKKGQ
57. YGGFMTSEKSQTPLVTL

* The one letter code for the amino acids is as given by Dayhoff in Atlas of Protein Sequence and Structure (National Biomedical Research Foundation, Silver Spring, Md., U.S.A., 1972), see also Table II. Also included in the compilation of χ_n values were additional chromatographic data for 15 amino acids and homologues.

by Meek [6,7]. With the set of peptides listed in Table I, the predicted k´ (apparent) values were then calculated by summing the group retention contribution for each amino acid and end groups. After calculating the correlation coefficient between the predicted and observed k´ (apparent) values, 0.2 (or any other value which can be specified in the imput of programme 1) was added to the χ-value of an amino acid or end group. The predicted k´ (apparent) values of all peptides were then recalculated and new correlation coefficients between these and observed k´ (apparent) values were again calculated. If the new correlation coefficients improved, i.e. greater than the correlation coefficient before modification, then 0.2 (or another appropriate value) was added to the χ-value of that amino acid or end group, otherwise the orginal χ-value was kept. After all 26 amino acids and end groups were tested to see whether modification of the original χ-value was needed, 0.2 (or another appropriate value) was sequentially subtracted from each amino acid χ-value in turn and correlations were again calculated after each substraction to decide whether the change was needed. These two cycles were repeated for the times specified in the input of programme 1, which in general, involved at least 20 repeat entries. At the end of the cycling procedure the slopes of the plots of k'_{app} (observed) versus k'_{app} (predicted) were calculated, the χ-values normalised [6] and the above cycles repeated until optimal correlation was obtained. Fig. 1, shows the plot of k'_{app} (observed) versus k'_{app} (predicted) for the peptide data analysed in this way.

Multiple Linear Equation Approach.

In this approach the group retention contribution of each amino acid is treated as an unknown x_i. Peptides with observed retention times and known amino acid compositions are listed as a series of equations by the computing programme 2 in the form

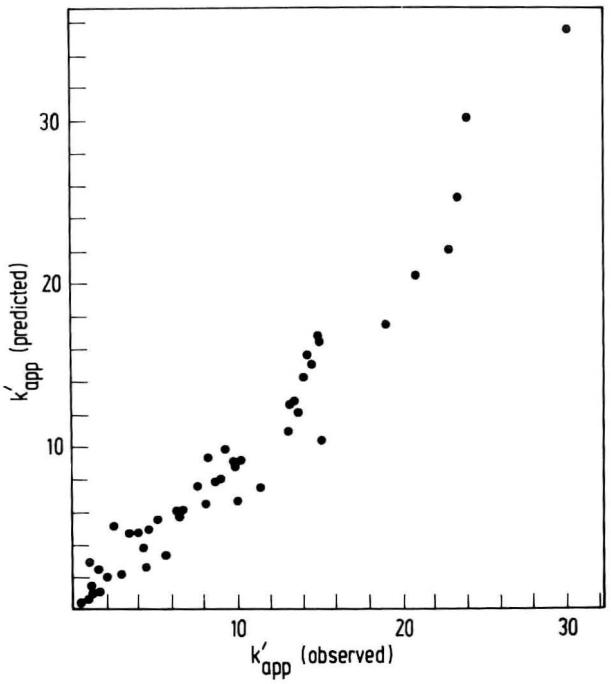


Figure 1. Correlation of observed k´ (apparent) versus k´ (apparent) predicted from the summation of the group retention contributions for amino acids and end groups. Values for the group retention contributions were computed via a forcing approach using chromatographic data for peptides listed in Table 1.

$$a_{11}x_1 + a_{12}x_2 + \ldots + a_{1n}x_n = b_1$$

$$a_{21}x_1 + a_{22}x_2 + \ldots + a_{2n}x_n = b_2$$

$$\vdots$$

$$a_{m1}x_1 + a_{m2}x_2 + \ldots + a_{mn}x_n = b_n$$

Computing programme 3 rewrites these equations in a more usual mathematical form: AX=b where

$$A = \begin{bmatrix} a_{11} & a_{12} & \cdots & a_{1n} \\ & \vdots & & \\ a_{m1} & a_{m2} & \cdots & a_{mn} \end{bmatrix}$$

$$X = \begin{bmatrix} x_1 \\ \vdots \\ x_n \end{bmatrix}$$

$$b = \begin{bmatrix} b_1 \\ \vdots \\ b_n \end{bmatrix}$$

Here we have more equations than the number of unknowns, therefore a unique solution will not usually exist. Because the observed retention times are subject to experimental errors, instead of choosing a particular number, n, from this set of equations, the method of least squares was applied to find a solution. This method uses all the information in the complete set of equations. The solution is obtained by solving $(A^TA)x = (A^Tb)$, which is a set of linear equation with n equations and n unknowns [26]. These equations can then be solved by standard procedures, e.g. by the Gaussian elimination procedure. Fig. 2 shows the plot of k'_{app} (observed) versus k'_{app} (predicted) using the calculated χ_n values for the amino acids and end groups obtained by this procedure.

Comparison of Predicted Versus Observed Retention Behaviour of Peptides.

Several studies have concluded [2,4,5] that the peptide chain proper makes only a very small contribution to the retention process for peptides on reversed phases eluted under

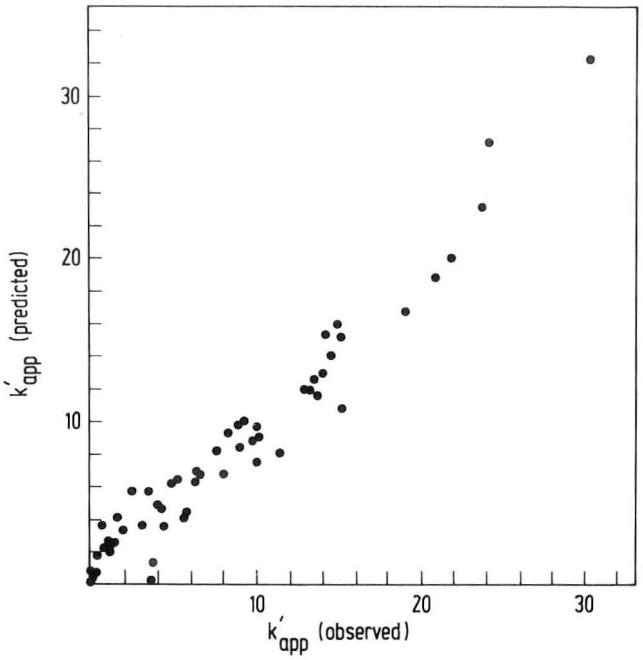


Figure 2. Correlation of observed k´ (apparent) versus k´ (apparent) predicted from the summation of the group retention contributions for amino acids and end groups. Values for the group retention contributions were computed via the multiple linear equation approach using chromatographic data for peptides listed in Table 1.

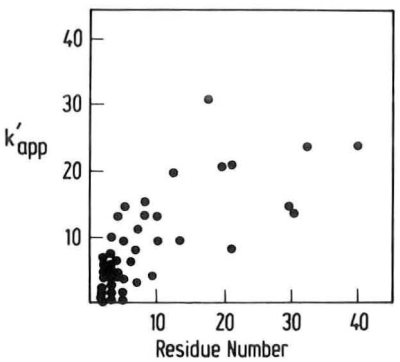


Figure 3. Plot of observed k´ (apparent) versus number of amino acid residues.

low pH conditions with aquo-organic solvent eluents. Furthermore, it has been concluded [2,5,28] that peptide size, per se, has little effect on retention which depends rather on the effective hydrophobic contact area. The trends evident from the data shown in Figs. 1-3 provide further support for these conclusions and the corollary that retention behaviour of a particular peptide follows the extent of its ionisation. For example, as the pH of the mobile phase was increased over the range pH 2.5-7.0 the retention of peptides rich in acidic amino acid residues, e.g. peptides 37, 41, tended to decrease whilst peptides rich in basic amino acid residues, e.g. peptides 6, 30, tended to have increased k′ (apparent) values. Because peptides containing basic amino acid residue experience diminished retention with phosphate mobile phases of low pH, it is likely that the phosphate ion acts via hydrophilic pairing ion effects augmented by the usual dependency of capacity factor on ionic strength.

As is evident from Fig. 1 and 2, a high degree of correlation is obtained between k'_{app} (observed) and k'_{app} (predicted) using the values of χ_n (Table II) derived by both methods of numerical analysis. It is apparent from these and associated studies reported by Meek and coworkers [6,7] that amino acid side chain hydrophobicities are the major solute factor in determining peptide retention to alkylsilicas with low pH eluents containing hydrophilic buffer ions. However, as can be seen from Figs. 1 and 2, and as manifested by the correlation coefficients of 0.973 and 0.972 obtained by the forcing and the multiple linear equation approaches, the fit of the data points to a straight line falls short of the expectations based solely on the solvophobic model. Several of the assumptions made in these calculations may be responsible for the observed divergence from the predicted linear relationships. For instance, no correlation terms have been included in the

TABLE II.

Group Retention Contributions for Amino Acids Residues

Entry	Code	Name	Frequency	χ_n(forcing)	χ_n(solving)
1.	A	Ala	23	-0.296	-0.121
2.	B	nor Val	-	-	-
3.	C	Cys	11	-1.500	-1.252
4.	D	Asp	20	2.171	1.918
5.	E	Gln	28	0.454	0.442
6.	F	Phe	47	2.782	2.522
7.	G	Gly	27	-0.770	-0.816
8.	H	His	13	-3.276	-2.673
9.	I	Ile	17	6.196	5.800
10.	J	nor Leu	-	-	-
11.	K	Lys	24	-0.405	-0.532
12.	L	Leu	53	3.424	3.160
13.	M	Met	9	3.562	3.566
14.	N	Asn	19	-1.687	-1.757
15.	O	hydroxy Pro	-	-	-
16.	P	Pro	19	-0.306	-0.396
17.	Q	Glu	22	-0.651	-0.764
18.	R	Arg	21	-1.184	-1.370
19.	S	Ser	36	0.405	0.664
20.	T	Thr	28	-0.987	-0.790
21.	U	pGlu	-	-	-
22.	V	Val	25	1.253	1.079
23.	W	Trp	2	-0.099	-0.278
24.	X	homo Ser	-	-	-
25.	Y	Tyr	34	1.145	0.896
26.	Z	hydroxy Lys	-	-	-
27.	@ [	NH_2, COOH end group	57	0.829	1.537

calculations to accomodate differences in specific electrostatic and hydrogen bonding interactions which are known to arise during the distribution of ionised peptides between polar mobile phases and hydrocarbonaceous silicas. The heterogeneity of the stationary phase surface of alkyl-bonded silicas has been examined [24,25] in several detailed investigations on peptide selectivity. Even with well 'capped' alkylsilicas of high carbon coverage, unprotected peptides show dual retention behaviour typified by the concave binodal dependence of $\ell n\ k'$ on the volume fraction of water in the mobile phase. This dual retention behaviour has been attributed to composite solvophobic-silophilic interactions. In addition, specific solvation effects dependent on the nature and concentration of the organic solvent modifier can lead [5,9] to individual selectivity divergencies for some peptides. From the scattering of the data points, it is unlikely that these electrostatic, hydrogen bonding or solvation components in the sorption process remain constant for different peptides. A further assumption, as yet little discussed in the literature on peptide separation by gradient elution RP-HPLC, relates to the nature of the gradient shape. For linear solvent strength gradient elution, it is assumed that the plots of $\ell n\ k'$ for each solute S_i, S_j ... versus separation time, t, after the start of the gradient will be linear and will have the same slope. As is evident from gradient elution studies of tryptic digests of proteins [2,29,30] linearity of $\ell n\ k'$ versus t over a wide range of t cannot always be anticipated with peptides.

The results reported here, nevertheless, give ample evidence that the hydrophobic group retention contribution of the amino acid residues in small peptides have an essentially additive effect on peptide retention to octadecylsilicas. With larger peptides, where secondary and tertiary structural features are likely to be important, greater deviations from

an additive effect can be anticipated. With such solutes, the choice of mobile phase composition will have a profound effect on resolution and sample recovery. For example, it is a common experience with larger or more hydrophobic peptides and polypeptides that efficient chromatography can only be achieved on reversed phase silicas over a very narrow range of organic solvent percentages. Because of the participation of competing retention processes, desorption of the intercalated domains of polypeptides and proteins from the solvated surface of the stationary phase will only be possible with mobile phases of sufficient elutropic strength to overcome the sum of the hydrophobic interactions without augmenting polar solute-stationary phase interactions. Gradient elution conditions can generally be chosen to satisfy this requirement even with macroglobulins which show extreme dependencies of $\ell n\ k'$ on ψ. In an associated study, we have applied these RP-HPLC procedures, and attendant methods of selectivity analysis, to the separation of proteins, including thyroglobulins and protein hormones.

ACKNOWLEDGEMENTS

This work was supported in part by a grant made by the Medical Research Council of New Zealand. Boris Grego is a recipient of a University Grants Committee Postgraduate Fellowship.

REFERENCES

1. M.T.W. Hearn and B. Grego, J. Chromatogr., in press.

2. M.T.W. Hearn in Advances in Chromatography (J.C. Giddings, P.R. Brown, E. Grushka and J. Cazes, eds.), Marcek Dekker, New York, N.Y., vol. 20, in press.

3. M.T.W. Hearn, in HPLC, Advances and Perspectives, (Cs. Horvath, ed.) Academic Press, New York, N.Y., vol. 3, in press.

4. I. Molnar and Cs. Horvath, J. Chromatogr., 142, 623, 1977.

5. M.T.W. Hearn and B. Grego, J. Chromatogr., 203, 349, 1981.

6. J.L. Meek, Proc. Natl. Acad. Sci. U.S.A., 77, 1632, 1980.

7. J.L. Meek and Z. Rosetti, personal communication.

8. E.P. Kroeff and D.J. Pietrzyk, Anal. Chem., 50, 1353, 1978.

9. M.J. O'Hare and E.C. Nice, J. Chromatogr., 171, 209, 1979.

10. S. Terabe, R. Konaka and K. Inouye, J. Chromatogr., 172, 163, 1979.

11. B. Larsen, B.L. Fox, M.F. Burke and V.J. Hruby, Int. J. Pept. Protein Res., 13, 12, 1979.

12. V. Pliska and J.L. Fauchere in Peptides, Structure and Biological Function (E. Gross and J. Meienhofer) Pierce Chem. Co., Rockford, Ill., 1979, p.249-252.

13. R.F. Rekker, The Hydrophobic Fragmental Constant, Elsevier, Amsterdam, 1977, p.301-324.

14. L.C. Sander and L.R. Field, Anal. Chem., 52, 2009, 1980.

15. Cs. Horvath and W. Melander, J. Chromatogr. Sci., 15, 393, 1977.

16. C.H. Lochmuller and D.R. Wilder, J. Chromatogr. Sci., 17, 574, 1979.

17. R.M. McCormick and B.L. Karger, Anal. Chem., 52, 2249, 1980.

18. J.H. Knox and A. Pryde, J. Chromatogr., 112, 171, 1975.

19. R.P.W. Scott and P. Kucera, J. Chromatogr., 142, 213, 1977.

20. O. Sinanoglu in Molecular Associations in Biology, (B. Pullman, ed.) Academic Press, New York, N.Y. 1968, p.427.

21. Cs. Horvath, W. Melander and I. Molnar, J. Chromatogr., 125, 129, 1975.

22. C.M. Riley, E. Tomlinson and T.M. Jefferies, J. Chromatogr., 185, 197, 1979.

23. S.J. Su, B. Grego and M.T.W. Hearn, J. Liquid Chromatogr., in press.

24. B. Grego and M.T.W. Hearn, Chromatographia, in press.

25. K.E. Bij, Cs. Horvath, W.R. Melander and A. Nahum, J. Chromatogr., 203, 65, 1981.

26. M.T.W. Hearn and B. Grego, submitted for publication.

27. G. Dahlquist and A. Bjorck in Numerical Methods, Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 1974, p.196-199.

28. M.T.W. Hearn and W.S. Hancock, Trends in Biochem. Sci. 4, 58, 1979.

29. C.A. Bishop, W.S. Hancock, S.O. Brennan, R.W. Carrell and M.T.W. Hearn, J. Liquid Chromatogr., 4, in press.

30. M.T.W. Hearn and B. Grego, unpublished observations.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1765-1775 (1981)

PURIFICATION OF COMMERCIALLY PREPARED BOVINE TRYPSIN BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

M. Patricia Strickler, M. Judith Gemski
and Bhupendra P. Doctor
Division of Biochemistry
Walter Reed Army Institute of Research
Washington, D.C. 20012

ABSTRACT

A reverse phase high performance liquid chromatographic method for the purification of milligram amounts of commercially prepared bovine trypsin is described. Increased specific enzymatic activity is observed in the purified material. The advantages of using purified trypsin in protein digestions as well as the potential application of reverse phase high performance liquid chromatography for enzyme characterization are described.

INTRODUCTION

When using reverse phase high performance liquid chromatography (RP-HPLC) as the method for separating peptide fragments, arising from trypsin digestion, impurities in trypsin can interfere with obtaining a true peptide profile. These impurities which are co-eluted with the peptide fragments, may be detected when large amounts of trypsin are required for complete digestion of protein or when the peptide fragment separation is monitored at high levels of sensitivity.

Separation of peptide fragments is commonly used to "fingerprint" or demonstrate the similiarities or differences in the sequences of a series of proteins.(1) The separation process may also be employed to purify the peptide fragments for further characterization.(2) In either case the presence of trypsin impurities co-eluting with the peptide profile could be misleading.

A method is here-by described for the purification of milligram amounts of commercially prepared bovine trypsin.

MATERIAL AND METHODS[1]

Reagents

TCPK-treated trypsin was purchased from Millipore Corp., Bedford, MA, and DPCC treated trypsin from Calbiochem, La Jolla, CA, and Sigma Chemical Co., St. Louis, MO. Acetonitrile and trifluoroacetic acid of HPLC grade were obtained from Fisher Scientific, Silver Spring, MD. The N α- ρ-Tosyl-L- Arginine methyl ester hydrochloride (TAME) was obtained from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were of analytical reagent grade.

Instrumentation

A Waters Associates, (Milford, MA), liquid chromatograph consisting of two M6000A solvent delivery systems, a M660 solvent flow programmer, a U6K universal injector, a M440 absorbance detector set at 280nm, a M450 variable wavelength detector and a M730 data module was utilized. Enzymatic activity was measured using a Beckman Spectrophotometer UV 5230 equipped with an automatic sample changer.

Chromatographic Procedure

A 30 minute linear gradient was run at a flow rate of 2ml/min on a μ Bondapak C_{18} column (3.9mmx 30cm), Waters Associates. The mobile phase consisted of solution A, 0.1 % aqueous trifluoroacetic acid (TFA) and solution B, acetonitrile-0.1% TFA run from 100% A to 45% B. The column effluent was monitored at 215nm and 280nm. All separations were carried out at ambient temperature.

Enzymatic Activity Procedure

A stock solution of trypsin at a concentration of 5mg/ml was prepared in 0.001N HCl. From this stock 200 μl (0.84mg as calculated by the absorbance at 280nm, E_{280}=14.3) was injected into the liquid chromatograph and eleven fractions were collected as described in the Results and Discussion. After lyophilization the fractions were reconstituted in 1.2ml of 0.001N HCl and the protein concentration of each was calculated as described above. The fractions were then diluted with 0.001N HCl to a concentration range between 10-20 μg/ml. Aliquots (0.3ml) of the substrate TAME, 0.001M in distilled water, and (2.6ml) of 0.046M Tris-HCl buffer, pH = 8.1, mixed with 0.0115M $CaCl_2$ were incubated at 25°C for 3 minutes to establish a baseline at 247nm. At zero time, 0.1ml of the diluted protein fraction was added and reaction rates determined after 3 minutes by the change in absorbance. Activity was calculated in units/mg of protein using the following equation:

$$U/\text{mg protein} = \frac{\Delta A_{247}/\text{min} \times 3 \times 1000}{540 \times \text{mg Trypsin}} \qquad (3)$$

RESULTS AND DISCUSSION

The source of the spurious peaks in the peptide fragment separations was traced to impurities in trypsin by chromatographing solutions of trypsin with concentrations of trypsin equal to that being used in the protein digestions. Three representative commercial solutions of trypsin, at concentrations of 1mg/ml were analyzed by HPLC to detect the presence of impurities. All three samples, exhibited similar impurities as shown in Figures 1, 2 and 3. A

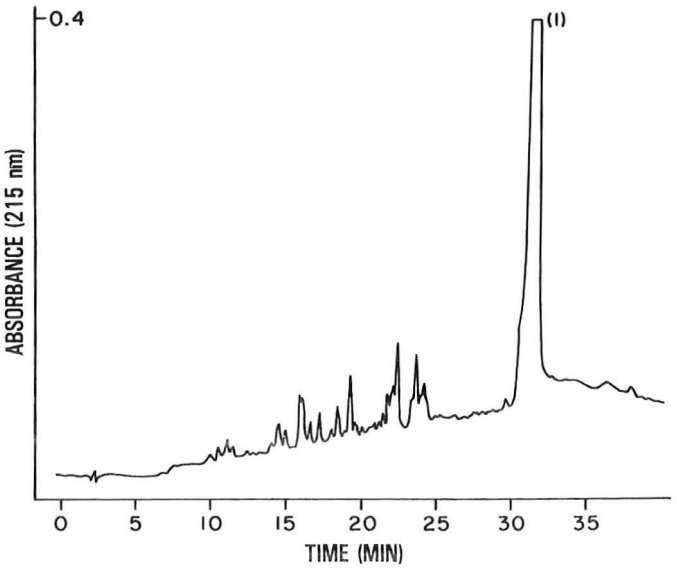


FIGURE 1

Separation of 100 µg of Bovine Trypsin (Millipore), (1) trypsin. Column: µ Bondapak C_{18} (3.9mm x 30cm), Gradient: 30 min., linear. Flow rate: 2.0ml/min. Mobile Phase: 100% aqueous, 0.1% trifluroacetic acid (TFA) to 45% acetonitrile, 0.1% TFA / 55% aqueous 0.1% TFA. Columnm temperature: ambient.

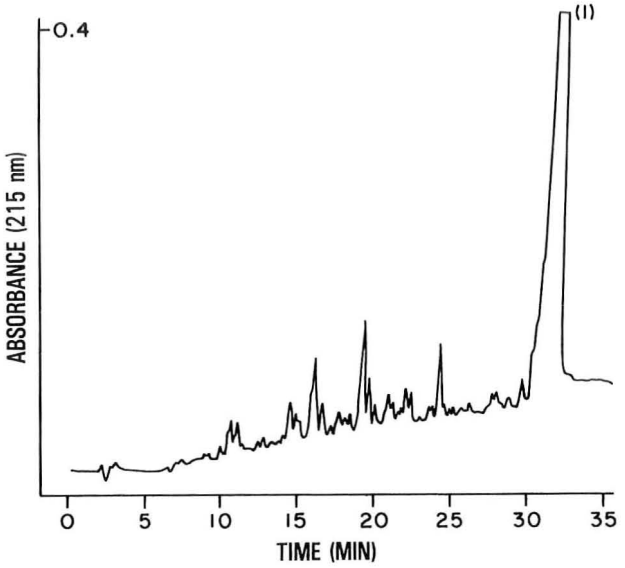


FIGURE 2

Separation of 100 μg of Bovine Trypsin (Sigma), (1) trypsin.

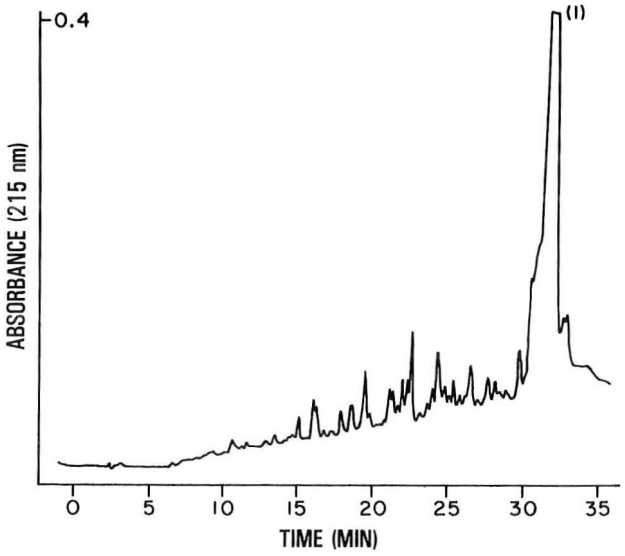


FIGURE 3

Separation of 100 μg of Bovine Trypsin (Calbiochem), (1) trypsin.

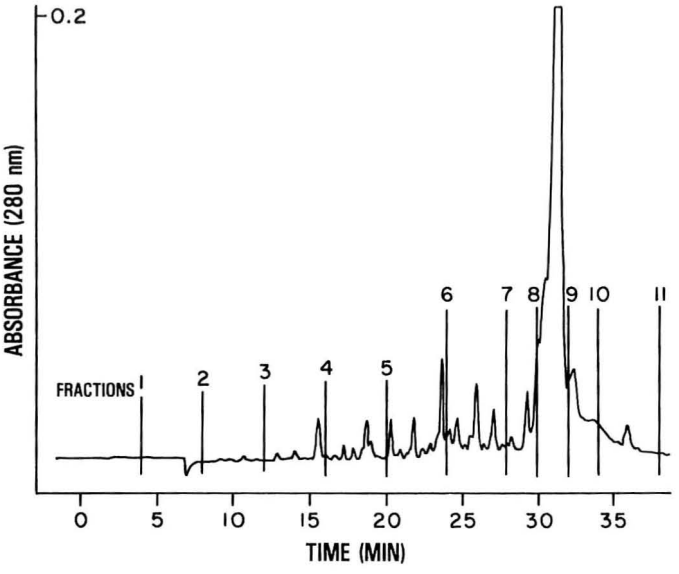


FIGURE 4

Separation of 1mg of Bovine Trypsin (Sigma), collected fractions are indicated by numbers 1-11.

milligram of trypsin (Sigma) was chromatographed (Figure 4) and four milliliter fractions were collected for 28 minutes followed by two milliliter fractions during the next six minutes. After lyophilization and reconstitution the amount of protein in each fraction was calculated using the absorbance at 280nm. The fractions were also assayed for enzymatic activity using the TAME procedure, which is specific for trypsin. The results are summarized in Table 1.

TABLE 1
Protein Concentrations and Enzymatic Activities of the Fractionated Trypsin.

Fraction Number	Time (min)	Concentation (mg/ml calc)	Activity (units/mg protein)
1	4	0.0168	
2	8	0.0133	
3	12	0.0091	
4	16	0.0175	3.7
5	20	0.0231	8.3
6	24	0.0245	
7	28	0.0280	
8	30	0.0637	53.1
9	32	0.2541	151.5
10	34	0.0203	45.1
11	38	0.0147	47.8
Starting sample	———	0.8484	135.8

Fraction 9, which contains the major peak, showed a higher specific activity than the original sample as indicated in Figure 5. A 57% recovery (calculated on the basis of the absorbance at 280nm) was obtained from the chromatographic separation. An aliquot of fraction 9 was rechromatographed to confirm its purification (Figure 6). The purified material used in subsequent digestions gave clean tryptic blank runs.

