# JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 7 NUMBER 13 1984

Editor: DR. JACK CAZES
Associate Editor: DR. HALEEM J. ISSAQ

CODEN: JLCHD8 7(13) i-vi, 2513-2696 (1984)
ISSN: 0148-3919

# JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

November 1984

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

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

• Analytical Abstracts • ASCA • BioSciences Information Service of Biological Abstracts (BIOSIS) • Chemical Abstracts • Current Awareness in Biological Sciences • Current Contents/Life Sciences • Current Contents/Physical and Chemical Sciences • Engineering Index • Excerpta Medica • Journal of Abstracts of the All-Union Institute of Scientific and Technical Information of the USSR • Physikalische Berichte • Science Citation Index

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

**Subscription Information.** *Journal of Liquid Chromatography* is published in fourteen numbers and two supplements in January, February, March (2 numbers), April, May (2 numbers), June, July (2 numbers), August, September, October (2 numbers), November, and December by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 7 (1984), containing fourteen numbers and two supplements, is $350.00 per volume (prepaid). The special discounted rate for individual professionals and students is $175.00* per volume. To secure this special rate, your order must be prepaid by personal check or may be charged to MasterCard or VISA. Add $40.00 for surface postage outside the United States. For airmail to Europe, add $72.32; to Asia, add $91.52.

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



Contributions to this journal are published free of charge. Application to mail at second-class postage rates is pending at New York City, New York and additional mailing offices.

***THIS REFLECTS A 50% DISCOUNT GIVEN TO INDIVIDUAL SUBSCRIBERS.**

Circle Reader Service Card No. 104

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 7, Number 13, 1984

*CONTENTS*

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2513-2524 (1984)

# CHROMATOGRAPHIC SEPARATIONS OF OLIGOVINYLPYRIDINES 1. ANALYTICAL-SCALE CHROMATOGRAPHY OF RADICALLY OLIGOMERIZED 2-VINYLPYRIDINE BY HPLC AND TLC

Heinz Hilgers and Franz P. Schmitz
Lehrstuhl fÜr Makromolekulare Chemie der
RWTH Aachen, Worringer Weg 1, D-5100 Aachen,
Fed. Rep. Germany

## ABSTRACT

Oligomer mixtures of 2-vinylpyridine prepared by radical oligomerization were separated by TLC and HPLC. With TLC, using diethyl ether as the eluent, good separations could be achieved up to a degree of oligomerization of n=3. TLC could therefore be used as a rapid test for the presence of oligomers in the reaction mixture. For HPLC-separations pentane and methanol were used as eluents, applying gradients in eluent composition with increasing methanol content. Corresponding to different end groups and isomerism of the oligomers, additional separations within a given degree of oligomerization were observed.

## INTRODUCTION

Poly(vinylpyridine)s show interesting features: owing to their basic and nucleophilic properties they are able to catalyze



 0148-3919/84/0713-2513$3.50/0

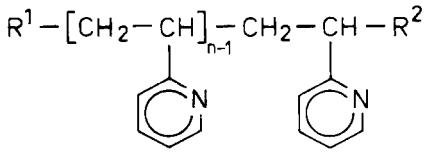


Fig. 1: Structure of oligo-2-vinylpyridines.
$R_1$: end group caused by initiation reaction,
$R_2$: end group caused by termination reaction.

a number of reactions (1-3), and they can furthermore be used as complexing agents (4-6). For detailed studies of these features, investigation of the correlation between degree of polymerization and structure on one hand and properties on the other would be of interest, employing individual, defined oligomers.

To our knowledge, only one of the numerous investigations on oligo(vinylpyridine)s included chromatographic analysis (7), using an eluent system consisting of hexane, methylene chloride and methanol and applying gradient elution. By this technique, separations of isomers (as was demonstrated for the trimer) of oligo(2-vinylpyridine)s from anionic oligomerization could be achieved.

We report here on analytical-scale chromatographic separations of oligo(vinylpyridine)s prepared by radical oligomerization. Analyses of oligomers prepared by anionic initiation and preparative-scale separations will be reported in subsequent papers.

## EXPERIMENTAL PART

Radical oligomerization by means of azo-bis(isobutyronitrile), AIBN:

390 mg AIBN were added to a solution of 0,513 ml 2-vinylpyridine in 50 ml dry toluene at 60°C. After stirring for 4 h most of the solvent was removed by distillation. Products of higher molecular

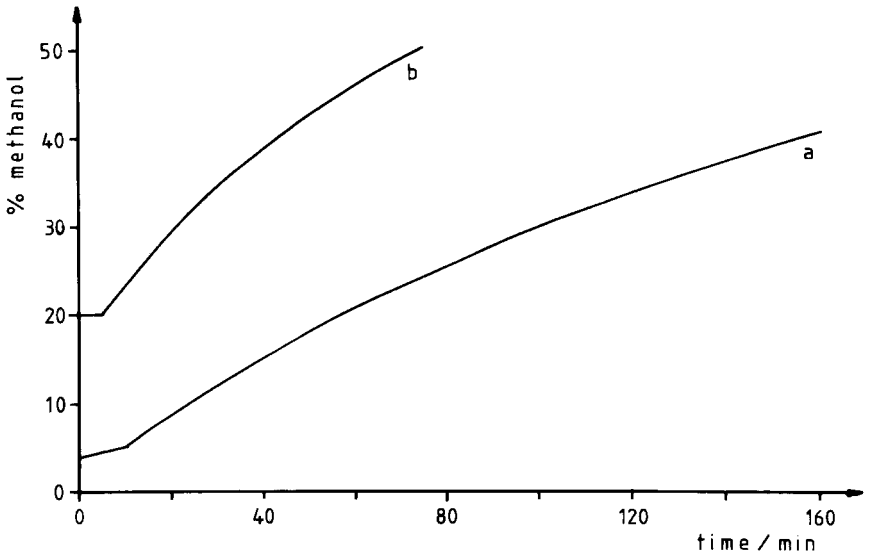



Fig. 2: Eluent composition gradients applied for the chromatograms shown in Figs. 6-9, 11 (a) and in Fig. 12 (b).

weights were obtained by precipitation from diethyl ether. The degree of oligomerization was controlled by varying the monomer/ initiator ratio.

Chromatography

TLC: Aluminum foils, coated with Kieselgel 60/Kieselgur F 259, layer thickness 0,2 mm (Merck, West Germany), with fluorescence indicator, were used. Eluents were diethyl ether and methanol, both dried and distilled before use.

LC: Glass columns of length 50 cm and internal diameter 2,8 cm were packed with silicagel (LiChroprep Si 100, 40-63 µm) or aluminum oxide (150 basic, 63-200 µm) (both from Merck); the eluent was methanol. UV-detectors, recorders and fraction collectors from LKB were used.

HPLC: The separations were carried out with an instrument 1084B from Hewlett Packard. Columns of stainless steel of lengh 25 cm and internal diameter 4,6 mm were packed with silicagel (LiChrosorb Si 100, 10 µm, Merck) using a slurry method. Eluents were pentane and methanol; the chromatographic runs were carried out by rising the methanol content (Fig. 2); the total flow rate was 1,5 ml/min. Eluents were distilled from sodium and degassed before use.

## RESULTS AND DISCUSSION

By properly chosing the monomer/initiator ratio, oligomeric 2-vinylpyridine samples could easily be obtained by radical oligomerization. Both the $^{1}H$- and the $^{13}C$-NMR spectra of the products thus obtained showed no indications for branched or even cross-linked products, whereas such side reactions can often be observed with oligo-(2-vinylpyridine)s obtained by anionic oligomerization (8).

For a rapid determination if - and to what extent - oligomers have been formed thin-layer chromatography was found to be suitable. Using diethyl ether as the eluent, only oligomers up to a degree of oligomerization of n=3 are able to migrate along the TLC-plate, whereas products of higher molecular weight remain at the starting point (Fig. 3). Separations within the regions for the respective degrees of oligomerizations are due to products with different end groups and/or isomerism. Adding methanol to the eluent results in improved transport properties of the eluent mixture, as is shown in Fig. 4.

However, the quality of the separation decreases with increasing methanol content; finally, with pure methanol as the eluent, resolution is almost completely lost in the thin-layer chromatograms of the radically oligomerized samples.

The eluent system pentane/methanol turned out to be most suitable for separating oligo-2-vinylpyridines by HPLC. As is usu-

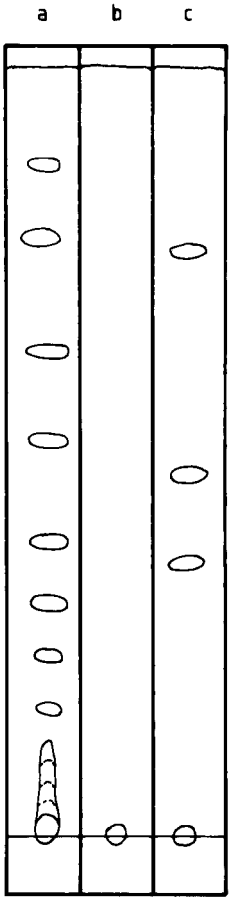



Fig. 3: Thin-layer chromatograms,
a: from radical oligomerization,
b: from radical polymerization,
c: from a mixture of test substances (cf. Fig. 6).

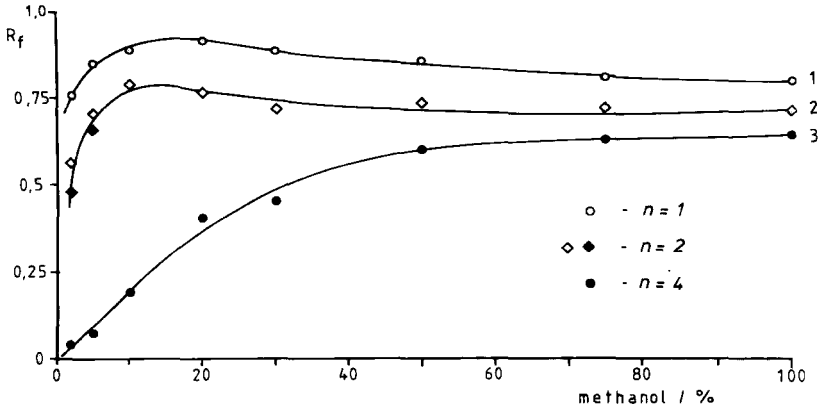



Fig. 4: Dependence of $R_f$-values on eluent composition, determined by means of a mixture of test substances (cf. Fig. 6). Left: 100% diethyl ether; right: 100% methanol.

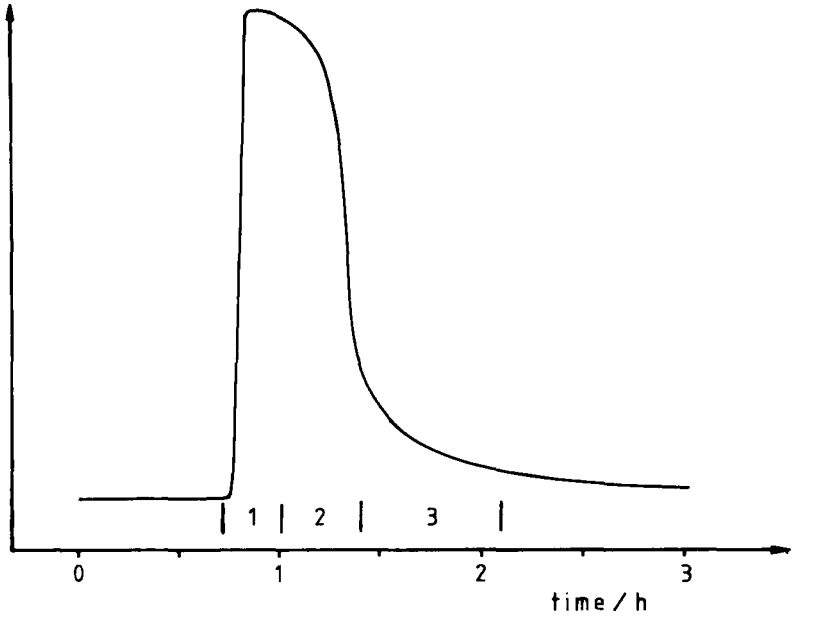



Fig. 5: Typical liquid chromatogram of an oligo(2-vinylpyridine) sample. Stationary phase: silica gel; mobile phase: methanol. The numerals correspond to the fractions collected for HPLC-separation.

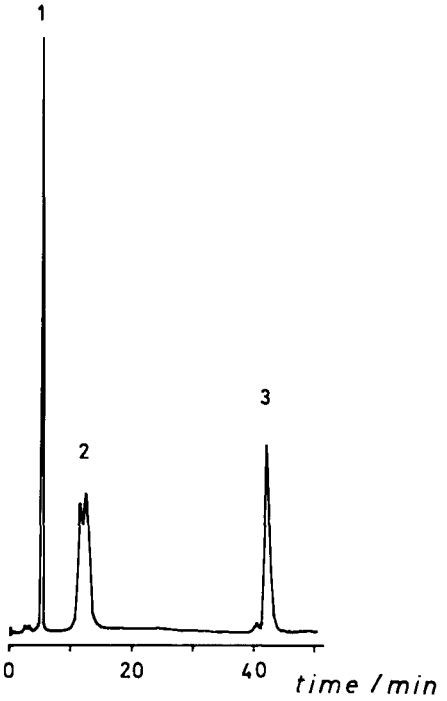



Fig. 6: HPLC-chromatograms of test substances. Conditions see text. 1: Ethylpyridine (n=1, $R_1$=H, $R_2$=H); 2: Dimer (n=2, $R_1$=H, $R_2$=$CH_3$); 4: Tetramer (n=4, $R_1$=H, $R_2$=$CH_3$).

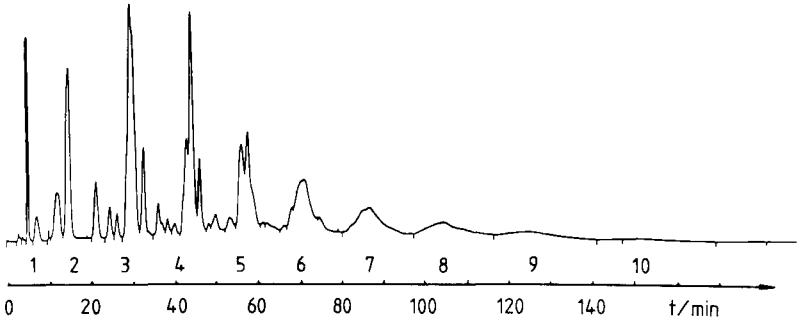



Fig. 7: HPLC-chromatogram of the first LC-fraction of an oligomer sample with monomer/initiator ratio of 1:1. Conditions see text. The numerals below the detector trace correspond to the degrees of oligomerization n.

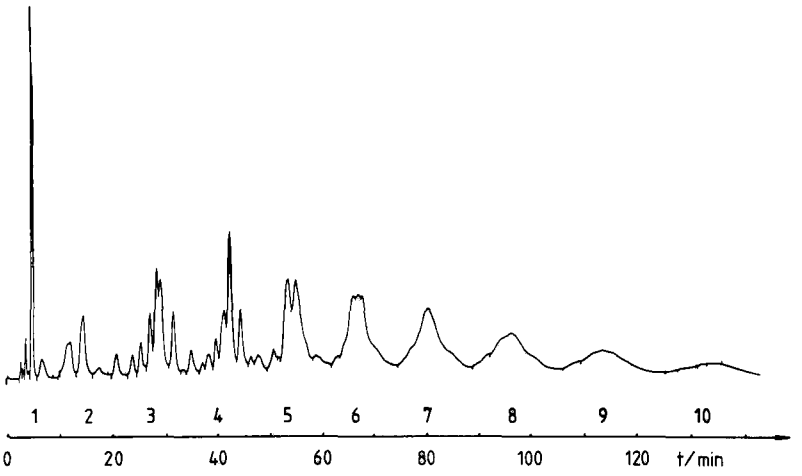



Fig. 8: HPLC-chromatogram of the first LC-fraction of an oligomer sample with monomer/initiator ratio of 1:2. For explanations see Fig. 7.

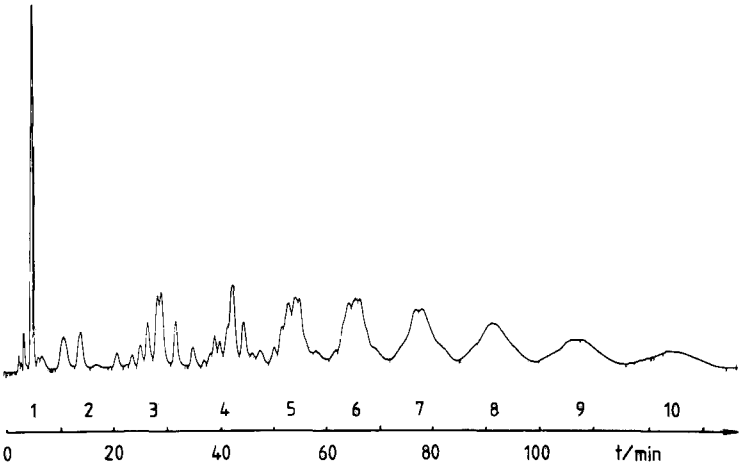



Fig. 9: HPLC-chromatogram of the first LC-fraction of an oligomer sample with monomer/initiator ratio of 1:4. For explanations see Fig. 7.

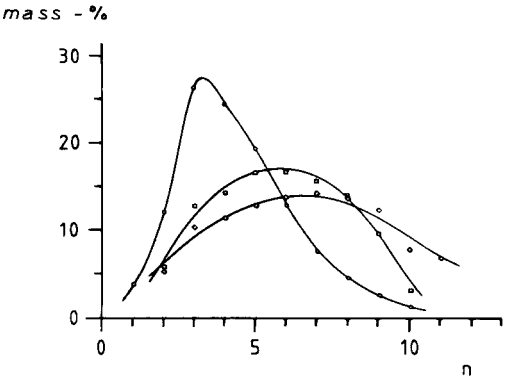



Fig. 10: Mass distribution plots for the three oligomer samples shown in Fig. 7 to 9. Monomer/initiator ratio: 1:1=(o), 1:2=(□) and 1:4=(◇).

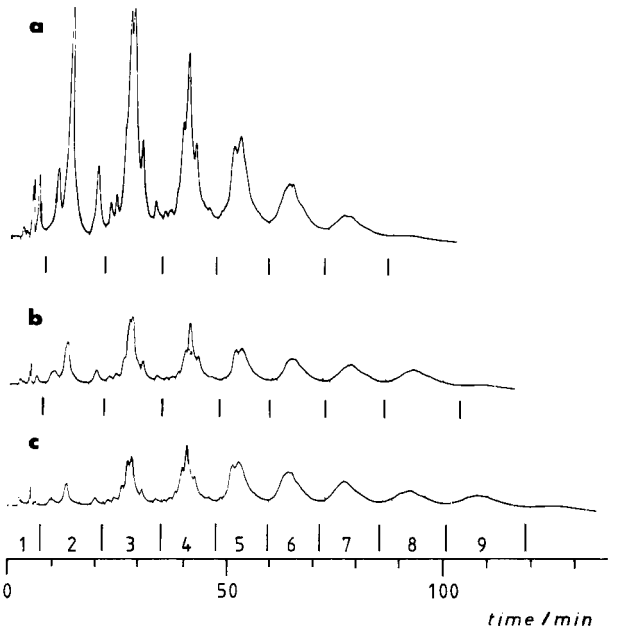



Fig. 11: HPLC-chromatograms of three subsequent fractions of an LC-separation of an oligo(2-vinylpyridine) sample with monomer/initiator ratio 1:1. For explanations see Fig. 7.

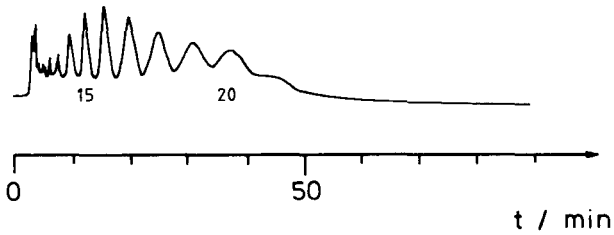



Fig. 12: HPLC-chromatogram of a lately eluting LC-fraction. For explanations see Fig. 7.

ally neccessary for the separation of oligomers, an eluent composition gradient had to be applied, in this case with a methanol content increasing from 4 to 40 % for oligomer samples of lower molecular weight. Figs. 7 to 9 show the HPLC analysis of the first of three LC-fractions (cf. Fig. 5) of a series of samples possessing increasing average degrees of oligomerization $\bar{n}$. The tentative assignments of the peaks to degrees of oligomerization are based on chromatograms of well-defined oligomers (Fig. 6).

Especially for the lower oligomers a remarkable separation within the oligomer regions of a given n was achieved. The individual peaks within these regions are due to configurational isomers and possibly different end groups. In accordance with the increasing number of possible isomers with increasing degree of oligomerization n, for n=1 to n=2 two peaks each, for n=3 at least 6 peaks and for n=4 at least 9 peaks can be observed. With increasing n and increasing peak numbers the peaks overlap, which finally results in only one broad signal for the respective degree of oligomerization. At present, the configurational and structural assignments of the oligomer peaks are not known.

Fig. 10 shows the correlation between monomer/initiator ratio and molecular weight distribution calculated from the HPLC-chromatograms: As to be expected, the distribution curves are shifted to higher $\bar{n}$-values with increasing monomer/initiator ratio. An analogous trend can be derived from the HPLC-chromatograms of three

successive LC-fractions (Fig. 11), where the relative increase of the peak areas for the higher oligomers is clearly visible.

Starting with higher methanol contents and applying steeper gradients results in rapid elution of higher oligomers (Fig. 12). The conditions for the chromatogram shown in Fig. 12 were chosen such that for each degree of oligomerization only one peak is observed.

## ACKNOWLEDGEMENT

We wish to thank Prof. E. Klesper for kindly supporting our work, Prof. T.E. Hogen-Esch for two oligomers of 2-vinylpyridine, Mr. B.Lorenschat for technical aid and the Deutsche Forschungsgemeinschaft for financial support.

## REFERENCES

1) Lupinski, J.H., Kopple, K.D. and Hertz, J.J., New Class of Film-Forming Electrically Conducting Polymers, J. Polym. Sci., Part C 16, 1561 (1967)

2) Seely, G.R., Chlorophyll-Poly(vinylpyridine) Complexes, J. Phys. Chem., 71, 2091 (1967); Bach, D. and Miller, I.R., Interaction of Deoxyribonucleic acid with Poly-4-vinylpyridine, Biochim. Biophys. Acta, 114, 311 (1966)

3) Clear, J.M., Kelly, J.M. and Vos, J.G., Polyvinylpyridine Complexes of Ruthenium (III) Chloride, Makromol. Chem., 184, 613 (1983)

4) Tsuchida, E., Kaneko, M. and Nishide, H., The Catalytic Effect of the Poly(vinylpyridine)-Ligand in the Oxidative Polymerization of Phenols, Makromol. Chem., 164, 203 (1973)

5) Tazuke, S. and Okamura, S., Vinylpyridine Polymers, in: Mark, H.F., Gaylord, N.G. and Bikales, N.M. (eds.), Encyclopedia of Polymer Science and Technology, Vol. 14, Interscience Publishers, New York-London-Sydney-Toronto, 1971, p. 637

6) Smid, J., Tan, Y.Y. and Challa, G., Effects of Poly(2-vinylpyridine) as a Template on the Radical Polymerization of Methacrylic Acid, Eur. Polym. J., 19, 853 (1983)

7) Huang, S.S., Mathis, C. and Hogen-Esch, T.E., Oligomerization of Vinyl Monomers, Part 9, $^{13}C$-NMR and Chromatographic Studies of Oligomers of 2-Vinylpyridine, Macromolecules, 14, 1802 (1981)

8) Hilgers, H. and Schmitz, F.P., to be published

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2525-2533 (1984)

# CHROMATOGRAPHY OF AC-ASP-TYR-MET-GLY-TRP-MET-ASP-$NH_2$ ON THE HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE AND THE HIGH-SPEED MULTI-LAYER COIL PLANET CENTRIFUGE

M. Knight, Y. Ito*, A.M. Kask, C.A. Tamminga and T.N. Chase
Experimental Therapeutics Branch
National Institute of Neurological and
Communicative Disorders and Stroke
Building 10, Room 5C106
National Institutes of Health
Bethesda, Maryland 20205 and
*Laboratory of Technical Development
National Heart, Lung, and Blood Institute
Bethesda, Maryland 20205

## ABSTRACT

The peptide Ac-Asp-Tyr-Met-Gly-Trp-Met-Asp-$NH_2$ was purified by countercurrent chromatography in the horizontal flow-through coil planet centrifuge. The solvent system used was ammonium acetate, pH 8.5 and n-butanol (1:1 by volume). The high pH served to maintain the peptide in solution. When the upper phase was utilized as the mobile phase better separation of the peptide from impurities resulted. The peptide was also chromatographed in a new apparatus, the high-speed multi-layer coil planet centrifuge. With the lower phase mobile and at a higher temperature, the peptide was fractionated very rapidly in 30 min compared to 7 hr on the other instrument.

## INTRODUCTION

Ac-Asp-Tyr-Met-Gly-Trp-Met-Asp-$NH_2$ is an N- and C-terminal protected cholecystokinin fragment that is important in the study of the mechanism of action of cholecystokinin (CCK) because the sulfated peptide is a CCK antagonist in the pancreas and interacts



 0148-3919/84/0713-2525$3.50/0

with CCK receptors in pancreas and brain (2). We previously reported the synthesis and purification of this peptide on the horizontal flow-through coil planet centrifuge (3,4), but have recently modified the purification procedure. The solvent system used previously, an n-butanol, acetic acid system, caused a large loss of peptide which came out of solution during the run. Since the peptide proved to be highly soluble in dilute basic solutions we changed to an n-butanol, ammonium acetate system and modified the pH. In addition, purification of the synthetic peptide at high speeds was tried on the multi-layer coil planet centrifuge (MLCPC), a new apparatus for countercurrent chromatography (CCC) which is designed for rapid fractionation of substances in two phase solvent systems with a large retention of stationary phase at higher rotation rates and flow rates. Details of the design of the MLCPC apparatus are described elsewhere (5,6).

## METHODS

Solvents used in synthesis and purification were reagent grade. Water was deionized and charcoal filtered (Hydro Service and Supplies, Durham, NC) and solvents for chromatography were HPLC grade. The peptide was synthesized by solid-phase procedures (2) on a Beckman Model 990B synthesizer (Beckman, Palo Alto, CA). The synthesis was started with 1 mm Boc β-Benzyl-L-Aspartyl benzhydrylamine resin to which 2.5 mm Boc amino acids were coupled for 2 hr with equimolar dicyclohexylcarbodiimide in the desired

sequence. After each coupling the peptide was deprotected with 25% trifluoroacetic acid in methylene chloride and neutralized with 10% triethylamine in methylene chloride. After coupling with the N-terminal aspartate residue the peptide was acetylated with acetylimidazole. The peptide was deblocked and cleaved from the resin by liquid hydrogen fluoride. A Kontes prototype flow-through coil planet centrifuge (serial no. 2) (Vineland, NJ) equipped with 2.6 mm i.d. PTFE tubing for a total volume of 260 ml was used for countercurrent purification (7,4). The solvent system of n-butanol and 0.2 M ammonium acetate, pH 8.5 (adjusted with $NH_4OH$) (1:1) was used with either the upper or lower phase mobile. The column-coil was rotated at 400 rpm. The effuent was monitored at 280 nm. Fractions of 15 min or 6 ml were collected as previously reported (4).

The peptide was also chromatographed on a table top model of a MLCPC (Laboratory of Technical Development, NHLBI, Bethesda, MD) equipped with a controlled heating system that maintained the coil at $45^{o}C$ during the run. For the n-butanol solvent systems carryover of stationary phase occurred during the chromatography at room temperature. This was observed to be related to the longer separation time of the phases. The increase in temperature shortened the separation time by decreasing the viscosity of the solvent system (8). Another modification of the instrument was the substitution of a metal gear in place of the plastic gear which became deformed during the high temperature conditions. The multilayer coil is comprised of 1.6 mm i.d. PTFE tubing with a total capacity of 285 ml coiled concentrically around a 3-in wide spool-

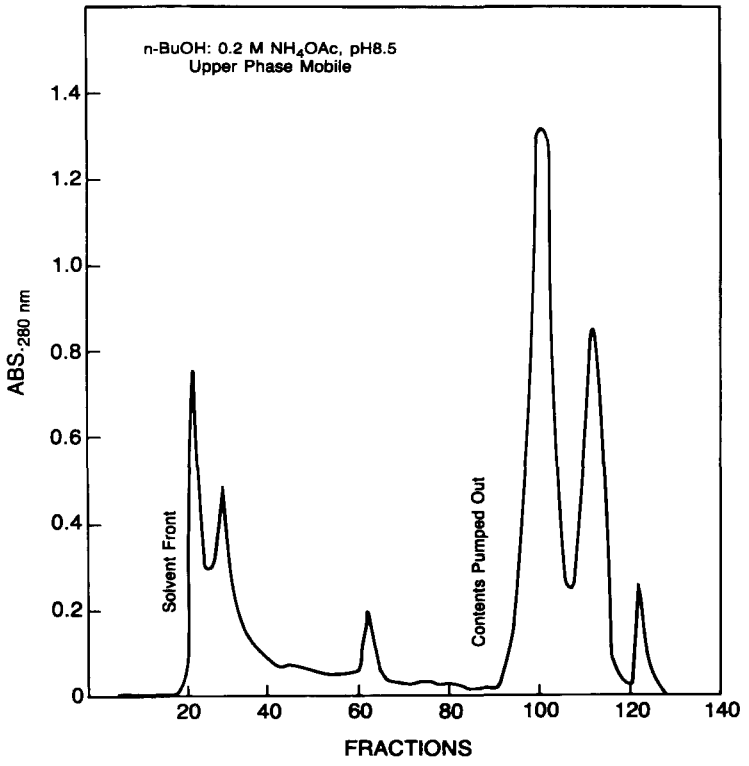



FIGURE 1

Chromatography of 300 mg peptide on the HFCPC in 0.2 M ammonium acetate pH 8.5 and n-butanol, upper phase mobile. Solvent front emerged at fraction 20. Rotation was stopped at fraction 86. Fraction (94-106) contained purified peptide. Absorbance of the fractions diluted 1/200 is shown.

shaped column holder which was mounted 4-in away from the central axis of revolution. The solvent system was equilibrated at 50°C in a water bath and during the separation both the coiled column and the mobil phase reservoir were maintained at the same temperature. These conditions produced an extremely high retention of the stationary phase at 82% of the total column capacity. Apparently the

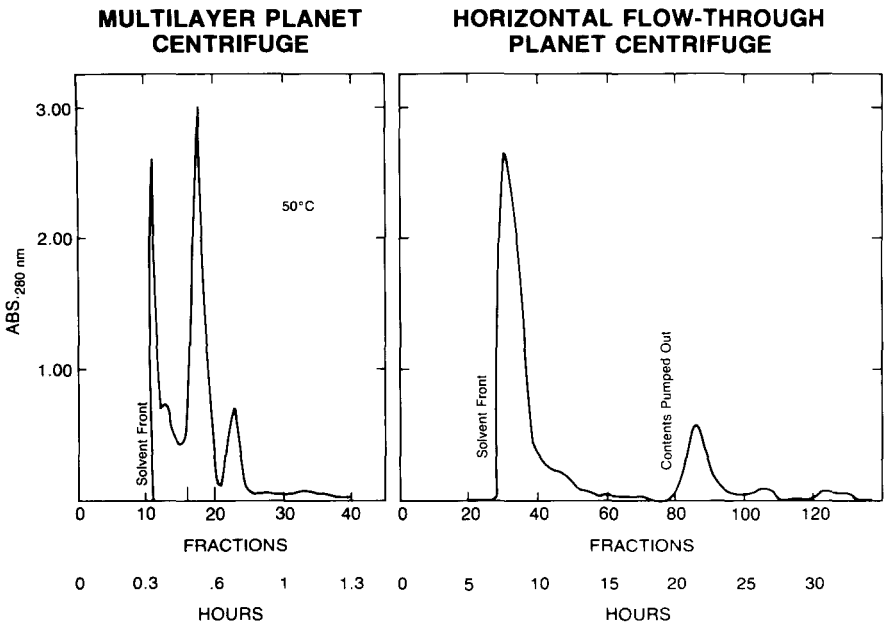



FIGURE 2

Comparison of the chromatography of synthetic peptide on the MLCPC (left) and HFCPC (right). The absorbance of the fractions is shown. The fractions are indicated by tube number and time. Lower phase of ammonium acetate system was mobile in both experiments. Pure peptide was contained in fractions (16-20) and (33-39) respectively.

hydrodynamic behavior of this volatile solvent system in the high-speed MLCPC is different from other more hydrophobic solvent systems which commonly contain chloroform. The basis for these modifications of the operating conditions of high-speed CCC are described in detail by Ito and Conway (8). The flow rate was 150 ml/hr and rotational speed was 800 rpm. The effluent was monitored by U.V. absorbance and fractions of about 6 ml or 2 min were collected. The purified peptide was sulfated by pyridine-

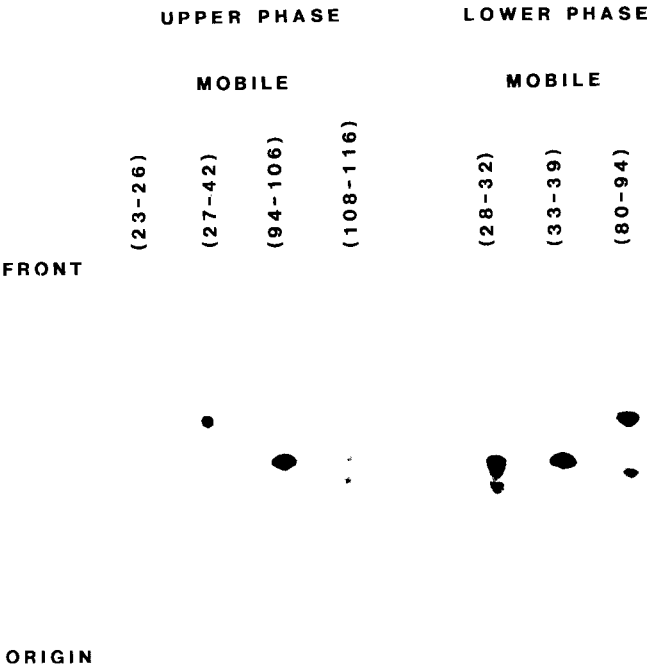



FIGURE 3

TLC (Silica gel, BAW system 4:1:1 by volume) of the content of the fractions from HFCPC run in both conditions of eluting with upper and lower phases of the ammonium acetate system, pH 8.5. Compounds were revealed by the Ehrlich spray.

sulfur trioxide and repurified according to previously described procedures (2).

## RESULTS

From a 1 mmole synthesis about 600 mg of product resulted. A sample of 300 mg was chromatographed on the HFCPC in ammonium

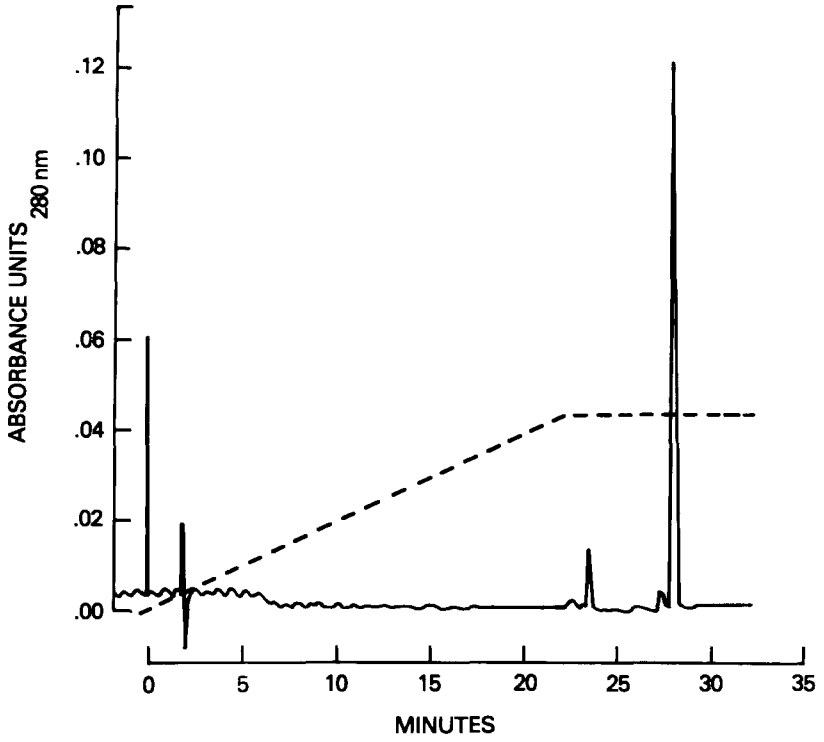



FIGURE 4

HPLC of MLCPC-purified peptide. A sample of 10 µg was chromatographed in 0.1% phosphoric acid on a $C_{18}$ µ Bondapak column (Waters 0.4 x 30 cm) and a linear gradient of acetonitrile (dotted line) from 7% to 30% in 25 min at a flow of 2 ml/min. Absorbance at 280 nm is shown. Full scale is 0.02 absorbance units.

acetate, pH 8.5 and n-butanol with the upper phase mobile (Fig. 1). The rotation was ceased at fraction 86 and contents were pumped out. The yield of purified peptide in fraction (94-106) was 140 mg. The rest of the sample was run with lower phase mobile (Fig. 2, right panel) but these conditions resulted in less resolution of the material. Fraction (33-39) contained 170 mg of peptide which was

relatively pure by TLC (Fig. 3), $R_f$ = 0.5. Fraction (28-32), material which eluted ahead with the solvent front, contained the same peptide plus impurities. The yield of purified peptide from the solid-phase synthesis was 32%. Amino acid analysis gave the molar ratios: Asp, 2.09; Gly, 1.08; Met, 1.86; Tyr, 0.97.

