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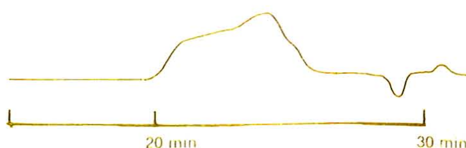

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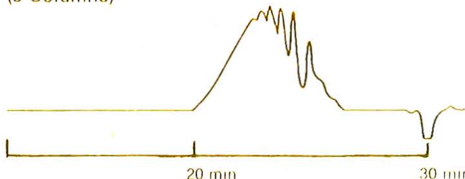

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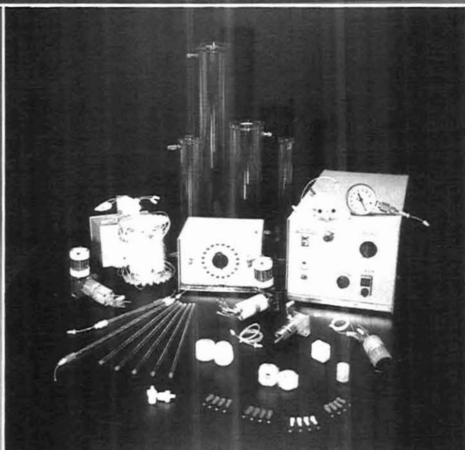

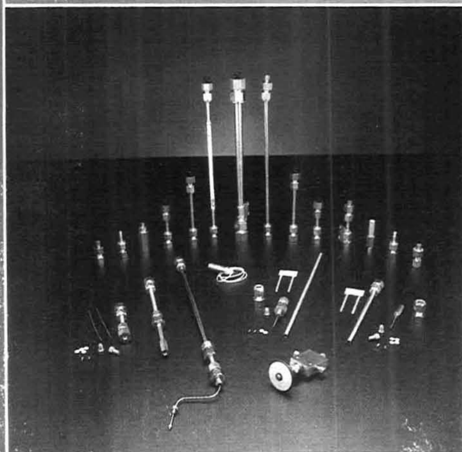

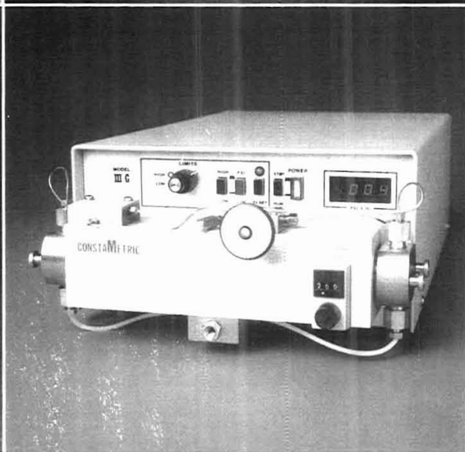

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QUANTITATION OF ALKYL SULFONATES USING UV DETECTOR SENSITIVE "ION-PAIR" REAGENTS IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

Amplification of detector response by means of detector-sensitive ion-pairing reagents demonstrates good sensitivity, linearity, and precision for the quantitation of alkylsulfonate ions by means of ultraviolet absorbance detection at 254 nm.

INTRODUCTION

UV detection of non-UV-absorbing samples was recently shown to be feasible in reversed-phase LC systems by using detector-sensitive "ion-

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pairing" reagents in the eluent (1-5). This visualization technique is accomplished without the aid of any reaction type detector or extraction step, and provides novel methods for controlling both retention and detectability which are of value to analytical chemistry methodology from both practical and mechanistic points of view. The explanation of UV visualization in this reversed-phase system is based upon the ion-interaction model (1, 4, 6) which has recently been supported by others (7, 8). This paper identifies the constraints in the use of the UV visualization as a quantitative tool and suggests some guidelines for its successful application.

EXPERIMENTAL

The chromatographic system has been described elsewhere (6) and consisted of a Model 6000A pump (Waters), 4-cm x 4-mm i.d. Porapak C_{18} precolumn (Waters), and a 30-cm x 4-mm i.d. μBondapak C_{18} main column (Waters). The UV detector signal was digitized by a Model ADC-12QZ analog-to-digital converter (Analog Devices) interfaced to a Model 9830A digital computer (Hewlett-Packard) (9). Chromatograms were drawn from the digitized data on a Model 9862A plotter (Hewlett-Packard).

The mobile phase was prepared from HPLC grade methanol and distilled water containing stated amounts of phenethylamine (Fisher) or p-ethyl benzenesulfonate (Rutgers-Nease). The mobile phase was adjusted with hydrochloric acid or perchloric acid (1<u>M</u>) to a nominal stated pH as measured by a glass electrode. Sodium pentane- and hexanesulfonate samples (Eastman Red Label) were usually prepared in the eluent. At 25°C and

Response Sensitivity Of Method

A mobile phase consisting of 6 mM phenethylammonium ion in methanol-water 35:65 was used to visualize non-UV-absorbing straight chain alkyl sulfonates. Figure 1A shows the retention behavior of increasing amounts

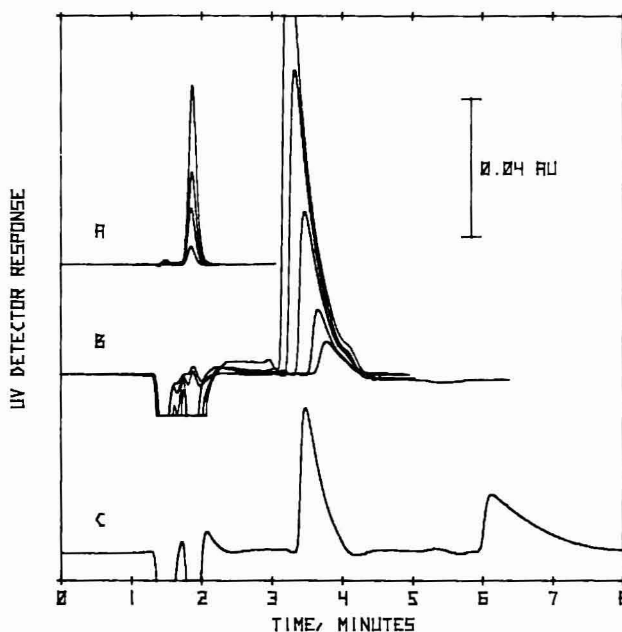

Figure 1. Visualization of alkylsulfonates. UV detector, 254 nm. Mobile phase was methanol-water 35:65, 6 mM phenethylammonium ion, pH = 3 with hydrochloric acid. All samples were made up in the mobile phase. A: Chromatograms obtained for 1, 3, 5, and 10 µL of 10 mM phenethylammonium ion. B: Chromatograms obtained for 1, 2, 5, 10, and 15 µL of 50 mM pentanesulfonate. C: Chromatogram obtained for 10 µL of 25.0 mM each pertanesulfonate (3.5 min) and hexanesulfonate (6.0 min).

of phenethylammonium ion injected as a sample. Figure 1B shows chromatograms obtained for increasing amounts of pentanesulfonate injected as a sample. The positive peak at about 3.5 minutes is caused by the co-elution of excess UV-absorbing phenethylammonium ion with the non-UV-absorbing pentanesulfonate (10). The two deficiency peaks at approximately 1.5 and 1.8 minutes are a result of the depletion of phenethylammonium ion from the bulk mobile phase. Note that the second deficiency peak occurs at the same retention time as phenethylammonium ion in Figure 1A. Response sensitivity from the injection of phenethylammonium ion (Figure 1A) was 2.62 AU-sec/μmole. Sensitivity to pentanesulfonate (Figure 1B) was 2.08 AU-sec/μmole, suggesting that approximately equal numbers of phenethylammonium and pentanesulfonate ions co-elute. Finally, Figure 1C shows the UV detector responses for the separation of pentanesulfonate and hexanesulfonate using phenethylammonium ion as the UV-absorbing ion-pairing reagent.

Calibration Methods

The chromatograms of Figure 2A are the results of constant volume injections of serial dilutions of 25.0 m*M* each of pentanesulfonate and hexanesulfonate. The samples were made up in and diluted with the mobile phase. Chromatograms in Figure 2B resulted from different volume injections of the 25.0 m*M* sample of pentanesulfonate and hexanesulfonate. Both the deficiency peak areas and the positive peak areas are seen to be related to the amount of sample injected and not to the concentration or volume injected. Integration of peak areas showed that for a given injection, positive and negative peak areas were equal within measurement

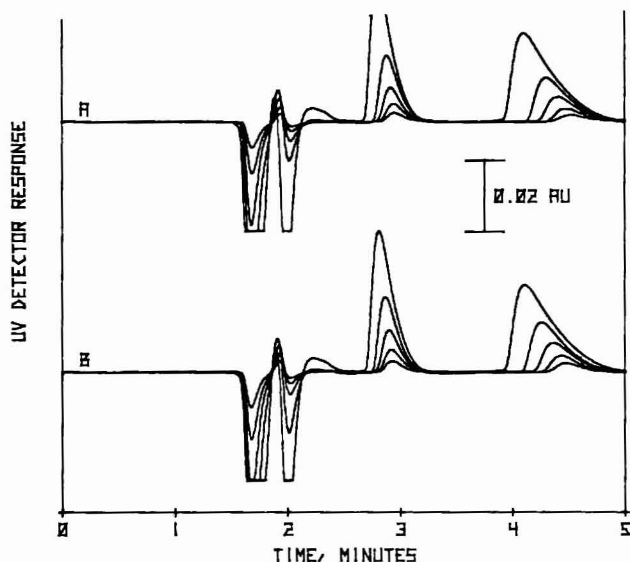

Figure 2. Comparison of constant volume and constant concentration injections of pentanesulfonate (2.8 min) and hexanesulfonate (4.3 min). UV detector, 254 nm. Mobile phase was methanol-water 35:65, 5 mM phenethylammonium ion, pH = 3 with perchloric acid. All samples were made up in the mobile phase. A: 16 µL of 1.6, 3.1, 6.3, 12.5, and 25.0 mM each pentanesulfonate and hexanesulfonate. B: 1, 2, 4, 8, and 16 µL of 25.0 mM each.

Comparison of the peak retention times of Figure 2 (5 mM phenethylammonium ion) and Figure 1 (6 mM phenethylammonium ion) indicates increased retention of the sulfonates with increased concentration of phenethylammonium ion as expected (6, 9).

Table I contains regression parameters (11) for eight sampling situations, showing the sensitivity linearity, and precision obtained in each case. Figure 3 is a representative regression plot of peak area vs.

TABLE 1

Regression of Peak Areas Vs. Amount Injected[a]

	Slope (AU-Sec/µmoles)		Intercept (AU-Sec)	
	Constant Concentration[b]	Constant Volume[c]	Constant Concentration	Constant Volume
entanesulfonate				
alone (n=11)	1.73±0.008[d]	1.73±0.007	0.015±0.002	0.010±0.002
n mixture(n=18)	1.31±0.006	1.33±0.020	0.016±0.001	-0.001±0.005
exanesulfonate				
alone (n=10)	1.82±0.020	1.83±0.030	0.008±0.005	0.006±0.012
n mixture(n=18)	1.35±0.080	1.37±0.030	0.011±0.002	-0.010±0.008

- Mobile phase was methanol-water 35:65, 5 m<u>M</u> phenethylammonium ion, pH=3 with perchloric acid. All samples were made up in the mobile phase.

- 1, 2, 4, 8, and 16 µL injections of 25 m<u>M</u>.

- 16 µL injections of 1.6, 3.1, 6.3, 12.5 and 25.0 m<u>M</u>.

d Uncertainties expressed as one standard deviation.

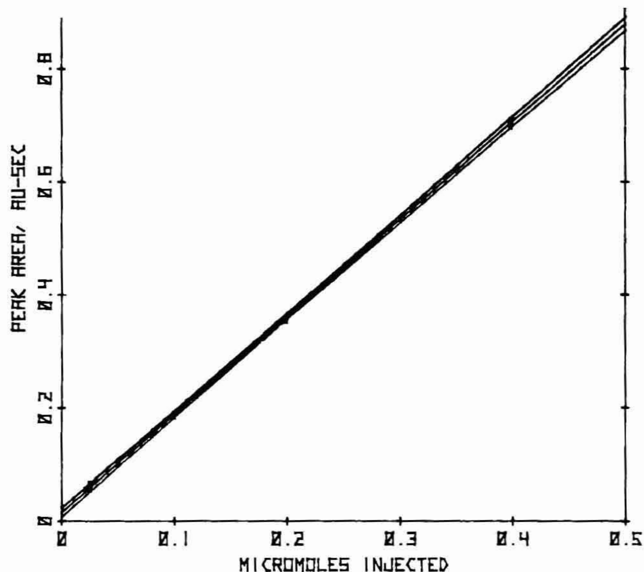

Figure 3. Regression plot of peak area vs. µmoles injected. Data from 16 µL injections of 1.6, 3.1, 6.3, 12.5, and 25.0 mM pentanesulfonate. UV detector, 254 nm. Mobile phase was methanol-water 35:65, 5 mM phenethylammonium ion, pH = 3 w'th perchloric acid. All samples were made up in the mobile phase. Confidence bands are for the 99.9% level.

µmoles of sample injected (11) for constant concentration injections of pentanesulfonate. At higher detection sensitivity, a relative imprecision (standard deviation/mean) of 3% at 3 nanomoles of pentanesu fonate was obtained. It can be seen from Table I that the slope is not affected by the mode of calibration (constant concentration or constant volume); however, constant volume calibrations appear to give smaller absolute intercept.

It has been reported that in UV visualization, variation of $\underline{k}'$ with sample size has limited quantitation when using peak heights (5). This data confirms that observation, but suggests that the use of peak area can improve the quantitation. Furthermore, the $\underline{k}'$ change as sample concentration increases seems to be independant of the charge on the "ion-pairing" reagent. Figure 4 demonstrates the applicability of the visualization

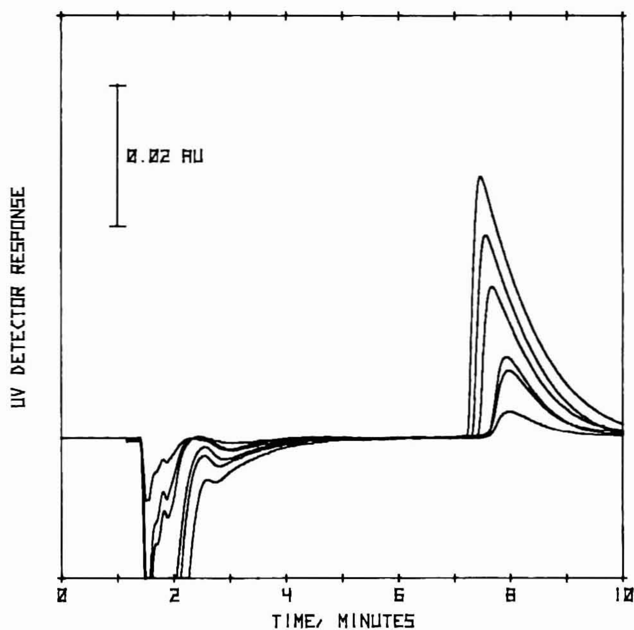

Figure 4. Visualization of octylammonium ion by p-ethylbenzenesulfonate ion. UV detector, 254 nm. All samples were made up in the mobile phase. Mobile phase was methanol-water 50:50, 5 $\underline{mM}$ p-ethylbenzenesulfonate, pH = 3 with hydrochloric acid. Chromatograms obtained for 2, 5, 6, 10, 15, and 20 μL of

technique in a system of interchanged charges; i.e., a negatively charged "ion-pairing" reagent and a positively charged sample. UV chromatograms for a sample of octylammonium ion were obtained for a mobile phase that contained 5 mM UV-absorbing p-ethylbenzenesulfonate in methanol-water 50:50 at pH 3.

Table I also shows that the slope of the calibration curve for an ionic species is decreased by the presence of other alkyl sulfonates in the sample. Therefore, when using this technique for quantitative analysis, it will be important to investigate the contribution of other ions, both inorganic and organic, present in the sample matrix.

Figure 5 is a plot of the detection sensitivities for pentane- and hexanesulfonate vs. the square root of the conductivity of eluents containing 5 mM phenethylammonium ion and added amounts of potassium perchlorate. The lower ionic strength mobile phases give more sensitive visualization. The slope of the line for hexanesulfonate is about twice that for pentanesulfonate indicating that in this study the less retained compound is less affected by increased ionic strength.

Deficiency Peaks

Figure 6 is a set of chromatograms for constant volume injections of different concentrations of the single sample, hexanesulfonate. As the concentration of hexanesulfonate is increased, both of the negative deficiency peaks increase in area, and the ratio of the first deficiency peak to the area of the second deficiency peak remains constant. Again, in each chromatogram the two deficiency peaks have the same total area as the positive peak. This indicates that the two deficiency peaks are not an effect arising from the number of sample components but instead are characteristic of paired-ion chromatography. It is believed that the first

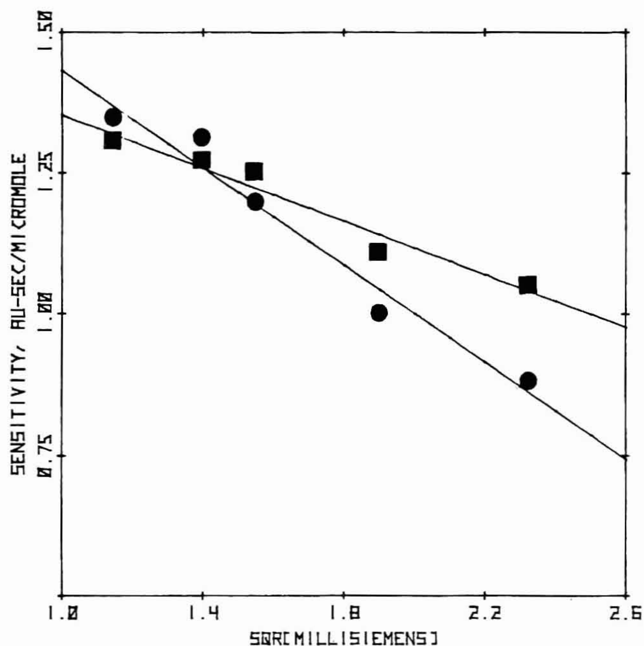

Figure 5. Sersitivity vs. conductivity of eluent. Mobile phase was methanol-water 35:65, 5 mM phenethylammonium ion, pH = 3 with perchlorate. ■ = pentanesulfonate (in mixture), ● = hexanesulfonate (ir mixture).

peak is induced by equilibration of the local eluent composition upon injection, and that the second deficiency peak is a vacancy peak for the "ion-pairing" reagent (6, 12, 13).

Mixed Ion-Pair Reagents

In reversed-phase ion-pair LC, mixed reagents have been used to control retention. However, in UV visualization the presence of additional

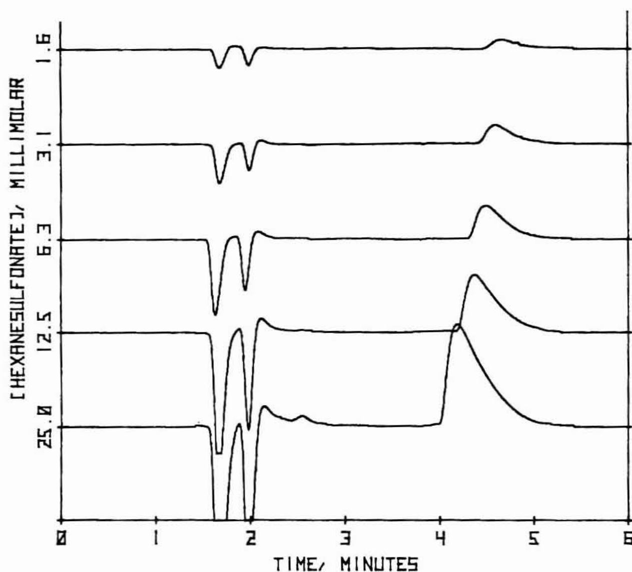

Figure 6. Individual chromatograms for 16 µL injections of 1.6, 3.1, 6.3, 12.5, and 25.0 m$\underline{M}$ hexanesulfonate. UV detector, 254 nm. All samples were made up in the mobile phase. Mobile phase was methanol-water 35:65 5 m$\underline{M}$ phenethylammonium ion, pH = 3 with perchloric acid.

ion-pairing reagent to adjust the retention time of the sample and a second UV-absorbing ion-pairing reagent to visualize the sample. Mobile phases composed of methanol-water 35:65 with 5m$\underline{M}$ octylammonium ion (the retention adjusting reagent) and either 1.0 m$\underline{M}$ or 2.0 m$\underline{M}$ phenethylammonium ion (the visualizing reagent) were prepared. The sensitivities in Table 2 confirm that both octylammonium ion and phenethylammonium ion participate in the retention of alkyl sulfonate (4,10). Thus, less of the UV-active phenethylammonium ion co-elutes with the alkyl sulfonate, which results in a decreased sensitivity for the alkyl sulfonate (6).

Table 2

Effects of Mixed "Ion Pairing" Reagents Upon the Visualization Sensitivity

(Octylammonium ion), m*M*	(Phenethylammonium ion), m*M*	Pentanesulfonate sensitivity, AU-Sec/μmole
5.0	1.0	0.058±0.010
5.0	2.0	0.121±0.003
0.0	5.0	1.31 ±0.01
0.0	6.1	1.59 ±0.04

The use of detector-sensitive ion-pairing reagents for the quantitation of otherwise non-detectable ionic compounds is an alternative to pre- and post-column derivatization techniques. Because only compounds of opposite charge are visualized by a UV-absorbing ion-pairing reagent, this technique enjoys an additional selectivity: the detection of neutral and similarly charged compounds will not be enhanced (2, 6). Although this work has used UV-absorbing ion-pairing reagents to demonstrate the amplification of detector response in reversed phase systems, the technique is applicable to other forms of detection as well, (e.g., electrochemical or fluoresence detection). This visualization technique offers a unique mechanistic probe into the fundamental nature of ionic interactions in liquid chromatography.

REFERENCES

1. B. A. Bidlingmeyer, S. N. Deming, W. P. Price, B. Sachok, and M. Petrusek, Advances In Chromatography, 14th International Symposium, Lausanne, Switzerland, September 24-28, 1979.

2. N. Paris, Anal. Biochem., 100, 250 (1979).

3. B. A. Bidlingmeyer, S. N. Deming, B. Sachok, 13th International Symposium On Chromatography, Cannes, France, July 2, 1980.

4. B. A. Bidlingmeyer, J. Chromatogr. Sci., 18 525 (1980).

5. N. Paris, J. Liquid Chromatogr., 3 (11), 1743 (1980).

6. B. A. Bidlingmeyer, S. N. Deming, S. N. Price, W. P. Sachok, M. Petrusek, J. Chromatogr., 186, 419 (1979).

7. J. K. Knox and R. H. Hartwick, J. Chromatogr., 204, 3 (1981).

8. S. N. Deming and R. C. Kong, Advances In Chromatography, 16th International Symposium, Barcelona, Spain, September 28 - October 1, 1981.

9. S. N. Deming, M. L. H. Turoff, Anal. Chem., 50, 546 (1978).

10. E. Tomlinson, T. M. Jefferies, C. M. Riley, J. Chromatogr., 159, 315 (1978).

11. S. N. Deming, S. L. Morgan, Clin. Chem., 25 (6), (1979).

12. C. N. Reilley, G. P. Hildebrand, J. W. Ashley, Jr., Anal. Chem., 34, 1198, (1962).

13. K. Slais, M. Krejci, J. Chromatogr., 91, 161 (1974).

SULFATED OLIGOSACCHARIDES FROM K-CARRAGEENAN AND OLIGOGALACTURONIC ACIDS SEPARATION BY H.P.L.C.

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SUMMARY

Sulfated oligosaccharides produced from enzymic hydrolysis of K-carrageenan have been separated up to DP 13 on a column of μ-Bondapak C-18 or a Radial Pak cartridge "Dextropak" with sodium nitrate as the mobile phase. Moreover, the separation of oligogalacturonic acids has been performed by the ion pair method on the same supports with 0.05 % dodecyltributylammonium chloride in a sodium nitrate solution as eluent.

INTRODUCTION

High performance liquid chromatography has been widely used in determination of sulfated disaccharides produced from chondroitin sulfates (1-3) or heparan sulfate and heparin (4). Meanwhile, there is no paper on the separation of the sulfated oligosaccha-

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rides from kappa-carrageenan . Recently, we have shown that fractionation up to DP 10 was obtained on a column of Bio-Gel P6 (5) but classical gel permeation chromatography is time consuming ; so it was interesting to solve this problem with a rapid and performant method. In a same way, we have tried to separate by H.P.L.C. the oligogalacturonic acids. These oligosaccharides have been studied with several chromatographic systems, and the most results have been summarized in a large review dealing with the separation of mono and oligosaccharides by liquid chromatography (6) ; high performance liquid chromatography has never been used for this fractionnation.

EXPERIMENTAL

Materials

Kappa-Carrageenan is a copolymer $(ab)_n$ purified as previously described (7-8). The enzymic hydrolysis was performed at pH 8.2 in 0.1M NaCl - 0.005M $NaHCO_3$ at 40°C with a kappa-carrageenase kindly given by Dr. Yaphe from Mc Gill University (Canada). The oligogalacturonic acids were obtained as a much appreciated gift from J.F. Thibault (Nantes - France) (9).

Apparatus

All separations were made using Waters Associates equipment :

was a Servotrace instrument (Sefram - France). The chromatographic supports were a prepacked column (4 x 300 mm) of a μ-Bondapak C-18 and a Radial Pak cartridge "Dextropak" for the radial compression system RCM-100 manufactured by Waters.

RESULTS AND DISCUSSION

Separation of sulfated oligosaccharides

In gel permeation chromatography process when a charged solute is chromatographed on different supports an electrostatic exclusion effect has to be taken into account and it is necessary to screen it by addition of an electrolyte to the eluent (10-12). The same phenomenom is observed on a reverse phase support ; so with the aim to suppress ionic effect and to obtain the same properties of the C-18 phase as found in the separation of neutral mono and oligosaccharides (13) a sodium nitrate solution has been used as the mobile phase in the concentration range 5.10^{-2}M - 1M. The chromatograms obtained are reproduced in Figure 1. The resolution is improved by increasing the ionic strength and in a 1 M $NaNO_3$ eluent sulfated oligosaccharides of kappa-carrageenan can be obtained up to DP 13. In opposite, there is no separation on the Dextropak cartridge when the salt concentration of the eluent is under 1M $NaNO_3$; the results are shown on Figure 2. The mechanism of separation of carbohydrate on a reverse phase column is due to hydrophobic interactions (13). Considering

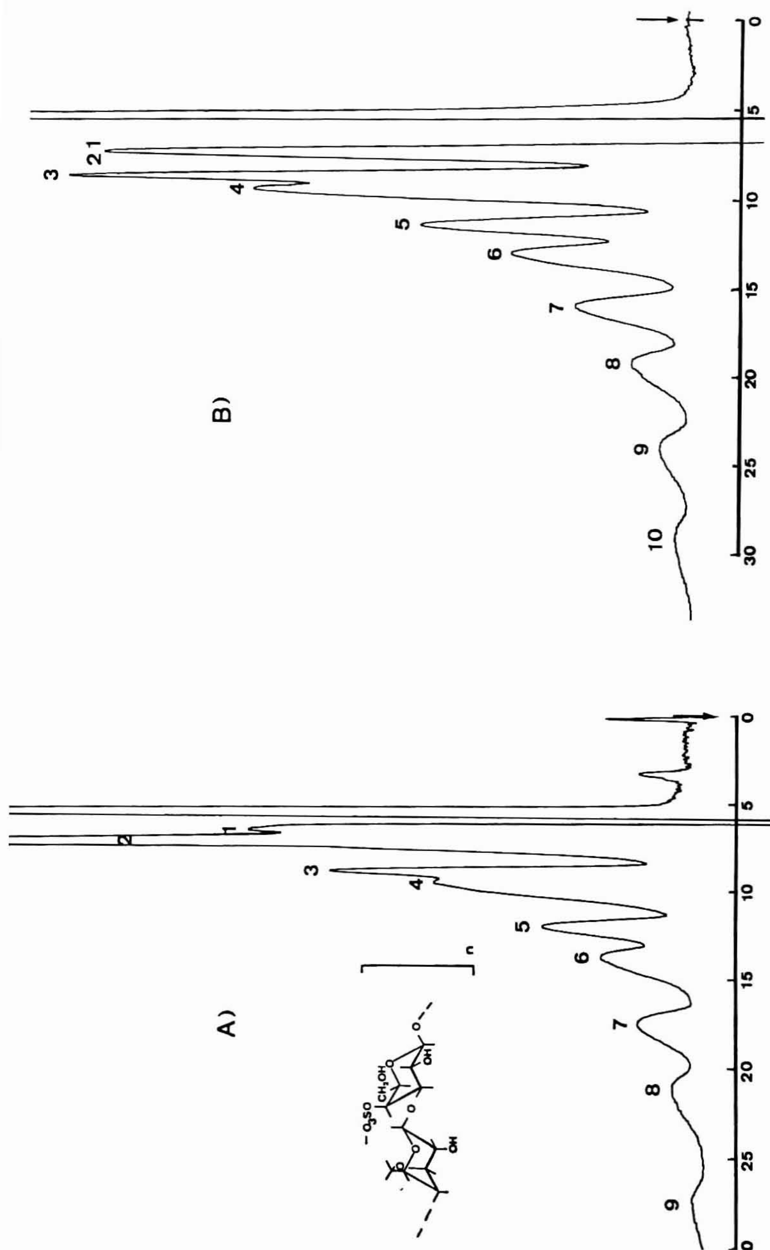


Figure 1 : Separation of K-carragennan oligosaccharides on C-18 μ-Bondapak column.

A - eluent : 0.1 M $NaNO_3$; flow rate : 0.5 ml/mn ; T = 20°C

B - eluent : 1 M $NaNO_3$; flow rate : 0.5 ml/mn ; T = 20°C.

The degree of polymerization is indicated by numbers over the peaks.

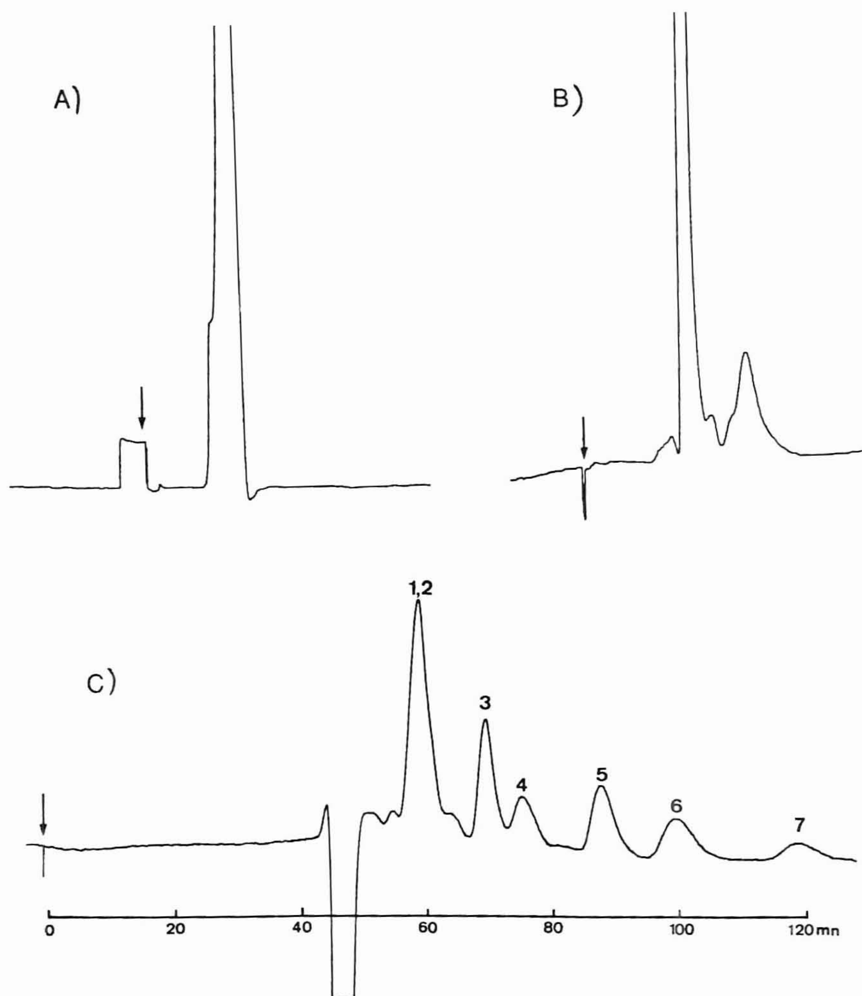


Figure 2 : Separation of K-Carrageenan oligosaccharides on "Dextropak" cartridge.

A - eluent : H_2O ; flow rate 1 ml/mn ; T = 20°C.

B - eluent : 0.1 M $NaNO_3$; flow rate 1 ml/mn ; T = 20°C

C - eluent : 1 M $NaNO_3$; flow rate 0.1 ml/mn ; T = 20°C

The degree of polymerization is indicated by numbers over the peaks.

the structure of the molecule (Figure 1) the hydrophobic anhydrogalactopyranosyl unit promotes the interactions ; the interaction strength with the support is controlled by the salt concentration. The sulfated polysaccharide is not kept back on the phase but it is excluded and eluted before the DP 1. In this case it seems that the charge density is too high and the hydrophobic interactions do not control the elution.

The behaviour observed on both columns can be explained by differences in hydrophobic interactionsand structure of the two phases. In Dextropak, the paking is formed by more regular µ-spherical silica particles ; the surface silanol groups are modified by reacting with dimethyloctodecylchlorosilane but µ-Bondapak C-18 is more hydrophobic (14) due to an additional treatment with trimethylsilyl group on residual silanol groups.

As it has been found, hydrophobic bonds are enhanced when the salt concentration is increasing (15) ; so in 1 M $NaNO_3$ eluent, the hydrophobicity of both paking should become nearly the same.

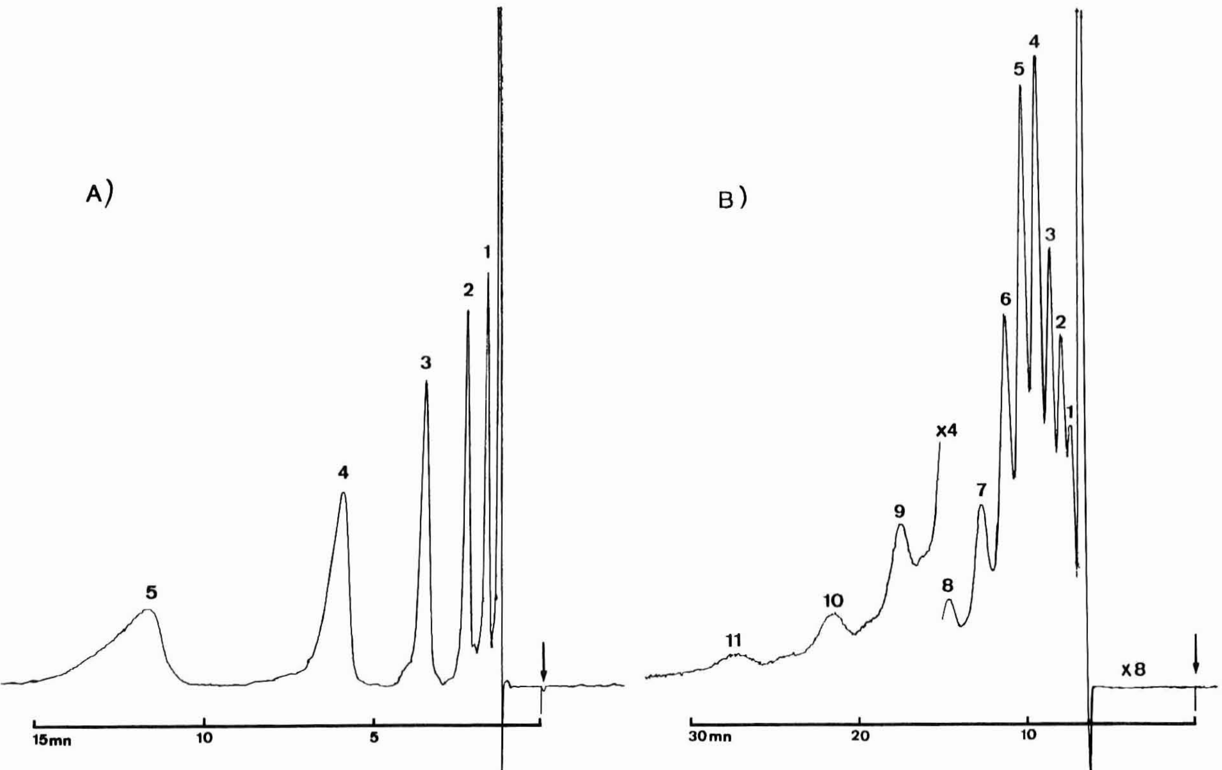
Separation of oligogalacturonic acids :

The galacturonic acid unit has a weak hydrophobic character, due to the presence of the carboxylic sites, so no separation occurs in $NaNO_3$ media. The reverse phase system based on the ion-pair chromatography (16) can be applied. In this method, the ionic form of the solute is suppressed by addition of an appropriate

counterion to the mobile phase. Generally the retention time is depending on the pH of the eluent and the nature and the concentration of the counterion used. All experiments have been performed on the "Dextropak" cartridge. We have tested, without success, tetramethylammonium and tetrabutylammonium ions ; whatever the pH is, there is no retention. A counterion more hydrophobic has been tried : with 0.05 % dodecyltributylammonium ion in water, the complex formed is so strong and so hydrophobic that he can't be eluted from the column. So we have eluted with 0.05 % dodecyltributylammonium chloride in sodium nitrate solution. The results obtained in 0.1 M and 0.2 M $NaNO_3$ are illustrated in Figure 3. When the concentration of the sodium nitrate is 0.1 M, the first five DP are resolved. In 0.2 M, the resolution of the upper DP is increased but in 1 M $NaNO_3$ there is no more separation. It was concluded that, by adding electrolyte, the complex is destabilized by a competition between the electrolyte and the hydrophobic counterions.

CONCLUSION

High performance liquid chromatography on C-18 supports has been proved to be an excellent method to analyse the oligosaccharides produced from kappa-carrageenan by enzymic hydrolysis or to fractionate a mixture of oligogalacturonic acids. In the first case, the mechanism is based on hydrophobic interactions between C-18 chains and the anhydrogalactosyl unit after suppression of the ionic exclusion with elution by a sodium ni-



ure 3 : Separation of oligogalacturonic acids on "Dextropak" cartridge

A - eluent : 0.1 M $NaNO_3$ + 0.05 % dodecyltributylammonium chloride ; flow-rate : 2 ml/mn.

B - eluent : 0.2 M $NaNO_3$ + 0.05 % dodecyltributylammonium chloride ; flow-rate : 0,4 ml/mn.

The degree of polymerization is indicated by numbers over the peaks.

trate solution. This system is not available with oligogalacturonic acids, but the separation can be performed by the ion-pair method ; the hydrophobic counterion used is dodecyltributylammonium. A concentration of 0.05 % in a sodium nitrate as the mobile phase allows to get good results, the resolution of different DP being checked by the ionic strength.

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REFERENCES

1. G.J.L. Lee, H. Tieckelmann. Anal. Biochem. 94, 231 (1979)

2. A. Hjerpe, C.A. Antonopoulos, B. Engfeldt. J. Chromatogr. 171 339 (1979).

3. N. Ototani, N. Sato, Z. Yosizawa. J. Biochem. 85, 1383 (1979).

4. G.J.L. Lee, H. Tieckelmann. J. Chromatogr. 195, 402 (1980)

5. C. Rochas, A. Heyraud. Polymer Bulletin 5, 81 (1981)

6. A. Heyraud, M. Rinaudo. J. Liq.Chromatog.(special Issue) 175 (1981).

7. C. Rochas, M. Rinaudo. Biopolymers 19, 1675 (1980)

8. C. Rochas, M. Rinaudo, M. Vincedon. Biopolymers 19 2165 (1980).

9. J.F. Thibault. J. Chromatogr. 194, 315 (1980)

10. A. Domard,M. Rinaudo, C. Rochas. J. Polym. Sci. Polym. Phys. Ed. 17 673 (1979).

11. C. Rochas;A.Domard,M. Rinaudo. Polym. J. 16 135 (1980).

12. M. Rinaudo, J. Desbrières. Eur. Polym. J. 16 849 (1980)
M. Rinaudo, J. Desbrières, C. Rochas. J. Liq. Chromatog. 4 (8), 1297 (1981).

13. A. Heyraud, M. Rinaudo. J. Liq. Chromatog . 3, 171 (1980)

14. Séminaire Waters, Paris, (1981).

15. C. Horvath, W. Melander; Chromatographia 11, 262 (1978)

16. G. Seroussi, R. Barbes, J.D. Dauchy, X. Delamare, C. Israelian, M. Laparra. Spectra 2000, 65, 29 (1981).

USE OF A PROGRAMMABLE POCKET CALCULATOR FOR DATA REDUCTION IN GPC: CALCULATION OF MOLECULAR WEIGHTS AND MOLECULAR WEIGHT DISTRIBUTION

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ABSTRACT

A short program was written for a pocket programmable calculator (HP-29C), to reduce data from a Gel Permeation Chromatogram. The output of this program consists of weight- and number-average molecular weights, polydispersity, and normalized weight distribution. All were uncorrected for dispersion. Mathematical approximation of the GPC calibration curve was made by exponential fit, also performed on the programmable calculator. The program and its application to NBS 706 and one narrow-molecular weight distribution (NMWD) polystyrene standards are presented. With slight modification, the program can be used on newer, more powerful calculators such as the HP-41C, on which dispersion correction subroutines could be performed.

INTRODUCTION

The advent of low-cost minicomputers has made the use of on-line GPC data acquisition and reduction economical and practical in the laboratory. However, these systems cost several thousand dollars. An inexpensive alternative to these systems is the use of a programmable pocket calculator, such as the Hewlett-Packard 29C.

A GPC calibration curve was generated, also on the calculator, by exponential fit of elution volumes of narrow-molecular weight polystyrene standards (NMWD) vs. peak molecular weight for each standard. Use was made of Hewlett-Packard's Curve Fitting Program.

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The expression thus generated was of the form:

$$MW_i = a \times e^{(b \times Ve)} \qquad (1_a)$$

$$\text{or:} \quad \ln MW_i = \ln a + b\, Ve \qquad (1_b)$$

where: a = intercept
b = slope
Ve = elution volume, ml.

The constants (a and b) were used to initialize the GPC program. In use, chromatogram heights (H_i) are entered in succession, immediately following the display of the molecular weight (MW_i) corresponding to the elution volume (Ve_i). When the last chromatogram height entered is zero, Mw, Mn, and the polydispersity are displayed, followed by a normalized cummulative distribution.

The equations used in the program were[1]:

$$Mw = \frac{\Sigma(H_i \times M_i)}{\Sigma H_i} \qquad (2)$$

$$Mn = \frac{\Sigma H_i}{\Sigma(H_i/M_i)} \qquad (3)$$

$$\text{Polydispersity} = Mw/Mn \qquad (4)$$

The normalized distribution is defined as:

$$\text{Normalized distribution} = \frac{H_i}{\Sigma H_i} \times 100 \qquad (5)$$

METHOD

Narrow molecular weight polystyrene standards, purchased from Waters Associates (Milford, Mass.), were used as received. The set consisted of PS 3600, 50000, 240000, and 2700000. NBS 706 (Office of Reference Materials, National Bureau of Standards, Washington, D.C.), was also used as received.

of BHT in 10.0 ml. of u.v.-grade THF (J.T. Baker, Co.). NBS 706 was used as a solution of 25 mg. in 10 ml. THF.

The GPC column set consisted of four μStyragel columns of nominal exclusion range 10^5 Å, 10^4 Å, 10^3 Å, and 10^2 Å. The solvent used was uninhibited tetrahydrofuran (THF), purchased from the J.T. Baker, Co.