During the development of the purification method for trypsin several interesting observations were made. A comparison of chromatographic profiles of freshly prepared solutions of trypsin with ones that had been stored showed the major peak, representing trypsin, to change in shape and decrease in area whereas some of the peaks, representing impurities increased in area. Similar

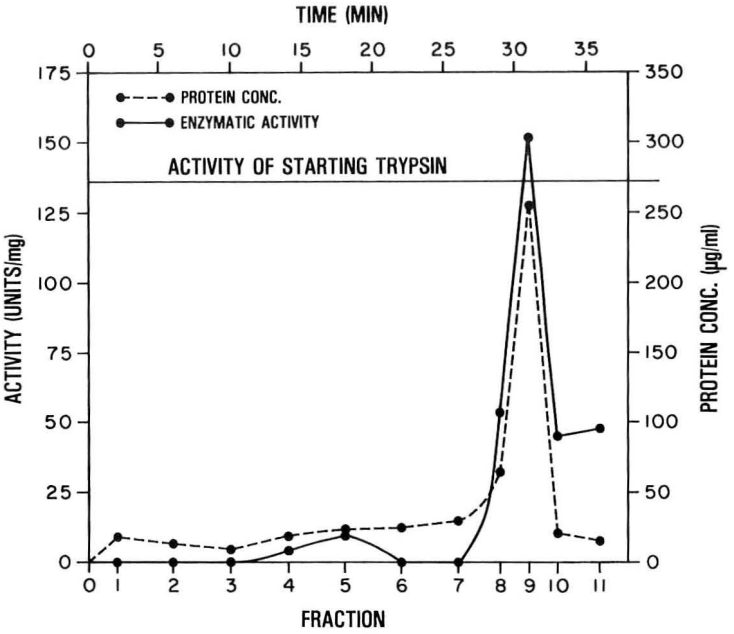


FIGURE 5

Fractionation of Trypsin by HPLC.

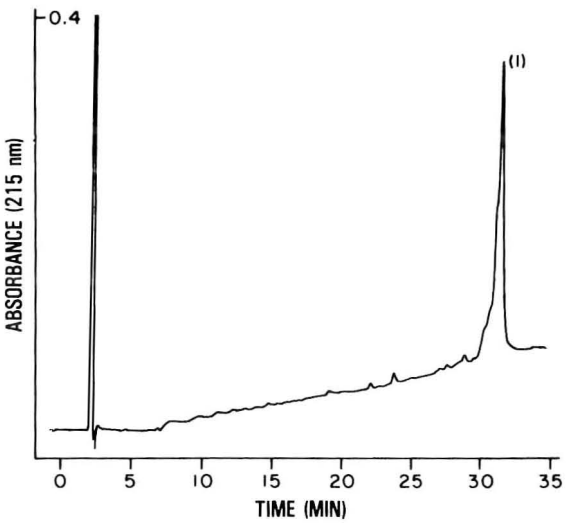


FIGURE 6

Rechromatography of fraction 9, (1) trypsin.

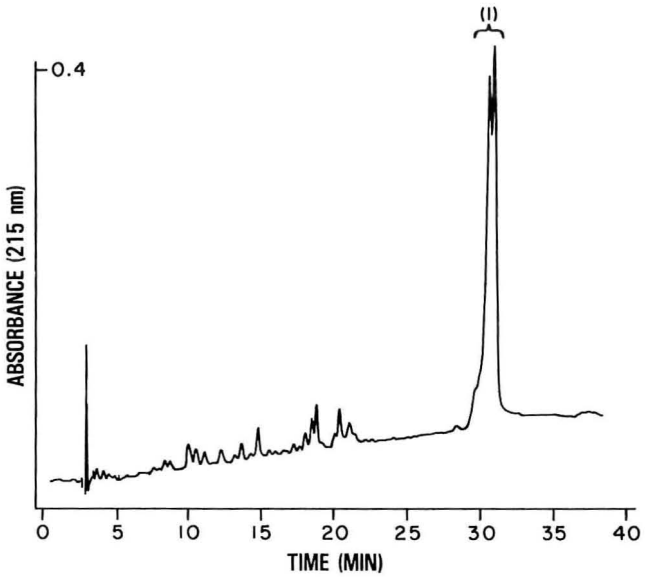


Figure 7

Chromatogram of 50 μg of trypsin (Millipore), (1) possibly the and forms of trypsin. Column: μ Bondapak C_{18} (3.9mm x 30cm), Gradient: 30 min., linear. Flow rate: 1.5ml/min. Mobile Phase: 12% acetonitrile, 0.1% TFA / 88% aqueous, 0.1% TFA to 45% acetonitrile, 0.1% TFA / 55% aqueous, 0.1% TFA. Column temperature: ambient.

chromatographic profiles were also seen in comparing lyophilized samples stored for less than one month to material maintained for four months at 0°C. These observations are consistent with the fact that trypsin not only undergoes autodigestion in solution but in lyophilized samples as well. (4, 5)

The change in shape of the major trypsin peak strongly suggested that this peak was not homogenous. By adjusting the chromatographic conditions, as shown in Figure 7, two distinct peaks were resolved from the single trypsin peak. Multiple forms of trypsin have been separated by others using column chromatography on SE-Sephadex. This technique has demonstrated that the predominant forms, α and β, are always present in commercially available trypsin. (5)

The method described in this paper provides a rapid and simple means of further purifying commercially prepared trypsin. The use of purified trypsin in protein digestions yields a true chromatographic peptide profile. This is especially crucial when large amounts of trypsin are needed for protein digestion or when monitoring at high sensitivities on HPLC. The procedure also demonstrates the potential of RP-HPLC for the final purification of other enzymes as well as the separation of different protein forms and peptide chains.

ACKNOWLEDGEMENTS

We thank Mr. Nesbitt D. Brown and Dr. Richard W. Travis for their technical assistance and Mr. Stewart Galloway for secretarial support.

REFERENCES

1. Hearn, T. W. and Hancock, W. S., Biological/Biomedical Applications of Liquid Chromatography, Hawk, G. L., ed., Marcel Dekker, New York, 1979, p. 243.

2. Fullmer, C. S. and Wasserman, R. H., J. Biol. Chem., 254, 7208, 1979.

3. Decker, L. A., ed., Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, N. J., 1977, p. 221.

4. Perlmann, G. E. and Lorand, L., eds., Methods in Enzymology, Vol XIX, Academic Press, New York, 1970, p. 49.

5. Schroeder, D. D. and Shaw, E., Chromatography of Trypsin and Its Derivatives, J. Biol. Chem., 243, 2943, 1968.

1. The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1777-1795 (1981)

REDUCTIVE MODE THIN-LAYER AMPEROMETRIC DETECTOR FOR LIQUID CHROMATOGRAPHY

Karl Bratin and Peter T. Kissinger
Department of Chemistry
Purdue University
West Lafayette, Indiana 47907

and

Craig S. Bruntlett
Research Laboratories
Bioanalytical Systems Inc.
West Lafayette, Indiana 47906

ABSTRACT

An electrochemical detector for liquid chromatography is described based on the use of a mercury film electrode in a thin-layer cell with the auxiliary electrode placed across the channel from the working electrode. Considerations relevant to optimizing performance of the detector are described including removal of dissolved oxygen from the sample and mobile phase. Detection limits are reported for a number of reducible organic compounds of interest in environmental and biomedical research. Detection limits at the picomole level are readily achieved for easily reduced nitro compounds. Typical applications are illustrated for the determination of parathion in field runoff water and chloramphenicol in human plasma.

INTRODUCTION

Amperometric detectors for liquid chromatography are in widespread use for the trace determination of easily oxidized

organic compounds of environmental, clinical, and pharmaceutical interest. Recent technological advances and applications in this area have been reviewed (1-5). Relatively little attention has been paid to electrochemical detection of easily reducible organic compounds because, until recently, it has been difficult to prepare a reliable mercury electrode, and carbon electrodes have not been seriously considered for this application due to their relatively low hydrogen overvoltage. Progress in this area has been slow due to problems associated with dissolved oxygen and metal ion impurities, but perhaps a more important reason is that many substances which are readily reduced on a mercury-film electrode are also good candidates for detection by optical absorption methods.

Since the introduction of the dropping mercury electrode (DME) for LCEC detection of easily reducible compounds in 1952 (6), several investigators have worked to improve the design of the DME based detector (7-10). These devices have shown only a limited use in a few academic laboratories due to the time dependent surface area, the expense and toxicity of mercury, the poor tolerance to a rapidly moving solution, and awkward cell construction. Improvements in tolerance to a moving solution have been accomplished by reducing the flow rate and by decreasing the drop time of the DME (7,10). This was accomplished at the expense of higher detection limits. Nevertheless, even though the problems are serious, several promising applications of DME-based detectors have recently been reported (11-14). Carbon-based electrode materials including glassy carbon (15), a basal plane pyrolytic graphite (16), and carbon impregnated silicone rubber (17) have been evaluated for reductive LCEC. These materials hold promise for many applications especially because preparation of the electrode surface is notably simplified. Nonetheless, mercury continues to be the material of choice for most electrochemical reductions.

Our laboratory (5,18) and others (19,20) have successfully used amalgamated gold, silver, and platinum electrodes. This approach results in easily prepared and mechanically rigid electrodes which provide adequate performance for monitoring trace amounts of many easily reducible compounds. Nevertheless, in certain specilized applications, DME-based transducers will remain useful because they possess two distinct advantages over other electrodes: a more negative potential range and a constantly renewable surface.

In the present paper we discuss various factors influencing the successful utilization of reductive mode LCEC for determination of organic compounds.

MATERIALS AND METHODS

Apparatus

A model LC-304 liquid chromatograph (Bioanalytical Systems) equipped with a PM-20 solvent delivery system, 70-10 fixed volume (20 or 100 μL) rotary sample injection valve, LC-4 electronic controller and LC-19 transducer package was used for all experiments. The columns were 15 cm x 3 mm i.d. packed with 10 μm LiChrosorb RP-18, 15 cm x 2 mm i.d. packed with 10 μm LiChrosorb RP-2, or 25 cm x 4.6 mm i.d. 5 μm Biophase C_{18} (Bioanalytical Systems Inc.).

Chemicals

Reagent grade acetonitrile, n-propanol, and triple distilled mercury from Fisher Scientific were used as purchased. Technical grade methanol and deionized water were distilled in an all glass apparatus prior to solution preparation. Citric acid, sodium phosphate (dibasic), monochloroacetic acid, sodium acetate, and glacial acetic acid purchased from Fisher Scientific

were used without further purification. All mobile phases were filtered through 0.22 μm cellulose acetate Millipore filters (Millipore Corp., Bedford Mass). Parathion and methyl parathion were purchased from Analabs Inc. (North Haven, CT). RDX, TNT, and PETN were gifts from Dr. Dietz (Bureau of Alcohol, Tobacco, and Firearms, Department of the Treasury, Cincinnati, OH). Diazepam, chlordiazepoxide and nitrazepam were received as gifts from Dr. R. P. W. Scott, Hoffman La-Roche Co., Nutley, NJ, XAD-2 resin, 120 mesh (Rohm and Hass) was conditioned according Paschal et al. (21).

Preparation of Mercury-Film Electrode

Preparation of Gold Substrate

It is very important to remove any residue present on the gold surface, in order to obtain an electrode with low background and noise characteristics. The old amalgam surface must be removed before polishing the gold substrate. This can be easily achieved by placing a few drops of 6 M nitric acid on the amalgam surface. After 2-3 minutes the electrode surface was washed with distilled water. Surface residues were removed by rubbing the electrode cube over a 600 grit silicone carbide abrasive paper. A mirror-like finish was obtained by rubbing the electrode cube over a microcloth (Buehler) polishing pad which was covered with Gamma Alumina (Fisher Scientific). Water was used as a lubricant. Alumina and gold residues were removed by sonicating the electrode cube in water/ethanol solution.

Preparation of Mercury-Film Electrode Surface

The mercury-film surface was prepared by two methods.

Method I. A small amount of mercury is placed on the polished gold surface with a mirror-like finish (making sure that the entire gold surface is covered by mercury).

After 2-3 minutes the excess mercury is removed into a beaker using a computer card. The mercury layer is then "smoothed" out using a dry tissue. The electrode is now ready to be used.

Method II. Electrolytic amalgam preparation is carried out in two steps. In the first step a solution containing 0.01 M $Hg(NO_3)_2$, 0.5 M KCl, and 0.1 M HCl is passed slowly through the cell using a 10 mL syringe. The syringe is connected directly to the flow cell. The potential of the working electrode is set at -0.7 V vs. Ag/AgCl for a period of 10-15 minutes. In the second step a solution containing 0.5 M KNO_3 and 0.1 M HNO_3 is pumped through the cell and the potential of the working electrode is set at -1.5 V for a period of three minutes. After rinsing the electrode surface with water the electrode is ready to be used.

Oxygen Removal

Two modifications of the LC system were made for reductive LCEC. The entire LC system was constructed with 316 stainless steel tubing and an oxygen removal apparatus (schematically shown in Figure 1) was placed in line with the pump and sampling valve.

The dissolved oxygen in the mobile phase was removed by modifying a procedure of Michael and Zatka (7). The mobile phase was heated to 60-70°C and nitrogen gas was vigorously bubbled through the mobile phase for approximately 20-30 minutes before initiating flow through the LC system (helium or argon have also been used). After starting the LC pump, a gentle flow of nitrogen gas was maintained over the surface of the mobile phase in order to prevent oxygen from diffusing back into the mobile phase, and the temperature of the mobile phase

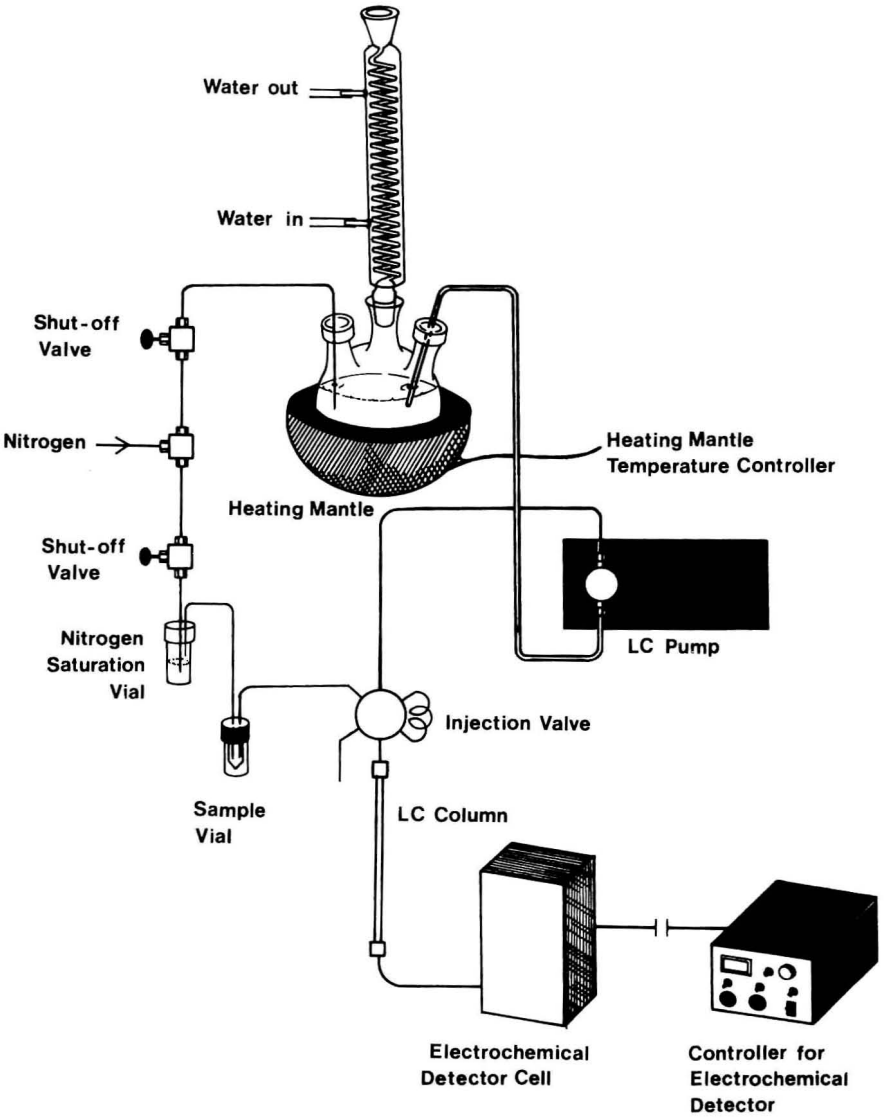


FIGURE 1. Schematic diagram of the reductive mode LCEC system.

was lowered to 35-40°C. The mobile phase was heated and a flow of nitrogen was maintained throughout the course of all LC experiments. Valuable time was saved when deoxygenation of the mobile phase was performed overnight at minimum flow (0.2 to 0.3 mL/min) through the LC system.

Sample deoxygenation was achieved by bubbling nitrogen gas through a sample solution. In order to prevent evaporation of solvent from a sample solution, dry nitrogen gas was passed through a saturation chamber which contained the same solvent as the sample solution. The deoxygenated sample solution was pulled into a sample loop without exposure to the ambient atmosphere. This sampling procedure was more convenient and effective than injecting deoxygenated solution into a sample loop with a syringe.

RESULTS AND DISCUSSION

Performance of the Detector

The utility of reductive mode LCEC detection is greatly limited if the dissolved oxygen is not removed from the mobile phase and sample solution prior to injection. Even at relatively low potentials, very large background currents are observed when oxygen is not removed from the mobile phase as illustrated in Figure 2. As mentioned previously, the LC system was modified to prevent diffusion of oxygen through the teflon tubing which is widely used in making low pressure connections in LC systems. It is not necessary to complicate the design of the reductive mode LCEC system by enclosing it in a box purged with nitrogen, if all teflon connections are replaced with a stainless steel tubing.

The solution resistance (R) can also be a concern when large background currents (> 50 nA) are encountered especially when the transducer schematically shown in Figure 3a was used

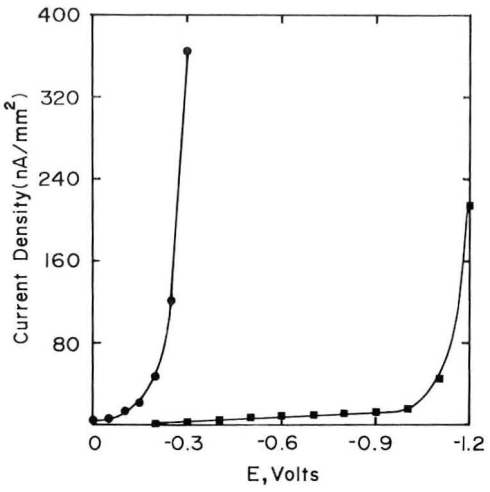


FIGURE 2. The observed background current on a Au/Hg electrode with (■) and without (●) deoxygenation of the mobile phase.

in preliminary experiments. This transducer performs adequately under low background current conditions (low negative potentials and also in oxidative LCEC; background current plus response due to analyte $\lesssim$ 150 nA) otherwise the product of current and resistance (referred to as IR drop) significantly diminishes the potential across the working electrode. The direct result of this IR drop is a decrease in the dynamic range of the detector as discussed in detail elsewhere (18). The solution resistance in the thin-layer channel can be decreased by increasing the ionic strength of the buffer, increasing the thickness of the channel, and decreasing the concentration of non-aqueous solvents. Increasing the ionic strength can also cause an increase in background current because salts often contain easily reducible impurities such as transition metal ions. Metal deposition on the mercury-surface decrease the negative potential range of the detector by decreasing the hydrogen overvoltage which in turn increases the background current.

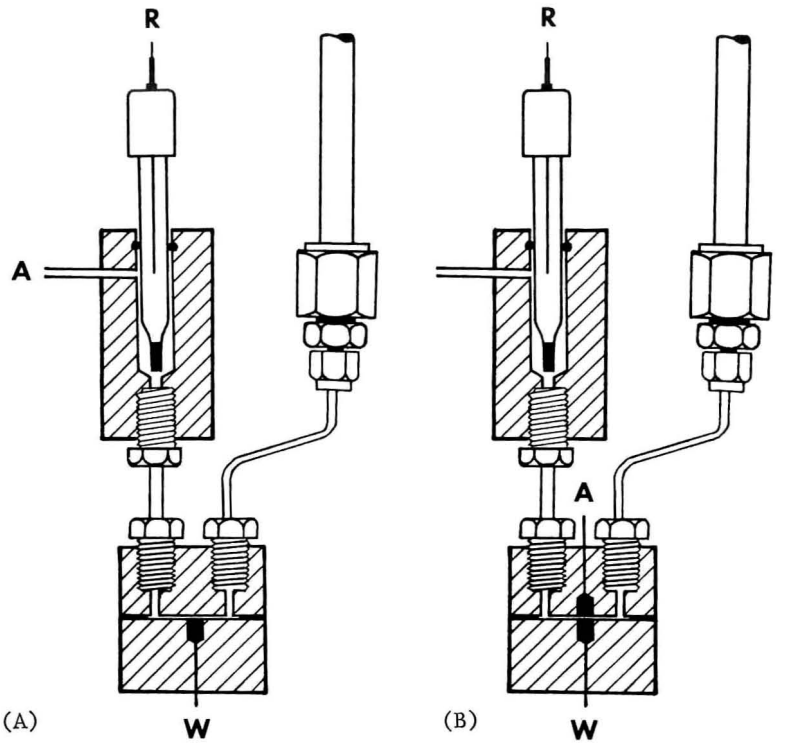


FIGURE 3. Common designs of thin-layer amperometric transducer.

The modified transducer illustrated in Figure 3b offers several important advantages over the more widely used design. The auxiliary electrode is placed in the top block directly across the channel from the working electrode. The path of current (from working electrode to auxiliary) is across the thin channel. The solution resistance even in low ionic strength solvents is extremely small and no longer influences the potential of the working electrode, resulting in extension of the linear dynamic range of the transducer by two orders of magnitude. A linear dynamic range of 5 1/2 orders of magnitude was found (50 pA → 2.5 μA) using picric acid as a model compound with the detector set at -0.8 V vs Ag/AgCl. The upper limit

of the linear dynamic range was not caused by the IR drop, but was due to saturation of the operational amplifier in the current-to-voltage converter. The modified transducer also allows the use of mobile phases with low ionic strength and high concentration of non-aqueous solvents such as acetonitrile, THF, or propanol.

It is very important to achieve a good amalgam formation around the edge of the gold substrate in order to minimize random noise spikes. It is not necessary to remove the old amalgam surface every time a new mercury surface was required. Usually an old amalgam surface was used 5 or 6 times as a substrate for the electrode surface regeneration, before it had to be removed with nitric acid. Slightly lower noise and long term stability at high detector sensitivity (especially at potentials greater than -0.8 V) was observed when the electrolytic method (Method II) was used to prepare a new electrode surface. Nevertheless,

TABLE 1

Typical Detection Limits of Easily Reduced Organic Compounds at S/N = 3 on a Au/Hg Electrode

Compounds	Detection Limits, picomoles
Picric Acid	0.13
2,5 - Dinitrophenol	0.16
p-Nitrophenol	0.72
RDX (Cyclonite)	0.76
Isosorbide dinitrate	1.3
Nitrazepam	1.8
Chlordiazepoxide	3.3
Diazepam	7.0
N-Nitrosopyrrolidine	10

Method I was generally preferred because it was less time consuming.

Representative detection limits for several easily reduced compounds are listed in Table 1. As expected, the detection limits for nitroaromatic compounds (especially for polyaromatic compounds) are lower because they undergo multielectron reduction processes, typically 4-12 electrons depending on the number of nitro groups, pH of the mobile phase, and the nature of the other substituents on the ring. Higher detection limits were observed for compounds with fewer electrons transferred and/or a higher reduction potential (diazepam and n-nitrosopyrolidine). Lower detection limits were obtained when the chromatographic and detector conditions were optimized for detection of a specific compound of interest. An LCUV system usually offers adequate sensitivity for nitro aromatic compounds, but the determination of several nanograms of nitrate esters per injection is difficult as illustrated in Figure 4. Crouthamel and Dorsch (22) reported LCUV detection limits of 30 ng for nitroglycerin. The UV detector performed better than expected in the case of TNT and RDX explosives as evident from a healthy response to 6 ng of RDX and 1.4 ng of TNT.

It was clearly evident from our determinations of the minimum detectable quantities that the background current and the quality of amalgam surface strongly affects the noise level of the detector. Two types of noise typically observed with a mercury film electrode were high and low frequency noise and random "spike" noise. As expected, the high frequency repetitive noise had a good correlation with the level of the background current as illustrated in Figure 5. The random spike noise had no correlation with the background current and was difficult to reproduce on a day-to-day basis and almost impossible to eliminate. Occasionally, when noise levels (typically due to spike noise) were unacceptably high (>2 nA), a new mercury

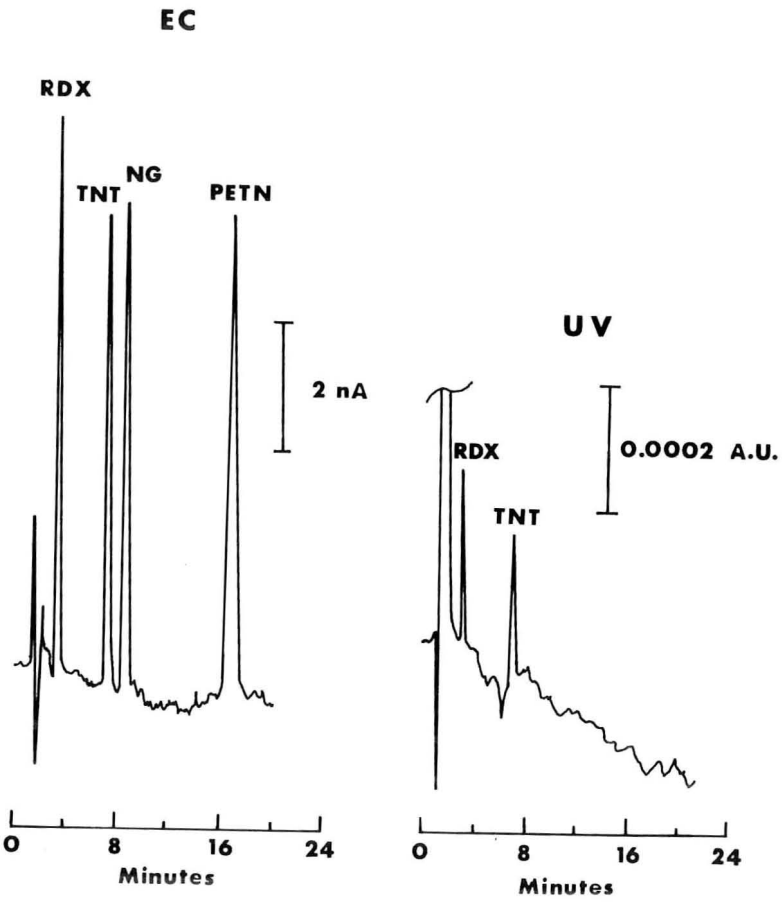


FIGURE 4. Comparison of EC (on the left, E = -1.0 V vs Ag/AgCl) and UV (on the right, at 254 nm) detectors for a mixture of explosive compounds. 27 pmoles of RDX, 6.2 pmoles of TNT, 41 pmoles of NG and 54 pmoles of PETN were separated on a 25 cm Biophase column. Mobile phase composition: 0.02 M monochloroacetic acid, 0.0146 M sodium acetate, 0.001 M EDTA, 16%(V/V) 1-propanol, and 5%(V/V) ethanol at 2 mL/min.