A small sample of the synthetic product, 15 mg, was chromatographed in the MLCPC in the conditions described in the Methods with lower phase mobile (Fig. 2). The contents of fraction (16-20) were 10 mg and were highly purified (90%) as determined by HPLC (Fig. 4). The solvent front emerged at tube 11 and the peak tube of the major material was fraction 18.

## CONCLUSIONS

The modification in the pH of the aqueous phase of n-butanol and ammonium acetate increased the solubility of the negatively charged peptide. The peptide salt was maintained in solution under these conditions. In an earlier experiment when 365 mg was chromatographed in the BAW system with the lower phase mobile 60 mg or 16.4% was recovered (3). In these conditions the recovery was much better, 46.6%. In the HFCPC elutions with the upper phase resulted in better chromatography which took 24 hr. With the lower phase mobile the peptide emerged in 5 hr but was less well fractionated.

Chromatography of the peptide on the MLCPC in the conditions of lower phase mobile showed fractionation in a much shorter time, 30 min. This is probably due to the greater retention of stationary phase in this instrument design. If more experiments show a

significant degree of purification in such fast conditions then this method which does not require a solid support would be very promising as a rapid purification process.

## REFERENCES


1. Abbreviations used: BAW = n-butanol, acetic acid and water; Boc = t-butyloxycarbonyl; CCC = countercurrent chromatography; CCK = cholecystokinin; HFCPC = horizontal flow-through coil planet centrifuge; HPLC = high performance liquid chromatography; MLCPC = multi-layer coil planet centrifuge; TLC = thin layer chromatography; PTFE = polytetrafluoroethylene.

2. Gardner, J.D., Knight, M., Sutliff, V., Tamminga, C.A., and Jensen, R.T., Am. J. Physiol. 246, G-292 (1984).

3. Knight, M., Kask, A.M., and Tamminga C.A., Peptides Structure and Function, eds. Hruby, V.J. and Rich, D.H., Pierce Rockland, Ill., 1984, p. 759.

4. Knight, M., Kask, A.M., and Tamminga, C.A., J. Liq. Chrom. 7, 351 (1984).

5. Ito, Y., Sandlin, J.L., and Bowers, W.G., J. Chrom. 244, 247 (1982).

6. Ito, Y., U.S. Patent No. 4,430,216.

7. Ito, Y., Anal. Biochem. 100, 271, (1979).

8. Ito, Y, and Conway, W.D., J. Chrom. (1984) in press.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2535-2543 (1984)

# A COMPUTER PROGRAM FOR THE SELECTION OF GRADIENT ELUTION IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Haleem J. Issaq*
Program Resources, Inc.
NCI-Frederick Cancer Research Facility
P.O. Box B
Frederick, MD 21701

and

Karen L. McNitt and Nina Goldgaber
Information Management Services
NCI-Frederick Cancer Research Facility
P.O.Box B
Frederick, MD 21701

## ABSTRACT

A computer program is presented for the selection of a gradient mobile phase that will give the same resolution for all the component pairs in the mixture. Each pair is treated as a separate experiment. The computer is used to compile these experiments and to recommend an optimum gradient.

## INTRODUCTION

Many studies, using statistical (1-4) and graphical (5) approaches for the selection of an isocratic mobile phase that will give optimum separation have been published. Most of these methods require the use of computers

*Author to whom correspondence should be addressed.



 0148-3919/84/0713-2535$3.50/0

for data handling, and storage which makes the chromatographers' job faster and easier. In HPLC this means calculation of the area under the peaks, the width of the peaks at any specified height, the retention times and the resolution between the peaks. Also, computers have been used for the prediction of peak elution order (6,7) and for the sequencing of experiments with different parameters for each. The parameters that could be changed are mobile phase composition, flow rate and temperature.
In this study we extend the use of computers for the selection of a gradient mobile phase that will give optimum separation.

## DISCUSSION

Gradient elution is mainly used in two cases, (a) when an isocratic mobile phase fails to give adequate resolution of all the components in a mixture, due to the component's properties; and (b) when an isocratic mobile phase gives satisfactory resolution of all the components but those peaks eluting last are wide and far from each other. In the first case (a) gradient elution is used to facilitate the separation while in the second case (b) it is used to bring the late eluting peaks closer together, which results in better sensitivity and shorter analysis time. For detailed discussion of gradient elution see ref. (8). The gradient shapes generally used are linear, convex or concave, see fig. 1. Although these gradient shapes may improve resolution for each adjacent pair of components, if not impossible. Gradient separations that result in the same resolution between the adjacent pairs will be defined as optimum gradient, figure (2). To achieve an optimum gradient the chromatographer should treat each adjacent pair of components as a separate experiment, to optimize the separation between the first pair, then the second pair and so on, so that the sum of these series of pair optimization experiments is the

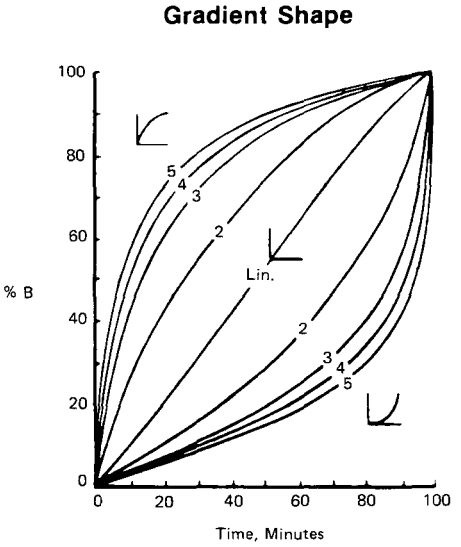



Figure 1. Gradient shapes used in high performance liquid chromatography

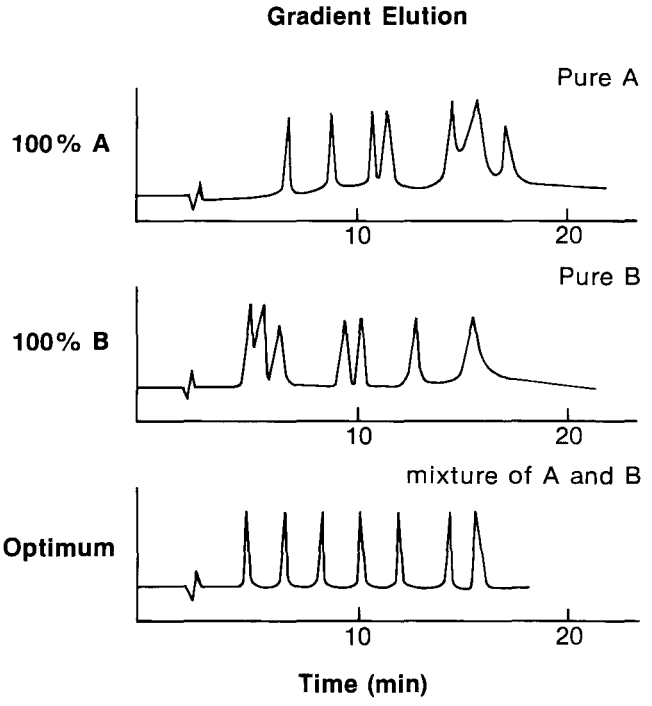



Figure 2. Gradient elution of a mixture using pure solvent A and B and optimum gradient mixture of solvents A and B.

optimum gradient. This seems to be very complex, but it is not if a computer is used to store the results of these series of experiments and to select the mobile phase for each pair based on the empirical data and to compile the final mobile phase. It is clear from the above that the gradient shape will be different from those shown in figure 1. The gradient shape predicted will consist of a series of straight lines (Figure 3). Each straight line represents a mobile phase composition that will result in the same resolution between all the pairs in the mixture. These straight lines may or may not have the same slope. To achieve an optimum gradient, a series of experiments are needed, the number of which depends on the complexity of the sample (number of components) and difficulty of separation. An average procedure will require five runs. The runs are of two types and it is up to the chromatographer to select the one he prefers. The first type is shown in figure 4, whereby the mobile phase composition is constant but the time period is not. Assume that the gradient is linear and will run from 100%A (the weak solvent) to 100%B (the strong solvent) in 10 min., the next gradient will run from 100%A to 100%B in 20 min., the third one from 100%A to 100%B in 30 min., and so on until all the components in the mixture are resolved. Solvents A and B should be miscible. Figure 5 shows the second type of gradient mobile phase composition experiments needed to determine the optimum gradient. In this case the mobile phase remains constant. The linear gradient is run from 0%B to 20%B (100%A to 80%A) in 30 min., the next time it is run from 0%B to 40% in 30 min., the third run from 0%B to 60%B in 30 min, and so on. In each case the computer will calculate the resolution between the peaks and print it in a table at the end of the experiment. The chromatographer will specify in the computer program the resolution needed between the adjacent pairs. The computer will at the end of the run search for the mobile phase composition that will give the predetermined resolution between each

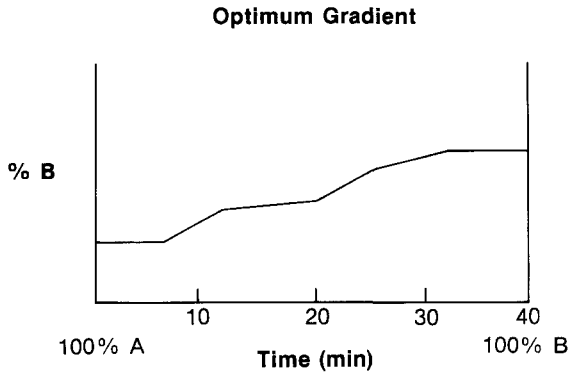



Figure 3. A graphical representation of an optimum gradient curve.

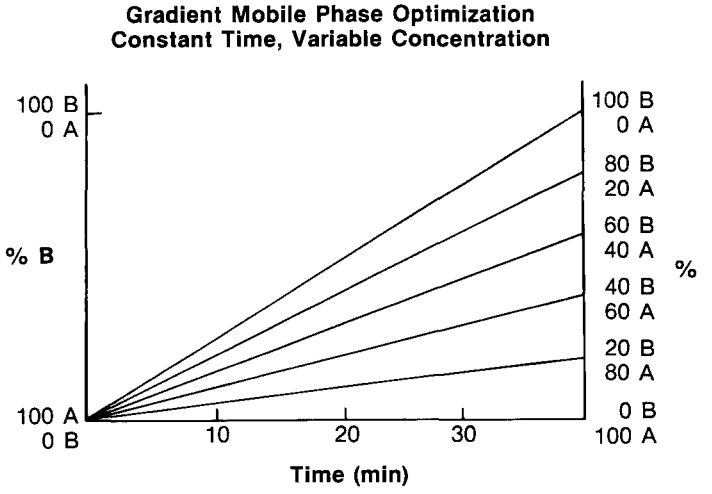



Figure 4. Gradient mobile phase optimization using constant time, 40 min., but variable mobile phase composition.

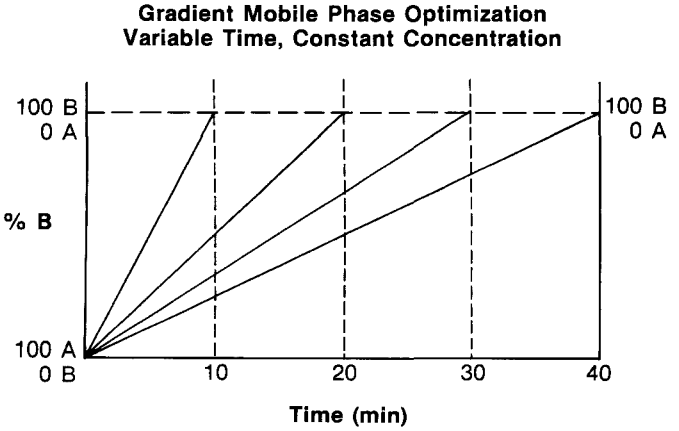



Figure 5. Gradient mobile phase optimization using variable time but the same gradient, 100%A (0%B) to 100%B (0%A).

adjacent pair and print it in a table and in a graphical form as shown in figure 3.

## COMPUTER PROGRAM

The computer program is available on request from the authors. It is written in Lab Basic for a Hewlett Packard 3354 Lab Data System. A flow chart of the program is presented in figure 6. The program assumes the peak areas and retention times are saved on the system's processed data file and the signal from the instrument is available on the system's raw data files. Due to the limitations of 32K of core on the HP3354, the program is actually split into two sections, though this split is invisible to the user.

The program operates as follows: for each run the user enters the processed data file name, the peak's elution order, in case of peak crossover and the final concentration of solvent A where the starting

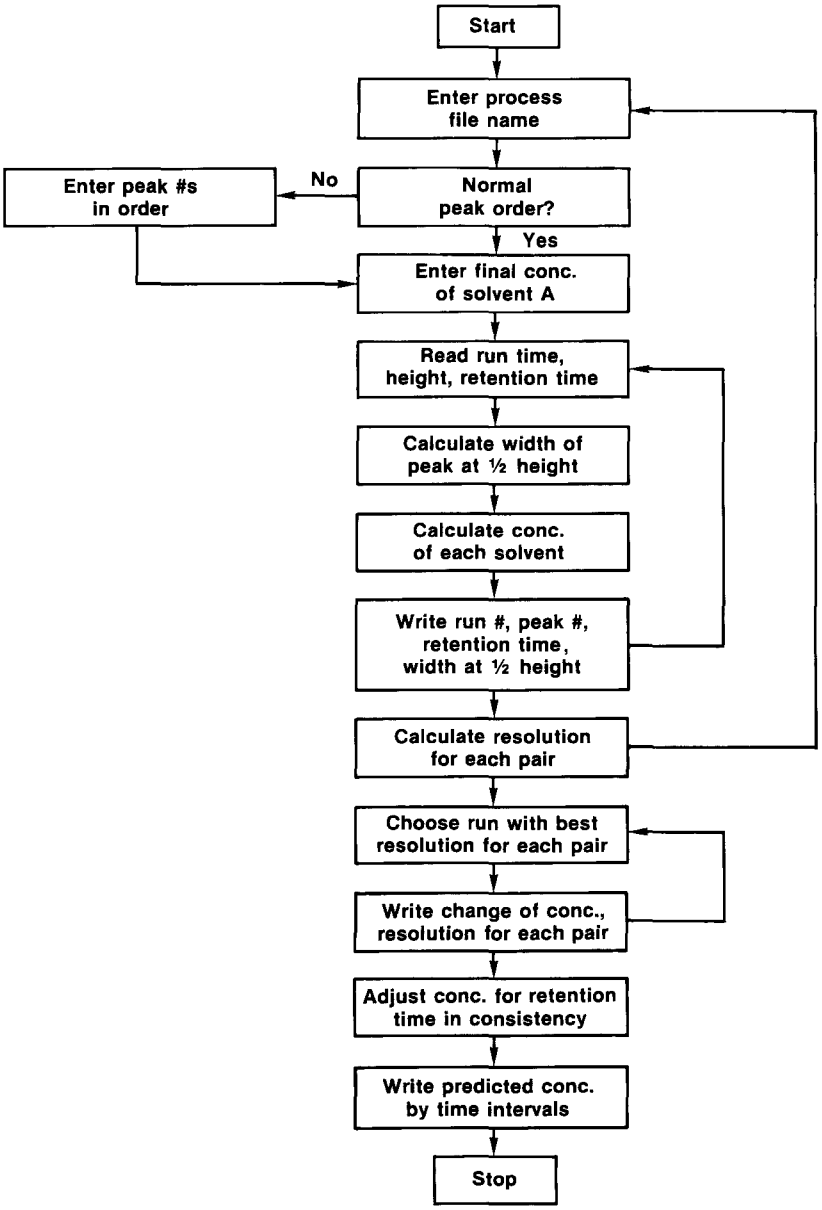



Figure 6. Flow chart of the computer program.

mobile phase composition is 0%A and 100%B. The processed data file gives the runtime and the peak retention times and heights. The peak width at half height is calculated from the raw data file. The concentration for each solvent at the peak retention time is computed and the resolution between each peak pair is calculated. Data for up to 10 runs with 10 peaks per run can be processed, this can be easily expanded to a larger number of peaks. For each peak pair the retention times and mobile phase compositions which produced the best resolution are selected as recommended at those times. If there are retention time conflicts between adjacent peaks (i.e. the best resolved retention times between peak 1 and 2 overlap the retention times for peaks 2 and 3) then a regression is fit to the four time-concentration points to smooth the data. However, no corrections are done on the mobile phase compositions if there are no retention time conflicts, which may result in inconsistant composition recommendations.

## ACKNOWLEDGEMENTS

This work was supported by Contract No. NO1-CO-23910, with the National Cancer Institute, NIH, Bethesda, MD 20205.

## REFERENCES

1. Belinky, B.R., Analytical Technology and Occupational Health Chemistry, ACS Symposium Series, Volume 220, pp. 149-168, American Chemical Society, Washington, DC. (1980).
2. Issaq, H.J., Klose, J.R., McNitt, K.L., Haky, J.E., and Muschik, G.M., J. Liq. Chromatogr. 4, 2091 (1981).
3. J.L. Glajch, J.J. Kirkland, M.K. Squire, and J.M. Minor, J. Chromatogr. 199, 57 (1980).

4. Sachok, R., Kokng, R.C., and Deming, S.H., J. Chromatogr. 199, 317 (1980).

5. Issaq, H.J., Muschik, G.M., and Janini, G.M., J. Liq. Chromatogr., 6, 259 (1983).

6. Issaq, H.J. and McNitt, K.L., J. Liq. Chromatogr. 5, 1771 (1982).

7. Issaq, H.J., American Laboratory, pp.41-46, February, 1983.

8. Synder, L.R., Gradient elution in High Performance Liquid Chromatography, Vol. 1, pp. 207-316, Academic Press (1980).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2545-2560 (1984)

# DETERMINATION OF THE MONO AND DIETHANOLAMIDES OF PALMITIC ACID AND OF SOYBEAN OIL FATTY ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Albert A. Ben-Bassat, Tamar Wasserman and
Avraham Basch*.
Israel Fiber Institute,
P.O. Box 8001, Jerusalem, Israel.

## ABSTRACT

HPLC separations and quantitative analyses are described for palmitic and soya monoethanolamides and diethanolamides synthesized from free fatty acids, methyl esters and triglycerides. Both reverse phase and adsorption techniques were employed, using a differential refractometer for detection. Starting materials and crude reaction products are analyzed without treatment or preliminary separations. The methods described are simple and rapid and can be used to monitor the course of condensation reactions as well as final products compositions. In some instances separation of homologous fatty acid alkanolamides was achieved, enabling comparison of the yields obtained from individual fatty acid precursors.

## INTRODUCTION

Direct determination of the content of surface active compounds in industrial products is very important. This simplicity saves tedious manipulations and time operation needed for isolation and purification of the components in the crude product. HPLC technique is an advance in the efforts to improve conventional methods.

---

* Author to whom correspondence should be addressed.



 0148-3919/84/0713-2545$3.50/0

Fatty alkanolamides are a widely used class of nonionic surface active agents. Particularly certain fatty diethanolamides are popular as foam builders for alkylaryl sulfonates and condensation products of higher fatty acids with diethanolamine have become important in surface active systems (1). These products are found in formulations ranging from laundry detergents, industrial cleaners to high-quality shampoos (2). The diethanolamides obtained from coconut fatty acids and from lauric acid have been incorporated into commercial products which find greatest use in the textile and cosmetic fields (3).

The fatty alkanolamides are products of condensation between an alkanolamine and a fatty acid or its derivative (methyl ester, triglyceride). The synthesis path of a monoethanolamide can be described as follows:

a) $R'COOH + NH_2CH_2CH_2OH \longrightarrow R'CONHCH_2CH_2OH + H_2O$

b) $R'COOR'' + NH_2CH_2CH_2OH \longrightarrow R'CONHCH_2CH_2OH + R''OH$

---

R' - long hydrocarbon chain

R" - methyl or ethyl group

Condensation of a carboxylic acid with diethanolamine is not a simple process because of the presence of three functional groups on the diethanolamine (4). Examination of the products has indicated that, in addition to the expected alkanolamide, they contain amine-esters (5,6). Sometimes at high temperatures formation of tertiary amines is known to occur (4,7,8). A second process which leads to formation of N-ethanolamides is amidation of fatty esters (practically methyl ester) in the presence of sodium methoxide (9,10). In 1958 G.C. Tesoro reported a method of using the ester or glyceride of the fatty acid (11). This synthesis is based on the preparation of N-substituted aliphatic amides by heating the ethyl ester of the corresponding acid with the desired amine (12).

In this study we report a satisfactory separation and determination of ethanolamides by using the HPLC technique. Measurements conditions such as eluent composition and flow rate, column packing material were empirically determined for each type of reaction product.

## EXPERIMENTAL

### Materials

The chemicals were used without further purification. Tetrahydrofuran and acetonitrile (HPLC grade) were obtained from Bio-Lab Ltd. Laboratories, Jerusalem, Israel. N-hexane, 2-propanol and acetic acid (analytical grade) were purchased from Frutarom, Laboratory Chemicals Ltd., Haifa, Israel. Deionized water was twice distillated. The first distillation was carried out in the presence of potassium permanganate and followed by a simple distillation. Perchloric acid (analytical grade, Frutarom) was used to adjust the pH of the water to 2.6. Monoethanolamine, palmitic acid (E. Merck, Darmstadt, W. Germany) and sodium methoxide (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) were synthesis grade. Diethanolamine (B.D.H., Chemical Ltd., Poole, England) was technical grade. Methylated soybean oil and glyceryl tripalmitate were obtained from Shemen Co., Israel. Soybean oil purified was purchased commercially.

The reference compounds were purchased as follows: methyl palmitate (92-94%) and methyl stearate (92-94%) from Henkel KGaA, Düsseldorf, W. Germany; methyl oleate, methyl linoleate and methyl linolenate (all approx. 99%) from Sigma Chemicals Co., St. Louis Mo., U.S.A.

The examined products were prepared according to methods reported in the literature (2,3,4,10,11). When the starting material was glyceryl tripalmitate, methyl esters or triglycerides of soybean fatty acids - 0.3% W/W of sodium methoxide was used as catalyst.

HPLC Procedure

The qualitative and quantitative analyses were run on a Varian 5030 HPLC system. Differential Refractometer - R401, Waters Associates was used to detect the separated and eluted compounds. In the present research different columns were used in order to achieve good separation of the components in various products:

A) Reverse-phase type Varian Micro Pak MCH-10 steel column (30cm x 4mm) packed with a monomeric $C_{18}$ (octadecyl) bonded onto 10μ silica gel. This column was used when the starting material was palmitic acid or glyceryl tripalmitate.

B) Reverse-phase type RP-18 steel column (30cm x 4mm) prepacked with 10 microns LiChrosorb, Cat. No. 9333 Merck, Darmstadt, W. Germany. This column was used for separation of ethanolamides prepared from methylated soybean oil.

C) Varian Micro Pak Si-5 (packed with 5μ silica) column (30cm x 4mm) was used in the case of soybean oil (mixture of triglycerides) as starting material.

The columns we operated at ambient temperature. Components were eluted isocratically at a pressure of about 115 psi. Optimum eluent compositions were arrived at experimentally, in consideration of the column type and nature of the substances determined. The examined samples were dissolved in eluent or THF (2-5% W/V) and 20 μl of the solution were injected via loop injector. In certain cases solution was achieved by gentle warming.

The detector was connected to a Hewlett-Packard 3390 A integrator to record peak areas, retention times and percentage compositions. The detector response was not calibrated for the different materials measured due to the lack of pure reference materials. The refractive indices of the eluents were close to 1.37, while those of fatty acids and their derivatives range from 1.44-1.47 (13). Replicate measurements showed that the

values for retention time and for percentage amount varied in a range of ± 2%.

Small peaks, representing less than 2% of the products, appeared in some of the chromatograms but were ignored for the purpose of this report. Free ethanolamine and diethanolamine eluted with the solvent front and were not determined.

In order to assure that the elution of the components is complete, for each series of HPLC measurements one or two samples were analyzed up to 30 minutes.

## RESULTS AND DISCUSSION

### HPLC Results

#### 1. Palmityl Monoethanolamide

The content of this alkanolamide was determined in products obtained through two different ways:

1a. Reaction of palmitic acid with monoethanolamine (1:1.1 molar ratio) under the following conditions: 3 hours at 160°C.

1b. Reaction of glyceryl tripalmitate with monoethanolamine (1:3.3 molar ratio) under the following conditions: 2 hours at 100°C.

HPLC measurements conditions for 1a and 1b: for each case the reaction product was dissolved in tetrahydrofuran (5% W/V) and analyzed on column A. The components were eluted with tetrahydrofuran, acetonitrile and water (47.5:36.5:16 V/V). The corresponding results are presented in Tables I and II.

The results presented in Tables I and II show clearly that in the case of glyceryl tripalmitate a greater amount of amine-ester was obtained.

#### 2. Palmityl Diethanolamide

The content of this alkanolamide was determined in products obtained by two different reactions:

TABLE I

HPLC Analysis Data for the Product of the Reaction Between Palmitic Acid and Monoethanolamine (a)

| Component | Retention Time (min) | Amount % |
|---|---|---|
| Palmityl monoethanolamide | 2.6 | 94.2 |
| Palmitic acid | 3.0 | 2.4 |
| Amine-ester (possibly) (b) | 6.9 | 3.4 |

(a) The flow rate of the eluent was 1.3 ml/min.

(b) Broad peak.

TABLE II

HPLC Analysis Data for the Product of the Reaction Between Glyceryl Tripalmitate and Monoethanolamine (a)

| Component | Retention Time (min) | Amount % |
|---|---|---|
| Palmityl monoethanolamide | 2.6 | 81.4 |
| Palmitic acid | 3.0 | 2.5 |
| Amine-ester | 6.4 | 16.0 |
| Glyceryl mono(di)palmitate | 7.0 | traces |

(a) The flow rate of the eluent was programmed: 1.3 ml/min for two minutes then increased at a rate of 0.1 ml/min for 28 minutes.

2a. Reaction of palmitic acid with diethanolamine (1:1.1 molar ratio) under the conditions: 3 hours at 160°C.

2b. Reaction of glyceryl tripalmitate with diethanolamine (1:3.3 molar ratio) under the conditions: 3 hours at 145°C.

The products were examined under the following conditions: the crude product was dissolved in tetrahydrofuran (5% W/V) and separated on column A. The components were eluted with tetrahydrofuran, acetonitrile and water (47.5:36.5:16 V/V). The results are presented in Tables III and IV (see also Figure 1).

3. In the present work HPLC measurements were also carried out for separation and determination of monoethanolamides or diethanolamides prepared from methylated soybean oil (a mixture of the methyl esters of soybean fatty acids). The principal soybean fatty acids are: linoleic, oleic, palmitic, linolenic and stearic. The chromatograms obtained enabled to calculate the ethanolamides content in the crude reaction product. For information and comparison of the results the starting material (methylated soybean oil) was analyzed by HPLC (Table V). The products of the following reactions were examined:

3a. Reaction of methylated soybean oil with monoethanolamine under the conditions: one hour at 95°C.

3b. Reaction of methylated soybean oil with diethanolamine under the conditions: three hours at 130°C.

The HPLC conditions were: the examined product was without further treatment dissolved in the eluent and separated on column B. The components were eluted with tetrahydrofuran, acetonitrile and water (43:43:14 V/V). The results are presented in Tables V, VI (see Figure 2) and VII.

The results obtained show that in addition to the corresponding ethanolamides, the examined products contain also amounts of the starting material and of amine-esters. It may be concluded that the monoethanolamides were obtained in a higher yield than the corresponding diethanolamides.

TABLE III

HPLC Analysis Data for the Product of the Reaction Between Palmitic Acid and Diethanolamine (a)

| Component | Retention Time (min) | Amount % |
|---|---|---|
| Palmityl diethanolamide | 5.2 | 60.4 |
| Palmitic acid | 6.4 | 1.6 |
| Amine-ester | 11.6 | 32.0 |
| Undefined (b) | 13.6 | 2.0 |

(a) The flow rate of the eluent was 0.6 ml/min increased to 1.0 ml/min during 15 minutes.

(b) Broad peak.

TABLE IV

HPLC Analysis Data for the Product of the Reaction Between Glyceryl Tripalmitate and Diethanolamine (a)

| Component | Retention Time (min) | Amount % |
|---|---|---|
| Palmityl diethanolamide | 2.4 | 63.1 |
| Palmitic acid | 2.9 | 2.3 |
| Amine-esters | 5.7 | 25.0 |
| Glyceryl mono(di)palmitate | 6.8 | 2.4 |

(a) The flow rate of the eluent was programmed: 1.4 ml/min for two minutes increased to 3.0 ml/min during additional 18 minutes and constant flow of 3.0 ml/min for further 10 minutes.

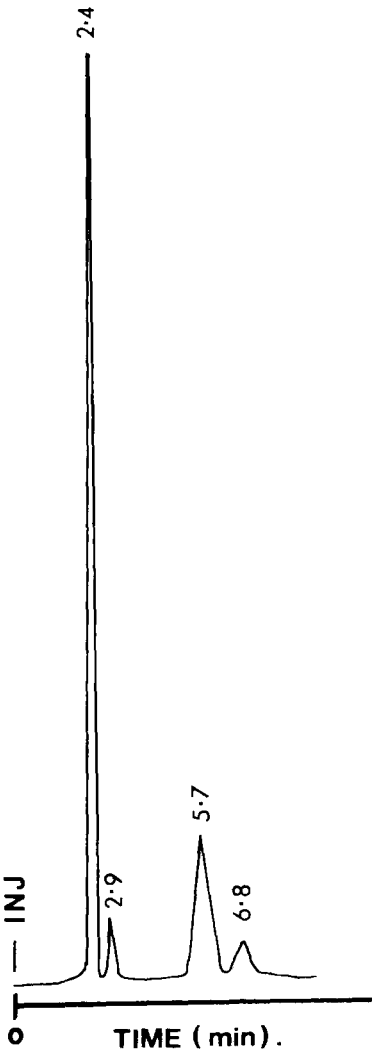



FIGURE 1: Reversed-phase HPLC chromatogram of the product of the reaction between glyceryl tripalmitate and diethanolamine (see Table IV).

TABLE V

HPLC Analysis of Methylated Soybean Oil (a)

| Component | Retention Time (min) | Amount % |
|---|---|---|
| Methyl linolenate | 6.5 | 8.5 |
| Methyl linoleate | 7.4 | 56.5 |
| Methyl palmitate ) | 9.0 | 31.0 |
| Methyl oleate ) | | |
| Methyl stearate | 11.7 | 3.0 |

(a) The flow rate of the eluent was: 0.6 ml/min increased to 1.0 ml/min in the first 15 minutes and 1 ml/min for further 5 minutes.

TABLE VI

HPLC Analysis Data for the Product of the Reaction Between Methylated Soybean Oil and Monoethanolamine (a)

| Component | Retention Time (min) | Amount % |
|---|---|---|
| Linolenyl monoethanolamide | 4.2 | 8.0 |
| Linoleyl monoethanolamide | 4.5 | 55.7 |
| Palmityl monoethanolamide ) | 5.1 | 28.4 |
| Oleyl monoethanolamide ) | | |
| Stearyl monoethanolamide | 5.9 | 6.4 |
| Methyl esters of soybean fatty acids | 7.2-8.9 | 1.4 |

(a) The flow rate of the eluent was 0.7 ml/min.

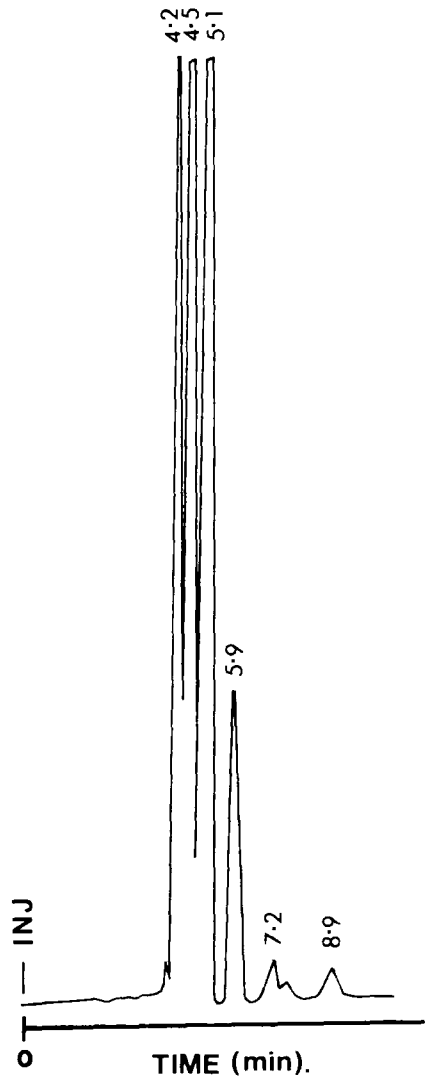



FIGURE 2: Reversed-phase HPLC chromatogram of the product of the reaction between methylated soybean oil and monoethanolamine (see Table VI).

TABLE VII

HPLC Analysis Data for the Product of the Reaction Between Methylated Soybean Oil and Diethanolamine (a)

| Component | Retention Time (min) | Amount % |
|---|---|---|
| Linolenyl diethanolamide | 4.4 | 5.0 |
| Linoleyl diethanolamide | 4.8 | 45.4 |
| Palmityl diethanolamide ) Oleyl diethanolamide ) | 5.3 | 21.0 |
| Stearyl diethanolamide | 6.2 | 5.0 |
| Methyl esters of soybean acids | 7.0-10.0 | 16.0 |
| Amine-esters | 13.2-16.6 | 4.0 |

(a) The flow rate of the eluent was programmed: 0.6-1.0 ml/min for 15 minutes followed by a constant rate of 1 ml/min for additional 5 minutes.

The mono- and-diethanolamides elute, as expected, in the same relative order as the corresponding methyl esters but at lower retention volumes, due to their greater polarities. Confirmation of the identities of the ethanolamide peaks can be found in their relative abundances compared with the starting methyl esters. These are equal ($\pm$ 2%) for the methyl esters, mono- and di-ethanolamides, implying incidentally similar reactivities for the various fatty methyl esters with ethanolamines.

4. Additional application of HPLC in the present work was the determination of mono or diethanolamides derived from purified soybean oil. This oil is a mixture of the triglycerides of the corresponding fatty acids (above mentioned).

TABLE VIII

HPLC Analysis Data for the Product of the Reaction Between Soybean Oil and Monoethanolamine (a)

| Component (group) | Retention Time (min) | Amount % |
|---|---|---|
| Triglycerides | 3.4 | 0.4 |
| Diglycerides | 3.8-4.3 | 2.4 |
| Amine-esters | 4.8 | 4.1 |
| Monoglycerides | 6.9 | 1.7 |
| Monoethanolamides (b) | 8.4 | 91.1 |

(a) The eluent composition was: n-hexane, isopropanol and acetic acid (80:20:1 V/V).

(b) Overlapping resulted in a broad peak.