A Waters Associates HLC/GPC 244 Liquid Chromatograph was used in the analysis. Flow rate was 2.0 ml./min.; detection was with the Model 440 U.V. Absorbance Detector (254 nm), 1.0 AUFS. All solutions were filtered through a 0.5 μm Teflon filter prior to analysis, using Waters' Sample Clarification Kit. All analyses were performed at room temperature. A modified internal standard method was used to correct for slight fluctuations in flow rate[2].

THE MOLECULAR WEIGHT PROGRAM AND ITS USE

First, a GPC calibration curve was generated, using data from the liquid chromatograph. For this purpose, Hewlett-Packard's Exponential Curve Fit Program was used, entering elution volumes vs. molecular weight for each standard (see Table 1). The calibration data were as follows:

The Exponential Curve Fit Program then gave the following expression, after Equation (1_b):

$$\ln MW_i = \ln 5.3489455 \times 10^{10} - 0.468683685 \times Ve \quad (6)$$

with $r^2 = 0.9994$ (correlation coefficient), and

$a = 5.3489455 \times 10^{10}$

$b = -0.468683685$

TABLE 1.

Elution Volumes and Corresponding Molecular Weights

Ve, ml.	PS Peak MW
21.10	2.7 E6
26.34	2.4 E5
29.74	5.0 E4
34.90	3.6 E3
41.34	2.22 E2 (BHT)

Both a and b will be needed to initialize the GPC program. All decimals should be carried out, for improved fit of the data to the regression curve.

The program keystrokes and their corresponding key codes are listed below. These must be entered with the calculator set to PROGRAM mode ("PRGM").

STEP	KEY ENTRY	KEY CODE
1	g LBL 2	01 15 13 02
2	RCL 2	02 24 02
3	GSB 3	03 12 03
4	RCL 1	04 24 01
5	x	05 61
6	g e^x	06 15 42
7	RCL 3	07 24 03
8	x	08 61
9	STO 4	09 23 04
10	f FIX 0	10 14 11 00
11	R/S	11 74
12	g x=0	12 15 71
13	GSB 1	13 12 01
14	STO i	14 23 22
15	STO +7	15 23 51 07
16	x	16 61
17	STO +5	17 23 51 05
18	RCL i	18 24 22
19	RCL 4	19 24 04
20	÷	20 71
21	STO +6	21 23 51 06
22	9	22 09
23	RCL 0	23 24 00
24	f x>y	24 14 51
25	g DSZ	25 15 23
26	1	26 01
27	.	27 73
28	5	28 05
29	g 1/x	29 15 74
30	STO +2	30 23 51 02
31	GSB 2	31 12 02
32	g LBL 1	32 15 13 01
33	RCL 5	33 24 05
34	RCL 7	34 24 07
35	÷	35 71
36	R/S	36 74
37	RCL 7	37 24 07
38	RCL 6	38 24 06
39	÷	39 71
40	R/S	40 74
41	÷	41 71
42	f FIX 2	42 14 11 02
43	R/S	43 74
44	2	44 02
45	9	45 09
46	STO 0	46 23 00
47	0	47 00
48	STO 1	48 23 01
49	g LBL 5	49 15 13 05
50	RCL i	50 24 02

STEP	KEY ENTRY	KEY CODE	STEP	KEY ENTRY	KEY CODE
51	g LBL 7	51 15 13 07	64	RCL i	64 24 22
52	RCL 7	52 24 07	65	R/S	65 74
53	÷	53 71	66	g DSZ	66 15 23
54	1	54 01	67	GTO 5	67 13 05
55	0	55 00	68	g LBL 8	68 15 13 08
56	0	56 00	69	RCL 1	69 24 01
57	x	57 61	70	R/S	70 74
58	STO +1	58 23 51 01	71	GTO 7	71 13 07
59	f PSE	59 14 74	72	g LBL 3	72 15 13 03
60	9	60 09	73	RCL 8	73 24 08
61	RCL 0	61 24 00	74	+	74 51
62	f x=y	62 14 71	75	g RTN	75 15 12
63	GTO 8	63 13 08	76	R/S	76 74

The GPC program is "initialized" (prepared to run), with the calculator in "RUN" mode, as follows:

INSTRUCTION	INPUT	KEYS	OUTPUT
1. Set all memory registers to zero:		f REG	0
2. Set program to step 0:		g RTN	0
3. Enter calibration curve parameters:			
a) slope b in Register 1(R_1):	b	STO 1	b
b) first Ve at $H_i \neq 0$, in R_2:	$H_{i(1)} \neq 0$	STO 2	$H_{i(1)} \neq 0$
c) intercept a in R_3:	a	STO 3	a
4. Enter indirect register address in R_0:	29	STO 0	29
5. Store correction to Ve (Sample) in R_8:	(±)Ve (correction)	STO 8	(±)Ve

[if Ve (Internal Standard Sample) > Ve (Internal Standard, Run 1), then correction is negative.]

The calculator is now ready to execute the program. By pressing R/S, the MW_i corresponding to the first non-zero chromatogram height is displayed (the molecular weights will be displayed start-

ing with the higest, and proceeding through intermediate MW's, to lowest MW's). The first $H_{i(1)}$ is entered, and R/S is again pressed:

	INSTRUCTION	INPUT	KEYS	OUTPUT
6.	Press R/S:		R/S	First MW_i at $H_{i(1)} \neq 0$
7.	Enter chromatogram height ($H_{i(1)}$) corresponding to MW_i (and $Ve_{(1)}$) displayed:	$H_{i(1)} \neq 0$	R/S	Second MW_i
8.	Enter remaining chromatogram heights, until last $H_{i(last)} = 0$:	$H_{i(2)}$ . . .	R/S	Third MW_i . . .
9.	When $H_{i(last)} = 0$, Mw is displayed:	$H_i = 0$	R/S	Mw
10.	Calculate Mn:		R/S	Mn
11.	Calculate Mw/Mn:		R/S	Mw/Mn
12.	Start display of % polymer fraction and % cummulative. Program pauses after % fraction is displayed, and stops after each % cummulative:		R/S	% cummulative
13.	If the number of data points is greater than 20, "9" is displayed after the 20th data point is entered. Reenter the 20th point, and continue with the remaining H_i's:	H_i	R/S	% cummulative
14.	Final % cummulative must always be 100.00%:		R/S	100.00

Memory register contents are as follows:

R_0: 29. Indirect register address. Controls Registers 29 through 9 for storage of chromatogram heights, $R_{29} \rightarrow R_9$.

R_1: a) GPC Calibration curve slope (b), for calculation of Mw's.
b) Cummulative % (during MWD calculation).

R_2: Ve_1, initially set to elution volume for which $H_i \neq 0$.

R_3: GPC Calibration curve intercept (a).

R_4: MW_i.

R_5: $\Sigma(MW_i \times H_i)$.

R_6: $\Sigma(H_i \div MW_i)$.

R_7: ΣH_i.

R_8: Ve correction from internal standard, (first run - current run).

R_9 to R_{29}: Chromatogram heights, starting at R_{29} for first non-zero H_i.

The GPC program can be summarized as follows:

STEP NO.	to STEP NO.	FUNCTION
1	11	Generate MW_i.
12	13	If last $H_i = 0$, then calculate Mw, Mn, and Mw/Mn (Subroutine 1).
14		Store H_i in $R_{29} \rightarrow R_9$, by using indirect address "i".
15		ΣH_i.
16	17	$MW_i \times H_i$, and $\Sigma(MW_i \times H_i)$.
18	21	$H_i \div MW_i$, and $\Sigma(H_i \div MW_i)$.
22	25	Memory register automatic decrement, from $R_{29} \rightarrow R_9$.
26	30	Automatic counter. Permits selection of specific time intervals for which H_i's will be collected.
31		Return to Subroutine 2, to calculate next MW_i, if $H_i \neq 0$.
32	43	Calculate Mw, Mn, and Mw/Mn, and display each.
44	48	Reset indirect address, to start calculation of % polymer and % cummulative distribution.

STEP NO.	to	STEP NO.	FUNCTION
49		61	Calculate % polymer and % cummulative.
62		65	If register is R_9, go to Subroutine 8 and enter excess H_i's manually (if > 20 heights).
66		67	If register is > R_9, then decrement register number, and continue with MWD calculation.
68		71	If register = R_9, enter excess H_i's manually.
72		75	Correction to Ve, due to flow rate variations.

RESULTS: APPLICATION OF THE GPC PROGRAM TO NBS 706 & PS 110,000

As Table 2 shows, excellent agreement between the calculator-generated molecular weights and the published molecular weights was obtained. The number average molecular weights are off somewhat, since no corrections for dispersion were made;

TABLE 2.

Calculator-Generated Molecular Weights: NBS 706 & PS 110,000

	Mw	Mn	Mw/Mn
NBS 706 (GPC):	256,990	119,997	2.14
	257,800[a]	136,500[b]	
	259,464[d]	124,789[d]	2.08[d]
PS 110,000(GPC):	112,376	103,601	1.08
	110,000[c]	111,000[b]	
	116,447[d]	106,447[d]	1.09[d]

a: NBS value from light scattering measurements.

b: NBS value from osmometry.

c: GPC peak molecular weight, from Waters Associates.

The calculation time per sample, including chromatogram handling, is between ten and fifteen minutes.

Tables 3 and 4 show the worksheets used to calculate the molecular weights and MWD.

Initially, only Ve and MW_i make up the tables. H_i's are obtained from the Gel Permeation Chromatogram, and the GPC Program generates the % polymer and % cummulative values. As these tables also illustrate, the data point interval can be changed readily, to accommodate various polydispersities.

TABLE 3.

NBS 706

Ve, ml.	H_i	MW_i	% polymer	% cummulative
22.00	0	1,779,262	0.00	0.00
22.67	0.8	1,301,794	0.40	0.40
23.33	4.2	952,455	2.00	2.51
24.00	9.5	696,861	4.77	7.29
24.67	16.5	509,857	8.29	15.58
25.33	23.7	373,036	11.91	27.49
26.00	28.9	272,931	14.52	42.01
26.67	30.3	199,689	15.23	57.24
27.33	27.3	146,102	13.72	70.95
28.00	21.6	106,896	10.85	81.81
28.67	14.1	78,210	7.08	88.89
29.33	8.3	57,222	4.17	93.07
30.00	5.1	41,866	2.56	95.63
30.67	3.5	30,584	1.76	97.39
31.33	2.4	22,411	1.21	98.59
32.00	1.4	16,397	0.70	99.30
32.67	0.8	11,997	0.40	99.70
33.33	0.4	8,778	0.20	99.90
34.00	0.2	6,422	0.10	100.00
34.67	0.0	4,699	0.00	100.00

TABLE 4.

PS 110,000

Ve, ml.	H_i	MW_i	% polymer	% cummulative
26.00	0	272,931	0.00	0.00
26.33	0.4	233,455	0.35	0.35
26.67	2.2	199,689	1.95	2.31
27.00	6.8	170,807	6.03	8.34
27.33	14.7	146,102	13.04	21.38
27.67	22.5	124,971	19.96	41.35
28.00	25.1	106,896	22.27	63.62
28.33	20.2	91,435	17.92	81.54
28.67	12.0	78,210	10.65	92.19
29.00	5.4	66,898	4.79	96.98
29.33	2.2	57,222	1.95	98.94
29.67	0.8	48,946	0.71	99.65
30.00	0.3	41,866	0.27	99.91
30.33	0.1	35,811	0.09	100.00
30.67	0.0	30,631	0.00	100.00

Although the GPC program is written to accept a large number of data points, the interval must be varied to keep the number of points between twenty and thirty. So long as only the molecular weight averages are desired, regular data entry is required. But, for a number of data points greater than twenty, manual re-entry of each H_i in excess of twenty must be done if the MWD is desired.

As Table 2 shows, the number average molecular weights differ somewhat from published values. However, as long as the GPC work is done on a comparative basis, obtaining a perfect match for Mw, Mn, and Mw/Mn is not necessary. The GPC program presented here

CONCLUSIONS

It has been shown that a pocket programmable calculator is a versatile and convenient tool in the reduction of Gel Permeation chromatography data. The short program presented here can be used as a back-up for existing laboratory minicomputers, or as a totally portable system. The GPC program could be modified slightly for use on newer, more powerful calculators such as the HP-41C, on which dispersion correction subroutines could be easily performed.

REFERENCES

(1) Cazes, J., Gel Permeation Chromatography, J. Chem., Ed., 43, A625 (1966).

(2) Kohn, E., and Ashcroft, R.W., Liquid Chromatography of Polymers and Related Materials, Chromatographic Science Series, 8, p. 105, Dekker, 1977.

CHARACTERIZATION OF DEXTRANS BY SIZE EXCLUSION CHROMATOGRAPHY USING DRI/LALLSP DETECTOR SYSTEM

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ABSTRACT

Herein is reported an experimental investigation of the molecular weight characterization of dextrans by aqueous SEC with a DRI/LALLSP detector system. Methodology for the determination of the molecular weight calibration curve and peak broadening parameter σ^2 (variance of a Gaussian instrumental spreading function) across the chromatogram have been developed. A blend of dextran standards permits, with one injection, measurement of the molecular weight calibration curve over a wide range of retention volumes. Measurements with salt-free water as mobile phase have confirmed that some dextran chains may have a negative charge.

INTRODUCTION

The use of an LALLSP detector with SEC is a relatively recent development[1-8]. In fact, to date, use of aqueous SEC with a LALLSP detector with dextrans has been reported only once[8]. Dextran was chosen as a test solute to develop the technique as previous studies showed that aqueous SEC of these polymers was

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relatively straightforward with the absence of significant ion exclusion and adsorption and with a wide range of well characterized broad MWD standards available commercially[9]. Generalized equations which correct for dispersion in the detector cell and which are applicable for the case of a nonlinear molecular weight calibration curve and peak broadening parameters which change with retention volume and molecular size have recently been published[10,11]. These correction equations form the basis for the method of determining the molecular weight calibration curve and peak broadening parameters across the chromatogram of a broad MWD polymer developed herein.

THEORY

It is assumed that Tung's integral equation adequately describes peak broadening in the aqueous SEC of dextrans.

$$F(v) = \int_0^\infty w(y)\, G(v,y)\, dy \tag{1}$$

A distribution function $w(v,y)$ which describes peak broadening in the detector cell is now defined. $w(v,y)\, dvdy$ is the area under the detector response at retention volume, $v-v+dv$ due to species with mean retention volume, $y-y+dy$. This distribution function has the following properties.

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

$$\int_0^\infty w(v,y)dy = F(v) \tag{3}$$

$$\int_0^\infty w(v,y)dv = w(y) \tag{4}$$

It is now assumed that the instrumental spreading function, $G(v,y)$ is Gaussian with a variance, $\sigma^2(v)$ which depends on retention

$$w(v,y) = \frac{F(v)}{\sqrt{2\pi}\ \bar{\sigma}(v)^2} \exp\left((v - \bar{y}(v))^2/2\ \bar{\sigma}(v)^2\right) \tag{5}$$

The mean $\bar{y}(v)$ and variance $\bar{\sigma}(v)^2$ of $w(v,y)$ can be related to $\sigma(v)^2$ the variance of the instrumental spreading function and to $D_2(v)$ the local slope of the molecular weight calibration curve which is given by

$$M(y) = D_1(v) \exp(-D_2(v)y) \tag{6}$$

as follows

$$\bar{y}(v) = v + \frac{1}{D_2(v)} \ln\left(\frac{F(v + D_2(v)\ \sigma(v)^2)}{\sqrt{F(v-D_2(v)\sigma(v)^2)\cdot F(v+D_2(v)\sigma(v)^2)}}\right) \tag{7}$$

$$\bar{\sigma}(v)^2 = \sigma(v)^2 + \frac{1}{D_2^2(v)} \ln\left(\frac{F(v-D_2(v)\sigma(v)^2)\cdot F(v+D_2(v)\sigma(v)^2)}{F(v)^2}\right) \tag{8}$$

It is clear that $\bar{y}(v)$ and $\bar{\sigma}(v)^2$ should not depend on $D_2(v)$ and indeed this has been shown[11]. For computational convenience equations (7) and (8) are used with any convenient value of $D_2(v)$. Once $\bar{y}(v)$ and $\bar{\sigma}(v)^2$ are known, the corrected response for the mass concentration detector is given by

$$w(v) = F(v)(\sigma(v)/\bar{\sigma}(v))\exp(-(v - y(v))^2/2\bar{\sigma}(v)^2) \tag{2a}$$

The correction equation for the weight-average molecular weight in the detector cell follows.

$$\frac{\bar{M}_w(v,uc)}{M(v)} = \frac{F(v - D_2(v)\sigma(v)^2)}{F(v)} \exp((D_2(v)\sigma(v))^2/2) \tag{9}$$

This equation is later used to determine $\sigma(v)$ across the chromatogram and then equations (7), (8) and (9) are used to determine the corrected detector response, $w(v)$.

EXPERIMENTAL

Operational details of the aqueous SEC chromatograph employed follow:

Packing: 3/8 inch ID columns (4 foot long approx.) dry-packed with CPG10 200/400 and 120/200 mesh glass packings

Mobile phase: 0.05 M K_2HPO_4 (NaOH to pH = 7.0) in deionized distilled water

Flowrate: 1ml/min.

Sample size: 0.4 ml at 0.5-1.0 wt% solution

Temperature: ambient

Inline filter: 0.22 micron Millipore

Detectors: Waters R-403 DRI and Chromatix KMX-6 with angle $6\text{-}7^{o}$ and field stop 0.15.

Polymer Samples: Pharmacia dextran standards and special blends of these standards with a desired molecular weight distribution.

A published specific refractive index increment (dn/dc = 0.1378) for dextran in 0.05 M K_2HPO_4 (pH = 7.0) at a wavelength λ = 632.8 nm was used in this investigation[12]. A second virial coefficient ($A_2 = 0.41 \times 10^{-3}$ ml/gmole) was determined for dextran standard T250. Setting $A_2 = 0$ in the measurement of $\bar{M}_w(v,uc)$ introduced an error of less than 2%, however. This correction for polymer solute concentration was therefore neglected for all the dextran samples characterized.

RESULTS AND DISCUSSION

To optimize pore size for the dextran standards, single columns containing one pore size were calibrated. Molecular

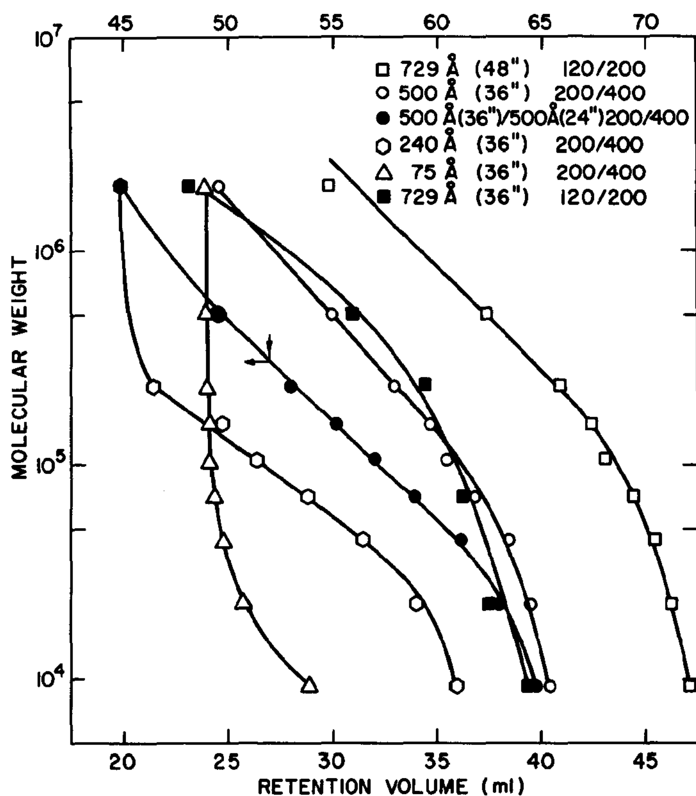

Figure 1. Molecular weight calibration curves obtained using Pharmacia dextran standards with CPG10 packings, mobile phase - 0.05M K_2HPO_4(pH=7.0) at 1.0 ml/min.

investigation[13] pore sizes of 700Å, 500Å, 240Å and 75Å gave suitable peak separation and were used in this investigation. With a proper choice of column length for each pore size a column combination was selected which gave an almost linear molecular weight calibration curve over a wide molecular weight range. To construct the calibration curve the root-mean-square ($\sqrt{\bar{M}_N \cdot \bar{M}_w}$) or $\bar{M}_{rms}$ molecular weight is assigned to the peak position. This procedure is in general not valid for all broad MWD standards, however it does provide a good first guess of the molecular weight

calibration curve. The molecular weight calibration curves for the chosen column combination using $\bar{M}_{rms}$ at the peak position and SEC/DRI/LALLSP are shown in Fig. 2. The $\bar{M}_{rms}$ was calculated using $\bar{M}_N$ and $\bar{M}_W$ values supplied by Pharmacia. The SEC/DRI/LALLSP calibration curve was found by setting $M(v) = \bar{M}_w(v,uc)$ at the peak position of the broad MWD standards. An examination of equation

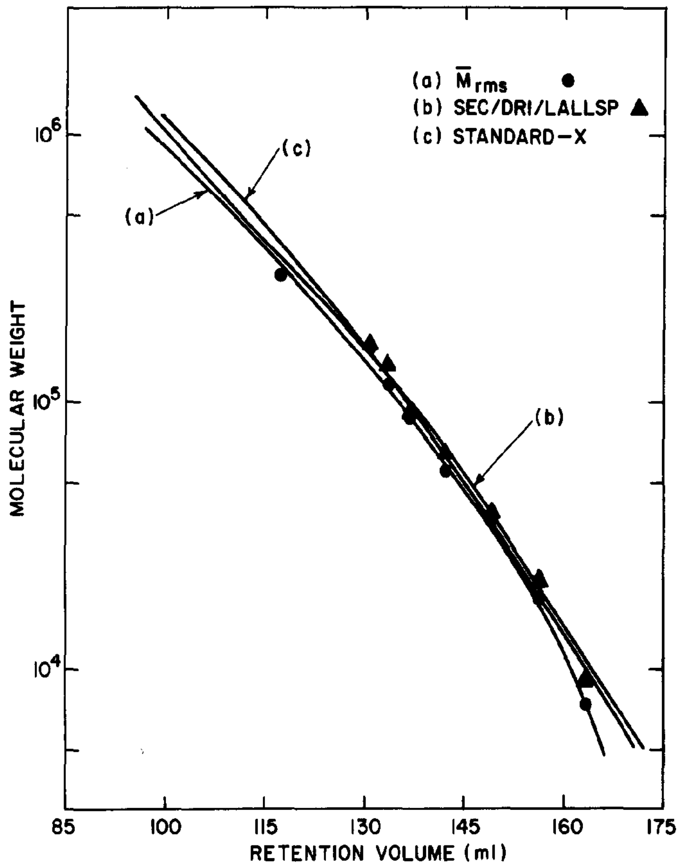

Figure 2. Molecular weight calibration curves for optimized column combination of CPG10 packings using Pharmacia dextran standards ($\bar{M}_{rms}$, SEC/DRI/LALLSP) and dextran standard-X (SEC/DRI/LALLSP).

(9) reveals that the correction for peak broadening is small near the peak position justifying the assumption, $M(v) = \bar{M}_w(v,uc)$.

Typical responses obtained from SEC/DRI/LALLSP are shown in Fig. 3. The noise in the LALLSP response clearly shows that particulate matter and deformable micelles pass through the inline 0.22 μm filter and enter the detector cell. To obtain a relatively smooth LALLSP response the mobile phase is pumped continuously at a low flowrate (0.1 ml/min) when the chromatograph

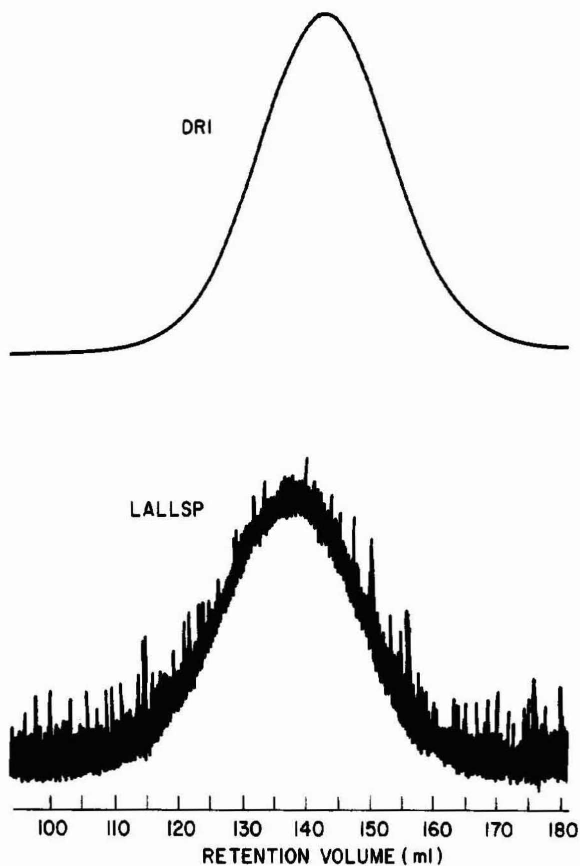

Figure 3. SEC/DRI/LALLSP chromatograms for Pharmacia dextran standard T70.

is not being used. To prepare the system for use the flowrate is increased to the desired level (1 ml/min in this study) and run for 5-6 hours before a polymer sample is injected. We have observed that with the initial startup of an SEC/DRI/LALLSP from zero flowrate, it can take up to a week of operation before an LALLSP response has an acceptable signal-to-noise ratio.

The calculational procedure for finding σ(v) across the chromatogram of a single broad MWD standard is now discussed. The calibration curve M(v) and its slope $D_2(v)$ have already been determined (SEC/DRI/LALLSP in Fig. 2). $\bar{M}_w(v,uc)$ is measured by SEC/DRI/LALLSP leaving one unknown σ(v). σ(v) was found using a single-variable search (Fibonnaci search in this study) across the chromatogram. Typical results for the two detector responses and σ(v) are shown in Fig. 4. It is encouraging that the magnitude of σ(v) per unit column length and the dependence of σ(v) on

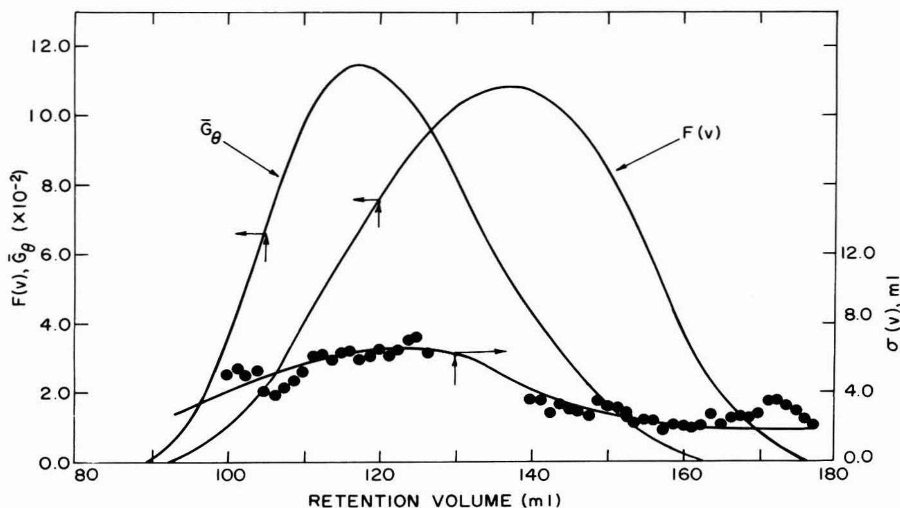

Figure 4. SEC/DRI/LALLSP chromatograms for a blend of Pharmacia dextran standards (71% T250 and 29% T40) and calculated peak broadening parameter σ(v).

We now discuss a technique of finding the true molecular weight calibration curve using one very broad MWD standard having a designed chromatogram shape. The broad MWD standard is obtained by mixing a number of broad MWD Pharmacia standards to obtain the desired chromatogram shape. An observation of equation (9) reveals the required shape. One would like the ratio $\bar{M}_w(v,uc)/M(v)$ to be as close to unity as possible over a wide range of retention volumes so that an $\bar{M}_w(v,uc)$ measurement gives a point on the molecular weight calibration curve. The exponential factor is always greater than unity and it is therefore desirable that the pre-exponential factor be less than unity hopefully to almost compensate and give a ratio close to one. A very broad MWD standard (STANDARD-X) was made by mixing T250(53wt.%), T40(29wt.%) and T10(18wt.%). The SEC/DRI/LALLSP responses are shown in Fig. 5. Standard X is now used to determine the molecular weight calibration curve. The molecular weight calibration curve is taken to be linear over a narrow retention volume range (v_i to v_{i+1}). Equation (9) is now rearranged to give

$$\ln\left(\frac{\bar{M}_w(v_i,uc)}{\bar{M}_w(v_{i+1},uc)}\right) = D_2(v_{i+1} - v_i) + \frac{D_2^2}{2}(\sigma_i^2 - \sigma_{i+1}^2) + \ln\left\{\frac{F(v_i - D_2\sigma_i^2)\,F(v_{i+1})}{F(v_{i+1} - D_2\sigma_{i+1}^2)F(v_i)}\right\} \qquad (9a)$$

Given σ_i across the chromatogram, one can solve equation (9a) for D_2 and then equation (9) for D_1 across the chromatogram. The molecular weight calibration curve is thus established. The drawback of having to know σ_i is not a serious one. In fact, the determination of the molecular weight calibration curve with specially prepared Standard-X is very insensitive to σ_i as is shown in Table 1. The first column of $\bar{M}_w(v,uc)/M(v)$ values were

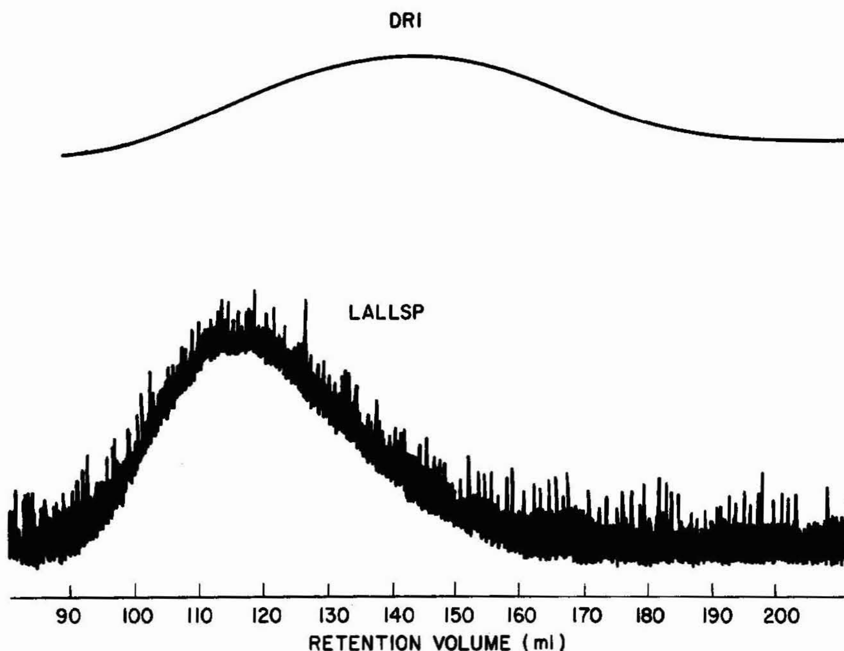

Figure 5. SEC/DRI/LALLSP chromatograms for a blend of Pharmacia dextran standards called standard-X (53% T250, 29% T40 and 18% T10).

obtained using the previously measured $\sigma(v)$ values. To illustrate the lack of sensitivity of this ratio to $\sigma(v)$, values of up to 50% lower and 50% higher were employed and $\bar{M}_w(v,uc)/M(v)$ calculated. These calculations clearly indicate that the designed shape of the chromatogram for Standard-X has served the purpose of giving $\bar{M}_w(v,uc)/M(v)$ ratios close to unity over a wide range of retention volumes. They also indicate that when Standard-X is used to obtain the molecular weight calibration curve a precise knowledge of $\sigma(v)$ is not required. In other words peak broadening parameters are not required when using Standard-X to obtain the molecular weight calibration curve ($\sigma(v)$ can be set equal to zero with small error).

TABLE 1

Standard-X - Sensitivity to Peak Broadening Parameter

Retention volume (ml)	$\bar{M}_w(v,uc)/M(v)$				
	$\sigma(v)$ actual	0.8 $\sigma(v)$	0.5 $\sigma(v)$	1.2 $\sigma(v)$*	1.5 $\sigma(v)$*
110.6	0.922	0.951	0.981	-	-
115.6	0.915	0.952	0.983	-	-
120.6	0.972	0.983	0.994	0.961	-
125.6	1.011	1.006	1.002	1.019	1.036
130.6	1.036	1.024	1.010	1.052	1.076
135.6	1.028	1.018	1.007	1.032	1.057
140.6	1.032	1.021	1.008	1.045	1.067
145.6	1.032	1.020	1.008	1.045	1.072
150.6	1.026	1.016	1.006	1.038	1.060
155.6	1.018	1.011	1.004	1.026	1.041
160.6	1.017	1.011	1.004	1.024	1.038
165.6	1.018	1.012	1.005	1.026	1.040
170.6	1.019	1.012	1.005	1.027	1.042
175.6	1.025	1.016	1.006	1.036	1.059
180.6	1.015	1.010	1.004	1.021	1.031

* Calculations at low retention volumes were not possible because of excessively large $\sigma(v)$.

The molecular weight calibration curve obtained using Standard-X was fitted by equation (10)

$$\ln M(v) = 17.80 - 1.695\times10^{-2}\ V - 2.158\times10^{-4}\ V^2 \tag{10}$$

and is shown plotted in Fig. 2.

The use of the analytical solution for w(v) (given as equation (2a)) for Pharmacia standard T70 is shown in Fig. 6. As

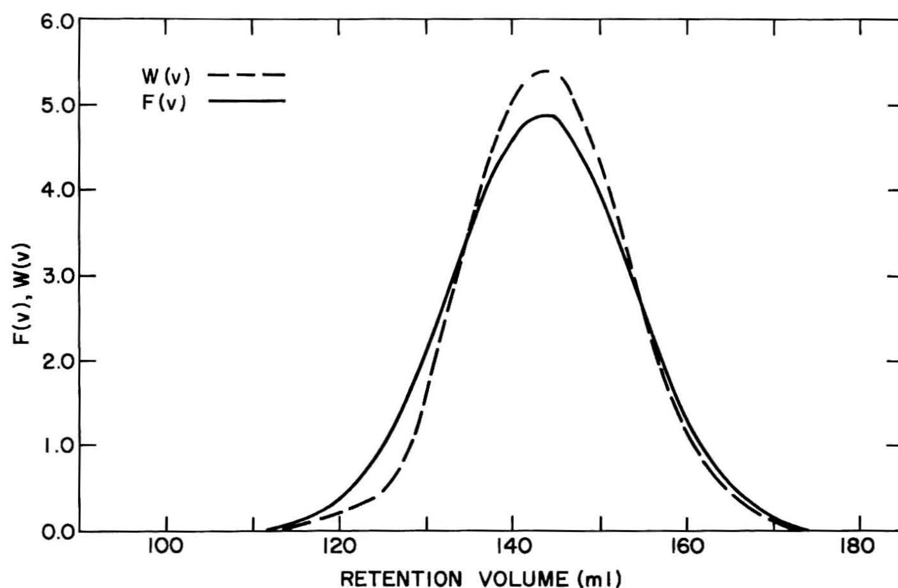

Figure 6. SEC/DRI chromatograms (raw and corrected for peak broadening) for Pharmacia dextran standard T70.

expected a greater correction for peak broadening is required at the high molecular weight end of the chromatogram. The $\sigma(v)$ values used for the correction are those shown in Fig. 4.

Molecular weight characterization data ($\bar{M}_N$ and $\bar{M}_w$) measured for a number of Pharmacia dextran standards are presented in Table 2. Molecular weight averages for the whole polymers corrected for peak broadening ($\bar{M}_N(c)$, $\bar{M}_w(c)$) were calculated using two calculation paths.

The first calculation path employed equations (11a) and (11b)

$$\bar{M}_N(c) = \left(\int_0^\infty F_N(v)/\bar{M}_N(v,uc)dv\right)^{-1} \quad (11a)$$

$$\bar{M}_w(c) = \int_0^\infty F_N(v)\bar{M}_w(v,uc)dv \quad (11b)$$

TABLE 2

Molecular Weight Characterization of Dextrans by SEC/DRI/LALLSP Using Two Calculation Paths (equations (11a,b) and equations (13a,b)).

Sample	$\bar{M}_N$	$\bar{M}_W$	$\bar{M}_N$	$\bar{M}_W$	$\bar{M}_N$	$\bar{M}_W$
	Pharmacia		(Equations (11a,b))		(Equations (13a,b))	
		$x10^{-3}$		$x10^{-3}$		$x10^{-3}$
T250	112.5	231.0	100.3	226.0	90.0	256.0
T150	86.0	154.0	76.9	141.0	69.6	139.1
T110	76.0	106.0	79.2	100.5	72.3	100.3
T70	42.5	70.0	43.0	70.4	40.1	69.4
T40	28.9	44.4	25.6	42.7	25.7	42.2
T20	15.0	22.3	16.9	22.7	14.7	22.7

where $F_N(v)$ is the normalized DRI detector response and $\bar{M}_W(v,uc)$ is the weight average molecular weight measured by SEC/DRI/LALLSP with no corrections for peak broadening. $\bar{M}_N(v,uc)$ was calculated using equation (12).

$$\bar{M}_N(v,uc) = \bar{M}_W(v,uc)\left(\frac{F(v)^2}{F(v+D_2(v)\sigma(v)^2)\cdot F(v-D_2(v)\sigma(v)^2)}\right) \exp\left(-(D_2(v)\sigma(v))^2\right) \quad (12)$$

where $D_2(v)$ is obtained from the molecular weight calibration curve and $\sigma(v)^2$ with the peak broadening calibration procedure described earlier.

The second calculation path employed equations (13a) and (13b).

$$\bar{M}_N(c) = \left(\int_0^\infty w(v)M(v)^{-1}dv\right)^{-1} \quad (13a)$$

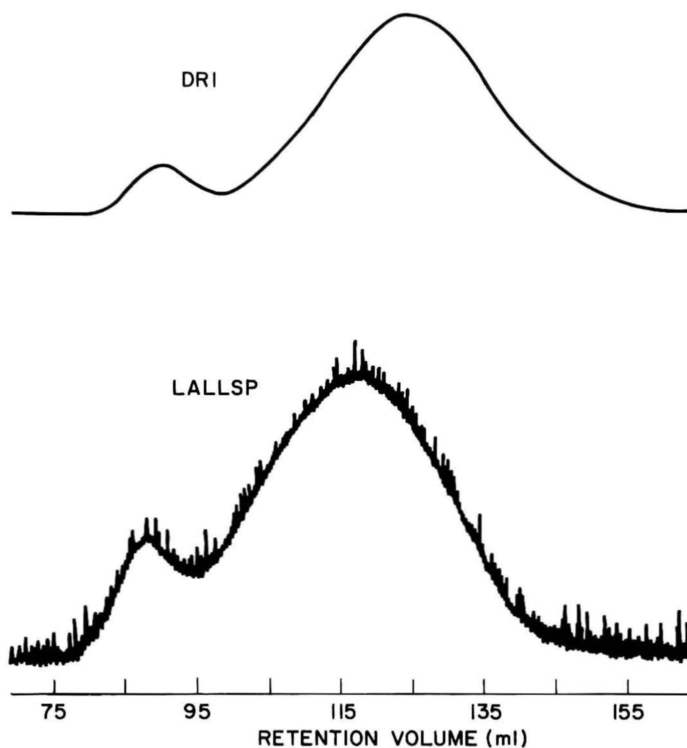

Figure 7. SEC/DRI/LALLSP chromatograms for Pharmacia dextran standard T150 with water free of salt as the mobile phase showing the so called "ghost" peak.

$$\bar{M}_W(c) = \int_0^\infty w(v)M(v)dv \tag{13b}$$

where w(v) is the DRI detector response corrected for peak broadening via equation (2a) and normalized. The values for $\bar{M}_N$ and $\bar{M}_W$ found using the two calculation paths are in good agreement (probably within experimental error). The recommended procedure is to use SEC/DRI/LALLSP to determine the molecular weight calibration curve and the peak broadening parameter $\sigma(v)^2$ across the retention volume range of interest as described and then for

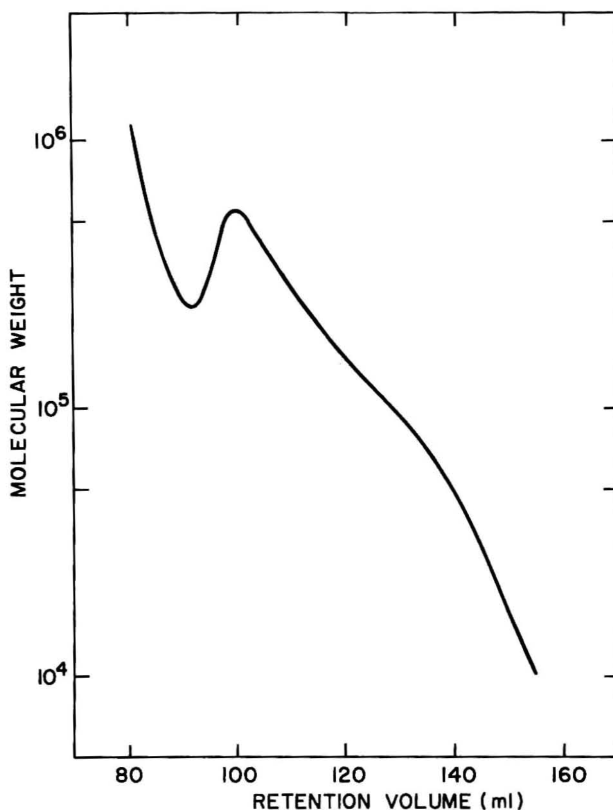

Figure 8. $\bar{M}_w(v,uc)$ measured by SEC/DRI/LALLSP for Pharmacia dextran standard T150 with water free of salt as the mobile phase showing the effect of the "ghost" peak.

further dextran characterizations to use SEC/DRI and equations (13a,b). The use of LALLSP online with SEC need only be made for the initial calibration.

Finally, it was decided to investigate the so called "ghost" peaks found with dextrans and SEC when pure water free of salt is used as mobile phase[9]. Typical detector responses for SEC/DRI/LALLSP using water free of salt as mobile phase are shown in Fig. 7. The apparent high molecular weight peak had $\bar{M}_w(v,uc)$ values which were smaller than expected on the basis of a molecular size separation (see Fig. 8). Apparently, some dextran

chains may have a negative charge and experience ion exclusion from the CPG10 packing.

SUMMARY

A methodology for the use of aqueous SEC/DRI/LALLSP with dextrans has been developed. This includes procedures for the determination of the molecular weight calibration curve using a broad MWD standard with a designed chromatogram shape. Also included are procedures for the determination of the peak broadening parameter, σ^2 (variance of a Gaussian instrumental spreading function) over a wide retention volume range using a single broad MWD standard.