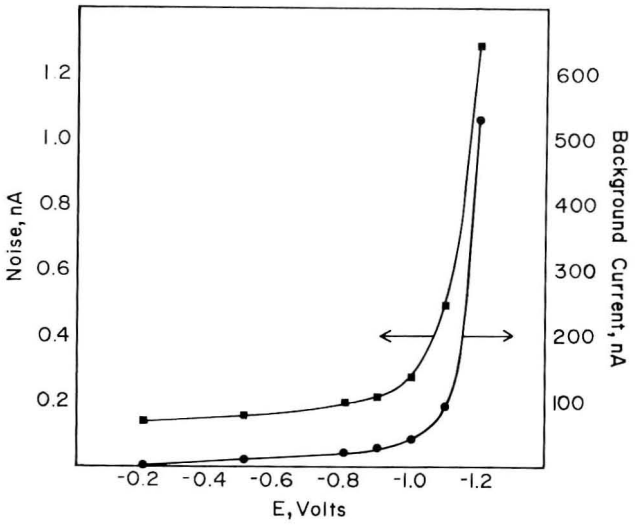


FIGURE 5. The dependence of the high frequency noise (■) and background current (●) on the applied potential of the Au/Hg electrode.

surface had to be prepared and extreme care was exercised in order to obtain a mirror-like gold substrate free of scratches and residue (formed during the nitric acid treatment of old amalgam). The lifetime of the electrode surface was dependent on the operating potential, nature of the sample, and amount of analyte injected.

Applications

Determination of Parathion and Methylparathion from Runoff Water

Parathion and methylparathion are organophosphorous insecticides which have been found to be effective replacements for extremely toxic organochlorine compounds such as DDT and Aldrin. Even though these pesticides accummulate very slowly in biological food chains because of their relatively rapid decomposition, they are still very toxic in their native form.

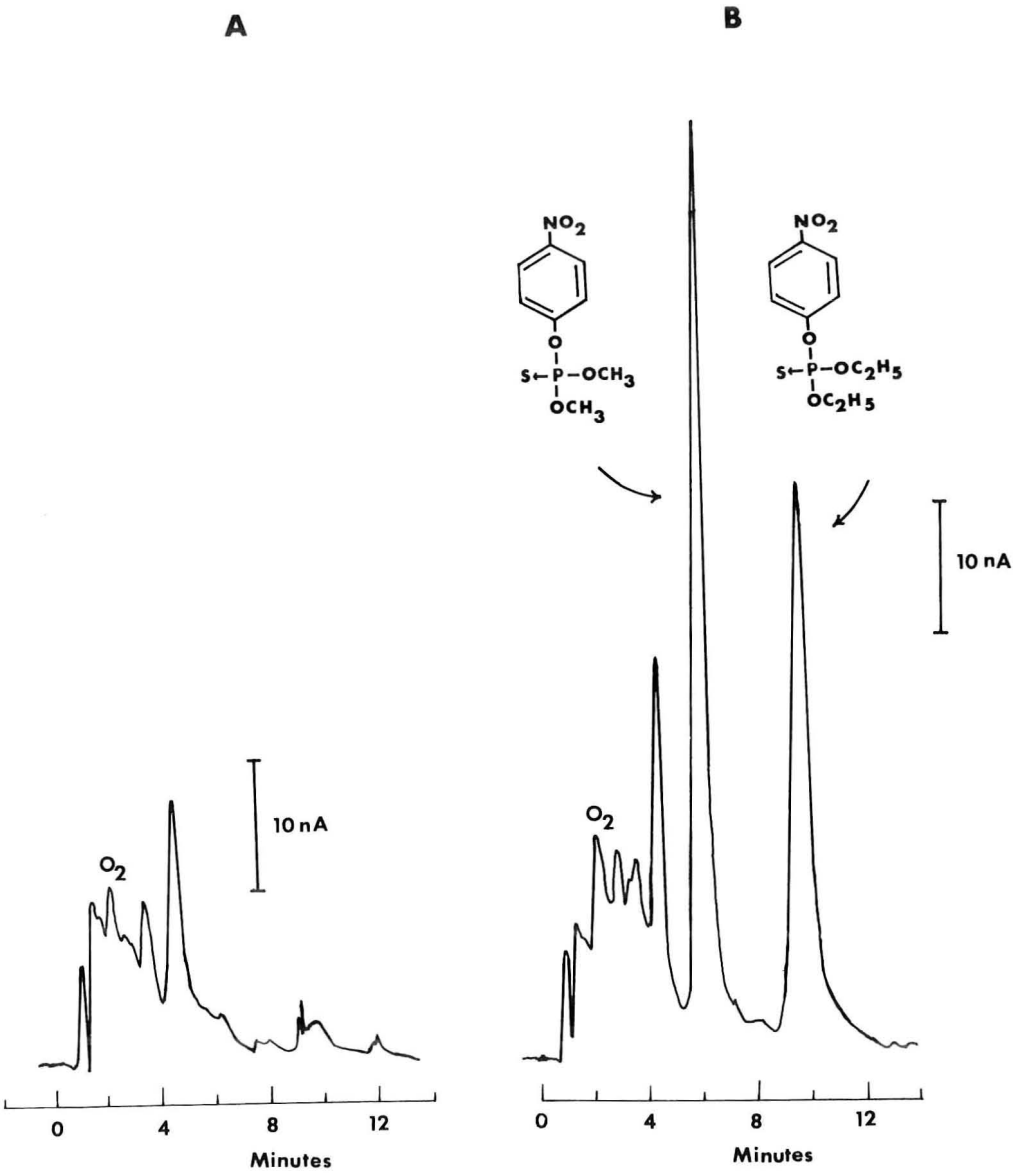


FIGURE 6. Representative chromatograms of runoff water. (A) Runoff water blank. (B) Runoff water spiked with 40 ng/mL of methyl parathion and parathion. Separation was achieved using a 15 cm column packed with 10 um LiChrosorb RP-2 material, mobile phase composition: 0.005 M acetate buffer pH 4 and 45% (V/V) acetonitrile at 0.4 mL/min. Au/Hg working electrode was set at -1.05 V vs Ag/AgCl.

The isolation and quantitation of parathion and methylparathion were chosen as an example for illustrating the utility of the thin-layer gold/mercury electrode as a reductive mode detector.

Parathion and methylparathion were determined in spiked runoff water collected from a stagnant farm pond in West Lafayette. The preliminary isolation was according to Paschal et al. (21) with minor modifications. Final separation and quantitation was achieved using a microparticulate reverse-phase LC column with a TL-9A electrochemical transducer. Two 25 mL runoff water samples were analyzed in parallel; one was used as a sample blank and the second was spiked with 1 μg of each of the two pesticides (40 ng/mL). Each sample was passed through a 5 cm isolation column packed with XAD-2 resin. The pesticides were eluted from the resin with 25 mL of diethyl ether. After evaporating the ether under a stream of nitrogen gas at ambient temperature, the residue was dissolved in 400 μL of the mobile phase. A 20 μL aliquot was injected onto the LC column and the chromatograms of the blank (A) and spiked sample (B) illustrated in Figure 6 were obtained. Chromatographic and detector conditions are given in the figure caption.

Determination of Chloramphenicol in Serum

Chlorampenicol, a broad-spectrum antibiotic, was originally derived from Venezuelan strain of streptomycetes. This antibiotic is responsible for bringing thyphoid fever under the control since the end of World War II. More than fifty million people have been treated with this antibiotic in the past three decades. Many analogs of chloramphenicol have been synthesized leading to the observation that the nitro-group in the para position on the phenyl ring is essential to the antibacterial activity of the drug.

Chloramphenicol is easily reduced at a mercury film electrode. A chromatogram of a diethyl ether extract of plasma

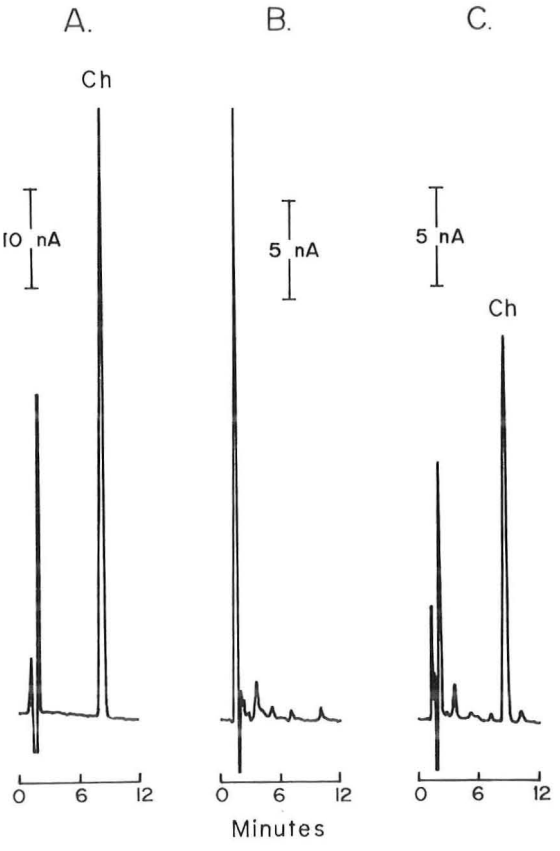


FIGURE 7. (A) Chromatogram of 295 pmoles of chloramphenicol (Ch), (B) Representative chromatogram of plasma blank, (C) Chromatogram of plasma spiked with 0.71 μg/mL of chloramphenicol. Chromatographic conditions: 25 cm Biophase column with C_{18} packing, mobile phase contained 0.02 M monochloroacetic acid, 0.001 M EDTA, and 0.0146 sodium acetate, and 9%(V/V) 1-propanol. 2 mL/min, Detector potential: -0.85 V vs AgCl.

is illustrated in Figure 7. One mL of plasma spiked with appropriate amounts of chloramphenicol was deprotenized with 50 µL of 4 M perchloric acid and centrifuged for 5 minutes at 2000 rpm. 300 µL of the resulting supernatant was loaded on an extraction column (Clin Elute Mode No. 1000-M). Chloramphenicol was eluted with 3 mL of diethylether. After evaporation of the ether, the residue was dissolved in 300 µL of a mixture of water: ethanol (80:20) and 50 µL of this reconstituted solution was injected onto a reverse phase column.

CONCLUSION

Reductive electrochemical detection has been developing very slowly over nearly 30 years. In the past several years progress has accelerated rapidly as applications to organometallic (19,20) and organic compounds (5,11-18) have been developed. It is now clear that reductive LCEC experiments can be made routine for a number of important problems. A new approach is therefore available in the arsenal of tools for trace determinations by liquid chromatography.

ACKNOWLEDGMENTS

We are grateful to Dr. W. Dietz for samples of explosive compounds and Dr. R. P. W. Scott for samples of benzodiazepines. This work was supported by the National Science Foundation and the National Institute of General Medical Sciences.

REFERENCES

1. Kissinger, P.T., Amperometric and Coulometric Detectors for High Performance Liquid Chromatography, Anal. Chem., 49, 447A, 1977.

2. Heineman, W.R. and Kissinger, P.T., Analytical Electrochemistry: Methodology and Applications of Dynamic Techniques, Anal. Chem., 52, 138R, 1980.

3. Rucki, R.J., Electrochemical Detectors for Flowing Liquid Systems, Talanta, 27, 147, 1980.

4. Bratin, K., Felice, L.J., Kissinger, P.T., Miner, D.J., Preddy, C.R., and Shoup, R.E., Introduction to Detectors for Liquid Chromatography, Kissinger, P.T., ed., BAS Press, West Lafayette, IN, 1981.

5. Kissinger, P.T., Bratin, K., King, W.P., and Rice, J.R., Electrochemical Detection of Picomole Amounts of Oxidizable and Reducible Residues Separated by Liquid Chromatography, ACS Symposium Series, 136, 57, 1981.

6. Kemula, W., Chromato-Polarographic Studies. I. General Considerations and the Description of the Set of Apparatus, Roczniki Chem., 26, 281, 1952.

7. Michel, L. and Zatka, A., An Electrochemical Detector with a Dropping Mercury Electrode for High Performance Liquid Chromatography, Anal. Chim. Acta., 105, 109, 1979.

8. Hanekamp, H.B., Voogt, W.H., Bos, P., and Frei, R.W., Evaluation and Characterization of an Electrochemical Detector for HPLC, Anal. Lett., 12, 175, 1979.

9. Hanekamp, H.B., Bos, P., Brinkman, U.A.Th., and Frei, R.W., A Comparison of Different HPLC Detector Designs Based on the Dropping Mercury Electrode Principle, Z. Anal. Chem., 297, 404, 1979.

10. Kutner, W., Debowski, J., and Kemula, W., Polarographic Detection for High-Performance Liquid Chromatography Using a Flow-Through Detector, J. Chromatogr., 191, 47, 1980.

11. Vohra, S.K., and Harrington, G.W., The Evaluation of a Polarographic Detector for High Performance Liquid Chromatography in the Determination of N-Nitrosamines, J. Chromatogr. Sci., 18, 379, 1980.

12. Schieffer, G.W., Reversed-Phase High-Performance Liquid Chromatography with Differential Pulse Polarographic Detection for Assaying Drugs in Feed. Stability-Indicating Assay of Diacetolol, J. Chromatogr., 202, 405, 1980.

13. Samuelsson, R. and Osteryoung, J., Determination of N-Nitrosamines by High-Performance Liquid Chromatographic Separation with Voltammetric Detection, Anal. Chim. Acta., 123, 97, 1981.

14. Hackman, M.R. and Brooks, M.A., Differential Pulse Amperometric Detection of Drugs in Plasma Using a Dropping Mercury Electrode as a High-Performance Liquid Chromatographic Detector, J. Chromatogr., 222, 179, 1981.

15. Lund, W., Hannisdal, M., and Greibrokk, T., Evaluation of Amperometric Detectors for High-Performance Liquid Chromatography: Analysis of Benzodiazepines, J. Chromatogr., 173, 249, 1979.

16. Wightman, R.M., Paik, E.C., Borman, S., and Dayton, M.A., Evaluation of the Basal Plane of Pyrolytic Graphite as an Electrochemical Detector for Liquid Chromatography, Anal. Chem., 50, 1410, 1978.

17. Joynes, P.L. and Maggs, R.J., The Monitoring of Liquid Chromatographic Columns: A New Approach, J. Chromatogr. Sci., 8, 426, 1970.

18. Kissinger, P.T., Bruntlett, C.S., Bratin, K., and Rice, J.R., Liquid Chromatography with Electrochemical Detection. State of the Art and Future Directions, National Bureau of Standards Special Publication 519, 705, 1979.

19. MacCrehan, W.A. and Durst, R.A., Measurement of Organomercury Species in Biological Samples by Liquid Chromatography with Differential Pulse Electrochemical Detection, Anal. Chem., 50, 2108, 1978.

20. MacCrehan, W.A., Differential Pulse Detection in Liquid Chromatography and its Application to the Measurement of Organometal Cations, Anal. Chem., 53, 74, 1981.

21. Paschal, D.C., Bicknell, R., and Dresbach, D., Determination of Ethyl and Methyl Parathion in Runoff Water with High Performance Liquid Chromatography, Anal. Chem., 49, 1551, 1977.

22. Crouthamel, W.G. and Dorsch, B., Specific High-Performance Liquid Chromatographic Assay for Nitroglycerin in Dosage Forms, J. Pharm. Sci., 68, 237, 1979.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1797-1805 (1981)

ISOLATION BY MEANS OF PREPARATIVE REVERSED-PHASE LIQUID CHROMATOGRAPHY OF EPIMERIC ALCOHOLS FORMED UPON REDUCTION OF PREGNENOLONE AND PROGESTERONE

S. Allenmark and H. Borén
Clinical Research Centre and IFM/Department of Organic Chemistry
Linköping University, S-581 85 Linköping, Sweden

ABSTRACT

A method is described which permits complete separation on a preparatory scale of the 20R and 20S epimeric alcohols obtained from lithium aluminium hydride and sodium borohydride reduction of pregnenolone and progesterone, respectively. The retention behaviour and resolution obtained on chromatography of the epimers on C-18 bonded phase material and elution with different acetonitrile/water and methanol/water mobile phases were studied. The order of retention is in both cases in accordance with ^{1}H-NMR chemical shift data which indicate a stable conformation with a more exposed 20-OH group in the 20S (=20α) epimer. Deviations from the elution order expected for true reversed-phase retention mechanisms were found on elution with mobile phase systems of reduced water content.

INTRODUCTION

Studies on certain enzymatic conversions (1) of pregnenolone (I), prompted us to synthesize tritium labelled pregn-5-ene-3β,20α-diol (=pregn-5-ene-3S,20S-diol) (IIa) by lithium aluminium hydride (LAH) reduction of labelled I. This, in turn, necessitated a method for the separation of the epimeric 3S,20S and 3S,20R (IIb) -diols formed in this reaction. Attempts to change the epimer ratio in favour of IIa by LAH/$AlCl_3$-reduction under conditions favouring

equilibration resulted in a rather complex mixture of steroids (2). For identification purposes pregn-4-ene-20α-ol-3-one (pregn-4-ene-20S-ol-3-one) (IVa) and its 20b (20R) epimer (IVb) were prepared by sodium borohydride reduction of progesterone (III). The conditions for optimal preparative separation, by means of reversed phase high performance liquid chromatography (HPLC), of the epimeric pairs of alcohols formed in these reactions were therefore the primary object for this study.

EXPERIMENTAL

Reduction of pregnenolone and progesterone

A detailed description of the reaction conditions used for metal hydride reduction of these ketones has recently been reported elsewhere (2).

HPLC

Columns (analytical 4.6x200 mm and preparative 10.0x250 mm) were prepared by upward slurry packing with Nucleosil C-18 5μ (Macherey and Nagel, Düren, G.F.R.). The liquid chromatograph was composed of an Altex mod. 100 constant flow solvent pump, a Rheodyne mod. 2710 injector valve, an LDC Spectromonitor III variable wavelength UV-VIS detector and a Hitachi mod. 561 potentiometric recorder.

For the analytical work a 20 μl loop volume was used which was changed to 50 μl in combination with preparative columns. With the latter system amounts of the order 1-2 mg were loaded onto the column. Collection of the compounds eluted during preparative runs was effected manually during observation of the UV-absorbancy registered on the recorder. 10-20 repeated injections were sufficient to permit isolation of the amounts necessary for identification by means of melting point, NMR, GC/MS and optical rotation (2).

The HPLC solvents used were acetonitrile HPLC grade S from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland) and methanol of p.a. quality from Merck (Darmstadt, G.F.R.). Glass distilled water was used to prepare the various mobile phases.

Radiochromatograms from analytical HPLC of the product mixture obtained on reduction of tritiated pregnenolone were obtained by continuous fraction collection using 15 sec/fraction, except for the first and last 10 min of the chromatogram, where 1 min/fraction was used. The collected fractions were then mixed with Aquasol (NEN Chemicals, Dreieich, G.F.R.) and the tritium activity determined by liquid scintillation counting.

RESULTS AND DISCUSSION

The reduction of I by LAH results in a mixture of IIa and IIb in an approximate ratio of 1:4. This is shown by Fig. 1 which originates from an experiment where tritium labelled I was used. The complete separation of the epimeric diols obtained, encouraged attempts to scale up the method to suit preparative purposes. A 10x250 mm steel column packed with 5 μ C-18 bonded phase material was run with 70% acetonitrile as the mobile phase and permitted injected amounts up to ca. 2 mg without any significant signs of overload. The separation result after repeated injections and eluate collection is shown by Fig. 2, which demonstrates the purity obtained as monitored by analytical HPLC. If a certain sacrifice in yield is permitted, the purity of the 20S-epimer can be further increased.

The complex reaction mixture from reduction of I by LAH/$AlCl_3$ under conditions of equilibration analyzed by HPLC at two different wavelengths is shown by Fig. 3. The peaks remaining at 235 nm were suggested to originate from reaction products having a conjugated carbonyl chromophore, most likely the pregn-4-ene-3-one system.

A sodium borohydride reduction of III gave, besides a large amount of unreacted III, the two epimeric alcohols IVa and IVb. These were readily separated on a preparatory scale, Fig. 4. When the reaction mixture was analyzed by HPLC using 60 % methanol as the mobile phase, IVa was completely overlapped by III. The superimposition of peaks from UV absorption at 247 nm for identification purposes is well illustrated by Fig. 5.

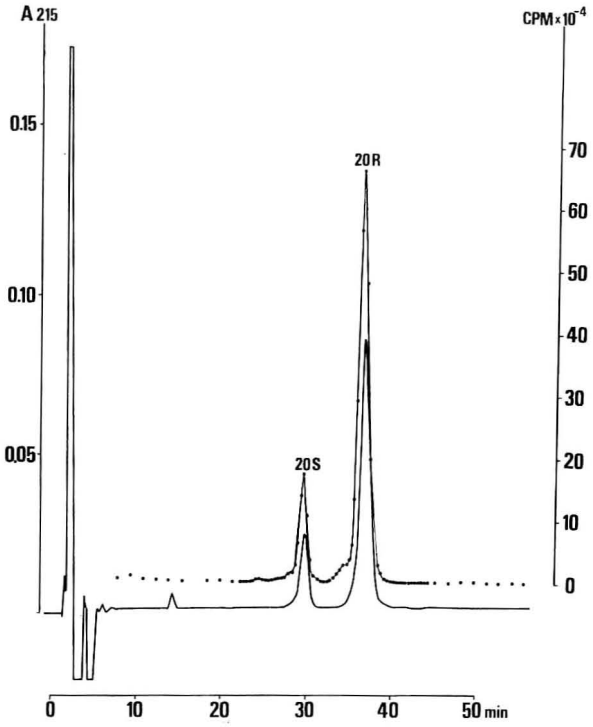


FIGURE 1. Resolution of the 20R and 20S epimeric diols obtained after LAH-reduction of tritiated pregnenolone. Analytical column, 50% acetonitrile, flow rate 1.0 ml/min. The dots refer to the fractions taken to liquid scintillation counting of tritium activity.

A summary of chromatographic data is given in Table 1, which shows the capacity ratios and resolution factors of the compounds of interest found in different solvent systems. The results show that the 20R-epimers behave as the more hydrophobic forms, in complete agreement with the reports by Heftmann et al. (3,4) of normal-phase TLC and HPLC as well as reversed-phase HPLC data. The resolving power of reversed bonded phase systems as applied to epimeric alcohols is well demonstrated by Fig. 6.

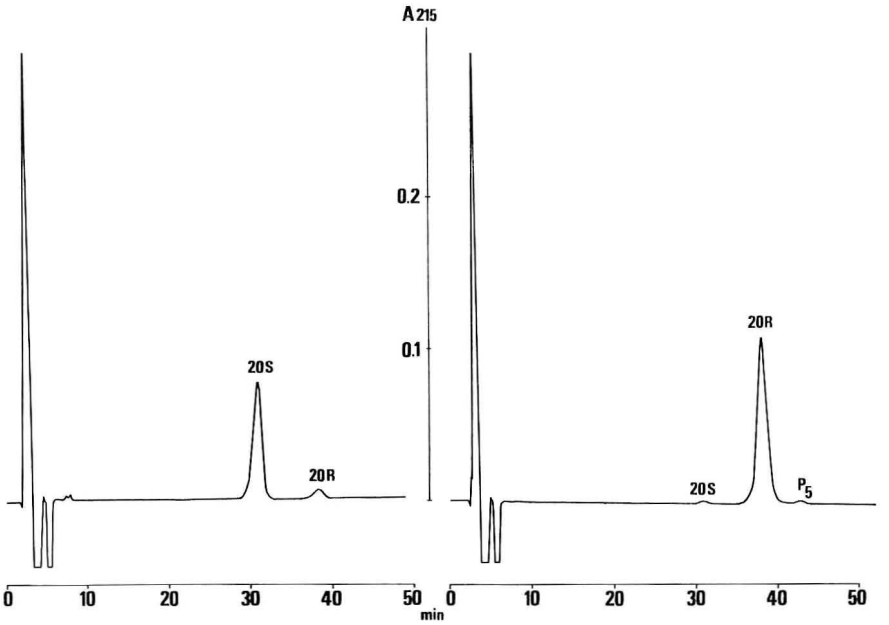


FIGURE 2. Results from a preparative HPLC separation of pregn-5-ene-3S, 20S-diol and pregn-5-ene-3S,20R-diol, as shown by analysis of purity. Analytical column, 50% acetonitrile, flow rate 1.0 ml/min.

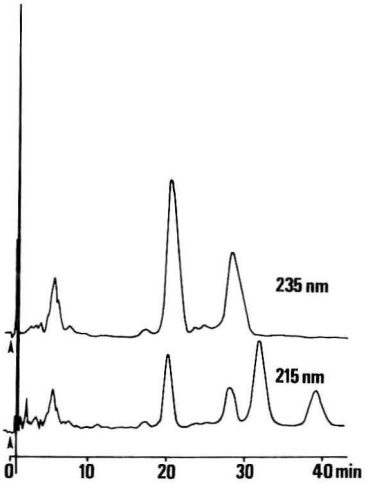


FIGURE 3. Illustration of the use of different UV-detector wavelengths to probe the presence of compounds with conjugated carbonyl group chromophores. Product mixture obtained on LAH/$AlCl_3$-reduction of pregnenolone.

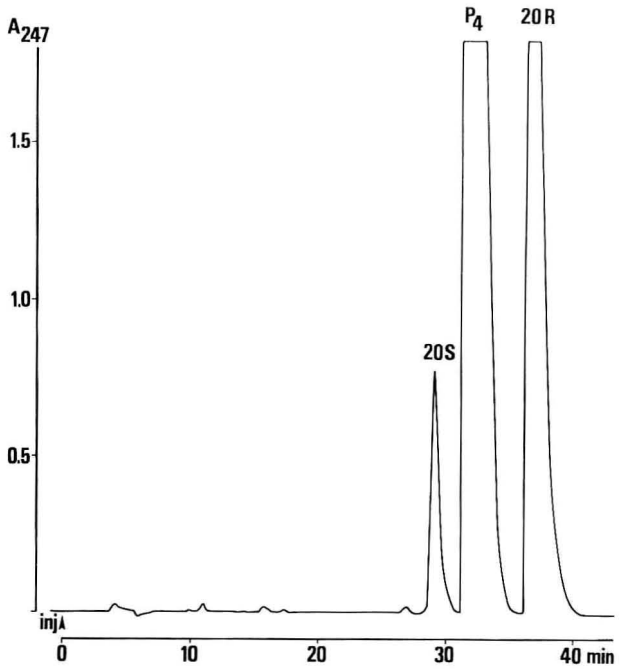


FIGURE 4. Resolution of pregn-4-ene-20S-ol-3-one and pregn-4-ene-20R-ol-3-one obtained on sodium borohydride reduction of progesterone. Preparative column, 70% acetonitrile, flow rate 2.4 ml/min, ca. 1 mg injected.

It is interesting to note that the chromatographic results correlate well with ^{1}H-NMR chemical shift data of the epimeric pairs. The downfield shift of 0.09 ± 0.01 ppm for the H-21 resonance signal and the same upfield shift for the H-18 signal found for the 20S relative to the 20R epimers can be taken as strong evidence for a stable conformation with a less exposed 20-OH group in the 20R epimer (5), which accordingly should be the more hydrophobic species. It can also be found from Table 1 and Fig. 4 that the elution order predicted for true reversed-phase chromatography, where the compounds are eluted in order of hydrophobicity, i.e. in this case IIa, IIb, I and IVa, IVb, III, is only fulfilled when mobile phase systems of sufficient water content are used. Obviously, the deviation from this order of

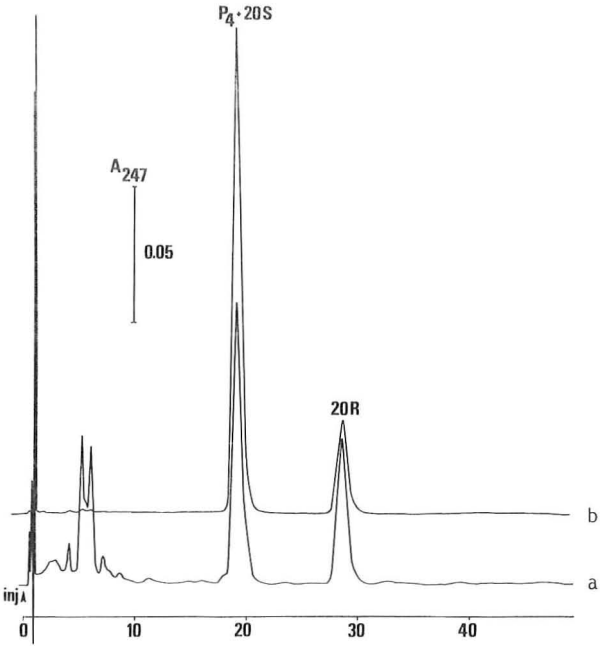


FIGURE 5. Chromatographic identification of the pregn-4-ene-3-one system by UV-monitoring at 247 nm. Analytical column, 60% methanol, flow rate 2.25 ml/min. a) Product mixture obtained on LAH/$AlCl_3$-reduction of pregnenolone. b) Products resulting from sodium borohydride reduction of progesterone.