TABLE IX

HPLC Analysis Data for the Product of the Reaction Between Soybean Oil and Diethanolamine (a)

| Component (group) | Retention Time (min) | Amount % |
|---|---|---|
| Triglycerides | 3.4 | 0.4 |
| Diglycerides | 3.7-3.8 | 2.9 |
| Fatty acids | 4.3 | 2.9 |
| Amine-esters | 5.2 | 22.2 |
| Monoglycerides | 6.1 | 4.3 |
| Undetermined | 7.4 | 1.8 |
| Diethanolamides (b) | 10.8 | 65.4 |

(a) The eluent composition was: n-hexane, isopropanol and acetic acid (75:25:1 V/V).

(b) Overlapping resulted in a broad peak.

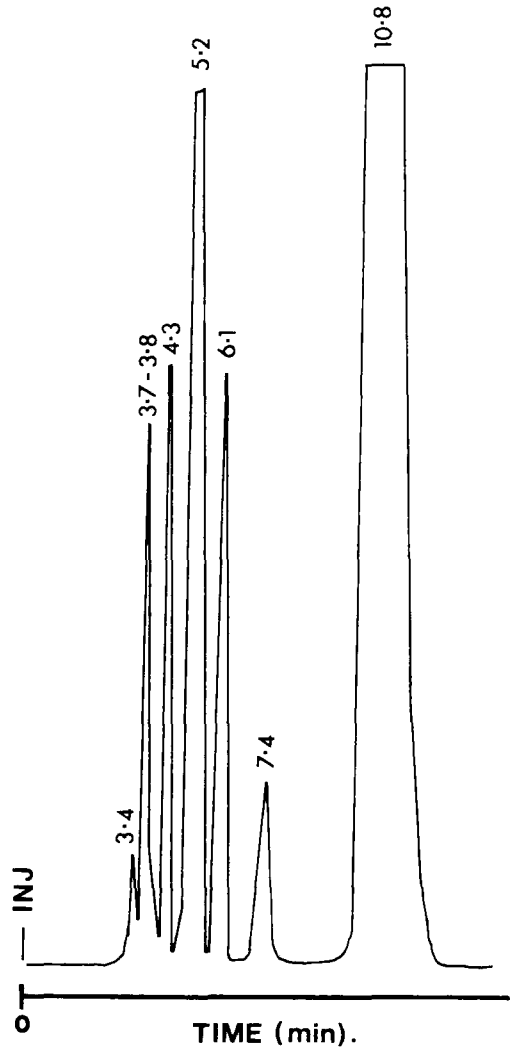



FIGURE 3: HPLC chromatogram of the product of the reaction between soybean oil and diethanolamine (see Table IX).

The products were analyzed without further treatment. The chromatograms obtained showed that in addition to the corresponding ethanolamides partially hydrolyzed glycerides and amine-esters were detected. Under the present reported HPLC conditions broad (overlapping) peaks were obtained for each group of components and integration of the total area was performed.

The products of the following reactions were analyzed:

4a. Reaction of soybean oil with monoethanolamine at 108°C for a period of two hours.

4b. Reaction of soybean oil with diethanolamine at 145°C for a period of three hours.

HPLC Determinations

In each case the crude product was dissolved in the eluent and separated on column C. The flow rate was 0.8 ml/min. The ethanolamides were eluted last without separation and their total amount was recorded. The results are presented in Tables VIII and IX (see also Figure 3).

Commercial soybean oil analyzed under these conditions contained 96.8% triglycerides (eluted as one peak) and 3.2% diglycerides.

## SUMMARY

HPLC conditions are described for the analysis of fatty acid mono-and-diethanolamides and associated impurities and by-products encountered in typical syntheses. Separations and quantitation were achieved for the homologous fatty acid methyl esters as well as the corresponding ethanolamides. These methods afford simple and rapid analyses for these complex mixtures and can be used to follow the course of synthesis and to characterize commercial products.

## ACKNOWLEDGEMENTS

This work was supported in part by Koor Foods Ltd., Israel.

## REFERENCES

1. Schwartz, A.M. and Perry, J.W., Surface Active Agents, Interscience Publishing Company, N.Y., 1949, p. 212.

2. Monick, J.A., JAOCS, 39, 213, 1962.

3. Kroll, H. and Nadeau, H., JAOCS, 34, 323, 1957.

4. Kritchevsky, W. (Ninol Laboratories), US Patent 2,089,212 (1937).

5. Masci, J.N. and Poirer, N.A. (to Johnson & Johnson), US Patent 2,680,753 (June 8, 1954).

6. Trowbridge, J.R., Falk, R.A. and Krems, I.J., J. Org. Chem., 20, 990, 1955.

7. Malkemus, J.D. (to Colgate-Palmolive-Peet Co.), US Patent 2,636,033 (April 21, 1953).

8. Bauer, E., US Patent 2,575,041 (November 13, 1951).

9. Meade, E.M. (Lankro Chemicals Ltd.), British Patent 631,637 (1949).

10. Meade, E.M. (Lankro Chemicals Ltd.), US Patent 2,464,094 (1949).

11. Tesoro, G.C. and Ferry, D. (to Onyx Oil & Chemical Co.), US Patent 2,844,609 (July 1958).

12. D'Alelio, G.F. and Reid, E.E., JACS, 59, 111, 1937.

13. Rosen, M.J. and Goldsmith, H.A., Systematic Analysis of Surface-Active Agents, second edition, Wiley-Interscience, N.Y., 1972, p. 531-539.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2561-2577 (1984)

# INVESTIGATION OF THE BEHAVIOR OF OXIDIZED PTERINS IN LIQUID CHROMATOGRAPHIC SYSTEMS

Craig E. Lunte and Peter T. Kissinger

Department of Chemistry

Purdue University

West Lafayette, Indiana 47907

## ABSTRACT

Cation-exchange, reverse-phase, and "ion-pair" reverse-phase chromatography were evaluated for the separation of several oxidized pterins. Chromatographic parameters effecting the retention of the pterins in these chromatographic systems were investigated. "Ion-pair" reverse-phase chromatography was found to be the most satisfactory system and a succesful separation using this system was developed.

## INTRODUCTION

The pterins are a family of compounds of biological interest. A reduced pterin is a required cofactor for the enzymatic hydroxylation of phenylalanine, tyrosine, and tryptophan (1). Abnormal pterin concentrations have been associated with several diseases, including; phenylketonuria (2,3), rheumatoid arthritis (4), kidney dysfunction (4), Parkinson's



 0148-3919/84/0713-2561$3.50/0

disease (5), and senile dementia (6). Elevated concentrations of the pterin neopterin have been proposed as a diagnostic marker for neoplasia (7) and Aquired Immunodeficiency Syndrome, AIDS (8).

For these reasons, determination of pterins in biological samples has recieved much attention recently. The most popular techniques for determination of pterins involve liquid chromatography with either electrochemical detection (9-11) or fluorescence detection (12-15). In the development of separation methods for the pterins, reverse-phase (13-15), "ion-pair" reverse-phase (9-11), and cation-exchange chromatography (12) have all been employed. In the course of developing an analytical methodology based on liquid chromatography/electrochemistry (LCEC), these approaches were evaluated for the separation of several oxidized pterins.

Several chromatographic parameters effecting the retention of pterins in these chromatographic systems were investigated. These parameters included the mobile phase pH, ionic strength, and organic modifier concentration. The effect of varying the chromatography column temperature was also studied. For the "ion-pair" reverse-phase system, the effect of the mobile phase ion-pairing reagent concentration was investigated. From these investigations a separation of several oxidized pterins was achieved.

## MATERIALS AND METHODS

Reagents. Pterin, pterin-6-carboxylic acid, and xanthopterin were purchased from Sigma Chemical Co. (St. Louis, MO). Biopterin was obtained from Calbiochem-Behring (La Jolla, CA). Neopterin was obtained from Fluka (Basle, Switzerland). 6-Hydroxymethylpterin was prepared by the method of Thijssen (18). Octyl sodium sulfate was purchased from Eastman Kodak (Rochester, NY). All other chemicals were reagent grade or better and used without purification.

Apparatus. The chromatographic system consisted of an Altex 110 pump with a Rheodyne 7125 injection valve with a 20 uL sample loop. A Biophase ODS 5u column (2.5 cm x 4.6 mm) was used in the reverse-phase and "ion-pair" reverse-phase experiments. For the cation-exchange experiments, a Zorbax 300 SCX 10u column (2.5 cm x 4.6 mm) was employed. The column temperature was controlled by an LC-23 column heater and an LC-22 temperature controller (Bioanalytical Systems, Inc., West Lafayette, IN). Detection was with a Bioanalytical Systems LC-4B amperometric detector using a glassy carbon working electrode and a Ag/AgCl reference electrode. The electrochemical detector was operated at -700 mV versus the Ag/AgCl reference.

Mobile Phase Preparation. All mobile phases were prepared from distilled, deionized water and glass distilled methanol. Each mobile phase was filtered through a 0.22 um filter (Millipore, Milford, MA) prior to use. Oxygen was removed by continuous purging with nitrogen and maintaining the mobile phase reservoir at 40°C. A flow rate of 1.0 mL/minute was used in all experiments.

## RESULTS AND DISCUSSION

Reverse-phase Chromatography. Reverse-phase chromatography has been the most widely used method to separate the pterins using fluorescence detection (12-15). Electrochemical detection puts two constraints on the system not experienced with fluorescence detection. First, the mobile phase must contain an electrolyte, preferably a buffer. In order to minimize solution resistance, the ionic strength should be approximately 0.1 molar, although lower concentrations have been employed successfully. Second, for optimal electrochemical detection, the mobile phase should be acidic in order to lower the reduction potentials of the oxidized pterins.

To investigate the behavior of the pterins in a reverse-phase system, the pH of the mobile phase was varied from 2.0 to 3.6 (Figure 1). It has previously been shown that above pH 5.0 the retention of the pterins is relatively independent of pH (15). For all of the pterins studied, except pterin-6-carboxylic acid, retention increased as the mobile phase pH increased. This is as expected since the pterins are protonated at lower pH (pKa's 1-2) and therefore have little affinity for the hydrophobic stationary phase. Pterin-6-carboxylic acid is zwitterionic, having both an acidic (carboxyl) and basic (amine) functionality. As the pH is increased the carboxyl group dissociates and thereby decreases retention.

Another parameter which was investigated was the mobile phase ionic strength. Figure 2 illustrates the strong

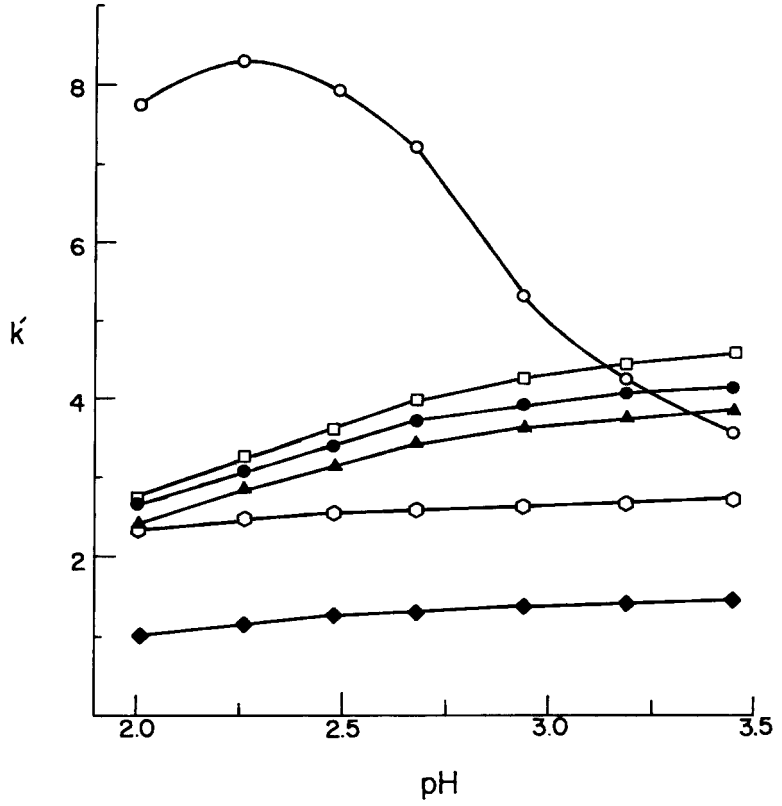



Figure 1.

Effect of pH on reverse-phase retention. ▲ = Biopterin, □ = 6-Hydroxmethylpterin, ◆ = Neopterin, ● = Pterin, ○ = Pterin-6-carboxylic acid, ⬡ = Xanthopterin.

effect increased ionic strength has on the retention of the pterins. From this result it is not surprising that the majority of reports of reverse-phase separations of the pterins have been achieved in unbuffered mobile phases (13,14).

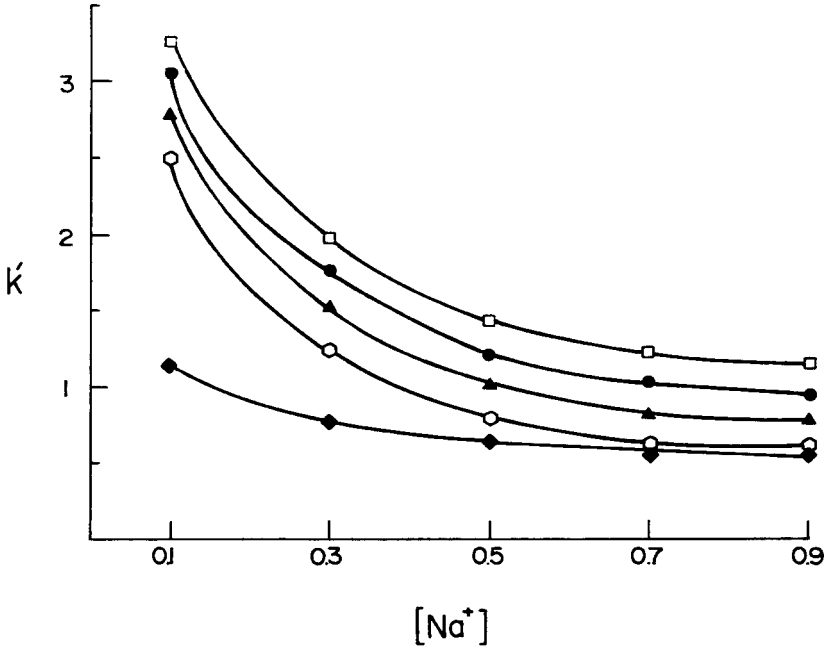



Figure 2.

Effect of ionic strength on reverse-phase retention. Symbols as in Fig. 1.

Overall, the largest capacity factor achieved for any pterin, other than pterin-6-carboxylic acid, was less than five. Therefore, the reverse-phase system lacks the flexibilty for adjusting the separation as needed for a complex sample. Figure 3 illustrates the optimum separation achieved with this system. It can be seen that xanthopterin elutes as a broad peak, most likely due to a mixed retention mechanism involving silanol groups on the packing material. It appears that a reverse-phase system is unsatisfactory for the separation of all of the pterins

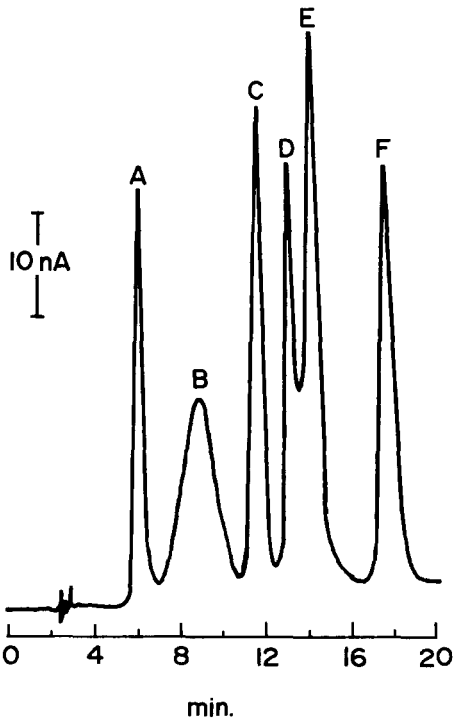



Figure 3.

Reverse-phase separation of pterins. Mobile phase; 0.1 M sodium phosphate buffer, pH 3.0. Peak identities: A, Neopterin; B, Xanthopterin; C, Biopter D, Pterin; E, 6-Hydroxymethylpterin; F, Pterin-6-Carboxylic Acid.

studied. The conditions could, however, be optimized to provide satisfactory separation of a sample containing only one or two pterins.

Cation-exchange Chromatography. At low pH the pterins are protonated and therefore amenable to cation-exchange chromatography. Figure 4 illustrates the effect of mobile phase pH on the retention of the pterins on a cation-

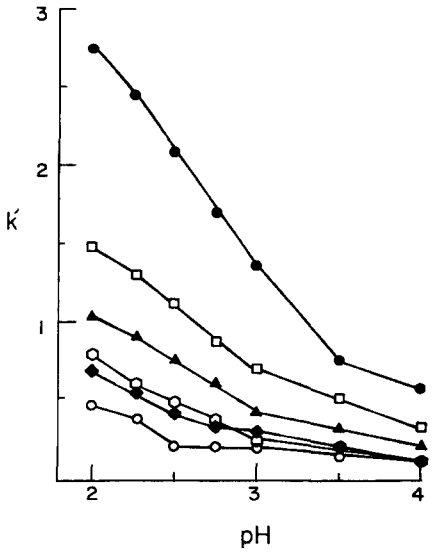



Figure 4.
Effect of pH on cation-exchange retention. Symbols as in Fig. 1.

exchange column. In contrast to reverse-phase chromatography, for cation-exchange chromatography an increase in mobile phase pH causes a decrease in the retention of the pterins. In this system, xanthopterin elutes as a symmetric peak. However, as for the reverse-phase system, the largest capacity factor is less than five. Indeed, at no pH studied was pterin-6-carboxylic acid removed from the void response. As can be seen from Figure 5, even with the optimal conditions, xanthopterin and neopterin are not well resolved. This small retention and poor resolution makes cation-exchange chromatography unsuitable for the separation of a mixture of several pterins.

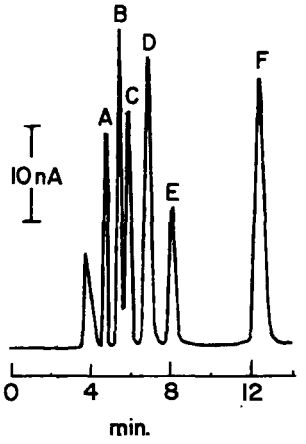



Figure 5.

Cation-exchange separation of pterins. Mobile phase; 0.1 M sodium phosphat buffer, pH 2.0. Peak identities: A, Pterin-6-Carboxylic Acid; B, Neopterin C, Xanthopterin; D, Biopterin; E, 6-Hydroxymethylpterin; F, Pterin.

"Ion-pair" Reverse-phase Chromatography. The advantages of both reverse-phase chromatography and cation-exchange chromatography can be realized through the addition of an ion-pairing reagent, octyl sodium sulfate, to the mobile phase. This gives the hydrophobic column cation-exchange properties. This can be seen in Figure 6 where the effect of mobile phase pH on the retention of the pterins is shown. As in the case of cation-exchange chromatography, retention decreases as the mobile phase pH increases. However, in the limit of high pH the retention is more similar to that for reverse-phase chromatography.

"Ion-pair" reverse-phase chromatography provides an additional chromatographic parameter, namely the ion-pairing

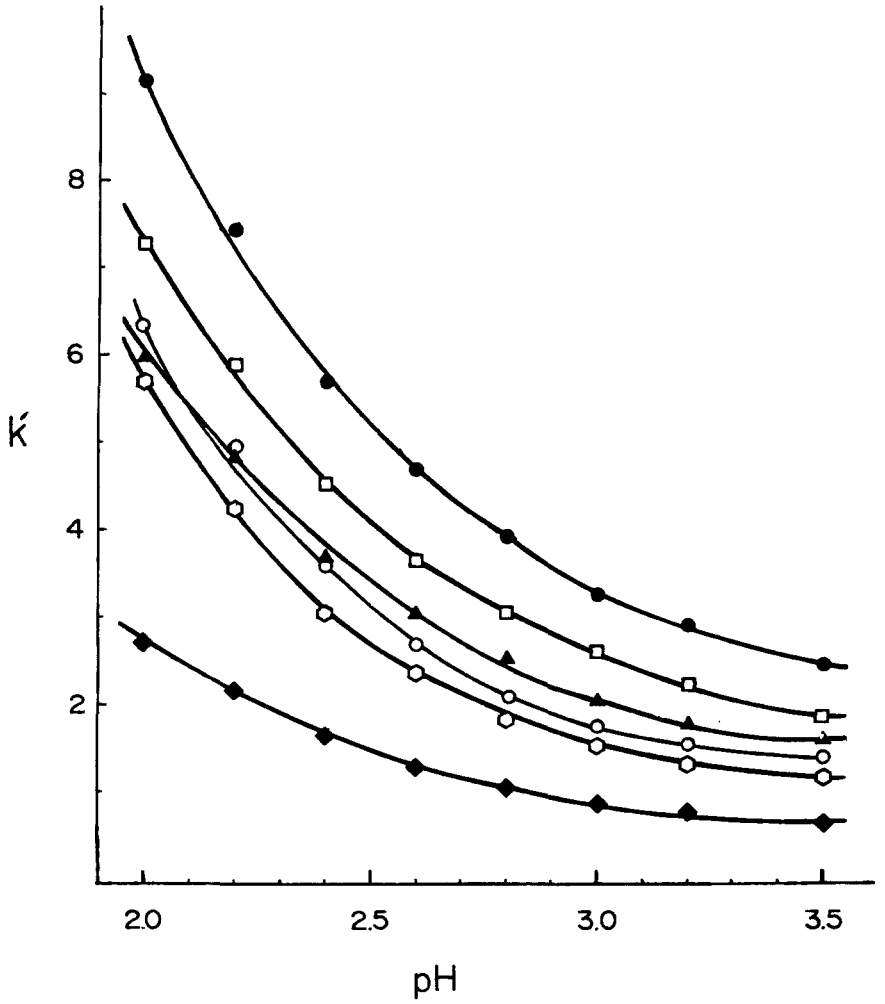



Figure 6.

Effect of pH on reverse-phase "ion-pair" retention. Symbols as in Fig. 1.

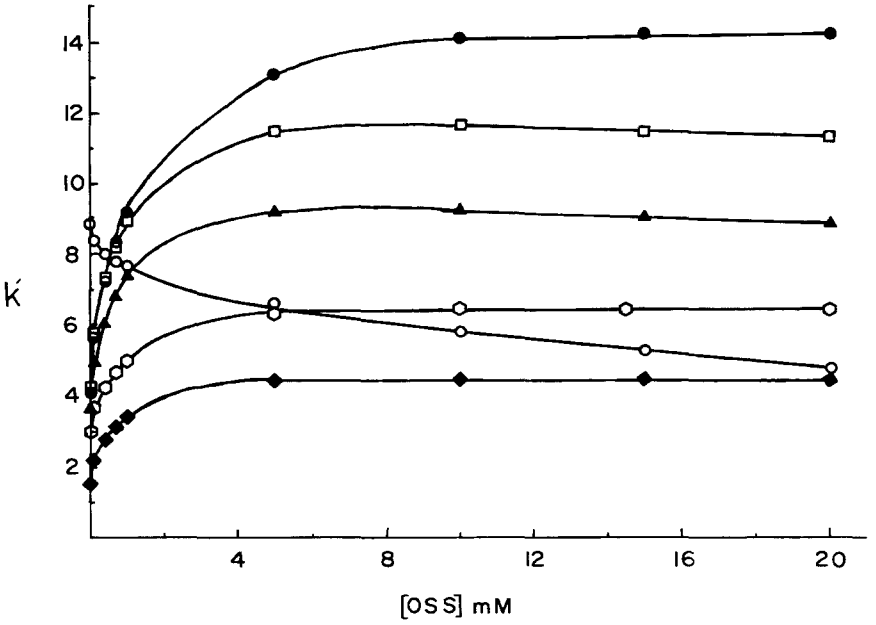



Figure 7.

Effect of ion-pairing reagent concentration on retention. Symbols as in Fig. 1. [OSS] = octyl sodium sulfate concentration.

reagent concentration in the mobile phase. Figure 7 shows the effect of ion-pairing reagent concentration on retention. Retention increases as the concentration of ion-pairing reagent increases, however, pterin-6-carboxylic acid is an exception. Pterin-6-carboxylic acid's behavior is likely due to charge repulsion between the ion-pairing reagent's sulfate group and the carboxyl group of this pterin.

Retention is a function of the surface concentration of adsorbed ion-pairing reagent, which in turn is related to

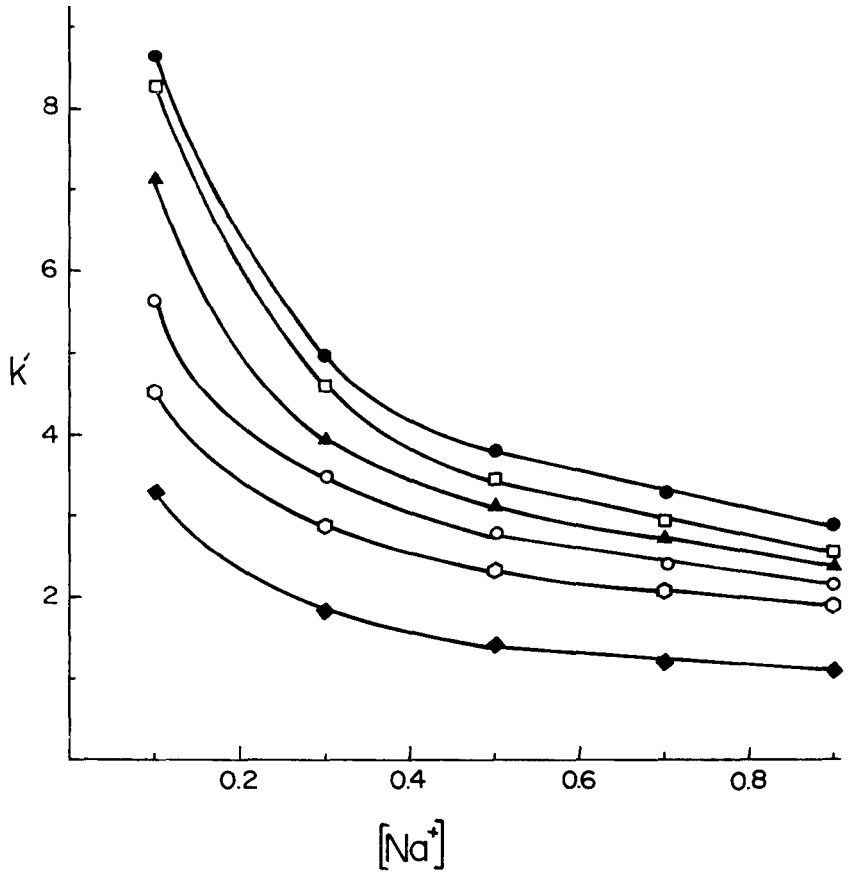



Figure 8.

Effect of ionic strength on reverse-phase "ion-pair" retention. Symbols as in Fig. 1.

the mobile phase ion-pairing reagent concentration by a Langmuir adsorption isotherm (16). The curves of Figure 6 do show the shape expected of a Langmuir isotherm. It should be noted that ion-pairing reagent concentrations above 5 mM have little effect on retention. This is due to saturation of the stationary phase with ion-pairing reagent.

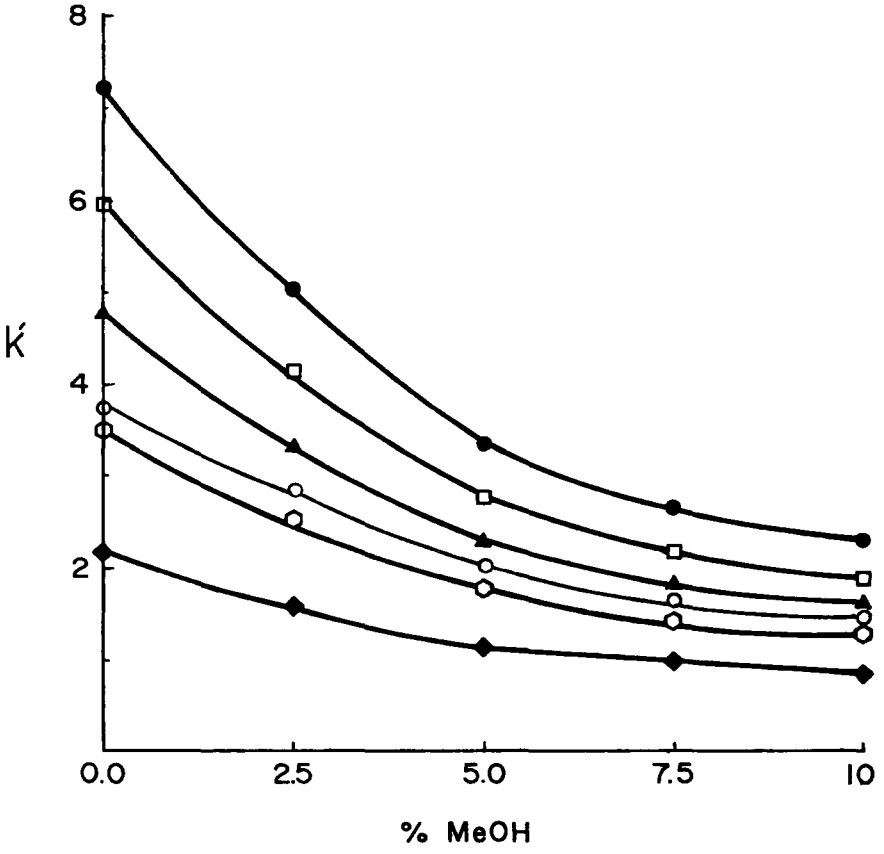



Figure 9.

Effect of methanol concentration on reverse-phase "ion-pair" retention. Symbols as in Fig. 1.

Two other parameters which effect the retention of pterins in this ion-pairing system are the mobile phase ionic strength and organic modifier (methanol) concentration. Increases in both of these parameters decrease retention with little effect on resolution (Figures

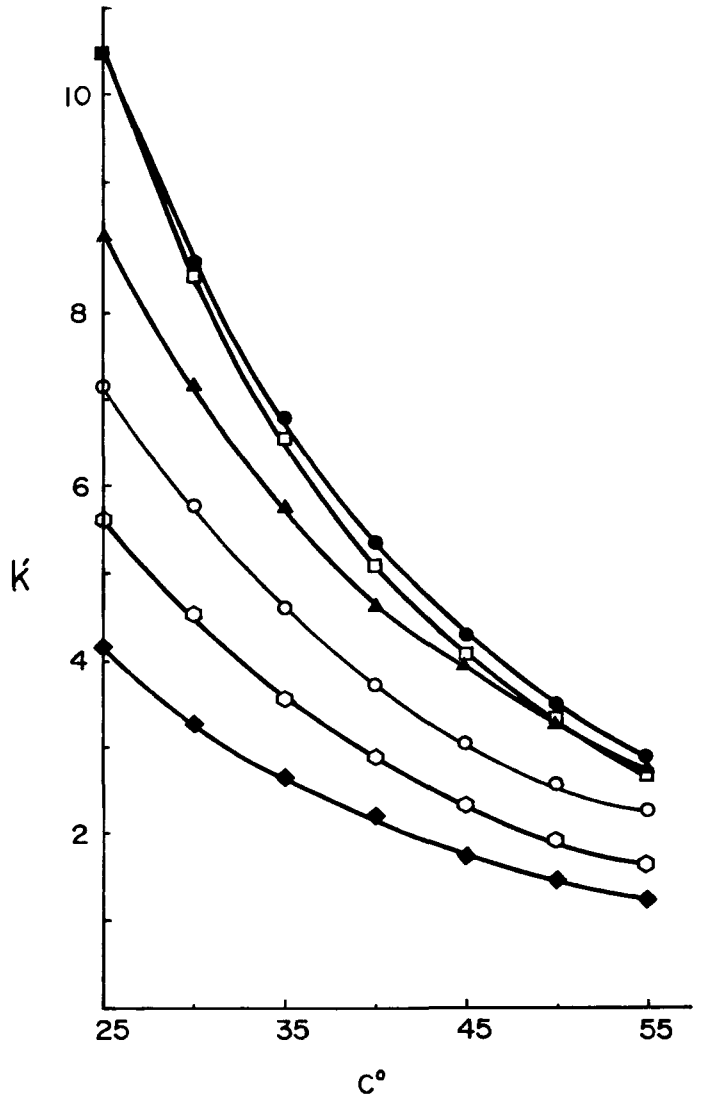



Figure 10.

Effect of column temperature on reverse-phase "ion-pair" retention. Symbols as in Fig. 1.

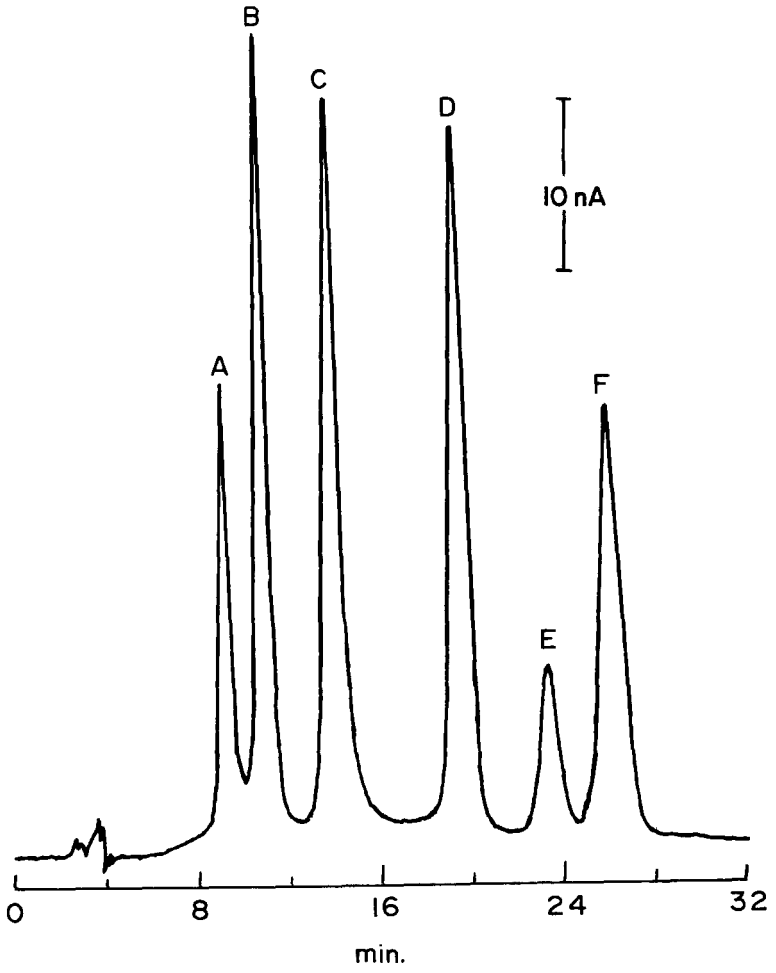



Figure 11.

Reverse-phase "ion-pair" separation of pterins. Mobile phase: 3 mM octyl sodium sulfate in 0.1 M sodium phosphate buffer, pH 2.5, at 30° Peak identities: A, Neopterin; B, Xanthopterin; C, Pterin-6-Carboxylic Acid; D, Biopterin; E, 6-Hydroxymethylpterin; F, Pterin.

8 and 9 respectively). Variation of either mobile phase ionic strength or methanol concentration is an excellent method of controlling the solvent strength without effecting the separation.

A final parameter which effects the "ion-pair" reverse-phase separation of pterins is the column temperature. The effect of column temperature on the retention of pterins is shown in Figure 10. For most pterins, as temperature increases retention decreases with little change in relative retention. Howevr, temperature has a much more pronounced effect on 6-hydroxymethylpterin than on the other pterins studied. Changes in column temperature can be used to "fine-tune" the retention of 6-hydroxymethylpterin to achieve the optimal resolution. Using "ion-pair" reverse-phase chromatography the separation shown in Figure 11 was achieved.

## CONCLUSION

Neither reverse-phase chromatography or cation-exchange chromatography proved adequate to separate a mixture of several oxidized pterins. "Ion-pair" reverse-phase chromatography was found to combine the advantages of the other two techniques to give a satisfactory separtion. In addition, the "ion-pair" reverse-phase system offers the flexibility to readily modify the separation for specific analytical requirements.

## REFERENCES

1. Kaufman, S.; Fisher, D.B., in "Molecular Mechanisms of Oxygen Activation" O. Hayaishi, ed., Academic Press: New York, 1974, p. 285.