REFERENCES

1. A.C. Ouano and W. Kay, J. Polym. Sci., A-1, 12, 1151 (1974).

2. A.C. Ouano, J. Chromatog., 118, 303(1976).

3. A.E. Hamielec and A.C. Ouano, J. Liquid Chromatog., 1, 111(1978).

4. A.E. Hamielec, A.C. Ouano and L. Nebenzahl, J. Liquid Chromatog., 1, 527(1978).

5. T.B. McRury and M.L. McConnell, J. Appl. Polym. Sci., 24, 651(1979).

6. D. Axelson and W. Knapp, J. Appl. Polym. Sci., 25, 119(1979).

7. T. Kato, A. Kanda, A. Takahashi, I. Noda, S. Maki and M. Nagasawa, Polymer J., 7, 575(1979).

8. R.C. Jordan, J. Liquid Chromatog., 3, 439(1980).

9. S.N.E. Omorodion, A.E. Hamielec and J.L. Brash, J. Liquid Chromatog., 4, 41(1981).

10. A.E. Hamielec, J. Liquid Chromatog., 3, 381(1980).

11. A.E. Hamielec, H.J. Ederer and K.H. Ebert, "Size Exclusion Chromatography of Complex Polymers-Generalized Analytical Corrections for Imperfect Resolution", J. Liquid Chromatog., in press (1981).

12. Chromatix KMX-6 Application Note LS7 Appendix 1, Chromatix, Sunnyvale, Calif.

13. A.M. Basedow, K.H. Ebert, H.J. Ederer and E. Fosshag, J. Chromatography, 192, 259(1980).

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PREPARATIVE LIQUID CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS AND PEPTIDES ON AMBERLITE XAD-4

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ABSTRACT

Amberlite XAD-4, a polystyrene-divinylbenzene copolymic reversed phase adsorbent which has a 750 m^2/g surface area and 50Å porosity, was used as the stationary phase for the preparative liquid chromatographic separation of amino acids and peptides. Mixtures of > 40 mg and > 100 mg sample load were separated on 8.0 and 20.5 mm i.d. columns, respectively. Mixed solvent and acidic and basic solutions which cannot be used with silica and alkyl-modified silica, were evaluated as mobile phases. Mixtures of amino acids, diastereomeric di-and tri-peptides, diastereomeric dipeptides obtained from the reaction of tert-butyloxycarbonyl-L-amino acid-N-hydroxysuccinimide esters with D,L-amino acids, and enkephalin peptides were separated. Major and minor sample components were isolated.

INTRODUCTION

High performance liquid chromatography (LC) has become an increasingly powerful analytical cool for the separation and determination of amino acids (AA), AA derivatives, and peptides.

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Successful analytical liquid chromatography (ALC) of these compounds has been achieved on alkyl-modified silicas (1-4). Recently, it has been shown that many of these mixtures are separable on porous, high surface area polystyrene-divinylbenzene copolymers which are nonpolar adsorbents and are useful stationary phases for reversed phase chromatography. The most useful ones are Amberlite XAD-2 and -4 (5) and Hamilton PRP-1 (6); only the latter is commercially available as bulk form or prepacked columns. In general, the LC trends are similar for these stationary phases, however, the level of retention for a given mobile phase may differ due to difference in surface area. If XAD (7) and PRP-1 (6) microparticles are used efficiencies approaching that of alkyl-modified silicas of similar particle size are obtained.

A major advantage of the copolymers over silica or alkyl-modified silica, which are the two most used stationary phases in PLC, is that the copolymers are stable throughout the pH range of 1 to 13. Thus, strongly acidic and basic mobile phases, which often are the optimum eluting conditions for AA and peptide separations and cannot be used with the silica type stationary phases, are readily used on the copolymers. Other useful factors in PLC applications are: 1) the copolymers have large loading capacities, 2) eluting conditions on the copolymers have been well characterized (5,6), 3) prep columns are relatively inexpensive, and 4) efficiencies can be increased if required by reducing the copolymer particle size.

This report describes the use of XAD-4 for the PLC separation of AA and peptides. The XAD-4 surface area is the largest and thus it provides the highest loading capacity of the available XADs. Optimization of XAD-4 column variables such as flow rate, column diameter, particle size, packing density, volume and mass overload, capacity, recovery, and eluting conditions (8) is discussed elsewhere. Mixtures that have been

purified on prep XAD-4 columns include organic acids, bases, and polyaromatics (8), drug metabolites (9), and 2':3'-, 3':5'-cyclic nucleotides (10).

The separations described here were chosen to: 1) illustrate the range of useful mobile phase conditions with an emphasis on those that cannot be used with silica and alkyl-modified silica and 2) identify the ones which are especially useful in peptide chemistry. Often the success of a peptide synthesis is determined by development of separation procedures, removal of unwanted peptide chains, and/or purification of a single peptide from the synthetic process.

MATERIALS

The AA and peptides were obtained from Sigma Chemical Co. Several dipeptides were synthesized by the reaction of an amino acid or peptide with tert-butyloxycarbonyl-L-amino acid-N-hydroxy-succinimide ester (11). Ethanol (95%) and HPLC grade CH_3CN was obtained from Matheson, Coleman, and Bell. Distilled water was further treated by passage through a mixed bed ion exchanger, charcoal, and a 0.2 μm millipore filter disk. Solvent composition is expressed as per cent by volume. All inorganic reagents were analytical grade and used as received. Amberlite XAD-4 was obtained from Mallinckrodt Chemical Works. Procedures for cleaning, crushing, sizing, and column packing the 37-44 μm (325 to 400 mesh) and 75-105 μm (140 to 200 mesh) XAD-4 particles are described elsewhere (8). Columns used were 8.0 mm i.d. (3/8 in o.d.) x 25 cm and 20.5 mm i.d. (1 in o.d.) x 32 cm, respectively, equipped with 15 μm end fittings (Jones Chromatography, Inc.).

Instrumentation

Instrumentation used was described previously (8). ALC was done on an Altex Gradient LC Model 332 equipped with an Altex 210

injector using either a 8.0 mm i.d. x 25 cm, 37-44 μm XAD-4 column or a 4.1 mm i.d. x 15 cm, 10 μm Hamilton PRP-1 column.

Procedures

Mixtures were prepared from standards using water or mixed solvents (EtOH-H_2O) and acid and base only when necessary except for samples obtained from the Boc reaction (11). Literature LC data (5,6) and separations with analytical level (μg quantities) samples were used to establish the eluting conditions prior to scale up of the procedure to a preparative level. Conditions for the separations are listed in the Figures and/or discussion and represent an optimum eluting condition for the amount and type of mixture being separated.

RESULTS AND DISCUSSION

Mobile Phase Conditions

AA and peptides are ampholytes whose ionic form changes from cation, to zwitterion, to an anion as a function of pH. If the side chain groups contain ionization sites these also can contribute to the overall charge depending on their ionization constants and the pH.

Retention of AA and peptides on analytical XAD and PRP-1 columns is dependent on the ionic form of the sample (4-6). Thus, retention is high in acidic and basic solution where the sample is a cation and anion, respectively, and at a minimum at the isoelectric pH where it is the zwitterion form. If the side chain has an acidic or basic group the formation of an additional cationic (acid solution) or anionic (basic solution) site significantly reduces the retention. If all the ionization steps are accounted for, capacity factor, k', can be quantitatively related to mobile phase pH and ionization constants of the acid-base groups; this has been experimentally verified for AA and peptide

retention on the XAD and PRP-1 copolymers (5,6). Side chain polarity will have a significant effect on retention. Thus, retention changes in the order Leu > Val > Ala > Gly which correlates to the change in hydrophobicity for the side chain group; these effects are discussed in detail elsewhere (3-6). These same trends were observed when determining pH effects on retention of AA and peptides at large mass loadings on the prep XAD-4 column. Thus, the ALC data (5,6) are an excellent guide for predicting mobile phase conditions on the prep XAD-4 column.

Column selectivity (α) for a given pair of AA or peptides changes as a function of pH (5,6). In some cases the elution order can be reversed when switching from an acidic to basic mobile phase. The adjustment of mobile phase pH can then be used to make α as large as possible and thus permit a higher column loading. Often a strongly acidic or basic mobile phase, which are conditions that cannot be used with silica or alkyl-modified silica, rather than one with an intermediate pH provides the optimum α.

Addition of buffers, acids, or bases to the mobile phase might be undesirable for certain AA and peptide preparative separations. Resolution can often still be achieved by careful control of the type and amount of organic solvent in the organic solvent-water mobile phase. Even a completely aqueous mobile phase can be useful. In the absence of pH control, chromatographic bands tend to be broader and complete resolution for compounds with closely related k' values is often more difficult to obtain than in the presence of pH control. The addition of salts and/or counterions to the mobile phase sharpens chromatographic peaks on XAD-4 (12), but their presence also complicates recovery of the AA or peptide. Retention of AA and peptides on the prep XAD-4 column, like ALC on XAD-4 (4,5), increases as the percent organic solvent in the mobile phase decreases. The effect on eluting power follows the order CH_3CN > EtOH > MeOH. An elutropic order for other solvents has also been established (4-7, 13).

In general, k' on the prep XAD-4 column does not change appreciably if the AA or peptide mass load is well below the mass overload limit providing a volume overload is not present. When comparing different AA and peptides in a given mobile phase, the mass overload limit decreases as the k' for each increases. Furthermore, increasing the sample load above the limit decreases k'. These observations are consistent with those observed for the separation of other samples on the XAD-4 prep column (8). For AA and peptide separations at an acceptable resolution it is therefore important to consider the maximum amount to be loaded, the k' at this loading level, and the mobile phase conditions when attempting to establish the optimum separation conditions. The separations cited here focus on the advantages of the absence and presence of pH control in the mobile phase particularly at pHs where silica and alkyl-modified silica cannot be used, use of mixed solvents in the mobile phase, separation of closely related components, isolation of minor and major components, and separation of modest to gross mass overloads. Not all separations were done at or above the mass overload limit nor was it always determined. As is often the case in PLC the limiting factor is the solubility of the sample in the injection volume used and not the overload limit.

PLC of Amino Acids

Figure 1 illustrates PLC separation of two AA mixtures using an acidic mobile phase where the weight ratio of DL-Dopa, L-Tyr, and D-Phe (total weight of about 12 mg) is about 10:1:0.1 and 0.1:1:10, respectively. The k' values for the mobile phase using an analytical load are 3.6, 6.4, and >20, respectively. In the former mixture the last two AA are well below the mass overload limit while the first is at the mass overload limit. The opposite is the case for the latter mixture. The first two AA are the most difficult to separate (they differ by a -OH group in position 3 on the side chain) and the resolution of

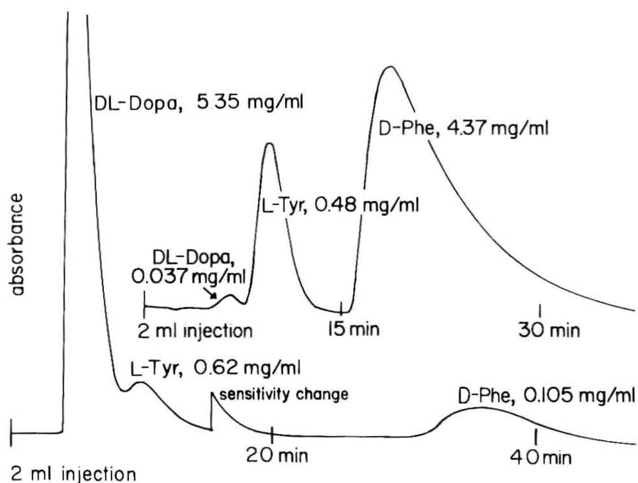


FIGURE 1

PLC of an Amino Acid Mixture

A 8.0 mm i.d. x 250 mm, 37-44 μm XAD-4 column was used with a 1.25% EtOH (95%)-98.75% H_2O, pH = 2 (H_3PO_4 buffer or 0.01M HCl), 0.1M ionic strength mobile phase at a flow rate of 5.6 ml/min. Sample injection was 2 ml.

this pair can be improved by decreasing the % EtOH in the 0.01M H_3PO_4 mobile phase; this also permits greater loading. If the mobile phase is at the zwitterion pH or in the absence of buffer or salt, resolution is significantly reduced. A basic pH is probably the optimum eluting pH (6, 7, 13) because of the acidic sites on the side chains of DL-Dopa and Tyr. This can't be used since DL-Dopa rapidly oxidizes in a strongly basic solution. A stepwise elution of increased % EtOH can be used to reduce the separation time for Phe removal.

DL-Dopa and Tyr are not very soluble. The sample load in Figure 1 was obtained by dissolving the mixture in a HCl solution. Since the sample solvent is a weaker eluent than the one used for the separation, it has little effect on peak shapes. The mixtures in Figure 1 also illustrate examples of the separa-

tion of a major and a minor sample component. In one case the DL-Dopa is about 88% of the sample while the Phe is at a 1.6% level. In the other the DL-Dopa is the minor constituent while the Phe is the major one. A separation of an Ala-Ile-Tyr mixture at a ratio of 21:7:0.1 using total loading levels of 7.1 and 14.2 mg was also carried out on the 8.0 mm i.d. XAD-4 column with an acidic mobile phase (8).

Figure 2 illustrates that PLC of more closely related AA is possible and that this can be done at high loads. At the conditions used and at an analytical load k' values in Figure 2A are ~0, 0.5, 2.1, and 16, respectively, while in Figures 2B and C they are ~0, 0.36, 1.2, and 1.9, respectively. Increasing the

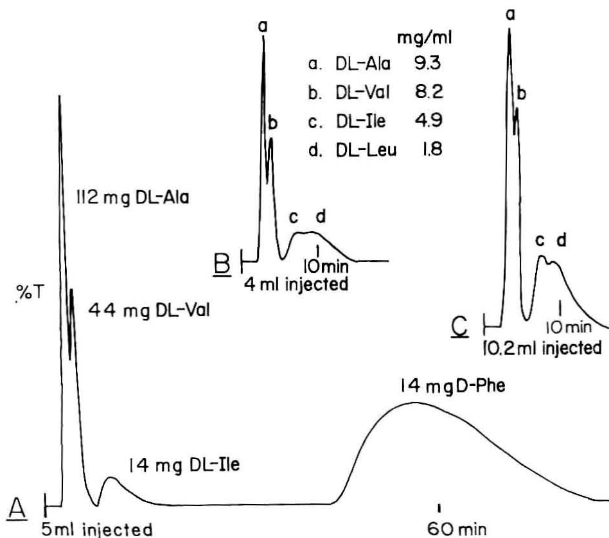


FIGURE 2

PLC of Amino Acid Mixtures on a Large Diameter Column

A 20.5 mm i.d. x 320 mm, 75-105 μm XAD-4 column was used for A, B, and C. An aqueous 0.02M H_3PO_4 mobile phase was used for A while a 0.02M Na_2HPO_4 mobile phase was used for B and C. Flow rates were 22 ml/min.

column diameter to 20.5 mm permits larger loading. The total load is 184, 96.8, and 248 mg in Figures 2A, 2B, and 2C.

In Figure 2A only the highly retained Phe is above the mass overload limit. This accounts for its elongated peak shape. The Phe elution time can be reduced and its peak shape improved by increasing the pH or adding more EtOH to the mobile phase stepwise after the appearance of Ile, or initially, if resolution of the first three AA is not desired. It should be noted that Phe, which is about 7.7% of the sample, can be easily separated from the mixture in a single pass at even much lower levels.

In Figures 2B and C all AA are below the mass overload limit even at the higher sample loading. The inability to obtain a better resolution is due to their similarity in k' values. It should be noted that Ile and Leu are isomeric and differ from Val and Ale by only one and two side chain carbons, respectively. Using an acidic eluent did not improve resolution while a more basic eluent than used in Figures 2B and C gave a slight improvement.

PLC of Peptide Diastereomers

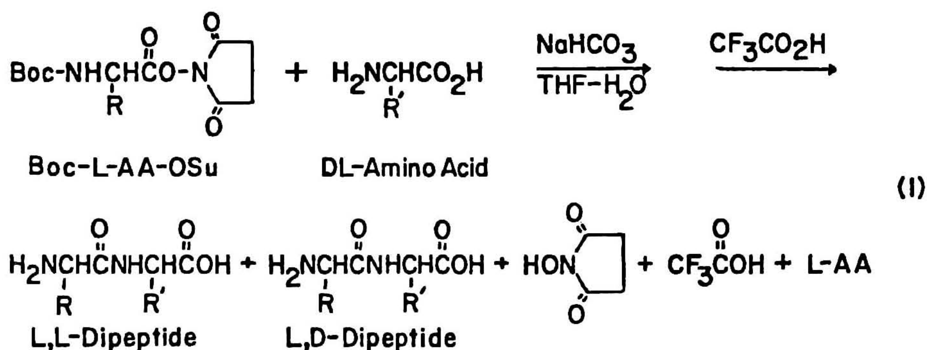
The ALC data for dipeptide diastereomers is readily available (4-6, 11). For all examples studied the L-L and D-D dipeptides always coelute first and are followed by the coelution of the L-D and D-L dipeptides. This order has been explained by considering the spatial arrangement around the chiral centers.

A mixture of dipeptide diastereomers, made by mixing pure L-Leu-L-Tyr (10 mg) and D-Leu-L-Tyr (4 mg), was separated on a 8.0 mm i.d. XAD-4 column. A 5:95 95% EtOH-H_2O mixture without pH control was used as the mobile phase. This sample loading is well above the mass overload limit. Since retention ($k'_{LL} \cong 7$ and $k'_{LD} \cong 13$) is high for this eluent the mass overload limit for the column is reduced and the peaks tend to

broaden. If the sample load is reduced resolution and peak shape are improved. Decreasing the % EtOH also increases resolution. However, this also increases the separation time and lowers the mass overload limit particularly for the second component.

A commercially available DL-Ala-DL-Val sample was also separated on the 8.0 mm i.d. XAD-4 column (9). An acidic aqueous mobile phase was used and quantities as high as 48 mg total dipeptide were separated into D-D, L-L and D-L, L-D components in a single pass through the column.

The reaction between an AA or peptide and the appropriate tert-butyloxy-carbonyl-L-amino acid-N-hydroxysuccinimide ester is often used in peptide synthesis to increase the chain length by one unit (11). This is illustrated in reaction 1 where a DL-amino acid is the starting material. Since the Boc reagent



introduces a L-amino acid, the final mixture should contain an equivalent amount of L-L and L-D dipeptide. Reaction 1 is useful in peptide synthesis because it goes to completion rapidly without loss of optical activity and many Boc reagents are commercially available. The reaction has even been used for the determination of optical purity of D,L-amino acids and diastereomeric dipeptides (11).

Several Boc reactions where carried out and the products separated on the prep XAD-4 column to illustrate the column's usefulness in peptide synthesis. To obtain the dipeptides from reaction 1 it is necessary to separate them from each other, from the blocking groups and other reagents used in the synthesis, from the L-amino acid introduced from the Boc reagent if used in excess or if the reaction is incomplete, and from the amino acid or peptide reactant if it is used in excess or if the reaction is incomplete. Figure 3 illustrates the PLC for three

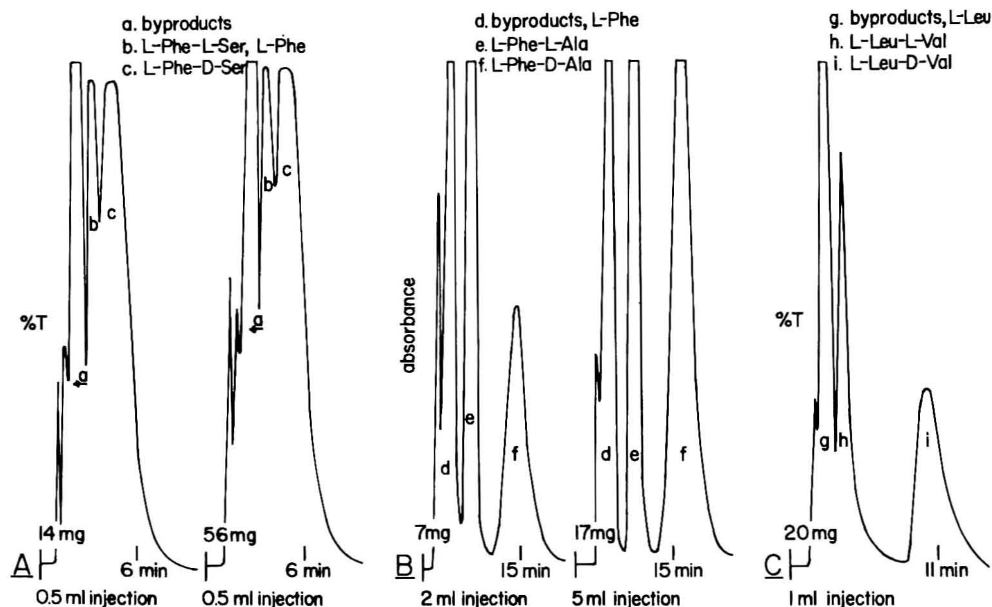


FIGURE 3

PLC of Three Different Boc Reaction Mixtures

The column in Figure 1 was used for all three separations. The mobile phases were 100% H_2O for A (k' = 1.3 for L-Phe-L-Ser and 2.3 for L-Phe-D-Ser), 5:95 95% EtOH:H_2O for B (k' = 1.7 for L-Phe-L-Ala and 3.8 for L-Phe-D-Ser), and 1:9 95% EtOH:H_2O for C (k' = 1.4 for L-Leu-L-Val and 4.2 for L-Leu-D-Val) at a flow rate of 5.6 ml/min.

different mixtures based on reaction 1. ALC separations with standards verified the peak designation.

In Figure 3A the reactants were DL-Ser and Boc-L-Phe-OSu and after workup a 0.5 ml sample containing 14 or 56 mg of the dipeptides (assuming 100% conversion) was injected and separated. The L-Phe is due to the excess Boc-L-Phe-OSu used in the reaction and appears in the L-Phe-L-Ser peak. No attempt was made to separate the L-Phe-L-Ser from the L-Phe, however, this mixture has been separated at an analytical level using pH control (5,6). A lower % EtOH will improve the separation of the dipeptides from the by-products but since the bands tend to broaden due to overloading resolution of the two dipeptides is not improved. At lower loading the resolution can be improved. In Figure 3B DL-Ala and Boc-L-Phe-OSu was used in reaction 1. After workup a 2 and 5 ml aliquot containing about 7 and 17 mg of dipeptides, respectively, (assuming 100% conversion) was injected and separated. Even at the larger mass overload a baseline separation of the dipeptides was obtained. Consequently, it should be possible to separate the mixture on the 80 mm i.d. column at a gross overload. In Figure 3C the sample was obtained from the reaction of DL-Val and Boc-L-Leu-OSu. After workup a 1 ml aliquot containing about 20 mg of dipeptides (assuming 100% conversion) was injected and separated. Resolution of L-Leu-L-Val from the by-product peaks can be increased by reducing the EtOH in the mobile phase, however, the retention time and peak broadening of the L-Leu-D-Val is significantly increased.

The prep separations in Figure 4 were carried out with an aqueous-EtOH mobile phase. The inorganic compounds are from the reaction mixture and appear in the by-product peak. Thus, recovery of the peptides from appropriately collected fractions is simplified. If pH control is used, resolution in most cases can be markedly improved (5,6), however, this requires an additional step to remove the buffer salts from the peptide.

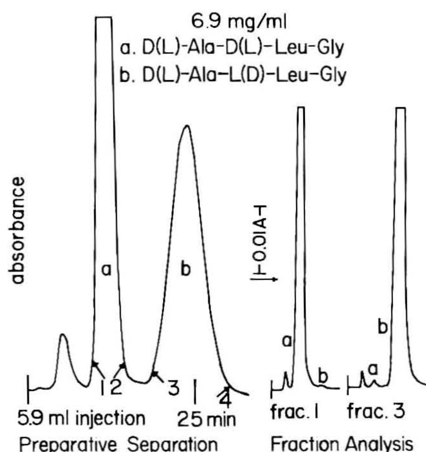


FIGURE 4

PLC of a Diastereomeric Tripeptide

The preparative separation was done on the column used in Figure 1 using a 5:95 $CH_3CN:H_2O$ mobile phase at a flow rate of 5.6 ml/min. The fraction analysis was done on a 4.1 mm i.d. x 150 mm, 10 µm PRP-1 column using a 3:97 $CH_3CN:H_2O$, pH = 6 (phosphate buffer), 0.1M ionic strength mobile phase at a flow rate of 1.4 ml/min.

Often this can be achieved on the same XAD-4 column using a salt free mobile phase.

Figure 4 illustrates the PLC separation of a commercially available sample of the tripeptide, DL-Ala-DL-Leu-Gly, on a 8.0 mm i.d. XAD-4 column. The L-L and D-D tripeptides (k' = 6.3) coelute as the first peak while the L-D and D-L tripeptides (k' = 15.7) coelute as the second peak. The sample was injected as 5.9 ml of 6.93 mg DL-Ala-DL-Leu-Gly/ml. At this 40 mg loading level, which is a gross mass overload, a baseline separation is obtained, because of the large difference in k' values. (At lower loadings resolution and peak shapes are even better.) Fractions were collected and these are indicated by number in the prep chromatogram in Figure 4. Purity of these fractions

was established by ALC on a PRP-1 column (6). Only the analytical chromatograms for fractions 1 and 3 are shown in Figure 4. The major peak is off scale and the chromatogram focuses on the trace quantity. By comparing peak heights or peak weights, the purity of the major diastereomer is estimated to be greater than 99% after a single pass through the prep column. No attempt was made to recycle the peak to improve purity. Since resolution is so favorable even a greater mass overload than shown in Figure 4 would be tolerable. Also, the option of improving peak shape and resolution through pH control of the mobile phase is still available.

PLC of Peptides

The enkephalins are peptides that have been isolated from brain extracts and are reported to be natural opiate receptor agonists. Figure 5 contains an analytical and preparative level chromatogram, where the total load is 27 mg (9 mg of each peptide), for the separation of three enkephalin peptides. The mixture was prepared from commercially available enkephalins. Since retention for the three enkephalins is high, k' values are 4.2, 7.6, and 12.2, respectively, the extent of mass overload increases as the peptide retention increases. This accounts for the increased broadening of the peaks as the retention times for the three enkephalins increases. To demonstrate that the three enkephalins can be isolated in high purity and good yield, fractions of the prep-chromatogram were collected; these fractions are identified by number in Figure 5. Analysis of each fraction was done by ALC. Only the chromatograms for the key fractions, numbers 1, 3, and 6, which correspond to each of three enkephalins, are shown in Figure 5. Clearly, the separation in terms of yield and purity is favorable in a single pass through the prep column. To improve enkephalin yield, the cross contaminated fractions (fraction 2 for the first pair of enkephalins and fractions 4 and 5 for the second pair of enkephalins) could be recycled.

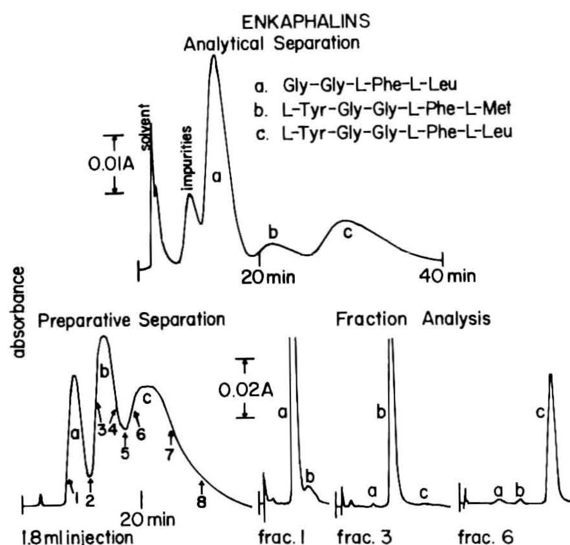


FIGURE 5

PLC of a Mixture of Enkaphalin Peptides

The preparative and analytical separation was done on the column used in Figure 1 using a 17:83 $CH_3CN:H_2O$, pH = 2.2 (H_3PO_4 buffer), 0.1M ionic strength mobile phase at a flow rate of 5.6 ml/min. The fraction analysis were done on a PRP-1 column (see Figure 7) using the above mobile phase at a flow rate of 1.4 ml/min.

Using a mobile phase without pH control would be preferred if the enkephalin is to be isolated. However, suitable resolution at a reasonable loading was not obtained in the absence of pH control. A basic pH will not only decrease the level of retention but also it will change the elution order to $b < c < a$. This is due to the influence of the additional charge site on the Tyr that forms in basic solution. Depending on which peptide is being sought the basic mobile phase might be preferred.

Separation of the three enkephalins is rapidly achieved at an analytical level even though the peptides are very similar.

For example, the Leu enkephalin and Met enkephalin (b and c in Figure 5) are 5-unit peptides that differ only in the fifth unit, the former contains L-Leu at unit-5 while the latter contains L-Met at unit-5. The difference in polarity between these unit-5 side chains accounts for the elution of the methionine enkephalin prior to the Leu enkephalin. The [des Tyr^1]-Leu enkephalin (a in Figure 5) is a 4-unit peptide and is similar to unit-2 to unit-5 in the Leu enkephalin. The additional side chain provided by the Tyr at unit-1 in the Leu and Met-enkephalins accounts for their greater retention over the [des Tyr^1]-Leu enkephalin. The correlation of a significant change in chromatographic retention with a modest change in peptide structure has been observed before (3-6).

The dipeptide separations shown in Figure 6 clearly illustrate a key advantage of a prep XAD-4 column in peptide separations, namely its utility with either a strongly acidic or basic mobile phase. Neither of these can be used with silica or alkyl-modified silica columns. Also changing the mobile phase pH from an acidic to basic one often leads to an elution order reversal.

In the 0.01M HCl mobile phase the k' values for L-Phe-L-Ser and L-Ser-L-Phe at an analytical load are 1.0 and 3.5, respectively, while in the 0.01M NaOH mobile phase retention is reversed so that their k' values are 3.1 and 1.4, respectively. The prep chromatograms for the separation of these two dipeptides for the two mobile phases are shown in Figure 6.

In Figure 6A, where the acidic mobile phase is used, L-Phe-L-Ser is separated from L-Ser-L-Phe at ratios of 400:1 and 1:100. The same separations, but with a reversed elution order due to the basic mobile phase, are shown in Figure 6B. It is readily seen that if recovery of L-Phe-L-Ser as the minor component is desired the basic mobile phase is best, while for recovery of L-Ser-L-Phe as the minor component the acidic mobile

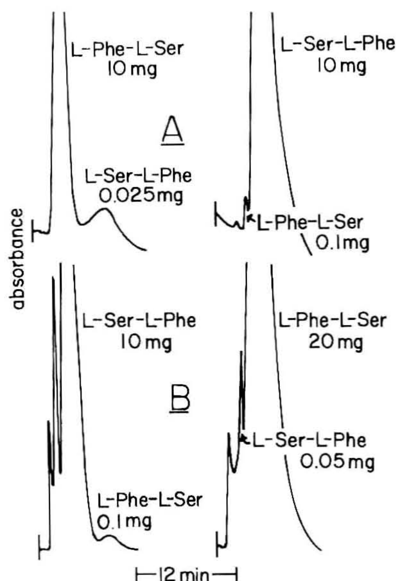


FIGURE 6

PLC of a Dipeptide Mixture by a Strongly Acidic and Basic Mobile Phase

The column in Figure 1 was used with a 0.01M HCl 5:9 95% EtOH:H_2O mobile phase in A and a 0.01M NaOH 5:95 95% EtOH:H_2O mobile phase in B. Flow rate was 5.0 ml/min.

phase is best. For major component recovery the reverse is best, that is, an acidic mobile phase for L-Phe-L-Ser and a basic one for L-Ser-L-Phe. The chromatograms in Figure 6 suggest that larger total mass loadings could still be separated. However, the limitation is sample solubility in the injection volume used rather than effects due to excessive mass overloading. Although not shown here reversals in elution order for other peptide mixtures are possible on the prep XAD-4 column.

Summary

Preparative LC columns packed with 37-44 μm XAD-4 particles can be used to separate a variety of AA and peptide mixtures at

large mass loadings for purposes of purification, and are viable alternates to silica and alkyl-modified silica columns. Of particular significance is that only the XAD-4 can be used with strongly acidic and basic mobile phases which often are the ones that provide the best resolution of AA and peptide mixtures. The acid, base, or buffer can then be usually removed from the isolated AA or peptide by passing the collected fraction through a XAD-4 column using a mixed solvent mobile phase where only the AA or peptide is retained.

In general mass overloading occurs at about 0.23% and 0.25% wt/wt for the 8.0 and 20.5 mm i.d. XAD-4 column, respectively, at k' values of about 2. Useful column efficiencies are still obtained even when k' > 10 and often at modest to gross overloads particularly when k' for the sample components differ appreciably. Depending on the mobile phase the 37-44 μm XAD-4 columns at preparative mass loadings develop efficiencies of about 500 to 1500 plates/meter. Increased efficiencies can be obtained by using smaller XAD-4 particles. Not all separations can be done at or above a mass overload because, as is often the case in PLC, sample solubility in the injection volume is not high enough to permit the large overload.

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REFERENCES

1. Walton, H. F., Ion Exchange and Liquid Column Chromatography, Anal. Chem. 52, 15R-27R (1980).

2. Krummen, K. and Frei, R. W., Quantitative Analysis of Nonpeptides in Pharmaceutical Dosage Forms by HPLC, J. Chromatogr. 132, 429-436 (1977).

3. Molnar, I. and Horvath, C., Separation of Amino Acids and Peptides on Non-Polar Stationary, Phases by HPLC, J. Chromatogr. 142, 623-640 (1977).

4. Kroeff, E. P. and Pietrzyk, D. J., HPLC Study of the Retention and Separation of Short Chain Peptide Diastereomers on a C-8 Bonded Phase, Anal. Chem. 50, 1353-1358 (1978).

5. Kroeff, E. P. and Pietrzyk, D. J., Investigation of the Retention and Separation of Amino Acids, Peptides, and Derivatives on Porous Copolymers by HPLC, Anal. Chem. 50, 502-511 (1978).

6. Iskandarani, Z. and Pietrzyk, D. J., Liquid Chromatographic Separation of Amino Acids, Peptides, and Derivatives on a Porous Polystyrene-Divinylbenzene Copolymer, Anal. Chem. 53, 489-495 (1981).

7. Baum, R. G., Saetre, R., and Cantwell, F. F., Liquid Chromatography Columns of Microparticle Amberlite XAD-2, Anal. Chem. 52, 15-19 (1980).

8. Pietrzyk, D. J. and Stodola, J. D., Characterization and Applications of Amberlite XAD-4 in Preparative Liquid Chromatography, Anal. Chem. 53, 1822-1828 (1981).

9. Dieterle, W., Faigle, J.W., and Mory, H., Preparative Reversed Phase Chromatography of Polar and Nonpolar Metabolites on Columns Packed with Micronized XAD-2 Resin, J. Chromatogr. 168, 27-34 (1979).

10. Uematsu, T. and Sasaki, T., Separation of 3':5'-and 2':3'-Cyclic Ribonucleotides from Their Related Mononucletoides by Amberlite XAD-4 Column Chromatography, J. Chromatogr. 179, 229 (1979).

11. Cahill, W. R., Kroeff, E. P., and Pietrzyk, D. J., Applications of tert-Butyloxycarbonyl-L-Amino Acid-N-Hydroxysuccinimide Esters in Chromatographic Separation and Determination of D,L-Amino Acids and Diastereomeric Dipeptides, J. Liq. Chromatogr. 3, 1319-1334 (1980).

12. Rotsch, T. D., Cahill, W. R., Pietrzyk, D. J., and Cantwell, F. F., Ionic Strength Effect on Liquid Chromatographic Retention of Benzenesulfonates on Nonpolar Adsorbents, Can. J. Chem. 59, 2179-2183 (1981).

13. Robinson, J. L., Robinson, W. J., Marshall, M. A., Barnes, A. D., Johnson, K. J., and Salas, D. S., Liquid Solid Chromatography on Amberlite XAD-2 and Other Styrene-Divinylbenzene Adsorbents I. Development of a Solvent Eluotropic Scale, J. Chromatogr. 189, 145-167 (1980).

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(3), 463-478 (1982)

METAL CATION/ANION SPECIATION VIA PAIRED-ION, REVERSED PHASE HPLC WITH REFRACTIVE INDEX AND/OR INDUCTIVELY COUPLED PLASMA EMISSION SPECTROSCOPIC DETECTION METHODS

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ABSTRACT

Conventional high performance liquid chromatography instrumentation and packing materials can be inexpensively and rapidly utilized for the qualitative and quantitative analysis of various metal cations or anions. The final approaches utilize reversed phase HPLC in the form of paired-ion separations. The detection of individually eluted, fully resolved metal cations or anions is possible *via* conventional refractive index or inductively coupled plasma emission spectroscopic detection. In many cases, unresolved mixtures of metal cations, eluted as a single peak on HPLC, can be resolved and identified *via* the use of ICP detection. Both metal cations and anions can be easily resolved, according to oxidation states, using paired-ion techniques, in combination with ICP detection. Final data representation can be in the form of conventional, continuous RI and/or ICP chromatograms, *via* pulsed data ICP presentations, and/or *via* tabular ICP data presentation.

INTRODUCTION (1)

Inorganic metal toxicity has long been an area of intense biological, toxicological, and medical interest. The apparent toxic properties of most,

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if not all, metals (free metals, cationic/anionic valences, chelates, and/or organometals) have been elucidated and extensively described for several decades (2-6). Unfortunately, the vast majority of published metal toxicity studies have never involved appropriate analytical methodology. Wherein analytical methods have been used as part of the biological studies, these provided total metal concentrations or levels, rather than the more desirable specific metal speciation profiles for the particular biological, toxicological, or medical samples of interest (7-17). Some of the more commonly employed instrumental methods of analysis for total metal determinations have been: direct flame/flameless atomic absorption spectroscopy (FAA, GFAA), direct current (DCP) or inductively coupled plasma (ICP) emission spectroscopy, flame emission spectroscopy, atomic fluorescence spectroscopy, anodic stripping voltammetry, pulse polarography, spark source mass spectrometry, X-ray fluorescence, and others (18, 19).

Within recent years it has become apparent to most scientists and some decision makers that partial or complete metal speciation of environmental, biological, toxicological, and/or medical type samples must be undertaken on a regular basis. That is, only when all or most metal species present in any sample are accurately known, can we then appropriately describe and ascribe biological, medical, or toxicological properties to that particular mixture of metals and/or metallic compounds/ions. Many metals exist as the free metal, various cationic and/or anionic oxidation states, organically bound chelates/complexes, and/or organometals. Obviously, the final speciation of metals in a complex sample matrix, often in the presence of other metals and their species, must involve some sort of an initial separation process. Considerable attention has been devoted in recent years towards the HPLC separation and detection of various metal species, usually employing UV, AA, ICP, or electrochemical detection (8-17, 20-31). Initially, HPLC was applied to the separation and identification of mainly organometals and/or metal chelates/complexes. Within the past few years, a tremendous interest has emerged towards inorganic metal cation/anion analyses, especially in the use of ion chromatography (IC) and/or high performance ion-exchange type chromatography, together with conductivity and/or electrochemical detection (15, 20-22, 32-39). It has become obvious that IC, especially *via* the use of commercially available instrumentation, has become quite popular, very useful, and can be readily utilized with pre-concentration methods for trace environmental and/or toxicological studies. Unfortunately, such chemical instrumentation has become quite expensive ($15,000-$25,000/unit), and must be dedicated to the performance of inorganic/organic cation or anion type analyses. IC does not lend itself to the performance of conventional HPLC separations *via*

liquid-solid, liquid-liquid, bonded phase, reversed phase, paired-ion, or gel permeation techniques and approaches.

Unfortunately, relatively little non-IC or non-ion-exchange type work has been reported in recent years, especially with regard to utilizing any commercial type HPLC instrumentation for performing inorganic metal cation/anion type analyses. Quite obviously, this situation should be remedied, and the work described here, in part, has been designed with this goal in mind. All of this work has utilized conventional reversed phase type packing materials, C_{18}, with paired-ion modified mobile phases. In the recent past, others have briefly reported on the use of such approaches for the separation of various cations and anions, but such work was most often not interfaced with the latest advances in ICP detection. That work which has used ion-exchange HPLC and/or chelation chromatography has generally used conductivity detection, atomic absorption spectroscopy, and/or electrochemical detection (8-16, 20-39). Very little work has been described with regard to metal cation or anion separations *via* conventional reversed phase HPLC interfaced with ICP detection. The work of Gast *et al*. is a notable exception to this last statement (30). Valenty and Behnken have described the use of C_{18} type columns together with paired-ion reagents for the complete separation of certain positively charged ruthenium complexes, followed by UV detection (40). Some inorganic anions have recently been analyzed using paired-ion reversed phase HPLC approaches, using UV, RI, and/or graphite furnace AA detection (11, 12, 26, 41-45). Perhaps Molnar *et al*. have reported the most extensively on the possible applications of paired-ion RP-HPLC for the separation of inorganic cations and anions, but this particular work involved conductivity detection (45). The overall detection limits in this study were quite impressive (ppb).

We describe here results utilizing paired-ion RP-HPLC techniques for metal cation and anion separations, wherein this is interfaced with RI and/or ICP detection methods. Paired-ion RP-HPLC allows for the separation of each group of cations combined with speciation within a cation group *via* ICP detection. This same approach also allows for the separation and ICP speciation of various arsenic containing oxyanions, as in the original work of Gast *et al*. (30). Thus, by applying these different separation methods together with RI and/or ICP detection, it is now possible to quickly and easily separate and speciate for a large number of metal cations and anions.

EXPERIMENTAL

Reagents

Inorganic salts, reagent grade, were obtained from the following sources: Baker analyzed reagents from VWR Scientific, Inc. (Boston, Mass.); Fisher

ACS certified grade from Fisher Scientific, Inc. (Medford, Mass.); Pfaltz & Bauer, Inc., grade unspecified (Stamford, Conn.); and Alfa/Ventron Inorganics, Inc., grade unspecified (Danvers, Mass.). The ion-pairing reagents, PIC A or PIC B, were obtained from Waters Associates, Inc. (Milford, Mass.), and the mobile phase water was purchased from the J.T. Baker Chemical Co. (Phillipsburg, N.J.), or used directly from a Corning Mega-Pure still (Corning Corp., Corning, N.Y.).

Apparatus

We have utilized a number of HPLC instrumental arrangements for the present work, and all have proven satisfactory. A typical HPLC arrangement consisted of a Laboratory Data Control (LDC) (Riviera Beach, Fla.) Model 711 solvent delivery system, modified with a special pulse dampener (Analabs, Inc., North Haven, Conn.), or a newer LDC Constametric III pump, a Rheodyne Model 7125 syringe injection valve (Rheodyne Corp., Cotati, Calif.), an Altex/Beckman variable wavelength UV-VIS detector (Altex/Beckman Corp., Irving, Calif.), a Waters Model 401 RI detector (Waters Assocs.), or a Micromeritics Model 771 RI detector (Micromeritics Corp., Norcross, Ga.), a modified Instrumentation Laboratory Model Plasma-100 inductively coupled plasma emission spectrometer (Instrumentation Laboratory, Inc., Wilmington, Mass.), and a Linear Corp. (Irvine, Calif.) or Honeywell Corp. (Minn., Minn.) dual pen recorder. The RI/ICP data were obtained *via* a dual pen recorder, and/or a separate ICP print-out from the Plasma-100 system. Often, both the recorder ICP chromatogram and the tabular data format from the ICP were obtained at the same time. At other times, the tabular data presentation could be manually used to reconstruct a pulsed type or continuous type HPLC-ICP chromatogram. This was done knowing the timed integration sequence of the tabular data presentation. In later runs, the Plasma-100 system was operated to present both pulsed type chromatograms, as below, and simultaneous tabular data presentations. This provided additional confirmation of the ICP results for the final metal speciation.

In the work described, the HPLC columns were all of the C_{18} type, and were usually obtained commercially, as follows: 1) Hibar II RP-18 pre-packed column (4.6mm x 25cm)(MCB Chemicals, Inc., Cinc., Ohio); 2) Alltech C-18 (4.6mm x 25cm)(Alltech Assocs., Inc., Deerfield, Ill.); 3) Altex/Beckman Ultrasphere ODS (4.6mm x 15cm)(Altex/Beckman Corp.); or 4) slurry packed in-house columns using Lichrosorb RP-18 (4.6mm x 25cm)(MCB Chemicals, Inc.).