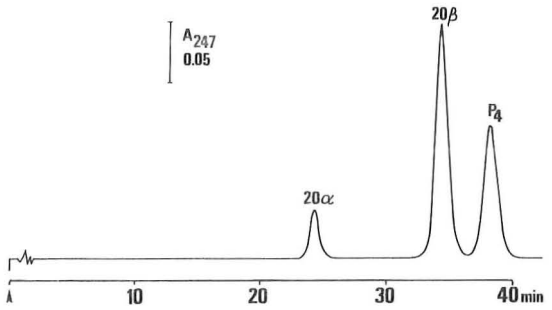


FIGURE 6. Illustration of the C-18 reversed-phase separation efficiency with 40% acetonitrile as the mobile phase. Product mixture obtained on sodium borohydride reduction of progesterone. Analytical column, flow rate 2.0 ml/min, UV 247 nm.

TABLE 1. Capacity Ratios (k'), Separation Factors (α) and Resolution (Rs) Obtained with Some Different Mobile Phases

Compound no.:	IIa	IIb	III	IVa	IVb	Mobile phase
k'	17.0	23.4	26.85	16.7	24.0	
α	1.375			1.434		A
Rs	4.56			5.06		
k'	9.0	12.1	14.1	9.0	12.7	
α	1.338			1.402		B
Rs	4.07			4.64		
k'	6.05	7.9	9.5	6.0	8.3	
α	1.308			1.380		C
Rs	3.62			4.29		
k'	20.2	26.6	13.1	13.9	19.6	
α	1.317			1.407		D
Rs	3.92			4.65		
k'	9.4	11.9	6.2	6.2	8.7	
α	1.275			1.404		E
Rs	3.40			4.43		
k'	5.1	6.3	3.5	3.5	4.7	
α	1.227			1.341		F
Rs	2.76			3.66		

Mobile phase systems: Acetonitrile in water: A = 40%, B = 45%, C = 50%. Methanol in water: D = 65 %, E = 70 %, F = 75 %

elution observed in other cases is the result of a competitive retention mechanism based on adsorption to polar groups of the stationary phase.

REFERENCES

1. Allenmark, S., Å:son Berg, A., Hammar, M. and Lindström, E., Combined Paper and Reversed-phase High-performance Liquid

Chromatography Method for the Study of Pregnenolone and Progesterone Metabolites, J. Chromatogr. Biomed. Appl., in press.

2. Allenmark, S. and Borén, H., Lithium Aluminium Hydride Reduction of Pregnenolone under Conditions of Assumed Kinetic and Equilibrium Control, Acta Chem. Scand., in press.

3. Heftmann, E., Behaviour of Stereoisomeric Steroids in Liquid Chromatography, J. Liquid Chromatogr. 2, 1137 (1979).

4. Lin, J.-T., Heftmann, E. and Hunter, I., High-performance Liquid Chromatography of the Reduction Products of Progesterone, J. Chromatogr. 190, 169 (1980).

5. Lee, H. and Wolff, M.E., C-18 Functional Steroids and D-Homo Steroids, J. Org. Chem. 32, 192 (1967).

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1807-1815 (1981)

ANALYSIS OF DEHYDROABIETIC ACID IN KRAFT MILL EFFLUENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R.K. Symons
Environment Protection Authority
Laboratory Services Branch
P.O. Box 41, East Melbourne, Victoria, 3002

ABSTRACT

A rapid high-performance liquid chromatographic method for the determination of dehydroabietic acid in kraft mill effluent is described. Selective solvent extraction of the samples with dichloromethane was performed. The samples were isocratically analysed on a Rad-Pak C18 column using 75% acetonitrile in water (0.1% acetic acid added). Detection was carried out at 220 nm and 267 nm on a variable wavelength detector with a detection limit of 0.01 mg/L.

INTRODUCTION

Toxic components of pulp and paper effluents are complex mixtures of organic and inorganic moieties either naturally occurring, added or formed during the pulping process.(1) Many of those components have been tested for toxicity.(2,3) Dehydroabietic acid (DHAA) is one of several naturally occurring resin acids extracted from softwood trees during the kraft process and is a major contributor of toxicity in non-bleached pulp effluent.(4-7) Its 96-h LC 50 (static bioassay) for

juvenile freshwater coho salmon is 0.75 to 1.8 mg/L.(1,2,5,6,8) Concentration of DHAA in mill effluents is highly variable ranging from 0.4 to 51.8 mg/L.(1)

Colorimetric (9) and gas chromatographic (GC) (10-13) procedures for the analysis of resin acids in mill effluents have been widely used. However, each suffers certain inadequacies with respect to separation, detection and identification of DHAA from other resin acids. The non-specificity and insensitivity of the colorimetric procedure to DHAA has already been noted,(2) while incomplete derivatization due to the bulk of involatile materials present, interference from decomposition of labile substances, and lengthy analysis times has hindered quantitative GC analyses. (15)

High-performance liquid chromatography (HPLC) has been developed for the analysis of a wide range of trace organic compounds in waters and waste waters, (14,16) and is free of many of the problems associated with GC.

By using a selective solvent extraction procedure and HPLC, a rapid and quantitative method has been developed for the determination of trace levels of DHAA in mill effluents at a detection limit of 0.01 mg/L.

MATERIAL AND METHODS

Glassware

The glassware used was a Kuderna-Danish evaporator with 3 mL concentrator tubes; 50 and 100 mL volumetric flasks; and 2000 mL separatory funnels. All glassware was soaked in a synthetic detergent, rinsed and dried. Prior to use all glassware was rinsed with dichloromethane.

Chemicals

All solvents used were specially purified for HPLC (Ajax Chemicals, Melb., Aust.).

Dehydroabietic acid was obtained from Dr. A.A. Sioumis (CSIRO, Div. Chem. Tech., Melb., Aust.) and a purity of 95% was determined by GC/MS of the TMS derivatives.

Apparatus

The chromatography was conducted using a Laboratory Data Control (Riveria Beach, Florida, USA) liquid chromatograph with dual Constametric III solvent delivery pumps, a Gradient Master, Rheodyne 7125 loop injector, and a Waters Assoc. (Bedford, Mass., USA) Radial Compression Separation System, Rad-PAK A (10 cm x 5 mm, C_{18}) column. Detection was accomplished via a Spectromonitor III variable-wavelength absorbance detector.

Retention times and peak areas were electronically acquired on a Hewlett-Packard (Avondale, PA, USA) HP3388A reporting integrator.

Chromatographic Conditions

The separations were achieved isocratically with a mobile phase composition of 75% acetonitrile in water (0.1% acetic acid) at a flow rate of 2.0 mL/min, and detection was carried out at 220 nm and 267 nm. The radial compression module was at ambient temperature in its fully compressed mode. The injections were made with a 20 µL sample loop.

Preparation of Standards and Samples

The standard solution of DHAA was prepared by dissolving 10 mg in a small amount of dichloromethane and diluting to 100 mL

with acetonitrile. This stock solution and subsequent working standards were stored at 4°C.

Samples of 2-L volumes were collected in plastic bottles, delivered to the laboratory on ice, and were stored at 4°C prior to extraction.

The sample, 500 mL, was adjusted to pH 12 with 10M NaOH and was extracted with three successive 60 mL volumes of dichloromethane to produce a base-neutral fraction. The dichloromethane extracts containing neutral compounds, were discarded.

The pH of the aqueous fraction was adjusted to 2 with conc. HCl and extracted with dichloromethane (3 x 60 mL). The acid-extractable fraction containing the DHAA and other resin acids were combined in a Kuderna-Danish evaporator which was connected to a rotary evaporator and the volume reduced under vacuum to approximately 2 mL. The concentrate was evaporated to dryness under a stream nitrogen and the residue dissolved in 1.0 mL acetonitrile before injection into the HPLC.

Identification and Quantitation of Peaks

Peaks observed in the sample chromatographic profiles were identified by (i) retention times in comparison with standards; (ii) coinjection with standards; (iii) peak area ratios at two different wavelengths (220 and 267 nm); and, (iv) GC/MS of the TMS derivatives of fractions collected from the HPLC. Both peak height and area measurements were used for the quantitation of DHAA in the samples.

RESULTS AND DISCUSSION

The HPLC profiles of DHAA obtained with UV detection at 220 nm and 267 nm shown respectively in Figures 1 and 2

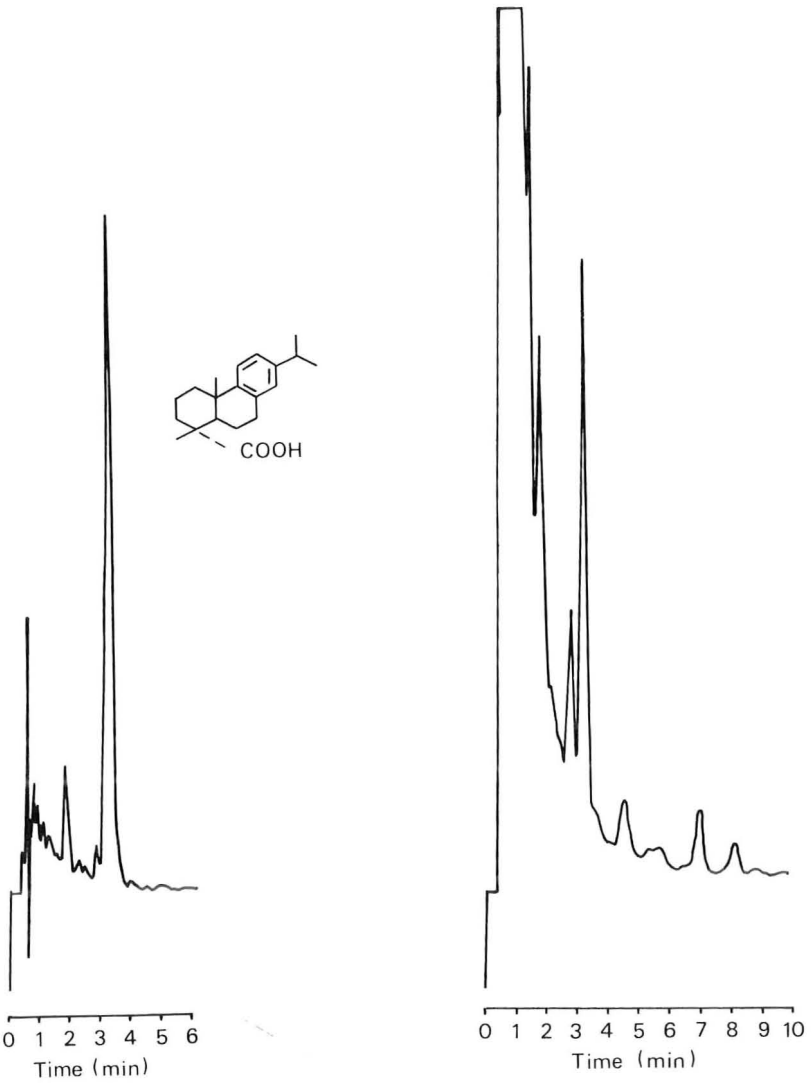


FIGURE 1

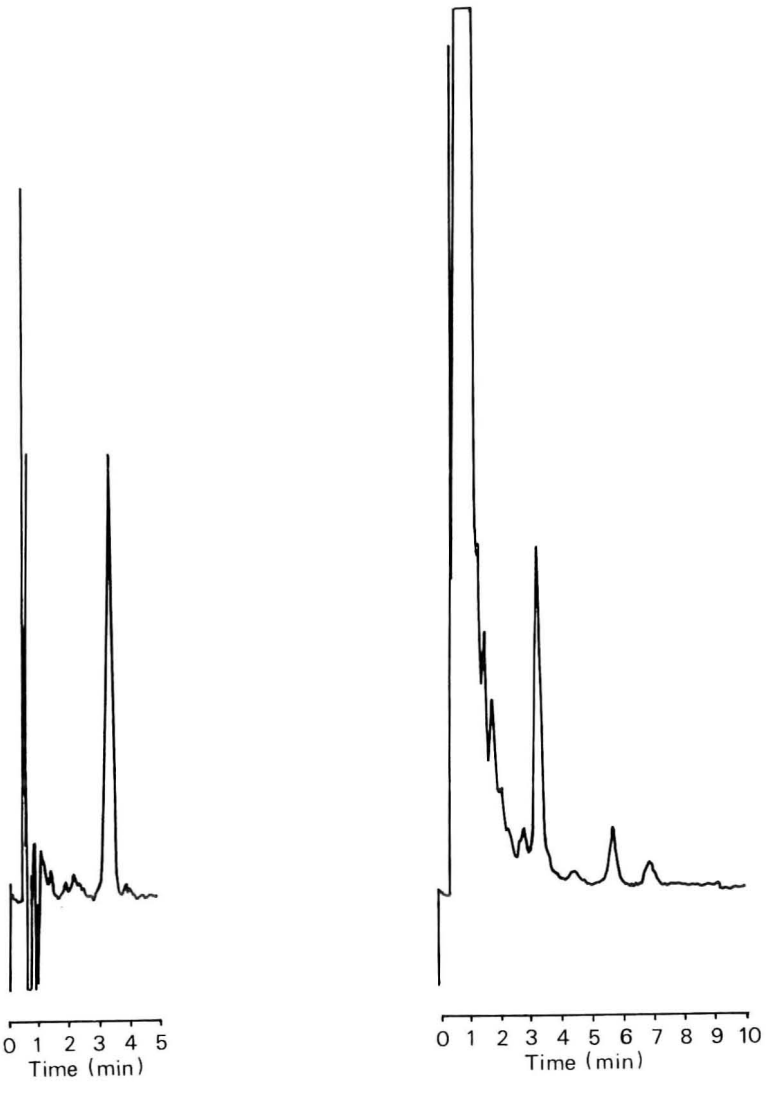


FIGURE 2

show good separation from other extractives with a retention time for DHAA of 3.25 minutes. It was also observed that DHAA exhibits UV absorption at both 220 and 267 nm wavelength, the main difference being in their relative intensities, with the 220 nm wavelength exhibiting a five-fold increase in sensitivity. The use of absorbance ratios to identify compounds in samples has been used, (17) but cannot be relied upon if interferences are present.

Any tendency for sample components to dissociate while being chromatographed, as in the case of resin acids, frequently leads to excessive peak broadening or a tailing peak. The addition of 0.1% acetic acid to the mobile phase decreases the pH sufficiently to suppress dissociation making the sample component less polar and more amenable to analysis, i.e., a much improved peak shape results. (18)

No attempt was made to quantitate any of the other compounds present however, results of GC/MS analysis suggested the presence of several resin acids of the abietic and pimaric-acid type. Further investigations relating to the identification of these resin acids by HPLC and their extraction from kraft mill effluent by Sep-Pak C18 cartridges (Waters Assoc.) is being carried out.

ACKNOWLEDGEMENTS

I would like to thank Dr. D. Burke for performing and interpreting the GC/MS data and Dr. A. Sioumis for supplying the resin acid standards.

REFERENCES

1. Hutchins, F.E., Toxicity of Pulp and Paper Effluents - A Literature Review, US EPA - 600/3-79-013, (1979).

2. Leach, J.M. and Thakore, A.N., Isolation of Toxic Constituents of Kraft Pulp Mill Effluents, CPAR, Can. For. Serv., Proj. Rept., 11-5, (1974).

3. Rogers, I.H., Isolation and Chemical Identification of Toxic Components of Kraft Mill Wastes, Pulp Paper Mag. Can. 74, 9, 111. (1973).

4. Leach, J.M. and Thakore, A.N., Toxic Constituents in Mechanical Pulping Effluents, Tappi, 59, 2, 129, (1976).

5. Leach, J.M. and Thakore, A.N., Identification of the Toxic Materials in Sulphite Pulp Mill Effluents, CPAR, Can. For. Serv., Proj. Rept., 407-3, (1978).

6. Iwama, G.K., Greer, G.L., and Larkin, P.A., Changes in Some Hematological Characteristics of Coho Salmon in Response to Acute Exposure to Dehydroabietic Acid at Different Exercise Levels, J. Fish. Res. Board Can., 33, 2, 285, (1976).

7. Rogers, I.H., Mahood, H.W., James, B.C., Servizi, J.A. and Gordon, R.W., Identifying Extractives Toxic to Aquatic Life, Pulp Paper Mag. Can., 80, 9, 94, (1979).

8. Davis, J.C. and Hoos, R.A.W., Use of Sodium Pentachlorophenate and Dehydroabietic Acid as Reference Toxicants for Salmonoid Bioassays, J. Fish. Res. Board Can., 32, 411, (1973).

9. Carpenter, W.L., "Colourimetric Measurement of Resin Acid Soaps in Pulp and Paper Mill Wastes", Tappi, 48, 11, 669 (1965).

10. Nestler, M.F.H. and Zinkel, D.F., "Quantitative Gas-Liquid Chromatography of Fatty and Resin Acid Methylesters", Anal. Chem., 39, 10, 1118, (1967).

11. Jaye, N.M., Proveaur, A.T., and Lawrence, R.V., "Improved Procedure for the Analysis (GLC) of Resin Acids", J. Am. Oil Chem. Soc., 51, 5, 195, (1974).

12. Holmpom, B., Avela, E., Pekkala, S., "Capillary Gas Chromatography - Mass Spectrometry of Resin Acids in Tall Oils Resin", J. Am. Oil Chem. Soc., 51, 9, 397, (1974).

13. Hasson, J.C. and Kulkarni, M.V., "Open-Tube-Column Gas Ghromatography of Resin Fluxes", Anal. Chem., 44, 9, 1586 (1972).

14. Horvath, C., and Melander, W., "Liquid Chromatography with Hydrocarbonaceous Bonded Phases; Theory and Practice of Reversed Phase Chromatography", J. Chromat. Sci., 15, 393, (1977).

15. Keith, L.H., "Identification of Organic Compounds in Unbleached Treated Kraft Paper Mill Wastewaters", Environm. Sci., Techol., 10, 6, 555, (1976).

16. Crathorne, B., Watts, C.D., and Fielding, M., "Analysis of Non-volatile Organic Compounds in Water by High-performance Liquid Chromatography", J. Chromat., 185, 671, (1979).

17. Krstulovic, A.M., Rosie, D.M. and Brown, P.R., "Selective Monitoring of Polynuclear Aromatic Hydrocarbons by High-Pressure Liquid Chromatography with a Variable Wavelength Detector", Anal. Chem., 48, 1383 (1976).

18. Majors, R.E., "Recent Advances in High-Performance Liquid Chromatography Packings and Columns, J. Chromat. Sci., 15, 334, (1977).

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1817-1829 (1981)

ISOCRATIC SEPARATION OF FATTY ACID DERIVATIVES BY REVERSED PHASE LIQUID CHROMATOGRAPHY. INFLUENCE OF THE SOLVENT ON SELECTIVITY AND RULES FOR ELUTION ORDER.

J.P. ROGGERO and S.V. COEN
Faculté des Sciences
33, rue L. Pasteur
84000 AVIGNON
France

ABSTRACT

The p-bromophenacyl esters of 16 fatty acids (C_{12}-C_{22}) have been separated by isocratic chromatography on a Radial Pak A cartridge (Reverse phase C_{18} material). The separation factors α were measured using two solvent mixtures of comparable strength and the superiority of methanol-water to acetonitrile-water becomes evident.

Five precise rules are established, which indicates the retention of every fatty acid. They explain the chromatographic process i.e. elution order, resolution and selectivity.

INTRODUCTION

HPLC is now widely employed instead of GLC to separate long chain fatty acid mixtures. After some experiments with Corasil II (1,2) or ion-exchange (3) columns the reverse mode, more versatile and of higher efficiency, became commonly used with C_8,C_{18} and in some cases C_{30} stationary phases (4-12).

The use of an UV detector, sensitive and ideal in gradient elution, is required for trace amounts of fatty acids (in the nanogram range). Then, several simple, rapid and exhaustive methods of derivatization are available giving benzyl, naphtacyl,

phenacyl and substituted phenacyl esters, all UV-absorbing. Another advantage of these derivatives is to permit direct quantitation of molar ratios based on peak areas. (1,4,6,13,14).

In the first analyses only simple mixtures were separated but in 1975 BORCH, employing a 90 cm μ Bondapak C-18 column, was able to resolve 24 fatty acids with a step-change program of solvent using a mixture of acetonitrile and water.

This mixture was also used by many authors and seems superior to tetrahydrofuran-water or dioxane-water, but PEI et al (7) or CHAN et al (8) employed methanol-water and D'AMBOISE et al (11) a ternary solvent mixture of acetonitrile, methanol and water. In most cases the gradient elution technique permitting the separation of acids from C_2 to C_{24} (9) has been used, but the isocratic mode is also possible (11).

The most important features of these separations are now well known :

The retention volume is :

- widely increased by chain enlargement,
- decreased by unsaturation,
- larger for the trans-unsaturated acid

Some authors also noted that small changes in position of the double bond affect the interaction between the fatty acid ester and the reverse phase.

Some separations are particularly difficult : those of isomers (α and γ linolenic...) but also palmitoleic (16:1) arachidonic (20:4) and myristic (14:0) acids. We note the bad resolution of oleic and vaccenic acids (18:1), arachidic (20:0) and erucic (22:1) acids, behenic (22:0) and nervonic (24:1) acids. In some cases elaidic (18:1 9t) and vaccenic (18:1 11c) acids co-elute and the separation of oleic and palmitic (16:0) acids is not always satisfactory.

No search has been done to obtain a precise evaluation (in given conditions) of the chromatographic factors k' or α which

are of considerable importance in the understanding of the retention rules. Such a determination needs the isocratic mode with a sufficiently wide variety of compounds ; this is now possible with a column of low back pressure and high efficiency (5-7,000 plates) such as the Radial Pak cartridge (Waters Associates).

MATERIALS AND METHODS

Reagents :

Fatty acids were purchased from Sigma Chemical Company and used without further purification ; p-bromophenacyl bromide and diisopropyléthylamine obtained from Fluka were also of satisfactory grade.

Acetonitrile and methanol of good quality were carefully rectified and a fraction of very low absorbance employed. Water was distilled from a glass still and all solvents filtered through a HA 0.45 (or FH 0.5) filter (Millipore Corp.).

Preparation of the chromatographic samples :

The derivatization was made according to the method of COOPER and ANDERS (4,14) using p-bromophenacyl bromide as reagent. Complete conversion requires heating at 60°C for 2 hr. For analysis of a standard mixture the only treatment before injection was a filtration on Millipore FH 0.5 µ. For application to a very small amount of saponified lipid a sample preparation by treatment on a Sep Pak C_{18} cartridge (Waters Associates) eliminates the high polarity compounds arising from the derivatization reaction and provides a large improvement of the baseline.

Instrumentation :

We used a Waters Associates Model 204 U/45 chromatograph equipped with a M.45 solvent delivery system, a U6K injector and a M 440 absorbance detector operating at 254 nm. The column was a

Radial Pak A cartridge (10μ reverse phase C_{18}) pressurized in a compression module RCM 100.

The efficiency of our columns was 5,000-7,000 plates and v_o from 1.7 to 2.0 ml.

Eluents :

Two solvent mixtures of comparable "strength" (15) have been used : 87-13 acetonitrile-water (azeotropic mixture) and 90-10 methanol-water.

Although k' values for linoleic acid were close ($V_R \simeq 45$-50 ml) these solvents are different in polarity (P' = 6.4 for acetonitrile-water and 5.6 for methanol-water) and present large differences in interaction with the solute.

Flow rate, 2 ml/min, was increased to 4 ml/min. after elution of oleic acid. Experiments with other flow rates gave no modification of results.

Analyses :

Mixtures of 4-6 sufficiently different fatty acid derivatives were injected into the chromatograph and elution times carefully noted. α values were firstly related to oleic acid for convenience, that acid eluting after 35-45 minutes. Recalculations gave α related to stearic acid. When two acids co-eluted they were reinjected separately, but always with the reference.

RESULTS

Experimental results :

Although the capacity factor k' of a particular fatty acid derivative differs from a column to another and decreases when after some analyses degradation has occurred, the separation factor α (relative retention) of two species never varies on a given

column and does not differ significantly when measured with another one. Reproducibility is better than 1%.

Table I shows the separation factors related to stearic acid in each case (α_A and α_M). Evidence of the greater selectivity of acetonitrile-water appears and is better noted with the results shown in tables 2 and 3 (dividing two values of table 1 gives the separation factor between the chosen acids). In all cases chain lengthening and increase in unsaturation cause larger modifications of retention when using acetonitrile-water as solvent.

But another feature of the separation of fatty acid derivatives using acetonitrile-water in isocratic mode is the very bad resolution between two major components of all lipids : palmitic (16:0) and oleic (18:1 9c) acids ; in our conditions 15,000 plates

TABLE 1

Separation Factors Related to Stearic Acid.

Num. Ref.	Acid derivatives	α_A	α_M	α_A/α_M
12 : 0	Lauric	0.138	0.148	0.932
14 : 0	Myristic	0.264	0.278	0.950
16 : 0	Palmitic	0.510	0.527	0.968
18 : 0	Stearic	1	1	1
14 : 1	Myristoleic	0.145	0.173	0.838
16 : 1	Palmitoleic	0.270	0.309	0.874
18 : 1 9c	Oleic	0.519	0.560	0.927
18 : 1 9t	Elaidic	0.571	0.610	0.936
18 : 1 6c	Petroselenic	0.551	0.593	0.929
18 : 2	Linoleic	0.303	0.372	0.814
18 : 3 ω_3	α Linolenic	0.192	0.265	0.725
18 : 3 ω_6	γ Linolenic	0.196	0.258	0.760
20 : 3 ω_3	Eicosatrienoic 11.14.17	0.357	0.479	0.745
20 : 3 ω_6	Eicosatrienoic 8.11.14	0.356	0.451	0.789
20 : 4 ω_6	Arachidonic	0.243	0.338	0.719
22 : 6 ω_3	Docosahexaenoic	0.194	0.314	0.618

would be required for R_S = 0.6. The separation of myristic (14:0) and palmitoleic (16:1) acids is also difficult.

These resolutions being excellent with methanol-water the better solvent is thus the less "selective".

That anomaly is in fact due to the similar changes in retention caused by a first unsaturation (table 3) or by decrease in chain length. Methanol-water being more sensitive to the second factor provides a better result.

A more precise survey of tables 1, 2 and 3 shows other interesting features which suggest many questions :

- Methanol-water resolves perfectly the two isomers of eicosatrienoic acid (20:3), not the linolenic acids (α values are 1.06 and 1.03 respectively, the second one being too low for a convenient resolution). With acetonitrile-water the isomers are always unseparable.

- The ω_3 family seems more sensitive to chain lengthening than the ω_6 one (table 2).

- A change in position of the double bond affects the retention (as in case of oleic and petroselenic acids). This result is apparent in table 3 when we remind us that myristoleic (14:1)

TABLE 2

Relative Retentions Between Homologs. Effect of Chain Lengthening.