2. Kaufman, S., Science 128, 1506, 1958.

3. Milstein, S.; Kaufman, S.; Summer, G.K., Pediatrics 65, 806, 1980.

4. Leeming, R.J.; Blair, J.A.; Melikian, V.; O'Gorman, D.J., J. Clin. Pathol. 29, 444, 1976.

5. Robinson, D.S.; Levine, R.; Williams, A.; Stathern, N., Psychopharmacol. Bull. 14, 49, 1978.

6. Leeming, R.J.; Blair, J.A.; Melikian, V., Lancet 215, 1979.

7. Hausen, A.; Wachter, H., J. Clin. Chem. Clin. Biochem. 20, 593, 1982.

8. Fuchs, D.; Hausen, A.; Hauber, CH.; Knosp, O.; Reibenegger, G.; Spira, T., Hoppe-Seyler's Z. Physiol. Chem. 364, 1345, 1983.

9. Lunte, C.E.; Kissinger, P.T., Anal. Biochem. 129, 377, 1983.

10. Lunte, C.E.; Kissinger, P.T., Anal. Chem .55, 1458, 1983.

11. Brautigam, M.; Dreesen, R.; Herken, H., Hoppe-Seyler's Z. Physiol. Chem. 363, 341, 1982.

12. Stea, B.; Halpern, R.M.; Smith, R.A., J. Chromatogr. 168, 385, 1979.

13. Fukushima, T.; Nixon, J.C., Anal. Biochem. 102, 176, 1979.

14. Woolf, J.H.; Nichol, C.A.; Duch, D.S., J. Chromatogr. 168, 385, 1979.

15. Andondonskaja-Renz, B.; Zeitler, H-J., Anal. Biochem. 133, 68, 1983.

16. Sorel, H.A.; Hulshoff, A., in "Advances in Chromatography Vol. 21" J.C. Giddings, E. Grushka, J. Cazes, P.R. Brown, eds., Marcel Dekker: New York, 1983, p. 87.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2579-2590 (1984)

# A RAPID LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF FLECAINIDE IN HUMAN BLOOD PLASMA USING ULTRAVIOLET DETECTION

J. Boutagy, F.M. Rumble and G.M. Shenfield

Department of Clinical Pharmacology,
Royal North Shore Hospital,
St. Leonards, N.S.W. 2065, Australia.

## ABSTRACT

A liquid chromatographic method for the assay of the antiarrhythmic drug flecainide in plasma has been developed. The method is rapid, simple and with sufficient detection sensitivity to render it suitable for therapeutic drug monitoring. Flecainide and added internal standard, a non-fluorinated analogue, were extracted by a single ether extraction from alkalinized plasma followed by a back-extraction of the ether with dilute phosphoric acid. A portion of the acid extract was then applied directly to a 30 cm ODS column eluting isocratically with 30% acetonitrile in water containing 0.01M dibutylamine phosphate. Monitoring was by ultraviolet detection at 214 nm and the total run time was 8 min. This method is specific and can quantitate plasma levels to less than 30 ng/ml (free base) from 0.5 ml of plasma without interference from antiarrhythmic drugs commonly used in therapy.

## INTRODUCTION

Flecainide acetate (R-818) is a relatively new antiarrhythmic drug recently introduced into clinical medicine for evaluation (1-3). It is an orally active drug effective in suppressing ventricular arrhythmias and has been designated a Class 1 antiarrhythmic agent (4-7). The pharmacological properties of



 0148-3919/84/0713-2579$3.50/0

flecainide derived from clinical and animal studies have been well documented in a recent symposium devoted to this drug (8).

To date, there is no established therapeutic range in man for flecainide plasma levels although there is mounting evidence that there is an effective plasma concentration range within which the drug is clinically active. This range is between 200 and 900 ng/ml (10,11) but as more data becomes available these values may be modified. It is therefore likely that as for other Class 1 antiarrhythmic drugs the routine monitoring of plasma flecainide levels will be a useful adjunct to therapy.

There are methods described for the assay of flecainide in blood or plasma. These include a spectrophotometric method (9) which suffers the disadvantage of interference from quinidine and propranolol, gas-liquid chromatography methods requiring derivatization (10-11) and several high-performance liquid chromatographic (HPLC) methods (12-16). The methods by HPLC all use fluorescence detection for selectivity and sensitivity and most require one millilitre of plasma. Of the two more simple of these procedures one method (14) uses a phenyl column to achieve separation together with a fluorinated isomer of flecainide as the internal standard but without good baseline resolution of standard and drug. Also with that chromatographic procedure diazepam co-elutes with flecainide becoming a potential source of interference should ultraviolet detection be used (15). The second method (16) uses normal-phase chromatography requiring small plasma volumes. In our own drug assay laboratory where an HPLC instrument used for

routine drug assay is dedicated to reverse-phase chromatography the constant switching to normal-phase would become inconvenient. We have therefore developed a simple and rapid assay for the analysis of flecainide in plasma using reverse-phase chromatography and ultraviolet detection at 214 nm giving sufficient sensitivity and selectivity to enable use in routine drug monitoring. The procedure is essentially a modification of the method used in our laboratory for the analysis of most commonly used antiarrhythmic drugs (unpublished data). The method consists of a single extraction of alkalinized plasma with diethyl ether followed by a back extraction into dilute aqueous acid. The chromatography is performed on a standard C18 reverse-phase column eluting with acetonitrile-water mobile phase containing di-butylamine phosphate as a modifier and monitoring using a fixed wavelength detector with a zinc lamp and filter for 214 nm output. The internal standard used for the assay is the non-fluorinated analog of flecainide.

## MATERIALS

Flecainide acetate, 2,5-bis-(2,2,2-trifluoroethoxy)-N-(2-piperidylmethyl) benzamide monoacetate and the internal standard 2,5-diethoxy-N-(2-piperidylmethyl) benzamide hydrochloride (S-15177) were supplied by Riker Laboratories (Thornleigh, N.S.W. Aust). Acetonitrile was L.C. grade (Ashland Reagent, Ashland Chemical Co., Columbus, OH. U.S.) Water was double distilled through an all glass still and filtered through a 0.45 μm filter (Millipore). Dibutylamine phosphate was purchased as D-4 reagent

(Millipore-Waters Associates, Lane Cove, N.S.W., Aust.) All other reagents were A.R. grade. Drug free plasma was obtained from pooled blood supplied by the Blood Bank at this hospital.

## METHODS

### Chromatography

Analysis was performed on a Varian Model 5000 liquid chromatograph coupled to a Vista 401 controller. Detection was with a Waters Model 441 U/V detector operating with zinc lamp at 214 nm (0.02 auf). Sample injection was by a Rheodyne injector or by an automated injector (Wisp Model 710B, Waters Associates). The chromatography was performed isocratically on a 30 cm, 10 μm μ-Bondapak C18, reverse-phase column (Waters Associates) in line with a Bondapak-C18/Corasil pre-column. The mobile phase was acetonitrile-water (30:70), the water containing 0.01 mole/litre dibutylamine phosphate. The pH of the mobile phase was 3.2 and was not adjusted. The mobile phase was pumped at a flow rate of 1.5 ml/min and the chromatography was performed at ambient temperature.

### Standard Solutions for Assay Calibration

A stock solution of flecainide acetate in water containing 10 mg/100 ml (8.73 mg/100 ml of flecainide base) was prepared and stored at 4 degrees C. Similarly a stock solution of the internal standard (8 mg/100 ml) was prepared in water and stored at 4 degrees. To prepare standard solutions in plasma, 0.25 ml of flecainide stock solution was made up to 20 ml with drug free plasma giving a final concentration of 1092 ng/ml of flecainide as

free base. This was further serially diluted with drug free plasma to give a series of concentrations to 27.3 ng/ml of flecainide base. The standard curve was constructed from drug free plasma containing added flecainide in the range between these two concentrations.

Plasma Extraction and Assay Calibration

To either standard control or patient plasma samples (0.5 ml) in 15 x 115 mm glass tubes was added internal standard solution (0.2 ml) made by diluting the stock internal standard 1:50 with water. Aqueous sodium hydroxide (2M, 50 µl) was then added and the samples were extracted once with diethyl ether (3 ml) by vortex for 30 sec. After centrifugation at 3000 rpm for 5 min the ether extract was transferred to a second tube containing 100 µl 0.1M phosphoric acid. The mixture was again vortexed for 30 sec and after centrifugation the ether layer was removed as completely as possible and discarded. A gentle stream of nitrogen was blown for approximately one minute over the remaining aqueous acid extract to remove traces of volatile material and an aliquot 20 or 50 µl) was injected into the liquid chromatograph. The assay was calibrated from serial plasma samples containing added flecainide at concentrations of 27.3 to 1092 ng/ml (free base) by measuring peak area ratio of flecainide to that of internal standard.

## RESULTS AND DISCUSSION

The presence of dibutylamine phosphate as a modifer in the mobile phase considerably enhanced the chromatography, and the

monitoring at 214 nm gave excellent response. Monitoring at this wavelength was made because of the enhanced intensity of absorption by flecainide at shorter wavelength compared to the intensity at the absorption maxima at 295 nm. The chromatograms obtained following extraction of control and patient plasma are shown in Figure 1. These are largely free from endogenous interference and the flecainide response from the lowest calibrator 27 ng/nl plasma is well quantitated above background. The total run time for the assay was 8 minutes. The non-fluorinated analog of flecainide was a suitable standard being well separated from the parent. The fluorinated isomer (2,3-bis analog of flecainide) has been used as an internal standard (14,15) but under the conditions of this assay it co-eluted with flecainide and was therefore not suitable.

Calibration

The calibration of the assay from plasma containing added flecainide produced a linear calibration curve from 27.3 to 1092 ng/ml (free base) (Figure 2). These values are well within the apparent therapeutic range and from the regression data generated for each calibration the concentration of flecainide in unknown samples was determined.

Recovery

To determine extraction efficiency known amounts of flecainide in 0.5 ml of plasma were extracted with ether as previously described followed by back-extraction in 0.1 M

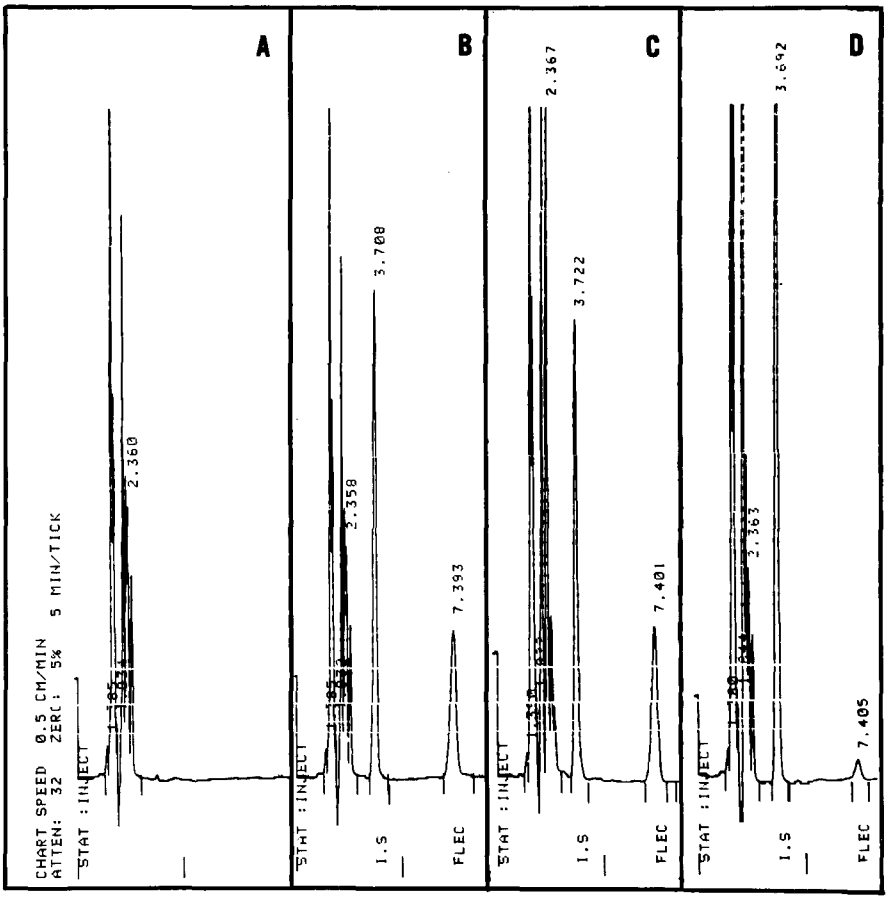



FIGURE 1. Chromatograms obtained following plasma extraction and injection of 20 ul aliquots. A = drug free human plasma with no added internal standard (I.S). B = Control plasma with flecainide (FLEC) concentration of 365 ng/ml. C = Patient plasma sample found to contain 394 ng/ml flecainide. D = Lowest calibrator plasma 27.3 ng/ml (50 µl aliquot injection).

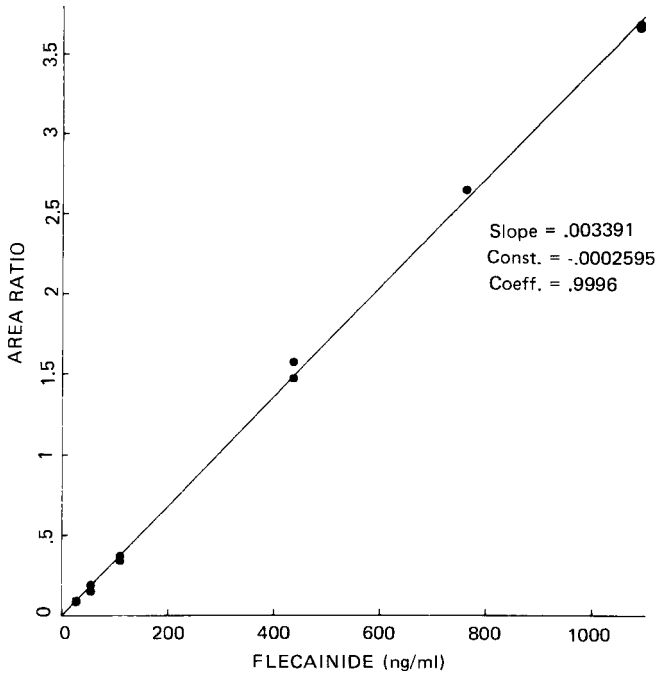



**FIGURE 2. Calibration curve from human plasma with duplicate measurements of area ratio of flecainide to internal standard (arbitary units) versus flecainide concentration (ng/ml).**

phosphoric acid. The acid extract was then adjusted to a known volume and aliquot injected onto the chromatograph. The response was then compared to that obtained from equivalent amounts of flecainide dissolved in the same volume of phopshoric acid. The results obtained are shown in Table 1. As can be seen the recovery of flecainide is consistently good at different concentration values indicating good extraction efficiency with ether at elevated pH followed by back-extraction into aqueous acid. There is also good recovery of the internal standard.

TABLE 1

Recovery of Flecainide and Internal Standard from Plasma. Extraction Efficiency for Flecainide was Calculated at Four Different Concentrations. For the Internal Standard, Recovery was at the Concentration Added to the Plasma (n = 5 for Each Concentration).

| Compound | Concentration (ng/ml) | Recovery (Mean % ± S.D.) |
|---|---|---|
| Flecainide | 109 | 88.7 ± 4.1 |
| | 218 | 90.5 ± 3.2 |
| | 436 | 93.1 ± 3.0 |
| | 873 | 94.8 ± 3.0 |
| Internal Std. | 640 | 86.0 ± 2.9 |

## Precision

The reproducibility and precision of the assay following repeated determinations at different concentration values are shown in Table 2. Within-run precision was determined by analysing five samples from the same specimen. The day-to-day precision was determined by analysing samples from three control specimens eight times at random periods over two months.

## Selectivity

Potential interference with the assay would arise from other commonly used antiarrthymic drugs which may be concomitantly administered and would extract from plasma to varying degrees under conditions of the assay. The relative retention times of these compounds and related substances are shown in Table 3. It

TABLE 2

Assay Reproducibility. Within-Run Precision (n = 6 for each concentration) and the Day-to-Day Precision (n = 8) Calculated for Flecainide.

| | Mean Conc. Found (ng/ml ± S.D.) | C.V. % |
|---|---|---|
| Within-Run | 26 ± 1.5 | 5.7 |
| | 96 ± 2.0 | 2.1 |
| | 194 ± 5.7 | 2.9 |
| | 582 ± 6.7 | 1.2 |
| | 962 ± 19.6 | 2.0 |
| Day-to-Day | 45 ± 3.0 | 6.7 |
| | 140 ± 8.0 | 5.7 |
| | 461 ± 19.7 | 4.3 |

TABLE 3

Relative Retention Times of Commonly used Antiarrhythmic Drugs and Related Compounds.

| Compound | Relative Retention Time (Flecainide = 1.0) |
|---|---|
| Procainamide | 0.23 |
| N-Acetylprocainamide | 0.26 |
| Tocainide | 0.30 |
| Lidocaine | 0.31 |
| Mexiletine | 0.37 |
| Quinidine | 0.38 |
| Quinine | 0.38 |
| Dihydroquinidine | 0.41 |
| Internal Standard | 0.50 |
| Propranolol | 0.61 |
| Flecainide | 1.0 |

can be seen that each is resolved from both flecainide and internal standard and do not interfere with the assay. Also tested were the basic drugs cimetidine and ranitidine as well as theophylline, diazepam and amiodarone. None of these had retention times close to flecainide or the internal standard and would not interfere.

## Conclusion

The procedure described in this paper is a practical method from the standpoint of simplicity, reproducibility and relative speed. The chromatograms produced are clear with sufficient sensitivity using ultraviolet detection to carry out routine analysis of this drug. The method has now been incorporated in our laboratory as a standard procedure for a drug with a rapidly increasing clinical use.

## REFERENCES

1. Schmid, J.R., Seebeck, B.D., Henrie, C.L., Banitt, E.H. and Kvam, D.C. Some Antiarrhythmic Actions of a New Compound, R-818, in Dogs and Mice. Fed. Proc. 34 775 1975.

2. Welter, A.N., Schmid, J.R. and Kvam, D.C. Cardiovascular Profile of R-818, Flecanide, a New Antiarrhythmic. Fed. Proc. 36 1003 1977.

3. Banitt, E.H., Bronn, W.R., Coyne, W.E. and Schmid, J.R. Antiarrhythmics 2. Synthesis and Antiarrhythmic Activity of N-(Piperidylalkyl) Trifluoroethoxybenamides. J. Med. Chem. 20 821 1977.

4. Hodess, A.B., Follansbee, W.P., Spear, J.F. and Moore, E.N. Electrophysiologic Effects of a New Antiarrhythmic Agent, Flecainide, on the Intact Canine Heart. J. Cardiovasc. Pharmacol. 1 427 1979.

5. Anderson, J.L., Stewart, J.R., Perry, B.A., Van Hamersveld, D.D., Johnson, T.A., Conard, G.J., Chang, S.F., Kvam, D.C. and

Pitt, B. Oral Flecainide Acetate for the Treatment of Ventricular Arrhythmias. New Engl. J. Med. 305 473 1981.

6. Duff, H.J., Roden, D.M., Maffucci, R.J., Vesper, B.S., Conard, G.J., Higgins, S.B., Oats, J.A., Smith, R.A. and Woosley, R.L. Suppression of Ventricular Arrhythmia by Twice Daily Dosing with Flecainide. Am. J. Cardiol. 48 1133 1981.

7. Hodges, M., Haugland, J.M., Granrud, G., Conard, G.J., Asinger, R.W., Mikell, F.L. and Krejci, J. Suppression of Ventricular Ectopic Depolarization by Flecainide Acetate, a New Antiarrhythmic Agent. Circulation 65 879 1982.

8. Bigger, Jr. J.T. Ed. Symposium on Flecainide Acetate. Am. J. Cardiol. 53 1984.

9. Chang, S.F., Miller, A.M., Jernberg, M.J., Ober, R.E. and Conard, G.J. Measurement of Flecainide Acetate in Human Plasma by an Extraction Spectrophotofluorometric Method. Arzneim-Forsch/Drug Res. 33 251 1983.

10. Conard, G.J., Carlson, G.L., Frost, J.W. and Ober, R.E. Plasma Pharmacokinetics of Flecainide Acetate (R-818), a New Antiarrhythmic, Following Single Oral and Intravenous Doses. Clin. Pharmacol. Therap. 25 218 1979 (abstract).

11. Somani, P., Antiarrhythmic Effects of Flecainide. Clin. Pharmacol. Therap. 27 464 1980.

12. De Jong, J.W., Hegge, J.A.J., Harmsen, E. and De Tombe, P.Ph. Fluorometric Liquid Chromatographic Assay of the Antiarrhythmic Agent Flecainide in Blood Plasma. J. Chromatogr. 229 498 1982.

13. Chang, S.F., Welscher, T.M., Miller, A.M. and Ober, R.E. High Performance Liquid Chromatographic Method for the Quantitation of Flecainide, A New Antiarrhythmic in Human Plasma and Urine. J. Chromatogr. 272 341 1983.

14. Chang, S.F., Miller, A.M., Fox, J.M. and Welscher, T.M. Determination of Flecainide in Human Plasma by High Performance Liquid Chromatography with Fluoresence Detection. J. Liq. Chromatogr. 7 167 1984.

15. Chang, S.F., Miller, A.M., Fox, J.M. and Welscher, T.M. Application of a Bonded-Phase Extraction Column for Rapid Sample Preparation of Flecainide from Human Plasma for High-Performance Liquid Chromatographic Analysis - Fluorescence or Ultraviolet Detection. Therap. Drug Monit. 6 105 1984.

16. Bhamra, R.K., Flanagan, R.J. and Holt, D.W. High performance Performance Liquid Chromatographic Method for the Measurement of Mexiletine and Flecainide in Blood Plasma or Serum. J. Chromatogr. 307 349 1984.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2591-2603 (1984)

# BILE ACIDS. LXX. PREPARATIVE SEPARATION OF KRYPTOGENIN FROM COMPANION SAPOGENINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Daniel M. Tal, Ping H. Patrick and William H. Elliott*
E.A. Doisy Department of Biochemistry
St. Louis University School of Medicine
1402 South Grand Boulevard
St. Louis, MO 63104

## ABSTRACT

Commercial samples of kryptogenin or its acetate can be purified by preparative high performance liquid chromatography on a PrepPAK-500/Silica cartridge. The free alcohol is separated from accompanying sapogenin with a mixture of chloroform:methanol (50:1), whereas the acetates are separated well with a mixture of methylene chloride:hexane (2:3). The companion sapogenins, diosgenin and yamogenin, 25R- and 25S- isomers, were separated by analytical HPLC with hexane-isopropanol (100:1) or as the acetates with hexane:isopropanol (250:1). Characterization of kryptogenin and yamogenin was completed with $^{1}$H-NMR, IR and MS spectrometry.

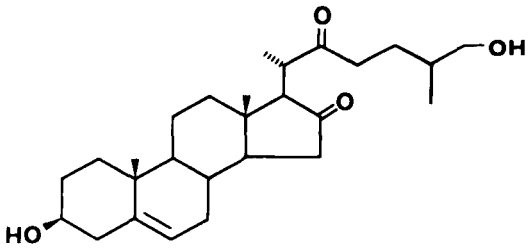
## INTRODUCTION

The need for pure kryptogenin (1) [(25R)-3β,26-dihydroxy-5-cholestene-16,22-dione] (Fig. 1) for the synthesis of a radioactive 26-cholestanoic acid (1) led us to seek a source of pure material. Usually kryptogenin is obtained from Dioscorea plants, the largest and most important genus of the family Dioscoreaceae.

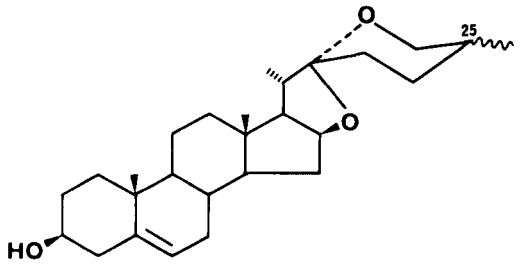
*To whom correspondence is to be addressed.



 0148-3919/84/0713-2591$3.50/0




1




2: 25R

3: 25S

Figure 1: Structures of Steroids. 1 = Kryptogenin, (25R)-3β,26-dihydroxy-5-cholestene-16,22-dione; 2 = Diosgenin, (25R)-5-spirosten-3β-ol; 3 = Yamogenin, (25S)-5-spirosten-3β-ol.

They thrive in Southeast Asia, Africa and Central and South America, with only a few species indigenous to Europe and North America (2).

Marker et al. (3) had found that species from the United States are richer in diosgenin (2) [(25R)-5-spirosten-3β-ol] while in plants from Mexico the sapogenin fraction consists of a mixture of steroidal compounds difficult to separate directly by

crystallization. Subsequently, they proposed two different methods of acetylation and crystallization to separate the acetates of kryptogenin (1), diosgenin (2) and yamogenin (3) [(25S)-5-spirosten-3β-ol]. Steroidal sapogenins are of economic importance as precursors of many medicinally useful hormones and contraceptives, and subsequently their extraction from Dioscorea species has been the subject of extensive reviews (2,4,5). 26-Hydroxycholesterol obtained from the pure kryptogenin (6) is an important metabolite of cholesterol as well, and can be prepared also from diosgenin (7) or from yamogenin (3) by acidic isomerization (2) to compound (2). It provides an avenue to 5α-sterols with a stereospecific (25R)-configuration, or to 5α-bile acids.

A sample of commercial kryptogenin (1), gratiously supplied by Syntex S.A., was received containing its companions (2) and (3). Therefore, the development of a good method of separation of tens of grams was necessary. Conventional techniques such as repetitive recrystallization (4) are time-consuming, tedious and fail to yield compounds of high purity. On the other hand, adsorption column or preparative layer chromatography are not economically suitable for separation of gram amounts. The use of high performance liquid chromatography (HPLC) on preparative silica columns as reported here offers improved versatility and efficiency in addition to shorter separation times.

## EXPERIMENTAL

### Materials

Preparative HPLC was performed with a Waters PrepLC/System 500 with a universal refractive index detector and a Waters PrepPAK-500/SILICA cartridge (5.7x30 cm) (8); fractions of 200 ml were collected. All solvents were HPLC grade or were redistilled

in glass before use. Samples of kryptogenin and its acetate were obtained from Syntex, S.A., Mexico City, Mexico. Diosgenin was a product of Sigma Chemical Co., St. Louis, MO.

## Analytical Instruments

$^1$H-NMR spectra of $CDCl_3$ solutions were recorded on a Jeol FT-100MHz spectrometer, with tetramethylsilane as internal standard. Mass spectra were obtained with an LKB Model 9000 mass spectrometer. Analytical HPLC was carried out with a Waters Model ALC-201 apparatus with a Model 401 refractive index (RI) detector, a Waters 760 Data Module and a Model 480 uv detector set at 212 nm, using a μPorasil column, (10 μ, 3.9 mm x 30 cm), and the conditions indicated; $t_R$ = retention time in min; $t_0$ = time in min of the solvent of the solute(s); k' = capacity factor. Thin layer chromatography (tlc) was done on Merck Silica Gel 60 $F_{254}$ pre-coated on aluminum sheets (5x20 cm x 0.2 mm). The ratios of solvents for tlc are given as v/v. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Infrared spectra were recorded on a Model 21 Perkin-Elmer double beam spectrophotometer as potassium bromide pellets. Gas chromatograms were obtained with an HP 402 High Efficiency Gas Chromatograph (GC) fitted with a six foot glass column (0.25 in O.D.) packed with 3% SP-2100 (8); relative retention times (RRT) are related to 5α-cholestane.

## Characterization of Compounds

### Kryptogenin

mp: 184-7°C from methanol (reported (9) m.p. 184-6°C); $^1$H-NMR: δ (ppm) 5.35 (1H, broad d, J=5 Hz, C6), 3.54 (1H, m, C3), 3.46 (2H, d, J=5.5 Hz, C26), 1.04 (3H, s, C19), 0.94 (3H, d, J=6.5 Hz, C27), 0.81 (3H, s, C18); MS: at 20 eV by direct insertion probe, m/z (%, fragment) 412 (71, M-$H_2O$), 397 [22,

M-($H_2O$+$CH_3$)], 394 (19, M-2$H_2O$), 181 (79, C15→C17+C20→C27-$H_2O$), 180 (100, 181-H), 166 [49, (181-$CH_3$) and/or (180-$CH_2$)], 139 (30, C17+C20→C27-$H_2O$), 125 (56, C20→C27-$H_2O$), 124 (81, 125-H); HPLC (flow rate, 1 ml/min, RI detector, $CHCl_3$:$CH_3OH$ / 50:1) k'=1.04 [$t_R$=47, $t_o^{CHCl_3}$=23]; tlc: ($CH_2Cl_2$:$CH_3OH$ / 37:3) $R_f$ 0.53.

Kryptogenin Diacetate

mp: 153-4°C from methylene chloride (reported (9) m 153°C); $^1$H-NMR: δ (ppm) 5.29 (1H, broad d, J=5 Hz, C6), 4.48 (1H, m, C3), 3.87 (2H, d, J=5.5Hz, C26), 2.03 (3H, s, OAc), 2.02 (3H, s, OAc), 1.04 (3H, s, C19), 0.95 (3H, d, J=6.5Hz, C27), 0.81 (3H, s, C18); MS: at 70 eV by direct insertion probe, m/z (%, fragment) 514 (<1, $M^+$), 455 (18, M-AcO), 454 (32, M-HOAc), 435 [16, M-(HOAc+$H_2O$+H)], 394 (89, M-2HOAc), 384 [17, M-(C23→C27)], 378 (14, 438-HOAc), 356 [22, M-(C22→C27+H)], 342 [20, M-(C22→C27+$CH_3$)], 325 [21, M-(C23→C27+HOAc)], 324 [43, M-(C23→C27+HOAc)], 298 [19, M-(C22→C27+HOAc+H)], 297 [44, M-(C22→C27+HOAc)], 296 [51, M-(C22→C27+H+HOAc)], 115 (100, C24→C27).

Yamogenin

mp: 200-202°C from acetone (reported (10,11) m.p. 200-201°C). $^1$H-NMR: δ (ppm) 5.35 (1H, broad d, J=5 Hz, C6), 4.54-4.30 (1H, m, C16), 3.96 (1H, double d, $J_{gem}$=11 Hz, $J_{vic}$=2 Hz, eq. H-C26), 3.51 (2H, m, C3+OH), 3.30 (1H, d, $J_{gem}$ = 11 Hz, ax.H-C26), 1.03 (3H, s, C19), 0.79 (3H, s, C18), 0.78 (3H, d, J=6.5 Hz, C27); MS: at 20 eV by direct insertion probe (comparable to a spectrum reported (12) at 70 eV, but with different intensities) m/z (%, fragment), 414 (5, $M^+$), 396 (3, M-$H_2O$), 342 [9, M-(C24→C27+O)], 300 [19, M-(C23→C27+2 O)], 282 [M-(C23→C27+2 O+$H_2O$)], 271 (18, M-side chain), 267 [13, M-(C21→C27+2 O+$H_2O$)], 253 (9, [M-(side chain + $H_2O$)]), 139 (100,

[M-C17+C20→C27+O]); HPLC (1 ml/min, RI detector, $CHCl_3$:$CH_3OH$ / 50:1), k'=0.13 [$t_R$=26, $t_o^{CHCl_3}$=23]; (1ml/min, UV detector at 212 nm, 0.1 AUFs, hexane:isopropanol / 100:1), k'=6.66 [$t_R$=33, $t_o^{CH_2Cl_2}$=4.31]; tlc: ($CH_2Cl_2$:$CH_3OH$ / 37:3) $R_f$ 0.66; GC: (220°C), RRT = 2.76.

Yamogenin Acetate

mp: 182-4°C from acetic anhydride (reported (10,11) m.p. 182°). MS: at 70 eV by direct insertion probe, m/z (%, fragment), 456 (2, $M^+$), 396 (53, M-HOAc), 381 [4, M-(HOAc+$CH_3$)], 368 [5, M-(HOAc+28)], 337 [13, M-(HOAc+C25→C27+O)], 324 [21, M-(C24→C27+HOAc)], 282 [100, M-(C23→C27+2 O+HOAc)], 267 [24, M-(C21→C27+2 O+HOAc)], 253 [34, M-(side chain + HOAc)], 139 [75, M-(C17+C20→C27+O)]; IR: comparable to reported values (13); HPLC (1 ml/min, UV detector at 212 nm, hexane:isopropanol / 100:1), k'=0.15 [$t_R$=23, $t_o^{CH_2Cl_2}$=20]; tlc: (hexane:isopropanol / 4:1) $R_f$ 0.79; GC: (210°C), RRT=4.72.

## RESULTS

### Separation of Samples of the Alcohols (1), (2) and (3)

Analytical HPLC separation of small samples of commercial kryptogenin were carried out successfully on a μPorasil column with a solvent mixture of chloroform:methanol (50:1). The method was scaled up to 5 g of the mixture utilizing Prep HPLC; 75 ml of solvent (chloroform:methanol / 50:1) dissolved 5 g of solid which was injected into the PrepPAK 500 column and eluted with a flow rate of 250 ml/min; chart speed, 2 min/cm; relative response R.I. detector = 50. Fractions of 200 ml were collected. Fractions 2-5 contained sapogenins (0.47 g, $R_f$ 0.66), fractions 6-9 contained 120 mg of unidentified material and fractions 10-17 contained kryptogenin (1) (4.39 g, $R_f$ 0.53, methylene choride: methanol / 37:3) (Fig. 2). A total of 4.98 g was recovered

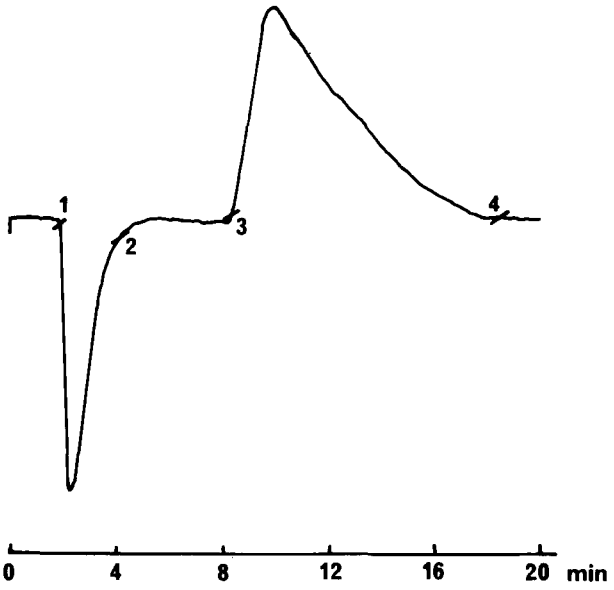



Figure 2: Prep HPLC separation of sapogenins (2 and 3) from kryptogenin (1). Commercial kryptogenin (5g) in 75 ml of chloroform:methanol (50:1) was injected onto a Waters PrepPAK-500/SILICA cartridge and eluted with the above solvent under the following conditions: flow rate 250 ml/min; chart speed, 2 min/cm; twenty fractions of 200 ml each were collected. Between Chart Marks 1 and 2, sapogenins (0.47 g) were identified; between Marks 3 and 4 kryptogenin (4.39 g) was obtained.

(99.7%) of which 88% was kryptogenin. A sample of kryptogenin was crytallized from methanol to provide a product with m.p. 184-7°. Further characterization was achieved as reported in Experimental. Separation and characterization of the sapogenins in fractions 2-5 will be discussed below.

## Separation of Acetates

By analytical HPLC a sample of commercial kryptogenin diacetate dissolved in methylene chloride was shown to separate as two major peaks with seven minor constituents using a solvent mix-

tures of hexane:isopropanol (100:1). The sharp peak eluted immediately after $CH_2Cl_2$ (k' 0.22) contained monoacetates whereas the constituent of the largest peak (k' 4.5) was shown to be kryptogenin diacetate. With a change of the ratio of solvents to 80:1, the k's were 0.17 and 1.33, respectively. With this information a 5 g sample of commercial kryptogenin diacetate was subjected to preparative liquid chromatography utilizing a solvent mixture of hexane:isopropanol (75:1), flow rate of 250 ml/min and the recorder speed at 2 min/cm. Since the solid did not dissolve satisfactorily in 200 ml of the solvent, an addition of 20 ml of $CH_2Cl_2$ partially solved the problem, but the solid precipitated on the column. After stripping the column with methanol, successive experiments showed that solvent mixtures of methylene chloride: hexane (1:1) or (2:3) provided suitable separations; good recoveries were obtained with the latter solvent mixture with samples of 19.5, 28.5 or 30.7 g of kryptogenin diacetate. Fig. 3 shows the separation of 30.7 g of commercial acetate; fractions 3-5 were shown to contain diosgenin and yamogenin acetate and fractions 7-18 contained kryptogenin diacetate.