Methods

In all studies involving paired-ion RP-HPLC, using the PIC B-5 or PIC B-8 counter-ions, the mobile phase consisted of the PIC reagent (0.005M)

prepared exactly according to the manufacturer's directions, at a final pH= 2.9-3.0. It would appear that a low pH is necessary for successful metal cation separations by a paired-ion RP-HPLC approach. Use of the PIC A reagent (0.005M) for arsenic oxyanion separations involved its preparation also according to the manufacturer's directions, with a final pH = 7.15. Specific flow rates, effluent split ratios, and more specific HPLC-detector operating conditions are presented below. An approximately 50:50 effluent split ratio was used in almost all of the dual detector studies, making use of a fixed ratio "T" type splitter (Alltech Assocs., Inc.).

In all of the paired-ion work, a mobile phase saturation, silica gel pre-column was used, on-line and just before the injection valve, in order to extend column lifetimes. The recommendation of Waters Assocs. was followed with regard to washing the C_{18} columns at the end of each day with 50:50 MeOH:HOH. Columns protected in these ways have lasted for at least six months, with no apparent change in overall column efficiencies or retention times. The analytical column was always thermostated in a constant temperature water bath at or about 25°C, in order to improve retention time reproducibility and decrease ambient temperature effects on capacity factors.

Detection limits by RI were determined directly from the resulting chromatograms, using minimum settings possible on the detector together with a signal:noise ratio of at least 3:1 in each case. Detection limits by ICP were determined using a 2:1 signal:noise ratio from the tabular data format, wherever this was feasible and practical.

RESULTS AND DISCUSSION

The use of an organic counter ion in the mobile phase to perform paired-ion reversed phase (RP)-HPLC has been described for many years, but almost exclusively with regard to organic ion separations (46-48). This method has proven to be an extremely useful alternative to traditional ion-exchange type HPLC for organic compounds that are able to form ion-pairs in aqueous solutions. Its use for inorganic cation/anion HPLC separations has been described much less, and the recent work of Molnar _et al_. just indicates some of the potentials which this approach holds (45). Wherein this approach has been described for inorganic ions, it has mostly been applied to the separation of anions, with much less work having been reported for metal cations (41-45).

We have been quite interested in demonstrating the possible uses of ion-pairing for metal cation and anion separations, and with the eventual interfacing of such approaches to ICP detection (49). All of the work that

we describe here has used a commercially available C_{18} type reversed phase column, of various dimensions, with mobile phases consisting of either PIC-B or PIC A type ion-pairing reagents at a single concentration. Quite obviously, other combinations of column packings, column dimensions, mobile phases, mobile phase concentrations, and final flow rates could be used in the future to improve or modify the final separations. Table I summarizes some of the results obtained using both the PIC B-8 and PIC B-5 type reagents, both for univalent and divalent metal cations. These RI results have been confirmed in almost all instances via ICP detection under similar HPLC conditions. In the case of the PIC B-8 reagent, it was not possible to get the divalent metal species to elute within a reasonable amount of time after observation of the univalent species. We therefore investigated the use of the analogous PIC B-5 reagent, Table I, wherein the univalent cations elute just after the solvent front, as a group, and the divalent species now elute at about 8.0 mins (t_r), again as a single peak on the RI. It is to be emphasized that in all of this metal cation ion-pairing work, we have not yet attempted to resolve either the +1 or +2 species from one another, but rather we have emphasized the overall group separations of +1 from +2.

TABLE I

Paired-Ion Reversed Phase HPLC of Metal Cations With RI Detection

Mobile Phase	Metal Cation	t_r (mins)	k' (cap. factor)
PIC B-8[a]	Na^+	8.25	6.2
	K^+	8.6	6.5
	Li^+	8.15	6.1
	Cu^+	8.2	6.1
PIC B-5[b]	K^+	2.85	0.36
	Cu^+	2.85	0.36
	Cu^{+2}	7.8	2.7
	Zn^{+2}	7.9	2.8
	Cd^{+2}	8.0	2.8
	Fe^{+2}	8.1	2.9
	Pb^{+2}	7.6	2.6

a. HPLC-RI conditions: Alltech RP-18 column (4.6mm x 25cm) with a mobile phase of 0.005M octane sulfonic acid (Waters PIC B-8), pH = 2.9, flow rate of 3.0ml/min.

b. HPLC-RI conditions: Alltech RP-18 column (4.6mm x 25cm) with a mobile phase of 0.005M octane sulfonic acid (PIC B-5), pH=2.85, flow rate 2.0 ml/min, Waters Assocs. Model 440 RI detector at 4X.

Thus, as in Table I, we have not attempted to resolve Cu^{+2} from Zn^{+2} or Cd^{+2}, but instead have tried to obtain useful group separations of all the +1 cations from the +2 cation group. Molnar _et al_. in their earlier work, described the successful resolution of Na^{+1}, K^{+1}, and NH_4^{+1} from each other, using similar paired-ion techniques, but these workers had to use three C_{18} type columns placed in series to achieve this resolution (45). Their use of a non-selective conductivity detector necessitated the initial chromatographic resolution of each +1 species, in order to qualitatively and quantitatively identify each species present. Our desire to interface this HPLC method with ICP detection does not demand complete HPLC resolution of either the +1 or +2 species, as demonstrated below.

Fig. 1 illustrates a typical paired-ion RP-HPLC-RI separation of Cu^{+1} and Cu^{+2}, injected initially as 26.8ug Cu^{+1} in the form of CuCl (cuprous chloride). This was an old sample of CuCl from the Chemistry stockroom at the University, prepared in the mobile phase, which apparently existed more as Cu^{+2} than the expected Cu^{+1}. Clearly, the reading of bottle labels does not guarantee the contents of a bottle are as represented at the time of sale. A recently purchased sample of CuCl showed _via_ this method almost no Cu^{+2} ion present. When a new sample of cupric acetate ($Cu(OAc)_2$) was prepared and analyzed, this sample existed almost entirely as Cu^{+2}, with only a very small contribution from the peak at t_r = 2.85 mins, which is that of Cu^{+1}. Also, when solutions of Cu^{+1}, prepared from old CuCl were analyzed by paired-ion RP-HPLC-RI-ICP techniques, the ICP clearly showed the presence of two distinct forms of copper, as expected from the above.

We have observed that under certain sample preparation conditions, depending on which acid may be used to dissolve the initial copper salts, it is possible to observe three distinct copper containing ionic species _via_ paired-ion RP-HPLC-ICP conditions. In order to dissolve certain copper salts, we have used, in some instances, hydrochloric or sulfuric acids (dilute concentrations), and these final solutions were then used for the paired-ion analyses. In certain instances, we have clearly observed _via_ both RI and ICP detection, three distinct copper containing species. Two of these are as already indicated, Figure 1, and a third copper containing species has a retention time in between those for the Cu^{+1} and Cu^{+2} species. It is possible that this third species is a mixed ligand, Cu^{+2}, tripartite ion species, perhaps containing only one counter-ion derived from the PIC B-5 reagent and the remaining ion Cl^{-1}.

It has, of course, now been possible to interface a large number of paired-ion RP-HPLC separations of metal cations, such as those in Table I, directly to the ICP. In this manner, element specific ICP chromatograms can

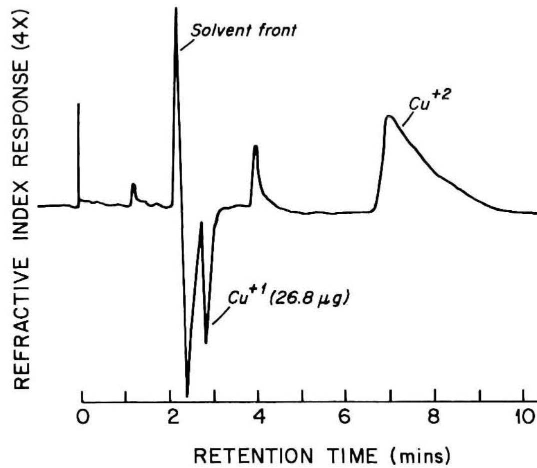

FIGURE 1

be readily obtained, either in continuous print-out or tabular formats. These tabular formats are then readily converted into either a pulsed or continuous type chromatogram, and this can be done manually or instrumentally. The true analytical capabilities for metal cations or anions resides, in part, in the multi-element capabilities of the ICP detector. That is, in order to determine how much of each possible valence state a metal may exist as in any given sample, it is really only necessary for the analyst to physically separate all of the +1 species from the +2s, or the +2s from the +3s, and so forth. Once the different valence states are all resolved from each other, chromatographically or otherwise, then the ICP can resolve each individual metal species present within a mixture of +1s, +2s, +3s, etc. Indeed, using this approach, but purely as an illustration of the possible capabilities and potentials of HPLC-ICP analyses for metal ions, Figure 2 indicates a synthetic mixture of three separate divalent metal cations. These are Fe^{+2}, Cd^{+2}, and Zn^{+2}, injected as a mixture of all three, at the levels indicated, Figure 2, using a split of the column eluent to both RI and ICP detectors. Naturally, the RI detector shows only a single peak for all three divalent species, since these are not chromatographically distinguishable from each other on the non-selective RI. Indeed, if one only used the non-selective RI detector, then it would be impossible to know that more than one species was present under the single chromatographic peak. However, the ICP is able to monitor a number of emission wavelengths, in rapid sequence, and to measure each light intensity at these various wavelengths as a function of

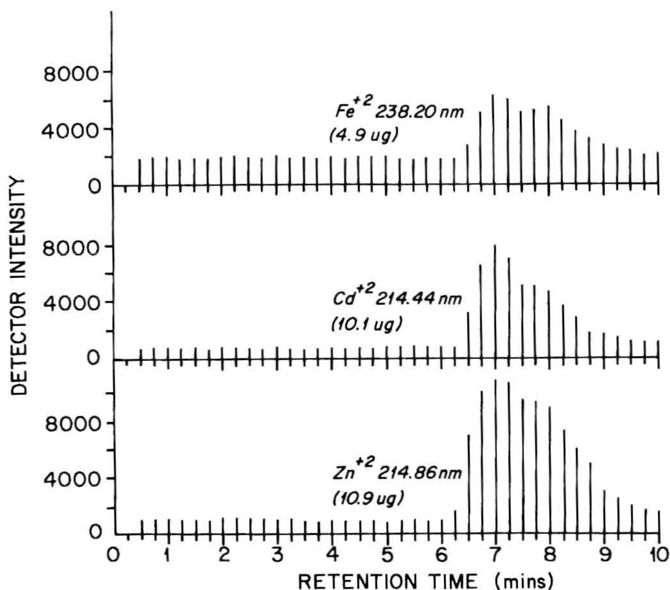

FIGURE 2

time. It is then able to store this information, as pre-determined by the particular program that operates the ICP, and to integrate each light emission at the appropriate wavelengths every one, two, five, or x seconds during the entire HPLC process. One therefore obtains an ICP print-out, Figure 2, here re-constructed manually from the tabular format, for each metal species wavelength of emission. The wavelengths used here are indicated, wherein we have previously determined no spectral interferences for any of the three metal wavelengths involved. In essence, one has three distinct HPLC-ICP chromatograms for all of the metal cations present in the initial sample. We have performed analogous, illustratuve studies, using other +1 and +2 species, wherein all of the +1s are separated from the +2s, and where the ICP is then able to determine how much of each +1 and +2 species were present in the original, simulated mixture of metal ionic species. This is therefore true metal speciation, wherein the analyst now has the ability to determine a very large number of metallic cations present in the same sample matrix, all with a bare minimum of sample work-up, handling, and preparation prior to the HPLC-ICP. This method avoids any form of sample derivatization before injection onto the HPLC-ICP system.

We, as well as others, have investigated the capabilities of paired-ion RP-HPLC-ICP for performing metal anion analyses, with the initial emphasis here on the speciation of various arsenic containing oxyanions. We have also applied these same techniques for the speciation of chromium ions, viz., chromate and chromic ions, but this work will be presented elsewhere (50). The analysis-speciation of arsenic containing compounds is of intense current interest, as evidenced by the number of literature references within recent years (11-14, 25, 26, 30, 51). Most of the reported work with arsenic oxyanions has involved the use of ion-exchange packings in conventional HPLC, but some work has been done using paired-ion RP-HPLC in combination with graphite furnace AA (11) or ICP (30, 51). We have investigated the paired-ion RP-HPLC resolution of three distinct arsenic species, viz., sodium arsenite ($NaAsO_2$), sodium dimethyl arsenate (sodium cacodylate, $Na(CH_3)_2AsO_2.3HOH$), and sodium arsenate ($Na_2HAsO_4.7HOH$). Using the PIC A type reagent, it has been possible to satisfactorily resolve all three arsenic oxyanions within about 10 mins. HPLC-RI, Figure 3, indicates the presence of at least three compounds, but there are a number of additional RI peaks present in the synthetic mixture of standards. Using RI alone, it would not be possible to assign individual peaks to any of the arsenic oxyanion materials. However, once the paired-ion RP-HPLC separation is interfaced with ICP detection, it becomes apparent precisely which chromatographic peaks are indeed the correct arsenic containing species. Figure 3 is a typical, manually re-constructed HPLC-ICP chromatogram of the three arsenic species involved, all clearly baseline resolved within about 10 mins. The arsenic emission was monitored here at a wavelength of 228.83nm, in combination with an integration time of about 1 sec. Each intensity reading at these 1 sec intervals was then used, after background subtraction, to produce the final ICP chromatogram.

We have intentionally omitted any discussion here of minimum detection limits via HPLC-ICP or HPLC-RI. However, from the data presented thus far, it should be apparent that our detection limits in either detection mode do not fall below the ppm levels for any of the metals studied here. In other work, we have made direct comparisons of direct-ICP and HPLC-ICP detection limits, especially for cadmium and chromium (50). Because of the band broadening present within all HPLC-detector interfacing, due to the nature of the HPLC process and the interfacing hardware dead volume, there must always be a worsening of the detection limits in the interfaced mode. One would automatically expect that this difference should be about 15-25 fold worse for the HPLC-ICP case, but we and others have seemingly found this difference to be somewhat more than this estimated value. In some instances, the overall difference between direct-ICP and HPLC-ICP can be as much as 2-3 orders of

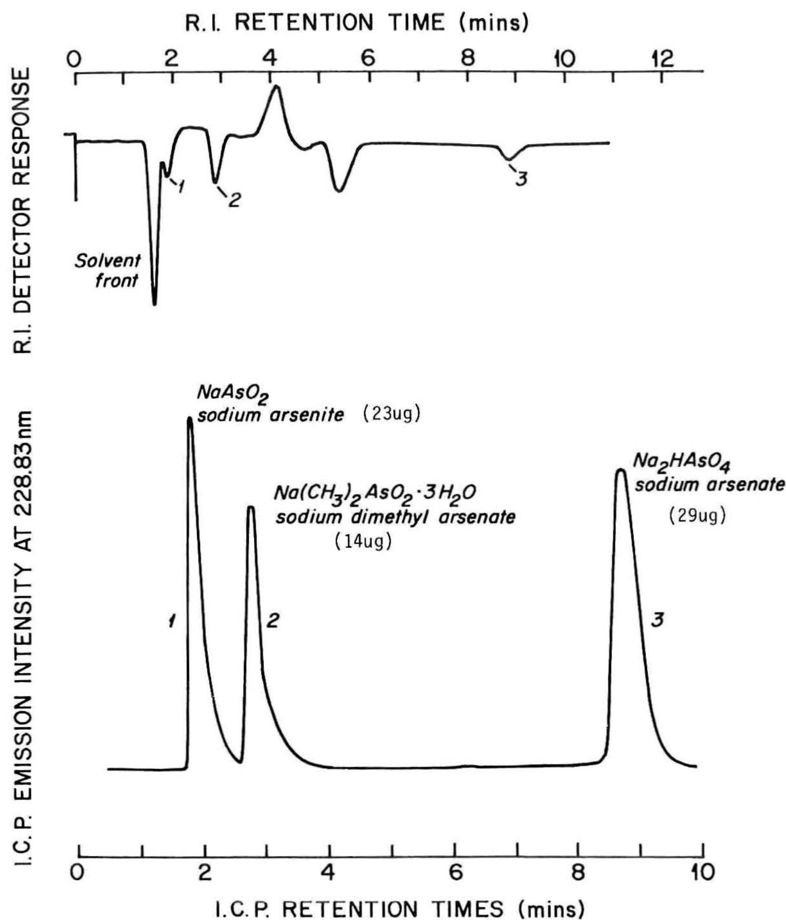

FIGURE 3

magnitude, and we are currently trying to understand the specific reasons for this effect. Obviously, for any HPLC-ICP method to have value in real world applications, there must be detection limits which rival or equal those already possible *via* direct-ICP. At the same time, we are somewhat surprised and truly impressed by those reports in the literature which report HPLC-ICP detection limits which are equal to or, in some cases, better than those possible/reported for direct-ICP. What is called for in these studies is a direct comparison of direct-ICP vs. HPLC-ICP for the same analytes and absolute amounts, but such studies are somewhat lacking in the existing literature (30, 51).

CONCLUSION

We have attempted to develop and perfect a variety of HPLC approaches for the successful and efficient resolution of a wide variety of inorganic, metallic cations and anions. We have especially wanted to use the tremendous amount of information and expertise that has evolved over the past few years with regard to paired-ion RP-HPLC methods/applications. There is every reason to believe that alternative methods of performing inorganic ionic separations will provide just as much useful, overall results as those described here. However, in the case of low pressure ion chromatography, the question remains of the general applicability of this approach for performing inorganic, metal ion analyses in combination with ICP, wherein this would mean the virtual exclusion of all other forms of HPLC separations. On the other hand, the reversed phase columns that we have utilized for RP-HPLC-RI-ICP are still readily available for performing other types of organic HPLC analyses, using the exact same type of HPLC instrumentation and hardware. This would appear to be a very significant advantage in using reversed phase HPLC approaches for inorganic, ionic or organometallic type separations.

The combination of ionic chromatography and element specific ICP detection is quite obviously an extremely powerful method for inorganic type analyses. ICP apparently has fewer matrix interference and salt interference problems as compared with either flame AA or graphite furnace AA methods of today. Most importantly, ICP has true multi-element capabilities something which conventional AA and/or GFAA do not really possess. ICP can also be quite compatible with high salt concentrations in the HPLC mobile phase (49, 50), without producing spectral or sample matrix interferences for the metal of interest. This may not be the case with FAA and/or GFAA approaches. Also, ICP would appear to be somewhat more compatible with gradient elution HPLC methods, especially with those which would involve the use of methanol, ethanol, or isopropanol. We and others have noted this potential for using mixed aqueous:organic type solvents in ICP operations, at times with increased signal responses for particular analytes. A number of other organic HPLC solvents may also prove compatible with routine ICP operation, and considerable more work remains to be undertaken in this area (16, 30, 52). Obviously, it would be extremely advantageous to undertake gradient elution RP-HPLC separations in combination with ICP detection, for this approach would then allow for a complete metal speciation profile determination for a very large number of metal elements/species. Quite clearly, our work here is but one step in that direction.

We have only briefly discussed detection limits in the HPLC-ICP mode, but the literature does already contain a certain amount of information in

this regard. It is our belief that, in general, an insufficient amount of work has been devoted to maximizing HPLC-ICP sensitivity and minimizing its detection limits. These must be brought more in line with the corresponding HPLC-GFAA or direct GFAA limits/sensitivities. Thus far, most reports of HPLC-GFAA detection limits indicate a significant advantage over the analogous HPLC-ICP detection limits for the same samples and HPLC conditions. Unfortunately, there are very few meaningful direct comparisons of HPLC-GFAA and HPLC-ICP for the same HPLC conditions and analytes of interest. It is our hope, as well as that of others, that the future will bring forth greatly improved limits of detection for HPLC-ICP methods, and that this approach will take its rightful place as a highly versatile and quite useful method of trace element analysis and speciation.

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REFERENCES

1. Abbreviations used: HPLC = high performance liquid chromatography; ICP = inductively coupled plasma emission spectroscopy; RI = refractive index detection; RP = reversed phase; PIC B = alkyl sulfonate counter-ion; PIC A = tetrabutylammonium counter-ion; UV = ultraviolet detection; FAA = flame atomic absorption spectroscopy; GFAA = graphite furnace atomic absorption spectroscopy; DCP = direct current plasma emission spectroscopy; IC = ion chromatography; MeOH = methanol; t_r = retention time; k' = capacity factor; ppb = parts-per-billion.

2. Berman, E., Toxic Metals and Their Analysis, Heyden & Son, Ltd., London, 1980.

3. Hammond, B.P. and Beliles, R.P., in Toxicology - The Basic Science of Poisons, Second Edition, Doull, J., Klaasen, C.D., and Amdur, M.O., eds., MacMillan Publishing Co., Inc., New York, 1980, p. 468.

4. Sigel, H., ed., Metal Ions in Biological Systems. Carcinogenicity and Metal Ions, Vol. 10, Marcel Dekker, New York, 1980.

5. Some Metals and Metallic Compounds. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Volume 23. International Agency for Research on Cancer, Lyon, 1980.

6. Harrison, R.M. and Laxen, D.P.H., Chemistry in Britain, 316 (June, 1980).

7. Scheutzle, D., ed., Monitoring Toxic Substances, ACS Symposium Series No. 94, American Chemical Society, Washington, D.C., 1979.

8. Van Loon, J.C., Anal. Chem., 51, 1139A (1979).

9. Van Loon, J.C., American Laboratory, 47 (May, 1981).

10. Parks, E.J., Brinckman, F.E., and Blair, W.R., J. Chrom., 185, 563 (1979).

11. Brinckman, F.E., Jewett, K.L., Iverson, W.P., Irgolic, K.J., Ehrhardt, K. C., and Stockton, R.A., J. Chrom., 191, 31 (1980).

12. Jewett, K.L. and Brinckman, F.E., J. Chrom. Sci., in press (1981).

13. Anonymous, Chem. & Eng. News, 29 (Feb. 16, 1981).

14. Ricci, G.R., Shepard, L.S., Colovos, G., and Hester, N.E., Anal. Chem., 53, 610 (1981).

15. MacCrehan, W.A., Anal. Chem., 53, 74 (1981).

16. Krull, I.S. and Jordan, S., American Laboratory, 21 (October, 1980).

17. Miyazaki, A. and Barnes, R.M., Anal. Chem., 53, 364 (1981).

18. Pinta, M., Modern Methods for Trace Element Analysis, Ann Arbor Science Publishers, Inc., Ann Arbor, Mich., 1978.

19. Kuwana, T., ed., Physical Methods in Modern Chemical Analysis, Vols. 1 and 2, Academic Press, Inc., New York, 1978, 1980.

20. Schwedt, G., Chromatographic Methods in Inorganic Analysis. Separation Methods and Systems, Detection Methods, Applications in Inorganic Trace Analysis, A.H. Verlag Publishers, Heidelberg, 1981.

21. Schwedt, G., in Topics in Current Chemistry, Boschke, F.L., ed., Vol. 85, Springer-Verlag Publisher, Berlin, 1981, p. 160.

22. Cassidy, R.M., in Trace Analysis, Lawrence, F.J., ed., Academic Press, New York, in press, 1981.

23. Kahn, N. and Van Loon, J.C., J. Liq. Chrom., 2, 23 (1979).

24. Brinckman, F.E. and Blair, W.R., in National Bureau of Standards Special Publications No. 556. National Bureau of Standards, Wash., D.C., 1979, p. 25.

25. Grabinski, A.A., Anal. Chem., 53, 966 (1981).

26. Woolson, E.A. and Aharonson, N., J. Assoc. Off. Anal. Chem., 63, 523 (1980).

27. Slemer, D.C., Koleel, P., Haworth, D.T., Taraszewski, W.J., and Lawson, S.R., Anal. Chem., 51, 575 (1979).

28. Uden, P.C., Bigley, I.E., and Walters, F.H., Anal. Chim. Acta, 100, 555 (1978).

29. Uden, P.C., Paper presented at the International Winter Conference on Developments in Atomic Plasma Spectrochemical Analysis, Puerto Rico, January, 1980, in press, Heyden & Son, New York, 1981-2.

30. Gast, C.H., Kraak, J.C., Poppe, H., and Maesen, F.J.M.J., J. Chrom., 185, 549 (1979).

31. Koropchak, J.A. and Coleman, G.N., Anal. Chem., 52, 1252 (1980).

32. Cassidy, R.M. and Elchuk, S., J. Chrom. Sci., 18, 217 (1980).

33. Mulik, J.D. and Sawicki, E., eds., Ion Chromatographic Analysis of Environmental Pollutants, Ann Arbor Science, Inc., Ann Arbor, Mich., 1979.

34. Girard, J.E., Anal. Chem., 51, 836 (1979).

35. MacDonald, J.C., American Laboratory, 45 (January, 1979).

36. Wetzel, R.A., Anderson, C.L., Schleicher, H., and Crook, G.D., Anal. Chem., 51, 1532 (1979).

37. Smith, F.D., Jr. and Chang, R.C., CRC Critical Reviews in Anal. Chem., 197 (August, 1980).

38. Pohl, C.A. and Johnson, E.L., J. Chrom. Sci., 18, 442 (1980).

39. Gjerde, D.T., Schmuckler, G., and Fritz, J.S., J. Chrom., 187, 35 (1980).

40. Valenty, S.J. and Behnken, P.E., Anal. Chem., 50, 834 (1978).

41. Chiu, G., Anal. Chem., 52, 1157 (1980).

42. Brinckman, F.E., Blair, W.R., Jewett, K.L., and Iverson, W.P., J. Chrom. Sci., 15, 493 (1977).

43. Reeve, R.N., J. Chrom., 177, 393 (1979).

44. Kamiura, T. and Tanaka, M., Anal. Chim. Acta, 110, 117 (1979).

45. Molnar, I., Knauer, H., and Wilk, D., J. Chrom., 201, 225 (1980).

46. Snyder, L.R. and Kirkland, J.J., Introduction to Modern Liquid Chromatography, Second Edition, J. Wiley & Sons, Inc., New York, 1979, Chapter 11.

47. Bidlingmeyer, B.A., Deming, S.N., Price, W.P., Sachok, B., and Petrusek, M., J. Chrom., 186, 419 (1979).

48. Bidlingmeyer, B.A., J. Chrom. Sci., 18, 525 (1980).

49. Krull, I.S., Bushee, D., Young, D., and Smith, S.B., Jr., Paper presented at the 1981 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, N.J., March, 1981. Abstract No. 580.

50. Krull, I.S., Bushee, D., Smith, S.B., Jr., Savage, R.N., and Schleicher, R., Anal. Letters, submitted for publication (1981).

51. Morita, M., Uehiro, T., and Fuwa, K., Anal. Chem., 53, 1806 (1981).

52. Boorn, A.A. and Browner, R.F., Anal. Chem., in press (1982).

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DETERMINATION OF GLUTAMIC ACID DECARBOXYLASE ACTIVITY IN SUBREGIONS OF RAT BRAIN BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A quantitative high pressure liquid chromatographic (HPLC) assay has been developed for the determination of glutamic acid decarboxylase (GAD) activity in subregions of rat brain. GAD activity was determined indirectly by measurement of gamma-aminobutyric acid (GABA). Fluorimetric detection was made possible by derivatization with ortho-phthalaldehyde and the limit of detection was 11 ng GABA.

INTRODUCTION

Gamma-aminobutyric acid (GABA) has been identified as a principle inhibitory neurotransmitter in the central nervous system of veterbrates (1). However, GABA is distributed in glial cells as well as within neurons, while glutamic acid decarboxylase (GAD) the enzyme that decarboxylates glutamic acid (GA) to GABA,

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is found only within the neurons (1). Therefore GAD activity is used as a marker of GABAergic neurons. GABA projections from neostriatum to globus pallidus (GP), entopeduncular nucleus (EP), and substantia nigra (SN) (2,3) as well as from SN to ventromedial thalamus (VM) (4) have been reported. Decreased GAD activity has been found in the striatum and SN of postmortem brains of humans afflicted with Parkinson's disease and Huntington's chorea (5,6).

A number of procedures have been developed for the indirect analysis of GAD activity via measurement of GABA formation. A radiometric method for GAD activity by measurement of $^{14}CO_2$ from the decarboxylation of ^{14}C-labeled GA has been described by Roberts and Simonsen (7). Lowe et al (8) reported a fluorimetric procedure for the determination of GAD activity in neural tissue by measurement of GABA formation. Recently, Holdiness et al (9) reported a modification of the Lowe et al (8) method for measurement of GAD activity in subregions of rat brain. Values reported for GAD activity were 7.91 $\pm$ 1.47 (GP), 6.87 $\pm$ 2.07 (EP), 3.38 $\pm$ 0.69 (VM), 13.80 $\pm$ 2.14 (SN_M) and 8.23 $\pm$ 2.26 (SN_L) ug GABA/hour/mg protein.

In this paper is reported an HPLC method for determination of GAD activity in rat brain tissue using fluorescent detection.

EXPERIMENTAL

Materials

The chemicals used in this study were sodium-L-glutamate (Pfaltz and Bauer, Inc., Stamford, CN), trichloroacetic acid, gamma-aminobutyric acid and 5-aminovaleric acid (Sigma Chem. Co., St. Louis, MO). Pyridoxal 5-phosphate, triton x-100 (scintillation grade), orthophthalaldehyde and 2-mercaptoethanol were procurred from Eastman Kodac Co. (Rodchester, NY). The internal standard was prepared by dissolving 2.0 mg of 5-aminovaleric acid in 25 ml of a solution consisting of 0.50 M KCL, 0.010 M EDTA, 0.5% triton x-100 and 0.40 M sodium phosphate buffer pH 6.40.

Instrumentation

A Waters Associates 202 HPLC with a 6UK injector was used with a stainless steel column (1 m by 4.6 mm I.D.) packed with Zipax strong cation exchange resin (DuPont, Wilmington, DE), 10 um particle size. Fluorescence was accomplished with a Perkin-Elmer MPF-4 spectrofluorimeter with an excitation wavelength of 335 nm (6 mm slit width) and as emission wavelength of 450 nm (10 mm slit width) and a dynode voltage of 500 volts. Post column derivatization was accomplished with a second Waters 6000A solvent delivery pump which connected after the column by a three way tee union. Thirty

feet of teflon tubing was required between the union and the spectrofluorimeter to allow for complete derivatization. The tubing, column and 30 ul quartz flow cell were maintained at 40°C.

Operating Procedures and Conditions

The mobile phase consisted of 0.50 M sodium acetate buffer adjusted to pH 5.0 and degassed for 30 minutes. The derivatizating agent was prepared by mixing 220 mg ortho-phthalaldehyde in a minumin amount of methanol with 200 ul of 2-mercaptoethanol dissolved in 0.50 M sodium phosphate buffer adjusted to pH 10. The flow rate was set at 2 ml/min and 0.10 ml/min for the mobile phase and derivatizating agent, respectively.

Sample Collection and Preparation

The sample collection procedure has been previously described (9). From each 1 mm thick brain tissue slice, two tissue punches were taken from symmetrical locations in the left and right hemispheres. Each set of punches was immediately transferred to a 12 ml polypropylene Eppendorf tube containing 100 ul of internal standard solution and sonicated for 10 seconds under low power sonication. After sonication, 50 ul of this solution was transferred to an identical tube containing 20 ul of 3% trichloroacetic acid (TCA). This second tube served as a blank and its concentration of GABA was

subtracted from the orginal sample. The substrate-buffer was prepared as previously described (9) and 50 ul of this solution was added to both the sample and blank tubes before they were incubated for two hours at 38°C. The enzyme was inactivated by addition of 20 ul of 3% TCA to the orginal sample tube and all tubes were centrifuged at 950 x g for 20 minutes. Injections of 40 ul of the supernate were made into the HPLC. The precipitated tissue was analyzed for protein as described by Lowery et al (10) and GAD activity has been reported in ug GABA/hour/mg protein.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram of GABA and internal standard isolated from rat brain. The retention times for GABA and internal standard are 4.70 and 6.80 minutes, respectively. Positive identification was achieved by peak superimposition, i.e., by addition of GABA and 5-aminovaleric acid standards (300 ng) to the extracts and observing increased peak height at the corresponding retention times.

The lower limit of detection (2/1 signal to noise) of this procedure is 11 ng GABA. Repetitive injections of standards gave good reproducibility of retention times (standard deviation $\pm$ 2%) and peak heights (standard deviation $\pm$ 2.5%). Standard curves were

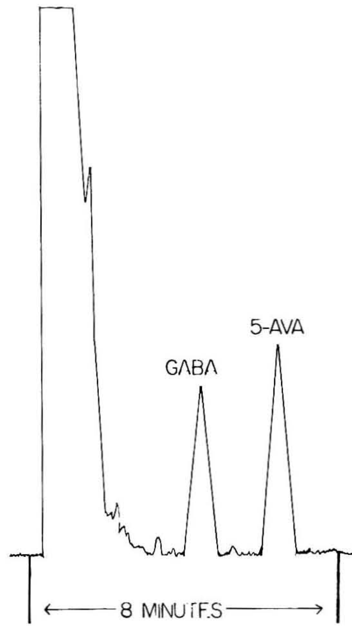

Figure 1. HPLC chromatogram of GABA and 5-aminovaleric acid (internal standard) extracted from rat brain tissue. The retention times of GABA and internal standard are 4.70 and 6.86 minutes, respectively.

linear in the range of 10 to 3000 ng and day to day reproducibility varied less than 3.1% (standard deviation). The recovery for GABA was 96 $\pm$ 6% and overall 1 to 3 fold GABA increases were observed for samples in this procedure.

The tissue punch placement is presented in Figure 2. All tissue slices are 1 mm thick and left and right hemispheric punches are combined for each nuclei. The first punch is the GP at AP 7.0 and the other locations are AP 6.0 (EP and VM) and AP 3.0 (SN_M and SN_L) based

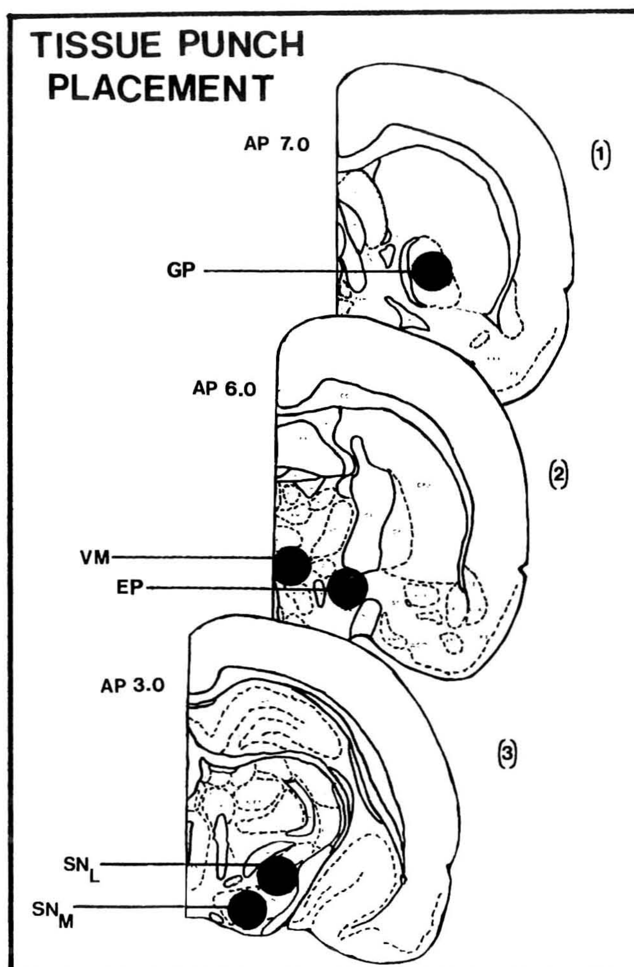

Figure 2. Tissue punch placement. The first punch was from a 1 mm thick tissue slice containing the globus pallidus (GP). The second slice contains the entopendunclar nucleus (EP) and ventromedial thalamus (VM). The third slice contains the substantia nigra medial (SN_M) and lateral (SN_L) punches. The numbers on the left refer to the anterior-posterior (AP) axis coordinates in the brain atlas of Pellegrino and Cushman (11). Punch diameter, 1.30 mm.

upon the atlas of Pellegrino and Cushman (11). An average weight of representative tissue samples from combined left and right brain punches (n = 20) is 2.30 $\pm$ 0.33 mg tissue (wet weight) with an average protein content of o.110 mg protein/mg tissue. The average GAD activity values ($\pm$ standard deviation) found by this method in each brain region (n = 5) are 7.81 $\pm$ 1.08 (GP), 6.73 $\pm$ 1.58 (EP), 3.75 $\pm$ 0.71 (VM), 13.70 $\pm$ 1.75 (SN_M), and 8.17 $\pm$ 1.68 (SN_L). These values are in close agreement with the previously reported activities determined by the fluorimetric procedure (9).

In conclusion, the HPLC method described is sufficiently sensitive and specific for analysis of GAD activity in subregions of rat brain tissue.

REFERENCES

1. Walaas, I., and Fonnum, F. F., GABA-Neurotransmitters: Pharmacochemical, Biochemical, and Pharmacological Aspects, P. Krogsgaard-Larsen, J. Scheel-Kruger, and H. Kofod, (editors), Academic Press, New York, (1978), 61.

2. Fonnum, F. F., Gottesfeld, A., and Grofova, I., Brain Res., 143 125 (1978).

3. Nagy, J. I., Carter, D. A., and Fibiger, H. C., Brain Res., 158, 15 (1978).

4. DeChaira, G., Proceddu, M. L., Morelli, M. Mulas, M. L., and Gessa, G. L., Brain Res., 176, 273 (1979).

5. McGeer, P. L., McGeer, E. G., Wada, J. A., and Jung, E., Brain Res., 32, 425 (1971).

6. McGeer, E. G., and McGeer, P. L., J. Neurochem., 15, 65 (1976).

7. Roberts, E., and Simonsen, D. G., Biochem. Pharmacol., 23, 113 (1974).

8. Lowe, I. P., Robins, E., and Eyreman, G. S., J. Neurochem., 3, 8 (1958).

9. Holdiness, M. R., Justice, J. B., Neill, D. B., and Salamone, J. D., Anal. Lett., 13, 1330 (1980).

10. Lowery, O. H., Rosebrough, N. J., Farr, A. L., and Randell, R. J., J. Biol. Chem., 193, 265 (1951).

11. Pellergrino, L. J., and Cushman, A. J., A Stereotaxic Atlas of the Rat Brain, Appleton-Century-Crofts, New York, (1967).

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EVALUATION OF NEW MICROPARTICULATE PACKINGS FOR AQUEOUS STERIC EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Two types of high performance aqueous size exclusion columns have recently been developed, one a rigid spherical silica-based packing containing a new hydrophilic bonded phase (MicroPak TSK Gel Type SW) and the other an organic-based, semi-rigid gel (MicroPak TSK Gel Type PW). Characteristics of MicroPak TSK SW and PW columns were compared to other commercially available aqueous SEC columns packed with similar supports. Chromatographic performance of pre-packed columns containing microparticulate support materials were compared for exclusion separations of water-soluble organic polymers, biopolymers, and small water-soluble oligomers. Amino acid probes were used to investigate non-exclusion effects of MicroPak TSK SW and PW columns.

INTRODUCTION

Steric exclusion chromatography (SEC) carried out in aqueous mobile phases, traditionally referred to as gel filtration chromatography, is widely used for the separation and characterization of natural and synthetic water-soluble polymers. The technique has been primarily employed to study biopolymers using crosslinked dextrans (e.g., Sephadex™) and agaroses (e.g., Sepharose™, Bio-Gel A™). Such

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gels are of limited utility in HPLC because their inherently low compressive strengths require operation at low pressures and flow velocities.

Controlled-pore glasses (CPG) and silica packings allow analysis at pressures and flow velocities typical of HPLC. Microparticulate silica supports offer greatly reduced separation times compared to the soft gels traditionally used in aqueous SEC; however, their active surface sites often result in adsorption and other non-exclusion effects (2). Silica-based packings which contain a chemically bonded phase such as glyceryl propyl ether (diol) reduce adsorption (i.e., chemically deactivate the silica) but often exhibit low pore volumes compared to carbohydrate gels, thus reducing resolution.

New HPLC packings for aqueous SEC have utilized both microparticulate, hydrophilic polymer gels (3,4) and silica supports containing hydrophilic bonded phases (5,6). Several of these silica-based supports have recently been characterized by Pfannkoch, Lu, Regnier, and Barth (7) from the standpoint of both SEC performance and attendant non-exclusion effects. A comprehensive review of commercially available aqueous exclusion packings and chromatographic practices has been published by Barth (8).

Two types of microparticulate aqueous exclusion packings have been developed by Toyo Soda Mfg. Co. (Tokyo, Japan). One is a rigid, silica-based packing with a new hydrophilic bonded phase (TSK Gel Type SW) and the other a hydrophilic, polymer-based, semi-rigid gel (TSK Gel Type PW) (9, 10, 11).

In this report, the chromatographic performance of MicroPak TSK columns containing these two support materials has been compared for SEC separations of water-soluble organic polymers, biopolymers, and small water-soluble molecules. Column characteristics and chromatographic performance have also been contrasted with other commercially available aqueous SEC supports of similar nature. Amino acid probes were used to investigate non-exclusion effects on MicroPak TSK SW type and TSK PW type columns.

EXPERIMENTAL

Chromatography was performed on Varian Model 5000 LC systems equipped with a refractive index detector and a UV-50 variable wavelength absorbance detector. Chromatographic separations were carried out at 25°C using Varian MicroPak TSK SW and TSK Gel PW type columns (7.5 mm x 30 cm) at a mobile phase flow rate of 1 ml/min. Sample injection volumes were 100 μl using a Valco manual loop injector. Solvents used were 0.01 M KH_2PO_4 (pH 6.8) for amino acid probe samples, 0.1 M KH_2PO_4 + 0.1 M KCl (pH 6.8) for proteins, and deionized water for polyethylene glycol standards.

Samples of polyethylene glycol (PEG) standards were obtained from Toyo Soda Mfg. Co. Ltd. (Tokyo, Japan) and Jefferson Chemical Co. (Austin, Texas). Aqueous solutions 0.1% w/v were used throughout this study. PEG standards above MW 10,000 were dissolved in aqueous solutions containing 0.5% ethanol to aid dissolution and retard chain scission of the standard (12). Detection of PEG standards was accomplished by a refractive index detector.

Proteins and amino acid probe samples were obtained from Sigma Chemical Co. (St. Louis, Missouri). Detection of proteins was at 280nm and detection of amino acids at 210nm in the UV.

RESULTS AND DISCUSSION

Characteristics of Commercially Available Aqueous SEC Microparticulate Supports

A. Surface-Modified, Silica-Based Packings

Tables 1 and 1a list all commercially available columns packed with surface-modified, silica-based aqueous SEC supports. Characteristics for each column type specify chemical nature of the bonded phase (or surface modification), packing name, available pore sizes, particle size and shape of the silica, molecular weight separation ranges, and a list of manufacturers and suppliers. Several of the support materials in this table are marketed by a variety of suppliers under different trade names. For example, SynChropak GPC

Table 1.
Characteristics of Microparticulate, Surface-Modified, Silica-Based Packings for Aqueous SEC

Chemical Type of Silica Surface Modification	Packing Name	Supplier
Glycerol propyl-type bonded phase; ($SiCH_2CH_2CH_2OCH_2CHOHCH_2OH$).	SynChropak GPC 100	1,2 (sold as Aquapore),
	SynChropak GPC 300	3 (sold as Aquapore),
	SynChropak GPC 500	4 (sold as Bio-sil GFC).
	SynChropak GPC 1000	
	SynChroPak GPC 4000	
	LiChrosorb Diol 100	5
	LiChrospher Diol 100	5
	LiChrospher Diol 500	
	LiChrospher Diol 1000	
	LiChrospher Diol 4000	
Polyether-type bonded phase; ($Si(RO)_nCH_3$); base silica μPorasil.	μBondagel E-125	6
	μBondagel E-500	
	μBondagel E-1000	
	μBondagel E-High Å	
	μBondagel E-Linear	
Bonded phase structure has not been published.	Waters Protein Column I-60	6
	Waters Protein Column I-125	
	Waters Protein Column I-250	
Glycol ether-type bonded phase; surface covered with hydroxyl groups, exact structure has not been published.	TSK Gel Type 2000SW	10,4 (sold as Bio-Sil TSK),
	TSK Gel Type 3000SW	7 (sold as MicroPak TSK SW),
	TSK Gel Type 4000SW	8 (sold as μSpherogel SW),
		9 (sold as Ultropak TSK SW).