Numeral representations	MeCN-H_2O	MeOH-H_2O
14 : 0 / 12 : 0	1.91	1.88
16 : 0 / 14 : 0	1.93	1.89
18 : 0 / 16 : 0	1.96	1.89
16 : 1 / 14 : 1	1.86	1.79
18 : 1 / 16 : 1	1.92	1.81
20 : 3 ω_3 / 18 : 3 ω_3	1.86	1.81
20 : 3 ω_6 / 18 : 3 ω_6	1.82	1.75

TABLE 3

Relative Retentions Between Acids of Same Chain-Length. Effect of Unsaturations.

Numeral representations	MeCN-H_2O	MeOH-H_2O
14 : 0 / 14 : 1	1.82	1.61
16 : 0 / 16 : 1	1.89	1.71
18 : 0 / 18 : 1 9c	1.93	1.79
18 : 0 / 18 : 1 6c	1.81	1.69
18 : 1 / 18 : 2	1.71	1.51
18 : 2 / 18 : 3 ω_3	1.58	1.40
18 : 2 / 18 : 3 ω_6	1.55	1.44
20 : 3 ω_6 / 20 : 4 ω_6	1.47	1.33

and palmitoleic (16:1) acids have not the double bond in the middle of their chain.

- The largest effect produced by the introduction of a third unsaturation into a C_{18} chain (i.e. the transformation of 18:2 into 18:3) is obtained with the ω_3 compound (α linolenic) when using acetonitrile-water. Elution with methanol-water reverses that result (table 3).

Rules for elution order :

We suggest to lay down five simple rules providing a satisfactory interpretation to all these experimental results. The first three are readily perceived.

First Rule

Other things being equal the trans unsaturated derivative elutes after the cis isomer.

For instance elaidic acid is more retained than oleic acid. PEI et al (7) also noted the case of palmitelaidic acid (16:2 9t).

Second Rule

For saturated acids, all chain lengthening by two carbon atoms causes the retention volume to be multiplied by a nearly constant factor. (Nevertheless that factor slightly increases with chain-length when using acetonitrile-water : table 2).

Third Rule

Unsaturation lowers retention. That effect is decreasing when unsaturation increases (table 3).

The other rules relate to position isomerism and necessitate further developments. Let us consider that the double bonds as a whole divide the fatty chain in two parts : the internal chain I and the terminal chain II :

$(CH_3 \ldots .) (CH = CH - CH \ldots CH = CH) (\ldots .)COOH$

Chain II Chain I

TABLE 4

Lengths of the Internal (I) and Terminal (II) Chains of Unsaturated Fatty Acids.

Num. Ref.	Fatty acid derivative	I	II
14 : 1 9c	Myristoleic	7	4
16 : 1 9c	Palmitoleic	7	6
18 : 1 9c	Oleic	7	8
18 : 1 6c	Petroselenic	4	11
18 : 2 9c 12c	Linoleic	7	5
18 : 3 ω_3	α Linolenic	7	2
18 : 3 ω_6	γ Linolenic	4	5
20 : 3 ω_3	Eicosatrienoic 11.14.17	9	2
20 : 3 ω_6	Eicosatrienoic 8.11.14	6	5
20 : 4 ω_6	Arachidonic	3	5
22 : 6 ω_3	Docosahexaenoic	2	2

These chains are shown in table 4. We find :

Fourth Rule

Other things being equal the retention is generally minimal if chains I and II are equal. As a consequence the retention is more increased by an enlargement of the longest chain.

That rule applies perfectly to interpret a previously noted difference between ω_3 and ω_6 families : a more important α factor for 20:3 versus 18:3 is observed in the first case in which the longest chain is involved (table 4)

One fact, however, needs interpretation : as previously noted in table 3, the α values between linoleic acid and the linolenic acid isomers are inverted by a change of solvent. That result suggests the last rule :

Fifth Rule

When eluting with acetonitrile-water a very short terminal chain II may cause a large decrease in retention. In some cases that effect prevails (ω_3 effect).

DISCUSSION

The foregoing rules may receive some theoretical support in considering the interactions involved in the chromatographic process.

In reversed phase chromatography the eluent is frequently a binary mixture of water and of a miscible less-polar solvent ; thus a change in that second solvent generally provides limited modifications in selectivity.

However some significant changes related to solute-solvent interactions may be observed. In our work, methanol-water, less-polar, elutes more quickly the saturated species but the retentions of polyunsaturated acids are larger. Table 5 shows an example to illustrate this fact.

TABLE 5

Retention Volumes of some Fatty Acid Derivatives (Consecutive Analyses using the same Column).

Numeral reference	$(v-v_o)$ ml CH_3CN-H_2O	$(v-v_o)$ ml CH_3OH-H_2O
18 : 0	162	127
16 : 0	83	67
14 : 0	43	35
18 : 1	84	71
18 : 2	49	47
18 : 3 ω_3	31	34
20 : 4 ω_6	39	43
22 : 6 ω_3	31	40

Methanol is essentially a strong hydrogen acceptor and has no important specific interaction with the fatty acid moiety of the solute. Thus the main effect of unsaturation is to decrease the fatty chain responsible for retention.

Rule 4 shows the large importance of a long continuous chain and, for example, the change from 14:0 to 14:1 generates less modification in retention than from 18:0 to 18:1, 9c (see tables 3 and 4).

Although that effect remains while using acetonitrile-water, we must consider the dramatic change in α values for unsaturated species.

In the last column of table 1 is shown the α_A/α_M ratio which is an interesting factor in such a survey. Taking into account the foregoing considerations the α_M factor may be accepted as "normal," thus a decrease in α_A/α_M ratio reveals some specific dipole-dipole interaction between the solute and acetonitrile. We observe that α_A/α_M ratio is increased by chain lengthening, the solute becoming less polar, and decreased largely by increasing unsaturation. The ω_3 effect (rule 5) significantly lowers α_A/α_M as compared to ω_6

isomers, showing an enhancement of the solvent-solute interaction at the level of the terminal double bond permitted by a lack of steric hindrance.

This effect is strong enough to make α linolenic acid slightly inferior to γ linolenic in retention. In case of eicosatrienoic acid isomers the chain lengthening prevails and rule 4 applies but the compounds are extremely close and no separation occurs whereas methanol-water provides an efficient resolution.

CONCLUSIONS

Although giving larger separations between homologs or between differently unsaturated fatty acids, acetonitrile-water provides less satisfactory resolution than methanol-water in analysis of a complex mixture using the isocratic mode. Giving a better repartition of peaks in a shorter analysis methanol-water is the best solvent in these conditions (fig. 1)

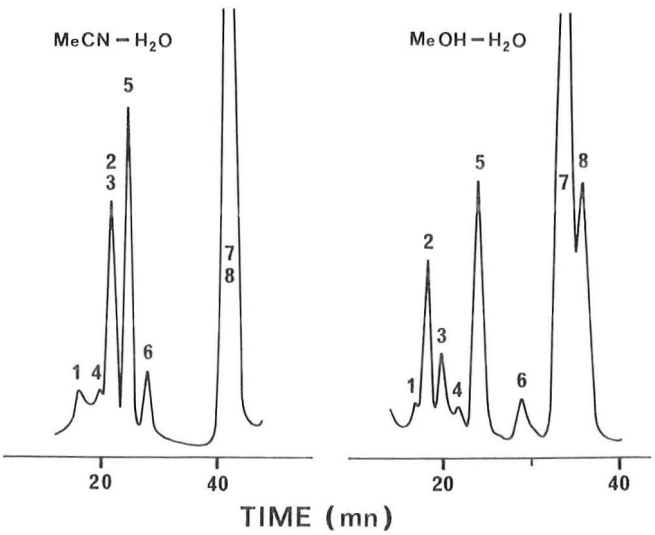


FIGURE 1 : Parts of the actual chromatograms of fatty acids extracted from the phospholipids of a fungus (unpublished results).

1: α linolenic - 2:myristic - 3:palmitoleic - 4:arachidonic
5: linoleic - 6:eicosatrienoic (ω_6) - 7:palmitic - 8:oleic.

Some simple empiric rules, confirmed by theoretical condiderations upon solute-solvent interactions, may permit to predict elution order and separation, facilitating the choice of solvent.

Use of our technique for the analysis of fatty acids from phospholipids of fungi will be reported elsewhere.

ACKNOWLEDGMENT

The authors thank Mrs Monique JOUBERT for her technical assistance.

REFERENCES

1. Politzer, I.R., Griffin, G.W., Dowty, B.J. and Laseter J.L. Enhancement of Ultraviolet Detectability of Fatty Acids for purposes of Liquid Chromatographic Mass Spectrometric Analyses. Anal. Lett. 6, 539 (1973).

2. Mikes, F., Schuring, V. and Gil-Av., E., Complex Forming Stationary Phases in High Speed Liquid Chromatography. J. Chromatogr. 83, 91 (1973)

3. Takata Y. and Muto G., Flow Coulometric Detector for Liquid Chromatography. Anal. Chem., 45, 1864 (1973).

4. Cooper, M.J. and Anders M.W., Determination of Long Chain Fatty Acid as 2-naphtacyl Esters by High Pressure Liquid Chromatography and Mass Spectrometry. Anal. Chem., 46, 1849 (1974).

5. Cooper, M.J. and Anders, M.W. High Pressure Liquid Chromatography of Fatty Acids and Lipids. J. Chromatogr. Sci. 13, 407 (1975).

6. Borch, R.F. Separation of Long Chain Fatty Acids as Phenacyl Esters by High Pressure Liquid Chromatography. Anal. Chem. 47, 2437 (1975).

7. Pei, P.T.S., Kossa, W.C. Ramachandran, S, Henly, R.S. High Pressure Reverse Phase Liquid Chromatography of Fatty Acid p-Bromophenacyl Esters. Lipids 11, 814 (1976).

8. Chan, H.W.S. and Levett, G. Silver Nitrate in Reversed-Phase High Performance Liquid Chromatography. Separation of cis- and trans- Isomers of monoenoic Fatty Acid p-Bromophenacyl Esters. Chem. & Ind. 578 (1978).

9. Jordi, H.C. Separation of Long and Short Chain Fatty Acids as Naphtacyl and Substituted Phenacyl Esters by High Performance Liquid Chromatography. J. Liquid. Chromatogr. 1, 215 (1978).

10. Mell, L.D. Jr, Joseph S.W. and Bussell, N.E. Cellular Fatty Acid Composition of Vibrio parahaemolyticus by Reversed-Phase High-Performance Liquid Chromatography. J. Liquid Chromatogr. 2, 407 (1979).

11. D'Amboise, M. and Gendreau M. Isocratic Separation of Human Blood Plasma Long Chain Free Fatty Acid Derivatives by Reversed Phase Liquid Chromatography. Anal. Lett. 12, 381 (1979).

12. Takayama, K, Jordi, H.C. and Benson, F. Separation of Fatty Acids as their p-Bromophenacyl Esters on a C_{30}-Bonded Silica Column by High Performance Liquid Chromatography. J. Liquid Chromatogr. 3, 61 (1980).

13. Durst, H.D., Milano, M., Kikta, Jr E.J., Connelly, S.A. and Grushka, E. Phenacyl Esters of Fatty Acids via Crown Ether Catalysts for Enhanced Ultraviolet Detection in Liquid Chromatography. Anal. Chem. 47, 1797 (1975).

14. Lawrence, J.F. and Frei, R.W., Chemical Derivatization in Liquid Chromatography. Elsevier. Amsterdam 1976. p 127.

15. Snyder, L.R. and Kirkland, J.J. Introduction to Modern Liquid Chromatography. Second Edition. J. Wiley, New-York 1979, p. 264.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1831-1845 (1981)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF D-GLUCOSE IN ERYTHROCYTES

Steven H. Wong, George Kalache and Edward E. Morse

Department of Laboratory Medicine
University of Connecticut Health Center
Farmington, Connecticut 06032

ABSTRACT

A procedure is presented after several attempts with different modes of chromatography for measuring high concentrations of d-glucose in erythrocytes. The procedure utilizes rapid deproteinization of hemolysate by mixing with acetonitrile. The supernatant is analyzed by strong cation exchange chromatography, using an Organic Analysis Column. Separation conditions are: eluent = 0.01 N H_2SO_4, flow rate = 0.6 ml/min, detection = 195nm at 0.05 AUFS, sample size = 20 µl and temperature = ambient. The coefficients of variation for 5 mg/ml samples were (within-run) 6.7%, and (day-to-day) 7.1%. This study shows the presence of a high concentration (1900 mg/dl) of d-glucose within the erythrocytes as a result of a high external d-glucose concentration (2000 mg/dl) in plasma, and suggests that d-glucose is rapidly transported into the cell.

INTRODUCTION

An increase in electronic mean corpuscular volume (MCV) induced by marked hyperglycemia was recently reported by Morse, et al. (1) A patient showed a high glucose serum concentration of 2250 mg/dl (22.5 mg/ml) with MCV of 128 μ^3. Postulations

included the displacement of Na^+ and Cl^- ions as a result of the association of glucose with the cell membrane, or the volume change within the cell membrane caused by glucose induced osmotic effect. The same report noted that sucrose did not induce any volume changes. In order to determine the validity of the latter postulation, intra-erythrocytic monosaccharide concentration measurements might be useful.

Measurement of carbohydrates in blood may be accomplished by a wide variety of methods. These methods include chemical testing (2) (3), enzymatic methods (4) (5), thin-layer chromatography (6) (7) (8), and gas-liquid chromatography (9) (10). More recently, high-performance liquid chromatographic (HPLC) columns have been available to measure carbohydrates (11) (12) (13). For our investigation, it would be desirable to measure high concentration of glucose (20 mg/ml) in erythrocyte with minimum sample preparations. For these purposes, GLC assays which require extensive sample preparation were not attempted while HPLC was chosen for this study so that a simple deproteinization step was used for sample clean-up, followed by HPLC separation of d-glucose from endogenous interference peaks.

Separation of carbohydrate by HPLC is based upon three different kinds of mechanism as recently reviewed by Schwarzenbach (14). These are ionic exchange chromatography, partition chromatography on ion exchange resin and partition chromatography on chemically bonded phases. The present study outlines several

attempts to measure endogenous d-glucose in the red blood cell as well as exogenous d-glucose using one of the above three mechanisms.

EXPERIMENTAL

Reagents

D-glucose, 'Baker-Analyzed' reagent was purchased from J. T. Baker Chemical Co., (Phillipsburg, N. J. 08865). Acetonitrile, UV grade, was obtained from Burdick and Jackson Laboratories (Muskegon, Mich. 49442). Water was double distilled. Tetra-ethylene-pentyl amine (TEPA) was purchased from Aldrich Chemical Co., (Milwaukee, Wis. 53233).

High-Performance Liquid Chromatographs

Several HPLC were used in this study. Preliminary studies with a Radial-pak B column, consisting of silica gel, were performed by using a M6000A pump with a Model 450 variable wavelength detector from Waters Assoc., (Milford, Mass.). The separation was carried out by using a mixture of acetonitrile/water 75:25, TEPA (0.01%) as eluent at a flow rate of 2 ml/min, and at room temperature. The detector was set at 195 nm with an attenuation of 0.02 AUFS. The other preliminary studies with a carbohydrate column from Bio-Rad Laboratories, (Richmond, Calif.), were carried out by using a Model 5000 HPLC from Varian Associates, (Walnut Creek, Calif.), with a Rheodyne 7125 injector equipped with a 20 µl loop. The detector was a Vari-Chrom variable wavelength detector from

Varian Associates, set at 195 nm, 0.1 or 0.05 AUFS attenuation. The eluent was water. The separation was carried out at a flow rate of 0.3 ml/min and at a temperature of 85°C.

Analyses of normal and high concentration erythrocytic d-glucose were performed by using a Laboratory Data Control Constametric III Pump with a Spectro-Monitor III variable wavelength detector from Laboratory Data Control (Riveria Beach, Florida). The injector was a Rheodyne 7125 injector with a 100 µl loop. The column was an Organic-acid analysis column from Bio-Rad Laboratory (Richmond, Calif.). Column packing was made from strong cation ion exchange resin with proton as the counter ion. The guard column was a size-exclusion HPX-87 cartridge from Bio-Rad. The eluent was 0.005N H_2SO_4. Analyses were carried out at a flow rate of 0.3 ml/min at room temperature. Detector was set at 195 nm and at an attenuation of 0.01 AUFS. The established procedures, including calibration, precision and high d-glucose concentration studies, were carried out by using a Model 5000 HPLC from Varian Associates as described previously. The eluent was 0.01N H_2SO_4. Flow rate was 0.6 ml/min and the separation temperature was ambient.

Sample Preparation

The preliminary studies using the silica-gel, carbohydrate and organic acid analysis columns involved protein precipitation of normal and high d-glucose concentration samples by mixing with

acetonitrile (1:1 or 1:2) and spun for ten minutes. Aliquots of the clear supernatant were injected into the HPLC.

For the calibration and precision studies, the following protocol was followed. EDTA blood was spun at 4000 revolutions per minute for 5 minutes. Plasma and upper layer of cells were removed. One ml of packed red blood cells was transferred to a marked test tube, and 9 ml of distilled water was added. To this hemolysate preparation, d-glucose was added to yield a concentration of 20 mg/ml. By serial dilution with normal hemolysate, the following d-glucose hemolysate calibration samples were prepared: 0, 2, 8, 10 and 15 mg/ml. Five 5 mg/ml d-glucose hemolysate replicates were also included for the precision study. Preparation of the high external d-glucose concentration hemolysate involved incubation of d-glucose/whole blood (20mg/ml) at room temperature for one hour. Then, the hemolysate was prepared from this sample as described previously.

From the above hemolysate preparations, 200 µl was taken from each sample and mixed with 400 µl of acetonitrile. These mixtures were vortexed, followed by spinning at 2000 RPM for 10 minutes. Twenty µl of the clear supernatant was injected for HPLC analysis. Peak heights were plotted against d-glucose hemolysate concentrations. Linear regression analysis was carried out to determine the correlation coefficient, slope and intercept. From the peak heights of the precision studies (5mg/ml) samples

and the high concentration hemolysate, the d-glucose hemolysate concentrations were estimated from these plots.

Results and Discussions

Measurement of d-glucose in erythrocytes consisted of two parts: the preparation of the hemolysates from the red blood cells, and the analyses of the hemolysates by HPLC. In attempting to optimize the hemolysate preparation, the procedure was kept as simple as possible so that any enzymatic activity would be mini-

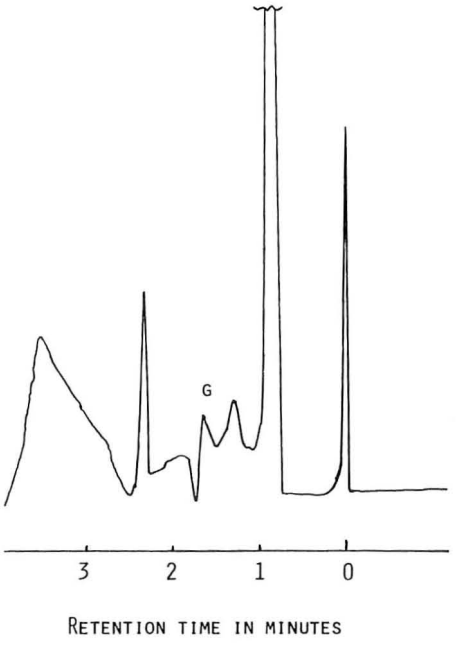


Figure 1: Chromatogram of a normal hemolysate sample. Column: RP-B, (Silica gel) Waters Associates, mobile phase: acetonitrile/water (75:25) with 0.01% TEPA, flow rate 2 ml/min, detection at 195 nm, 0.02 AUFS, injection volume 40 µl, and G = d-glucose.

mized. This was accomplished by protein precipitation by acetonitrile. Figure 1 shows the chromatogram of a normal hemolysate. The separation was carried out by using a Radial-Pak B column consisting of silica-gel packing, and the eluent was acetonitrile/water (75:25), TEPA (0.01%). D-glucose eluted at 1.5 minutes with retention volume (V_R) of 3 ml. The normal hemolysate chromatogram showed a peak with the same retention time as glucose. Endogenous peaks eluted before and after this glucose peak. Due to the small V_R = 3 ml and the negative peak following the d-glucose peak which resulted in inaccurate quantitation, this assay was not further developed. In addition, the many advantages of ion exchange resin over silica-gel packing, such as long column life and wide useable pH range (a.) 0 to 14 for cation exchange and (b.) 0 to 12 for anion exchange, led the investigation to ion exchange mode for assaying erythrocytic d-glucose.

Figures 2A and 2B show the chromatograms of a normal and high concentration (2 mg/ml) hemolysates. The column was a strong cation exchange column with Mg^{+2} as the counter ion. Mobile phase was water. Separation was carried out at 85°C and the detector was set at 195 nm. D-glucose eluted on the shoulder of an endogenous peak with retention time of 4.6 minutes, and V_R = 1.4 ml. The incomplete separation and small V_R indicated that these chromatographic conditions were not ideal to identify d-glucose in hemolysate. Thus, another form of ion exchange column packing was used.

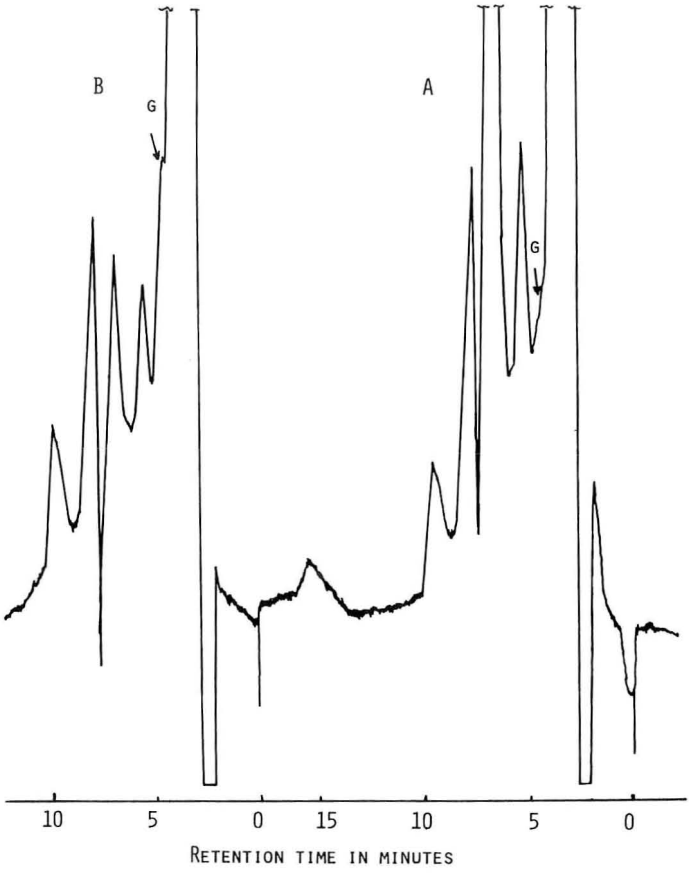
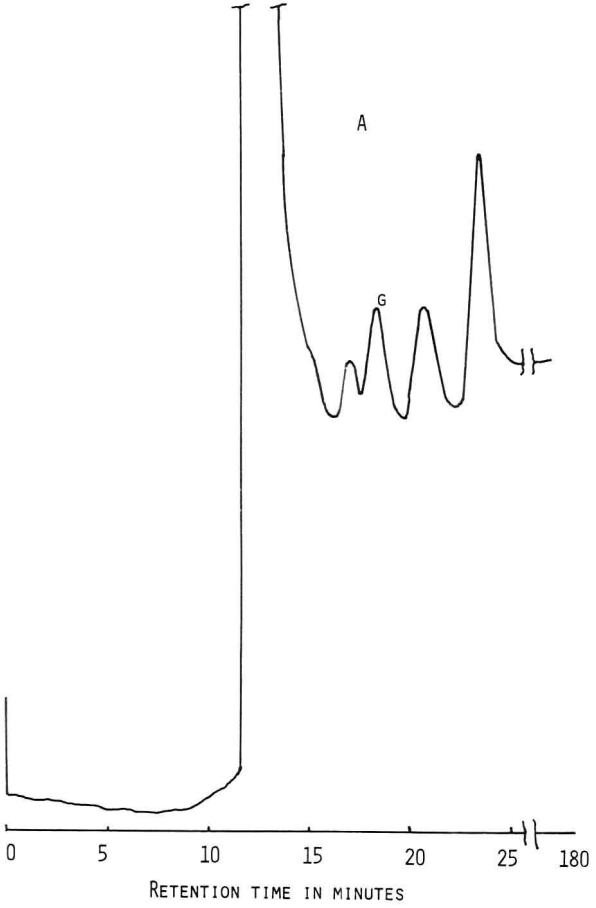


Figure 2A: and 2B: CHromatograms of a normal and high d-glucose hemolysate sample. Carbohydrate column (Bio-Rad Laboratories), mobile phase: water, flow rate 0.3 ml/min, detection at 195 nm, at 0.02 AUFS, temperature = 85°C, injection volume = 4 µl and G = d-glucose.

The column of choice was a newly available organic acid column with a strong cation exchange packing and a counter ion of proton. Figures 3A and 3B show the chromatograms of normal and high concentration (3 mg/ml above normal) hemolysate prepared by mixing plain or glucose-spiked red cells with an equal volume of acetoni-



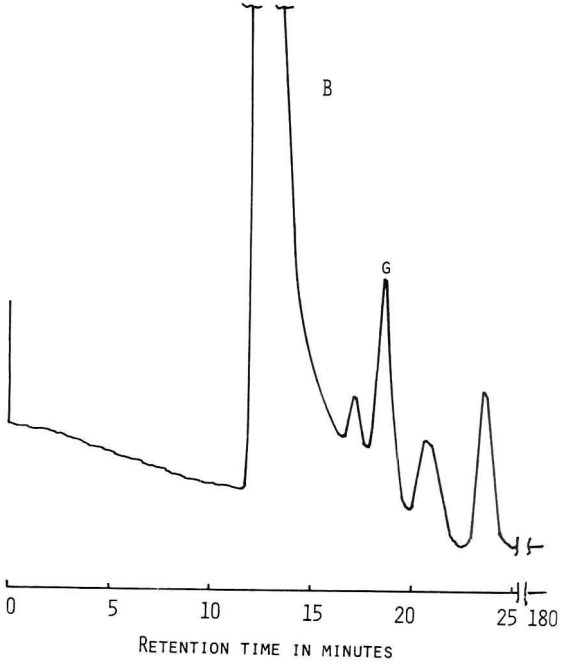


Figure 3A:
and 3B:

Chromatograms of a normal and high d-glucose hemolysate sample. Organic Acid Analysis Column (Bio-Rad Laboratories), mobile phase: 0.005 N H_2SO_4, flow rate 0.3 ml/min, detection at 195 nm 0.01 AUFS, injection volume 5 µl, and G = d-glucose.

trile for protein precipitation. The eluent was 0.005N H_2SO_4 at a flow rate of 0.3 ml/min with detection at 195 nm. The normal hemolysate showed the presence of a glucose peak at 19 minutes, V_R = 5.7 ml, while the high concentration hemolysate showed a higher peak at the same retention time. These two chromatograms suggested the presence of d-glucose in normal hemolysate. The glucose peak was followed by peaks eluting with retention time up to two and a half to three hours. These peaks were probably due to

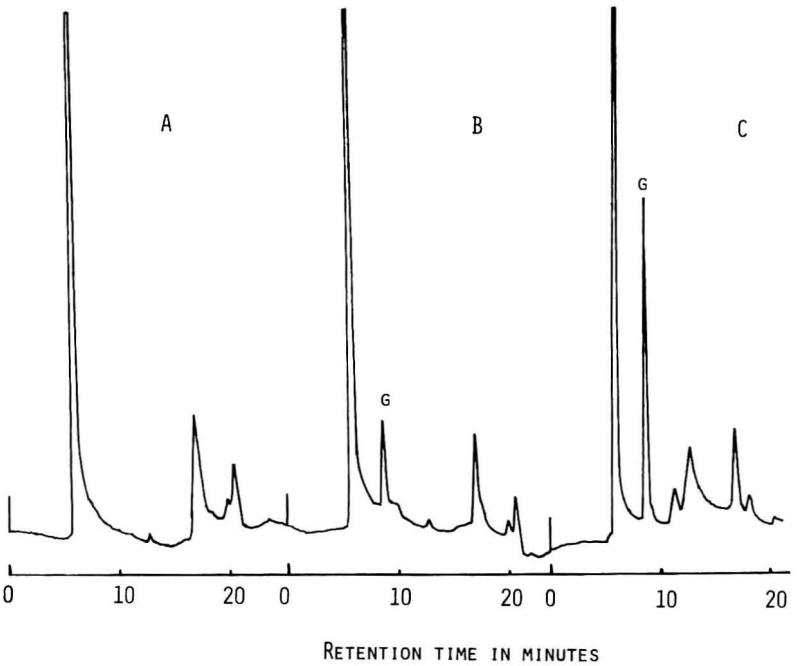


Figure 4A: Chromatograms of a normal, 2 mg/ml and 10 mg/ml d-
4B: glucose hemolysate sample. Organic Acid Analysis
and 4C: Column, (Bio-Rad Laboratories) mobile phase: 0.01 N H_2SO_4, flow rate 0.6 ml/min, detection at 195 nm, 0.05 AUFS, injection volume 20 µl, and G = d-glucose.

endogenous substances such as amino acids and nucleosides. Since the chromatographic separation time was lengthy before the column was completely regenerated for the next sample analysis, this method was not used for multiple samples analysis needed to assay d-glucose concentration in erythrocytes. Thus, chromatographic conditions were optimized to analyze the hemolysates within 30 minutes.