Alternatively, commercial kryptogenin (40 g) was acetylated with a mixture of 800 ml of acetic anhydride and 400 ml of dry pyridine with stirring at room temperature for 64 hrs, and was worked up in the usual manner to provide, by tlc a completely acetylated product. Preparative HPLC with a solvent mixture of methylene chloride:hexane (2:3) afforded separation analogous to that described above.

## Identification of the Components of the Sapogenin Fraction

Aliquots of fractions of "sapogenins" and of their acetates were analyzed by HPLC and GC (Table 1; Figs. 4A and B). Diosgenin acetate (m.p. 199-202°; reported (9) 202-204°) prepared from commercial diosgenin was mixed with a sample of the sapo-

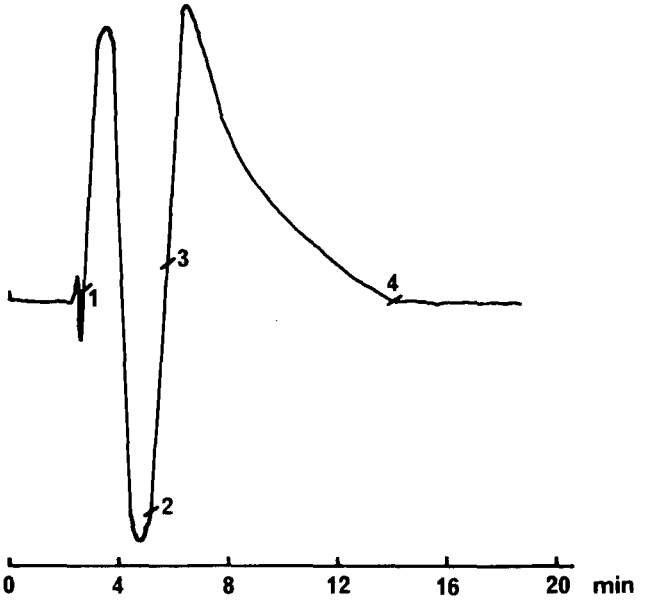



Figure 3: Prep HPLC separation of acetates of sapogenins (2 and 3) from diacetate of kryptogenin (1). Commercial kryptogenin diacetate (30.7 g) in a mixture of methylene chloride:hexane (2:3) was separated with that solvent mixture under the conditions detailed for Figure 2. Between Chart Marks 1 and 2 a mixture of the acetates of diosgenin and yamogenin was obtained; between Marks 3 and 4 kryptogenin diacetate was identified.

TABLE 1
Identification of Sapogenins

| | HPLC* | | | | GC* | |
|---|---|---|---|---|---|---|
| Sapogenin | Solvent Ratio** | Elution Time (min) | k' | RRT | Elution for 5α-Cholestane | Column Temp. |
| Diosgenin | 100:1 | 31.0 | 6.19 | 2.65 | 5.68 min | 220° |
| Yamogenin | 100:1 | 33.0 | 6.66 | 2.76 | 5.68 min | 220° |
| Acetates of | | | | | | |
| Diosgenin | 250:1 | 12.38 | 1.75 | 4.37 | 10.75 min | 210° |
| Yamogenin | 250:1 | 18.32 | 3.07 | 4.72 | 10.75 min | 210° |

*k' and RRT values are averages of 3-5 determinations.
**Solvent = hexane:isopropanol

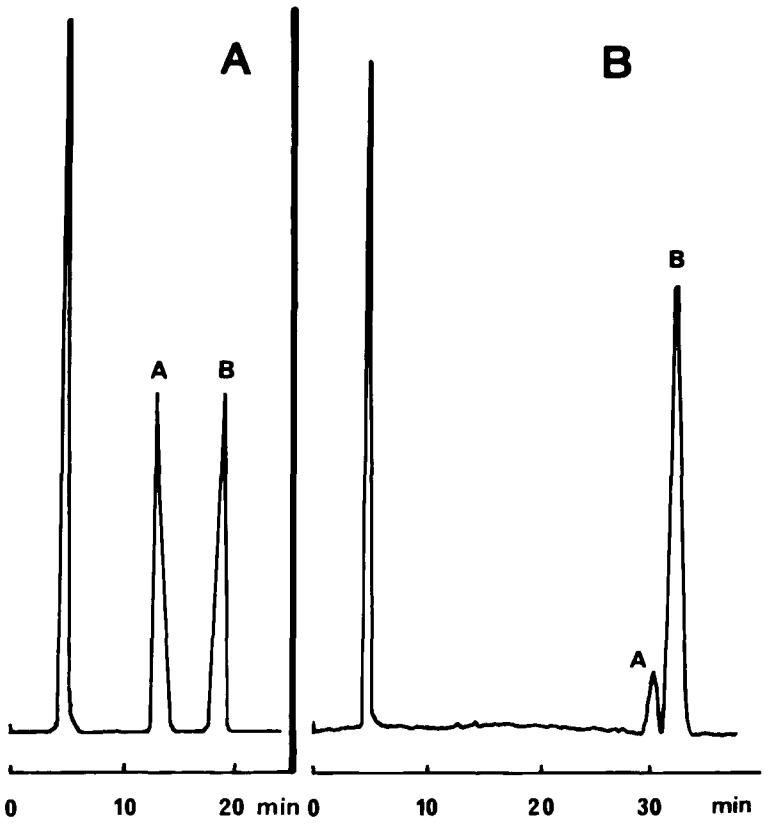



Figure 4A: Separation of the acetates of diosgenin (A) from yamogenin acetate (B) by analytical HPLC. A sample of commercial diosgenin was acetylated and added to a sample of the sapogenin acetates separated earlier from kryptogenin diacetate. After HPLC with a mixture of hexane:isopropanol (250:1) diosgenin acetate (peak A) consisted of the synthetic product and the companion of yamogenin acetate (B), now in approximately equal quantities, whereas the acetates of the sapogenin mixture were present in a ratio of approximately 1:10 (diosgenin:yamogenin).

Figure 4B: Separation of diosgenin (A) from yamogenin (B) by analytical HPLC. A Waters Model ALC-201 with a U6K injector, a Model 401 differential refractometer, a Model 480 u.v. detector set at 212 nm and a Model 760 Data Module were coupled with a μPorasil column (30 cm x 3.9 mm, 10μ particles) to separate diosgenin (A) from yamogenin (B) with a solvent mixture of hexane:isopropanol (100:1).

genin diacetates separated from kryptogenin acetate, and subjected to analytical HPLC with a mixture of hexane-isopropanol (250:1); the peak corresponding to diosgenin acetate of the sapogenin mixture. With the Waters Data Module, the relative quantities of the acetates were found to be 90.23% yamogenin and 9.73% diosgenin. This ratio was confirmed by a similar study of the free alcohols (Fig. 4B). A mixture of the sapogenins with a commercial sample of diosgenin showed co-elution of the peaks attributed to this sapogenin, comparable to that in Fig. 4A. The order of elution of these sapogenins in GC and their RRT's are comparable to those reported by Vanden Heuvel and Horning (14); mass spectral and NMR data, and melting points of the acetate and free sapogenin correspond to or are consonant with those data reported for yamogenin.

The presence of these relatively large amounts of yamogenin in preparations of kryptogenin may reflect concentrations in prior steps of purification and/or the choice of the particular Dioscorea plant from which this material was derived. Earlier reports of Marker et al. (9,10) and Takeda (2) dwell on this matter. This report demonstrates the relative ease of purification of kryptogenin or its acetate by prep HPLC in separation from companion sapogenins, as opposed to the difficulties discussed by Marker et al. (3,9,10). In our hands the use of the acetate was preferred because this material was the reactant for subsequent production of 26-hydroxycholesterol.

## ACKNOWLEDGEMENT

These studies were supported by NIH Grant HL-07878. The generosity of Syntex, S.A., Mexico 1, D.F., Mexico, in providing samples and NMR spectra for comparison made possible these studies. The able assistance of the Washington University NMR facility, of Mr. John Crimi and Mr. David Brown in mass spec-

trometry, and of Mr. Gregory Groshan in gas chromatography is gratefully acknowledged.

## REFERENCES

1. Tal, D.M. and Elliott, W.H. Bile Acids. LXXI. A New Radiochemical Synthesis of a Dihydroxy-5α-cholestanoic Acid. J. Labelled Compds Radiopharm. (submitted).

2. Takeda, K. Steroidal Sapogenins of the Dioscoreaceae. Prog. in Phytochem. 3, 287, 1973.

3. Marker, R.E., Wagner, R.B., Ulshafer, P.R., Wittbecker, E.L., Goldsmith, D.P.J. and Ruof, C.H. Steroidal Sapogenins. J. Amer. Chem. Soc. 69, 2167, 1947.

4. Heftman, E. Biochemistry of Steroidal Saponins and Glycoalkaloids. Lloydia 30, 209, 1967.

5. Hardman, R. Pharmaceutical Products from Plant Steroids. Tropical Science II, 196, 1969.

6. Scheer, I., Thompson, M.J. and Mosettig, E. 5-Cholestene-3β,26-diol. J. Amer. Chem. Soc. 78, 4733, 1956.

7. Arunachalam, T., MacKoul, P.J., Green, N.M. and Caspi, E. Synthesis of 26-halo-, 26-(phenylseleno)- and 26-indolyl Cholesterol Analogs. J. Org. Chem. 46, 2966, 1981.

8. Shaw, R. and Elliott, W.H. Bile Acids. LIX. Purification of 5α-anhydrocyprinol by Preparative High Performance Liquid Chromatography. J. Chromatogr. 177, 289, 1979.

9. Marker, R.E. and Lopez, J. Steroidal Sapogenins. No. 164 Nologenin and its Degradation Products. J. Amer. Chem. Soc. 69, 2386, 1947.

10. Marker, R.E., Wagner, R.B., Ulshafer, P.R., Wittbecker, E.L., Goldsmith, D.P.J. and Ruof, C.H. Sterols. CLVII. Sapogenins. LXIX. Isolation and Structures of Thirteen New Steroidal Sapogenins. New Structures for Known Sapogenins. J. Amer. Chem. Soc. 65, 1199, 1943.

11. Kessar, S.V., Gupta, Y.P., Mahajan, R.K., Joshi, G.S. and Rampal, A.L. Synthetic Studies in Steroidal Sapogenins and Alkaloids. V. Synthesis of Kryptogenin, Diosgenin and Yamogenin. Tetrahedron 24, 899, 1968.

12. Budzikiewicz, H., Wilson, J.M. and Djerassi, C. Mass Spectrometry and its Application to Structural and Sterochemical Problems. XV. Steroidal Sapogenins. Monatsh. Chem. 93, 1033, 1962.

13. Jones, R.N., Katzenellenbogen, E. and Dobriner, K. The Infrared Absorption Spectra of the Steroidal Sapogenins. J. Amer. Chem. Soc. 75, 158, 1953.

14. Vanden Heuvel, W.J.A. and Horning, E.C. Gas Chromatographic Separations of Sapogenins. J. Org. Chem. 26, 634, 1961.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2605-2610 (1984)

# RAPID AND SIMPLE TECHNIQUE FOR THE QUANTITATION OF POLYAMINES IN BIOLOGICAL SAMPLES

Rodney F. Minchin and Gayle R. Hanau
Pharmacology and Toxicology Section,
Laboratory of Experimental Therapeutics and Metabolism
Division of Cancer Treatment
National Cancer Institute
Bethesda, Maryland, 20205

## ABSTRACT

A rapid and simple technique has been developed to quantify putrescine, spermidine, and spermine in biological tissue. The method, based upon several published procedures, involves protein precipitation with perchloric acid followed by dansylation with 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride). After extraction on a Waters $C_{18}$ Sep-Pak cartridge, the samples are analyzed by high pressure liquid chromotography using a step solvent change and a 3μ $C_{18}$ reverse phase column. The chromotographic conditions allowed complete analysis of the three polyamines within 10 min with a total run time of 13 min (sample injection and re-equilibrium of column). Standard curves were linear up to 1 μg polyamine and the coefficient of variation for the assay ranged from 4% at 1 μg polyamine per sample to 11% at 50 ng polyamine per sample. The assay is therefore both rapid and simple. Moreover, unlike other available methods, the present technique does not require duel pumps, ion pairing agents, solvent extraction or a gradient control system. The concentrations of putrescine, spermidine and spermine in rat lung, liver and kidney are reported.

## INTRODUCTION

Polyamines are ubiquously distributed endogenous compounds that have been associated with the regulation of numerous biological functions such as DNA and RNA synthesis (1), cellular proliferation (2), differentiation (3), intracellular membrane fusion (4), protein kinase activity (5) and mitochondrial membrane activities (6). Moreover, elevated levels of polyamine have been associated with tissue injury (7,8) and certain forms of cancer in man (9), and it has been suggested that urinary or plasma polyamine levels may be a useful clinical diagnostic tool for the progression of such cancers (9).

In the present paper, a simple and rapid method for the simultaneous determination of putrescine, spermidine and spermine has been described.



 0148-3919/84/0713-2605$3.50/0

Several excellent high pressure liquid chromatographic techniques for polyamine determinations have been described (10,11). However, all available methods require elution with a solvent gradient and can take between 25 and 60 min for each assay (including re-equilibration of the column) often with poor resolution when the polyamines are extracted from biological tissue. The present technique does not require a solvent gradient system, ion pairing agents, buffers, solvent extraction or extensive reaction periods, and the chromatographic procedure is essentially complete in less than 10 min.

## METHODS

Extraction of polyamines - The lungs, liver and kidneys from 250 g male Sprague Dawley rats were homogenized in 9 vol 50 mM phosphate buffer using a teflon-glass homogenizer. An aliquot (1.8 ml) of each sample was vortexed with 0.2 ml 60% perchloric acid and centrifuged at 3000 g for 4 min. The resulting supernatant (0.9 ml) was mixed with 50 µl $K_2CO_3$ (400 mg/ml) and 50 µl 1,6-diaminohexane (Internal standard: 500 µg/ml) and centrifuged as above. The polyamines were then dansylated by adding 40 µl of supernatant containing the internal standard to 100 µl $K_2CO_3$, 800 µl distilled water and 2 ml dansyl chloride (2 mg/ml in acetone). The mixture was vortexed and incubated at 60°C for 60 min in the dark. The entire sample was then placed on a $C_{18}$ Sep-pak cartridge (Waters Assoc., U.S.A.) and washed with 9 ml 20% methanol in water. The dansylated polyamines were then eluted with 5 ml 100% methanol and 10-100 µl of this fraction was directly analyzed by HPLC.

Chromatographic conditions - An Altex 3µ-ODS (4.6 mm ID x 7.5 cm) column was equilibrated with 75% methanol in water. Following injection of sample, the solvent was changed to 95% methanol in water at 2.5 min and then back to 75% methanol at 8 min. The specific times for changing solvents were selected to minimize run time and was a function of the volume of the HPLC system. The dansylated polyamines were detected by flourescence using a Kratos FS950 detector fitted with an FSA 100 mercury lamp and FSA 403 excitation filter (Kratos Instruments, U.S.A.). A 470 nm emission filter was also used.

## RESULTS AND DISCUSSION

Figure 1 illustrates a typical chromatographic profile for putrescine, 1,6-diaminohexane, spermidine and spermine (1 µg in 40 µl initial sample). The

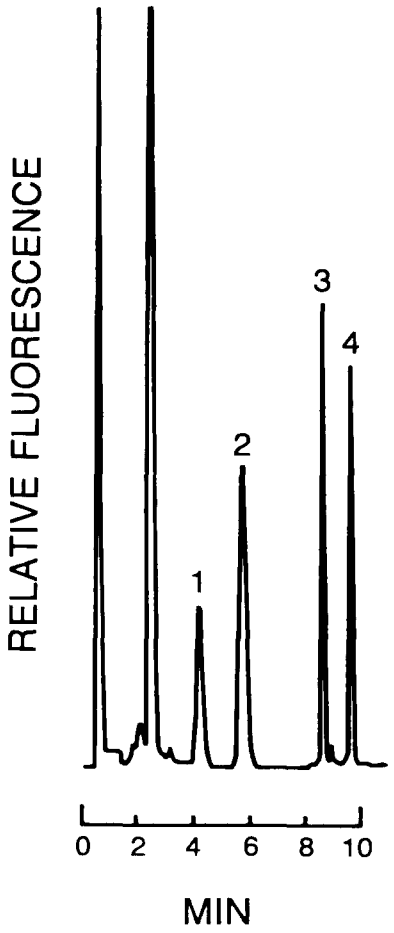



Figure 1. Chromatographic profile of polyamine standards. 1 = putrescine, 2 = 1,6-diaminohexane, 3 = spermidine, 4 = spermine.

retention times for each polyamine were: putrescine, 3.75 min; 1,6-diaminohexane, 5.46 min; spermidine, 8.43 min; spermine, 9.34 min. Standard curves constructed over the range of 50 ng - 1 μg/sample were linear and exhibited a coefficient of variation of 4% at the upper concentrations and 11% at the lower concentrations.

The dansylation of the polyamines was not enhanced by increasing the concentration of dansyl chloride or by extending the incubation period. The yellow color of the dansyl chloride had almost completely disappeared after 60

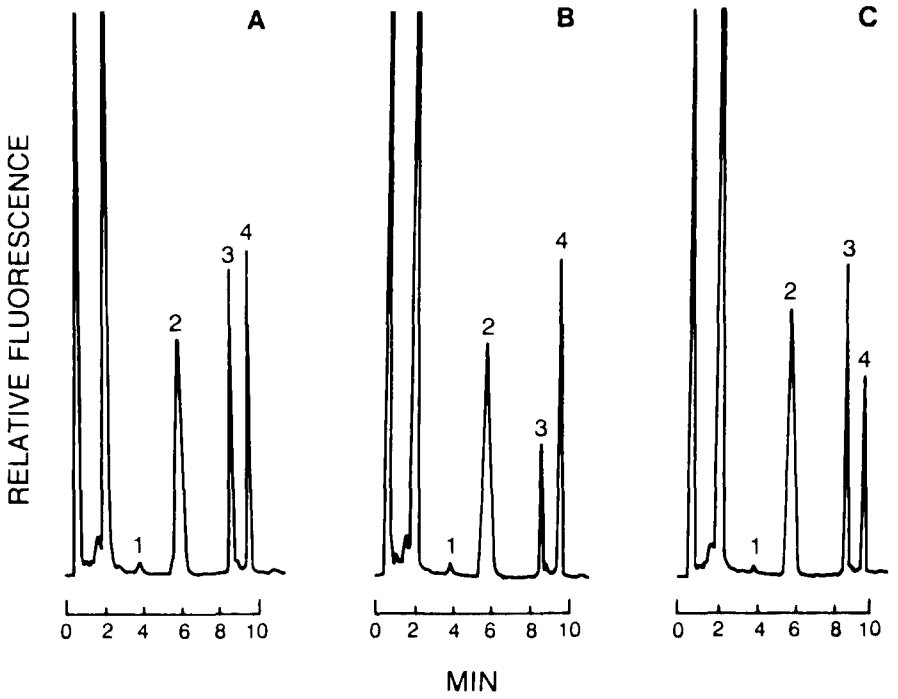



Figure 2. Chromatographic profile of polyamines extracted from rat liver (A), lung (B) and kidney (C). 1 = putrescine, 2 = 1,6-diaminohexane, 3 = spermidine, 4 = spermine.

min. It was found that termination of the reaction before this time resulted in the elution of an excessively large peak at 1.89 min which interfered with the chromatographic profile of putrescine. The change in solvents was optimized for minimum elution time, including the time necessary for the re-equilibration of the column, without compromising the resolution between peaks. Use of a 3μ $C_{18}$ column of only 7.5 cm in length and minimal tubing between the pump, injector, column and detector was also essential to limit the total time for each assay. Under the conditions described, a minimum of 13 min was required between each injection.

The chromatographic profiles for polyamines extracted from rat liver, lung and kidney are shown in figure 2. Putrescine levels were minimal compared to those of spermidine and spermine. The illustrated profiles could be readily

TABLE 1

Concentration of Polyamines in Rat Tissues

| Tissue | Putrescine (nmol/g) | Spermidine (nmol/g) | Spermine (nmol/g) |
|---|---|---|---|
| Liver | 72 ± 10* | 925 ± 51 | 813 ± 45 |
| Kidney | 66 ± 8 | 506 ± 45 | 855 ± 91 |
| Lung | 67 ± 6 | 709 ± 50 | 458 ± 70 |

Results are expressed as Mean ± S.E., n=4, except* where n=3.

enhanced by concentrating the dansylated polyamine solution under a stream of nitrogen. The estimated concentrations of putrescine, spermidine and spermine in each tissue are listed in table 1 and the reported values are within the range of published values (12).

The present study has developed a simple and reliable technique for the estimation of putrescine, spermidine, and spermine in biological tissue. The method is also rapid compared to established procedures but does not compromise peak resolution. The described technique may be useful for routine clinical determination of the polyamines for diagnostic purposes. Automation of the solvent changes can be readily achieved with an Autochrom solvent selector (Rainin Instrument, U.S.A.) and an appropriate time-dependent signal generator such as a standard HPLC data integrator.

## REFERENCES

1. Stevens, L. The biochemical role of naturally occurring polyamines in nucleic acid synthesis. Biol. Rev. 45, 1, 1970.

2. Goyns, M.H. The role of polyamines in animal cell physiology. J. Theor. Biol., 97, 577, 1982.

3. Erwin, B.G., Ewton, D.Z., Florini, J.R., and Pegg, A.E. Polyamine depletion inhibits the differentiation of L6 myoblast cells. Biochem. Biophys. Res. Commun. 114, 944, 1983.

4. Hong, K., Schuber, F., and Papahadjopoulos, D., Polyamines: Biological modulations of membrane fusion. Biochem. Biophysics. Acta. 732, 469, 1983.

5. Cochet, C., and Chambaz, E.M., Polyamine-mediated protein phosphorylations:

a possible target for intracellular polyamine action. Mol. Cell Endocrinol., 30, 247, 1983.

6. Byczkowski, J.Z., and Porter, C.W. Interactions between bis(quanyl-hydrazones) and polyamines in isolated mitochondria. Gen. Pharmacol., 14, 615, 1983.

7. Hacker, A.D., Tierney, D.F., and O'Brien, T.K. Polyamine metabolism in rat lung with oxygen toxicity. Biochem. Biophys. Res. Commun., 113, 491, 1983.

8. Anchus, S., Ingnen, T., Engelbrecht, C., Hafstrom, L., and Heby, O. Urinary polyamine excretion as related to cell death and cell proliferation by carbon tetrachloride intoxication. Exp. Mol. Path., 38, 255, 1983.

9. Schimpff, S.C., Levy, C.C., Hawk, I.A., and Russell, D.H. Polyamines-potential roles in the diagnosis, prognosis and therapy of patients with cancer. In: Polyamines in normal and neoplastic growth. Russell, D.H., eds., Raven Press, New York, 1973, p395.

10. Brown, N.B., and Strickler, M.P. Fentomolar ion-pair high-performance liquid chromatographic method for determining Dns-polyamine derivations of red blood cell extracts utilizing as automated polyamine analyzer. J. Chromatog., 245, 101, 1982.

11. Brossat, B., Straczek, J., Belleville, F., and Nabet, P. Determination of free and total polyamines in human serum and urine by ion-pairing high performance liquid chromatography using a radial compression module. J. Chromatog., 277, 87, 1983.

12. Bachrach, V. Function of naturally occurring polyamines. Academic Press, New York, 1973.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2611-2630 (1984)

# INDIVIDUAL CAROTENOID DETERMINATIONS IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C.C. Tangney
Section of Clinical Nutrition
Rush-Presbyterian-St. Luke's Medical Center
Chicago, IL 60612

## ABSTRACT

A high-performance liquid chromatographic technique is described to quantify beta carotene from alpha carotene and lycopene in human plasma. Total analysis time is 14.5 min. A reverse-phase column was employed with a mobile phase composed of 65% acetonitrile: tetrahydrofuran (90:10, v:v) in methanol. Use of the internal standard, beta-apo-8- carotenoic ethyl ester permitted a reliable way to quantify potential losses in plasma extractions. Plasma beta carotene levels obtained from subjects several days after supplement use were observed to increase three-fold or more.

## INTRODUCTION

Determinations of serum beta carotene in clinical laboratories have relied largely upon the measurement of total carotenoids via absorbance at a fixed wavelength (1-3). This nonspecific technique was employed in a recent study which examined serum carotenoids from patients who were diagnosed as having cancer in the subsequent five years and similar patients who remained cancer-free. Consequently, one could not ascertain which if any of the individual carotenoid level(s)



 0148-3919/84/0713-2611$3.50/0

were different in cases versus controls; no significant difference between these two groups were found with total carotenoid measurements. As Peto and others (5,6) have recommended, the epidemiologic evidence for the lung cancer-beta carotene hypothesis can only be tested if clinical studies incorporate more discriminatory analyses of carotenoids in foods and tissues. Secondly, research must establish whether a single blood determination (or dietary estimate) is truly reflective of the usual dietary intake patterns over the period of carcinogenesis.

The predominant carotenoids found in human plasma or sera include alpha carotene, beta carotene and lycopene along with possibly an oxy-carotenoid or xanthophyll named cryptoxanthin (7,8). Therefore, analyses of fluids should incorporate determinations of these compounds. Several earlier reports in the literature (9-11) have examined the correspondence between prescribed test meals or beta carotene supplement use and plasma/serum total carotenoids concentrations in a number of human subjects. A preliminary investigation was conducted in this laboratory to evaluate the relationship between short term supplement use and plasma levels with a method that quantifies beta carotene levels apart from those of other carotenoids.

Previously reported separations of carotenoids were primarily in food matrices by long, laborious open-column chromatographic techniques (12-14) or with rather complicated hardware setups for greater speed of analysis and normal phase high-performance liquid chromatography (15,16). With the advent of bonded phase columns, specifically, the so-called reverse phase columns, the analyses of complex mixtures of carotenoids and/or xanthophylls became possible (8, 11-19). Alpha and beta carotenes as geometric isomers are probably the most difficult pair of compounds to separate when considering human tissue specimens. The method described herein was developed for the quantitative analyses of these compounds in

such matrices. The method development was also intended to quantify other vitamins potentially protective against human cancer, in particular, the fat-soluble vitamins A and E. The following attributes have led to routine use of the method in our laboratory: 1) the resolution of beta and alpha carotenes; 2) the use of an internal standard structurally similar to the carotenoids of interest and; 3) a standard protocol to mitigate storage losses. To the best of our knowledge, only two reports in the literature use an internal standard (21,22) and neither include a scheme to separate alpha carotene from beta carotene.

## MATERIALS AND METHODS

### Materials

L-ascorbic acid, beta hydroxytoluene (BHT), all-trans beta carotene, all-trans alpha carotene, lycopene, retinyl acetate, and all-trans retinol were obtained from Sigma (St. Louis, MO). Alpha and beta carotene were of the highest purity available, types V and IV, respectively. Azobenzene and retinyl palmitate were obtained from Fluka (Hauppauge, NY). Alpha and gamma tocopherols (both d-), dl-alpha tocopheryl acetate, dl-tocol, canthaxanthin and beta apo-8-carotenoic acid ethyl ester (BcEE) were generous gifts from Dr. W.E. Scott, Hoffman LaRoche Inc. (Nutley, NJ). All standards dry or as solutions were stored in amber bottles under $N_2$ at - 20°C. Crystals of alpha and beta carotene, retinyl palmitate, and lycopene were first dissolved in hexane/acetone (60/40, w/v) solutions. Subsequent dilutions for working calibration mixtures were made with the mobile phase. For the tocopherols, initial dilutions were made with absolute ethanol, then with methanol. Retinol and retinyl acetate were dissolved in methanol directly. All solvents were HPLC-grade (Omnisolve, MCB Manufacturing Chemists, Inc., Gibbstown, NJ) and degassed by vacuum filtration prior to use.

Liquid Chromatography

The liquid chromatographic system included two reciprocating pumps (Model 510) and an automatic gradient controller (Model 680, Waters Associates, Milford, MA); a Rheodyne 7125 sample valve injector with a 50ul loop (Cotati, CA); a Perkin-Elmer LC-75 variable UV-visible spectrophotometer (Norwalk, CT) set at 292nm or 450nm and 0.04 aufs and; a Hewlett Packard 3390A reporting integrator (Avondale, PA). Two types of stainless steel columns were operated at ambient temperatures - a 5um Ultrasphere ODS column (Altex Scientific, Berkeley, CA), 250mm long x 4.6mm id and a 3um Supelcosil LC-18 (Supelco, Inc., Bellefonte, PA), 150mm long x 2.9mm, id. A Brownlee guard column holder with a 10um ODS cartridge (Santa Clara, CA) was placed in series between an inline solvent filter and the analytical column; cartridges were replaced monthly to obviate contamination of the analytical column.

Initial experiments were performed to achieve capacity ratios (K') between 2 and 10. K' was defined as $t_R$-$t_o/t_o$, where $t_R$ and $t_o$ were the retention times of the compound of interest and the unretained peak, respectively. Various strategies were tested in order to optimize the resolution (Rs) between alpha and beta carotene and/or alpha and gamma tocopherols. Rs was defined as follows:

$$Rs = (t_{R2} - t_{R1})/(w_2 + w_1)$$

where $t_{R2}$ and $w_2$ represented the retention time and peak width of beta carotene (or alpha tocopherol), respectively, and $t_{R1}$ and $w_1$, the corresponding parameters for alpha carotene (or gamma tocopherol).

The gradient program on the Ultrasphere column was as follows: pump 5% water in methanol (100% A solvent reservoir) for the first 14 min, change linearly to 4% tetrahydrofuran in methanol (100% B) over 2 min and maintain for another 14 min and return to 100% A over the next 3 min and maintain this

solvent composition for a minimum of 15 to 18 min for reequilibration. Eluents were monitored at 292nm for the first 22 min until the retinyl palmitate peak eluted; then the wavelength was quickly changed to 450nm for the carotenes. The total analysis time was 45 min. Isocratic analyses of retinol, retinyl acetate, and the tocopherols were also accomplished on the Supelcosil C-18 column within 14 min using 100% methanol mobile phase system described previously (23). Then isocratic analyses for carotenoids were conducted on the same Supelcosil column with 35% methanol in acetonitrile/tetrahydrofuran, (90/10, v/v); flow rates approximated 2ml/min.

Quantification and Calibration

A standard curve of peak height ratios (compound of interest to internal standard, BcEE or tocol) versus concentration ratios was calculated daily for every set of analyses. Calibration mixtures contained differing amounts of beta carotene, alpha carotene, and lycopene with a constant amount of the internal standard. Weight ratios were selected to reflect expected concentrations in samples studied. Linearity of calibration graphs was tested by analysis of variance using $R^2$ as the criterion of adequacy.

Extractions from Serum or Plasma, Recovery Efficiencies, and Storage Protocol

Serum or plasma samples (0.5ml) were placed in glass test tubes containing 0.1ml BcEE (10.56ug/ml), and 0.1ml 3% L-ascorbic acid in absolute ethanol. Three ml of 0.0125% BHT in hexane were added and contents were gassed under $N_2$, capped, mixed on a vortex mixer for 20s, and centrifuged to separate phases. The organic phase was removed and transferred to a new tube; the remaining aqueous infranatant was reextracted with 3ml of 0.005% BHT in hexane. The second organic layers were

pooled with the first and evaporated until almost dry under $N_2$. The residue was carefully rinsed with additional hexane, transferred to a microcentrifuge tube and evaporated to dryness with $N_2$. This concentrate was then quickly redissolved in 0.2ml mobile phase. All extractions were performed either under subdued light or red lights.

Dogs fed commercial lab chow were used for recovery experiments because no carotenoids were detected in their plasma. Recovery efficiencies were assessed by adding 1.225ug and 2.45ug beta carotene to dog plasma as well as plasma and sera from one human volunteer with detectable alpha and beta carotene. Six replicates were made at each level: beta carotene was added to three and not added to the other three.

Two specimens of fasting blood from one human volunteer, 20ml each were collected in EDTA-coated and anticoagulant-free vacutainer tubes. Plasma and sera aliquots were extracted as usual and analyzed by HPLC the same day (day 0). Remaining plasma and sera were divided into amber glass vials each containing approximately 1.5ml sample, gassed quickly with $N_2$ and frozen at $-20^{o}C$ for later analyses. Samples were extracted and analyzed again at 7, 18 and 48 days after venipuncture. No consistent differences were observed for beta carotene measurements analyzed at any time or between sera and plasma extracts. Broich et al (19) have reported similar observations. Stability of frozen sera and plasma with the additions of various antioxidants for longer time periods is currently being studied.

Description of Subjects and Supplementation Design

In attempt to determine what effect large doses of beta carotene have on plasma beta carotene levels of human volunteers, a time series-type design was established with four subjects. Fasting blood specimens were drawn on day zero just

prior to ingestion of one capsule containing 30mg Solatene R (Hoffmann-LaRoche, Inc. Nutley, NJ). This preparation was selected for its notably high bioavailability. No attempt was made to record or modify the subjects' dietary intakes. Subjects received either no, one, two or three capsules each morning from the investigator for a variable number of days. At several time points, subjects fasted 10-12 hr prior to the morning venipuncture. All samples were either extracted within 4 hr of venipuncture and analyzed the same day or gassed under a $N_2$ stream and frozen at -20°C until extraction and HPLC analyses could be performed (usually with 7 days).

## RESULTS AND DISCUSSION

### Chromatography

In order to simultaneously resolve compounds of similar as well as widely divergent lipophilicities, a gradient elution program was necessary. Figure 1 illustrates the separation a standard solution mixture of retinol, tocopherols, retinyl palmitate and the carotenes-alpha and beta. The Ultrasphere ODS column adequately resolved gamma and alpha tocopherols with a resolution (Rs) = 1.27, but the Rs between alpha and beta carotenes was somewhat poorer, Rs = 1.01. Note the proximity of the retinol peak to the solvent front ($t_o$). A small percentage of water (5%) was added to methanol (A reservoir) in order to retain the compound, K'>2. Greater retention was achieved by increasing the proportion of water, but the analysis time also increased. In addition, more water exacerbated the solubility limits for the carotene, leading to poorly shaped peaks and eventual pressure increments. The manual change in wavelength during the run and after the elution of carotenes also imposed several limitations. First the operator had to be present for the analyses. Second,

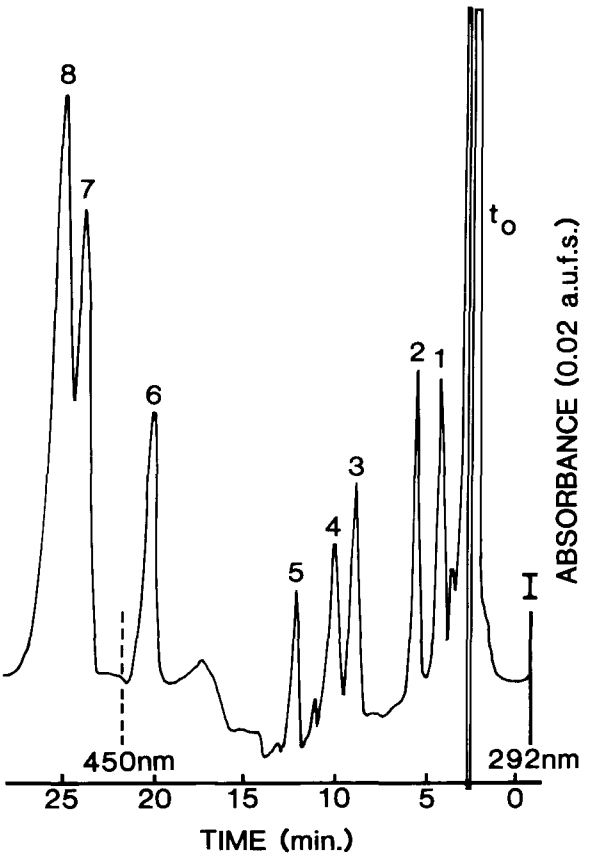



FIGURE 1: Representative HPLC profile of a standard mixture on the Ultrasphere ODS column. Conditions are described in text. $t_o$ = solvent front; 1 = retinol; 2 = retinyl acetate; 3 = gamma-tocopherol; 4 = alpha-tocopherol; 5 = alpha-tocopheryl acetate; 6 = retinyl palmitate; 7 = alpha-carotene; 8 = beta-carotene.

because the selected carotenoid-like internal standards were more polar than the carotenes, they eluted when the detector was set at 292nm and thus were not optimally detected. For this reason, dl-alpha-tocopheryl acetate was used as the internal standard for all peaks of interest. Third, the

analysis time was quite long. In the investigator's opinion, the best solution for these drawbacks is to use a dual wavelength detector which was not available in our laboratory. With such a detector, no baseline correction would be necessary. Most importantly, with a single chromatographic injection and sample preparation, quantification of at least 8 different compounds would be possible. Further, the most chemically similar and appropriate internal standards---retinyl acetate for retinol, dl-tocol for the vitamin E compounds, and BcEE for the carotenoids could be used without interference or overlapping peaks. These internal standards are ideal because such compounds would not be present in analyzed samples.