Suppliers:
1. SynChrom Inc. (Linden, IN)
2. Brownlee Labs (Santa Clara, CA)
3. Chromatix (Sunnyvale, CA)
4. Bio-Rad Laboratories (Richmond, CA)
5. E. Merck (EM Laboratories, Elmsford, NY)
6. Waters Associates (Milford, MA)
7. Varian Associates (Walnut Creek, CA)
8. Beckman-Altex Inc. (Berkeley, CA)
9. LKB Instruments Inc. (Rockville, MD)
10. Toyo Soda Manufacturing Co. Ltd. (Tokyo, Japan)

Table 1a.

Packing Name	Pore Size (Å)	Particle Size (μm) and Shape	Molecular Weight Separation Range Proteins (Aqueous Mobile Phase)	Polystyrene (THF Mobile Phase)
SynChropak GPC 100	100	10 (spherical particles)	3,000-300,000	<5-80,000
SynChropak GPC 300	300	"	-	<1.5-300,000
SynChropak GPC 500	500	"	10,000-5 million	<3-600,000
SynChropak GPC 1000	1000	"	100,000-20 million	<0.6-1.4 million
SynChropak GPC 4000	4000	"	-	<2.5-8 million
LiChrosorb Diol 100	100	10 (irregular particles)	10,000-100,000	-
LiChrospher Diol 100	100	10 (spherical particles)	-	<80,000
LiChrospher Diol 500	500	"	-	<600,000
LiChrospher Diol 1000	1000	"	-	<1.4 million
LiChrospher Diol 4000	4000	"	-	<8 million
μBondagel E-125	125	10 (irregular particles)	-	2,000-50,000
μBondagel E-500	500	"	-	5,000-500,000
μBondagel E-1000	1000	"	-	50,000-2 million
μBondagel E-High Å	N.A.	20 (irregular particles)	-	15,000-7 million
μBondagel E-Linear	Blend	10 (irregular particles)	-	2,000-2 million
Waters Protein Column I-60	60	10 (irregular particles)	1,000-20,000	-
Waters Protein Column I-125	125	"	2,000-80,000	-
Waters Protein Column I-250	250	"	10,000-500,000	-
TSK Gel Type 2000SW	130	10 (spherical particles)	500-60,000	-
TSK Gel Type 3000SW	240	"	1,000-300,000	-
TSK Gel Type 4000SW	450	13 (spherical particles)	5,000-1 million	-

NOTE: Data obtained from manufacturer's literature.

N.A. = Not available.

packing is also sold under the names of Aquapore and Bio-Sil GFC columns, and TSK Gel SW packing is sold under names of μSpherogel TSK SW, MicroPak TSK SW, and other column names.

SynChropak high performance modified silica was the first commercially available glycophase-type packing for aqueous SEC. Silylpropylglycerol bonded phases of this type also include LiChrosorb Diol and more recently LiChrospher Diol. Although largely employed in the separation and characterization of water-soluble biopolymers, all of these packings can be used with organic solvents for analysis of synthetic polymers and other applications.

μBondagel packing is a silica-based support containing an aliphatic ether bonded phase. Its primary application has been the characterization of synthetic polymers and polysaccharides in both aqueous mobile phases and organic solvents such as tetrahydrofuran (THF). Recently, Waters Associates has developed a proprietary Protein Column designed specifically for characterization of biopolymers such as proteins. The nature of the silica surface modification has not been published although it is known to contain a neutral, hydrophilic phase covalently bonded to silica.

TSK Gel Type SW packing is believed to consist of a glycol ether-type bonded phase similar in nature to the glycophases. The surface of TSK SW packing is known to be highly hydroxylated, although the exact packing structure has not been published. This surface-modified silica support exhibits little tendency for adsorption and high efficiency for analysis and separation of biopolymers such as proteins and enzymes.

B. Organic Gel-Based Packings

Characteristics of commercially available organic gel-based packings for aqueous SEC are listed in Tables II and IIa. The chemical nature of the support, packing name, available pore sizes, particle size and shape, molecular weight separation ranges, and pH range of operation are listed for each material along with a list of manufacturers and suppliers.

Spheron packing is a poly(hydroxyl methacrylate) copolymerized with ethylene dimethacrylate. The packing particles are aggregated to form macroporous beads capable of withstanding pressures up to 3000 psi. The support is compatible with both water and organic solvents, allowing a wide range of applications (53).

Shodex OHpak support is believed to be composed of a glycerol methacrylate copolymer although the exact structure of the packing has not been published. Shodex OHpak B-804 packing is the only pore size available of this type and has been employed in the characterization of both polysaccharides and biopolymers.

Shodex IonPak is a sulfonated poly(styrene divinylbenezene) gel offering high efficiency and a wide variety of pore sizes for aqueous SEC. Applications of this gel have focused on characterization of polysaccharides and neutral, synthetic polymers.

TSK Gel Type PW packing is a crosslinked, hydroxylated polyether gel whose exact structure has not been published. Most of the pore sizes available with this packing are known to contain residual carboxyl groups and the smaller pore sizes (1000PW and 2000PW) additionally contain residual amino groups. This support material has been employed in a wide variety of aqueous SEC applications and has been found to be particularly well suited to the analysis of water-soluble synthetic polymers, including polycations.

Comparative Characterization of MicroPak TSK SW and TSK PW Columns

A comparison of the characteristics of MicroPak TSK SW and TSK PW columns serves to illustrate some of the fundamental differences between silica-based and organic gel-based supports for aqueous SEC as well as provide additional information on these two column types. MicroPak TSK SW column packing is a rigid, hydrophilic, spherical, and porous silica that contains a chemically bonded phase thought to be a polyether-type coating. The surface of the packing is covered with hydroxyl groups (13). MicroPak TSK PW column packing is a semi-rigid, hydrophilic, crosslinked polymer-based gel containing the group $-CH_2CHOHCH_2O-$ as the main backbone component (14).

Table 2.
Characteristics of Microparticulate, Organic Gel-Based Packings for Aqueous SEC

Chemical Type of Gel	Packing Name	Supplier
Glycol methacrylate gel copolymer; poly(2-hydroxyethylmethacrylate-co-ethylene dimethacrylate).	Spheron P40 Spheron P100 Spheron P300 Spheron P500 Spheron P700 Spheron P1000 Spheron P100000	1
Methacrylate glycerol copolymer.	Shodex OHpak B-804	2,3
Sulfonated poly(styrene divinyl-benzene) copolymer.	Shodex IonPak S-801 Shodex IonPak S-802 Shodex IonPak S-803 Shodex IonPak S-804 Shodex IonPak S-805	2,3
Hydroxylated polyether copolymer; contains ($-CH_2CHOHCH_2O-$) groups as main backbone component. Also known to contain residual -COOH groups and $-NH_2$ groups (1000PW and 2000PW only); exact structure has not been published.	TSK Gel Type 1000PW TSK Gel Type 2000PW TSK Gel Type 3000PW TSK Gel Type 4000PW TSK Gel Type 5000PW TSK Gel Type 6000PW	7,4 (sold as MicroPak TSK PW), 5 (sold as μSpherogel PW), 6 (Bio-Gel TSK).

Suppliers:
1. LaChema (Brno, Czechoslovakia)
2. Perkin-Elmer Corp. (Norwalk, CT)
3. Showa Denko K.K. (Tokyo, Japan)
4. Varian Associates (Walnut Creek, CA)
5. Beckman-Altex Inc. (Berkeley, CA)
6. Bio-Rad Laboratories (Richmond, CA)
7. Toyo Soda Manufacturing Co. Ltd. (Tokyo, Japan)

Table 2a.

Packing Name	Pore Size (Å)	Particle Size (μm) and Shape	Molecular Weight Separation Range in Aqueous Mobile Phase: Polysaccharides	Polyethylene Glycols (PEGs)	pH Stability
Spheron P40	40	10-20 (macroporous beads)	20,000-60,000	-	1-12
Spheron P100	100	"	40,000-100,000	-	
Spheron P300	300	"	60,000-300,000	-	
Spheron P500	500	"	80,000-500,000	-	
Spheron P700	700	"	250,000-700,000	-	
Spheron P1000	1000	"	800,000-5 million	-	
Spheron P100000	-	"	<100 million	-	
Shodex OHpak B-804	N.A.	10 (spherical particles)	<400,000	-	4-12
Shodex IonPak S-801	55	10 (spherical particles)	< 1,000	-	2-11
Shodex IonPak S-802	100	"	< 5,000	-	
Shodex IonPak S-803	160	"	< 50,000	-	
Shodex IonPak S-804	220	∿15 (spherical particles)	<500,000	-	
Shodex IonPak S-805	350	"	<5 million	-	
TSK Gel Type 1000PW	-	10 (spherical particles)	-	∿100-1,000	2-12
TSK Gel Type 2000PW	50	10	-	200-5,000	
TSK Gel Type 3000PW	200	13	500-10,000	∿1,000-50,000	
TSK Gel Type 4000PW	500	13	1,000-700,000	2,000-300,000	
TSK Gel Type 5000PW	1000	17	10,000-2 million	4,000-800,000	
TSK Gel Type 6000PW	-	25	100,000-20 million	40,000-8 million	

NOTE: Data obtained from manufacturer's literature.
N.A. = Not available.

A comparison of the characteristics of MicroPak TSK SW and TSK Gel PW columns is shown in Table 3. Exclusion limits, typical efficiencies, ratio of pore volume (V_i) to interstitial volume (V_o), particle sizes, and pore sizes are listed for each column type. MicroPak TSK SW type columns have higher efficiencies and (V_i/V_o) ratios than the PW type columns, and therefore should offer higher resolution. The PW type columns operate over a larger molecular size range than do the SW columns due to the wider range of pore sizes available. MicroPak TSK PW columns also allow operation over a wider pH range (2 to 12) than the silica-based SW columns (2.5 to 7.5) (see reference 9).

Figure 1 displays a vanDeemter-type plot of linear velocity versus plate height (HETP) for some MicroPak TSK SW and PW type columns. Plate height increases with flow velocity and is found to plateau at high flow velocities. In comparing the 3000PW to the 4000SW column, it can be seen that the 4000SW column has a higher efficiency at all flow rates examined. The 3000SW column has a smaller particle size packing (10 μ) than either the 3000PW or 4000SW column packing (13 μ) and, consequently, lower plate height at all flow velocities. In practice, flow velocities of 0.04 to 0.06 cm/sec, roughly corresponding to 0.8 to 1.3 ml/min mobile phase flow rate, have been found to offer the best compromise between speed and efficiency for both column types.

Polyethylene glycol (PEG) calibration curves for SW and PW columns are shown in Figures 2 and 3. The slopes exhibited in the linear region of the calibration curve have been found to be indicative of the resolution attainable with steric exclusion columns (15). Lower slopes usually correspond to higher resolution values for a given column pore size.

<u>Comparative Performance of Aqueous SEC Columns</u>

Chromatographic column performance has been traditionally expressed in terms of the number of theoretical plates or efficiency:

$$N = 16\left(\frac{V_R}{W}\right)^2 \qquad (1)$$

TABLE 3.

Characteristics of MicroPak TSK Gel Type SW and TSK Gel Type PW Columns

Column Type	Particle Size*	Pore Size* (Å)	M.W. Exclusion Range PEG	M.W. Exclusion Range Protein	Theoretical Plates/Meter (N/M)	Pore to Interstitial Volume Ratio (V_i/V_o)
2000SW	10 ± 2 μ	130	20,000	100,000	21,000	0.92
3000SW	10 ± 2 μ	240	40,000	400,000	19,000	1.33
4000SW	13 ± 3 μ	450	200,000	1 million (est)	17,000	1.52
1000PW	10 ± 2 μ	-	1,000	-	16,000	0.89
2000PW	10 ± 2 μ	50	4,000	15,000	17,000	0.87
3000PW	13 ± 2 μ	200	50,000	450,000	15,000	0.83
4000PW	13 ± 2 μ	500	200,000	-	14,000	0.78
5000PW	17 ± 2 μ	1000	1 million (est)	>1 million	13,000	0.98
6000PW	25 ± 5 μ	-	8 million (est)	-	8,100	1.06

NOTE:

1. Method for calculation of theoretical plates: Sample: 1% w/v solution Ethylene Glycol
   Mobile Phase: 1 ml/min H_2O
   Detector: RI
   Injection Volume: 100 μl
2. V_i = Pore Volume; V_o = Interstitial Volume.

* Data supplied by Toyo Soda. Estimated pore size values obtained by comparison with packings of known pore sizes from calibration curve data.

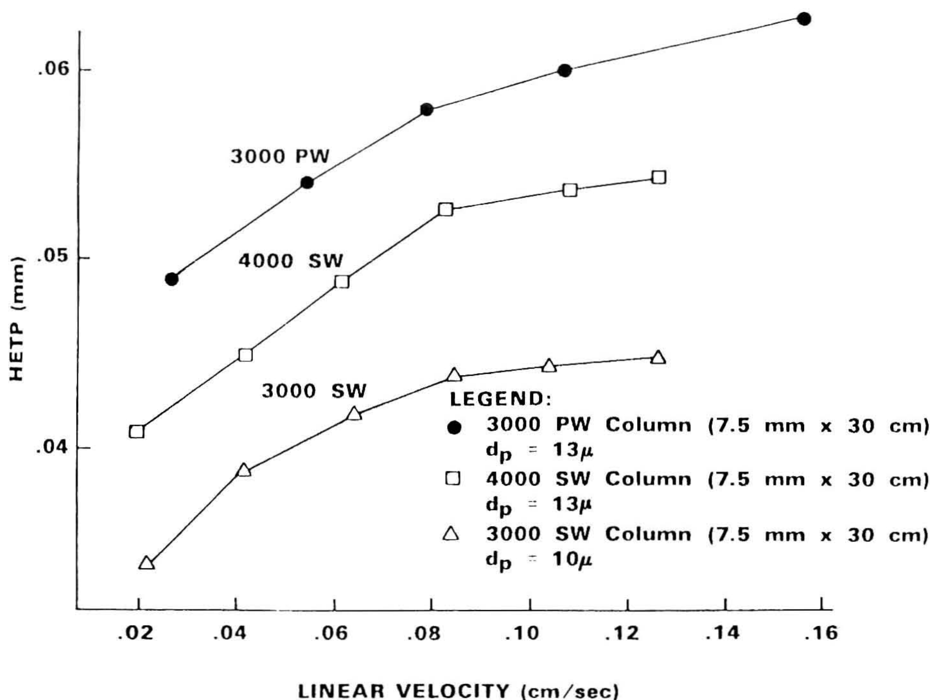

FIGURE 1. VanDeemter-type plot of linear velocity (cm/sec) versus plate height (HETP in mm) for MicroPak TSK 3000SW (d_p = 10 μ), 4000SW (d_p = 13 μ), and 3000PW (d_p = 13 μ) columns. Column dimensions: 7.5 mm x 30 cm; Mobile phase: H_2O

where V_R is peak retention volume and W the peak width at baseline as measured by a peak triangulation technique.

The resolution (or separation efficiency) of a two-component mixture has also been used as a column performance parameter as described by the following equation:

$$R_S = \frac{2(V_{R2}-V_{R1})}{W_1 + W_2} \qquad (2)$$

where V_{R1} and V_{R2} are the elution volumes of two solutes and W_1 and W_2 their respective peak widths.

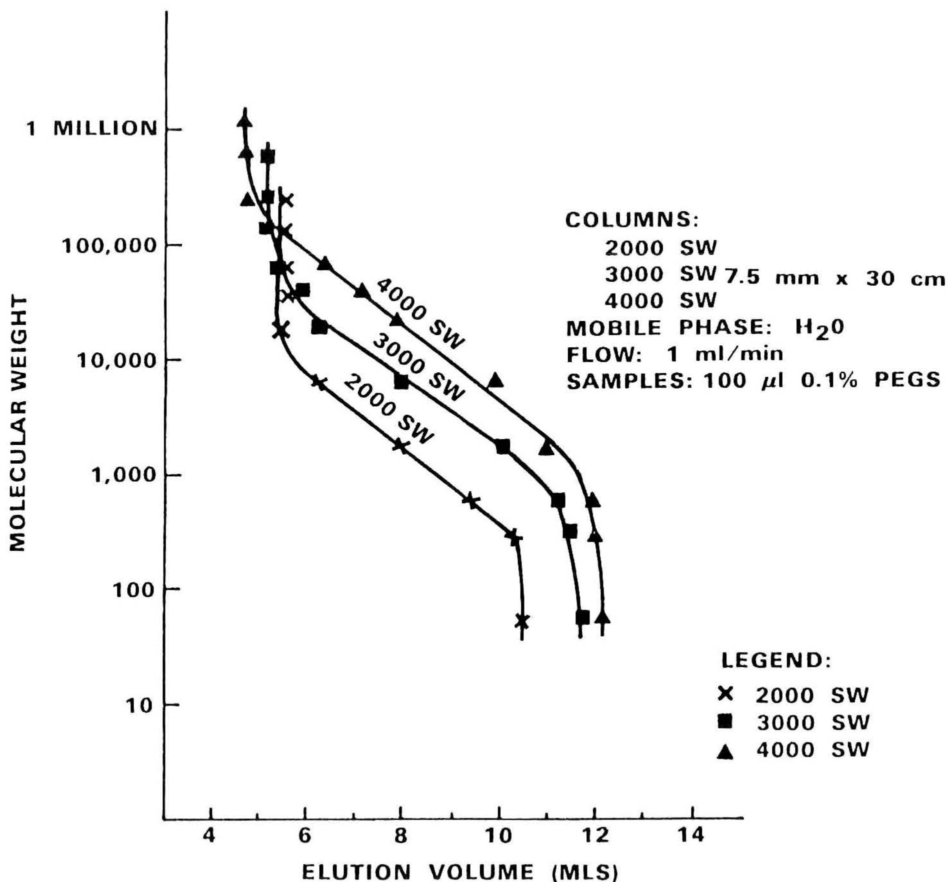

FIGURE 2. PEG standard calibration curves for MicroPak TSK SW type columns. Mobile phase: H_2O; Flow rate: 1 ml/min; Injection volume: 100 µl; Loading: 0.1% PEG. Column dimensions: 7.5 mm x 30 cm.

The values of R_S and N calculated by these equations, however, are highly dependent on column dimensions and on the solutes chosen to characterize performance. Additionally, in steric exclusion chromatography, it would be very desirable to relate chromatographic resolution to molecular weight since separation is based upon molecular size discrimination.

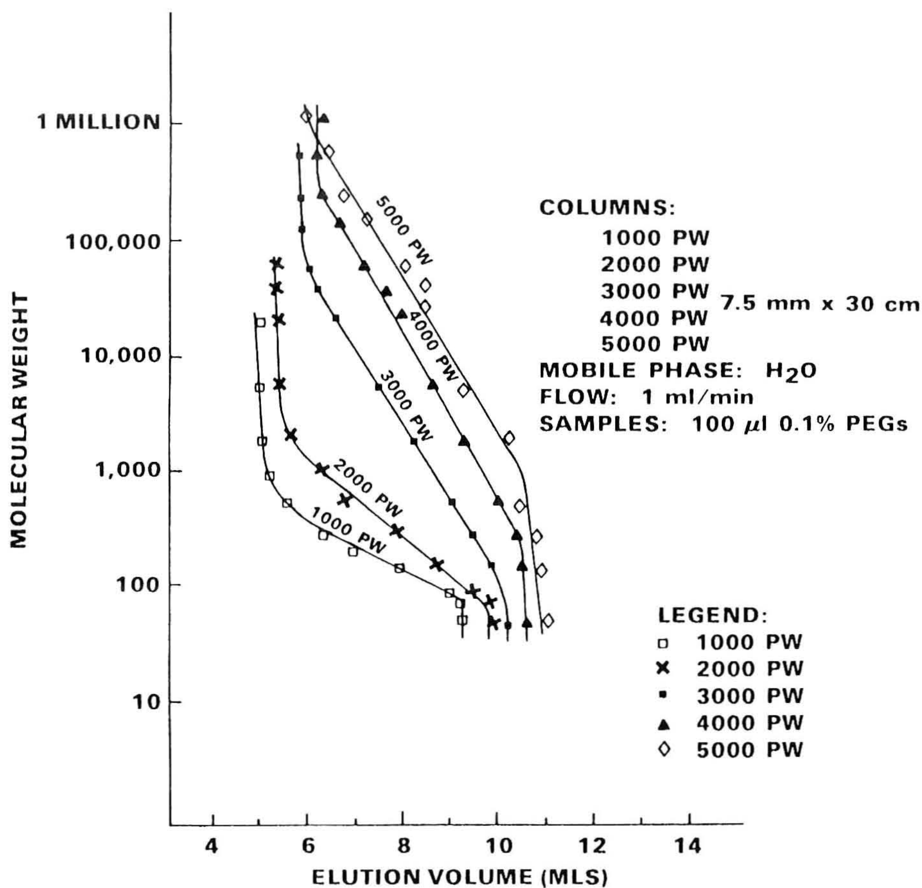

FIGURE 3. PEG standard calibration curves for TSK PW type columns. Mobile phase: H_2O; Flow rate: 1 ml/min; Injection volume: 100 µl; Loading: 0.1% PEG. Column dimensions: 7.5 mm x 30 cm.

The concept of specific resolution, R_{SP}, was introduced by Bly (16) who has shown that in the linear region of the calibration curve of log MW versus V_R for an exclusion column, resolution can be normalized and expressed as a function of molecular weights of a solute pair:

$$R_{SP} = \frac{2(V_{R2} - V_{R1})}{W_1 + W_2} \times \frac{1}{\log(MW_1/MW_2)} \qquad (3)$$

where MW_1 and MW_2 are the molecular weights of two solutes. For a pair of solutes with a decade difference in MW, equation (3) reduces to the expression for resolution. Additionally, outside the linear calibration range for a steric exclusion column, R_{SP} approaches zero.

Specific resolution, R_{SP}, is independent of the solute probes if the samples have very narrow molecular weight distributions (MWD) (17). Thus, R_{SP} is a more descriptive parameter for accurate performance comparison of steric exclusion column types. This parameter has been applied by Kirkland and Antle (18) to the performance characterization of high performance steric exclusion packings using organic mobile phases.

Table 4 displays a comparison of efficiency, specific resolution (R_{SP}), and pore volume data for several commercially available aqueous SEC columns packed with surface-modified, silica-based supports. In SEC, resolution is a function of both column efficiency and support pore volume (V_i). As can be seen from the data in this table, columns with high efficiency and large support pore volumes offer high resolution for SEC separations, as for example the TSK 3000SW column.

A comparison of pore volume and efficiency for several commercially available aqueous SEC columns packed with organic gel-based supports is shown in Table 5. Lack of published data on the performance of these packings precludes a more incisive comparison.

Specific resolution, R_{SP}, values were calculated with a series of narrow MWD polyethylene glycol standards and protein standards for both MicroPak TSK SW and PW columns. The average molecular weight for a pair of standards can be defined as follows:

$$\text{Average MW} = \frac{MW_1 + MW_2}{2} \qquad (4)$$

A plot of R_{SP} versus Average MW defines the molecular weight range of optimum resolution for a steric exclusion column and provides a practical performance criterion for column selection and comparison in steric exclusion analysis. Such plots have been used by Kato *et*

TABLE 4.
Comparison of Efficiency, Resolution, and Pore Volume for Commercially Available Columns Containing Surface-Modified, Silica-Based Supports for Aqueous SEC

Columns	Length	I.D.	(T.P./m)[a] Theoretical Plates/Meter	R_{SP}[b]	Pore Volume (V_i)
MicroPak TSK 2000SW	30 cm	7.5 mm	22,567	2.57	5.06 ml
MicroPak TSK 3000SW	30 cm	7.5 mm	30,720	3.32	6.78
MicroPak TSK 4000SW	30 cm	7.5 mm	17,000	-	6.84
SynChropak GPC 100	25 cm	4.6 mm	8,316	1.65	2.00
SynChropak GPC 300	25 cm	4.6 mm	16,800	2.08	-
Waters I-125	25 cm	7.8 mm	19,788	2.04	4.94
Waters μBondagel	30 cm	3.9 mm	⩾12,000	-	1.20

a) Measured using the peptide glycyltyrosine (K_D range 1.01 to 1.07 for columns tested) except TSK 4000SW (ethylene glycol) at aqueous mobile phase velocity of 0.33 mm/second, and μBondagel (from manufacturer's data measured at velocity of 1.8 mm/sec in THF).

b) Specific resolution factor computed for the peptide glycyltyrosine (MW=238) and the protein ovalbumin (MW=43,500).

NOTE: Based upon experimental data from reference 7.

Table 5.
Comparison of Efficiency and Resolution for Commercially Available Columns Containing Organic Gel-Based Supports for Aqueous SEC

Columns	Length	I.D.	(T.P./m) Theoretical Plates/Meter	Pore Volume
MicroPak TSK 1000PW	30 cm	7.5 mm	16,520[a]	4.54 ml
MicroPak TSK 2000PW	30 cm	7.5 mm	17,030	4.83
MicroPak TSK 3000PW	30 cm	7.5 mm	15,120	4.63
MicroPak TSK 4000PW	30 cm	7.5 mm	14,300	4.52
MicroPak TSK 5000PW	30 cm	7.5 mm	13,050	5.51
MicroPak TSK 6000PW	30 cm	7.5 mm	8,100	6.01
Shodex OHpak B-804	50 cm	8.0 mm	$\geqslant$10,000[b]	8.19
Spheron P-1000	25 cm	8.0 mm	9,000[c]	-
Shodex S-801/S	50 cm	8.0 mm	20,000[b]	-
Shodex S-802/S	50 cm	8.0 mm	20,000	-
Shodex S-803/S	50 cm	8.0 mm	20,000	-
Shodex S-804/S	50 cm	8.0 mm	14,000	-
Shodex S-805/S	50 cm	8.0 mm	14,000	-

a) Experimentally measured in aqueous mobile phase at a linear velocity of 0.4 mm/sec with ethylene glycol.
b) From manufacturer's literature.
c) Experimentally determined with 17 μm particles slurry packed into SS tubing. See reference 39.

al. to characterize protein separations on SW columns (19). Demonstration of the utility of these plots to chromatographic performance of MicroPak TSK SW and TSK PW columns has recently been performed (20).

Specific resolution curves for MicroPak SW columns using PEG standards are shown in Figure 4. In comparing the 2000SW and 3000SW columns, the 2000SW displays higher specific resolution values for solutes below MW 1000, while the 3000SW column displays higher resolution values for larger solutes. This fact has also been observed for protein separations using 2000SW and 3000SW columns (see reference 19). Although the 4000SW column operates over a wide range of solute molecular weights, specific resolution

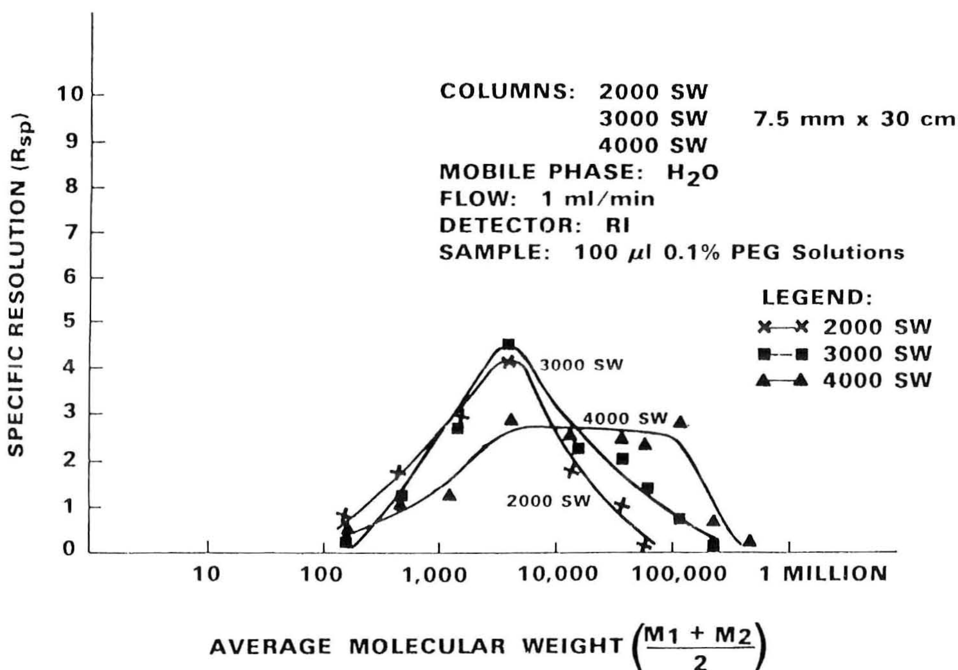

FIGURE 4. Specific resolution (R_{SP}) curves for MicroPak TSK Gel type SW columns using PEG standards. Mobile phase: H_2O; Flow rate: 1 ml/min; Injection volume: 100 µl; Loading: 0.1% PEG; Column dimensions: 7.5 mm x 30 cm.

values are much lower than those obtained with 2000SW and 3000SW columns for solutes less than 20,000 in molecular weight.

Specific resolution plots for MicroPak PW columns using PEG standards are displayed in Figure 5. As column pore size increases, specific resolution values decrease for PW columns. The extremely large values of specific resolution obtained with 1000PW and 2000PW columns reflect the advantage of these columns for analysis of small water-soluble molecules such as polyethylene glycol oligomers and oligosaccharides (21).

In comparing specific resolution curves for MicroPak TSK SW and PW columns, several points are noteworthy:

i. In molecular weight ranges applicable to both SW and PW columns, higher resolution is provided by operation with SW columns;

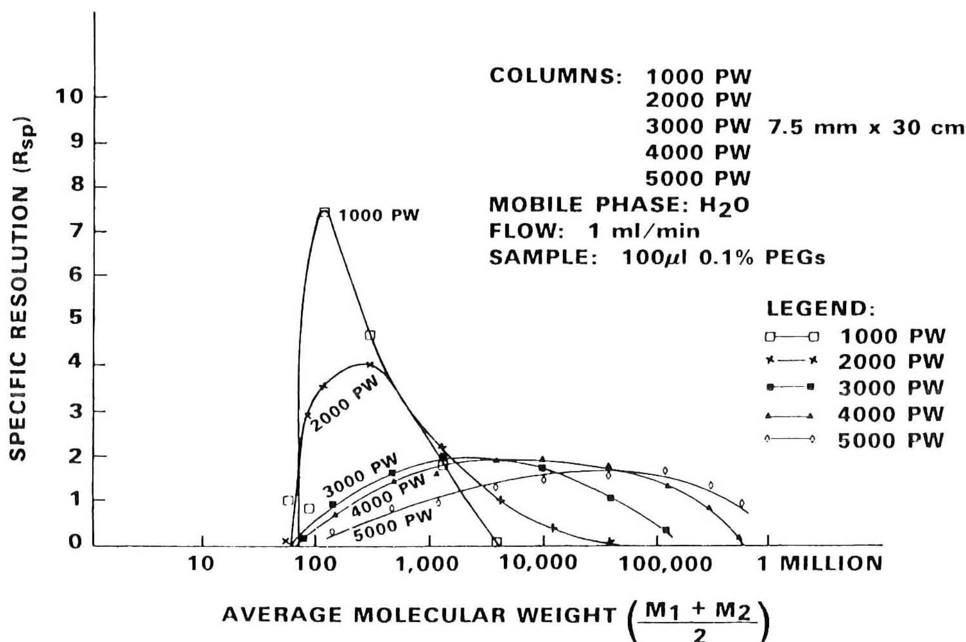

FIGURE 5. Specific resolution (R_{SP}) curves for MicroPak TSK Gel Type PW columns using PEG standards. Conditions same as in Figure 4.

ii. For solutes of molecular weight greater than 200,000 the PW columns offer a wider molecular weight range of operation and higher resolution values;

iii. 1000PW and 2000PW columns are best suited to the analysis of small water-soluble molecules.

Comparison of Microparticulate Aqueous SEC Columns for Analysis of Water Soluble, Synthetic Polymers

The influence of the mobile phase in aqueous steric exclusion chromatography is particularly important because of its effect on solute conformation and size. Ionic strength is critical in eliminating hydrophilic interactions between the column packing and charged solutes (polyelectrolytes) that lead to attendant problems of ion exclusion, ion inclusion, ion exchange, and adsorptive effects (22). For SW and PW type columns, ionic strengths greater than 0.1 μ are preferred, and polyvalent anions seem to be more effective in eliminating ionic effects for many polymers. Polysaccharides, poly(vinyl alcohol), and poly(vinyl pyrrolidone) polymers can be successfully chromatographed on both SW and PW type columns using low ionic strength mobile phases such as 0.02 M KH_2PO_4 (see reference 21).

Shodex IonPak columns have been shown to be effective in the characterization of high molecular weight polysaccharides, polyethylene glycols, and poly(vinyl alcohol) polymers utilizing water as a mobile phase (40). However, since the packing is ionic, the columns would be best employed in neutral to low pH mobile phases to avoid ion-exclusion or exchange separation mechanisms. In some cases ion-exclusion and hydrophobic interactions have been exploited to achieve separation of acidic from neutral compounds and small organic molecules (41).

μ-Bondagel and several glycophase-type packings have also been applied to the analysis of water-soluble polymers utilizing mobile phase ionic strengths of 0.1 μ or greater (42,43). Ionic interactions caused for example by residual silanol sites can be minimized

by higher ionic strength mobile phases; hydrophobic interactions with these supports can usually be controlled by adding organic solvent modifiers. Due to excellent mechanical strength of the supports, such packings can also be utilized for synthetic polymer analysis in organic solvents. μ-Bondagel has been used to characterize a number of water-soluble polymers, including sulfonated polystyrenes, poly(vinyl alcohol), and anionic polyelectrolytes (44).

For very polar synthetic polymers such as polyacrylamide, poly(acrylic acid), and polyethyleneimine, mobile phase ionic strengths greater than 0.3 μ give satisfactory results for MicroPak TSK PW type columns but SW type columns exhibit adsorption effects even at these ionic strengths, as do most other silica-based packings. The use of acetic acid as a mobile phase modifier has also been found to decrease non-exclusion interactions with PW type columns (23). In general, PW type columns give better performance than SW type columns for water-soluble organic polymers due to availability of larger pore size columns and minimal non-exclusion effects for polar, water-soluble polymers. Characterization of cationic polymers has also been reported on PW columns (45,46).

In exclusion chromatography, separation is achieved solely on the basis of effective molecular size of a solute only when no significant interactions take place between the stationary phase and the sample. For such a separation, the support material must be inert (i.e., contain no active surface sites) with the eluent used for analysis and the solute must be recovered with 100% efficiency. In practice, unwanted non-exclusion interactions or the inertness of a support material can be evaluated from solute elution volume and recovery in a given mobile phase. Recovery data for water-soluble polymers analyzed on MicroPak TSK 3000PW columns in various mobile phases is shown in Table 6 (24). Polymer mass recovery was defined as the peak area ratios of injections made with and without a column. Polymers studied were poly(vinyl alcohol) (PVA), poly(ethylene oxide) (PEO), poly(vinyl pyrrolidone) (PVP), poly(acrylamide) (PAM), and poly(acrylic acid) (PAA). Higher recoveries of

Table 6.
Recovery Data for Water Soluble Polymers on MicroPak TSK Gel PW Type Columns

Polymer	MW	3000PW (Water)	3000PW (0.08 M Tris)
Polyvinyl alcohol (PVA)	125,000	80%	98%
Polethylene oxide (PEO)	50,000	99	-
Polyvinyl pyrrolidone (PVP)	10,000	60	100
Polyacrylamide (PAM)	600,000	40*	80*
Polyacrylic acid (PAA)	90,000	-	84

Sample Loading: 50 to 300 μg
* 3000PW + 5000PW columns in series.

PVP and PAM polymers, both acidic polyelectrolytes, as well as PVA with 0.08 M tris eluent are due to reduction of hydrophilic interactions between packing and sample as a consequence of increased ionic strength of the mobile phase. No comparative synthetic polymer recovery data has been published utilizing similar microparticulate aqueous SEC supports.

Comparison of Microparticulate Aqueous SEC Columns for Biopolymer Analysis

High performance exclusion chromatography is a promising tool in protein chemistry for purification and molecular weight characterization of proteins. The utility of high speed SEC biopolymer separations depends upon adequate recovery of proteins in their native (e.g., active) form after passage through the column. Previous supports used for high speed SEC separations based on silica or controlled pore glass have suffered from poor recovery due to high densities of acidic silanol groups which contribute to adsorption and denaturation of proteins (25). Pfannkoch *et al.* (7) have measured recovery of enzyme activity from several new silica-based bonded phase support materials. Recovery of trypsin activity from such columns was greater than 86% for all supports tested and almost quantitative for some supports (LiChrosorb Diol; SynChropak GPC 100; and TSK 3000SW columns). Fukano *et al.* (26) using TSK 3000SW and 4000SW columns have reported almost quantitative mass

recovery of a number of proteins and quantitative recovery of activity for several enzymes.

Mass recovery of two proteins, cytochrome C (MW 13,500) and fluorescein conjugated rabbit anti-human IgG (MW 150,000) was determined using a MicroPak TSK 3000SW column (27). Protein recovery was defined as the peak area ratios of injections made with and without a column. As shown in Table 7, recoveries range from 84 to 88%.

To determine recovery of enzyme activity, β-galactosidase (grade IV from *E. coli*, Sigma Chemical Co.) was injected using the assay buffer (0.1 M KH_2PO_4 + 0.01 M KCl + 0.001 M $MgSO_4$ + 0.05 M β-mercaptoethanol, pH 7.0) as mobile phase. Enzyme activity was measured with and without a column using a modification of the ONPG hydrolysis procedure described by Miller (28). β-galactosidase, which has a molecular weight of about 520,000 daltons, elutes within the permeation volume from a MicroPak TSK 4000SW column. Activity recovery of ONPG units was 98%.

Recovery of cytidine kinase activity from MicroPak TSK 3000SW columns has also been determined (W. Kreis, Sloan-Kettering Institute, unpublished results). Two 7.5 mm x 30 cm 3000SW columns were used in series with 50 mM potassium phosphate (pH 6.8) as mobile phase flowing at 1.0 ml/min; 20 μl (containing 0.6 mg protein) of a

Table 7.
Protein Mass Recovery from MicroPak TSK 3000SW Column*

Sample	Detection	Chromophore	Recovery **
Anti-Human IgG, 31 μg	495nm	Fluorescein	88.1
Anti-Human IgG, 31 μg	280nm	Tyrosine, Tryptophan	84.1
Cytochrome C, 10 μg	280nm	Tyrosine, Tryptophan	84.9

* Conditions: Mobile Phase: 0.01 M KH_2PO_4 (pH 7.2) + 0.1 M KCl
Flow Rate: 1.0 ml/min
Temperature: 30°C

$$** \text{ Recovery} = \frac{\text{Total Area With Column}}{\text{Total Area Without Column}} \times 100$$

mouse Ascites homogenate was injected and fractions were collected, held on ice, and assayed for cytidine kinase activity after the method of Kreis et al. (29). Activity of the pooled fractions was 70%, 89.3%, and 97.4% of the uninjected sample for three separate injections. (Collected fractions were held on ice for two hours prior to assay, and run-to-run variation may reflect technical variations in the assay).

Calibration curves for MicroPak TSK SW columns using protein standards are displayed in Figure 6. The utility of these columns for biopolymer separations has been demonstrated by Wehr and Abbott (30). Kato et al. (31) demonstrated the TSK 3000SW column to be the most useful SW column for protein separations.

Protein calibration curves for selected MicroPak TSK PW type columns are shown in Figure 7. The adsorption effects of proteins on 3000PW and 5000PW columns are slightly greater than the SW type columns; however, recovery of proteins from these PW type columns is greater than 80% in most cases (32). Hashimoto et al. (33) have shown TSK 3000PW and 4000PW columns to be suitable for biopolymer separations. In general, the 3000PW column has been found to be the most useful PW column pore size for protein analysis.

To compare MicroPak TSK SW and PW columns for protein separations, specific resolution curves using protein standards were constructed for 3000SW and 3000PW columns as shown in Figure 8. These plots clearly show the 3000SW column to provide higher resolution over most of the molecular weight region covered by these columns for protein analysis.

Hara et al. (34) have utilized a TSK 5000PW column and a 3000SW column in series for the analysis of serum lipoproteins. Such a column set takes advantage of the higher molecular weight region over which the larger PW column pore sizes operate and the efficiency gained by use of SW columns.

Spheron packing has been applied to the separation of glycoproteins and protoglycans (47). Although hydrophobic interactions with the packing were found to be significant, such interactions in

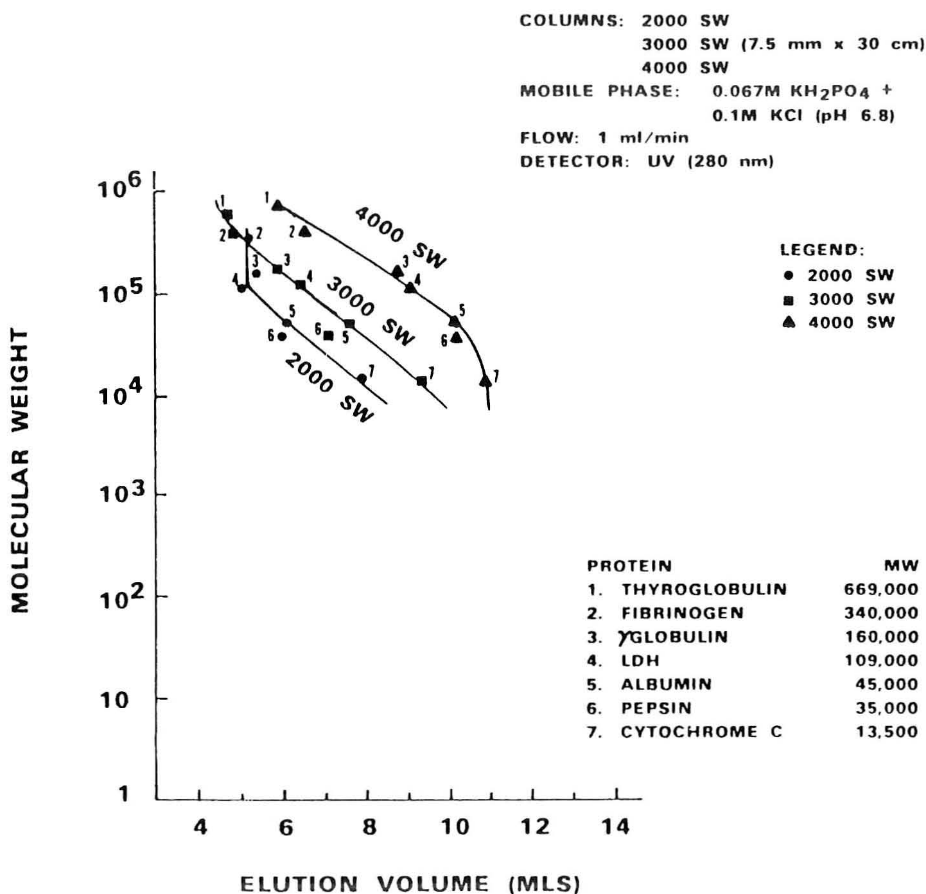

FIGURE 6. Protein standard calibration curves for MicroPak TSK gel type SW columns. Mobile phase: 0.067 M KH_2PO_4 + 0.1 M KCl (pH 6.8); Flow rate: 1 ml/min; Injection volume: 20 μl; Column dimensions: 7.5 mm x 30 cm; Detector: UV (280nm).

some cases were exploited for the separation of peptides, proteins, and nucleic acids (48,49).