Figure 4 shows the chromatograms of normal hemolysate, 2 mg/ml and 10 mg/ml spiked hemolysates. The hemolysate samples were prepared as outlined in the experimental section. Figure 4A shows the chromatogram of a normal sample. D-glucose was not found due to the dilution factor of thirty fold . Normal glucose concentration in whole blood is 65-110 mg/dl, i.e. 0.7 to 1.1 mg/ml. The maximum amount of d-glucose introduced for analysis was 0.6 µg, not detectable with the present detector and its appropriate attenuation.

Figures 4B and 4C show the chromatograms of hemolysate samples with 2 mg and 10 mg per ml of hemolysates respectively. Retention time of d-glucose was 9 minutes, with V_R = 5.4 ml. The d-glucose peak was well resolved from the endogenous peak at 6 minutes. Multiple peaks eluted after d-glucose. Analysis time for each sample was between 20 to 30 minutes.

Figure 5 shows that the plot of peak height of d-glucose versus d-glucose/hemolysate concentration was linear with correlation coefficient = 0.9977, slope = 0.878 and intercept = -0.020.

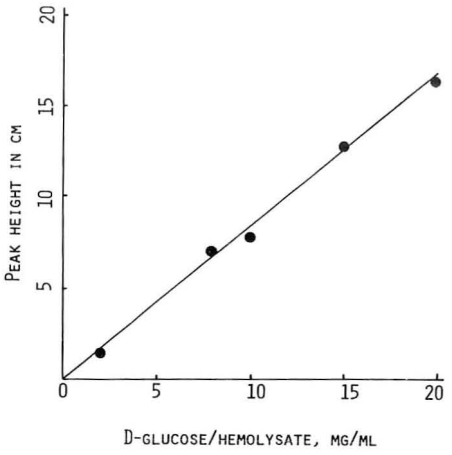


Figure 5: Calibration curve for d-glucose/hemolysate concentration versus d-glucose peak heights.

Precision studies showed that the within-run coefficient of variation (CV) for the 5 mg/ml samples was 6.7% (mean = 4.91, S.D. = 0.33 and number of samples = 5), and the day-to-day CV was 7.1% (mean = 5.04, S.D. = 0.36 and number of samples = 40). This assay was used to measure erythrocytic d-glucose with high d-glucose whole blood concentration. D-glucose was added to the whole blood so that the concentration was 2000 mg/dl above normal concentration. Figure 6 shows the chromatogram of the hemolysate of such a sample. D-glucose peak height was 1.6 cm, corresponding to 1.9 mg of d-glucose per ml of hemolysate, prepared by ten fold dilution of erythrocytes. This was equivalent to 19 mg/ml, or 1900 mg/dl of erythrocytes. This d-glucose concentration probably represented d-glucose inside the red blood cells as well as the d-glucose in the plasma trapping. Plasma trapping occurred as a

result of the separation of plasma from red blood cells by centrifugation. The amount of plasma trapped between the red blood cells was estimated to be about three to five percent.Thus the majority of the 1900 mg/dl d-glucose detected was due to the erythrocytic d-glucose.

The present study shows a method of simple measurement of d-glucose in hemolysate using a single deproteinization step, followed by strong cation exchange HPLC using an organic acid analysis column. The experience from three months of precision studies indicated that this column was stable. The absence of d-glucose response of normal hemolysate was due to the dilution

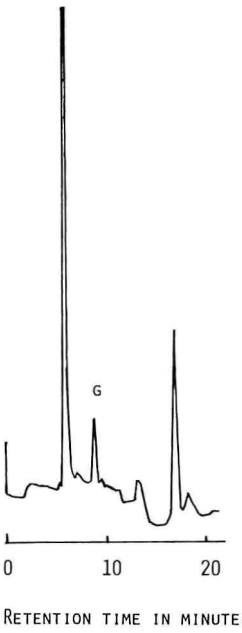


Figure 6: Chromatogram of a high external d-glucose hemolysate sample. Conditions same as in figure 4A.

factor. The assay utilized the UV detection of carbohydrate at 195 nm instead of a refractive index detector. This assay may be developed to measure metabolite of d-glucose such as sorbitol. The present study represents a new liquid chromatographic method to measure directly high d-glucose concentration inside the red blood cells.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of the following persons: Mr. Dale Jensen of the Waters Associates for providing the HPLC for the preliminary study, Mr. Mike Gray of the Bio-Rad Laboratories for providing the Organic Acid Analysis columns, Mr. Bill Joyce of the Varian Associates for loan of a Varichrom detector, and Mrs. Linda Karwoski for typing the manuscript.

REFERENCES

1. Morse, E. G., Kalache, G., and Stockwell, R., Annals of Clinical and Laboratory Science, 10(5), 450, 1980.
2. Hagedorn, H. C., and Jensen, B. N., Biochem Z, 135, 46, 1923.
3. Marais, J. P., deWit, J. L., and Quicke, G. V., Anal.Biochem. 15, 373, 1966.
4. Muller, D., Biochem Z, 199, 136, (1928).
5. Schmidt, F. H., Klin Wochschr, 39, 1244, (1961).
6. Gal, A. E., Anal. Biochem., 24, 452, 1968.
7. Mes, J., and Kamm, L., J. Chromatography, 38, 120, 1968.
8. Young, D. S., and Jackson, A., Clin. Chem., 16, 954, 1970.

9. Travis, S. F., Morrison, A. D., Clements, R. S., Jr., Winegrad, A. I. and Oski, F. A., J. of Clin. Investigation, 50, 2104, 1971.

10. Sweeley, C. C., Bentley, R., Makita, M., and Wells, W. W., JACS, 85, 2497, 1963.

11. Schwarzenbach, R., J. Chromatography, 117, 206, 1976.

12. Saunders, R. M., Carbohyd. Res., I, 76, 1968.

13. Rocca, J. L. and Rouehouse, A., J. Chromatography, 117, 216, 1976.

14. Schwarzenbach, R. in "Biological/Biomedical Applications of Liquid Chromatography II" Eds, Hawk, G. L., Champlin, P. B., Hulton, R. F., Jordi, H. C. and Mal, C., Marcel Dekker Inc., N. Y., 1978, p. 193.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1847-1854 (1981)

SELECTION OF A SUITABLE SOLVENT SYSTEM FOR THE ISOLATION OF TOXICOLOGICALLY ACTIVE COMPONENTS OF AN AFRICAN ARROW POISON OF PLANT ORIGIN

Michael Dennis* and Richard F. Ochillo**
Laboratories of Pharmacology and Toxicology
Xavier University of Louisiana
New Orleans, LA 70125

ABSTRACT

The efficiency of different solvent systems in the isolation of toxicologically active components of an african arrow poison of plant origin was investigated. The solvent systems were: de-ionized water; acetonitrile; acetonitrile/water (9:1); gradient elution with carbon tetrachloride/chloroform (10-50%); gradient elution with sodium chloride (0.02M) acetonitrile (45-95%) and; methanol/de-ionized water (98:2) and; chloroform and carbon tetrachloride (10-50%) gradient elution. The gradient elution with sodium chloride (0.02M)/ acetonitrile (45-95%) gave the best separation of four distinct peaks suggesting the presence of at least four components in the arrow poison. De-ionized water eluted the arrow poison from the column in one single fraction, whose peak height was linearly related to the amount of toxin (0.02 - 20.0 µg) suggesting that HPLC might also be useful in quantifying the toxin and/or its active components.

INTRODUCTION

Drug development has become very expensive and the need for exploring new and relatively cheaper approaches for developing drugs has become urgent as the debates for medicare cost containment reaches a new crescendo. Traditional african medi-

---

* Michael Dennis was a junior undergraduate when this investigation was carried out.

**To whom inquires and reprint requests should be directed.

cine is one of the possible new hopes and frontiers. African folk medicine provides a challenge for the development of new therapeutic agents based on phytotherapy, which has evolved over a long period of time. Accordingly, we have decided to toxicologically characterize an african arrow poison of plant origin by using modern chromatographic techniques to isolate the active component(s) and eventually investigate the mechanism of action of the toxin.

We previously used thin layer and ion exchange chromatographic methods to isolate the active components of the toxin (Cook, Dennis and Ochillo, 1979; Dennis, Cook and Ochillo, 1979; Cook, Dennis, Rolfs, Pugh and Ochillo, 1980; Cook, Dennis and Ochillo, 1981). Although these chromatographic techniques led to satisfactory separation of the active principles of the toxin, they are quite time-consuming and not readily subject to automatic methods of analysis. Therefore, we have used high performance liquid chromatography (HPLC) to separate toxicologically active components of the arrow poison and the results of our separation are reported in this manuscript. Also, we have attempted to use HPLC to quantify the toxin.

MATERIALS AND METHODS

Arrow poison is heat-extracted from plants by the natives of Kenya from where the sample used in this investigation was obtained. The procedures for preparation and the types of plants the poison is extracted from is in preparation and will be published elsewhere. However, the material under test was a dry extract. The chemical reagents used in this investigation were purchased from Sigma Chemical Company (St. Louis, MO) or from Burdick and Jackson Laboratories, (Muskegon, MI).

The acetonitrile, methanol, chloroform and carbon tetrachloride were used to elute the components of the toxin from the column as received from commercial sources. The sodium chloride solution was made from crystals as required. Appropriate elution solvent mixtures were prepared as follows: sodium chloride (0.02M)/acetonitrile (8:2); acetonitrile/deionized water (9:1) and; gradient elution of sodium chloride (0.02M)/acetoni-rile (45-95%), methanol/deionized water (98:2) and; chloroform and carbon tetrachloride (10-50%) gradient elution.

A 200mg portion of the poison (RFO-KMM-#1) was weighed and dissolved in de-ionized water and filtered through a millipore filter (pore size, 0.45 um). A 20 ul aliquot of the sample was injected into a Perkin-Elmer Series 3 HPL (Perkins-Elmer, Norwalk, Connecticut) equipped with a deuterium power supply, a digital programmer, an optical unit for detection of UV absorption at 260nm, a stainless steel HPLC column (25 x 0.26 cm) packed with Silica-A (HC-ODS-Sil-S, Lot 34) and a model 023 recorder. The flow rate was 1.0ml/minute (-cm/min.) with a pressure drop of 1800 psig at ambient temperature.

RESULTS AND DISCUSSION

The results of the separation of active principle(s) using HPLC and different solvent systems are presented in Figure 1-4. Since the separation using de-ionized water as a solvent led to the elution of only one peak, we decided to use this solvent to investigate the relation between the sample size injected into the column and the peak height. The relationship is shown in Fig. 5.

The results of our preliminary investigation indicated that the toxin is soluble in water, which is polar, suggest-

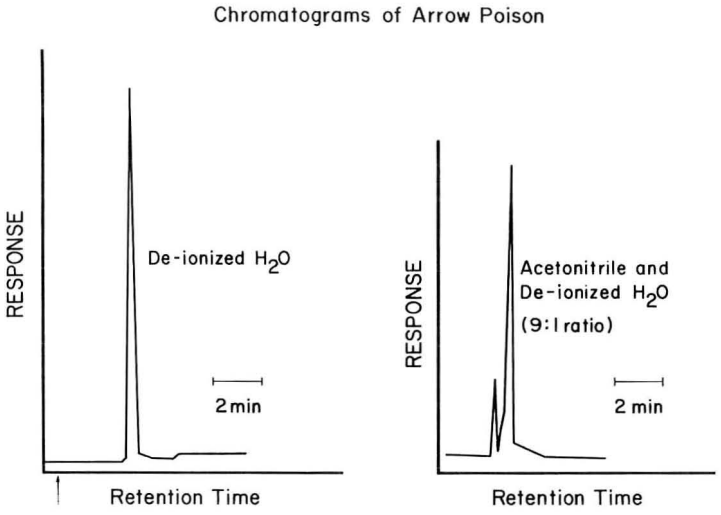


FIGURE 1.Chromatograms of eluted peaks of the arrow poison as a function of retention time. The solvent used for elution are shown in the figure.

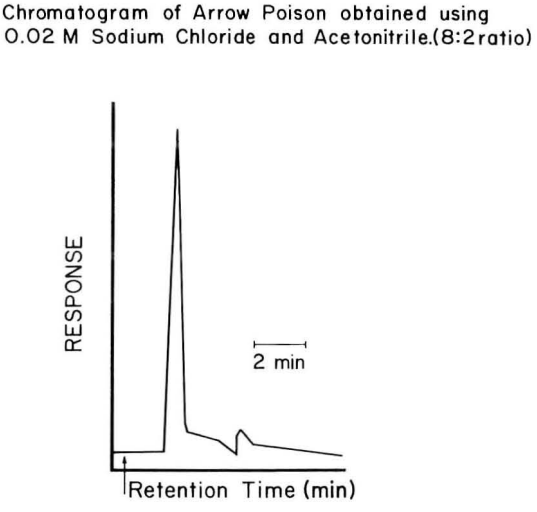


FIGURE 2.Elution pattern of arrow poison using sodium chloride and acetonitrile (8:2).

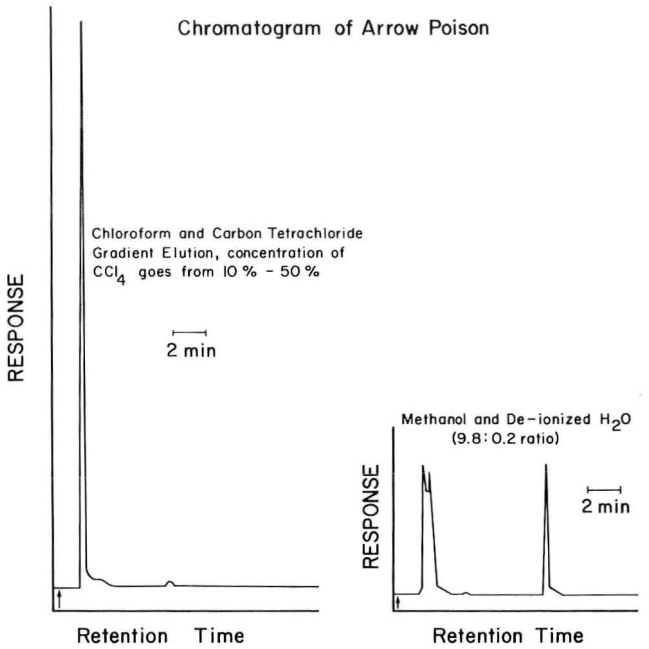


FIGURE 3. Elution profile using gradient elution of chloroform and carbon tetrachloride (10-50%) and; methanol and deionized water.

ing that the toxicologically active components therein are polar (Cooke et al. 1981.) Therefore, in our search for more suitable solvents for the separation of the active principles, we started with de-ionized water and varied the polarity of the solvent. This was accomplished by using different solvents and their mixtures such that polarity is changed until the appropriate solvent was identified. The best separation was achieved using gradient elution with 0.05M sodium chloride/ acetonitrile (45-95%) leading to a resolution of four distinct peaks (Fig. 4). In order of decreasing efficiency, the quality of separation achieved with the different solvents as shown in Figs. 1-4 can be arranged in the following order: gradient

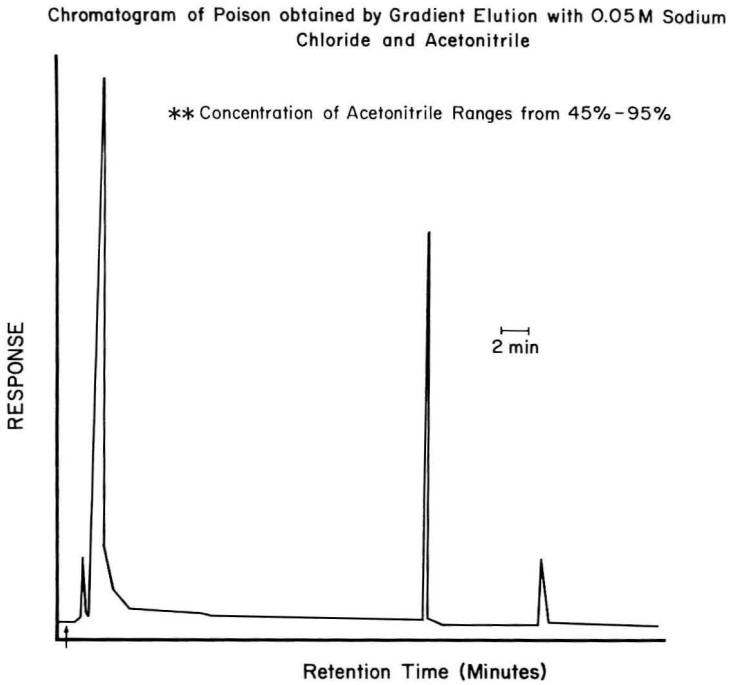


FIGURE 4.Elution profile using gradient elution with 0.05M sodium chloride and acetonitrile ranging in concentration from 45% to 95% (v/v).

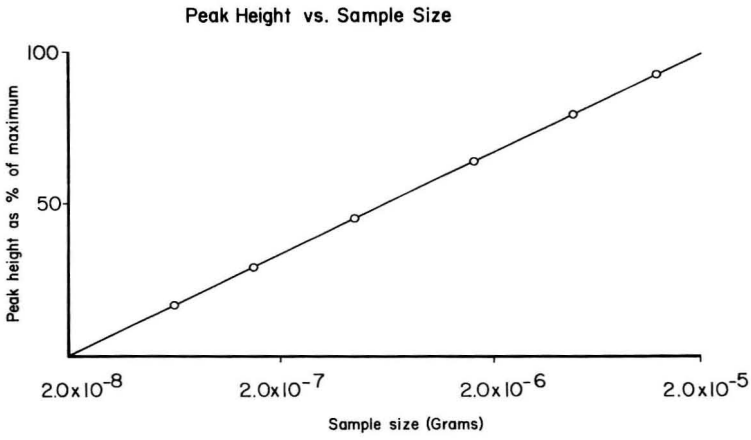


FIGURE 5.Calibration curve for solution of the arrow poison.

elution with 0.05 sodium chloride/acetonitrile (45-95%) > methanol and de-ionized water (98:2) > acetonitrile and de-ionized water 9:1) > gradient elution of chloroform/carbon tetrachloride (10-50%) > sodium chloride (0.02M) and acetonitrile (8:2) > de-ionized.

The relationship between the sample size and the peak height was linear for the toxin size ranging from 20ng to 20 µg (Fig. 5). This observation would provide evidence in support of the use of HPLC as a powerful tool for the separation and quantifying the arrow poison and its toxicologically active components. Currently, we are in the process of developing a sensitive bioassay within the same sensitivity range as the separated HPLC components; thus we can compare the two methods before deciding on which one might be most appropriate for investigating activities of the toxin.

ACKNOWLEDGEMENTS

This investigation was partially supported by Grant 2S06RR0800810 from the Division of Research Resources of the National Institute of Health, Bethesda, MD. Part of this work was presented at the Society of Pharmacology and Experimental Therapeutics Fall Meeting in Portland Oregon in August 1979. We thank Ms. Joan Milton for typing the manuscript.

REFERENCES

Dennis, Michael, Eddie B. Cook and Richard F. Ochillo. Application of high performance liquid chromatography in the separation of the active component(s) of an african arrow poison. Pharmacologist 21:143, 1979.

Cook, Eddie B., Mike Dennis and Richard F. Ochillo, Application of thin layer, ion exchange and high performance liquid chromatography to separate pharmacologically active components of an african arrow poison of plant origin. Journ. Liquid Chromatogr. 4(3),549-557, 1981.

Cook, Eddie B., Mike Dennis, Robert H. Rolfs, Duvie Pugh and Richard F. Ochillo. Preliminary pharmacological and toxicological studies of african arrow poison of plant origin. The Eight Annual Minority Biomedical Support Symposium. Division of Research Resources, National Institute of Health, Bethesda, Maryland. pg. 90. 1980.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1855-1867 (1981)

REVERSE PHASE HPLC DETERMINATION OF AZQ IN BIOLOGICAL FLUIDS

James A. Kelley and Edward D. Siu Chong
Laboratory of Medicinal Chemistry and Biology
National Cancer Institute, National Institutes of Health
Bethesda, Maryland 20205

ABSTRACT

A sensitive and specific reverse phase HPLC method employing a simple sample preparation procedure and utilizing an internal standard was developed to measure the new antitumor agent AZQ in biological fluids. A single chloroform extraction gave drug recoveries of greater than 88% from plasma, urine and CSF in the range of expected physiological concentrations (20-800 ng/ml). Isocratic reverse phase HPLC with UV detection at 340 nm resulted in a limit of quantitation of 5 ng/ml although smaller amounts of the drug could be detected. This assay was successfully applied to determine the single dose plasma pharmacokinetics of AZQ in rats. The potential of this method for determining AZQ disposition and pharmacokinetics in human subjects was demonstrated by analysis of patient CSF.

INTRODUCTION

AZQ (Figure 1) is a potential new antitumor drug that is currently undergoing Phase I and Phase II clinical trial. This aziridinylbenzoquinone is a rationally synthesized compound designed to allow central nervous system (CNS) penetration while retaining antitumor activity (1,2). AZQ shows a broad spectrum of activity against murine model tumor systems, including significant increases in life span in intracerebral L1210 and P388 and cures in intracerebral ependymoblastoma (1,3).

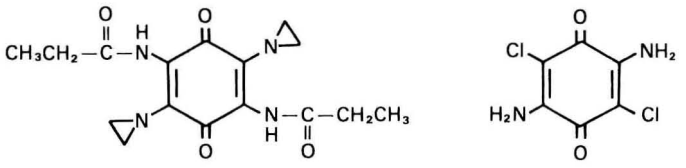


FIGURE 1. Structure of AZQ and the Internal Standard DADCQ.

A low optimum dose combined with appreciable animal toxicity indicated that human clinical trials of AZQ would have to start at doses of 0.5-1.0 mg/m^2. Thus a sensitive and specific analytical method to measure AZQ in plasma was required in order to allow determination of pharmacokinetics. Also of interest was the disposition of intact AZQ in other biological fluids, especially cerebrospinal fluid (CSF), since the drug was designed to enter the CNS. The high polarity and thermal instability of AZQ even after derivatization prohibited sensitive gas phase techniques such as gas chromatography and combined gas chromatography-mass spectrometry from being used to quantitate this compound (4). However, AZQ possessed a strong UV chromophore (λ_{max} = 340, log ε = 4.17) and a nonpolar nature, so high-performance liquid chromatography (HPLC) on a reverse phase column was a feasible alternative. Indeed, reverse phase HPLC provided a convenient method of analyzing formulation mixtures of AZQ (5). However, the concentrations of the drug encountered here were three orders of magnitude greater than those expected in vivo and AZQ did not have to be isolated from a complex biological matrix.

MATERIALS

AZQ (2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone, NSC 182986) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI and

used as received. The AZQ (Lot AJ58.1) was assayed at 96.5 ± 0.8% purity (4). 2,5-Diamino-3,6-dichloro-1,4-benzoquinone (DADCQ, Figure 1) was obtained from Dr. J. S. Driscoll of this laboratory and was recrystallized from hot ethyl acetate before use as an internal standard. Both AZQ and DADCQ were weighed on a Cahn 25 Automatic Electrobalance (Ventron Instruments Corp., Paramount, CA) before being dissolved in 25% CH_3CN/H_2O (v/v) to give standard solutions 1.175×10^{-5} M and 4.94×10^{-5} M, respectively. The standard solutions were stable for at least a month if they were refrigerated. Mono- and dibasic sodium phosphate (J.T. Baker Chemical Co., Phillipsburg, NJ) were used to make a pH 6.5 0.1 M buffer. Acetonitrile and chloroform were "distilled in glass" (Burdick and Jackson, Muskegon, MI) or HPLC grade (Fisher Scientific Co., Fairlawn, NJ) reagents. Chloroform was further purified to remove the 0.5-1% ethanol stabilizer by passage through a 15 mm ID column containing 25 g of basic alumina, activity grade I (AG-10; Bio-Rad, Richmond, CA). The chloroform was used immediately or stored under nitrogen and refrigerated. Acetonitrile and distilled water were filtered through the appropriate 0.45 µm solvent-resistant filters (Millipore Corp., Bedford, MA), before mixing to make the mobile phase.

METHODS

Separations were accomplished at ambient temperature (20°C) on either a 10-µm µBondapak C_{18} column (3.9 mm ID x 300 mm; Waters Associates, Milford, MA) or a 5-µm Ultrasphere-ODS column (4.6 x 250 mm; Altex Scientific Co., Berkeley, CA) using a mobile phase of 25% CH_3CN/H_2O (v/v). The analytical column was preceded by a Waters guard column that was repacked daily with 37-50 µm Bondapak C_{18}/Corasil. The remainder of the high pressure liquid chromatography system was comprised of a Waters Associates Model 6000A solvent delivery system, U6K injector and Model 440 UV absorbance monitor with a 340 nm filter. The mobile phase flow

rate was 1.0 ml/min and injections of standards and biological extracts (100 µl aliquot) were made using a 100 µl Waters gas tight syringe. Peak areas and heights were simultaneously determined on a SP4100 computing and recording integrator (Spectra-Physics, Santa Clara, CA).

Standards were made by adding 5 µl of DADCQ internal standard solution (51 ng) to 1.0 ml aliquots of the appropriate biological fluid in a 15 ml glass conical centrifuge tube and then by spiking with the required volume of AZQ standard solution. Pooled rat and human plasma, pooled monkey CSF (kindly provided by Dr. R. Riccardi, Pediatric Oncology Branch, NCI), and human urine were used. The spiked standards were gently vortexed and allowed to stand for 10 min in an ice bath before extractive workup. After addition of 0.5 ml 0.1 M pH 6.5 phosphate buffer, the standards were extracted with 1 x 5.0 ml $CHCl_3$ by vigorous vortexing for 2 min. The resulting emulsions were centrifuged at 2400 rpm for 5 min on a Dynac table top centrifuge (Clay Adams, Becton Dickinson and Co., Parsippany, NJ). If the emulsions were not completely broken, the samples were frozen in a dry ice-acetone bath and then slowly thawed. Centrifugation again for 5 min at 2400 rpm was sufficient to break the emulsions. The organic layer was transferred to a 10 ml capacity evaporative concentrator tube (Kontes, Vineland, NJ) and evaporated to dryness at room temperature under a stream of prepurified nitrogen. The residue was suspended in 0.5 ml 25% CH_3CN/H_2O by rapid vortexing, and the sample was filtered through a 0.5 µm Fluoropore filter using a stainless steel Swinny holder (Millipore Corp.) before chromatography. One milliliter biological samples were extracted in the same manner after addition of 5 µl DADCQ internal standard solution.