Because of these equipment limitations in our laboratory, the second chromatographic method developed for the determination of carotenoids in tissues is illustrated by the chromatograms in Figures 2 and 3. Figure 2 depicts a chromatogram of a standard solution mixture. At a flow rate of 2.0 ml/min, the total assay time was 14.5 min. Two conditions primarily facilitated the faster elution times - a shorter column and a mobile phase of higher solvent strength (nonaqueous solvents only). The latter also permitted better sample solubility and lower operating pressures. However, with such fast elution profiles, the internal standards first attempted (azobenzene and canthaxanthin) were not sufficiently retained. BcEE with a K' = 2.11 was selected. The Rs for alpha and beta carotene was consistently between 1.88 and 1.90 at flow rates of 2.0 - 2.5ml/min. Figure 3A depicts a chromatogram of a plasma extract in which BcEE was added. This extract was also prepared with no internal standard added; no other compounds were observed to co-elute with this compound (Figure 3B). Figure 4 demonstrates the similarity in absorbance patterns between these carotenoids. Because these standards were dissolved in the mobile phase, a slight shift in

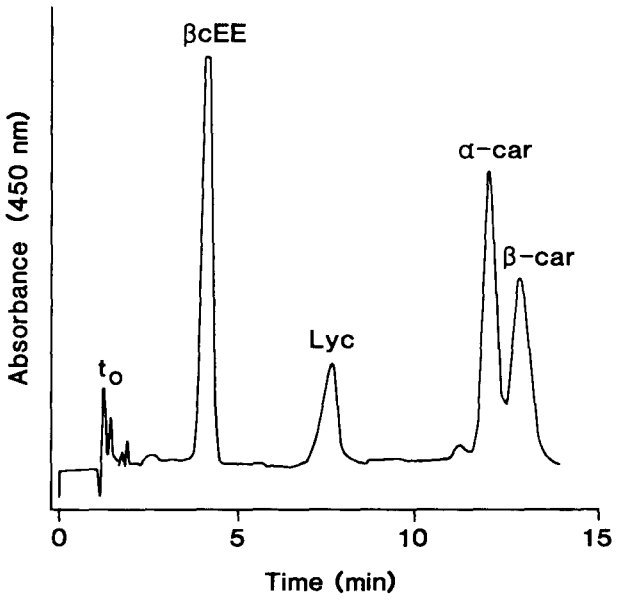



FIGURE 2: Representative HPLC profile of standard mixture on Supelcosil C-18 column. Conditions are described in text $t_o$ = solvent front; BcEE = beta apo-8-carotenoic ethyl ester; Lyc = lycopene; α-car = alpha-carotene; β-car = beta-carotene.

wavelength maxima was observed in comparison to reported values in other solvents (17,24). Several batches of commercially prepared standards were highly "contaminated" with cis isomers, as evidenced by secondary absorbance maxima between 320 and 340nm. Crystallization in hexane reverted much of this isomeric mixture to the all-trans form as evidenced by a loss in absorbance in the 320-340nm range (24). Peak identification of all compounds was tentatively confirmed by co-chromatography of standards in these two chromatographic schemes and by the absorption spectra in both the mobile phase and hexane solutions.

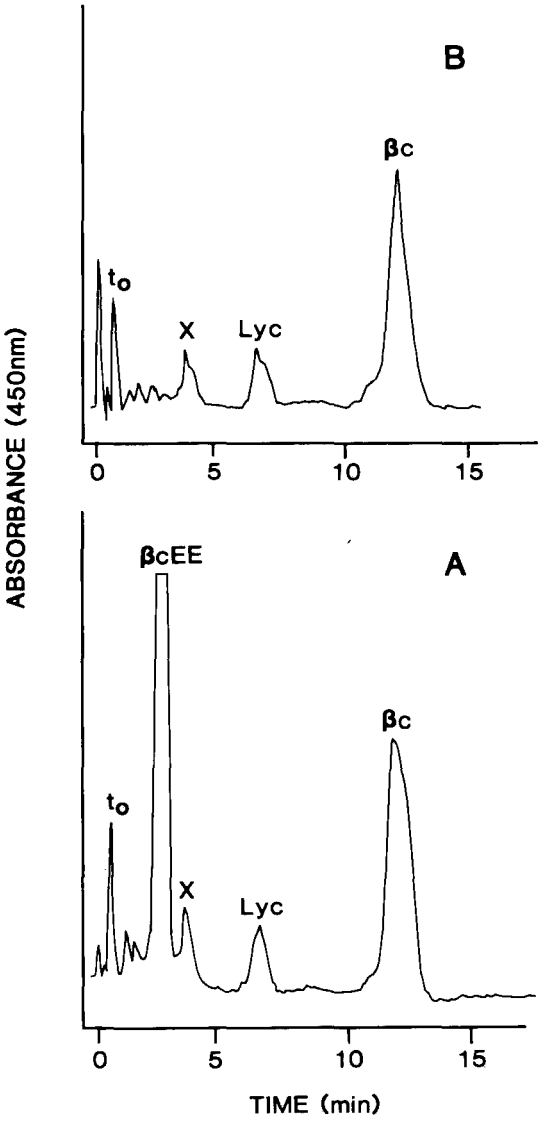



FIGURE 3: A. (lower) HPLC profile of a plasma sample extracted as described in text. The internal standard BcEE was added prior to extraction. B. (upper) HPLC profile of the same plasma sample extracted similarly but with no BcEE added.

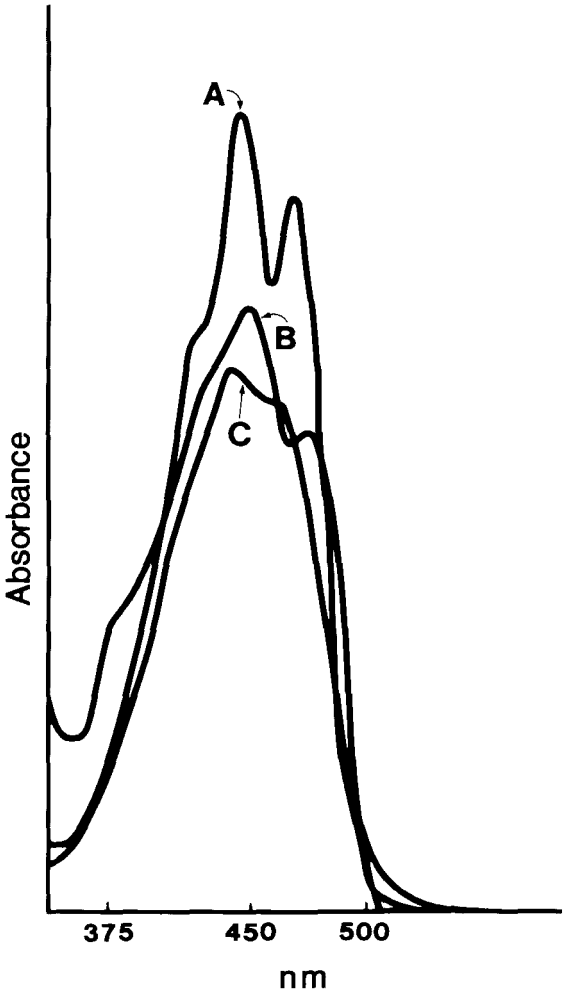



FIGURE 4: Absorption spectra of (A) alpha carotene, (B) beta carotene, and (C) beta-apo-8-carotenoic ethyl ester.

Calibration, Quantification and Recovery Experiments

Calibration curves for alpha and beta carotenes were linear from 20ng/ml to 10ug/ml; lycopene with a poorer absorptivity at 450nm had linear range from 45ng/ml to 8ug/ml. $R_2$ values for four or five standard solutions injected each day were greater than 0.995; sample analyses were not conducted unless this minimum value was exceeded. A comparison of slopes of calibration curves obtained for six days of analysis yielded a coefficient of variation of 4.78%.

Recoveries of spiked sera and plasma of dog or man ranged 84.80% to 91.30% (Table 1). Extraction efficiencies were significantly improved by the addition of 3% ethanolic ascorbic acid ($p<0.05$). No experiments were attempted however to assess the efficacy of 0.0125% BHT in hexane; this solution was used in all assays. Because there were no significant differences in recovery efficiencies between samples spiked with 1.225ug beta carotene and 2.45ug beta carotene, the means and coefficient of variation represent those of all six replicates. Nor was there a significant difference between percent recoveries obtained for human plasma and that of dog. Similar improvements in recoveries of labile vitamins A and E with these antioxidants were reported by Chow and Omaye (25), but slightly higher concentrations of ascorbic acid and BHT were used.

Plasma Concentrations in Human Volunteers

Plasma beta carotene levels of four subjects taking varying amounts of Solatene daily are presented in upper half of Figure 5. All subjects were consuming their "usual" diets at that time (late winter/early spring) but actual dietary intakes were not monitored. None of the subjects consumed other vitamin/mineral supplements. The investigator ascertained pill compliance by direct observation. The

TABLE 1

Recovery Efficiencies for Beta Carotene in Various Tissues.

| | Antioxidant | Percent Recovery | |
|---|---|---|---|
| Sample | Ethanol Phase | X | CV[a] |
| Plasma, human | 0 | 81.95 } d | 11.6/ |
| Plasma, human | 3% AA[b] | 91.30[c] } d | 3.67 |
| Sera, human | 0 | 79.97 } d | 7.29 |
| Sera, human | 3% AA[b] | 90.06[c] } d | 4.08 |
| Plasma, dog | 3% AA[b] | 84.80[c] | 0.92 |

[a] Coefficient of Variation, %.

[b] Ascorbic Acid.

[c] Values represent that of six replicates, that is the percent recoveries at both spiking levels - 1.225ug and 2.45ug beta carotene.

[d] $p < 0.05$

supplementation regimen is depicted graphically in the lower half of Figure 5 of each volunteer. Excluding the baseline determinations, the contribution of these supplements to plasma levels probably far outweighed that made by diet. Recent evidence from Shekelle et al (26), Willett and coworkers (27) and unpublished observations in this laboratory suggest that typical beta carotene intakes for the American population range from 1.5 to 7.0mg per day. Admittedly, these estimates are crude at best since the beta carotene content of foods were not directly determined but derived from indices based on food groups or partition coefficients. In spite of these limitations, it is likely that the volunteers in the current study consumed from 10 to 60 times as much as that usually obtained from diet. Baseline plasma determinations of three of the four volunteers were considerably higher than the average

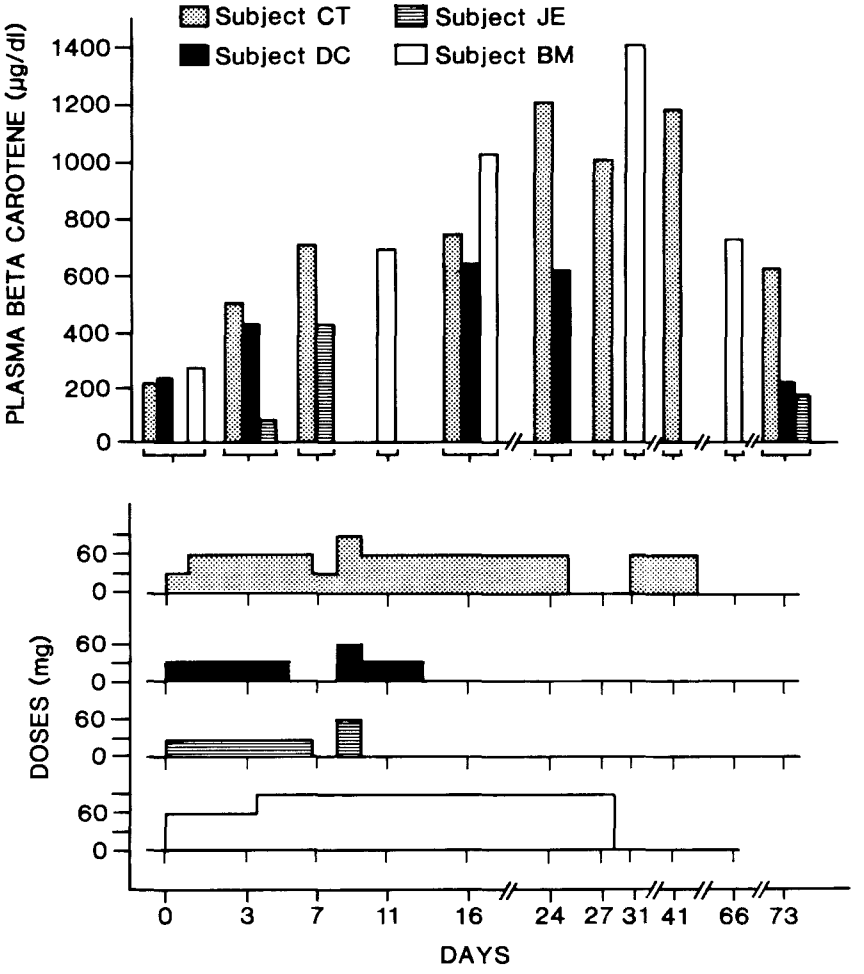



FIGURE 5: Plasma beta carotene levels of four subjects (upper graph) ingesting various amounts of Solatene (lower graph) over time.

beta carotene levels reported by Ross and Parker (28) but well within the "normal" ranges. All subjects exhibited barely detectable lycopene levels (0.51-4.55ug/dl); there was no consistent pattern of change in these levels concomitant with the supplement dose ingested. Detectable alpha carotene levels were observed for only one volunteer, C.T., and this concentration was only observed at one time only. The third subject, J.E., though exhibiting undetectable levels prior to supplementation, later evidenced higher levels that were apparently reponsive to supplementation. A fivefold increment in plasma beta carotene levels was observed from day 3 to day 7. The changes in concentration for the remaining three subjects with 30 or 60mg Solatene administration were approximately three- to fourfold from day 0 to day 16. In subject B.M. who ingested the highest dose (90mg) daily for 24 days, plasma beta carotene levels increased nearly sixfold from day 0 to day 31. The response to dose administration appeared quite rapid for all subjects, although the rate of response seems to be quite different from subject to subject. The actual rate of response is difficult to assess because blood samples were not obtained at earlier time points nor at the same time points with similar doses for subjects. In contrast to the seemingly rapid response in plasma levels with initial doses, the cessation of Solatene therapy was not accompanied by a precipitous drop in plasma levels (days 31-73). By days 66 or 73 (more than a month after supplementation was discontinued) levels were still elevated in three of the four subjects. Plasma levels of subjects B.M. and J.E. measured 45 days and 67 days later, respectively, were still much higher than baseline levels. The relative responsiveness of plasma levels to supplementation has also been examined by Bjornson and associates (29) and Mathews-Roth et al (30). In the former, several patients with diagnosed erythropoietic

protoporphyria ingesting 60 to 120mg Solatene demonstrated a similarly sharp rise in beta carotene levels with the initiation of therapy and more gradual lowering with discontinuation. A direct comparison is not possible here because of differences in patient characteristics, dose levels, time and analytical technique. Willett and coworkers (27) measured total serum carotenoids in 59 health adults consuming only 30mg of Solatene at 0, 8, and 16 weeks. These investigators found that plasma carotenoids nearly tripled by 8 weeks. Again, the study design was considerably different and no determinations were conducted following discontinuation of supplements. While prior literature confirms the basic trend observed in this pilot study, few if any have employed a chromatographic technique that is as specific for beta carotene or as reliable and fast. In future large scale trials in which supplement use or dietary intakes will be monitored by plasma determinations, it is essential that such a technique be available for investigators. Further research is necessary to quantitate the degree of association between dietary intakes and plasma levels using a specific measurement for beta carotene in tissues. These studies are presently ongoing in our laboratories.

## ACKNOWLEDGEMENTS

This investigation was supported in part by Grant #HL29690-02 from the National Institute of Health, a grant from Hoffmann LaRoche, Inc. and an Grant-in Aid at Rush-Presbyterian-St. Luke's Medical Center. The author is also indebted to Dr. Rick B. Shekelle and Dr. Phyllis E. Bowen for their advice regarding preparation of the manuscript.

## REFERENCES

1). Interdepartmental Committee on Nutrition for National Defense. Manual for Nutrition Surveys. 2nd Ed. U.S. Government and Printing Office. Washington, D.C., 1963.

2). Roels, O.A. and Trout, M. A method for the determination of carotene and vitamin A in human blood serum. Amer. J. Clin. Nutr. 7, 197, 1959.

3). Targan, S.R., Merrill, S. and Schwabe, A.D. Fractionation and quantitation of β-carotene and vitamin A derivatives in human serum. Clin. Chem. 15, 479, 1969.

4). Willett, W.C., Polk, F.B., Underwood, B.A., Stampfer, M.J., Rosner, B., Taylor, J.O., Schneider, K., and Hames, C.G. Relation of serum vitamins A and E and carotenoids to the risk of cancer. New England J. Med. 310, 430, 1984.

5). Peto, R., Doll, R., Buckley, J.D. and Sporn, M.B. Can dietary beta-carotene materially reduce human cancer rates? Nature 290, 201, 1981.

6). Simpson, K.C. and Chicester, C.D. Metabolism and nutritional significance of carotenoids. Ann. Rev. Nutr. 1, 351, 1981.

7). Bayfield, R.F., Falk, R.H. and Barrett, J.D. The separation and determination of α-tocopherol and carotene in serum or plasma by paper chromatography. J. Chromatog. 36, 54, 1968.

8). Nells, HJ, CF and DeLeenheer, A.P. Isocratic nonaqueous reversed-phase liquid chromatography of carotenoids. Anal. Chem. 55, 270, 1983.

9). Fujita, A and Morimoto, H. Absorption of carotene in man as observed from the increase of serum vitamin A and carotene levels. J. Vitaminol. 6, 278-290, 1960.

10). Urbach, C., Hickman, K. and Harris, P.L. Effect of individual vitamins A, C, E and carotene administered at high levels on their concentration in the blood. Exp. Med. Surg. 10, 7, 1952.

11). Feldman, E.B. and Adlersberg, D. Response to carotene loading in malaborption states. Amer. J. Med. Sci. 22, 730, 1959.

12). Purcell, A.E. Partition separation of carotenoids by silica-methanol columns. Anal Chem. 30, 1049, 1958.

13). Gebhardt, S.E., Elkins, E.R. and Humphrey, J. Comparison of two methods for determining the vitamin A value of clingstone peaches. J. Agric. Food Chem. 25, 629, 1977.

14). Rodriguez, D.B., Raymundo, L.C., Lee, T.C., Simpson, K.L. and Chichester, C.O. Carotenoid pigment in ripening Momordica charantia fruits. Ann. Bot. 40, 615, 1976.

15). Sweeney, J.P. and Marsh, A.C. Vitamins and other nutrients. Separation of the carotene stereoisomers in vegetables. J. Assoc. Off. Anal. Chem. 53, 937, 1970.

16). Stewart, I. High-performance liquid chromatographic determination of provitamin A in orange juice. J. Assoc. Off. Anal Chem. 60, 132, 1977.

17). Braumann, T. and Grimme, L.H. Reversed-phase high performance liquid chromatography of chlorophylls and carotenoids. Biochem. Biophys. Acta 637, 8, 1981.

18). Zakaria, M., Simpson, K.L., Brown, P., and Krstulovic, A. Use of reversed-phase high-performance liquid chromatographic analyses for the determination of provitamin A carotene in tomatoes. J. Chromatog. 176, 109, 1979.

19). Broich, C.R., Gerber, L.E. and Erdman, J.W. Determination of lycopene, $\alpha$- and $\beta$-carotene and retinyl esters in human serum by reversed phase high performance liquid chromatography. Lipids. 18, 253, 1982.

20). Bushway, R.J. and Wilson, A.M. Detection of $\alpha$- and $\beta$-carotene in fruit and vegetables by high performance liquid chromatography. Canad. Inst. Food Sci. Techn. J. 15, 165, 1982.

21). Driskell, W.J., Bashor, M.M. and Neese, J.W. Beta-carotene determined in serum by liquid chromatography with an internal standard. Clin. Chem. 29, 1042, 1983.

22). Stancher, B. and Zonta, F. High performance liquid chromatographic determination of carotene and vitamin A and its geometric isomers in foods: applications to cheese analysis. J. Chromatog. 238, 217, 1982.

23). Tangney, C.C. and Stibolt, T.B. Verification of bronchoaveolar lavage index for tocopherol assessment of lung. Federation Proc. 42, 923, 1983.

24). Davies, B.H. Chemistry and Biochemistry of Plant Pigments (Goodwin, T.W., ed.) Vol. 1, Academic Press, New York: 1976, p. 38.

25). Chow, F.I. and Omaye, S.T. Use of antioxidants in the analyses of vitamins A and E in mammalian plasma by high performance liquid chromatography. Lipids. 18, 837, 1983.

26). Shekelle, R.B., Liu, S., Raynor, W.J., Lepper, M., Maliza, D., Rossof, A.H., Paul, O., Shrcyock, A.M. and Stamler, J. Dietary vitamin A and risk of cancer in the Western Electric study. Lancet 2, 1186, 1981.

27). Willett, W.C., Stampfer, M.J., Underwood, B.A., Taylor, J.O. and Hennekens, C.H. Vitamins A, E. and carotene: effects of supplementation on their plasma levels. Amer. J. Clin. Nutr. 38, 559, 1983.

28). Ross, G. and Parker, J.G. Serum carotene concentration in normal individuals and its clinical interpretation. N.Y.S. J. Med. 24, 3584, 1962.

29). Bjornson, L.K., Kayden, H.J., Miller, E., Moshell, A.B. The transport of α-tocopherol and β-carotene in human blood. J. Lipid Res. 17, 343, 1976.

30). Mathews-Roth, M., Pathak, M.A., and Fitzpatrick, J.B., Harbor, L.C., and Kass, E.H. β-carotene as an oral photoprotective agent in erythrocytic protoporphyria. JAMA 228, 1004, 1974.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2631-2642 (1984)

# SUBSTITUENT EFFECTS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DIARYLACRYLONITRILES

S. Caccamese, V. Iacona, G. Scarlata and R M. Toscano
Dipartimento di Scienze Chimiche
Università di Catania
viale Doria 6, Catania, Italy

## ABSTRACT

Thirty diarylacrylonitriles, carrying various para substituents in the phenyl ring attached to the position 1 of the ethylenic bridge and various heterorings (thienyl, furyl, pyrryl, N-methylpyrryl) attached to the position 2, have been investigated on silica gel. An additional substituent is present in the thienyl derivatives. The capacity factors of all the compounds can be grouped in only five or six families, according to the variation in the para-phenyl substitution or in the heteroring moiety, respectively. The strongest effect in the enhancement of the capacity factors is given by a methoxy or nitro group in the para-phenyl position.

Other minor effects as well as the relationship between capacity factors and solvent mixtures polarity are discussed.

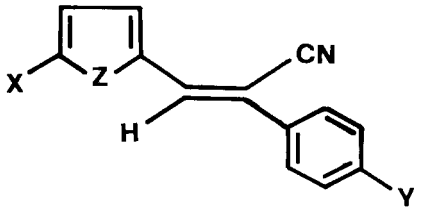
## INTRODUCTION

Recently it has been observed that the substitution of a hydrogen atom in the ethylenic bridge by a cyano group in stilbenes modifies the behaviour to the photoisomerization and dimerization(1). In this respect,we



 0148-3919/84/0713-2631$3.50/0

are concerned with heteroaryl analogs of general formula




| | | |
|---|---|---|
| X=H | Z=O,NH,$NCH_3$ | Y=H,$CH_3$,Cl,$OCH_3$,$NO_2$ |
| X=H,$CH_3$,Br | Z=S | Y=H,$CH_3$,Cl,$OCH_3$,$NO_2$ |

Thirty compounds of this class were available from previous studies of one of us on their infrared and ultraviolet (2), $^1H$ (3) and C 13 NMR (4) spectroscopies.

During a work on the solid-state irradiation of such compounds it was clear that photoisomerization rate was greatly effected by the X, Y and Z substituents pattern and, since the reaction was monitored by HPLC, a relationship between these substituents and the chromatographic behaviour on straight silica gel became apparent.

In this report, a rationalization for this correlation is given. Furthermore, the interaction of these compounds with solvent mixtures of different polarity is illustrated.

## EXPERIMENTAL

### Apparatus

The liquid chromatography used was a Varian Model 5060 equipped with a JASCO Model Uvidec-100 III UV spectrophotometer at 240 nm, cell volume 8 µl. The detector was connected to a Varian CDS 401 Data System with data

collection. The sample was introduced with a Valco air-actuated valve equipped with a 10 µl external loop.

Chromatographic conditions

Each compound was examined at least in duplicate in order to determine elution time and capacity factor. Separations were carried out on a LiChrosorb Si 100 column (25 cm. x 4 mm. I.D.) packed with 10 µm silica particles (Merck, Darmstadt, G.F.R.). The solvent flow-rate was 1.0 ml/min. Temperature was 18°C for the analyses done in methylene chloride-hexane mixtures and 26°C for the analyses done in tert-butylmethylether-hexane mixtures. Reproducibility of elution time was better than 0.4% at constant temperature. A temperature increase from 18° to 26°C, using the same solvent mixture, resulted in a decrease of about 5% in the elution times.

Chemicals and solvents

Compounds 1-30 were previously prepared by condensation of 2-heteroaromatic aldehyde with the appropriate acrylonitrile in the presence of bases and their physical characterization is reported elsewhere (3-5).

All compounds were of the Z configuration as previously shown (3), for many of them, by a $^{1}H$ NMR stereochemical study. They demonstrated a high purity and so were used without further purification. The methylene chloride and n-hexane were HPLC grade from J.T.Baker (Phillipsburgh, N.J., U.S.A.). The tert-butylmethylether was HPLC grade from Fluka (Buchs, Switzerland).

## RESULTS AND DISCUSSION

The capacity factors k' were calculated by the usual equation $k' = t_R - t_0/t_0$ where $t_R$ = elution time of compound, $t_0$ = the time of zero retention measured as the time of the non-retained solvent peak. They are listed in Table 1 for the solvent system methylene cloride-n-hexane (80:20). An example of the achievable resolution in the separation of an *ad hoc* prepared mixture of thienyl derivatives is illustrated in Figure 1.

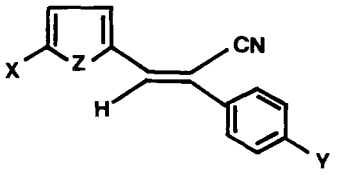
The substituents in the molecules induce extensive conjugation between X and cyano group as well as between an Y electron-releasing group and the cyano group. However, polar effects play a major role in the interaction of the functional groups with the silica adsorption sites competitively with the solvent molecules. Figures 2 and 3 illustrate, in fact, the correlation between the capacity factor and the substitution pattern in the acrylonitrile skeleton. These plots use log k' versus log $k'_{\varkappa}$

In fact, being the logarithm of a capacity factor proportional to the free energy change ($\Delta G$) associated with the chromatographic distribution process (6), this type of plot relates the $\Delta(\Delta G)$ obtained when an additional substituent is introduced (ordinate) in a sequence of $\varkappa$ compounds having a particular substitution pattern (abscissa).

A similar plot has already been used in HPLC (7). In Figura 2, $\varkappa$ refers to compounds 2,3,1,4,5 carrying as Y substituent Cl, $CH_3$, H, $OCH_3$ and $NO_2$ respectively. All thirty compounds reported in Table 1 are on six lines (A to F)

TABLE 1

Capacity Factors of
Z -[1-(Phenyl)-2-(Heteroaryl)] Acrylonitriles [a]




| Compound | X | Z | Y | K' |
|---|---|---|---|---|
| 1 | H | S | H | 0.26 |
| 2 | H | S | Cl | 0.21 |
| 3 | H | S | $CH_3$ | 0.24 |
| 4 | H | S | $OCH_3$ | 0.48 |
| 5 | H | S | $NO_2$ | 0.56 |
| 6 | Br | S | H | 0.15 |
| 7 | Br | S | Cl | 0.12 |
| 8 | Br | S | $CH_3$ | 0.13 |
| 9 | Br | S | $OCH_3$ | 0.31 |
| 10 | Br | S | $NO_2$ | 0.43 |
| 11 | $CH_3$ | S | H | 0.22 |
| 12 | $CH_3$ | S | Cl | 0.17 |
| 13 | $CH_3$ | S | $CH_3$ | 0.20 |
| 14 | $CH_3$ | S | $OCH_3$ | 0.45 |
| 15 | $CH_3$ | S | $NO_2$ | 0.53 |
| 16 | H | O | H | 0.32 |
| 17 | H | O | Cl | 0.27 |
| 18 | H | O | $CH_3$ | 0.31 |
| 19 | H | O | $OCH_3$ | 0.59 |
| 20 | H | O | $NO_2$ | 0.72 |
| 21 | H | NH | H | 0.41 |
| 22 | H | NH | Cl | 0.36 |
| 23 | H | NH | $CH_3$ | 0.39 |
| 24 | H | NH | $OCH_3$ | 0.78 |
| 25 | H | NH | $NO_2$ | 0.98 |
| 26 | H | N-$CH_3$ | H | 0.48 |
| 27 | H | N-$CH_3$ | Cl | 0.39 |
| 28 | H | N-$CH_3$ | $CH_3$ | 0.46 |
| 29 | H | N-$CH_3$ | $OCH_3$ | 0.94 |
| 30 | H | N-$CH_3$ | $NO_2$ | 0.99 |

[a] Solvent system n-hexane-methylene chloride (20:80) on LiChrosorb 100.

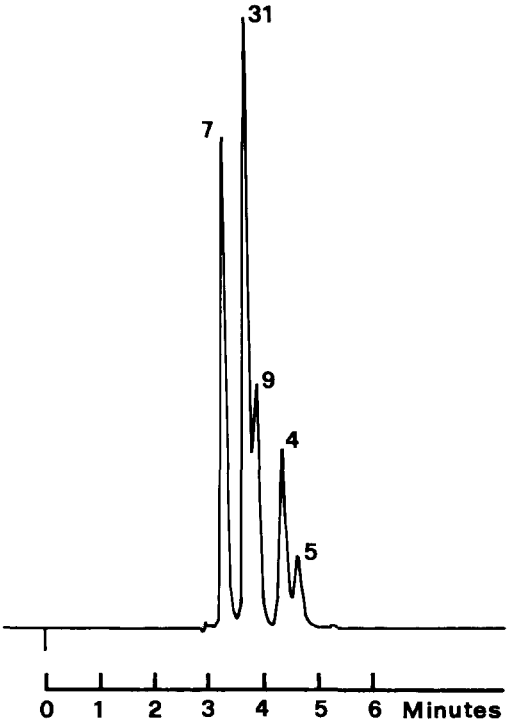



FIGURE 1. Separation of diarylacrylonitriles (for peaks 4, 5, 7 and 9 see Table 1) and E-[$\alpha$ -phenyl,$\beta$-2-thienyl)] ethylene (for peak 31 see text). For conditions see Table 1.

defining six acrylonitriles series each of them having the same heteroaryl moiety (X,Z = H,$NCH_3$; H,NH; H,O; H,S; $CH_3$,S; Br,S respectively). It can clearly seen that introduction of a methoxy or nitro group in para-phenyl position increases drastically the capacity factor, whereas introduction of a methyl or chloro group reduces slightly the capacity factor. This fact can be rationalized on the basis only of the local dipole moments induced either by a strong electron-demanding group (nitro) or by a strong

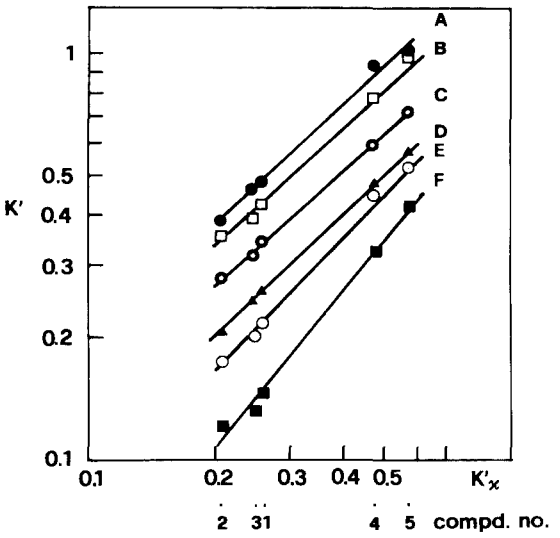



FIGURE 2. Effect of the heteroaryl moiety on the elution of diarylacrylonitriles. Logarithmic plot of k' vs. $k'_{x}$ with x = compounds 2, 3, 1, 4 and 5 for the solvent mixture hexane-methylene chloride (20:80). A, series with X=H and Z=$NCH_3$ (compounds 27, 28, 26, 29, 30); B, series with X=H and Z=NH (compounds 22, 23, 21, 24, 25); C, series with X=H and Z=O (compounds 17, 18, 16, 19, 20); D, series with X=H and Z=S (compounds 2, 3, 1, 4, 5); E, series with X=$CH_3$ and Z=S (compounds 12, 13, 11, 14, 15); F, series with X=Br and Z=S (compounds 7, 8, 6, 9, 10). Compounds in each series are listed according to increasing k' values.

electron-releasing group (methoxy). Conjugative effects cannot account for the "same-sense" strong k' increase given by these two groups. Particularly, if we plot the capacity factors k' for each X, Z series versus the $\sigma^+$ Hammett substituent constants of the Y groups we obtain a typical U-shaped curve in which methoxy and nitro groups are located at the upper opposite bounds.

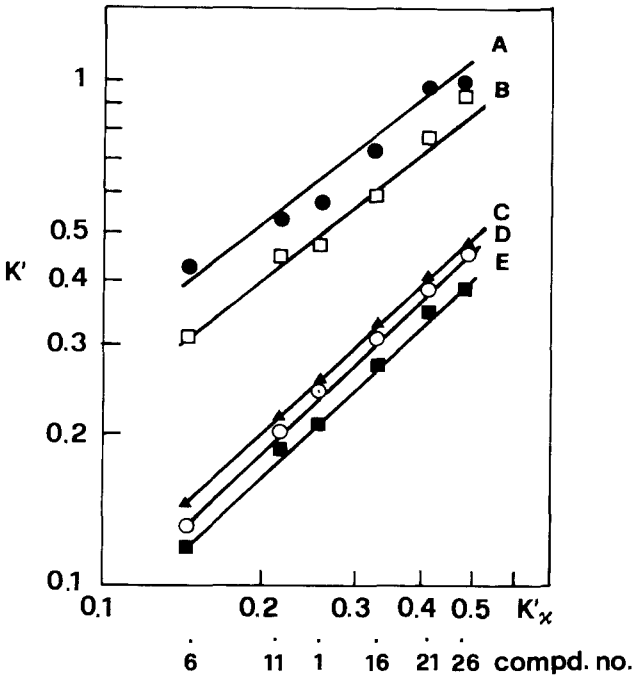



FIGURE 3. Effect of the para-phenyl substitution on the elution of diarylacrylonitriles. Logarithmic plot of k' vs. $k'_{x}$ with x= compounds 6, 11, 1, 16, 21 and 26 for the solvent mixture hexane-methylene chloride (20:80). A, series with Y=$NO_2$ (compounds 10, 15, 5, 20, 25, 30); B, series with Y=$OCH_3$ (compounds 9, 14, 4, 19, 24, 29); C, series with Y=H (compounds 6, 11, 1, 16, 21, 26); D, series with Y=$CH_3$ (compounds 8, 13, 3, 18, 23, 28); E, series with Y=Cl (compounds 7, 12, 2, 17, 22, 27). Compounds in each series are listed according to increasing k' values.

Selectivity between compounds with different X and/or Z substituents but with the same Y substituent remains constant, as it can be seen from the nearly parallel lines A,B,C,D and E. Only bromine substitution in the thiophene ring (line F) affects more markedly the increase in the elution time.