Significant hydrophobic interactions in aqueous mobile phases of Shodex OHpak has been employed in the separation of amino acids (50). This packing has also been applied to the analysis of some peptides.

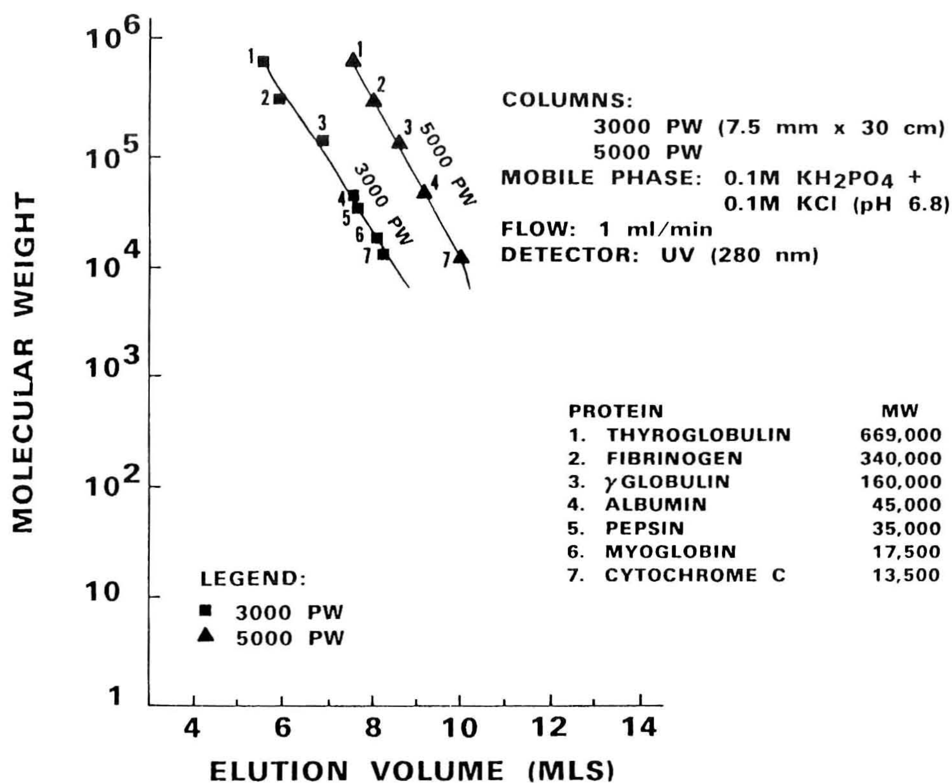

FIGURE 7. Protein standard calibration curves for MicroPak TSK 3000PW and 5000PW columns. Mobile phase: 0.1 M KH_2PO_4 + 0.1 M KCl (pH 6.8); Flow rate: 1 ml/min; Column dimensions: 7.5 mm x 30 cm; Detector: UV (280nm).

Due to hydrophobic interactions, ethylene glycol or SDS has been used as a mobile phase modifier with μBondagel columns for protein characterization (44,51). The Waters Protein Columns have been employed for separation of proteins with acidic to mildly basic isoelectric points and enzymes (51,52,54). Apparently, proteins with higher isoelectric points (> 8) experience varying degrees of adsorption with these columns.

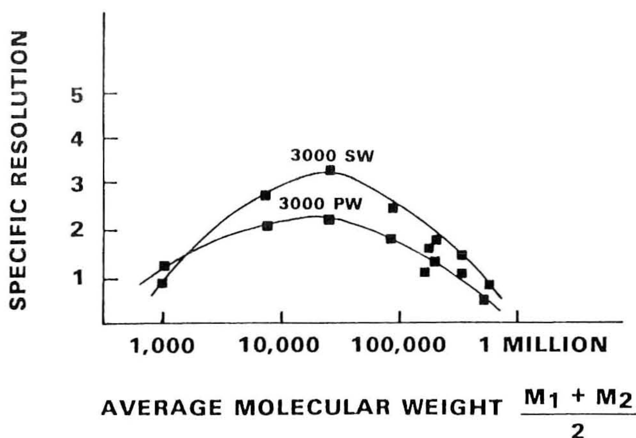

FIGURE 8. Comparison of specific resolution (R_{SP}) curves for MicroPak TSK 3000SW and 3000PW columns using protein standards. Mobile phase: 0.1 M KH_2PO_4 + 0.1 M KCl (pH 6.8); Flow rate: 1 ml/min; Injection volume: 100 μl; Column dimensions: 7.5 mm x 30 cm.

Characterization of Non-Exclusion Effects on MicroPak TSK SW and PW Columns

Non-exclusion effects resulting from solute-support interactions can be broadly classified as arising from hydrophilic interactions (ionic effects such as ion exchange, ion exclusion, ion inclusion) and hydrophobic interactions. Such interactions in aqueous SEC usually result in various degrees of adsorption (35,36).

Examples of hydrophobic interactions on a 2000SW column have been observed for xanthines (20) and pectins (37). In most cases, addition of small amounts of an organic modifier (5-10% MeOH) overcome adsorption effects of this type.

Hydrophobic and other non-exclusion interactions on SW and PW columns were characterized using a series of selected amino acids as test probes. These compounds were chosen in part due to a previously measured "hydrophobicity scale" defined by Rekker (38). Table 8 shows the summation of fragmental hydrophobicity constants for each

Table 8.

Hydrophobic Fragmental Constants of the Common Amino Acids

Amino Acid	Σf
Tryptophan	2.31
Phenylalanine	2.24
Leucine	1.99
Isoleucine	1.99
Tyrosine	1.70
Valine	1.46
Cystine	1.11
Methionine	1.08
Proline	1.01
Cysteine	0.93
Arginine	-
Alanine	0.53
Lysine	0.52
Glycine	0.00
Aspartic Acid	-0.02
Glutamine	-0.07
Histidine	-0.23
Threonine	-0.26
Serine	-0.56
Asparagine	-1.05
Glutamic Acid	-1.09

Where Σf = Summation of Fragmental Hydrophobic Constants.

amino acid. Negative numbers represent hydrophilic amino acids. Positive fragmental constants represent hydrophobic amino acids. The larger the positive summation, the "more hydrophobic" the amino acid. Tryptophan, phenylalanine, leucine, tyrosine, valine, and cysteine were chosen as test probes. To serve as controls, mono-, di-, tri-, and tetraglycine (MW $\sim$ 100 to 500) were analyzed on each column to ensure differences in amino acid retention were not due to molecular size differences. Glycine oligomers coeluted on all columns tested except the 2000PW column which exhibited some separation. It should also be noted that of the amino acid probes used, tryptophan, phenylalanine, and tyrosine are aromatic, and leucine, valine, and cysteine are non-aromatic compounds.

The amino acid probes were analyzed on MicroPak TSK 2000PW, 3000PW, and 5000PW columns as well as 2000SW, 3000SW, and 4000SW columns with a mobile phase of 0.01 M KH_2PO_4 (pH 6.8). Results are displayed in graph form in Figures 9 and 10. Summation of the hydrophobic fragmental constant is plotted versus k' for the amino acid probes. Several points are noteworthy:

i. A non-linear relationship exists between k' and hydrophobicity as measured by Σf;

ii. The PW columns seem very sensitive to aromatic compounds which exhibit greater retention (higher k's) than the non-aromatic

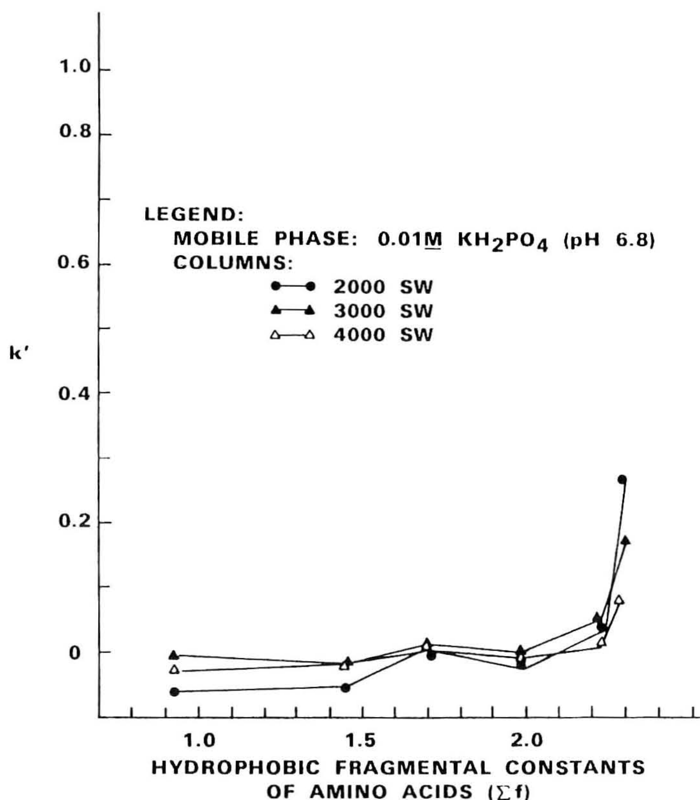

FIGURE 9. Comparison of hydrophobic interactions of MicroPak TSK SW type columns as measured with amino acid test probes. Mobile phase: 0.01 M KH_2PO_4 (pH 6.8); Flow rate: 1 ml/min; Injection volume: 100 μl; Column dimensions: 7.5 mm x 30 cm.

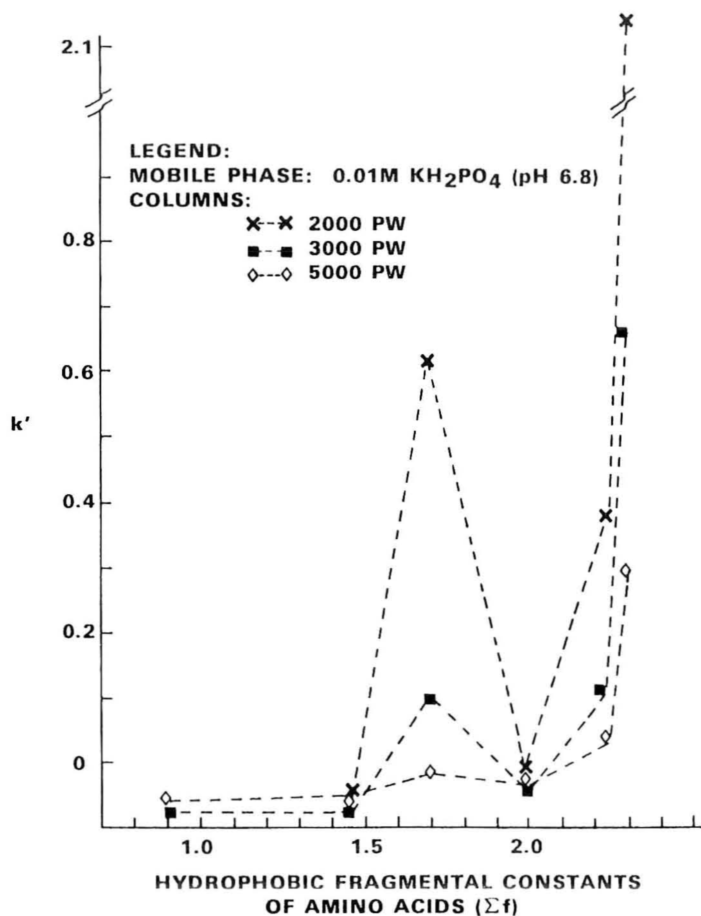

FIGURE 10. Comparison of hydrophobic interactions of MicroPak TSK Gel 2000PW, 3000PW, and 5000PW columns as measured with amino acid test probes. Conditions same as in Figure 9.

compounds. This is clearly demonstrated for tyrosine (Σf = 1.7);

iii. The sensitivity to aromatic compounds of the PW columns greatly increase with decreasing pore size.

Ion exchange and ion exclusion interactions were evaluated using charged and neutral amino acid test probes of similar molecular weights:

Pair 1: Methionine - neutral, MW 149
Lysine - net charge (+), MW 146
Pair 2: Threonine - neutral, MW 119
Aspartic acid- net charge (-), MW 133

These pairs of hydrophilic amino acid test probes were analyzed on PW and SW columns in mobile phases of increasing ionic strength. Ionic effects as measured by retention volume (k') as a function of ionic strength were negligible on both SW and PW columns using amino acid solute probes. However, Pfannkoch et al. (see reference 7) have shown TSK 3000SW column packing to have negative charge, resulting in ion-exclusion behavior with citric acid. Increasing mobile phase ionic strength above 0.12 M was shown to eliminate this effect.

CONCLUSION

Most commercially available microparticulate supports characterized and compared in this work exhibit varying degrees of non-exclusion effects in aqueous SEC separations. All the surface-modified, silica-based supports function as weak cation exchangers (see reference 7). All organic gel-based supports have some tendency toward hydrophobic interaction with a variety of solutes. In many instances, however, such non-exclusion effects can be exploited to achieve the desired chromatographic separation.

Chromatographic performance of commercially available aqueous SEC columns seems to be best judged by comparison of specific resolution values over the molecular weight range of operation. However, due to lack of comparative data of this type for most aqueous SEC columns, performance can be estimated based upon the slope of the calibration curve in the selective permeation range (linear molecular weight separation range) as well as efficiency and pore volume data.

A comparison of MicroPak TSK SW and PW columns reveals SW type columns have a more limited molecular weight separation range than PW type columns, although SW columns exhibit higher efficiency and resolution. Due to both limited pore size range and adsorption effects of polar, synthetic, water-soluble polymers on SW columns, PW

columns are recommended for the analysis of synthetic water-soluble polymers. SW type columns offer higher efficiency and resolution than PW type columns for protein analysis (3000SW most useful). Small pore size PW columns (1000PW and 2000PW) are best suited for small molecule analysis (MW 100 to 1000) as evidenced by large specific resolution values for PEG standards.

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REFERENCES

1. Porath, J., and Flodin, P., Gel Filtration Chromatography of Biopolymers, Nature 183, 1657 (1959).

2. Mizutani, T., and Mizutani, D., Prevention of Adsorption of Proteins on CPG Packings, J. Chromatogr. 111, 214 (1975).

3. Application Data on Shodex IonPak. Technical Data 77, Showa Denko K.K., Tokyo, Japan.

4. Application Data on Shodex OHpak. Technical Data 77,8, Showa Denko K.K., Tokyo, Japan.

5. Regnier, F. E., and Noel, R., Glyceryl Propyl Silane Bonded Phases in Aqueous Exclusion of Biopolymers, J. Chromatogr. Sci. 14, 316 (1976).

6. Becker, N., and Unger, K. K., Aqueous SEC Analysis of Proteins and Amino Acids on Bonded Amino Silica Packings, Chromatographia 12, 539 (1979).

7. Pfannkoch, E., Lu, K. C., Regnier, F. E., and Barth, H. G., Characterization of Several Commercial High Performance Aqueous SEC Columns, J. Chromatogr. Sci. 18, 430 (1980).

8. Barth, H. G., Practical Approach to Aqueous SEC and Compendium of SEC Columns, J. Chromatogr. Sci. 18, 409 (1980).

9. TSK Gel SW Type Column Technical Data and TSK Gel PW Type Column Technical Data, Toyo Soda Mfg. Co., Ltd., Tokyo, Japan (1978).

10. Fukano, K., Komiya, K., Sasaki, H., and Hashimoto, T., Evaluation of TSK SW Type Columns for High Pressure Aqueous SEC, J. Chromatogr. 166, 47 (1978).

11. Kato, Y., Komiya, K., Sasaki, H., and Hashimoto, T., High Performance SEC of Oligomers Using TSK PW Type Columns, J. Chromatogr. 153, 546 (1978).

12. Kato, Y., personal communication.

13. TSK Gel SW Type Column Technical Data, Toyo Soda Mfg. Co., Ltd., Tokyo, Japan (1978).

14. Hashimoto, T., Sasaki, H., Aiura, M., and Kato, Y., High-speed Aqueous GPC Using TSK PW Columns, J. Polymer Sci: Polymer Physics Edition 16, 1789 (1978).

15. deVries, A. J., LePage, M., Beau, R., and Guillemin, C. L., Evaluation of Porous Silica as Packing for GPC, Anal. Chem. 39, 935 (1967).

16. Bly, D. D., Resolution in GPC, J. Polymer Sci: Part C 21, 13, (1967).

17. Yau, W. W., Kirkland, J. J., Bly, D. D., and Stoklosa, N. J., Effect of GPC Column Performance on Accuracy of Molecular Weights Obtained from Exclusion Chromatography, J. Chromatogr. 125, 219 (1976).

18. Kirkland, J. J., and Antle, P. E., High Performance SEC of Small Molecules with Porous Silica Columns, J. Chromatogr. Sci. 15, 137 (1977).

19. Kato, Y., Komiya, K., Sasaki, H., and Hashimoto, T., Separation Range and Separation Efficiency in High-Speed GFC on TSK SW Type Columns, J. Chromatogr. 190, 297 (1980).

20. Alfredson, T. V., Wehr, C. T., and Tallman, L., Comparative Performance of Microparticulate Packings for Aqueous SEC. Presented at American Chemical Society Meeting, Las Vegas, 1980. Symposium on Polymeric Separation Media, Division of Polymer Chemistry, Plenum Press (in press).

21. Alfredson, T. V., and Majors, R. E., New Packings (TSK SW and PW) for Aqueous SEC. Paper presented at Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (1980).

22. Stenlund, B., Giddings, J. C., Grushka, E., Cazes, J., and Brown, P., Advances in Chromatography 14, 37 (1976).

23. Alfredson, T., unpublished results.

24. Herman, D. P., Field, L. R., and Abbott, S., Size Exclusion Behavior of Synthetic Water Soluble Polymers on Diol Bonded Phase Supports (Comparison to TSK PW Type Columns), J. Chromatogr. Sci. 19, 470 (1981).

25. Hiatt, C. W., Shelokov, A., Rosenthal, E. J., and Galimore, I. M., Treatment of CPG Packings to Prevent Adsorption, J. Chromatogr. 56, 362 (1972).

26. Fukano, K., Komiya, K., Sasaki, H., and Hashimoto, T., Evaluation of New Supports for Aqueous SEC: TSK SW Type Columns, J. Chromatogr. 166, 47 (1978).

27. Wehr, C. T., Klink, F. E., and Robinson, J., Recovery of Proteins from High Speed SEC Columns (TSK SW Type). Varian LC Application Note #102, Varian Instrument Division, Walnut Creek, CA.

28. Miller, J. H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, 1972, pp. 352-355.

29. Kreis, W., Hession, C., Soricelli, A., and Scully, K., Cancer Treatment Reports 61, 1355 (1977).

30. Wehr, C. T., and Abbott, S. R., High Speed SEC of Biopolymers Using MicroPak TSK SW Columns, J. Chromatogr. 185, 453 (1979).

31. Kato, Y., Komiya, K., Sasaki, H., and Hashimoto, T., Separation Range and Resolution in High-speed GFC on TSK SW Columns, J. Chromatogr. 190, 297 (1980).

32. Alfredson, T., unpublished data.

33. Hashimoto, T., Sasaki, H., Aiura, M., and Kato, Y., High Speed GFC of Proteins Using TSK PW Columns, J. Chromatogr. 160, 301 (1978).

34. Hara, I., Okazaki, M., and Ohno, Y., Rapid Analysis of Chlosterol of High Density Lipoprotein and Low Density Lipoprotein in Human Serum Using TSK SW and PW Columns, J. Biochem. 87, 1863 (1980).

35. Cooper, A. R., and Matinger, D. P., Aqueous GPC, Amer. Lab. 9 (1), 13 (1977).

36. Yau, W. W., Kirkland, J. J., and Bly, D. D., Modern Size Exclusion Chromatography, Wiley-Interscience, New York, 1979.

37. Klink, F., Selectivity in Aqueous SEC of Pectins Using SW Columns, Varian LC Applications Note #106, Varian Instrument Division, Walnut Creek, CA.

38. Rekker, R. F., The Hydrophobic Fragmental Constant, Elsevier, Amsterdam, New York, 1977.

39. Barker, P. E., Hatt, B. W., and Holding, S. R., Problems Associated With the Column Packings Used in the Characterization of Dextran by Aqueous Gel Permeation Chromatography, J. Chromatogr. 174, 143-151 (1979).

40. Miller, R. L., and Vandemark, F. L., Characteristics of Shodex Aqueous SEC Columns: The Analysis of Polyhydric Polymers, Perkin-Elmer Newsletter, vol. 8, no. 3, December, 1980, pp. 69-72.

41. Application Data on Shodex IonPak. Technical Data 77, Showa Denko K. K., Tokyo, Japan.

42. Barth, H. G., and Regnier, F. E., High-Performance GPC of Water-Soluble Cellulosics, J. Chromatogr. 192, 275-93 (1980).

43. Applications of Aquapore Columns. Chromatix, Sunnyvale, CA.

44. Vivilecchia, R. V., Lightbody, B. G., Thimot, N. Z., and Quinn, H. M., The Use of Microparticulates in Gel Permeation Chromatography, J. Chromatogr. Sci. 15, 424-433 (1977).

45. Dubin, P. L., and Levy, I. J., Polymer Preprints, 22(1), 1980. Also available from Bio-Rad Laboratories, Richmond, CA.

46. Kato, Y., and Hashimoto, T., High-Speed Aqueous GPC of Cationic Polymers, Toyo Soda Mfg. Co. Ltd., Tokyo, Japan. Available from Varian Associates, Walnut Creek, CA. Submitted to J. Chromatogr.

47. Vytasek, R., Coupek, J., Macek, K., Adam, M., and Deyl, Z., Application of the Sorption Properties of Spheron Gels in High Resolution Liquid Column Chromatography of Naturally Occuring Macromolecular Species, J. Chromatogr. 119, 549-56 (1976).

48. Strop, P., Mikes, F., and Chytilova, Z., Hydrophobic Interaction Chromatography of Proteins and Peptides on Spheron P-300, J. Chromatogr. 156, 239-54 (1978).

49. Strop, P., Basnak, I., and Farkas, J., Separation of Alkyl Derivatives of Uracil by Solvophobic Adsorption Chromatography on Spheron, J. Chromatogr. 138, 47-62 (1977).

50. Application Data on Shodex OHpak. Technical Data 77.8, Showa Denko K. K., Tokyo, Japan.

51. Hodge, J. L., and Rossomando, E. F., Separation of Enzymatic Activities in Dictyostelium Discoideum by HPGPC, Anal. Biochem. 100, 179-83 (1979).

52. Waters Associates, Milford, Mass., Bulletin 82967, January, 1980.

53. Coupek, J., Krivakova, M., and Pokorny, S., New Hydrophilic Materials for Chromatography - Glycol Methacrylates; Institute of Macromolecular Chemistry, Czechoslovakia Academy of Sciences, Prague 616, Czechoslovakia.

54. Pollak, J. K., and Campbell, M. T., Rapid Purification of the Unstable Enzyme Carbamoyl Phosphate Synthase by HPLC, J. Liquid Chromatogr. 4(4), 629-638 (1981).

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RAPID ISOLATION OF ECDYSTEROIDS FROM CRUSTACEAN TISSUES AND CULTURE MEDIA USING SEP-PAK C18 CARTRIDGES

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ABSTRACT

A liquid chromatographic method for isolating ecdysteroids from crab tissues and crustacean tissue culture media is reported. The method employs commercially-available Sep-Pak C18 cartridges containing a reversed phase packing that retains ecdysteroids. The technique is simple and rapid; it typically yields recoveries in the range of 85-90% for extractions of tissues, and 90-95% for extractions of media.

INTRODUCTION

Ecdysteroids are steroid hormones that regulate growth and molting in arthropods (cf. 1). Among the ecdysteroids, ecdysone, the apparent secretory product of arthropod molting glands, and ecdysterone, a 20-OH metabolite which stimulates the cellular events that lead to growth and molting, are of primary interest.

A reliable quantitative assay for ecdysteroids, in terms of specificity, resolution, and sensitivity, is UV absorbance at 254 nm coupled with high pressure liquid chromatography

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(HPLC) (2). However, the method applied to extracts of arthropod tissues requires removal of numerous contaminants prior to HPLC. The clean-up procedure, usually solvent partition, is tedious, time-consuming, and expensive, with losses of ecdysteroids attending the several steps.

We report a rapid, efficient method for isolating ecdysteroids from crustacean tissues and culture media using Sep-Pak C18 chromatographic cartridges. Coupled with HPLC, the procedure reduces the preparation steps by 50% and the time involved by 75%.

MATERIALS AND METHODS

Chemicals and Supplies

Sep-Pak C18 cartridges (approximately 1 cm x 1 cm, containing octadecylsilane bonded phase retained between two filters), were purchased from Waters Associates, Inc. (Milford, Mass.). High purity water and glass distilled MeOH were from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.). Ecdysone was from Simes (Milano, Italy) and ecdysterone from Rhoto Pharmaceutical (Osaka, Japan); both were purified on HPLC before use. Medium 199 (10X concentrate with Hank's salts and L-glutamine) was purchased from GIBCO (Grand Island, N.Y.). The salts used in Pantin's crustacean saline (3) were reagent grade. Fluoropore filters were from Millipore (Bedford, Mass.).

Experimental Animals

Cancer antennarius crabs (male, 5-10 cm in breadth) were purchased from Pacific Bio-Marine (Venice, Cal.). They were maintained in artificial sea water in compartmented marine trays, exposed to a photoperiod of 15L:9D, and fed pieces of fish every other day.

High Pressure Liquid Chromatography

A Waters Model 6000 pump with Waters U6K injector and ISCO UA5 absorbance monitor and Type 6 optical unit (ISCO, Lincoln, Neb.) were used for HPLC analysis. Quantitation of ecdysteroids was at 254 nm using a LDC Model 308 computing integrator (LDC, Riviera Beach, Florida). A uPorasil column from Waters was used with a solvent system of $CHCl_3$/ 95% EtOH (86:14) pumped at 2 ml/min (1200 psi). HPLC solvents were Burdick and Jackson glass distilled and were filtered before use.

Procedure

Standard procedure was to connect a Sep-Pak C18 cartridge to a luer-tip glass syringe, and to prime the cartridge by washing sequentially with 2 ml of methanol (MeOH) and 5 ml of H_2O. A sample was then placed in the syringe and injected through the cartridge. Subsequent washing and elution varied with experimental objective and is described below.

Recovery of ecdysteroids was monitored by adding ecdysone and ecdysterone standards to samples intended for extraction and Sep-Pak processing (all standards were made up in $CHCl_3$/ 95% EtOH (86:14)). Recoveries were calculated by dividing yield by the starting amount of standard, each determined by HPLC. In the case of blood and tissue extractions, a control blank (containing blood or tissue but not standards) was included; values of any endogenous ecdysteroid were subtracted from yields of the experimental samples before calculating recovery. Control blanks were not included with the two media extracted since repeated measurements beforehand showed the media did not contain endogenous ecdysteroids.

To determine the MeOH concentration required to elute ecdysteroids from Sep-Pak cartridges, 10 ul each of ecdysone

(181.5 ug/ml) and ecdysterone (179.0 ug/ml) were added to test tubes and allowed to air dry. Five ml of 1 of 11 test solvents were added to each tube: H_2O, MeOH diluted with water to concentrations of 10-100%. After priming a Sep-Pak cartridge, a test solvent was injected, and the resultant elutant collected, dried down on a rotary evaporator (BUCHI Rotavapor, Switzerland), and taken up in 1 ml $CHCl_3$/ 95% EtOH (86:14). Each sample was then injected through a 0.5 um fluoropore filter, dried under a stream of N_2, taken up in 250 ul $CHCl_3$/95% EtOH (86:14), and the recovery of ecdysteroids quantitated on HPLC.

For the extraction of ecdysteroids from Pantin's saline, standards were added in 3 quantities: 5, 10, or 15 ul each of ecdysone (181.5 ug/ml) and ecdysterone (179.0 ug/ml). After allowing the standards to dry, 5 ml of Pantin's saline were added to each tube and vortexed. The Sep-Pak procedure: 1) prime cartridge, 2) inject sample, 3) rinse with 10 ml of H_2O, and 4) elute ecdysteroids with 10 ml of 100% MeOH. The elutant was dried on a rotary evaporator and prepared for HPLC analysis as described above. Individual assays were performed on 5 separate tubes for each set of standards.

Three sets of standards were also used in the extraction of Medium 199: 10, 20, and 30 ul each of ecdysone (101.7 ug/ml) and ecdysterone (107.5 ug/ml). After the standards had dried, 2 ml of Medium 199 were added to each tube. The Sep-Pak procedure: 1) prime cartridge, 2) inject sample, 3) rinse with 10 ml of H_2O, 3) rinse with 15 ml of 20% MeOH, and 5) elute with 10 ml of 100% MeOH. Five trials were conducted on each set of standards.

For the extraction of ecdysteroids from crab tissues, a male _Cancer antennarius_ (intermolt, body wt.: 488.5 g) was exsanguinated and sacrificed. A window was cut in the

dorsal carapace with a bench dental drill (EMESCO, N.Y., N.Y.), and tissue samples dissected free and weighed on a Roller-Smith balance. Tissues were homogenized by hand in 10 ml of 100% MeOH using a ground glass tissue homogenizer. A one ml aliquot of the homogenate was pipeted into a centrifuge tube to which ecdysteroid standards had been added (20 ul ecdysone (101.7 ug/ml) and 20 ul ecdysterone 107.5 ug/ml)), and one ml was pipeted into a control tube to which no standards had been added. This was repeated 5X for each tissue, with vigorous vortexing between each pipeting. The homogenate was centrifuged for 15 min at 2000 RPM, and the supernatant removed and saved. After adding another ml of MeOH to the pellet, the sample was recentrifuged and the second ml of supernatant added to the first. The combined supernatants were dried on a rotary evaporator, and the dried residue taken up in 5 ml of H_2O and added to a primed Sep-Pak cartridge. After rinsing with 10 ml of H_2O and 10 ml of 20% MeOH, the cartridge was eluted with 10 ml of 100% MeOH and the elutant dried down and prepared for HPLC.

The crab blood was allowed to clot, centrifuged for 15 min at 2000 RPM, and the serum removed. One ml of serum was pipeted into a centrifuge tube to which standards had been added (20 ul ecdysone (101.7 ug/ml) and 20 ul ecdysterone (107.5 ug/ml)), and one ml was pipeted into a control tube. Blood samples were then injected into a primed Sep-Pak cartridge. The rinse and elution procedure was as for crab tissues.

RESULTS

The effect of MeOH concentration on the elution of ecdysteroids from Sep-Pak C18 cartridges is shown in Table 1. Water, 10% MeOH and 20% MeOH do not elute ecdysteroids

TABLE 1

The Effect of MeOH Concentration on the Elution of Ecdysteroids From Sep-Pak C18 Cartridges

Solvent		Percent Recovery	
		Ecdysone	Ecdysterone
H_2O		0	0
MeOH	10%	0	0
	20%	0	0
	30%	6.2 ± 8.3	24.0 ± 7.0
	40%	42.5 ± 2.8	74.2 ± 4.9
	50%	69.1 ± 2.3	83.1 ± 4.5
	60%	85.4 ± 3.2	92.1 ± 10.3
	70%	90.2 ± 5.9	93.9 ± 3.9
	80%	89.7 ± 13.9	90.2 ± 10.0
	90%	88.6 ± 0.2	95.5 ± 8.4
	100%	89.7 ± 0.6	90.2 ± 2.5

from the cartridges. Accordingly, these more polar solvents can be used to de-salt samples, and to wash components such as sugars, amino acids, hydrophilic proteins, and other polar organics from biological extracts that contain ecdysteroids.

As the MeOH concentration of the solvent is increased, the recovery of ecdysteroids in the elutant also begins to increase (TABLE 1). The difference in percent recovery between ecdysone and ecdysterone seen with solvents of intermediate polarity (e.g. 40% MeOH) is a reflection of the relative polarities of the two ecdysteroids, i.e., the less polar ecdysone is retained more strongly by the octadecylsilane bonded phase of the cartridge than is the more polar ecdysterone.

Concentrations of MeOH greater than 70% can be used to elute ecdysteroids quantitatively from Sep-Pak cartridges. (TABLE 1). 100% MeOH was chosen for use in future experiments

because, compared to solvents containing water, it dries more quickly and is less prone to loss of yield through bumping during rotary evaporation.

Pantin's saline and Medium 199 are among the culture media that have been utilized for in vitro studies on the synthesis and secretion of ecdysteroids by crustacean molting glands (4,5). That Sep-Pak cartridges can be used for the extraction of ecdysteroids from these media is shown in TABLE 2. The Sep-Pak rinse and elution procedure for Pantin's saline involves only a rinse with 10 ml of H_2O to remove the constituent salts, followed by the elution of ecdysteroids with 10 ml of 100% MeOH. Medium 199 is a complete, defined tissue culture medium; when only a water rinse is used before elution of ecdysteroids with 100% MeOH and subsequent HPLC analysis, a large contaminating

TABLE 2

Extraction of Ecdysteroids From Crustacean Culture Media

Culture Medium	Ecdysone		Ecdysterone	
	Standard (ug)	Percent Recovery	Standard (ug)	Percent Recovery
Pantin's Saline	0.9075	97.6 ± 6.9	0.8950	89.9 ± 1.9
	1.815	92.5 ± 5.9	1.790	96.4 ± 7.1
	2.7225	96.8 ± 3.8	2.685	97.2 ± 3.9
Medium 199	1.017	95.6 ± 9.7	1.075	102.7 ± 6.1
	2.034	91.0 ± 3.7	2.150	94.5 ± 5.7
	3.051	96.2 ± 3.1	3.225	87.9 ± 4.3

peak, migrating with a retention time similar to ecdysone standard, is found on the chromatographs. A wash with 15 ml of 20% MeOH effectively removes this contaminant and allows quantitation of the ecdysteroid standards.

Sep-Pak C18 cartridges can also be used to extract ecdysteroids from aqueous solutions of tissue extracts (TABLE 3). After injecting the aqueous solution through the cartridge, a rinse with 10 ml of H_2O, followed by 10 ml of 20% MeOH effectively cleans up these samples in preparation for HPLC. The decrease in percent recovery of ecdysteroids from tissue samples compared to the percent recovery from culture media is apparently due to the additional steps involved in tissue extraction per se (i.e., homogenization, centrifugation, evaporation and resuspension), which precede the Sep-Pak procedure itself.

DISCUSSION

The Sep-Pak technique is a simple and effective method for cleaning up biological samples prior to analysis of ecdysteroids by HPLC. It is a useful alternative to the usual solvent extraction and partition methods. Prior to the development of this technique, analysis of ecdysteroids in our laboratory was accomplished by the method outlined in FIGURE 1. This method was developed for use on crustacean tissues; it is a modification of the methods of Horn et al. (6) and of Kaplanis et al. (7), and is characteristic of similar techniques used by others. It requires a chloroform/methanol (2:1) extraction, two solvent partitions, a silica gel preparatory column preceding HPLC, and two HPLC steps. The method has proven effective, but is labor-intensive and extremely time-consuming. In our hands, recovery of ecdysteroids using this system is typically in the range

TABLE 3

Extraction of Ecdysteroids From Crab Tissues

Tissue	Wet Weight (mg)	Percent Recovery	
		Ecdysone	Ecdysterone
Gonad	893.0	92.9 ± 7.2	85.5 ± 6.6
Gill	943.6	87.2 ± 1.4	87.1 ± 5.1
Muscle	965.0	84.7 ± 2.6	83.7 ± 2.9
Hepato-pancreas	864.8	79.5 ± 15.0	88.2 ± 8.2
Blood	25.0[a]	90.0 ± 3.2	87.8 ± 9.5

[a]milliliters

of 40-50%. In contrast, the Sep-Pak procedure is rapid, easily performed, uses limited organic solvents, and requires minimal laboratory equipment. Moreover, recovery is typically 85-90% (TABLE 3). The utility of the method is even more apparent when one is extracting ecdysteroids from culture media. In this case, the preliminary solvent extraction can be eliminated, and the media injected directly through the cartridge. This decrease in the number of experimental steps is accompanied by an increase in recovery to 90-95%.

Because Sep-Pak C18 cartridges are relatively inexpensive, many workers will be dissuaded from attempting to reuse them. However, we have reused cartridges as many as five times with no decrease in recovery. Cartridges were routinely washed with 10 ml of 100% MeOH and equilibrated with 10 ml of H_2O before reuse.

The Sep-Pak method reported here should have widespread applications for both in vitro and in vivo studies on ecdysteroid physiology and arthropod growth and molting.

FIGURE 1. Extraction and purification of ecdysteroids for HPLC analysis using (a) solvent partition and (b) Sep-Pak C18 cartridges

REFERENCES

1. Riddiford, L.M., Insect endocrinology: action of hormones at the cellular level. Ann. Rev. Physiol., 42, 511, 1980.

2. Lafont, R., Somme-Martin, G., Mauchamp, B., Maume, B.F., and Delbecque, J.-P., Analysis of ecdysteroids by high-performance liquid chromatography and coupled gas-liquid chromatography - mass spectrometry. Progress In Ecdysone Research, Hoffman, J.A., ed., Elsevier, Amsterdam, 1980, p. 45.

3. Pantin, C.F.A., On the excitation of crustacean muscle. I. J. Exp. Biol., 11, 11, 1934.

4. Chang, E.S. and O'Connor, J.D., Secretion of α-ecdysone by crab Y-organs *in vitro*. Proc. Natl. Acad. Sci., 74(2), 615, 1977.

5. Keller, R. and Schmid, E., *In vitro* secretion of ecdysteroids by Y-organs and lack of secretion by mandibular organs of crayfish following molt induction. J. Comp. Physiol., 130, 347, 1979.

6. Horn, D.H.S., Fabbri, S., Hampshire, F., and Lowe, M.E., Isolation of crustecdysone (20R-hydroxyecdysone) from a crayfish (*Jasus lalandei H. Milne Edwards*), Biochem. J. 109, 399, 1068.

7. Kaplanis, J.N., Dutky, S.R., Robbins, W.E., Thompson, M.J., Lindquist, E., Horn, D.H.S., and Galbraith, M.N., Makisterone A: a 28-carbon molting hormone from the embryo of the milkweed bug. Science, 190, 681, 1975.

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PEPTIDE FORMATION IN THE PRESENCE OF METAL ION PROTECTING GROUPS. III. THE SEPARATION OF [$(NH_3)_5Co(III)$-] AMINO ACIDS AND PEPTIDES BY REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

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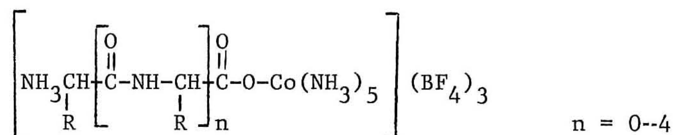
ABSTRACT

Pentaammine cobalt(III) amino acid and peptide complexes of the type



n = 0--4

can be separated rapidly under mild conditions by high pressure liquid chromatography (HPLC) using octadecyl silane derivatized columns (RP-18) with 0.2% sodium trifluoroacetate (NaTFA) in aqueous-methanol at pH 2.5. The retention time of these cobalt(III) complexes is a function of the hydrophobicity of the amino acid or peptide ligand and the total charge on the complex. In the trifluoroacetate media used for these separations, the hydrophobic ligand and the number of trifluoroacetate counter ions on the cobalt complex contribute to the selective elution of these complexes.

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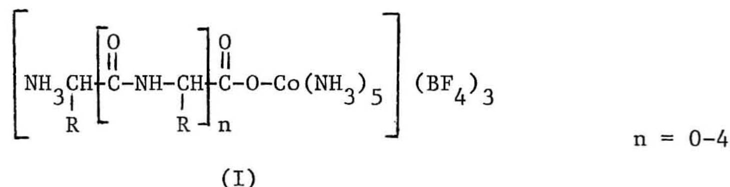
INTRODUCTION

A number of cobalt(III) complexes of amino acids and peptides of the general formula



have been synthesized using $[(NH_3)_5Co(III)-]$ as a carboxyl protecting group (1,2). In our earlier work we have used ion exchange (3) and gel filtration methods to purify the metal peptide complexes synthesized. These methods, although successful at times, were extremely time consuming especially when used at every step of the synthesis.

With the development of reverse phase high pressure liquid chromatography (HPLC) for the analysis of polar and charged molecules we became interested in developing methods to separate these metal-peptide complexes (I) using HPLC. The advantages of reverse phase HPLC over conventional ion exchange techniques for the separation of charged complexes are a) the speed of the analysis (ca few minutes), and b) the mild conditions used (ca pH 3, weakly acidic aqueous-organic solutions). Recently a number of chromatographic methods for the analysis of a variety of related cobalt(III) complexes using high pressure liquid chromatography (4-6) have been reported.

In this paper we present systematic methods that we have developed for the separation of $[(NH_3)_5Co(III)-]$ amino acid and peptide complexes (2), with hydrophobic and hydrophilic side chains, using reverse phase high pressure liquid chromatography. The cobalt peptide complexes have been synthesized by stepwise

peptide formation using $[(NH_3)_5Co(III)-]$ as a C-terminal protecting group (2).

RESULTS AND DISCUSSION

A number of methods using different salt media in methanol-water and acetonitrile-water solvents were attempted for the separation of the $[(NH_3)_5Co(III)-]$ amino acid and peptide complexes. In most cases broad unresolved peaks were obtained. In the following section some of these early methods are presented. These methods were found to be of only limited use. The separation method that we have adopted for the separation of cobalt(III) amino acid and peptide complexes is discussed under a separate heading.

All the separations have been obtained using octadecyl-silane derivatized silica gel columns (μ-Bondapak, 10 μ, Waters Assoc.) with the radial compression module.

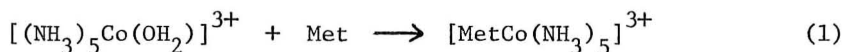
I. Early Methods

Ion Pairing with Sodium Heptanesulfonic Acid (NaHSA). At 0.1-0.2% NaHSA in aqueous-methanol the complexes $[(NH_3)_5Co(III)-L]$ where L = Gly, GlyGly, and GlyPhe were selectively eluted from the column with $[PheGlyCo(NH_3)_5]^{3+}$ retaining the most and $[GlyCo(NH_3)_5]^{3+}$ retaining the least. A similar separation for $[ProProCo(NH_3)_5]^{3+}$ and $[ProCo(NH_3)_5]^{3+}$ resulted in sharp peaks with longer retention times for $[ProProCo(NH_3)_5]^{3+}$. When this solvent system was used for other more hydrophobic cobalt peptides, e.g. $[PheLeuCo(NH_3)_5]^{3+}$, broad unresolved peaks were obtained. Addition of ammonium acetate (NH_4OAc) (1-2%) to NaHSA in aqueous-methanol extended the usefulness of this system, but here again, broad unresolved peaks were obtained for hydrophobic di- and tripeptides.

II. A General Method for the Separation of Hydrophobic and Hydrophilic Cobalt Peptide Complexes .

There are a number of reports on the separation of peptides on reverse phase high pressure liquid chromatography using trifluoroacetatic acid in aqueous organic solvents (7-10). By using trifluoroacetate anion (TFA) as a counter ion for the tripositively charged cobalt complexes, with, for example, 0.2% HTFA (pH adjusted to 2.5 with NaOH) in methanol-water mixtures varying from 20-60% methanol, we have been able to obtain good separation for a number of $[(NH_3)_5Co(III)\text{-amino acid}]$ and $[(NH_3)_5Co(III)\text{-peptide}]$ complexes of closely related structures. We have been able to resolve $[(NH_3)_5Co(OH_2)]^{3+}$ (2.6 min) from $[GlyCo(NH_3)_5]^{3+}$ (3.1 min); $[GlyGlyCo(NH_3)_5]^{3+}$ (3.2 min) from $[PheGlyCo(NH_3)_5]^{3+}$ (5.9 min) and $[ProProCo(NH_3)_5]^{3+}$ (6.3 min) with 20% methanol-water in 0.2% HTFA (pH 2.5) at a flow rate of 2.0 ml/min.