For study of AZQ pharmacokinetics in rat plasma, the drug was dissolved in N,N-dimethylacetamide (Fisher Scientific Co., Fairlawn, NJ) at a concentration of 20 mg/ml before being diluted

to a final concentration of 0.25 mg/ml with pH 6.5 0.01 M phosphate buffer. Male Sprague-Dawley rats, weighing 200-240 g (Taconic Farms, Germantown, NY), were anesthetized with sodium pentobarbital (35 mg/kg ip). A 1 mg/kg dose of AZQ was then given as a single bolus injection via an exposed femoral vein using a 25 gauge needle attached to a 1 ml syringe. Animals were decapitated at varying timed intervals after AZQ injection and blood from each animal was collected in separate heparinized glass beakers kept on ice. Blood was transferred to 15 ml glass centrifuge tubes and plasma obtained by a 15 min centrifugation. One milliliter aliquots of plasma were pipetted into separate 15 ml centrifuge tubes and the samples were either worked up immediately as described above or frozen in dry ice and stored at -20°C for future analysis.

Recoveries of AZQ and DADCQ from plasma, urine and CSF were determined by comparison of the absolute HPLC peak area or peak height of extracted spiked standards to calibration curves generated from comparable standards directly made up in 25% CH_3CN/H_2O. A blank of each different biological fluid was also run. The peak area or peak height ratio of AZQ to DADCQ internal standard was computed for each spiked standard and plotted against AZQ concentration for the range 20-880 ng/ml to generate a calibration curve (Figure 2). A calculator least-squares program (TI-55, Texas Instruments, Dallas, TX) was used to define the best straight line through each set of standard points in the body fluids examined. Initial pharmacokinetic parameters for AZQ were calculated from the rat plasma concentration curve by the method of residuals (6). The experimental data points were then fit to the biexponential function representing a two-compartment open model ($C = Ae^{-\alpha t} + Be^{-\beta t}$) by using MLAB, an on-line computer modeling laboratory utilizing an iterative, non-linear least squares program.

TABLE 1

Recovery of Spiked AZQ and DADCQ from Biological Fluids

Biological Fluid	AZQ Recovery	AZQ Range	DADCQ[1] Recovery
rat plasma	97 ± 9%	43-855 ng/ml	80 ± 6%
human plasma	88 ± 3%	22-880 ng/ml	88 ± 3%
human urine	92 ± 4%	21-214 ng/ml	88 ± 5%
monkey CSF	89 ± 7%	21-428 ng/ml	88 ± 3%

1. The concentration of DADCQ was 51 ng/ml in all cases.

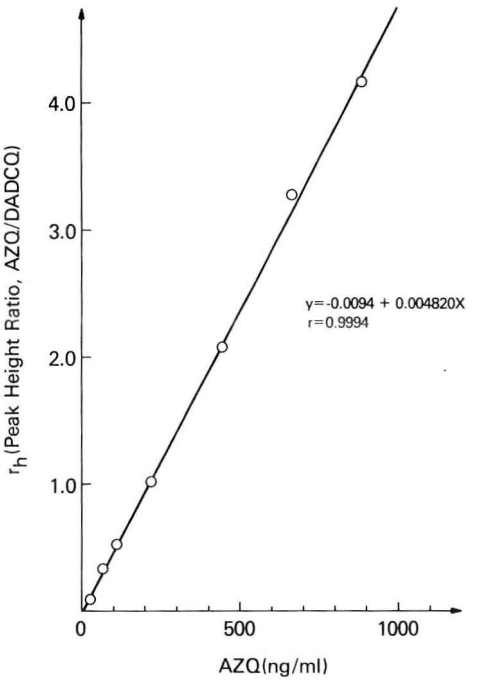


FIGURE 2. Typical Calibration Curve for Human Plasma.

RESULTS

Both AZQ and DADCQ could be easily and efficiently extracted from plasma, urine and CSF (Table 1). Recoveries were examined for the expected range of AZQ concentrations in rat plasma as determined from optimal therapeutic doses of ^{14}C-AZQ (7). Linear and reproducible calibration curves could be constructed from extracted spiked standards for all biological fluids examined (Figure 2):

rat plasma: $y = 0.0996 + 0.004934x$ ($r = 0.9998$)

human plasma: $y = -0.0094 + 0.004820x$ ($r = 0.9994$)

human urine: $y = -0.0073 + 0.004565x$ ($r = 0.9995$)

monkey CSF: $y = -0.0005 + 0.004344x$ ($r = 0.9976$)

HPLC analysis of extracted 1.0 ml aliquots of rat plasma from animals given a bolus injection of 1 mg/kg AZQ resulted in chromatograms similar to Figure 3. Because of detection at 340 nm

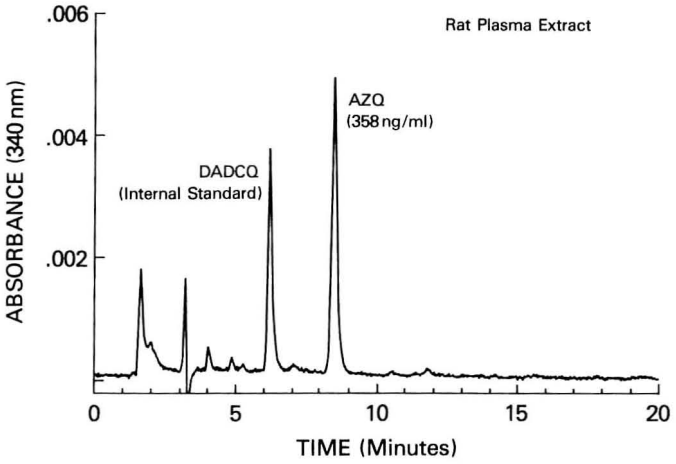


FIGURE 3. Representative HPLC Analysis of AZQ in Rat Plasma. Conditions are as described in Methods. A male Sprague-Dawley rat was intravenously administered a single 1 mg/kg dose of AZQ. The animal was sacrificed 60 min after drug injection and the plasma analyzed.

interfering artifacts were kept to a minimum, and the AZQ and DADCQ peaks could be readily integrated. The limit of quantitation was found to be about 10 ng/ml for rat plasma and 5 ng/ml for human plasma using a 1.0 ml sample size; the limit of detection was somewhat less. Rat plasma blanks consistently had a very small peak with almost the same retention time as AZQ. This accounted for the positive non-zero intercept in the calibration curve. No such interference was encountered with human plasma and urine or monkey CSF.

DISCUSSION

Extraction with chloroform was chosen as the means of isolating AZQ, a drug designed to have an adequate lipophilic character (1), because it was efficient, simple and applicable to all the body fluids under consideration. Since plasma lipids and other chloroform-soluble components could be expected to partition into the organic phase, assay sensitivity and specificity depended on UV detection at the μ_{max} of AZQ (340 nm) and adjustment of k' on the octadecylsilane column to achieve sharp peaks yet adequate separation. In a situation where low levels of drug were expected, extraction with a volatile organic solvent allowed facile concentration of the extract. In practice, a two-fold concentration was found adequate to attain sensitivity in the low nanogram range. With injection of a 100 µl aliquot, less than a nanogram of AZQ on-column could be detected.

A common problem in the use of an organic solvent to extract a biological medium is the formation of emulsions. This was encountered in almost all cases with plasma and on a more random basis with CSF and urine. However, application of the freeze-thaw technique outlined in Methods resulted in sharp phase separation for every sample. Evaporation of this chloroform plasma extract and attempted solution of the residue in 25% CH_3CN/H_2O resulted in a suspension where the undissolved materials were

probably lipids. This phenomenon was not so noticeable with CSF and urine extracts. Filtration through a 0.5 μm Fluoropore membrane eliminated most of this material. As an added precaution a guard column, which was repacked daily, was used to protect the analytical column from any strongly adhering components such as lipids.

Although AZQ is not ionized in the range of physiologically occurring pH's, it does undergo pH-dependent decomposition (4,5). Accordingly, all biological fluids were buffered to pH 6.5, the pH of maximum AZQ stability, before extraction.

Studies using ^{14}C-AZQ in rats and dogs have suggested that AZQ undergoes extensive metabolism (7). It should be emphasized that chloroform extraction will not isolate the more polar metabolites, nor will those less polar metabolites that are extracted be detected if the quinone chromophore is no longer intact. Thus the assay is quite specific for the parent drug; and only closely related contaminants, decomposition products or metabolites are likely to be detected in addition to AZQ.

The choice of an internal standard in an HPLC assay can be quite critical. The internal standard should have a chromatographic behavior that is similar but not identical to the compound(s) being determined and should be sensitive to the method of detection. The internal standard should also be chemically similar to the analyte, since minimization of sample handling errors requires that it be carried through the isolation procedure. If the chemical similarity is close enough, the internal standard might even be used as a carrier for very small amounts of the unknown during sample workup. Finally, the internal standard should be a compound that is readily available, either commercially or through a simple synthesis. DADCQ was chosen as the internal standard for this analysis because it met all of the above criteria. This tetrasubstituted benzoquinone is retained by an octadecylsilane column yet elutes before AZQ,

has strong absorbance at 340 nm, is chloroform extractable (see Table 1) and hydrolytically stable, and is readily available since it was an intermediate in the initial synthesis of AZQ (1,8).

This assay was applied to determine the single dose plasma pharmacokinetics of AZQ in rats receiving an optimum dose (1 mg/kg) of the drug (7). A typical chromatogram is shown in Figure 3. Both AZQ and the DADCQ internal standard are clearly defined with no obvious interferences. In those cases where the AZQ concentration was expected to exceed 1 µg/ml (i.e. immediately after drug injection) and be outside the range of the calibration curve, the amount of internal standard added to the sample before workup was doubled. Upon subsequent HPLC analysis, a 50 µl aliquot was injected and the calculated concentration doubled to obtain the true level. For maximum sensitivity the method required at least 1.0 ml of biological fluid. In a human such a sample size presented no problem, but for 200-240 g rats where the total blood volume was on the order of a few milliliters, serial sampling of the type necessary for pharmacokinetics would severely perturb the system. Therefore, rats of similar age, sex and weight were sacrificed at prescribed times after injection of AZQ and only a single sample was obtained from each animal. The data from these composite samples was then used to define the plasma pharmacokinetics.

AZQ exhibits biphasic behavior in rat plasma (Figure 4). A very rapid redistribution phase is followed by a slower yet still fast elimination phase with a half-life of 26.5 min. Because the half-life of the initial phase is on the order of 3-5 minutes, sampling was not rapid enough to accurately define it. Each point in Figure 4 represents multiple animals and the error bar represents the range of concentrations for these different animals. AZQ plasma concentrations could be easily determined over four half-lives. Indeed, in some instances the parent drug could

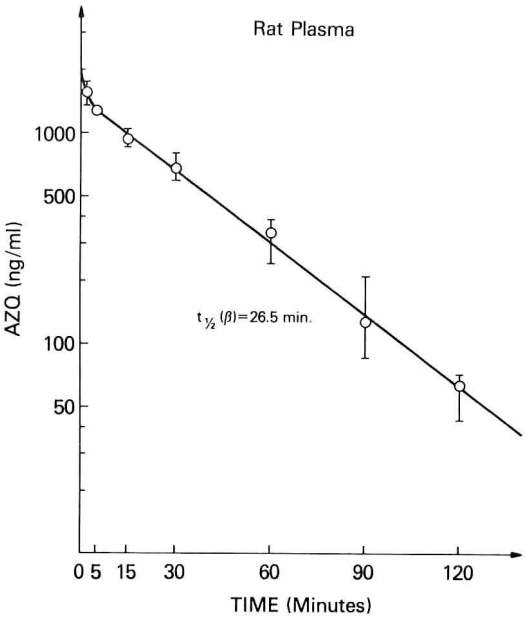


FIGURE 4. Plasma Pharmacokinetics of a Single 1 mg/kg Dose of AZQ in Male Sprague-Dawley Rats. Each point represents the mean AZQ concentration in 3 or more animals. The brackets about each point signify the range of measured AZQ concentrations.

still be detected after 4 hr at concentrations of less than 10 ng/ml.

CONCLUSION

The data presented above show that this HPLC assay is suitable for measuring AZQ concentrations in human patients. The method possesses sufficient sensitivity and specificity to determine single dose AZQ pharmacokinetics, even at the lowest initial dose (e.g. 1 mg/m^2) employed in a Phase I clinical trial. A primary question about the disposition of AZQ is the ability of the drug to enter the CNS, since it was specifically designed to do

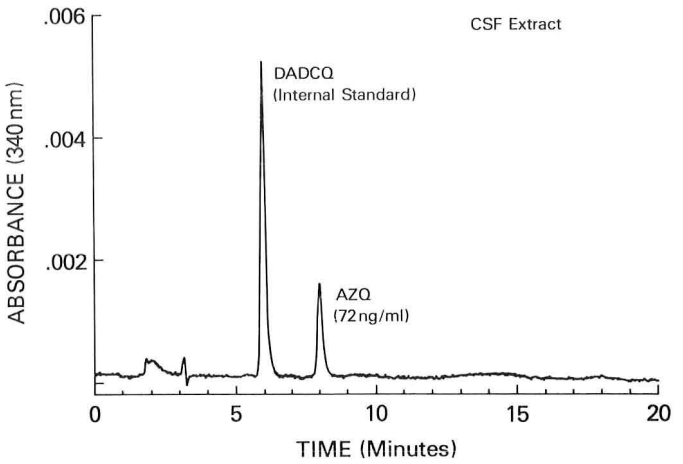


FIGURE 5. HPLC Analysis of AZQ in Human CSF. The sample was obtained by lumbar puncture 90 min after intravenous administration of a 15 mg/m^2 dose of AZQ.

this. AZQ penetration into CSF has been demonstrated in non-human primates (rhesus monkeys) by using ^{14}C-AZQ (9). Figure 5 presents the first evidence that intact AZQ likewise enters the CSF in humans. Here one can measure the unchanged drug directly without relying on radioactivity and the uncertainty generated by metabolism or decomposition. Determination of AZQ disposition and pharmacokinetics in human patients in Phase I clinical trials is currently in progress.

ACKNOWLEDGEMENTS

The authors thank Dr. John S. Driscoll of this laboratory for many helpful discussions concerning the chemistry of aziridinylbenzoquinones and Dr. Guiragos K. Poochikian, Pharmaceutical Resources Branch, NCI for his useful and timely advice on the chromatography of AZQ derivatives. The human CSF sample from a patient in the Phase I clinical trial of AZQ was kindly provided by Dr. Richard L. Schilsky, Medicine Branch, NCI.

REFERENCES

1. Khan, A.H. and Driscoll, J.S., Potential Central Nervous System Antitumor Agents. Aziridinylbenzoquinones. 1, J. Med. Chem., 19, 313, 1976.

2. Chou, F., Khan, A.H. and Driscoll, J.S., Potential Central Nervous System Antitumor Agents. Aziridinylbenzoquinones. 2, J. Med. Chem., 19, 1302, 1976.

3. Driscoll, J.S., Dudeck, L., Congleton, G. and Geran, R.I., Potential CNS Antitumor Agents VI: Aziridinylbenzoquinones III, J. Pharm. Sci., 68, 185, 1979.

4. Poochikian, G.K. and Kelley, J.A., 2,5-Diaziridinyl-3,6-bis-(carboethoxyamino)-1,4-benzoquinone II: Isolation and Characterization of Degradation Products, J. Pharm. Sci., 70, 162, 1981.

5. Poochikian, G.K. and Cradock, J.C., 2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone I: Kinetics in Aqueous Solutions by High-Performance Liquid Chromatography, J. Pharm. Sci., 70, 159, 1981.

6. Gibaldi, M. and Perrier, D., Pharmacokinetics, Marcel Dekker, New York, 1975.

7. Sui Chong, E.S., unpublished results.

8. DADCQ was the major synthetic impurity (1.5% w/w) in early batches of AZQ. This contaminant was not present in the later batches used in the clinical formulation because of a slightly different method of synthesis.

9. Gormley, P.E., Wood, J. and Poplack, D.G., Ability of a New Antitumor Agent, AZQ, to Penetrate into CSF, Pharmacology, 22, 196, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1869-1874 (1981)

SEPARATION OF MENTHOL ISOMERS BY NORMAL PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (1)

Stephen A. Haut and Margaret T. Core
Philip Morris USA, Research Center
P. O. Box 26583
Richmond, Virginia 23261

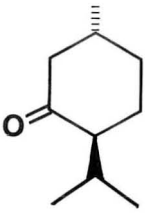
ABSTRACT

An HPLC method has been developed for the separation of the four isomers of menthol using isocratic and normal phase ethyl acetate/isooctane systems. This method has been used to detect and measure these isomers in peppermint oils. The method has several advantages over existing techniques. It is more rapid than GC which in addition requires unstable columns for similar analysis. Because solvent and column are normal phase and isocratic, the method and sample preparation are very simple.

INTRODUCTION

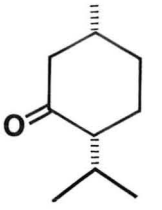
A variety of methods (2,3) have been devised for the GC determination of the isomers of menthone and menthol (1-6). Only one paper, however, has appeared using HPLC and that concerned itself with 1 and 2 (4). There have also been HPLC papers published dealing with p-menthane and monoterpene derivatives other than menthols in essential oils (5,6).

Recently we have developed an HPLC method which is useful for menthol isomers. These compounds can be rapidly identified



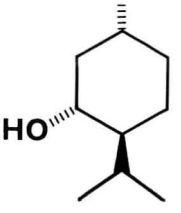
1

MENTHONE



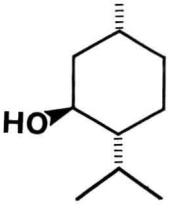
2

ISOMENTHONE



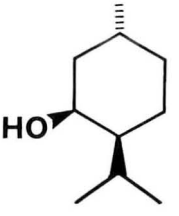
3

MENTHOL



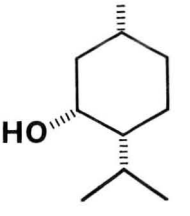
5

ISOMENTHOL



4

NEOMENTHOL



6

NEOISOMENTHOL

without the need for unstable GC phases (eg. Hyprose), expensive reverse phase HPLC systems, or tedious sample preparation.

EXPERIMENTAL

The instrumentation involved a Waters Associates Liquid Chromatograph, Model ALC-201, equipped with a U6K loop injector, a Radial Compression Separation System (10 cm x 0.8 cm Radial Pak B μ-Porasil cartridge, RCM-100 module) (7), and a Model 401 Differential Refractometer.

Menthones and menthols used for standards were obtained from SCM Organic Chemicals. In all cases HPLC identities were substantiated by GC[3] comparison to these authentic materials.

Liquid chromatography grade solvents obtained from Waters Associates were filtered through 0.45μ Millipore filters prior to use.

All samples were prepared for HPLC by dissolution in 3% ethyl acetate/isooctane and filtration through 0.45μ Millipore filters. Injection volumes were adjusted to apply about 0.1 mg of total sample to the HPLC column on each run.

DISCUSSION

As can be seen in Figure 1, each component is completely resolved using 3% ethyl acetate/isooctane.

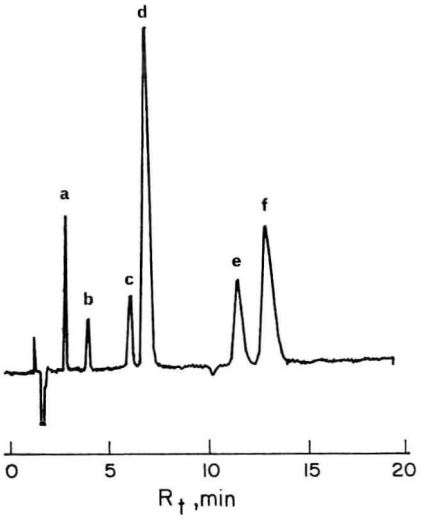


FIGURE 1 HPLC of Menthone and Menthol Isomers
Solvent = 3% ethyl acetate/isooctane, Flow Rate = 3mL/min
(a) Menthone (b) Isomenthone
(c) Neoisomenthol (d) Neomenthol
(e) Menthol (f) Isomenthol

In order to test the applicability of this method in determining the presence of 1-6 in a complex mixture, a sample of corn mint oil (George Uhe Company, Inc.) was run under the same conditions as Figure 1. The resulting chromatogram is shown in Figure 2. The presence of 1, 2, 3 and 4 can readily be determined.

Further work on similar isomeric separations is currently underway in our laboratories.

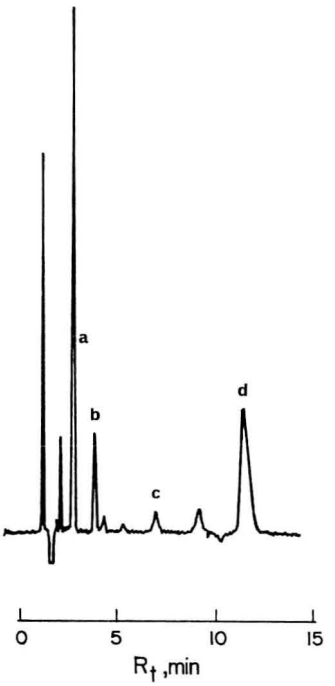


FIGURE 2 HPLC of Corn Mint Oil
Solvent = 3% ethyl acetate/isoctane, Flow Rate-3mL/min
(a) Menthone (b) Isomenthone
(c) Neomenthol (d) Menthol

ACKNOWLEDGMENTS

Our thanks to Ms. Mayada Logue for adapting the literature GC methods for our use and to Mr. Al Correia of Waters Associates for his cooperation.

REFERENCES

1. Presented in part at the 34th Tobacco Chemists' Research Conference, Richmond, Virginia, October 27, 1980.

2. Gillen, D. G.; and Scanlon, J. T.; J. Chromatogr. Sci., 10, 729 (1972).

3. Thieme, H.; and Benecke, R.; Zentrale Pharm. Pharmakother Laboratoriumsdiagn., 116, 139 (1977).

4. Bergman, N-A., and Hall, B.; Acta Chem. Scand., B33, 148 (1979).

5. Jones, B. B.; Clark, B. C.; and Iacobucci, G. A.; J. Chromatogr.; 178, 575 (1979).

6. Bicci, C.; Frattini, C.; J. Chromatogr. 190, 471 (1980).

7. The standard 3.9mm x 30cm μPorasil steel column works equally as well. Equilibration time is longer and pressures at similar flow rates are much higher. Use of the RCM Module allows for decreased analysis time by further increasing the flow rate if desired without serious resolution loss.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1875-1886 (1981)

DETERMINATION OF ZERO RETENTION TIMES (t_o) BY TEMPERATURE DEPENDENT REVERSED PHASE HIGH PERFOMANCE LIQUID CHROMATOGRAPHY

B. Neidhart, K.P. Kringe, W.Brockmann

Institut für Arbeitsphysiologie an der Universität Dortmund
Geschäftsführender Direktor: Prof.Dr. H.G. Wenzel
Ardeystr. 67, D-4600 Dortmund 1

ABSTRACT

For a given reversed phase HPLC system the zero retention time (t_o) can be determined by measuring brutto retention times at different temperatures. For calculation of t_o value temperature intervals must be equidistant in 1/T. If at least two compounds are separated on the column having almost the same sorption enthalpies a more simple intersecting point method can be practised. In this case only two temperature points are necessary for graphic evaluation. The presented methods for determination of t_o values are proved for noradrenaline and adrenaline under selected experimental conditions using aqueous solutions of alkaline perchlorates as mobile phases. Thus t_o values can be determined with an accuracy and a precision of less than $\pm$ 3 %.

INTRODUCTION

For a given separation problem a HPLC system can be described by a set of direct and derived chromatographic values which depend on various parameters like column length and diameter, particle type and size, flowrate, composition and pH of the mobile phase, temperature, etc. and of course, on the compounds to be separated. The most controversively discussed point is the zero retention time (t_o), which is necessary for calculation of mass distribution coefficient (k'), relative retention (α), resolution (R) and effective number of plates (N).

The latter values are important for identifications, selectivity and polarity control, consequently for rating and optimization of the whole chromatographic system (for general review see references cited by Berendsen et al.(1)).

The t_o value is defined as retention time of the compounds in the mobile phase, and should thus be temperature independent. Quite a few experimental methods are known, by which t_o can be evaluated.

Practised methods are:

- Injection of slightly modified mobile phase and measuring with a RI detector,
- injection of O_2 saturated mobile phase and measuring O_2 absorbance with an UV detector,
- doping of the mobile phase with a fluorophore (chininesulfate), injection of undoped mobile phase, and measuring the drop in fluorescence,
- injection of dissolved small molecules - so called "non-retarded" substances - and measuring by various detection methods.

Following recently published papers (1,2), these methods do not allow the determination of the exact t_o value because of its dependency on the porous structure of the stationary phase. Thus, most of the merely experimental determined t_o values are too high, which lead to smaller k' values. In order to overcome this problem a mathematical treatment of experimental data of homologous compounds is recommended. Up to now, except time consumption, this method seems to be the best procedure for the determination of t_o values. One disadvantage of this method is due to the fact, that for most of the practical separation problems simple homologous series do not exist. Consequently, there is still an urgent need for an universal method, which would allow to determine correct t_o values.

OBJECTIVES

Facing this aim, an investigation was started with the assumption, that chromatographic processes are strongly dependent on temperature whereas t_o is not. This led to a first work hypothesis: With increasing temperature the brutto retention time (t_R) should decrease approaching the real t_o value . Thus, by mathematical treatment of temperature dependent t_R values the calculation of t_o should be possible. A first series of temperature dependent t_R measurements for noradrenaline (NA) and adrenaline (A) (chromatographic conditions are described below) resulted in experimental curves (Fig. 1), which showed a monotone

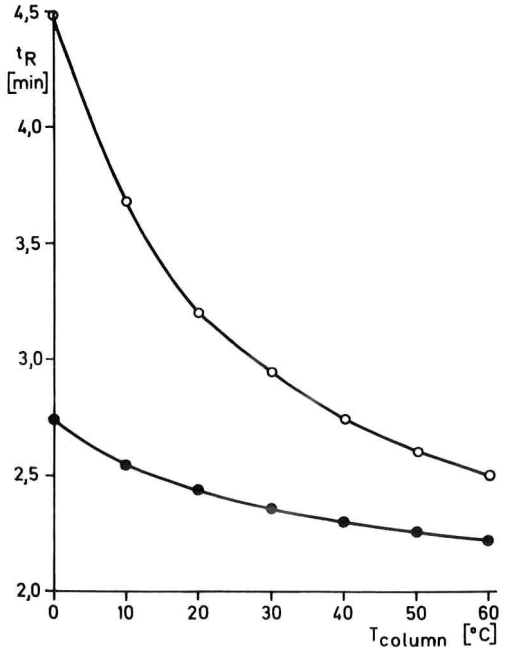


Figure 1: Influence of temperature on brutto retention times for noradrenaline (●) and adrenaline (o) (column: nucleosil 10-C_{18}; mobile phase: 0.1 M $HClO_4$)

decrease of t_R with increasing temperature and which, at least visually, approached the same limit value for both compounds. This result indicated, that the above stated hypothesis was possibly found to be correct. Such a method would be advantageous for direct determination of t_o with a particular chromatographic system under real experimental conditions. The systematic investigation was performed with catecholamines (NA and A), as in connection with occupational physiology studies a quantitative analysis of these compounds became necessary (3).