Furthermore, from Figure 2 it can be observed the sequence in the capacity factors 2-thienyl<2-furyl<2-pyrryl<2-N-methylpyrryl acrylonitriles. This fact cannot be rationalized on the basis of hydrogen-bonding interaction between the lone pair electrons of the heteroatom and the silica active hydroxy sites; probably the actual availability of these electrons, involved in conjugation, and the conformational preferences of the heteroring have to be taken into account.

In Figure 3, $\alpha$ refers to compounds 6,11,1,16,21 and 26. In this way, the abscissa is sensitive to the X substituent as well as to the Z substituent. Since compounds on each line (A to E) belong either to 5-substituted thienyl- or to heteroaryl-substituted acrylonitriles, it can be implied that both substitution patterns (X and Z) affect in an equal amount the capacity factor. Moreover, it can be again observed the marked effect on the increase in the capacity factor induced by the nitro (line A) and methoxy (line B) para-substituents in the phenyl ring.

The nearly parallel lines indicate that selectivity factor between compounds having different para-phenyl substituents but the same heteroaryl moiety remains constant.

Thus, these data support the hypothesis that the "pivoting" groups in the competition with the solvent for the adsorption silica sites are the strong polar para-phenyl substituents regardless of the direction of their dipole moments. A less marked influence on the adsorption equilibrium is due to the heteroaryl moiety.

In order to evaluate the influence of the cyano group, we have measured the capacity factor of the

E-[$\alpha$-phenyl,$\beta$-2-thienyl]ethylene, compound 31, $\underline{k}'$= 0.25, in the same solvent system, column ad temperature. If we compare this value with that of compound 1 ($\underline{k}'$= 0.26), we observe that -at least in this case- the introduction of the cyano substituent in the ethylenic bridge has not practical effect on the adsorption equilibrium on the silica.

Coming now to the effect of the solvent strength on the retention, Figure 4 shows, as expected (8), a logarithmic decrease in $\underline{k}'$ with increasing concentrations of methylene chloride in n-hexane. All the compounds, except 1, reported in Figure 4, carry a methoxy group in the para-phenyl-position and differ only in the heteroaryl moiety.

The nearly parallel lines among pyrryl $\underset{\sim}{24}$, furyl $\underset{\sim}{19}$ and thienyl $\underset{\sim}{4}$ derivatives indicate that the separation factor between these classes is independent of the methylene chloride content in the solvent, at least for the concentration studies. Slope of the line referring to compound 1, which lacks the polar methoxy substituent in the para-phenyl position, is instead less pronounced indicating a milder interaction of the solute with the methylene chloride.

Figure 5 shows the dramatic effect on the capacity factors of compounds 1, 4 and 31 of small concentration of tert-butylmethylether in n-hexane. This ether has been recently introduced in HPLC because of clear advantages over other aliphatic ethers (9). Although its solvent strength parameter ($\varepsilon°_{Al_2O_3}$ = 0.2) is low, we believe that its interaction with compounds shown in Figure 5 should be due to polar effects between the cyano group and the

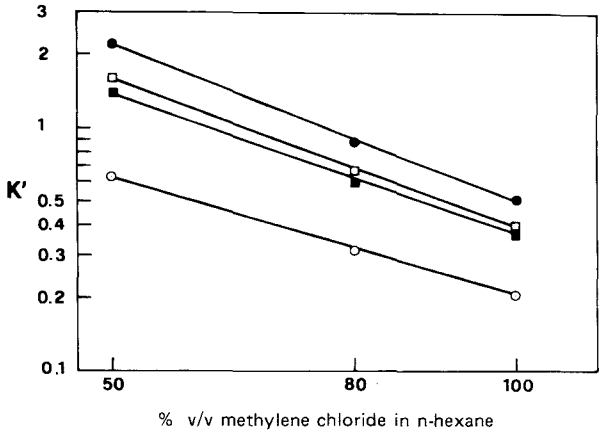



FIGURE 4. Effect of the methylene chloride concentration in hexane on the capacity factor of: ● compound 24; □ compound 19; ■ compound 4; ○ compound 1 (for numbers see Table 1).

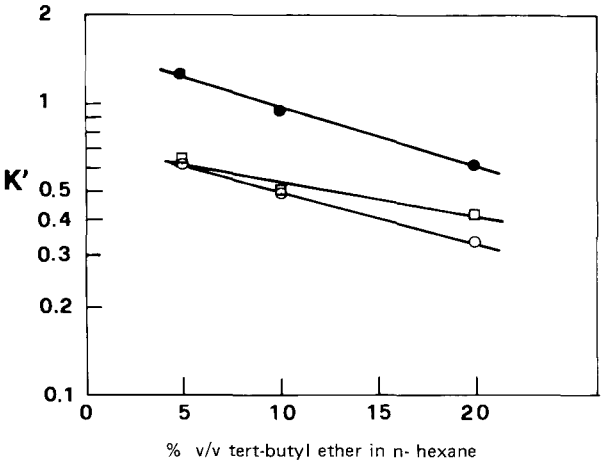



FIGURE 5. Effect of ter-butylmethylether concentration in hexane on the capacity factor of: ● compound 4; ○ compound 1; □ compound 31 (for numbers see Table 1 and text).

solvent. Compounds 31, lacking the cyano group, is in fact much less affected by the increasing concentration of tert-butylmethylether in n-hexane with respect to the other two.

Hence, it clearly appears that the order of elution of Z-[1-(p-substituted phenyl)2-(5-substituted-2-heteroaryl)acrylonitriles] is well related to their substitution pattern on the acrylonitrile skeleton.

## REFERENCES

1. Costanzo, L. L., Giuffrida, S., Pistarà, S., Scarlata, G. and Torre, M., J. Photochem., 18, 317, 1982.

2. Amato, M. E., Carbone, D., Fisichella,S. and Scarlata, G., Spectrochim. Acta, 38 A, 1079, 1982.

3. Bottino, F. A., Scarlata, G., Sciotto, D. and Torre, M., Tetrahedron, 38, 3713, 1982.

4. Ballistreri, F. P., Musumarra, G. and Scarlata, G., Chemica Scripta, 18, 214, 1981.

5. Work in progress.

6. Knox, J. H.,in Practical High Performance Liquid Chromatography, Simpson, C. F., ed., Heyden, London, 1978, p.44.

7. Bianchini, J. P. and Gaydou, E. M., J. Chromatogr., 259, 150, 1983.

8. Snyder, L. R., Principle of Adsorption Chromatography, Marcel Dekker, New York, 1968.

9. Little, O. J., Dale, A., Whatle, J. A. and Wickings, J. A., J. Chromatogr., 169, 381, 1979.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2643-2651 (1984)

# DETERMINATION OF FORMALDEHYDE IN THE POLYMERIZED RAGWEED ANTIGEN BY HPLC

Stanley K. Lam and Victoria A. Margiasso
Albert Einstein College of Medicine
Department of Laboratory Medicine
Bronx, NY 10461

## ABSTRACT

Formaldehyde is used in the polymerization of ragweed antigen for the desensitization treatment of hay fever. Because the polymerized ragweed antigen is used as a vaccine which stimulates the production of the desired immunological effects upon injecting into the body, trace formaldehyde in the injected material is a health concern. Two procedures for the determination of formaldehyde were developed. In the direct procedure, formaldehyde in the sample was reacted with 2,4-dinitrophenylhydrazine (2,4-DNP) in solution to yield a strongly UV absorbing derivative. In an alternate approach, formaledhyde in the sample in a sealed vial was allowed to diffuse in the gaseous form into a reaction chamber consisted of a culture tube insert in which formaldehyde was derivatized with 2,4-DNP reagent. Following extraction into methylene chloride, the derivative was injected into the liquid chromatograph, separated on a reversed phase column and detected at UV 254 nm.

## INTRODUCTION

Formaldehyde, an industrial toxin, is abundant in the atmosphere. It is present in the exhaust of gasoline and diesel engines as well as in tobacco smoke. Formaldehyde is also released into the air from certain synthetic polymers, i.e.



 0148-3919/84/0713-2643$3.50/0

phenolic resins, urea formaldehyde resins, and melamine formaldehyde. Formaledehyde is an ingredient in many domestic products. Exposure to formaldehyde results from the use of these resin products in insulators, textile and paper products; and also from a wide variety of household goods, including antiperspirants, wart remedies, mouth wash, and disinfectants, etc.

Exposure to formaldehyde in the concentration range of 1 to 5 ppm can produce primary irritation to the eyes, nose, and throat. Serious exposure can cause asthma and bronchitis (1). Further, contact with formaldehyde may cause skin irritation. The carcinogenicity of formaldehyde, however, is still a disputed issue. Because the high incidence of exposure to formaldehyde in the industrial community, trace level of this air pollutant is a serious enviromental problem. Both GC (2) and HPLC (3) procedures for the monitor of formaldehyde in ambient air have been reported.

Formaldehyde is used in the preparation of certain therapeutic substances. In the production of diphtheria and tetanus vaccines, formaldehyde is used to inactivate the organisms in the bacterial broth. The ragweed antigen, after polymerized with formaldehyde, is used as a vaccine for the desensitization treatment of hay fever. Injected into the body, the ragweed antigen vaccine produces the necessary immunogenicity without the allergenicty (4). However, trace of formaldehyde always remains in these vaccines. As a result of the required treatment with these vaccines, patients may also expose to formaldehyde. A procedure that measures the formaldehyde levels in these therapeutic agents is necessary for health regulatory purpose and consumer protection.

A colorimetric technique for the determination of formaldehyde using 2,4-DNP has been reported (5). However, the procedure is tedious and non-specific. When it is applied to measure the yellowish ragweed preparation, it requires an

accurate blank correction for the sample matrix. In this report, we describe two proceudres for the measurement of formaldehyde in the ragweed preparation. In the direct procedure, the formaldehyde sample was mixed and reacted with the 2,4-DNP solution. In the headspace procedure, formaldehyde in a ragweed sample placed in a sealed vial was allowed to diffuse in the gaseous form into a reaction chamber and derivatized with 2,4-DNP.

## EXPERIMENTAL

### Instrumentation

The liquid chromatograph consisted of an Altex Model 110A solvent pump and an Altex Model 153 UV detector (254 nm) (Altex Scientific Co., Berkeley, CA.). The chromatogram was recorded on a Linear recorder (Linear Instruments Corp., Irvine, CA). The sample was introduced by a Rheodyne 7125 injection valve onto a Supelcosil® column. The mobile phase consisted of 500 ml $H_2O$, 400 ml methanol and 0.2 ml $H_3PO_4$.

### Chemicals and Reagents

Methanol and 37% formalin solution were purchased from Polyscientific Corp., Bayshore, NY. 2,4-Dinitrophenylhydrazine was obtained from Eastman Kodak Co., Rochester, NY. Phosphoric acid was bought from J.T. Baker Chemical Co., Phillipsburg, N.J. Methylene Chloride was obtained from Fisher Scientific Co., Fairlawn, N.J.

### Procedure

A working derivatization reagent was prepared by dissolving 40 mg of 2,4-DNP in 25 ml of 2.5 N HCl. The direct determination of formaldehyde was accomplished by adding 25 ul of sample to 100 ul of 2,4-DNP working reagent in a 10 x 75 mm test tube. After

vortexing for several seconds, the reaction product was extracted into 200 ul of methylene chloride. A 25 ul aliquot of the extract was injected into the liquid chromatograph and the derivative was separated on a reversed phase column. The retention time of the formaldehyde 2,4-dinitrophenylhydrazone was 4.6 minutes. A calibration curve was constructed from a set of formaledhyde standards. The concentration of the sample corresponding to the peak height was obtained from the calibration curve.

The reaction set-up is shown for the headspace method (Figure 1). It consisted of a 4 ml screw cap vial with a 6x35 mm culture tube insert which served as the reaction chamber. One ml of the sample or standard to be analyzed was pipetted in the outer well and one hundred ul of 2,4-DNP solution in the inner. The vial was capped tightly and shaken gently to speed up the gaseous diffusion and the reaction of formaldehyde in the 2,4-DNP solution. A set of standards was run under the same experimental conditions. After 14 hours, 3.5% of the total formaldehyde in the sample reacted with the 2,4-DNP reagent in the inner well. At the end of the experiment, the reaction chamber was removed from the screw cap vial and 100 ul of methylene chloride was added to extract the hydrazone. A 25 ul aliquot of the extract was injected into the liquid chromatograph and analyzed as in the direct procedure.

## RESULTS AND DISCUSSION

The determination of formaldehye by gas chromatography is difficult because it is polar and reactive, and could be irreversibly adsorbed onto the solid supports. Although the unmodified formaldehyde can be monitored by the thermoconductivity detector, the sensitivity is inadequate for trace analysis

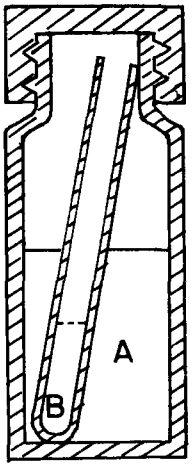



Figure 1. Set up of the headspace reaction vial. (A). Sample well - 4 ml screw cap vial. (B). Reagent well - 6x35 mm culture tube insert.

studies. With hydrogen flame ionization detectors, the signal responses to formaldehyde is insignificant because the absence of methyl carbon on the molecule. To enhance the detectability of formaldehyde by the flame detectors, derivatization, for example with 2,4-DNP, is necessary. However, among other problems associated with the derivatization technique (6), the major difficulty is thermal decomposition at the high temperature required to vaporize the modified formaldehyde.

Because its 2,4-DNP derivative absorbs strongly in UV, determination of formaldehye by HPLC is a simple procedure. Formaldehyde reacts rapidly with 2,4-DNP in acidic solution, and forms a precipitate. The precipitate is extracted into methylene chloride with an efficiency of 96%. Although an equally good extraction solvent, the use of ethyl acetate, which may contain traces of aldehyde or ketone which interferes with the assay, is discouraged. A 25 ul aliquot of the organic phase can be

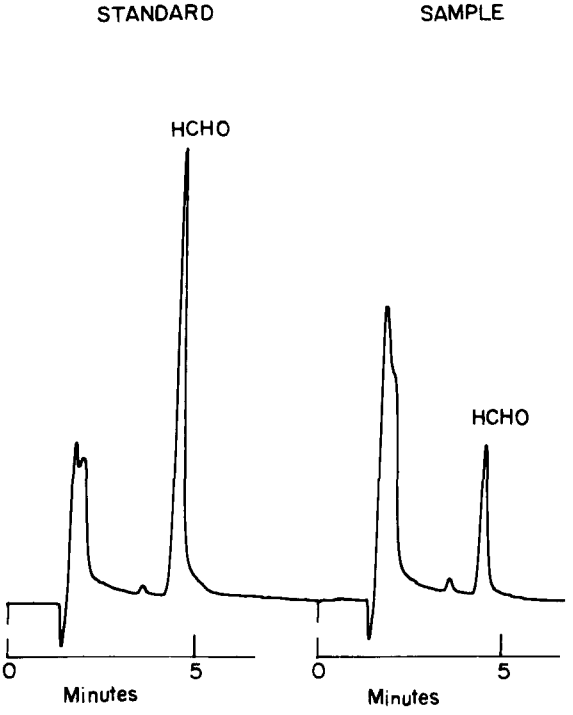



Figure 2. Chromatogram of formaledehyde, (A) in standard; and (B) in ragweed antigen sample.

injected into the liquid chromatograph without pre-concentration. The chromatograms of a formaldehyde standard and a ragweed antigen sample are shown (Figure 2). Low molecular weight carbonyls, acetaldehyde and acetone, evidently not interfere in this procedure are separated from formaldehyde (Figure 3). A calibration curve of formaldehyde at a ten inch full scale absorbance of 0.8 A.U. is shown (Figure 4). The limit of detection of 1 nanogram with a signal to noise ratio of 5 was achieved. By concentrating the organic extract, the detection limit can be improved at least by an order of magnitude. In the repeated analysis of 8 samples, a coefficient of variation of 2.5% was obtained.

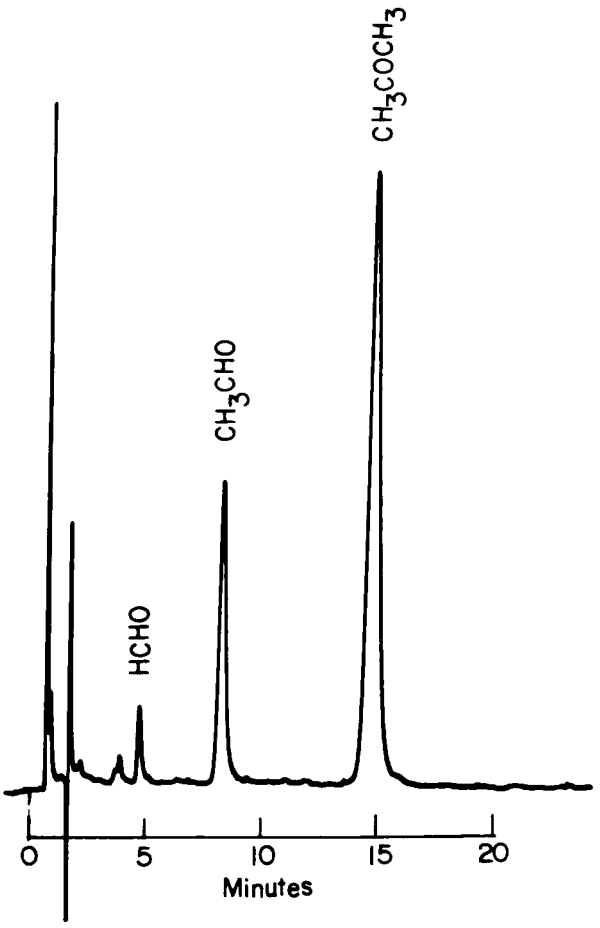



Figure 3. Chromatogram of low molecular weight carbonyls.

Since the ragweed sample may be sensitive to acid hydrolysis in the derivatization solution, formaldehyde which is used for the polymerization of the ragweed antigen could be released to give a falsely high result. A headspace procedure for the determination of formaldehyde was devised to investigate the possibility. The gas phase reaction set-up is shown (Figure 1).

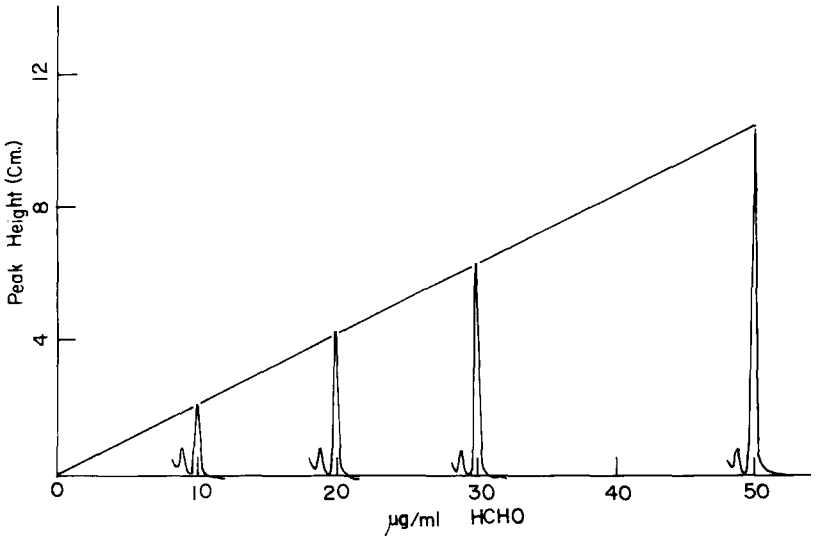



Figure 4. Standard curve by the direct procedure.

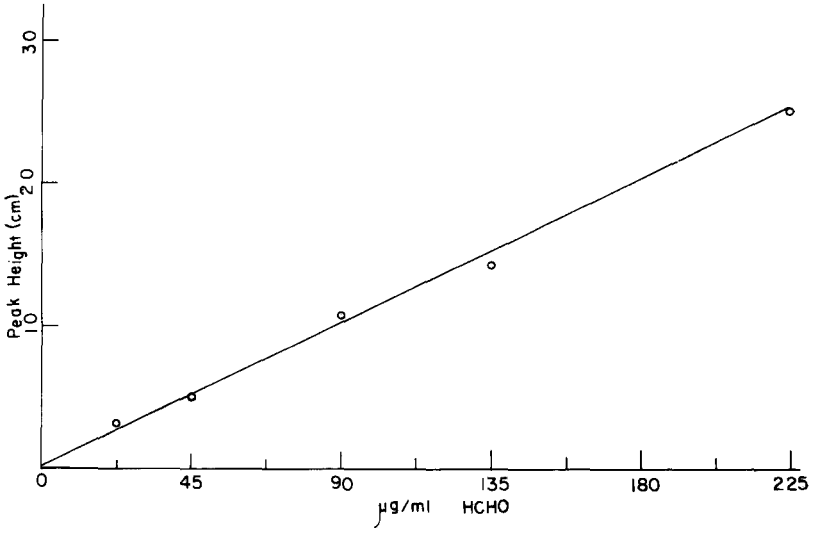



Figure 5. Standard curve by the indirect headspace procedure.

Because less sensitive in the headspace procedure due to incomplete reaction, the detector attenuation was set at 0.01 A.U.F., whereby a concentration of 1 ug/ml could be easily detected. A linear calibration curve that relates the concentration of formaldehyde to the peak height is shown (Figure 5). The headspace procedure gave results that agreed with the direct procedure and assured the integrity of the polymerized ragween antigen that the bonding was not disrupted under the 2.5N HCl derivatization condition.

In conclusion, two procedures for the determination of formaldehyde are described. The direct procedure is fast and sensitive while the diffusion procedure is more specific. The headspace procedure is an excellent alternative when the sample is sensitive to the derivatization reagent, or contains potential non-volatile interferences as in the determination of volatile aldehydes and ketones in serum which is abundant in non-volatile carbonyls such as sugars and ketoacids (7).

## REFERENCES

1. D.K. Harris, British J. Medicine, 10, 255 (1953).

2. Y.U. Yokouchi, T. Fujh, Y. Ambe and K. Fuwa, J. of Chromatogr., 180, 133 (1979).

3. K. Fung and D. Grosjean, Anal. Chem. 53, 168 (1981).

4. W.A. Check, JAMA 243, 1703 (1980).

5. M.B. Jacods, E.L. Eastman and D.L. Shepard, J. Am. Pharm. Assoc., XL, 365 (1951).

6. L.J. papa and L.P. Turner, J. Chromatogr. Science, 10, 744 (1972).

7. A.R. Stowell, K.E. Crow, R.M. Greenway and R.D. Batt, Anal. Biochem., 84, 384 (1978).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2653-2658 (1984)

# THE DETERMINATION OF METHYLENE BIS-THIOCYANATE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

R. Shustina and J. H. Lesser
Department of Analytical Research
Research and Development Division,
Makhteshim Chemical Works, Ltd.
Beer Sheva, 84100, Israel

## ABSTRACT

A fast, straightforward and specific method for the determination of methylene bis-thiocyanate (MBT) in technical materials and aqueous samples is described. Reversed phase high precision liquid chromatography using acetonitrile-water as eluent at a flow rate of 2 ml/min. gave a capacity factor of 2.73 for MBT. Using the method for aqueous samples allowed the determination of 2 ppm MBT by direct injection.

## INTRODUCTION

Methylene bis-thiocyanate (MBT) is a fungicide and slimicide used extensively in the wood-pulp industry. It can be synthesized by mixing dibromomethane with sodium thiocyanate in an appropriate solvent (1). Methods of analysis for thiocyanates in general include those involving conversion to heavy metal derivatives (2,3), or to cyanide ion (4). Additional methods include reaction of alkyl and aryl thiocyanates with sulfide (5) and a micromethod using polysulfide (6). These methods are general, work intensive and require several hours for completion. An infrared (7,8) as well as an NMR (9) method represent instrumental analysis reported, however neither is applicable to low concentrations or to aqueous thiocyanate samples.



 0148-3919/84/0713-2653$3.50/0

A rapid and specific analytical method for the determination of MBT applicable to technical, aqueous, and low concentration samples was required. This paper presents an HPLC method for the determination of MBT which meet the above requirements.

## EXPERIMENTAL

### Materials and Reagents

Acetonitrile Spectroscopic grade (Aldrich Chemical Co., U.S.A.), hexane, Analytical grade (Merck, Dramstadt, FGR) and dichloromethane (Analytical grade, Frutarom, Haifa, Israel) were used. Water was distilled and passed through a Norganic cartridge (Millipore Corporation, Massachussets, U.S.A.). Technical MBT samples were supplied by Makhteshim Chemical Works, Ltd. Aqueous solutions of MBT were prepared by dissolving a quantity of technical material in water and diluting to the desired concentration. Non-aqueous technical samples were prepared by dissolving MBT in acetonitrile prior to analysis.

### High Performance Liquid Chromatography

A Lichrosorb RP-18 octadecylsilane reverse phase column (Merck, Darmstadt, FGR), 12.5 cm 4.6 mm i.d. was used in a Varian model 5020 HPLC equipped with a Rheodyne model 7125 injector and UV-1 ultraviolet detector (254nm). MBT was eluted using acetonitrile: water (12:88) and a flow rate of 2 ml/min. Peak areas were integrated electronically using a Varian model CDS-111L electronic integrator and recorded using a Pantos Unicorder model U-228 (Pantos, Japan).

### Linearity Study

Into a 100 ml volumetric flask was weighed 0.222 g. of MBT and acetonitrile was added to the mark. From this stock solution a series of dilutions were made ranging from 0.148 mg/ml to 2.2 mg/ml. 10 µl of these solutions were injected onto the column in duplicate. The resultant peak areas were plotted against the concentration and are represented in Figure 1.

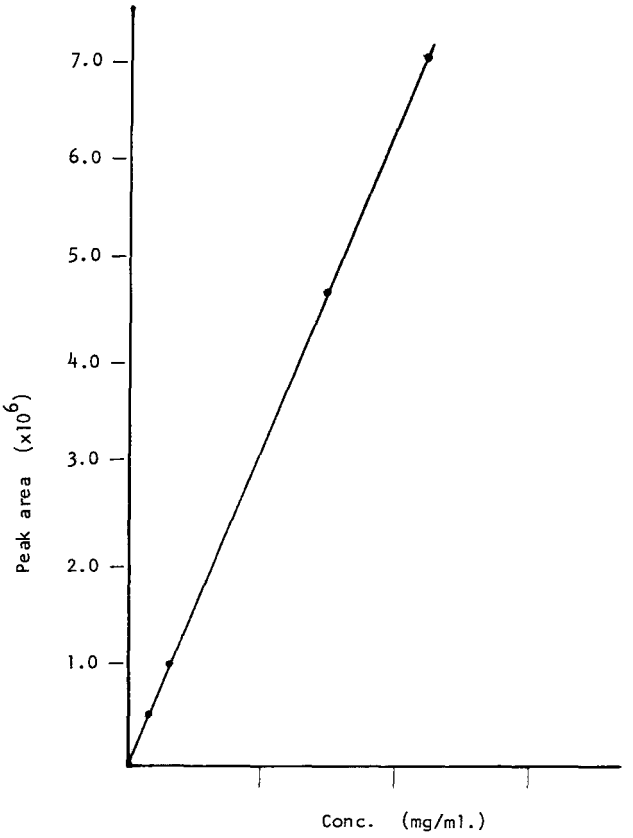



Figure 1. Curve obtained for MBT when peak area versus concentration injected was plotted.

Relative Standard Deviation

A solution of 0.139 g of MBT in 100 ml of acetonitrile was prepared and 13 successive 10 µl injections made. From the resultant peak areas the relative standard deviation was calculated.

## RESULTS AND DISCUSSION

A rapid specific method for the analysis of MBT in aqueous samples and technical materials has been developed using HPLC and

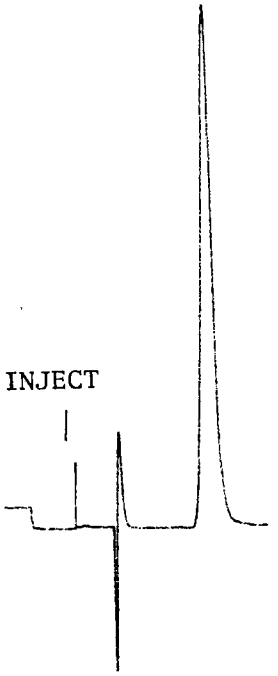



Figure 2. Typical Chromatogram of MBT

ultraviolet detection. An octadecylsilane reversed phase column was used with an acetonitrile; water mixture as eluant. A typical chromatogram obtained using this method is shown in Figure 2. Both thiocyanate and bromide ion elute rapidly from the system and their elution time was used as the $t_o$ value in the capacity factor (k') calculation. The k' was calculated and found to be 2.73. Unreacted dibromomethane did not interfere with the analysis. For technical samples the total analysis time was about 10 min. When peak areas were plotted against concentration the graph shown in Figure 1 was obtained showing a linear response curve from 0.148 mg/ml to 2.2 mg/ml sample concentration. Calcul-

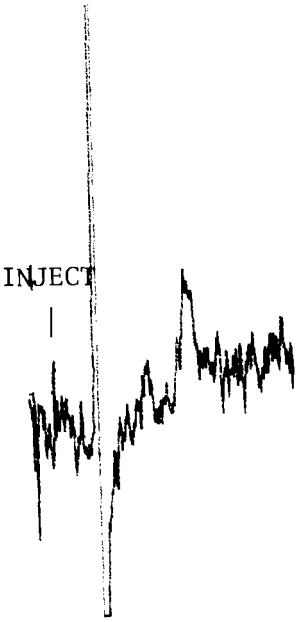



Figure 3. Chromatogram of 2 ppm MBT Aqueous Sample

ation of the relative standard deviation yielded a value of ± 0.6% based on peak areas.

In order to confirm that the chromatographic peak represented MBT alone several samples were chromatographed on a silica column using hexane; dichloromethane (10:90) as eluent. Only a single sharp peak was observed in these instances.

Aqueous samples were filtered and injected without further workup. As little as 2 ppm (S/N>2) could be detected in this manner as shown in Figure 3. The fungicides captan, foltan and captafol did not elute under the above conditions and therefore are not potential interferents.

In conclusion a method for the analysis of MBT, applicable to aqueous and non-aqueous samples, has been presented. Method

reproducibility, linear range, and limit of detection have been established under given experimental conditions.

## REFERENCES

1. Matt, J., "Sequesting solvents in ethylene bis (Thiocyanate) production", U. S. Pat. 3,524,871 (Nalco Chemical Co.) 18 Aug. 1970.

2. M. Schuessler, and R. Pohloudek Fabini, "Identification of organically bound thiocyanate III. Organic thiocyanates", Pharm. Zentralhalle 100, 452-8, 1961.

3. Mieczyslaw Wronski, "Mercurimetric determination of thiocyanates and isothiocyanates", Talanta 13 (8), 1145-9, 1966.

4. Kemp W. E. "The Analysis of Aliphatic and Aromatic Thiocyanates", Analyst (London), 64, 648-53, 1939.

5. Maija Saarivirta and Artturi T. Virtanen, "A method for estimating benzyl isothiocyanate, benzyl thiocyanate, and benzyl nitrile in the crushed, moistened seeds of Lepidium sativum", Acta Chem. Scand. 17, Suppl. 1, S74-S78, 1963.

6. R. Pohloudeic Fabini, D. Goeckeritz, and H. Brueckner, "Organic thiocyanate compounds XXXIII. Quantitative determination of organic thiocyanates", Mikrochim. Acta 3, 588-98, 1967.

7. R. B. Hannan, "Analysis of insecticides for thiocyanates by infrared spectroscopy", Chem. Specialties Mfrs. Assoc. Proc. Dec., 151-3, 1955.

8. N. G. McTaggart, E. Thornton, and A. D. Harford, "The determination of pyrethrins and other insecticidal compounds by infrared spectrometry", Pyrethrum Post 4, No. 4, 12-15, 1958.

9. A. Mathias, "Analysis of alkyl thiocyanates and isothiocyanates by N. M. R.", Tetrahedron 21 (5), 1073-5, 1965.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2659-2669 (1984)

# DETERMINATION OF FOLIC ACID IN COMMERCIAL DIETS BY ANION-EXCHANGE SOLID-PHASE EXTRACTION AND SUBSEQUENT REVERSED-PHASE HPLC

Gary W. Schieffer*, Glenn P. Wheeler, and Carolyn O. Cimino

Analytical Chemistry Department
Norwich Eaton Pharmaceuticals, Inc.
A Procter and Gamble Company
Norwich, New York 13815

## ABSTRACT

Folic acid at the μg/g level is determined in total nutritional diets by concentration on disposable commercial anion exchange columns followed by elution with a concentrated salt solution and subsequent reversed-phase HPLC with absorbance detection at 365 nm. The method is specific for folic acid with respect to some known degradants and electrochemically generated oxidation products.

## INTRODUCTION

Although folic acid (pteroylmonoglutamic acid) represents only a small percentage of the naturally occuring folates, its stability and availability account for its widespread use in the fortification of foods and the preparation of vitamin supplements and total nutritional diets.

Reversed-phase HPLC is generally the method of choice for folic acid determinations replacing microbiological, photometric,



 0148-3919/84/0713-2659$3.50/0

and ion-exchange HPLC techniques whenever possible (1,2). The reversed-phase HPLC methods are sensitive and, for stability-indicating purposes, selective with respect to known folic acid degradants such as the oxidation products pterine (I) and pterin-6-carboxylic acid (II)(3) and the cleavage product N-(p-aminobenzoyl)-L-glutamic acid (III)(4).

However, when an assay is sought for low levels of folic acid in complex preparations such as total nutritional diets, which characteristically contain proteins, amino acids, saccharides, lipids, and minerals, the sample must be cleaned up prior to HPLC.

Folic acid and other folates in food and human milk were solute-focused at the head of a microparticulate phenyl column equilibrated with eluent containing tetrabutylammonium ion-pairing agent (2). After elution of interfering substances, the column was reequilibrated with eluent free of ion-pairing agent, eluting the folates. Folic acid in infant formula was extracted by enzymatic digestion followed by concentration on DEAE cellulose anion-exchange columns packed in the laboratory (5). After elution from the column, the extract containing folic acid was injected on a microparticulate octyldecylsilane column with an acetonitrile-acetate gradient.

This report extends the anion-exchange clean-up technique to the selective determination of folic acid at the μg/g level in total nutritional diets. Sample handling is greatly facilitated by use of the newly introduced solid-phase extraction

system comprising small, disposable, prepacked columns of 40-60 μm bonded phase packing that are eluted under vacuum with a special manifold system.

## MATERIALS

### Reagents and Materials

Pterine (I), pterin-6-carboxylic acid (II), N-(p-aminobenzoyl)-L-glutamic acid (III), folic acid (FA), dihydrofolic acid (IV), dl-L-tetrahydrofolic acid (V), dl-N-5-methyltetrahydrofolic acid (VI), and p-aminobenzoic acid (VII) were purchased from Sigma Chemical Co., St. Louis, MO.

In addition to folic acid at 1.6 and 2.2 μg/g, respectively, powdered diets A and B contained maltodextrin, modified starch, L-leucine, L-glutamine, L-arginine acetate, magnesium gluconate, L-valine, L-isoleucine, calcium glycerophosphate, safflower oil, L-lysine acetate, L-aspartic acid, L-alanine, L-phenylalanine, L-proline, glycine, L-threonine, L-methionine, sodium glycerophosphate, L-histidine monohydrochloride monohydrate, L-serine, potassium chloride, potassium citrate monohydrate, L-tryptophan, sodium citrate dihydrate, potassium sorbate, L-tyrosine, choline bitartrate, ascorbic acid, ferrous gluconate, polyoxyethylene sorbitan monooleate, zinc acetate dihydrate, niacinamide, α-tocopherol acetate, calcium pantothenate, manganese glycerophosphate, riboflavin phosphate sodium salt, cupric citrate, pyridoxine hydrochloride, thiamine hydrochloride, ascorbyl palmitate, Vitamin A palmitate, biotin, sodium selenite, sodium molybdate anhydrous, potassium iodide,

chromic acetate monohydrate, Vitamin $K_1$, Vitamin $B_{12}$, and Vitamin $D_3$. Diet C contained glucose oligosaccharides in place of maltodextrin and modified starch, a higher proportion of amino acids, and folic acid at 0.55 μg/g.

All other reagents were reagent grade.

Apparatus

The extraction clean-up step was accomplished with disposable, bonded-phase, quaternary amine anion-exchange solid phase extraction columns having a 500 mg sorbent mass and a 2.8 ml column volume (Bond Elut®, Analytichem International, Harbor City, CA). A vacuum manifold (Vac Elut®, Analytichem International) capable of holding up to 10 disposable columns was used to greatly facilitate elution. The loss of collected eluent due to splattering was prevented by use of a vacuum controller/ release and an additional needle valve. Detachable 75-ml plastic solvent reservoirs were used to increase the solvent capacity of the columns.