A large number of cobalt peptide complexes have been separated using this trifluoroacetate aqueous-methanol solvent system. Some representative examples will be shown here. Figure 1 shows the facile separation of $[(NH_3)_5Co(OH_2)]^{3+}$ from $[MetCo(NH_3)_5]^{3+}$ (Met = L-methionine).
This separation allows one to monitor the formation of $[MetCo(NH_3)_5]^{3+}$ from $[(NH_3)_5Co(OH_2)]^{3+}$ and methionine (eq 1)(2).



$$[(NH_3)_5Co(OH_2)]^{3+} + Met \longrightarrow [MetCo(NH_3)_5]^{3+} \quad (1)$$

For a series of $[(NH_3)_5Co(III)\text{-}]$ complexes with neutral amino acids, the retention time was found to be proportional to the hydrophobicity of the amino acid side chain. The complex $[PheCo(NH_3)_5]^{3+}$ retained more than $[ProCo(NH_3)_5]^{3+}$, which in turn retained more than $[GlyCo(NH_3)_5]^{3+}$, however, the differences were small (ca fractions of a minute) when using

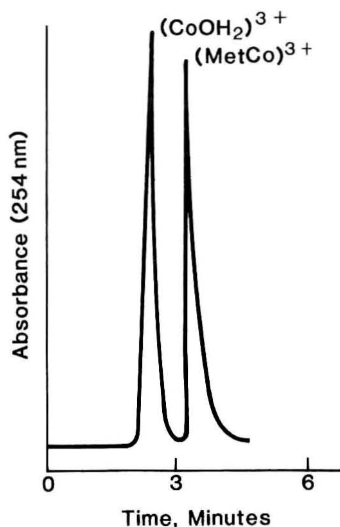


FIGURE 1.

Chromatographic Separation of $[(NH_3)_5Co(OH_2)]^{3+}$ from $[MetCo(NH_3)_5]^{3+}$. (0.2% TFA, pH 2.5, 20% methanol-water, 2 ml/min).

0.2% TFA , pH 2.5, 20% aqueous-methanol. No further separation of these $[(NH_3)_5$Co-amino acid] complexes was investigated.

For the cobalt(III)-peptide complexes, the retention time increased with the hydrophobicity of the peptide side chains. For example, $[ProProCo(NH_3)_5]^{3+}$ (6.3 min) retained significantly longer than $[GlyGlyCo(NH_3)_5{}^{3+}$ (3.2 min). Figure 2 shows the retention time of two different cobalt peptide complexes and the effect on the retention time of increasing the methanol concentration from 20 to 40%. In 40% methanol the retention time of the two peptide complexes decreased.

The biologically active pentapeptides Leu-enkephalin (TyrGlyGlyPheLeu) and Met-enkephalin (TyrGlyGlyPheMet) were synthesized by seqential peptide formation on $[(NH_3)_5$Co(III)-]

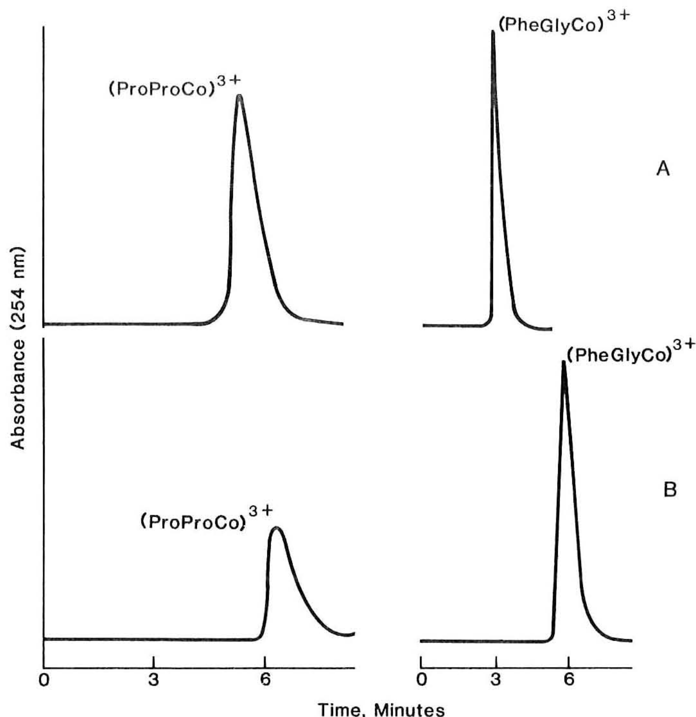


FIGURE 2.

Chromatograms of $[ProProCo(NH_3)_5]^{3+}$ and $[PheGlyCo(NH_3)_5]^{3+}$ in 40% methanol-water (A) and in 20% methanol-water (B). (Both in 0.2% TFA, pH 2.5, 2 ml/min).

and the fragments were subjected to amino acid analysis (2). The retention times of the peptide complexes increased with increasing number of amino acid residues (2). Figures 3a and 3b show a comparison between the retention times of the free peptides and the cobalt-peptides. It should be noted that in the trifluoroacetate solvent system used, the retention times of the cobalt-peptide complexes are longer than those of the free peptides.

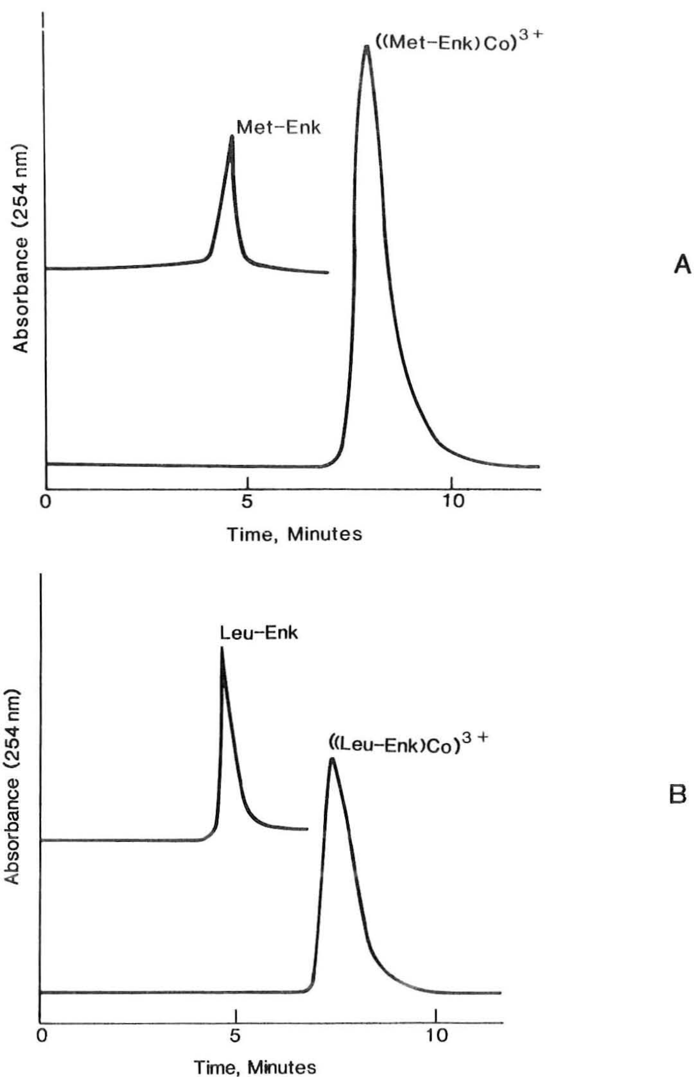


FIGURE 3.

Chromatograms of Free Met-enkephalin (Met-Enk) and its Corresponding $[(NH_3)_5Co(III)-]$ Complex (A) and Free Leu-Leu-enkephalin (Leu-Enk) and its Corresponding $[(NH_3)_5Co(III)-]$ Complex (B). (in 40% methanol-water, 0.2% TFA, pH 2.5, 2 ml/min). (Co = $[(NH_3)_5Co(III)-]$)

The trifluoroacetate solvent system was also used for the separation of hydrophilic peptide sequences during the synthesis of the tetrapeptide complex $[HisGlyHisGlyCo(NH_3)_5]^{5+}$. Figure 4 shows the chromatographic separation of the intermediates in the synthesis of $[HisGlyHisGlyCo(NH_3)_5]^{5+}$ (in a synthetic mixture prepared from all the pure components). The fragments $[GlyCo(NH_3)_5]^{3+}$, $[HisGlyCo(NH_3)_5]^{4+}$, $[GlyHisGlyCo(NH_3)_5]^{4+}$, and $[HisGlyHisGlyCo(NH_3)_5]^{5+}$ are well resolved. These highly charged species can be well resolved on reverse phase HPLC by

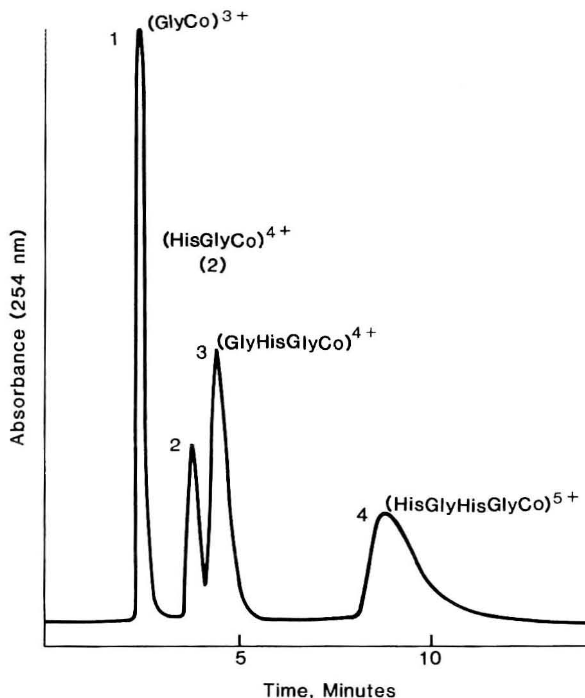


FIGURE 4.

Chromatographic Separation of the Peptide Fragments the Synthesis of $[HisGlyHisGlyCo(NH_3)_5]^{5+}$. (40% methanol-r, 0.2% TFA, pH 2.5, 2 ml/min). (Co = $[(NH_3)_5Co(III)-]$).

ion pairing with trifluoroacetate. It is noteworthy that the separation of a highly charged complex such as $[HisGlyHisGlyCo(NH_3)_5]^{5+}$ from its homologs is very time consuming by classical ion exchange techniques. In the present separation (Figure 4) the ion pairing with five trifluoroacetate anions increases the retention time of the cobalt-peptide complex and makes the separation of the tetrapeptide complex from the di- and tri-peptide complexes possible in a short period of time (ca minutes).

Retention Times of $[(NH_3)_5Co(III)-]$ Amino Acid and Peptide Complexes

In the trifluoroacetate solvent system (0.2% TFA, pH 2.5, aqueous-methanol) the retention time of the Co(III) complexes depends on at least two factors: a) the hydrophobicity of the amino acid or peptide bound to cobalt(III), and b) the ion pairing of the cobalt complexes as trifluoroacetate salts. The cobalt amino acid or peptide complexes with chloride, perchlorate, or fluoroborate counter ions do not retain on these C_{18} columns. Most of the free amino acids also do not retain appreciably with this solvent system. The interaction between the derivatized silica gel support and the $[(NH_3)_5Co(III)-]$ amino acid or peptide complexes involves both the hydrophobic amino acid or peptide moiety and the total number of trifluoroacetate counter ions with the cobalt complex as a result of its charge. The combination of these two factors contribute to the selective retention times that these cobalt(III) complexes exhibit. The separation techniques used in this study are not unique to Co(III) and should also prove useful to the separation of amine complexes of other charged, substitution-inert metal ions such as Rh, Ru, Ir, and Os complexes. In general the combination of the overall charge of the complex and the

hydrophobicity of the ligands on metal complexes can result in their rapid and selective elution from derivatized silica gel columns.

SUMMARY

The retention time of the pentaammine cobalt(III) amino acid or peptide complexes on reverse phase C_{18} columns is a function of two factors: a) the hydrophobicity of the sixth ligand (amino acid or peptide) bound to cobalt and b) the charge on the Co(III) complex which is ion paired to the trifluoroacetate counter ion.

EXPERIMENTAL

Materials and Methods

All solvents were HPLC grade (purchased from Baker Chemical). House distilled water was further purified by passing it through organic purifiers and ion exchangers (Barnsted System). All chromatography was carried out at room temperature using isocratic elution conditions on a Waters HPLC system (two M 6000 A pumps, M 660 solvent programmer, Model 440 UV detector and a RCM-100 radial compression module) and a Perkin Elmer LC 75 variable wavelength detector. Radial-Pak C_{18} columns (8 mm ID x 10 cm) (Waters) were used for all the analyses. The flow rate was maintained at 1-3 ml/min. Detection of the cobalt-peptides was done at 254 nm and detection of the free peptides with aromatic side chains was also done at 254 nm. The syntheses of all the cobalt amino acid and peptide complexes are described in references 1 and 2.

ACKNOWLEDGEMENTS

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REFERENCES

1. S. S. Isied and C. Kuehn, J. Am. Chem. Soc 100, 6752 (1978).
2. S. Isied, A. Vassilian, and J. Lyon, manuscript submitted to J. Am. Chem. Soc.
3. G. H. Searle, Austr. J. Chem. 30, 2625 (1977).
4. a) B. D. Warner and J. I. Legg, Inorg. Chem. 20, 1625 (1981).
   b) ibid, 18, 1839 (1979).
5. D. A. Buckingham, C. R. Clark, R. Tasker, and M. Hearn, J. Liquid Chrom. 4, 689-700 (1981).
6. a) G. G. Strazza and C. M. Polcaro, J. Chromatogr. 147, 516 (1978).
   b) P. Heizmann and K. Bullschmiter, J. Chromatogr. 137, 153 (1977).
7. B. Currie, J. Chang and R. Cooley, J. Liquid Chromatogr. 3, 513-27 (1980).
8. C. Bishop, L. Meyer, D. Harding, W. Hancock, and M. Hearn, J. Liquid Chromatogr. 4, 661-680 (1981).
9. C. E. Dunlap, S. Gentleman, and L. Lowney, J. Chromatogr.160, 191-8 (1978).
10. D. Voskamp, C. Olieman, and H. Beyerman, Recueil, J. Roy. Neth. Chem. Soc. 99, 105 (1980) and references therein.

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A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF NAPROXEN AND DES-METHYL-NAPROXEN IN BIOLOGICAL SAMPLES

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ABSTRACT

A HPLC method for the quantitative analysis of naproxen and its major metabolite des-methyl-naproxen in biological fluid samples is described.

Two methods of detection are compared: U.V. spectrophometry and spectrophotofluorometry. In both procedures an internal standard is used: diflunisal in the U.V. procedure and the ethoxy-analog of naproxen during fluorometry. The sensitivity of the fluorometric detection is higher than that of the U.V. detection; the limit being respectively 0.1 µg and 2.0 µg per milliliter sample. The fluorescence detection procedure can also be applied to very small samples (0.05 ml) in the therapeutic concentration rang range. Both procedures have been applied to clinical and laboratory studies in which they appear to be very satisfactory because of their ease of handling and their suitability for routinely performed analysis.

INTRODUCTION

Naproxen ((+)-6-methoxy-α-methyl-2-naphtalene-acetic acid; Fig. 1.1) is a nonsteroidal anti-inflammatory drug which is used

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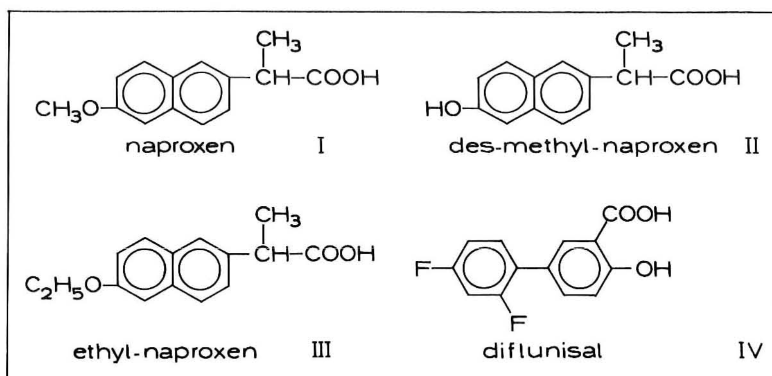

FIGURE 1

Structural formulas of naproxen (I), 6-0-des-methyl-naproxen (II), ethoxy-naproxen (III) and diflunisal (IV).

extensively for the treatment of arthritic diseases (1). In the course of a study on the clinical efficacy of several anti-inflammatory compounds, we are investigating also the pharmacokinetic profile of naproxen as related to its therapeutic effectiveness. For these studies we needed a rapid and accurate procedure for the quantitative analysis of naproxen in plasma and urine. Since it is known from literature data (2) that naproxen in man undergoes two major biotransformations, viz. demethylation to 6-0-des-methyl-naproxen (11) and conjugation with glucuronic acid, the procedure should differentiate between these compounds. The analysis of naproxen in biological samples has been the subject of various reports. Gas-liquid chromatography has been used (3, 4, 5) but this requires a derivatization step for estrification of the carboxylic acid function of naproxen. We have experience with gas-liquid chromatography of naproxen after methylation with diazomethane. Although this procedure gives excellent results in general, it has a major drawback in the fact that the des-methyl-metabolite (11) yields naproxen itself. Of course it is possible

to obtain a better specificity by using other derivatization techniques, e.g. butylation (3). An interesting and relatively sensitive analytical method for naproxen makes use of the strong fluorescent properties of naproxen (6, 7, 8). Direct spectrophotofluorometry however requires rather extensive procedures for extraction and sample preparation and purification. And even then, application of direct fluorometry to samples of patients, receiving sometimes comedication with a number of other drugs, carries a large risk of interferences.

Since we wanted to avoid complicated sample preparation and derivatization techniques we developed a specific high-performance liquid chromatographic method for the determination of naproxen and its des-methyl-metabolite in biological fluids. Although several methods have been described for the HPLC analysis of naproxen in biological samples (9, 10), in our hands the methods lack the sensitivity needed for thorough pharmacokinetic studies. In this paper we report the HPLC analysis of both naproxen and des-methyl with subsequent detection and quantification by spectrophotofluorometry. This method is compared with the standard U.V. detection and its advantages are discussed.

MATERIALS AND METHODS

Materials

Naproxen as well as 6-O-des-methyl-naproxen were kindly supplied by Syntex Research (Palo Alto, Calif., U.S.A.). Diflunisal (IV), used as an internal standard, was kindly supplied by Merck, Sharp and Dohme (Brussels, Belgium). The other internal standard, the ethoxy-analog of naproxen (III), was obtained from Syntex Research (Palo Alto, Calif., U.S.A.). The column packing material used was Lichrosorb RP 8 (5 μm) which was obtained from E. Merck

(Darmstadt, G.F.R.). All other chemicals were of analytical grade and also obtained from Merck.

Apparatus and Chromatography

A Hewlett-Packard 1081 B high-performance liquid chromatograph was used, equipped with an automatic sampling system and a HP fixed wave-length (254 nm) U.V. detector. The output of the detector was fed to the HP 3351 Lab Data System, which was operated in the internal standard mode. The fluorescence detector used was the Perkin Elmer model 3000 fluorescence spectrometer which was operated at an excitation wave-length of 235 nm (slit width 10 nm) and an emission wave-length of 350 nm (slit 5 nm). The column was stainless steel, 15 cm x 4.6 mm I.D. and was packed with Lichrosorb RP 8 (5 μm). The mobile phase was a mixture of methanol and citrate buffer (pH 6.5, 0.0 M). The methanol buffer volume ratio was 50 : 50 for plasma and 40 : 60 for urine. The flow rate was fixed at 1.5 ml/min and the column temperature at 30°C when U.V. detection was used. In case of fluorometric detection the flow rate was lower (1.0 ml/min) but the temperature higher (35°C). A major factor in this respect is the large injection peak in case of U.V. detection which is absent in fluorometric detection. These differences were desirable for obtaining optimal conditions in both cases. In the experiments with U.V. detection diflunisal was used as internal standard. The fluorescence detection necessitated the use of another standard, with fluorescence properties equal to those of naproxen; for that purpose the ethoxy-analog was used.

Sample Preparation

Plasma (0.5 ml) was pipetted into a stoppered test tube, containing the internal standard, along with hydrochloric acid (0.7 ml, 1.5 N). After homogenization by mechanical shaking for 30 min

the mixture was extracted with 5 ml of a mixture of diethyl-ether and n-hexane (1 : 1 volume ratio). After centrifugation for 15 min at 1500 g, the organic layer was transferred into another tube and evaporated at 30°C under a gentle stream of dry filtered air. The residu was reconstituted in 1 ml of the mobile phase and aliquots of 10 µl were injected into the column. When fluorometric detection was applied the reconstituted residu of samples containing over 10 µg/ml was diluted 20-fold prior to the injection of again 10 µl.

When urine samples (usually 0.25 ml) were analyzed the same procedure was followed, except for the acidification which was performed with acetic acid (4.2 N) instead of hydrochloric acid. The reason for this modification was the fact that des-methyl-naproxen, which is present in most urine samples but never in plasma samples in measurable amounts, appeared to be unstable during the sample preparation procedure when hydrochloric acid was used. For plasma samples we prefer acidification with hydrochloric acid because it results in a better precipitation of plasma proteins.

Calibration and Recovery

Calibration was performed by adding known amounts of naproxen to blanc plasma or blanc urine samples and by handling these according to the procedure outlined above. Since urine samples usually contain both naproxen and its metabolite des-methyl-naproxen also calibration for the latter had to be performed in the same way in case of urine samples. Calibration graphs were obtained by plotting the concentration of naproxen calculated by the data system against the concentration added. The data system was operated in the internal standard mode and calibrated with one of the known added concentrations, usually 50 µg/ml. For the calculations peak height ratios of naproxen to internal standard were

used. The overall recovery of naproxen and des-methyl-naproxen in the procedure was determined by comparing peak height ratios obtained after direct injection of standard solutions to peak height ratios of extracted naproxen solutions to standard solutions of the internal standard.

In order to estimate the overall precision of the method samples with various concentrations were analysed in ten times for calculation of the standard deviation of the measurement.

RESULTS AND DISCUSSION

Under the conditions described no interference from endogenous compounds in plasma or urine is encountered (Fig. 2a). Table I

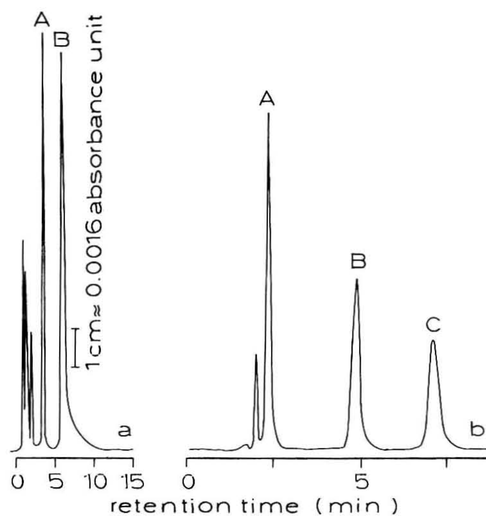

FIGURE 2a

HPLC chromatogram of naproxen (A) and diflunisal (B) after extraction from plasma, using ultraviolet detection.

FIGURE 2b

HPLC chromatogram of 6-O-des-methyl-naproxen (A), naproxen (B), ethoxy-naproxen (C) after extraction from urine, using fluorescence detection.

TABLE I

Retention Times of Naproxen and Some Other Anti-inflammatory Drugs in the Chromatographic System Described in the Text

	RT[a] (min)	RT[b] (min)
salicylic acid	1.65	
6-O-des-methyl-naproxen	1.70	2.30
oxyphenbutazon	2.55	
naproxen	3.60	4.80
ethoxy-naproxen	5.20	7.00
phenylbutazon	5.70	
diflunisal	6.25	
mefenamic acid	12.75	
flufenamic acid	17.60	

a: flow 1.5 ml/min, column temperature 30°C
b: flow 1.0 ml/min, column temperature 35°C

lists the retention times of naproxen, its des-methyl-metabolite and some other anti-inflammatory drugs in the chromatographic system employed. It is obvious that also these compounds are not likely to cause difficulties in the analysis of naproxen. It may be important to notice already here that in plasma samples we never were able to detect any des-methyl-naproxen. This metabolite hower is consistently found in urine samples. Probably the rate of excretion, augmented with the rate of further metabolism (conjugation to glucuronic acid) is so high that the metabolite is eliminated very shortly after it has been formed, so that no appreciable plasma levels are reached. The recovery of naproxen and des-methyl-naproxen taken through the whole procedure was 95% on the average,

without any systematic variations. The other chromatographic parameters are dependent upon the method of detection and therefore they are indicated separately in the following:

Ultraviolet detection

Calibration graphs were constructed for naproxen concentrations ranging from 1-100 µg/ml. A plot of naproxen concentration as calculated by the data system (y) versus the concentration of naproxen added (x) yielded a perfectly straight line, fitting the equation:

$$y = 0.949x + 1.511 \qquad (1)$$

(correlation coefficient = 0.9999, n = 6)

This equation results when calibration is performed with the 50 µg/ml sample. Since the data system neglects the intercepts of equation 1 it is obvious that optimal analytical results can only be obtained when the calibration point is as close as possible to the samples to be analysed.

The overall precision of the method was measured on the basis of samples with 3 different concentrations. The results of Table II clearly are illustrating the fact that accuracy is best for concentrations near to the point of calibration of the data system (50 µg/ml).

The sensitivity of the procedure is comparable to that of common G.C. procedures: when using 0.5 ml samples concentrations of 1 µg/ml can be determined.

Fluorometric detection

Under the circumstances of optimal fluorescence of naproxen the original internal standard diflunisal did not show any fluorescence at all. The fluorescence of diflunisal is optimal at an excitation wave-length of 235 nm and an emission wave-length of

TABLE II

Overall precision of the method with U.V. detection as illustrated by 10-fold analysis of three different concentrations of naproxen added to plasma samples

added (μg/ml)	found (μg/ml)	standard deviation
5.08	5.85	0.140 (2.40%)
50.80	50.49	0.361 (0.71%)
101.60	100.50	3.14 (3.13%)

410 nm. The second standard, the ethoxy-analog of naproxen, appeared to be suitable (Fig. 2b). Calibration in this case was performed with the use of two different amounts of internal standard: for the naproxen range from 0.1-10 μg/ml sample the amount of standard was 1.25 μg and for the naproxen range from 5-100 μg/ml sample an amount of 25 μg was employed. In both cases linear calibration graphs were obtained by plotting the amount of naproxen calculated (y) against the amount added (x): for the naproxen range 0.1-10 μg/ml sample (calibration 5 μg/ml)

$$y = 1.029x - 0.012 \qquad (2)$$

(correlation coefficient = 0.9993, n = 5)

and for the naproxen range 5-100 μg/ml sample (calibration 50 μg/ml)

$$y = 1.1013x + 0.215 \qquad (3)$$

(correlation coefficient = 0.9995, n = 5)

As compared with equation 1 it is obvious that the value of the intercepts in equations 2 and 3 are substantially smaller. This

indicates a higher sensitivity and better accuracy in the low concentration range. It is obvious that the slopes of equations 2 and 3 are very similar. In fact a very satisfactory calibration graph for the whole concentration range can be obtained by combining all data in one equation:

$$y = 1.017x + 0.537 \qquad (4)$$

(correlation coefficient = 0.9997, n = 10)

Although this equation confirms the overall linearity it is clear that accurate measurements in the lower concentration range can only be performed by using equation 2 based on addition of the lowest amount of internal standard.

The precision of the fluorometric method again was measured by repeated analysis of samples with different concentrations and appears to be at least as good as that of the U.V. procedure as can be seen in Table III. The sensitivity of this procedure is definitely higher than that of the U.V. procedure. When using 0.5 ml samples concentrations of 0.1 μg/ml can be determined.

TABLE III

Overall precision of the method with fluorometric detection of naproxen (cf. Table II)

added (μg/ml)	found (μg/ml)	standard deviation
0.154	0.149	0.006 (3.9%)
1.536	1.504	0.024 (1.6%)
10.24	10.14	0.14 (1.4%)
51.20	51.18	0.62 (1.2%)
102.40	103.87	1.24 (1.2%)

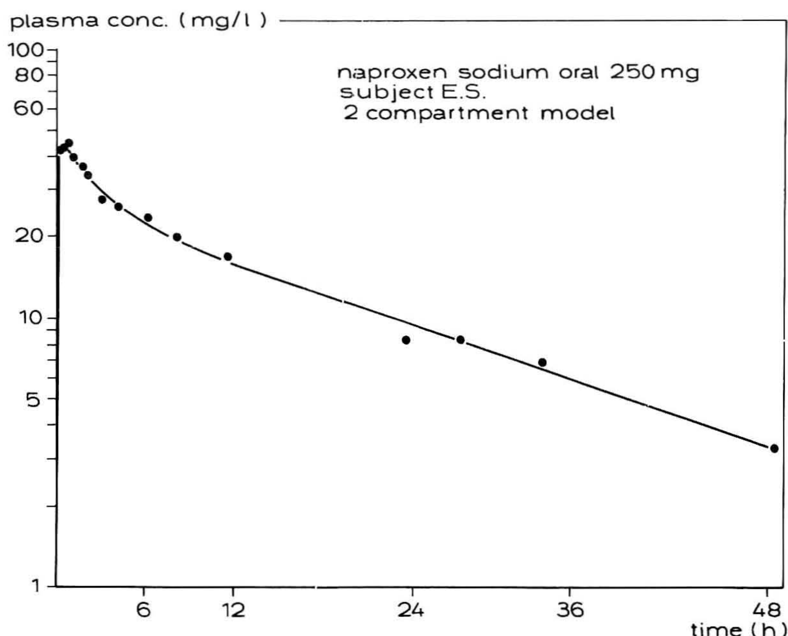

FIGURE 3

Computer fitting of a plasma curve of naproxen sodium, using a two-compartment model. The concentrations were assessed with the fluorometric detection method.

CONCLUSION

The procedures described combine ease of handling with accurate and sensitive analytical results. Furthermore they are suitable for routine measurements and can easily be adapted to automatic analysis. The procedure with U.V. detection in our experience is very suitable for the routine measurement of naproxen plasma levels of patients during chronic medication. Results of such measurements combined with their clinical and pharmacological significance will be reported elsewhere. When, however, studies of the detailed pharmacokinetics of naproxen after single dose are

TABLE IV

Comparative analysis of 9 plasma samples of a human volunteer after an oral dose of 250 mg of naproxen, by U.V. detection and fluorometric detection

time after administration (hr)	U.V. (g/ml)	fluorometry (g/ml)
0.5	65.62	67.16
1.0	60.45	60.96
2.0	49.97	50.59
3.1	42.89	44.28
4.2	37.04	38.47
6.3	30.74	31.97
8.0	28.58	28.78
12.0	19.31	19.60
24.3	12.29	12.22

concerned or in other cases where the accurate measurement of low concentrations of naproxen is required, or when only very small samples are available the fluorometric detection method becomes necessary when high-performance liquid chromatography is the analytical method of choice. As an example of such a situation Fig. 3 shows the plasma curve of naproxen after oral administration of a 250 mg dose to a human volunteer. The plasma curve appears to obey two-compartment kinetics, although in previous studies a kinetic analysis according to a single compartment model is proposed (11). Details of this pharmacokinetic behaviour will be published elsewhere. Finally, Table IV shows a set of experimental data, viz. plasma concentrations of naproxen in a volunteer, that have been calculated by high pressure liquid chromatography with

U.V. as well as fluorometric detection. These data indicate that both methods are equally reliable in the concentration range in which the U.V. procedure can be adequately applied.

REFERENCES

1. Brogden, R.N., Heel, R.C., Speight, T.M. and Avery, G.S., Drugs 18, 241 (1979).
2. Segre, E.J., J. clin. Pharmacol. 15, 316 (1975).
3. Wan, S.H. and Shaikh, M.B., J. Chromatogr. 170, 473 (1979).
4. Amos, B., Marple, L., Smithers, J. and Martin, S.B., J. Chromatogr. 206, 151 (1981).
5. Desager, J.P., Vanderlist, M. and Harvengt, C., J. clin. Pharmacol. 16, 189 (1976).
6. Antilla, M., J. Pharm. Sci. 66, 433 (1977).
7. Mortensen, A., Jensen, E.B., Petersen, P.B., Husted, S. and Andreasen, F., Acta Pharmacol. Toxicol. 44, 277 (1977).
8. Von Held, H., J. Clin. Chem. Clin. Biochem. 16, 579 (1978).
9. Slattery, J.T. and Levy, G., Clin. Biochem. 12, 100 (1980).
10. Nielsen-Hudsk, F., Acta Pharmacol. Toxicol. 47, 267 (1980).
11. Runkel, R.A., Kraft, K.S., Boost, G., Sevelius, H., Forchielli, E., Hill, R., Mogoun, R., Szakacs, J.B. and Segre, E., Chem. Pharmac. Bull. 20, 1457 (1972).

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RAPID SEPARATION OF TESTOSTERONE AND ITS MICROSOMAL METABOLITES BY REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography has been used to separate testosterone from its oxidative metabolites (7α-, 16α- and 6β-hydroxytestosterone and androstenedione) following *in vitro* incubation with rat liver microsomes. The separation was accomplished in less than 18 minutes on a radially compressed C_{18} reverse-phase column using isocratic elution with tetrahydrofuran:water (24/76, v/v).

INTRODUCTION

Testosterone is an endogenous substrate for hepatic microsomal monooxygenases (1,2). This steroid is metabolized by rat hepatic microsomes to products such as 7α-, 16α- and 6β-hydroxytestosterone and androstenedione in reactions that appear to require different forms of cytochrome P-450 (3). The hydroxylation of testosterone has been utilized in the characterization of several forms of purified hepatic cytochrome P-450 (4-6). Therefore, testosterone may be utilized as a single substrate

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to quantify simultaneously the activity of several different enzymes present within a microsomal incubation mixture. Enzymes catalyzing specific hydroxylations of testosterone have been induced selectively by agents such as phenobarbital and 3-methylcholanthrene (7). A rapid and sensitive method for quantitation of testosterone and its major microsomal metabolites could be employed in the study of the effects of inducers of microsomal monooxygenases, such as the environmental contaminants polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs), on the microsomal metabolism of testosterone.

Several different methods have been developed for the analysis of testosterone and its metabolites. Descending paper chromatography has been the analytical method of choice for several investigators (3,6,7). However, a major disadvantage of such a technique is the relatively long sample development time and the necessity for radiolabeled samples. In addition, the complete resolution of the 4 major microsomal metabolites and the parent compound cannot be accomplished in a single development. Recently, thin-layer chromatographic methods have been reported which can separate all metabolites in a single development (5,8). However, a radiolabeled substrate of high specific activity is required for quantitation with this method. Recently, high performance liquid chromatography (HPLC) has been used in the analysis of testosterone and its metabolites. Shaikh et al. (8) reported a normal phase system that requires a long elution time and lacks a high degree of sensitivity. The reverse phase system reported by van der Hoeven (9) requires gradient elution and does not adequately separate the 7α- and 6β-hydroxy metabolites. Therefore, our goal was to develop a highly sensitive rapid isocratic HPLC system for analysis of testosterone and the four major microsomal metabolites.

MATERIALS AND METHODS

Microsomal Incubations and Sample Preparation

Testosterone and cofactors (Steraloids, Inc., Wilton, NH; Sigma Chemical Co., St. Louis, MO) were added in 66 mM Tris-HCl, pH 7.4, to

open screw cap test tubes to give a final volume of 1.06 ml. The reaction mixture contained approximately 1.15 μ mole testosterone, 0.3 μ mol NADH, 0.4 μmol NADP, 0.3 μmol NADPH, 5.8 μ mol glucose-6- phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 3.0 μmol $MgCl_2$ and 0.8 mg microsomal protein. Following 1 min of preincubation, reactions were initiated by the addition of testosterone in 11 μ l of dimethyl sulfoxide. Reaction mixtures were incubated for 0-120 min, usually 30 min, at 37°C in a Dubnoff metabolic shaker. Reactions were terminated by addition of 5 ml of diethyl ether/chloroform (3:1, v/v). Incubation blanks were prepared by addition of diethyl ether/chloroform at zero time.

The reaction products were prepared for chromatography using the following method: samples were extracted with 3x5 ml of diethyl ether/chloroform (3:1, v/v); the organic fraction was dried by elution through anhydrous sodium sulfate, evaporated to dryness under N_2 and reconstituted in 1.0 ml of 95% ethanol for chromatography.

High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a Waters model M6000-A pump (Milford, MA) with a Model 440 uv detector set at 254 nm and a Model U6K injector. Samples were chromatographed on a Waters radial compression module with a 10 μm radial pak reverse phase (C_{18}) cartridge (8 mm x 10 cm) at ambient temperature. The solvent was tetrahydrofuran (THF):H_20 (24:76, v/v). The flow rate was maintained at 3.2 ml/min with a resulting column pressure of 800 psi. Peak areas were quantified using an electronic integrator (Shimadzu Seisakusho Ltd., Kyoto, Japan). Compounds were identified by their retention times relative to reference standards (Steraloids, Inc., Wilton, NH, and Medical Research Council, Steroid Reference Collection).

RESULTS AND DISCUSSION

Baseline separation of testosterone and the four major microsomal metabolites (7α, 16α and 6β -hydroxytestosterone and androstenedione) was

achieved with the THF-H_2O solvent system employed in this study. Interfering peaks were not present in the microsomal sample matrix as is evident from the chromatograph of the blank incubation (Fig. 1). Analysis of a single sample required less than 18 min; testosterone was the latest eluting peak in the chromatogram. Rat hepatic microsomes produced significant quantities of all four metabolites (Fig. 2). In addition hepatic microsomes from animals pretreated with PBBs (100 ppms) produced substantially more of the hydroxylated metabolites as well as several other

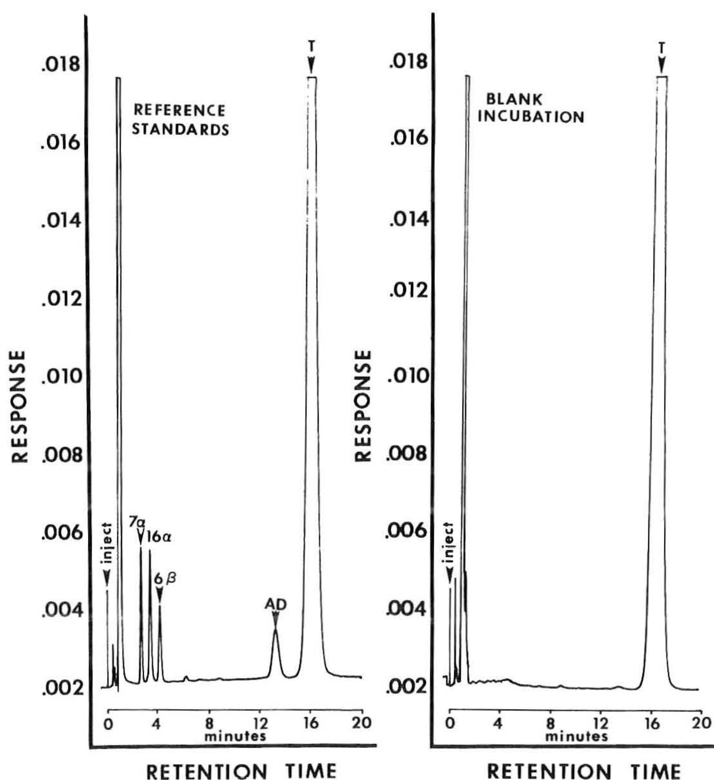

Fig. 1. HPLC profile of mixture of reference standards (7α-, 16α-, 6 β-hydroxytestosterone, androstenedione, and testosterone, left to right) and a blank incubation. An isocratic elution with THF/H_2O (24:76 v/v) was performed at a flow rate of 3.2 ml/min. UV absorbance was monitored at 254 nm at a full scale sensitivity of 0.02 absorbance units.

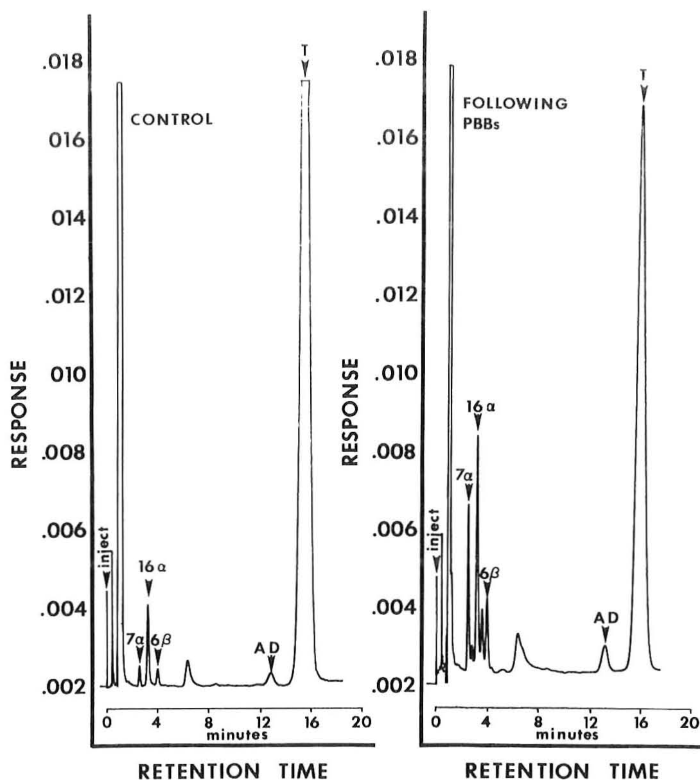

Fig. 2. HPLC profiles of microsomal incubations from untreated and PBB treated rats. HPLC conditions were the same as those described in the legend for Fig. 1.

unidentified peaks (9). However, even in the presence of these peaks, resolution was sufficient to allow quantitation of all three of the hydroxylated metabolites (Fig. 2).

Gas chromatography-mass spectrometry (GC/MS) of the methoxime-trimethylsilyl ethers derivative of HPLC peaks from actual incubation samples revealed that the major component of each HPLC peak had retention time and mass spectrum identical to the corresponding reference standard for the metabolite in question. No additional steroidal compo-

nents were evident by GC/MS analysis of the isolated HPLC peaks. Quantitation of testosterone and metabolites was linear over the range of values encountered in microsomal incubation mixtures. Amounts as low as 10 nanograms of steroid were easily quantified (Fig. 3).