EXPERIMENTAL

Apparatus: Waters model M 6000A was used as high pressure pump connected to a Rheodyne model 7010 injection valve with 100 µl sample loop. The columns were packed with nucleosil 10-C_{18}, 5-C_{18} and 10-C_8 (Macherey + Nagel) and column dimensions were SS 250/6.4/2.9, SS 200/6.0/4.0 and SS 250/6.0/4.0 resp.. For thermostatic control a Kryostat (Desaga Frigostat) was used and detection was performed with an Aminco-Bowman-Spectrophotofluorometer (8 µl flow through cuvette) connected to a Corning Recorder 840.

Reagents: All chemicals were of analytical grade. Aqueous solutions of $HClO_4$, $LiClO_4$, $NaClO_4$, $KClO_4$ and $KClO_3$ with concentrations of 0.0025, 0.005, 0.01, 0.02, 0.05, 0.1 and 0.5 M were used as mobile phases.

Procedure: The most important requirements for temperature dependent measurements are definite and constant temperatures of the chromatographic system during the experiment. In order to fulfil this prerequisite, the temperature gradient of the mobile phase along its pathway from injection valve to end of column was investigated for different isolation materials and segments using four thermocouples at a time. Thus, it could be shown, that at flow rates of 1 ml/min thermostatic control of at least 20 cm of the stainless steel capillary in front of the column is sufficient if the temperature of the column itself is regulated. In practice 30 cm of the capillary together with the column are incapsuled in a brass tube (i.d.2.4 cm) which is well isolated and

through which the circulating liquid (isopropanole/water:1/1) is pumped (14 l · min $^{-1}$). The experiments were run at temperatures of 0, 10, 20 up to 60^{o} C at a flowrate of 1 ml/min for the mobile phase. The pressure in the system was dependent on temperature and type of RP stationary phase and ranged between 400 psi for 10-C_8 at 60^{o} C and 3800 psi for 5-C_{18} at 0^{o} C. Concentrations of NA and A were 100 ng/ 100 µl and 50 ng/100 µl resp. and detection was performed fluorimetrically (excitation 275 nm/emission 317 nm). For better evaluation of the chromatograms the recorder was set to a velocity of 10 cm · min^{-1}.

MATHEMATICAL MODEL

A common equation of the Arrhenius type is:

$$k' = k_o \cdot e^{-\frac{\Delta(\Delta H)}{RT}} \qquad (1)$$

where k' : mass distribution coefficient
k_o : proportionality constant
$\Delta(\Delta H)$: difference in the sorption enthalpies of eluent and compound for sorption at the stationary phase (4)
R : universal gas constant
T : absolute temperature

but $$k' = \frac{t_R - t_o}{t_o} \qquad (2).$$

Substituting k' from eq. (1) by eq. (2) one gets

$$t_R = k_o \cdot t_o \cdot e^{-\frac{\Delta(\Delta H)}{RT}} + t_o \qquad (3).$$

Equation 3 indicates that at infinite temperature $t_R = k_o \cdot t_o + t_o$, which means, that with increasing temperatures t_R approaches t_o if k_o is negligibly smaller than t_o. For this case the stated work hypothesis is correct.

Under the assumption, that k_o and $\Delta(\Delta H)$ are temperature independent, which at least is justified within the experimental T interval, the zero retention time can be calculated (5):

$$t_o = \frac{t_R^2(T_1) - t_R(T_2) \cdot t_R(T_3)}{2t_R(T_1) - t_R(T_2) - t_R(T_3)} \qquad (4).$$

The $t_R(T)$ values are experimentally determined brutto retention times at different temperatures, with the condition, that T_1, T_2 and T_3 are chosen such, that the $1/T$ values become equidistant (e.g. 0^o C, 20^o C, 43^o C).

Thus, the error of t_o is determined by the precision of the experimental t_R values. In order to minimize this error, temperature intervals should be as wide as possible, and temperature should be exact and constant. The temperature interval, naturally given by the chromatographic system and the separation problem, ranges practically between 0^o C and 80^o C.

Using the above described procedure t_o can be determined with one component only. In practice, however, one has to deal mostly with multicomponent systems. For such cases a more simple method for the determination of t_o can be practised, if given conditions are fulfilled.

Starting with a more common equation for mass distribution coefficients

$$k'_A = a \cdot g_A(T) \text{ and } k'_B = b \cdot g_B(T) \qquad (5)$$

A,B : components A and B
a,b : temperature independent factors

and with the condition

$$g_A(T) = g_B(T) \text{ for all experimental temperatures}$$

follows

$$t_o = \frac{t_{R,A}(T_1) \cdot t_{R,B}(T_2) - t_{R,B}(T_1) \cdot t_{R,A}(T_2)}{t_{R,A}(T_1) + t_{R,B}(T_2) - t_{R,A}(T_2) - t_{R,B}(T_1)} \qquad (6)$$

$t_{R,A(B)}(T)$: brutto retention time for component A(B) at temperature T.

In this case the temperatures must not necessarily be equidistant in $^1/T$.

The identity of $g_A(T)$ and $g_B(T)$ means practically, that the sorption enthalpies of the two components are equal or at least very similar, which is the case for many chemically related substances. Hereof it results automatically, that the selctivity coefficient α is temperature independent.

For practical determination of t_o the following procedure is recommended:

1. measurement of $t_{R,A}$ and $t_{R,B}$ at two different temperatures (T_1, T_2)
2. plotting of $t_{R,A}$ and $t_{R,B}$ for both temperatures at arbitrary positions of the abzisses X_1 and X_2 respectively
3. Y value of point of intersection of the two straight lines for T_1 and T_2 is identical with the exact t_o value.

Again, in order to minimize the error of t_o the range between T_1 and T_2 should be as wide as possible.

RESULTS AND DISCUSSION

For proving the mathematical model quite numerous experiments were performed at various temperatures with different types and concentrations of the mobile phase and for three different RP materials. As the chosen compounds NA and A are chemically related, the intersecting point method was used for the calculation of zero retention times. An example for graphic evaluation of t_o is shown in Fig. 2.

In the following tables (1,2) mean t_o values are listed which were calculated from experimental $t_R(T)$ values using

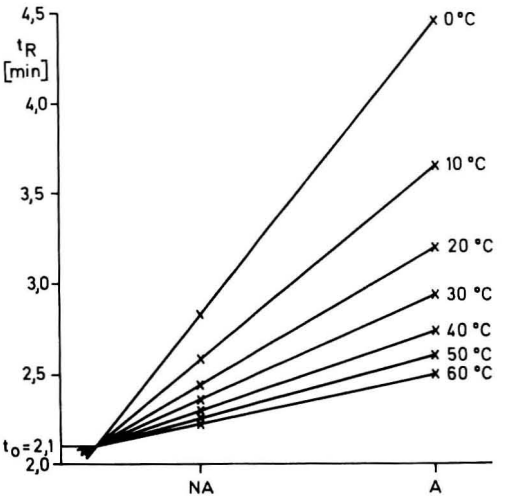


Figure 2: Graphic evaluation of t_o via temperature dependent t_R values of noradrenaline and adrenaline
(column: nucleosil 10-C_{18}; mobile phase: 0.1 M $HClO_4$)

equation 6. In order to keep the influence of errors of single t_R values small, the mean t_o values were calculated from intersecting points of at least 5 temperature pairs which were obtained by cyclic permutation of different experimentally given temperatures.

Tables 1 and 2 demonstrate, that the zero retention times of the systems under investigation are neither dependent on the mobile phase nor on its concentration; standard deviation is about $\pm$ 3 % rel.. A comparison of these t_o values with those calculated from equation 4 showed the results in excellent agreement (Tab. 3,4).

The mathematical model was proved by the experiments. By plotting ln k' versus $^1/T$ straight lines were obtained from which k_o values and $\Delta(\Delta H)$ values were calculated. k_o values were found to be in the range of 10^{-4}, which means,

TABLE 1

mean t_o values [min] calculated from eq. 6 for different types and concentrations of the mobile phase (column: nucleosil 10-C_{18}; standard deviation for $n \geq 10$)

conc. [M]	$LiClO_4$	$NaClO_4$	$KClO_4$	$KClO_3$
0.005	2.02 ± 0.06	2.06 ± 0.06	2.03 ± 0.04	2.09 ± 0.07
0.01	2.00 ± 0.05	2.10 ± 0.07	2.05 ± 0.03	2.06 ± 0.06
0.02	2.07 ± 0.05	2.06 ± 0.03		
0.05		2.11 ± 0.03	2.04 ± 0.04	2.07 ± 0.03
0.1	2.06 ± 0.06	2.08 ± 0.06		
0.5	2.05 ± 0.04	2.10 ± 0.05		

TABLE 2

mean t_o values [min] of different column types calculated from eq. 6 for various concentrations of $HClO_4$ as mobile phase (standard deviation for $n \geq 10$)

conc. [M]	10-C_{18}	10-C_8	5-C_{18}
0.0025			2.25 ± 0.05
0.005	2.03 ± 0.02	3.09 ± 0.05	2.26 ± 0.06
0.01	2.05 ± 0.03	3.08 ± 0.05	2.21 ± 0.05
0.02	2.04 ± 0.05	3.08 ± 0.06	2.23 ± 0.06
0.05	2.07 ± 0.02	3.08 ± 0.06	2.20 ± 0.06
0.1	2.09 ± 0.07	3.15 ± 0.07	2.25 ± 0.04
0.5	2.06 ± 0.03	3.12 ± 0.05	

TABLE 3

mean t_o values $[min]^*$ calculated from different temperature or concentration dependent equations for various types of mobile phases (column: nucleosil $10\text{-}C_{18}$; concentration see table 1)

	$LiClO_4$	$NaClO_4$	$KClO_4$	$KClO_3$
eq.6, T dependent	2.04 ± 0.02	2.09 ± 0.02	2.04 ± 0.02	2.07 ± 0.03
eq.6, c dependent	2.06 ± 0.06	2.08 ± 0.06	2.04 ± 0.04	2.10 ± 0.06
eq.4, T dependent	2.03 ± 0.06	2.11 ± 0.03	2.06 ± 0.04	2.08 ± 0.03

* weighted mean ($n \geq 3$) of the mean ($n \geq 10$)

TABLE 4

mean t_o values $[min]^*$ calculated from different temperature or concentration dependent equations for three column types and $HClO_4$ as mobile phase (concentrations see table 2)

	$10\text{-}C_{18}$	$10\text{-}C_8$	$5\text{-}C_{18}$
eq. 6, T dependent	2.05 ± 0.01	3.10 ± 0.02	2.24 ± 0.02
eq. 6, c dependent	2.06 ± 0.05	3.07 ± 0.06	2.28 ± 0.05
eq. 4, T dependent	2.08 ± 0.03	3.11 ± 0.04	2.26 ± 0.02

* weighted mean ($n \geq 3$) of the mean ($n \geq 10$)

that the stated work hypothesis is correct. $\Delta(\Delta H)$ values were found to be in the range of 15 kJ $\cdot$ $mole^{-1}$ and were almost identical for the two compounds under given experimental conditions (type and concentration of mobile phase, type of stationary phase), which in fact led to temperature independent α values.

As t_R values are also dependent on the concentration of mobile phase, one may think about the possibility of determining the zero retention times by concentration dependent RP HPLC. Using the intersection point method, t_o values were calculated on basis of eq. 6 after replacement of $t_R(T)$ by $t_R(c)$. Tables 3 and 4 show the results in quite good agreement, but standard deviation of these t_o values are obviously larger than those resulting from the temperature dependent measurements. It seems as if the temperature method is less sensitive to little changes in the chromatographic system than the concentration method.

For comparison t_o values were also determined using the experimental methods listed in the introduction. In some cases agreement was quite good but in other cases extremely bad. This confirms the statement, that "classical methods" must not necessarily lead to exact zero retention times.

CONCLUSIONS

For a given chromatographic RP system the zero retention time can be determined by measuring t_R values at different temperatures ($n_T > 2$). The temperature intervals must be equidistant in $^1/T$. If at least two compounds are separated on the column having almost the same sorption enthalpies a more simple intersecting point method can be practised. In this case only two temperature points are necessary for graphic evaluation.

The presented methods for determination of t_o values are, of course only proved for two compounds (NA and A) under selected experimental conditions. Although, further experiments are needed to elucidate the applicability and the

limitations of the methods, we believe, that this procedure can be utilized to very many practical problems. This will have the advantage of working with a given system without any changes of those chromatographic parameters, which might influence the zero retention time.

ACKNOWLEDGEMENT

The authors would like to thank Mrs. P. Deutschmann for conscientious performance of the experiments.

REFERENCES

1. Berendsen, G.E., Schoenmakers, P.J., Galan, L. de, Vigh, G., Varga-Puchony, Z. and Inczédy, J., J. Liquid Chromatogr. 3, 1669, 1980

2. Kaiser, R.E. and Oelrich, E., Optimierung in der HPLC, Hüthig, Heidelberg, 1979

3. Kringe, K.P. and Neidhart, B., Temperatureinfluß auf die Reversed Phase-HPLC der Katecholamine Adrenalin und Noradrenalin, 5. Diskussions-Tagung über Hochleistungs-Flüssigkeits-Chromatographie, Kaiser, U.J. and Franzen, K.H., eds., Waters GmbH, Königstein, 1980, p. 309

4. Ahr, G. and Engelhardt, H., Reproduzierbarkeit von k'-Werten beim Arbeiten mit Umkehrphasen, 5. Diskussions-Tagung über Hochleistungs-Flüssigkeits-Chromatographie, Kaiser, U.J. and Franzen, K.H., eds., Waters GmbH, Königstein, 1980, p.21

5. Hoerl, A.E., jr., Fitting Curves To Data, Chemical Business Handbook, Perry, J.H., ed., McGraw Hill, London, 1954, p.20-55

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1887-1888 (1981)

LC NEWS

HYDROPHOBIC DISPOSABLE FILTERS are designed to filter air, gas, and liquids. They have a polypropylene housing and a Teflon filter for a broad range of chemical resistance and are useful as vent filters for carboys, distilled water reservoirs, instruments, bioisolation chambers, etc. Gelman Sciences, Inc., JLC/81/10, 600 S. Wagner Rd., Ann Arbor, MI 48106, U.S.A.

ELECTROCHEMICAL DETECTOR for HPLC does not require a knowledge of eletrochemistry. Electrochemistry detection offers high sensitivity and selectivity. Glassy carbon electrodes are used which offer a high degree of conversion. Kipp Analytica, B.V., JLC/81/10, Ph. Foggstratt 24, 7821 AK Emmen, The Netherlands.

CARDIAC DRUG IN SERUM via HPLC is described in a recent technical bulletin. Levels of procainamide and its metabolite, N-Acetyl-procainamide are determined with high specificity, sensitivity, precision, and speed. Varian Instrument Group, JLC/81/10, 10060 Bubb Road, Cupertino, CA 95014, U.S.A.

LC COLUMN & PACKING GUIDE contains specifications and performance data for a complete line of products. In this new edition are recently announced preparative columns designed for operation at 40-50 cm^3/min. They are available in SIL, ODS, C-8, and NH_2 types. DuPont Company, JLC/81/10, Anal. Inst. Div., McKean Building-Concord Plaza, Wilmington, DE 19898, U.S.A.

COLUMN HEATERS for general purpose use with HPLC columns support either one or two columns within a thermally insulated aluminum block. Precision inserts accomodate most standard columns. The injector block may also be incorporated within the heating block. A digital temperature indicator covers the range from ambient to 150°C with control to within 0.1 degree. Thermoelectrically refrigerated models are also available. Eldex Labs, JLC/81/10, 3551 Haven Avenue, Menlo Park, CA 94025, U.S.A.

GRADUATE CREDITS IN CHROMATOGRAPHY are offered in a cooperative program between Tracor Instruments and Southwest Texas University. Candidates are required to attend a 4-1/2 day lab/lecture session at either location (depending upon the course) and then to write a thesis after completing this course. Faculty is from the University and Tracor. The University is located in San Marcos, Texas, and is an accredited university. Tracor Instruments, Inc., JLC/81/10, 6500 Tracor Lane, Austin, TX 78721, U.S.A.

BIORESEARCH AND CHROMATOGRAPHY HANDBOOK contains procedures for gas, liquid, and thin-layer chromatography, derivatisation methodologies, and special section on laboratory aids. The BioReserach section includes updated references for amino acid-protein analysis, protein sequencing, chemical modifications, peptide sequencing and synthesis, and affinity chromatography. Pierce Chem. Co., JLC/81/10, P.O. Box 117, Rockford, IL 61102, U.S.A.

REPLACING ODS COLUMNS can be accomplished without guesswork. "Mobile Phase Selection for Various Octadecyl Reverse Phase HPLC Columns," compares the mobile phase composition required for each of 8 manufacturers' ODS columns, discusses why the differences exist, and explains how a chromatographer can

adapt analyses from one brand of column to another. Supelco, Inc., JLC/81/10, Supelco Park, Bellefonte, PA 16823, U.S.A.

LINEAR-K REVERSED PHASE TLC PLATES are available in analytical and preparative layers. They provide fully silanized, reversed phase layer by bonding, via Si-O-Si-C bonds, octadecylsilane groups to a special silica gel. They provide excellent resolution, are fast, and can be heavily loaded, with sample capacity up to 100 micrograms for some compounds. Whatman, Inc., JLC/81/10, 9 Bridewell Place, Clifton, NJ 07014, U.S.A.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1889-1890 (1981)

LC CALENDAR

1981

August 30-September 5	"XI Int'l Congress, IV European Congress of Clinical Chemistry", Vienna, Austria. Contact: 11th Int'l Congress of Clinical Chem., P. O. Box 105, A-1014 Wien, Austria.
September 7-10	"4th Int'l Bioanalytical Forum", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.
September 10-12	Workshop: "Some Approaches to the Anal. of Biological Specimens", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.
September 15-19	Workshop: "Introduction to Determination of Drugs in Biological Fluids", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.
September 20-25	"8th Annual FACSS Meeting", Philadelphia, PA USA. Contact: R. A. Barford, USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118, USA.
September 28-October 1	"Chromatography-81: Int'l. Symposium on Advances in Chromatography" Barcelona, Spain. Contact: Dr. A. Zlatkis, Chem. Dept., University of Houston, Houston, TX 77004, USA.
September 30-October 2	Introduction to Liquid Chromatography, Santa Clara, CA, USA, Contact: G. Gilfillan, Hewlett-Packard Co., 1501 Page Mill Road, Palo Alto, CA 94304, USA.
October 1-2	"Japan Conference on Chromatography", Palace Hotel, Tokyo, Japan. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7, Canada.
October 4-9	"Symposium on Novel Separation Processes", at the 2nd World Congress of Chemical Engineering, Montreal, Canada. Contact: Congress Secretariat, 151 Slater Street, Suite 906, Ottawa, Ont., Canada, K1P 5H3.
October 4-7	ASTM-E19 Committee Meeting, Bar Harbor Hotel, San Diego, CA, USA. Contact: ASTM, 1916 Race St., Philadelphia, PA 19103, USA.
October 12-15	"EXPOCHEM '81", Houston, TX. Contact: Dr. A. Zlatkis, Chem. Dept., University Houston, Houston, TX 77004, USA.
October 22-23	LC/MS Workshop, sponsored by International Assoc. of Environmental Analytical Chem., Palais des Congres, Montreux, Switzerland. Contact: Prof. R. W. Frei, Free University, De Boelelaan 1083, 1018WV Amsterdam, The Netherlands.

October 27-29 "Petroanalysis-81", Cumberland Hotel, Marble Arch, London, England Contact: Miss I.A.McCann, Inst. of Petroleum, 61 New Cavendish St., London, W1M 8AR, England

November 16-17 International Symposium on HPLC of Proteins & Peptides, Washington, D.C. Contact: S. E. Schlessinger, Int'l. Symp. on HPLC of Proteins & Peptides, 400 E. Randolph, Chicago, IL 60601, USA.

November 19-20 "1981 International Chromatography Conference", Carillon Hotel, Miami Beach, Florida, USA. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7.

November 23-25 2nd International Congress on Analytical Techniques in Environmental Chemistry, Barcelona, Spain. Contact: Expoquimia, Plaza de Espana, Barcelona-4, Spain.

November 26-27 Workshop: Chem. & Anal. of Hydrocarbons in the Environment, Barcelona, Spain. Contact: J. Albaiges, Expochimia, Plaza de Espana, Barcelona-4, Spain.

1982

March 28-April 2 "National American Chem. Soc. Meeting", Las Vegas, NV USA. Contact: A. T. Winstead, Am. Chem. Sco., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

April 14-16 "12th Annual Symposium on the Anal. Chem. of Pollutants", Amsterdam, The Netherlands. Contact: Prof. R. W. Frei, Congress Office, Vrije Universiteit, P. O. Box 7161, 1007-MC Amsterdam, The Netherlands.

June 28-30 "Analytical Summer Symposium", Michigan State Univ., East Lansing, MI USA. Contact: A. I. Popov, Chem. Dept., Michigan State Univ., East Lansing, MI 48824, USA.

July 12-16 "2nd Int'l Symposium on Macromolecules", - IUPAC, University of Massachusetts, Amherst, Massachusetts, USA.

July 19-22 23rd Prague Microsymposium on Macromolecules: "Selective Polymeric Sorbents" - INPAC, Institute of Macromolecular Chemistry, Prague, Czechoslovakia. Contact: P.M.M. Secretariat, Institute of Macromolecular Chemistry, 162-06 Prague 616, Czechoslovakia.

August 15-21 "12th Int'l Congress of Biochemistry", Perth, Western Australia. Contact: Brian Thorpe, Dept. of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.

September 12-17 "National American Chem. Soc. Meeting", Kansas City, MO USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

1983

March 20-25 "National American Chem. Soc. Meeting", Seattle, WA USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements for inclusion in the LC Calendar to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 127, Hopedale, Massachusetts 01747, USA.

INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

Journal of Liquid Chromatography is a bimonthly publication in the English language for the rapid communication of liquid chromatographic research.

Directions for Submission

One typewritten manuscript suitable for direct reproduction, carefully inserted in a folder, and two (2) copies of the manuscript must be submitted. Since all contributions are reproduced by direct photography of the manuscripts, the typing and format instructions must be strictly adhered to. Noncompliance will result in return of the manuscript to the authors and delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards and securely bound before mailing.

Manuscripts should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

Reprints

Owing to the short production time for articles in this journal, it is essential to indicate the number of reprints required upon notification of acceptance of the manuscript. Reprints are available in quantities of 100 and multiples thereof. For orders of 100 or more reprints, twenty (20) free copies are provided. A reprint order form and price list will be sent to the author with the notification of acceptance of the manuscript.

Format of Manuscript

1. The general format of the manuscript should be as follows: title of article; names and addresses of authors; abstract; and text discussion.

2. Title and Authors: The entire title should be in capital letters and centered on the width of the typing area at least 2 inches (5.1 cm) from the top of the page. This should be followed by three lines of space and then by the names and addresses of the authors in the following way (also centered):

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN SURFACE WATERS BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakra Way
Salt Lake City, Utah 84108

3. Abstract: Three lines below the addresses, the title ABSTRACT should be typed (capitalized and centered on the page). This should be followed by a single-spaced, concise, abstract comprising less than 10% of the length of the text of the article. Allow three lines of space below the abstract before beginning the article itself.

4. Text Discussion: Whenever possible, the text discussion should be divided into such major sections as INTRODUCTION, MATERIALS, METHODS, RESULTS, DISCUSSION, ACKNOWLEDGMENTS, and REFERENCES. These major headings should be separated from the text by two lines of space above and one line of space below. Each heading should be in capital letters, centered, and underlined. Secondary headings, if any, should be flush with the left margin, underscored, and have the first letter of all main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. Paragraphs should be indented five (5) typewriter spaces.

6. Acknowledgment of collaboration, sources of research funds, and address changes for an author should be listed in a separate section at the end of the paper.

7. References (including footnotes) in the text will be numbered consecutively by numbers in parentheses. All references (and footnotes) should then be aggregated in sequence at the end of the communication. No footnotes should be shown at the bottom of pages. The reference list follows immediately after the text. The word REFERENCES should be capitalized and centered above the reference list. It should be noted that all reference lists should contain initials and names of all authors; *et al.* will not be used in reference lists. Abbreviations of journal titles and styles of reference lists will follow the American Chemical Society's Chemical Abstracts List of Periodicals. References should be typed single-spaced with one line space between each reference.

8. Each page of manuscript should be numbered lightly at the bottom of the sheet with a light blue pencil.

9. Only standard symbols and nomenclature approved by the International Union of Pure and Applied Chemistry should be used.

10. Any material that cannot be typed, such as Greek letters, script letters, and structural formulae, should be drawn carefully in black India ink (do not use blue ink).

Typing Instructions

1. The manuscript must be typewritten on good quality white bond paper measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). Do not use Corrasible bond or its equivalent. The typing area of the article opening page, including the title, should be 5½ inches wide by 7 inches deep (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches deep (14 cm x 21.6 cm).

2. In general, the chapter title and the abstract, as well as the tables and references, are typed single-spaced. All other text discussion should be typed 1½-line spaced, if available, or double-spaced. Prestige elite characters (12 per inch) are recommended, if available.

3. It is essential to use black typewriter ribbon (carbon film is preferred) in good condition so that a clean, clear impression of the letters is obtained. Erasure marks, smudges, creases, etc., may result in return of the manuscript to the authors for retyping.

4. Tables should be typed as part of the text but in such a way as to separate them from the text by a three-line space at both top and bottom of each table. Tables should be inserted in the text as close to the point of reference as possible, but authors must make sure that one table does not run over to the next page, that is, no table may exceed one page. The word TABLE (capitalized and followed by an Arabic number) should precede the table and be centered on the page. The table title should have the first letters of all main words in capitals. Titles should be typed single-spaced. Use the full width of the type page for the table title.

5. Drawings, graphs, and other numbered figures should be professionally drawn in black India ink (do not use blue ink) on separate sheets of white paper and placed at the end of text. Figures should not be placed within the body of the text. They should be sized to fit within the width and/or height of the type page, including any legend, label, or number associated with them. Photographs should be glossy prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn. Captions for the pictures should be typed single-spaced on a separate sheet, along the full width of the type page, and preceded by the word FIGURE and a number in arabic numerals. All figures and lettering must be of a size to remain legible after a 20% reduction from original size. Figure numbers, name of senior author, and arrow indicating "top" should be written in light blue pencil on the back or typed on a gummed label, which should be attached to the back of the illustration. Indicate approximate placement of the illustrations in the text by a marginal note in light blue pencil.

6. The reference list should be typed single-spaced although separated from one another by an extra line of space. Use Chemical Abstract abbreviations for journal titles. References to journal articles should include (1) the last name of all author(s) to any one paper, followed by their initials, (2) article title, (3) journal, (4) volume number (underlined), (5) first page, and (6) year, in that order. Books should be cited similarly and include (1) author, surname, first and middle initials, (2) title of book, (3) editor of book (if applicable), (4) edition of book (if any), (5) publisher, (6) city of publication, (7) year of publication, and (8) page reference (if applicable). E.g., Journals: Craig, L. C. and Konigsber, W., Use of Catechol Oxygenase and Determination of Catechol, Chromatogr., 10, 421, 1963. Books: Albertsson, P. A., Partition of Cell Particles and Macromolecules, Wiley, New York, 1960. Article in a Book: Walter, H., Proceedings of the Protides of Biological Fluids, XVth Colloquim, Pteeters., H., eds., Elsevier, Amsterdam, 1968, p. 367.