A major advantage of the use of reverse phase chromatography was the early elution of all the polar microsomal metabolites. This provided much greater sensitivity than previously reported normal phase systems. In

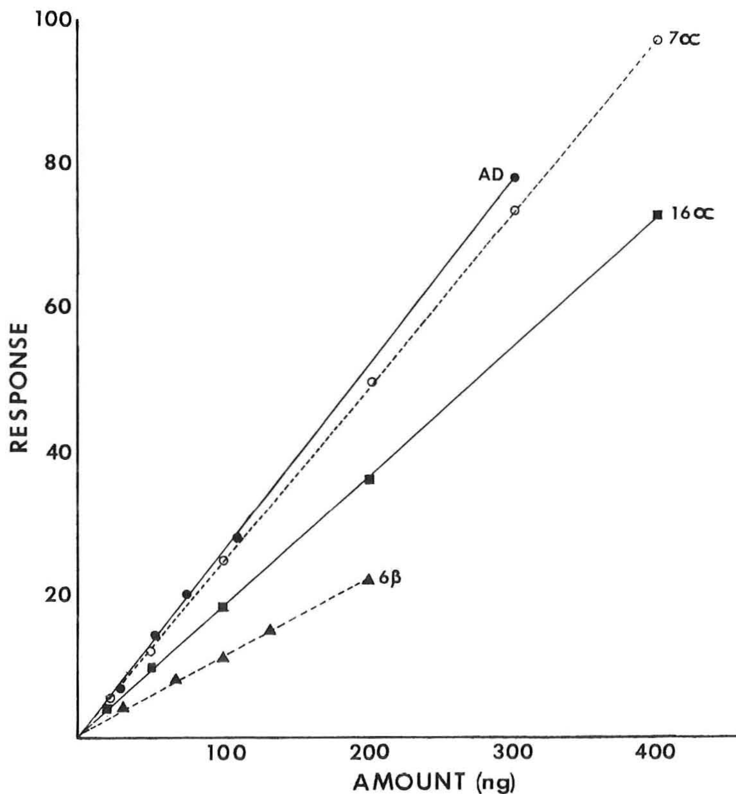

Fig. 3. Calibration curve for the four microsomal metabolites of testosterone expressed as peak area counts in mV·sec (response) as a function of varying amounts of metabolite. This curve covered the range of values encountered in the samples. HPLC conditions were the same as those described in the legend for Fig. 1.

addition, the late elution of testosterone in our system reduced the possibility that the metabolites may be obscured by the precursor in samples from a microsomal incubation mixture. Both acetonitrile and methanol have been used without success in the separation of all of the hydroxylated metabolites of testosterone (10). However, the separation of 7α- and 16α-hydroxytestosterone, androstenedione and testosterone by reverse-phase HPLC utilizing dioxane:water solvent gradient has been reported (11). Therefore, it is not surprising that another ether, tetrahydrofuran, provided baseline separation of all metabolites studied. Perhaps hydrogen bonding could have accounted for the selectivity gained by the use of THF. The rapid analysis time was an advantage gained by the high flow rates attainable with radial compression chromatography.

An HPLC method is now available for the rapid separation of the major hydroxylated metabolites of testosterone produced by rat hepatic microsomes. This method provides sufficient sensitivity for quantitation of these steroids in microsomal incubation mixtures containing less than one milligram of protein and incubated for less than 10 min under substrate saturated conditions.

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REFERENCES

1. Conney, A.H. and Schneideman, K., Enhanced androgen hydroxylase activity in liver microsomes of rats and dogs treated with phenylbutazone. J. Pharmacol. Exp. Ther. 146, 225, 1964.

2. Conney, A.H., Levin, W., Ideda, M., Kuntzman, R., Cooper, D.Y. and Rosenthal, O., Inhibitory effect of carbon monoxide on the hydroxylation of testosterone by rat liver microsomes. J. Biol. Chem. 243, 3912, 1968.
3. Conney, A.H., Levin, W., Jacobson, M., Kuntzman, R., Cooper, D.Y. and Rosenthal, O., Microsomes and Drug Oxidations, Gillette, J.R., Conney, A.H., Cosmides, G.J., Estabrook, R.W., Fouts, J.R. and Mannering, G.J., Eds., Academic Press, New York, 1969, pp. 279.
4. Shiverick, K.T. and Neims, A.H., Multiplicity of testosterone hydroxylases in a reconstituted hepatic cytochrome P-450 system from uninduced male rats. Drug Metab. Disp. 7, 290, 1979.
5. Chung, L.W.K. and Chao, H., Neonatal and hepatic cytochrome P-450. I. Comparison of testosterone hydroxylation in a reconstituted system between neonatally imprinted and nonimprinted rats. Mol. Pharmacol. 18, 543, 1980.
6. Haugen, D.A., van der Hoeven, T.A. and Coon, M.J., Purified liver microsomal cytochrome P-450. Separation and characterization of multiple forms. J. Biol. Chem. 250, 3567, 1975.
7. Conney, A.H. and Klutch, A., Increased activity of androgen hydroxylases in liver of rats pretreated with phenobarbital and other drugs. J. Biol. Chem. 238, 1611, 1963.
8. Shaikh, B., Hallmark, M.R., Issaq, H.J., Risser, N.H. and Kawalek, J.C., Use of high pressure liquid chromatography and thin layer chromatography for the separation and detection of testosterone and its metabolites from in vitro incubation mixtures. J. Liq. Chrom. 2, 943, 1979.
9. Newton, J.F., Braselton, W.E., Jr., Lepper, L.F., McCormack, K.M. and Hook, J.B., Effect of polybrominated biphenyls on metabolism of testosterone by rat hepatic microsomes. Toxicol. Appl. Pharmacol., in press.
10. van der Hoeven, T.A., Separation of metabolites of testosterone hydroxylation by high-performance liquid chromatography. J. Chrom. 196, 494, 1980.
11. O'Hare, M.J., Nice, E.C., Magee-Brown, R. and Bullman, H., High pressure liquid chromatography of steroids secreted by human adrenal and testis cells in monolayer culture. J. Chrom. 125, 357, 1976.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE QUANTITATION OF VITAMIN A PALMITATE IN LIQUID MULTIVITAMIN FORMULATIONS

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ABSTRACT

Several normal-phase high performance liquid chromatographic methods for the quantitation of Vitamin A palmitate in liquid multivitamin formulations using both radially-compressed and conventional silica columns are described. The methods are reproducible, require minimal sample preparation and resolve the predominant cis-trans isomers of vitamin A (13-cis, all-trans, 9-cis and 9,13-dicis). Good agreement is obtained between HPLC and USP assay for samples containing a small amount (6% or less) of the vitamin A as the 13-cis isomer.

INTRODUCTION

The current compendial method (1) for the determination of vitamin A esters - acetates, palmitates and propionates - involves saponification, extraction and subsequent spectrophotometric quantitation as the alcohol, using the Morton-Stubbs technique (2,3). The method is time consuming, operator dependent, does

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not account for the difference in extinction coefficients, nor for the difference in biological activities of the predominant cis-trans isomers of vitamin A (4-6).

Recently several high-performance liquid chromatographic methods for the determination of vitamin A, either separately (7-12) or simultaneously (13-18) with vitamins D and E, have been reported. The simultaneous methods, although much faster than the compendial techniques, still do not separate the predominant cis-trans isomers of vitamin A, and therefore may overestimate the available potency. Most of the single determination methods have achieved separation of the predominant cis-trans isomers of vitamin A. However, these methods, like the simultaneous determination techniques, are concerned only with vitamin A acetate. The two reported HPLC vitamin A palmitate methods (10,13) have not been applied to liquid multivitamin formulations, and only one (13) is capable of resolving the major isomers.

This report describes several normal-phase HPLC methods suitable for the quantitation of vitamin A palmitate in liquid multivitamin formulations. The methods require minimal sample preparation, are reproducible and can utilize both conventional steel and radially-compressed silica columns. Most importantly, the methods resolve the four predominant sterically unhindered (19) isomers of vitamin A (all-trans, 13-cis, 9-cis and 9,13-dicis) and anhydro vitamin A.

EXPERIMENTAL

REAGENTS - Hexane and methyl t-butyl ether were obtained from Burdick and Jackson. 1,4-Dioxane was obtained from Fisher Scientific and fluoranthene from Matheson, Coleman and Bell. All other reagents were analytical reagent grade. The standard is a vitamin A palmitate dispersed in oil, the potency of which is

about one million International Units (IU) per gram. It was characterized by the USP assay, by the direct spectrophotometric British Pharmacopeia (20) assay and by HPLC against a six-times recrystallized vitamin A palmitate reference standard. The standard, which has an all-trans to 13-cis ratio of about 95 to 5, was overlayed with nitrogen and stored under refrigeration.

INSTRUMENTATION - Two chromatographic systems were used throughout. One system consisted of a Perkin-Elmer Series 2/2 pump, a Rheodyne Model 7120-loop injector and a Perkin-Elmer Model LC-75 variable wavelength detector set at 325 nm.

The other system consisted of a Waters Associates Model M45 pump, a Model 710 automatic injector and a Model 440 UV detector set at 313 nm.

COLUMNS AND MOBILE PHASES - System I - several columns were used. These were: a Waters Associates μPorasil (30 cm x 3.9 mm), an E. S. Industries Chromegasorb 100R Silica, 10 μ (30 cm x 4.6 mm) and an E. M. Industries Lichrosorb SI-60, 5 μ (25 cm x 4.6 mm). The mobile phase was a 1% methyl t-butyl ether solution in hexane. The flow rate can be varied from 1.4 to 2.0 ml/min.

System II - A Waters Associates Radial Compression Module, RCM-100, fitted with a Radial-Pak B silica cartridge, 8 mm ID, was also used. The mobile phase, pumped at 1.4 ml/min, was a hexane: 1,4-dioxane: triethylamine mixture (997:3:0.02).

PROCEDURE - Low actinic glassware was used throughout. Extractions and dilutions were done with a 0.1% solution of butylated hydroxytoluene (BHT) in hexane.

Standard Preparation - Approximately 50 mg of accurately weighed vitamin A standard was dissolved and diluted to volume in a 50-ml volumetric flask (Standard Solution). Approximately 75.0 mg of fluoranthene were accurately weighed, dissolved and diluted to volume in a 50-ml volumetric flask (Internal Standard).

A 2.0 ml aliquot of the Standard Solution and 2.0 ml of Internal Standard were pipetted into a 50-ml volumetric flask and diluted to volume (Working Standard).

Sample Preparation - Water dispersed multivitamin formulations - A 2.0 ml sample aliquot was transferred into a 50-ml centrifuge tube. The pipet or volumetric flask was rinsed twice with 2 ml portions of dimethylsulfoxide (DMSO). The combined sample-DMSO mixture was extracted with 3 x 25 ml portions of 0.1% BHT in hexane with the aid of a mechanical shaker (approximately 5 minutes shaking time) and centrifuged. The clear supernatants were combined and diluted to 100 ml in a volumetric flask (Method I). Ten ml of this solution and 2.0 ml of the Internal Standard were pipetted into a 50-ml volumetric flask and diluted to volume (Sample Solution).

An alternate preparation (Method II) was also used. To the DMSO-sample mixture 25.0 ml of the BHT/hexane solution was pipetted. The mixture was shaken for 5 minutes on a mechanical shaker and centrifuged. Two ml of the supernatant and 2.0 ml of the Internal Standard were pipetted into a 50-ml volumetric flask and diluted to volume (Sample Solution).

Sample Preparation - Oil dispersed multivitamin formulations - Oil-dispersed formulations were treated in the same manner as the vitamin A standard. The sample solutions to be injected, however, were prepared to contain about 35 IU/ml of vitamin A palmitate.

System Suitability - A solution was prepared containing about 40 IU/ml of isomerized vitamin A palmitate (about 30% of the vitamin A exists as the 13-cis isomer) and 20 μl were injected. The system was considered suitable if baseline separation was obtained between the 13-cis and 9-cis vitamin A peaks. Alternately, the resolution factor between the all-trans and 13-cis peaks

should be greater than 3.2 if system suitability requirements are to be satisfied.

Quantitation - The vitamin A palmitate content was calculated using internal standard peak-area techniques and expressed in terms of IU per volume or weight. The total area of the vitamin A was expressed as the sum of the areas of the 13-cis and all-trans peaks. The extinction coefficients and biopotencies of both isomers were assumed to be equal. The validity of this assumption will be discussed in a later section. Twenty microliter injection volumes were made throughout.

RESULTS

An accurate analysis of vitamin A is a fairly difficult task. The instability of the molecule (5, 8, 9, 21, 22), the possible presence of several isomers and the lower relative biological activity (4-6) of these isomers all contribute to the difficulty.

The present methods minimize the above problems. The addition of BHT to the extracting solvent prevents both air oxidation (21) and possible column degradation/isomerization (8) of vitamin A palmitate. In addition by separating the most probable cis-trans isomers and utilizing the published extinction coefficients (6, 7), the calculation of sample potencies with increased accuracy will be possible once reference standard materials and reliable potencies become available.

Chromatograms of the working standard, and some sample solutions, obtained with system I, are shown in Figures 1-3. A chromatogram of a system suitability solution, obtained with system I, is similar to the one shown in Figure 3. A chromatogram of this same solution, obtained with system II, is shown in Figure 4. In all cases the 13-cis and all-trans vitamin A peaks are separated.

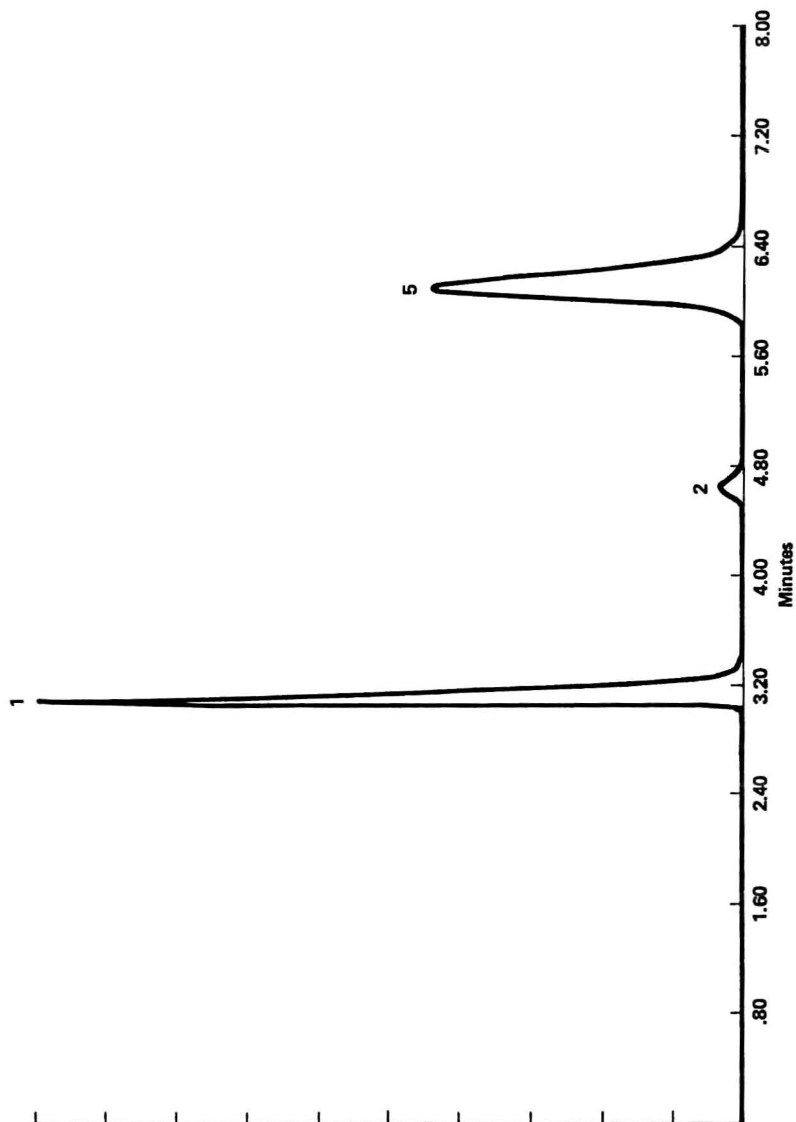

FIGURE 1. Chromatogram of the Working Standard at 313 nm. Column: Chromegasorb 100R. Mobile Phase: 1% Methyl-t-butyl ether in hexane. Flow rate: 2.0 ml/min. (1) Fluoranthene (Internal Standard); (2) 13-cis vitamin A palmitate; (5) all-trans vitamin A palmitate.

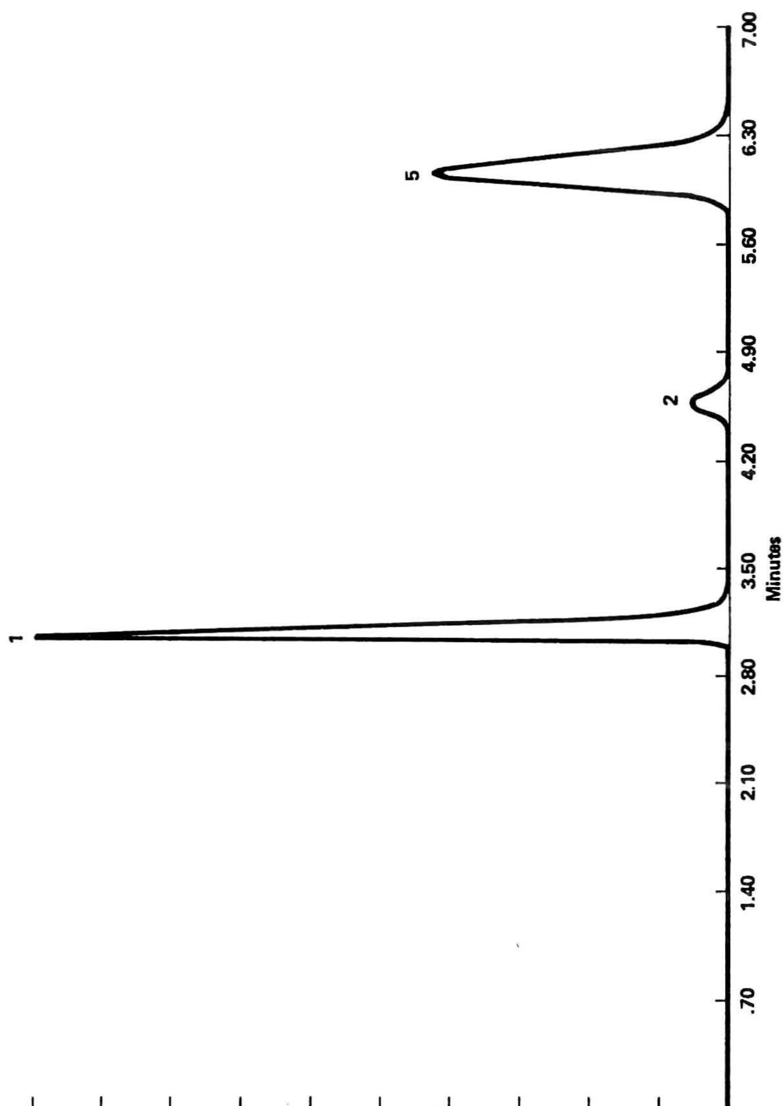

FIGURE 2. Chromatogram of a sample containing about 5% of the vitamin A as the 13-cis isomer. Conditions and explanation as in Fig. 1.

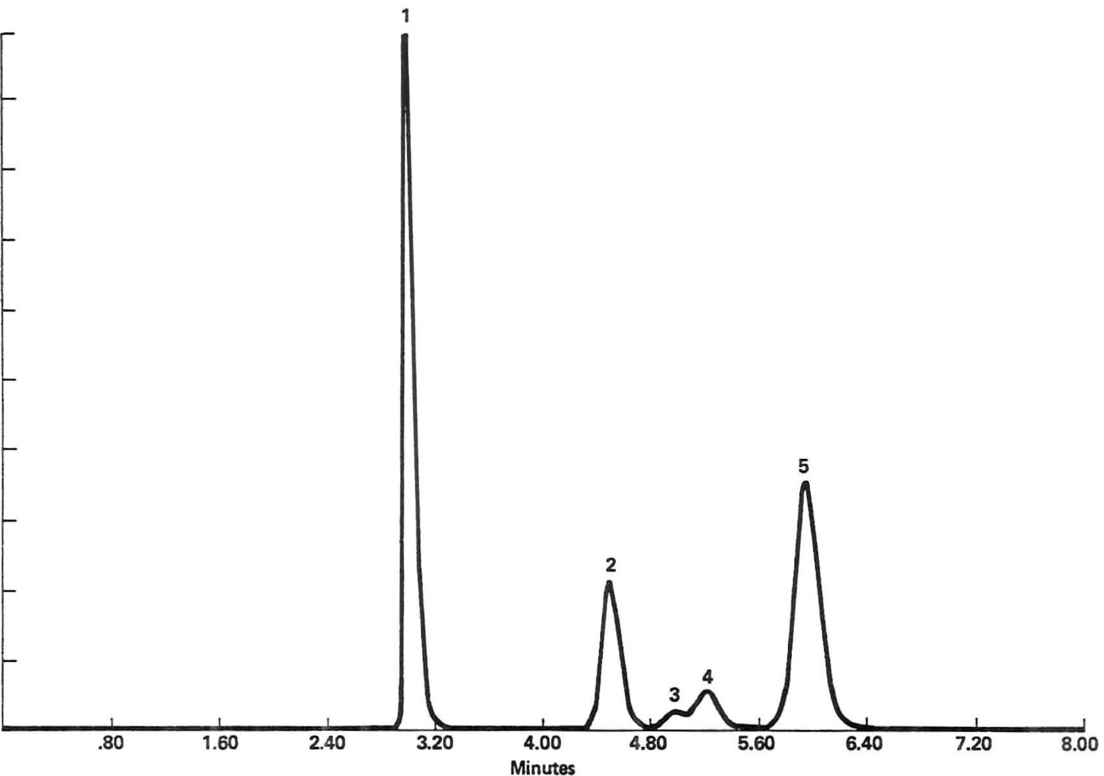

FIGURE 3. Chromatogram of a sample containing about 30% of the vitamin A as the 13-cis isomer. Conditions and explanation as in Fig. 1. (3) 9,13-dicis vitamin A palmitate; (4) 9-cis vitamin A palmitate.

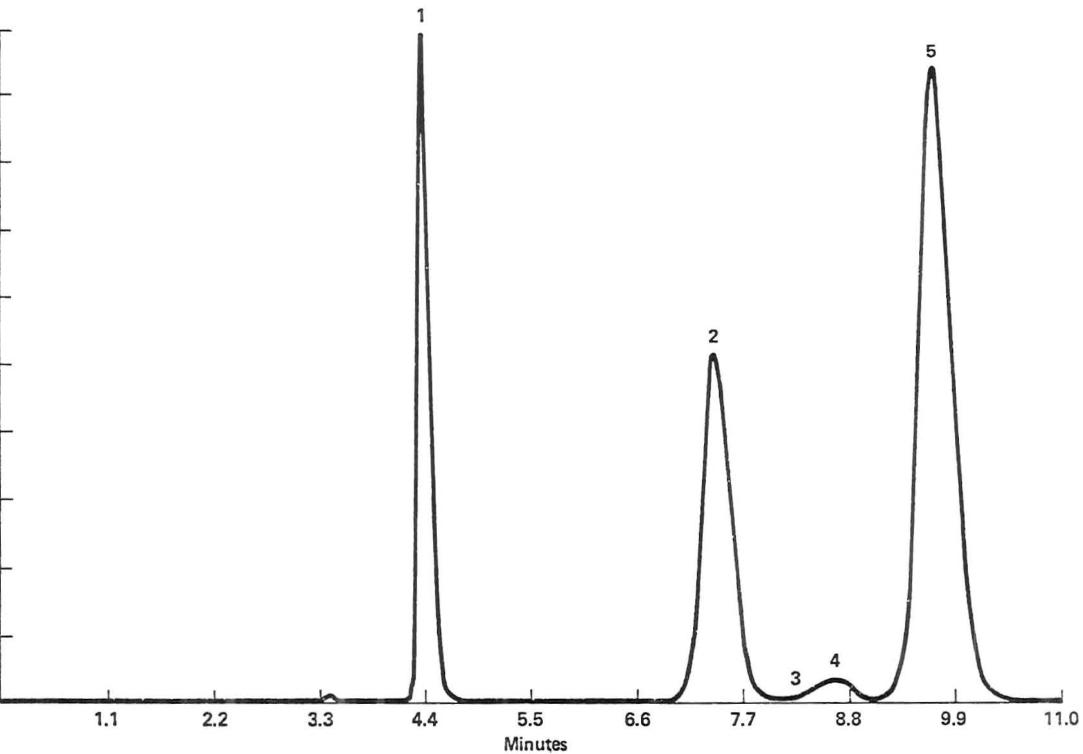

FIGURE 4. Chromatogram of a System Suitability solution at 325 nm containing internal standard (1). Column: Radial-Pak B (8 mm I.D.). Mobile phase: hexane-dioxane-triethylamine (997:3:0:0.02). Flow Rate: 1.4 ml/min. (2) 13-cis vitamin A palmitate; (3) 9,13-dicis vitamin A palmitate; (4) 9-cis vitamin A palmitate; (5) all-trans vitamin A palmitate.

Two other peaks corresponding to the 9-cis and 9,13-dicis isomers, are also separated from the main peaks. Both systems are capable of resolving the 13-cis and all-trans isomers from the low-potency 9-cis and 9,13-dicis isomers. System I, however, gives better separation between the 9-cis and 9,13-dicis isomers. The best separation between the isomers was obtained on the Lichrosorb 5 μ column.

Quantitation of vitamin A in the various samples was accomplished by adding the areas of the 13-cis and all-trans peaks. This method assumes equal extinction coefficients and biopotencies for these two isomers. A calculation based on published extinction coefficients (6, 7) and some available biopotencies (4-6) shows that this method of quantitation may overestimate by 4% the true potency of a sample containing 30% of the vitamin A as the 13-cis isomer (Figure 3). However, there is no overestimation in samples containing 4-6% of the vitamin as the 13-cis isomer (Figure 2) since these samples are similar to the standard. The areas of the low-potency 9-cis and 9,13-dicis isomers were not considered, even though they could amount to as much as 15% of the total vitamin A area in some very old samples (Figure 3). It should be noted that the USP method does not correct (6, 12) for the low biological activity of these isomers even though they do contribute to the measured absorbance.

Results of vitamin A palmitate HPLC assays in oil and water-dispersed multivitamin formulations are shown in Tables I and II.

Table I also shows the agreement between USP and HPLC assay results. Table II shows the HPLC assay results obtained under different experimental conditions: 325 nm versus 313 nm, single extraction versus triple extraction and system I versus system II. Identical results were obtained using the Lichrosorb and Chromegasorb columns.

Table I.

Comparison of HPLC and USP Vitamin A Assays of Various Formulations

Sample Description	Lot No.	% Label Claim USP	HPLC
Vitamin A and β-Carotene in Oil	1	110	106
	2	106	107
	3	110	107
Vitamin A, D_2 and β-Carotene in Oil	1	110	107
	2	107	109
	3	103	104
Water-dispersed vitamin A	1	110	107[a]
Water-dispersed vitamins A, D_2 and E	1	100	101[a]
Water-dispersed vitamin A and D_2	1	110	108[a]

[a]Single extraction, 313 nm

Table II.

HPLC Results of Vitamin A Assays on Water-dispersed Pharmaceutical Dosage Forms

Product	Lot	Months Shelf-Life	Age	Potency, % Claim System I	System II	
A	1	36	78	107[c]	105[a]	104[b]
A	2		60	120	116	116
A	3		40	123	121	
A	4		27	121	119	120
B	1	24	37	84	86	
B	2		11	108	114	114
C	1	24	36	99	96	
C	2		16	129	124	
D	1	36	43	127	129	
D	2		27	126	121	123

[a]Triple extraction technique, 325 nm
[b]Single extraction technique, 325 nm
[c]Triple extraction technique, 313 nm

Recovery studies were conducted by adding a solution of the standard vitamin A to previously assayed water-dispersed formulations and assaying as previously described. Recovery rates were well within the range of 100 ± 4%. Sample matrix interferences were found to be non-existent by stopped-flow spectral comparison and absorbance ratioing techniques.

The linearity of the chromatographic methods was determined between 15% and 230% of the working range by injecting vitamin A palmitate solutions containing a constant amount of internal standard. The relative standard deviation of the peak-area response ratio was less than 1.8% over the entire range.

The reproducibility of the chromatographic systems was determined by making six injections of the working standard and computing the peak-area response ratio. The relative standard deviation of the response ratio ranged from 0.3 to 1.6%.

Table III.

Results of Replicate Assays from Two lots of Water-dispersed Multivitamin Dosage Form

	% of Claim	
	System I[a]	System II[b]
	106	121
	107	126
	106	122
	106	122
	108	128
	108	124
Average	107	124
Standard Deviation	0.98	2.7
Rel. Std. Deviation	0.92%	2.2%

[a] Sample A, lot 1
[b] Sample C, lot 2

The reproducibility of the methods was determined by conducting six replicate assays of a water-dispersed multivitamin sample. The results and the relative standard deviations are shown in Table III.

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REFERENCES

1. United States Pharmacopeia, XX, National Formulary, XV, 1980, pp. 933-34.

2. R. A. Morton and A. L. Stubbs, Analyst, 71, 348 (1946).

3. R. A. Morton and A. L. Stubbs, Biochem. J., 41, 525 (1947).

4. P. L. Harris, S. R. James and J. H. Brinkman, J. Am. Chem. Soc., 73, 1252 (1951).

5. R. W. Lehman, J. M. Dieterle, W. T. Fisher and S. R. Ames, J. Am. Pharm. Assoc., 49, 363 (1960).

6. M. Kofler and S. H. Rubin, Vitam. Horm. (New York), 18, 315 (1960).

7. H. Steuerle, J. Chromatogr., 206, 319 (1981).

8. M. Vecchi, J. Vesely and G. Osterhelt, J. Chromatogr., 83, 447 (1973).

9. Y. Takashiwa, T. Nakajima, S. Tanaka, M. Washitake, T. Anmo and H. Matsumaru, Chem. Pharm. Bull., 27, 1563 (1979).

10. C. Jenkins and R. Stroz, Hoffmann-La Roche, Unpublished data.

11. R. Venteicher, R. Mejia and E. Calizaya, Hoffmann-La Roche, Manuscript in preparation.

12. J. Kryschuk and M. Munichman, Hoffmann-La Roche, Unpublished data.

13. M. C. Mulry, J. R. Kirk and R. H. Schmidt, presented at the 180th National Meeting of the ACS, Las Vegas, Nevada, Aug. 1980, Paper AGFD 108.

14. H. M. Abdou, F. M. Russo-Alesi and V. Fernandez, Pharm. Tech., 5, 40 (1981).

15. S. A. Barnett and L. W. Frick, Anal. Chem., 51, 641 (1979).

16. J. W. Dolan, J. R. Gant, N. Tanaka, R. W. Giese and B. L. Karger, J. Chromatogr. Sci., 16, 616 (1978).

17. D. Thorburn Burns and C. Mackay, J. Chromatogr., 200, 300 (1980).

18. M. Eriksson, T. Eriksson and B. Sorensen, Acta Pharmaceutica Suecica, 15, 274 (1978).

19. L. Pauling, Fortshr. Chem. Org. Naturstoffe, 3, 203 (1939).

20. British Pharmacopeia 1980, p. A95.

21. M. Hashmi, Assay of Vitamins in Pharmaceutical Preparations, John-Wiley and Sons, New York, 1973, p. 8.

22. D. Davalian and C. H. Heathcock, J. Org. Chem., 44, 4459 (1979).

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The HPLC Analysis of Caffeine and Theobromine in Animal Diets

by

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An HPLC method is described for the analysis of added caffeine and theobromine in animal diets using HPLC with samples extracted in $CHCl_3$ and interferences eliminated with a Sep-pak^{TM}. This method has good accuracy and precision but is not suitable for matrixes where the methylxanthines exist as a integral part of the matrix (e.g., foods).

The HPLC Analysis of Caffeine and Theobromine in Animal Diets

An animal diet is a complex matrix of many components consisting of various amount of protein, fat, carbohydrate vitamins and minerals used as a carrier for a wide variety of drugs and food components. The matrix by virtue of its complexity presents a formidable analytical challenge for the analyst. HPLC allows the analysis of a large variety of potential components with great accuracy and precision in a short analysis time.

A method is described for the rapid accurate analysis of the pure added methylxanthines) caffeine and theobromine in animal diet using HPLC with sample cleanup accomplished using a commercially available Sep-pak^{TM}.

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Methods and Materials

Extraction of caffeine and theobromine. Place 2.5 g ± 0.1 (weighed to 0.01 g) of animal diet into a flask add 100 ml of $CHCl_3$ and heat the solution to 60°C for 30 min. Cool extract to room temperature and bring up to weight with $CHCl_3$. Withdraw 10 ml of $CHCl_3$ extract and run through a Silica Sep-pak™. Elute caffeine or theobromine with 15 ml of CH_3OH. Depending on the level of the compound of interest the sample can be injected directly from the CH_3OH or concentrated to an appropriate volume.

The analysis was accomplished by HPLC using a Water's Radial Compression Module (RCM) with a C_{18} (Radial Pak A) cartridge. The mobile phase was 74/25/1 $H_2O/CH_3OH/HOAC$ at a flow rate of 3.0 ml/min. Detection was at 280 nm. Typical analysis for both compounds was less than 10 minutes. Standard concentrations were 0.5 μg/μl for theobromine and .025 μg/μl for caffeine. Figures 1 and 2 show sample chromatograms for standards and samples respectively.

Results

Precision studies of the method show a %Cv of less than 1% for standards (n = 10) and less than 2% for sample (n = 10). Recovery studies of additions to diet are summarized in Table 1.

An additional study of multiple extractions (n = 4) of a spiked sample (60 mg/100 g theobromine) gave excellent data with data showing differences to be not significant at a 95% confidence level.

An examination of two rabbit diets with added theobromine gave data that can be seen in Table 2.

This method provides a fast accurate analysis of added pure methylxanthines in animal diets. Although it is not suitable for use in systems where the methylxanthine is in a bound form (i.e. like a food system) because the energy input from the $CHCl_3$ is not sufficiently high as to extract

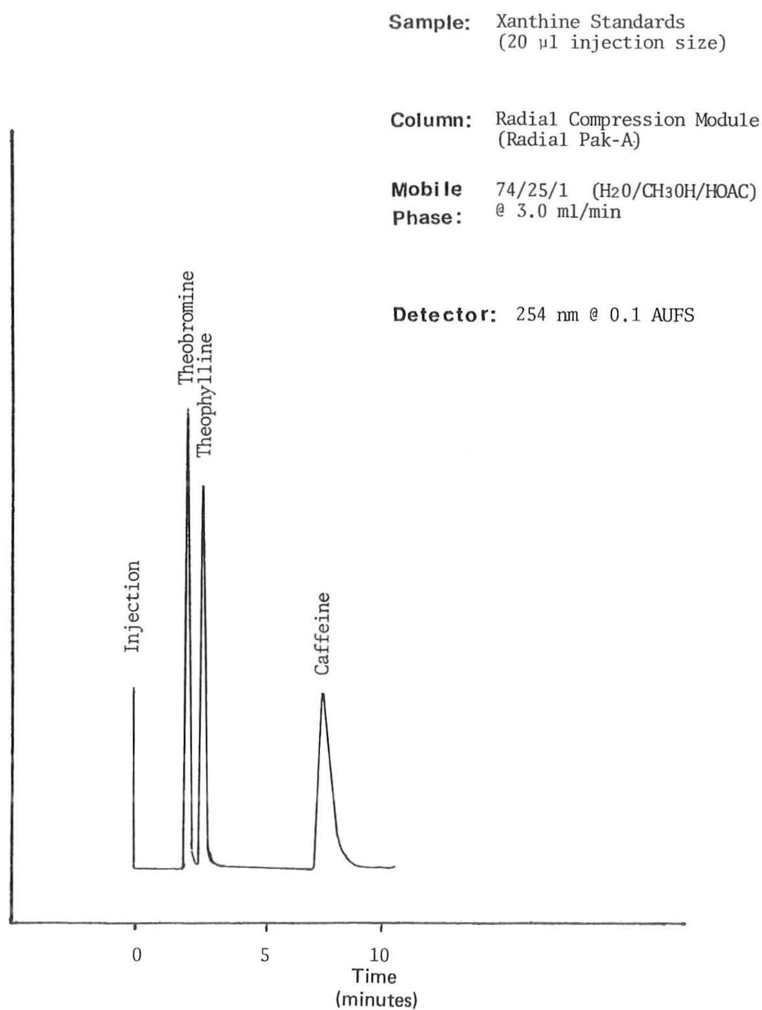

Figure 1. Analysis of Xanthine Standards, 20 ul inj. size; Column: Radial Compression Module, Rad-Pak-A; Mobile Phase: 74/25/1 ($H_2O/CH_3OH/HOAc$) @ 3.0 ml/min; Detector: UV/254 nm @ 0.1AUFS.

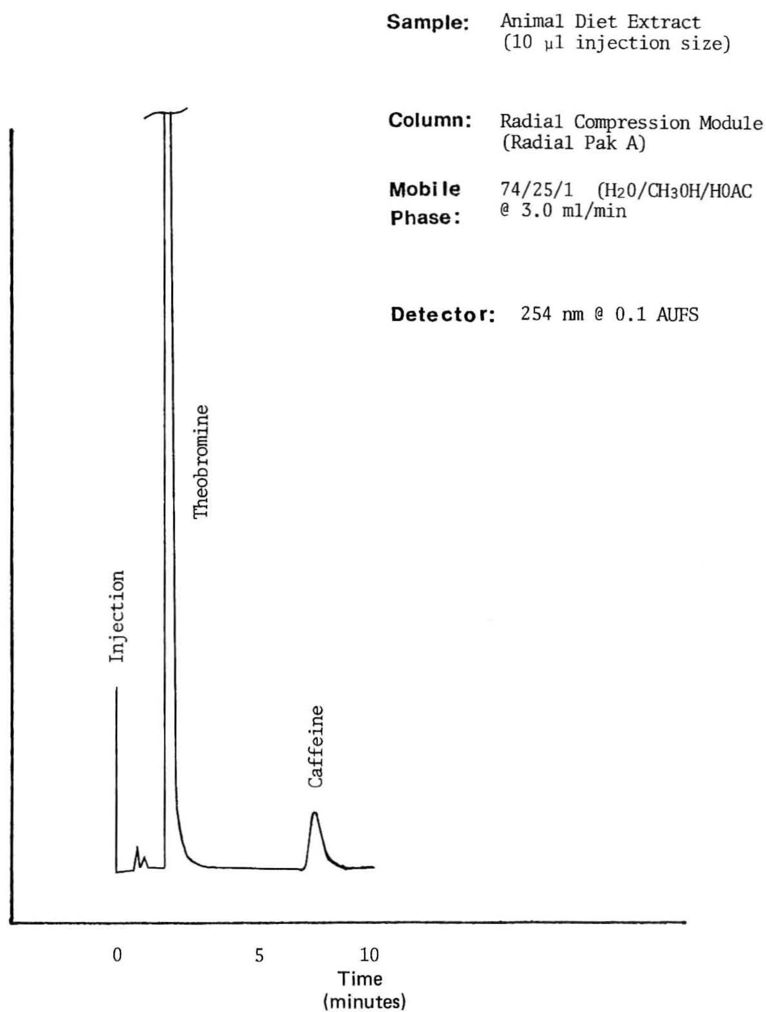

Figure 2. Analysis of Animal Diet Extract, 10 ul inj. size; Operating conditions as in Figure 1.

Table 1

Recovery Study of Added Caffeine and

Theobromine to Animal Diets

n = 2

% Theobromine Added	% Theobromine Rec.	% Recovery
+.027	.025	95.6
+.068	.067	98.6
+.135	.134	99.3
		x = 97.8

% Caffeine Added	% Caffeine Rec.	% Recovery
+.002	.0019	95.0
+.004	.003875	93.8
+.008	.00795	99.4
		x = 96.1

Table 2

Amount Added Theobromine	Amount Analyzed Theobromine
60 mg/100 g	57.6 mg/100 mg
120 mg/100 g	114 mg/100 mg

all of the bound xanthines. This method will extract 70-75% of the total compounds in the food system.

Acknowledgements

The authors wish to thank Hershey Foods for the opportunity to publish this research. Thanks to M. Sholly for manuscript typing and to Kevin Snyder for technical assistance and chromatogram preparation.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(3), 591-592 (1982)

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LC CALENDAR

1982

MARCH 8 - 12: Pittsburgh Conf. on Anal. Chem. and Appl. Spectroscopy, Atlantic City, NJ, USA. Contact: Pittsburgh Conf., Inc., P. O. Box 7780-1223, Philadelphia, PA, 19182, USA.

MARCH 28 - APRIL 2: National Amer. Chem. Soc. Meeting, Las Vegas, NV, USA. Contact: A. T. Winstead, Am. Chem. Soc., 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, The Free University, P. O. Box 7161, 1007-MC Amsterdam, The Netherlands.

APRIL 18 - 21: 66th Annual Meeting, Federation of Amer. Soc. for Exp't'l. Biol. (FASEB), Louisiana Superdome, New Orleans, LA, USA. Contact: FASEB, 9650 Rockville Pike, Bethesda, MD, 20014, USA.

MAY 16 - 18: LCEC Symposium: Biomedical Applications of LCEC and Voltammetry, Indianapolis Hyatt Regency. Contact: K Klippel, LCEC Symposium, P. O. Box 2206, W. Lafayette, IN, 47906, USA.

JUNE 7 - 11: 6th Internat'l. Symposium on Column Liquid Chromatography, Cherry Hill Inn, Cherry Hill, NJ. Contact: R. A. Barford, ERRC-SEA, USDA, 600 E. Mermaid Lane, Philadelphia, PA, 19118, USA.

June 20 - 23: Int'l. Conf. on Chromatography And Mass Spectrometry in Biomed. Sci., Bordighera,

Italy. Contact: Dr. Alberto Frigerio, Italian Group for Mass Spectrometry in Biochem. & Med., Via Eritrea 62, 20157 Milano, Italy.

JUNE 28 - 30: Analytical Summer Symposium, Michigan State University, East Lansing, MI, USA. Contact: A.I.Popov, Chem. Dept., Michigan State University, East Lansing, MI, 48824, USA.

JULY 12 - 16: 2nd Int'l. Symposium on Macromolecules-IUPAC, University of Massachusetts, Amherst, MA, USA.

July 19 - 22: 23rd Prague Microsymposium on Macromolecules: Selective Polymeric Sorbents - IUPAC, inst. of Macromolecular Chem., Prague, Czechoslovakia. Contact: P. M. M. Secretariat, Inst. of Macromolecular Chem., 162-06 Prague, Czechoslovakia.

AUGUST 2 - 5: Int'l. Conf. on Ion Chrom., at the 24th Rocky Mountain Conf., Denver, CO, USA. Contact: Dionex Corp., 1228 Titan Way, Sunnyvale, CA, 94086, USA.

AUGUST 15 - 21: 12th Int'l. Congress of Biochem., Perth, Western Australia. Contact: Brian Thorpe, Dept. Biochem., Faculty of Science, Australian National University, Canberra A.C.T. 2600, Australia.

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JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece. Contact: Dr. S.

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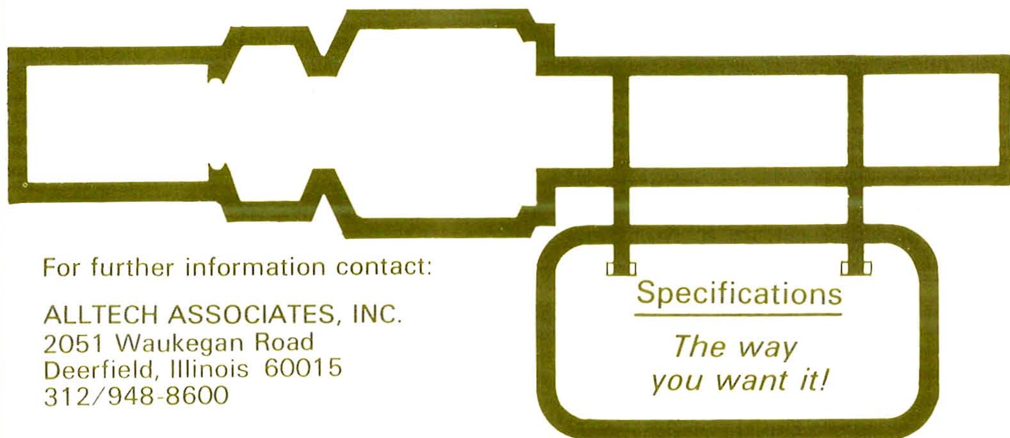

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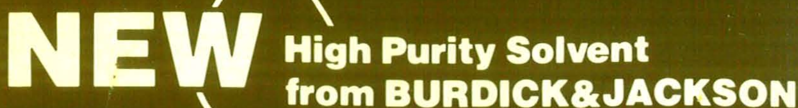

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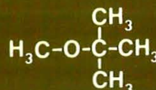

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