A high-performance liquid chromatograph (ALC/GPC 204, Waters Associates, Milford, MA) equipped with a 250 μl (diets A and B) or 500 μl (diet C) fixed-loop injector (Model 7125, Rheodyne, Berkeley, CA), a variable wavelength detector set at 365 nm (Model III, Laboratory Data Control, Riviera Beach, FL), a reciprocating pump (Model 6000A, Waters Associates), and a prepacked microparticulate reversed-phase column (μ-Bondapak® $C_{18}$, Waters Associates) was used. The column was pumped isocratically at 2.0 ml/min with a 6% acetonitrile 94% 0.1 *M*

sodium acetate buffer adjusted to pH 5.7. A diode array detector (HP 1040A, Hewlett Packard, Palo Alto, CA) and a precolumn coulometric cell (6) were used for the specificity study. Peak heights were measured manually.

## METHODS

### Procedure

Ten g of diet was mechanically shaken with 100.0 ml of a 0.01 *M* pH 7.4 phosphate buffer until a homogeneous dispersion resulted (about 20 min). The dispersion was filtered through Whatman GF/A glass fiber paper and 10.0 ml of the filtrate was loaded onto a disposable anion-exchange column that had been pretreated with one column-volume each of hexane, methanol, and water, respectively. The solution was pulled through the column under vacuum and the eluate was discarded. After washing with two column volumes of water, the folic acid was eluted from the disposable column with approximately 4.5 ml of a 10% sodium chloride, 0.1 *M* sodium acetate solution. The eluate was collected in a 5 ml volumetric flask, which was then diluted to volume with the sodium chloride-acetate solution. The sample was injected directly into the LC.

A reference standard stock solution was prepared by dissolving 15 mg of folic acid in a small volume of phosphate buffer and then diluting to 100 ml. This stock solution was further diluted with the phosphate buffer to yield a concentration of 1-5 µg/ml. Finally, 1.0-5.0 ml aliquots of the stock solution were diluted to 100.0 ml with sodium chloride-

acetate solution to prepare a standard response curve. Since folic acid standards were completely recovered from the anion-exchange column, a "method standard" was not needed.

The diet was also assayed using a standard addition technique. In this case, 20.0 g of diet was leached with 200.0 ml of phosphate buffer. Zero to 10.0 ml of a 1.5 μg/ml folic acid standard solution was added to 50.0 ml aliquots of the filtered leachate. The solutions were diluted to 100.0 ml with phosphate buffer, and extracted with the disposable columns as described above.

Specificity Studies

Solutions containing known degradation products and folate forms other than folic acid were prepared by dissolving 0.5 to 1 mg of each compound in 50 ml 0.01 *M* pH 7.4 phosphate buffer containing 0.3% mercaptoethanol. The solutions were injected directly and capacity factors were examined. In addition, specificity with respect to electrochemically generated oxidation products was examined with a precolumn coulometric cell (6) maintained at 2.0 V with respect to a silver/silver chloride (0.1 *M* in KCl) reference electrode.

## RESULTS AND DISCUSSION

Initially, a detector wavelength of 280 nm, near the absorption maximum for folic acid, was chosen. However, a strongly absorbing excipient eluted just following the folic acid peak. To avoid potential interference that might be observed with some columns, a wavelength of 365 nm, at which the excipient

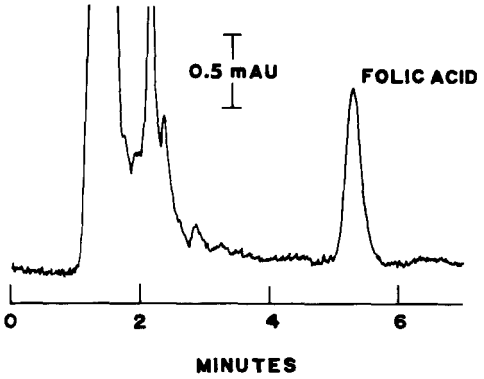



Figure 1. HPLC of a commercial diet extract. 2.0 ml/min flow rate. 250 µl injection volume. 365 nm detector wavelength.

was completely transparent, was chosen, yielding the chromatogram shown in Figure 1.

In addition, a sample volume of 20.0 ml was initially eluted through the ion-exchange columns. However, a plot of peak height versus sample weight yielded a negative deviation from linearity at weights greater than about 6 g, presumably due to elution of some of the folic acid from the column by the relatively high ionic strength sample matrix. When the sample aliquot was reduced to 10.0 ml, a plot of peak height versus sample weight yielded linearity (correlation coefficient 0.998 for n=5) to at least 14 g of sample.

An eight-point plot of peak height versus nanogram of standard folic acid injected yielded a straight line from about 40 to 200 ng injected (100 ng nominal) with a correlation coefficient of 0.9995, a % intercept [(y-intercept/$\bar{y}$)x100, where $\bar{y}$ is the average y] of 0.9%, and a % variation [($S_{y/x}/\bar{y}$)x100,

TABLE 1

Results for the Assay of Folic Acid in Three Commercial Diets.

| g sample | µg/g | % theory | g sample | µg/g | % theory |
|---|---|---|---|---|---|
| | Diet A | | | Diet B | |
| 5.0 | 2.03 | 90.6 | 7.5 | 1.68 | 102.4 |
| 7.0 | 2.02 | 90.2 | 10.0 | 1.58 | 96.3 |
| 7.0 | 1.98 | 88.4 | 10.0 | 1.71 | 104.3 |
| 9.0 | 2.10 | 93.8 | 10.0 | 1.69 | 103.0 |
| 10.0 | 1.99 | 88.8 | Av. | 1.66 | 101.5 |
| 10.0 | 2.01 | 89.7 | RSD | 3.5% | |
| 10.0 | 2.12 | 94.6 | | | |
| 10.0 | 1.99 | 88.8 | | Diet C | |
| 10.0 | 2.07 | 92.4 | | | |
| 10.0 | 2.10 | 93.8 | 5.0 | 0.55 | 100.5 |
| 11.0 | 2.05 | 91.5 | 8.0 | 0.54 | 97.7 |
| Av. | 2.04 | 91.1 | 11.0 | 0.51 | 93.3 |
| RSD | 2.4% | | 14.0 | 0.52 | 95.6 |
| | | | Av. | 0.53 | 96.8 |
| | | | RSD | 3.2% | |

where $S_{y/x}$ is the standard error of estimate] of 1.9%. Assays of three commercial diets yielded the data listed in Table 1.

Four-point standard addition yielded 2.03 µg/g for diet A, 1.64 µg/g for diet B, and 0.57 µg/g for diet C, in good agreement with the standard curve values listed in Table 1. Because of the large number of components in each diet, recoveries determined by using a diet specially prepared without folic acid would be difficult. Therefore, recoveries for each diet were estimated by calculating the ratio of the standard additions plot slope to the slope of the standard response curve (8). Recovery values of 101.4%, 102.6%, and 100.2% were obtained for diets A, B, and C respectively.

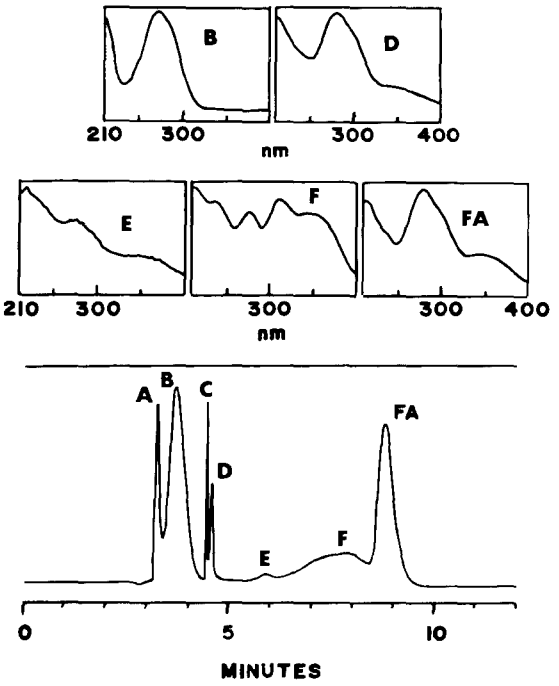



Figure 2. HPLC and UV spectra of electrochemically generated folic acid (FA) oxidation products. 1.0 ml/min flow rate. 250 µl injected. 280 nm detector wavelength. 80% coulometric yield. A yielded a spectrum nearly identical to that for B; C yielded a featureless spectrum.

## Specificity

If an assay is to be stability-indicating, known or postulated degradation products must not interfere with the determination of the analyte. Oxidation products I and II were found to elute just after the solvent front and do not interfere. The cleavage product III eluted just after the folic acid peak but is transparent at 365 nm. VII was not observed at all, and

VI eluted after folic acid and did not interfere. IV and V, which represent metabolites of folic acid and other folate forms present in foods but not in the total nutritional diet, did interfere; however, separation of these folates from folic acid was not deemed necessary for the present method. In any case, there are other reversed-phase systems capable of separating these compounds from folic acid (2,7).

Forced degradation was accomplished by *in situ* electrochemical oxidation (6) of 80% of the folic acid to yield the peaks shown in Figure 2. UV spectra of some of the peaks are also shown. No further attempt was made to identify the oxidation products although product F was observed at about 0.5 area % in the folic acid standard. Decreasing the flow rate to 0.4 ml/min resulted in an observable 99% decrease in the folic acid peak, indicating no interference from coeluting oxidation products.

## REFERENCES

1. Holcomb, I.J. and Fusari, S.A., Liquid Chromatographic Determination of Folic Acid in Multivitamin-Mineral Preparations, Anal. Chem., 53, 607, 1981.

2. Reingold, R.N., Picciano, M.F., and Perkins, E.G., Separation of Folate Derivatives by *In Situ* Paired-Ion High-Pressure Liquid Chromatography, J. Chromatogr., 190, 237, 1980.

3. Stokstad, E.L.R, The Vitamins, Vol. III, Sebrell, Jr., W.H. and Harris, R.S., eds., Academic Press, Inc., New York, 1954, pp. 96-97.

4. Strohecker, R. and Heinz, H.M., Vitamin Assay--Tested Methods, CRC Press, Cleveland, 1965, p. 183.

5. Hoppner, K. and Lampi, B., The Determination of Folic Acid (Pteromonoglutamic Acid) in Fortified Products by Reversed

Phase High Pressure Liquid Chromatography, J. Liq. Chromatogr., 5, 953, 1982.

6. Schieffer, G.W., Precolumn Coulometric Cell for High-Performance Liquid Chromatography, Anal. Chem., 53, 126, 1981.

7. Tafolla, W.H., Sarapu, A.C., and Dukes, G.K., Rapid and Specific High-Pressure Liquid Chromatographic Assay for Folic Acid in Multivitamin-Mineral Pharmaceutical Preparations, J. Pharm. Sci., 70, 1273, 1981.

8. Cardone, M.J., Detection and Determination of Error in Analytical Methodology. Part II. Correction for Corrigible Systematic Error in the Course of Real Sample Analysis, J. Assoc. Off. Anal. Chem., 66, 1283, 1983.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2671-2677 (1984)

# A RAPID METHOD FOR THE PREFRACTIONATION OF ESSENTIAL OILS. APPLICATION TO THE ESSENTIAL OIL OF BLACK SPRUCE [PICEA MARIANA (MILL.)BSP.]

SELMA HAJJI,JACQUES BELIVEAU,DAVID Z.SIMON,ROMANO SALVADOR
Faculty of Pharmacy,University of Montreal,Montreal.

CLAUDE AUBE,ALBERTO CONTI
Station de Recherches,Agriculture Canada,St.Jean,Que.

## ABSTRACT

A rapid method for the prefractionation of complex essential oils, prior to the identification of their components,is described.The use of "hybrid" flash chromatography,developed in our laboratories,in a specially constructed,jacketed column,which permits the use of low boiling solvents,makes it possible to effect satisfactory separations through a single column rather than through multiple columns as in classical methods,and in a fraction of the time required.The procedure enables a facile separation of hydrocarbons from oxygenated compounds and yields,in some instances,fractions of pure compounds.Enriched fractions of trace constituents are also obtainable for identification by gas chromatography.The method is rapid and inexpensive.

## INTRODUCTION

Essential oils are complex mixtures of terpenoids and phenylpropane derivatives.The number of components in any single oil may vary from few to over one hundred.The presence of that many individual components makes the analysis of the oil,by gas chromatography,very difficult because of overlap of peaks and similarity of retention times.Thus,prefractionation of the oil,prior to analysis is of utmost importance.

Various methods of prefractionation have been described, most using multiple columns.To our knowledge,Scheffer et al.



 0148-3919/84/0713-2671$3.50/0

(1,2)have developed one of the best fractionation techniques, involving liquid-solid chromatography,but their method requires two columns for the separation of hydrocarbons from oxygenated components,the gel must be specially treated and a mixture of gels of various sizes is required for the separation of each fraction.The procedure is time consuming varying from days to weeks in certain cases.The procedure we will describe permits a separation similar to that of Scheffer in only 45 to 60 minutes.

The basis of the process was described by Still et al.(3). In this method,called "flash chromatography" a column,the diameter of which varies with the quantity to be separated,is filled to 15 cm with silica gel(40 - 63 µm).The column is fitted, at its top,with a relief valve through which a flow of nitrogen is introduced.The pressure is regulated by means of the relief valve so that the elutant is forced through the column at the rate of 2.5 cm per minute.The composition of the elutant is that which gives a separation on a silica plate in TLC.This type of "three dimensional" TLC separates the mixture in 45 to 60 minutes,the separation following closely the Rf values on TLC.

We have combined the essentials of "flash chromatography" with the procedure of Scheffer to make possible the superior separation obtained by Scheffer in only 45 to 60 minutes on a single column.This technique,which we term "hybrid" flash chromatography,has made it possible to separate complex mixtures, to isolate some of the constituents in the pure state and to obtain enriched fractions of trace constituents in a very short time.In essence we have combined a gradient technique,similar to that of Scheffer,with the "flash chromatography"of Still.The order of elution,for the essential oils,is identical to that described by Scheffer.The hydrocarbons are first eluted with hexane and the remaining components are eluted with an increasing gradient of ether in hexane.The oxygenated compounds are eluted in the following order according to the functional group:oxides,

esters,ketones and alcohols.Using different solvent mixtures we have also applied this technique to the separation of alkaloids (4) and saponins(5).

## EXPERIMENTAL

### Instruments and Operating Conditions

The column for "hybrid" flash chromatography is a standard glass column,equipped with a water-cooled jacket,for its entire length,to allow the use of low boiling solvents.The nitrogen flow was regulated by the relief valve to force a drop of 2.5 cm per minute of the surface of the elutant.The construction of the relief valve was as described by Still.

Gas chromatography was done on a Perkin-Elmer Model 990, modified to accept a 60 m capillary column(Durawax fused silica; J&W DX4-60N) and FID.Nitrogen gas flow was 16.6 cm/sec.,with a 50:1 split ratio.The program was $90^{o}$ to final temperature $180^{o}$ at $3^{o}$/min. for the whole oil and for the hydrocarbons.For oxygenated compounds the program was $130^{o}$ to final temperature $180^{o}$ at $4^{o}$/min.Injector and manifold temperature as well as the FID was held at $250^{o}$.

The spruce oil was extracted by "hydrodiffusion" using a Shmid Hydrodiffuser(Switzerland) Model LS-500,kindly provided by PAL Hydrodiffusion(Canada) Inc., Ste.Adele, Quebec,Canada.

### Materials and Standards

Hexane(Fisher HPLC grade) and ether for anesthesia(Biopharm) were used as eluting solvents.Silica Gel 60(E.Merck No.9385 200-400 mesh) was heated at $105^{o}$ for 60 minutes and deactivated by addition of 5% water.

Ten standards were used:-bornyl acetate,limonene,myrcene, p-cymene,cineole, $\alpha$ terpineole,carvone,linalool,menthol(Aldrich Chemical) and fenchone(Fluka).

The black spruce oil was extracted in our laboratories,by hydrodiffusion,from identified species collected in March 1983 in the region north of Montreal(the Laurentides).

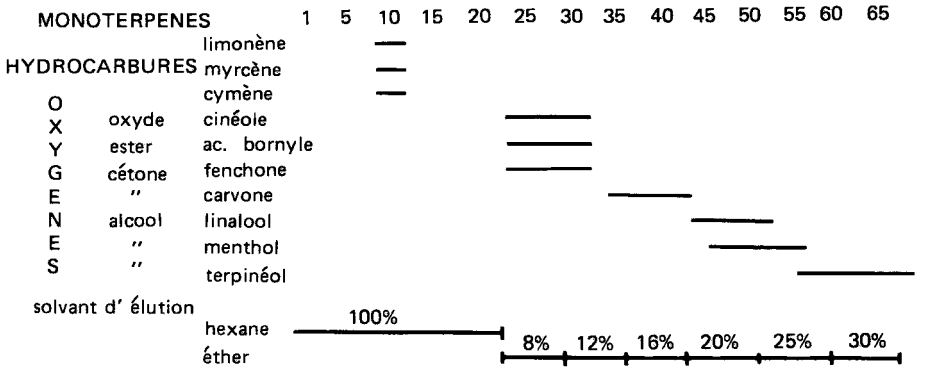



FIGURE 1 : Elution of test mixture showing separation of hydrocarbons from oxygenated compounds.Total time:- 55 minutes.

TABLE 1

Main Constituents of Black Spruce Oil Separated by "Hybrid" Flash Chromatography

| Constituent | RT Capillary Column | Peak No. | Percentage % |
|---|---|---|---|
| α-pinene | 9' 40 | 3 | 13.8 |
| Camphene | 11' 20 | 4 | 24.9 |
| β-pinene | 12' | 5 | 4.1 |
| myrcene | 13' 40 | 6 | 0.1 |
| limonene | 15' 40 | 8 | 0.5 |
| 1,8-cineole | 16' 10 | 9 | 4.9 |
| bornyl acetate | 36' 40 | 19 | 41.2 |
| borneol | 43' | 29 | trace |
| piperitone | 44' 40 | 32 | trace |

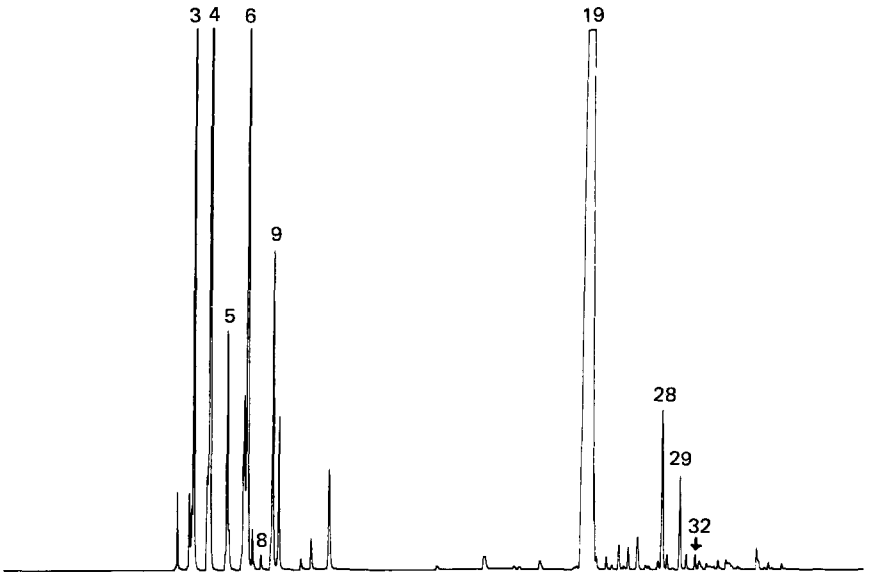



FIGURE 2 : GLC spectrum of Oil of Black Spruce.Numbered peaks are identified in Table 1.

## PROCEDURE

To verify the efficacy of the "hybrid" flash chromatography procedure,a test mixture was separated prior to its application to spruce oil.

### Preparation of Test Mixture

Each of the standards was dissolved in hexane as follows: carvone 7%;limonene,myrcene,cymene,cineole and terpineol 9%; bornyl acetate and fenchone 10%;linalool and menthol 12%.

A mixture of 0.2 ml of each(total 2 ml) was applied to the column containing 50 g(15 cm)of gel.

### Separation of Test Mixture

The column,containing 2 ml of the test mixture was eluted first with hexane under nitrogen pressure sufficient to force a drop in solvent level of 2.5 cm/min. Twenty-two fractions of 10 ml each were taken.Nitrogen pressure was removed and the hexane was replaced with a mixture of 8% ether in hexane.The pressure was restored and seven fractions of 15 ml were taken.

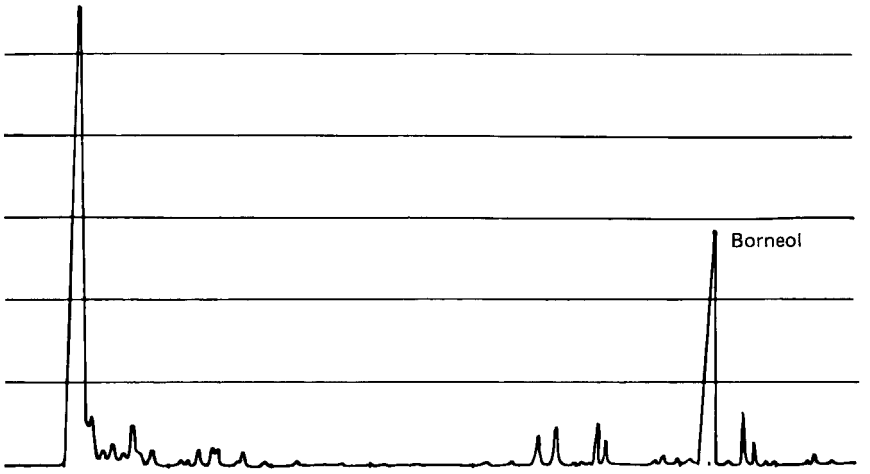



FIGURE 3 : Peak 29--Borneol.An enriched fraction.Compare with this same peak in Figure 2. (GLC)

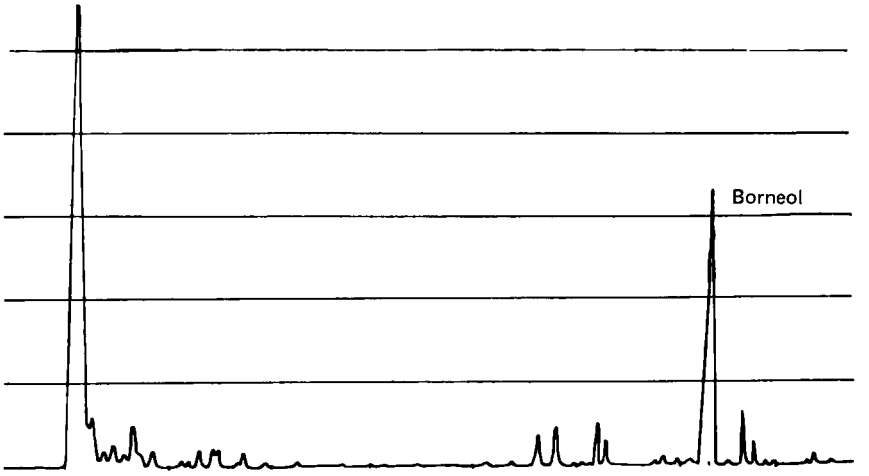



FIGURE 4 : Peak 29--Borneol.Identification by peak enrichment in GLC.

Similarly seven fractions of 15 ml were taken at each increased concentration of ether in hexane i.e. 12%,16%,20%,25%,30% and 50%.The fractions were evaporated to 1 ml for analysis by gas chromatography.The order of elution is the same as that reported by Scheffer(2).(Figure 1)

Chromatography of Black Spruce Oil

The separation of 2 ml of black spruce oil was done on the same column under identical nitrogen pressure and identical gradient,the same number of fractions of the same volume being taken. The entire separation was completed in 55 minutes.The fractions isolated were identified by gas chromatography by means of retention times and peak enrichment.The main constituents are reported in table 1 while the entire spectrum is shown in Figure 2. The identification of peaks 29 and 32 of Figure 2 borneol and piperitone respectively,both present in trace amounts,was made simple by combining fractions of several runs and thus obtaining enriched fractions which were used for the identification.The speed of the prefractionation makes multiple runs not at all tedious. (Figures 3 and 4)

## CONCLUSION

The application of the gradient technique to "flash chromatography provides a very rapid means for the separation of complex mixtures,with resolution as great or greater than the time consuming classical methods.Repeated runs for fraction enrichment are no longer to be dreaded.Equipment is not costly and unless low boiling solvents are to be used,an ordinary column fitted with a relief valve,as described by Still,and silica 200 - 400 mesh is all that is required.

## REFERENCES

1 Scheffer,J.J.C.,Koedam,A. and Svendsen,A.B.,Chromatographia, 9, 425 (1976)

2 Scheffer,J.J.C., Koedam,A.,Shusler,M.Th.I.W. and Svendsen, A.B., Chromatographia, 10, 669 (1977)

3 Still,W.Clark, Kahn,M. and Mitra,A., J.Org.Chem., 43, 2923 (1978)

4 Eloumi-Ropivia,J.,Beliveau,J. and Simon,D.Z., Master thesis of J.Eloumi-Ropivia.

5 Tedeneke,W.,Beliveau,J. and Simon,D.Z. Masters project of W.Tedeneke

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2679-2689 (1984)

# QUANTITATION OF SEROTONIN IN HUMAN PLASMA, SERUM AND CEREBROSPINAL FLUID SAMPLES BY HPLC-EC USING 6-HYDROXYTRYPTAMINE AS AN INTERNAL STANDARD

N. Narasimhachari
Departments of Psychiatry and Pharmacology
Medical College of Virginia/Virginia Commonwealth University
Richmond, Virginia 23298

## ABSTRACT

A simple method for sample clean up and concentration of serotonin (5-HT) in biological samples, such as human cerebrospinal fluid and serum, is described. To the sample 6-hydroxytryptamine (6-HT) is added as an internal standard and it is then absorbed either on $C_{18}$ SEP-PAK cartridge or Biorex-70 short column. 5-HT and 6-HT are then eluted from the column with methanolic formic acid. After evaporation, the residue is dissolved in the mobile phase and an aliquot is used for LC-EC quantitation.

## INTRODUCTION

In previous communication, the use of 5-hydroxyindole as an internal standard for the quantitation of 5-hydroxytryptophan, 5-hydroxytryptamine (serotonin, 5-HT), 5-hydroxyindoleacetic acid, homovanillic acid was described (1). In this instance, samples such as cerebrospinal fluid (CSF), blood platelets and tissue homogenates were directly injected into the LC-EC system with the internal standard (5-HI) added to the sample. However, the other biological samples samples such as serum or plasma, some tissue homogenates need extensive sample cleanup before quantitation by



 0148-3919/84/0713-2679$3.50/0

LC-EC. Extraction procedures or ion exchange columns where acidic and basic components are separated have been used for the clean up (2,3). Various types of compounds were used as internal standards by some investigators while others have used no internal standard at all (2,3,4). In our earlier work with a catechola- mine metabolite by GC and GC/MS successfully used isomeric compounds as internal standards (5). I now report the use of 7-hydroxytryptamine (6-HT) as an internal standard for the quantitation of 5-HT in biological samples.

## MATERIALS AND METHODS

Serotonin hydrochloride and 6-hydroxytryptamine creatinine sulfate were obtained from Sigma Chemical Company (St. Louis, MO). All solvents were HPLC grade from Burdick and Jackson or Baker Chemical Company. $C_{18}$ SEP-PAK cartridges were obtained from Waters Associates (Milford, MA). HPLC-EC system is from Bioanalytical Systems (BAS, West Lafayette, IN) with LC-4 detector. $C_{18}$ reverse phase columns, 5 um 15 x 0.46 cm, 3 um 10 x 0.46 cm from Rainin, 5 um 10 x 0.46 cm from Biorad, 5 and 3 um 15 x 0.46 cm from Supelco were used. Two mobile phases (1) 0.05 M Triethylamine phosphate (pH 3.0) with acetonitrile 3.5% and (2) 0.035 M ammonium acetate buffer (pH 4.6) containing 5% acetonitrile were used. All solvents were filtered through millipore filters and degassed before use. The ionization was set at 0.65 V.

### GC/MS Conditions:

A Finnigan 4000 GC/MS 6110 Data System was used for GC/MS analysis. A 3% OV-17 on Gas Chrom Q column (2m x 4mm i.d.) was used for GC at an oven temperature 180°C isothermal. All quantitative GC/MS analyses were carried out at ionization potential 70ev, separator temperature 250°C, ion source temperature 260°C

and ionization current 0.45 A. The standard method (6) using $d_4$-5-HT as internal standard and tri-trifluoracetyl (TFA) derivative was used for validating LC-EC results. Ions m/z 351 and 354 were monitored for 5-HT and internal standard.

Procedure for Sample Cleanup:
Plasma SEP-PAK Procedure:

To one milliliter of plasma, 200 ng of the internal standard (6-HT) was added, the sample alkalinized with 0.5 ml 10% sodium carbonate, and the mixture passed through a $C_{18}$ SEP-PAK cartridge activated prior to use (7). The plasma was followed by 1 ml of water wash, 5-HT and internal standard were eluted by passing 4 ml of methanolic formic acid (90 ml methanol + 10 ml formic acid) through the $C_{18}$ cartridge. The eluate was collected, evaporated under nitrogen and the residue redissolved in 0.2 ml of the mobile phase and 50 ul used for LC-EC.

A standard calibration curve was obtained by using 50, 100, 150 and 200 ng of 5-HT and 200 ng of 6-HT (internal standard and plotting peak height ratio vs. concentration of 5-HT.

The procedure was repeated on duplicate samples of plasma with 200 ng of $d_4$-5-HT added as internal standard. The eluate after evaporation under nitrogen was heated with trifluoroacetic anhydride for 15 min. in a reactive vial at 60°C. The reagent was removed under nitrogen, residue dissolved in 20 ul and 2 ul injected into GC/MS system. From a standard calibration curve obtained under same conditions 5-HT in plasma sample is calculated.

Plasma Ion Exchange Procedure:

A 2.5 cm long 0.5 cm diameter column of Biorex 70 ($H^+$ form) was prepared by pouring slurry of the resin into small chromatographic columns (Biorad) with sintered discs. The column was washed with deionized glass distilled water prior to use. To 1 ml

of plasma sample in a test tube 200 ng of 6-HT (internal standard) was added in the plasma and 1 ml of water wash pushed through the column. This was followed by 2 ml of distilled water wash and 1 ml of methanol wash. 5-HT and 6-HT were then eluted with 2 ml of methanolic formic acid (90 ml methanol and 10 ml 10% formic acid). The eluate was collected in a 5 ml centrifuge tube, evaporated under nitrogen, residue dissolved, in 200 ul of mobile phase and 40 ul of this injected into HPLC system.

A standard calibration curve was obtained with 1 ml each of water solutions containing 50, 100, 150, 200 ng of 5-HT and 200 ng of 6-HT as internal standard following the same procedure. Peak height ratios of 5-HT and 6-HT were plotted against 5-HT concentrations.

Platelet Samples:

Blood samples were collected in sodium citrate tubes, the samples centrifuged at low speed to obtain platelet rich plasma. After obtaining the platelet count (range 500,000 to 600,000 ul) 0.5 ml of the sample was diluted with an equal volume of mobile phase, 500 ng of internal standard added. The mixture was then sonicated for 30 sec and 25 ul injected directly into HPLC. The remaining sample was then divided into two equal parts and one part processed through SEP-PAK after adding 0.5 ml of carbonate buffer. The second part is adjusted to pH 6.5 with carbonate buffer (4 drops) and processed through Biorex-70 column as described above.

Cerebrospinal Fluid Samples:

To 2 ml of CSF sample 5 ng of 6-HT is added and the sample is processed through either SEP-PAK cartridge or Biorex-70. In the former case, the sample is made alkaline with 0.5 ml of carbonate buffer. The SEP-PAK cartridge is washed with 1 ml of hexane and

then eluted with 2 ml of methanolic formic acid. The ion exchange column is washed with 2 ml of water, 1 ml of methanol and then eluted with 2 ml of methanolic formic acid. The eluates are evaporated to dryness under nitrogen at 40°C, the residue is dissolved in 100 ul of mobile phase and 40 ul used for HPLC. A calibration curve is obtained using 1, 2, 3 and 4 ng of 5-HT standards in water following the same procedure.

## RESULTS

The separation of 5-HT and 6-HT standards and in a platelets sample by direct injection is shown in Figure 1. The retention data on different columns and mobile phases are shown in Table I. Samples run without the internal standard indicated no interference from any endogenous compound. Further, the SEP-PAK and Ion exchange procedures were standardized by using $d_4$-5-HT as internal standard and quantitating 5-HT by GC/MS method (1,6). The reproducibility by both methods was good (c.v. 1.7%, n=4). In four experiments using 1 ml aliquots of the same 5-HT/6-HT mixture, the ratio of peak heights was very reproducible; mean $\pm$ SD $\pm$ 0.03 by SEP-PAK method and 1.35 $\pm$ 0.03 by Biorex-70 method. A standard calibration curve with 4, 5, 6, and 8 ng of 5-HT and 10 ng 6-HT gave a value of 0.992. The values for 5-HT on some plasma serum and platelet rich plasma samples by both methods are shown in Table II. The results are in close agreement. Further, in platelet rich plasma samples the results are also comparable to the values obtained by direct injection of platelet samples (Table II). In all, we analyzed 30 control platelet rich plasma samples using either 6-HT or 5-hydroxyindole as internal standard, by direct injection method. The values by either of these methods range between 700 and 1300 ng/$10^9$ platelets with a mean 851 $\pm$ 120 ng/$10^9$ platelets in agreement with the value reported by

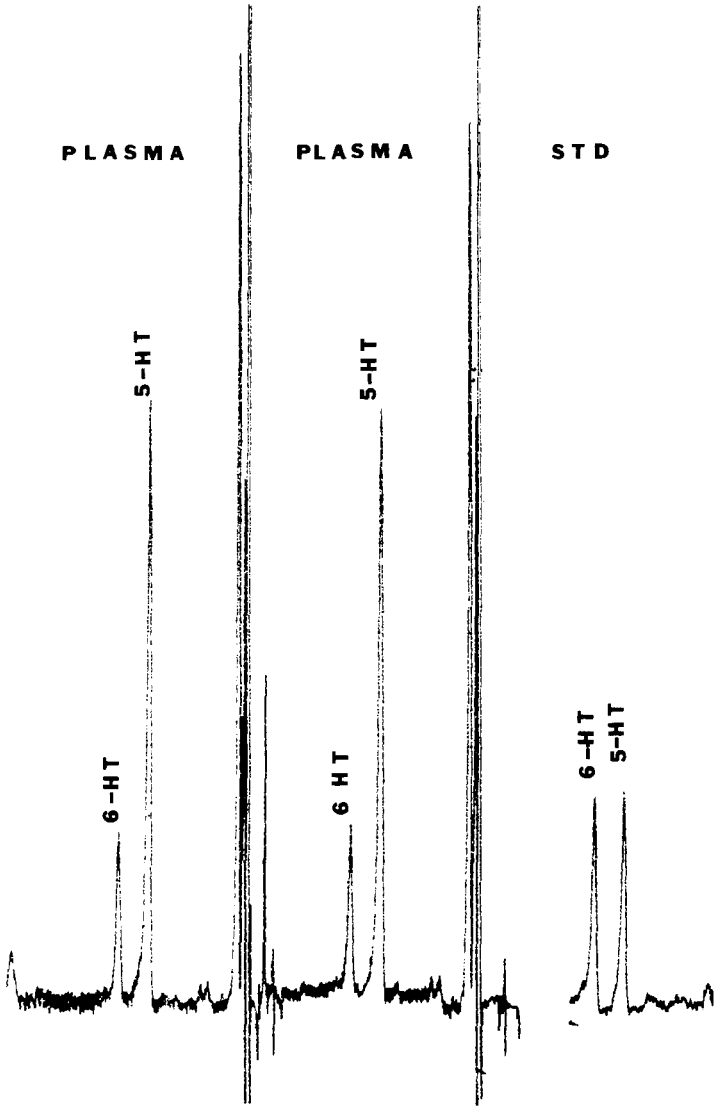



FIGURE 1A. Chromatograms of standard mixture of 5-HT and 6-HT (5 ng each) and plasma sample of phenelzine-treated subject using Biorex-70.

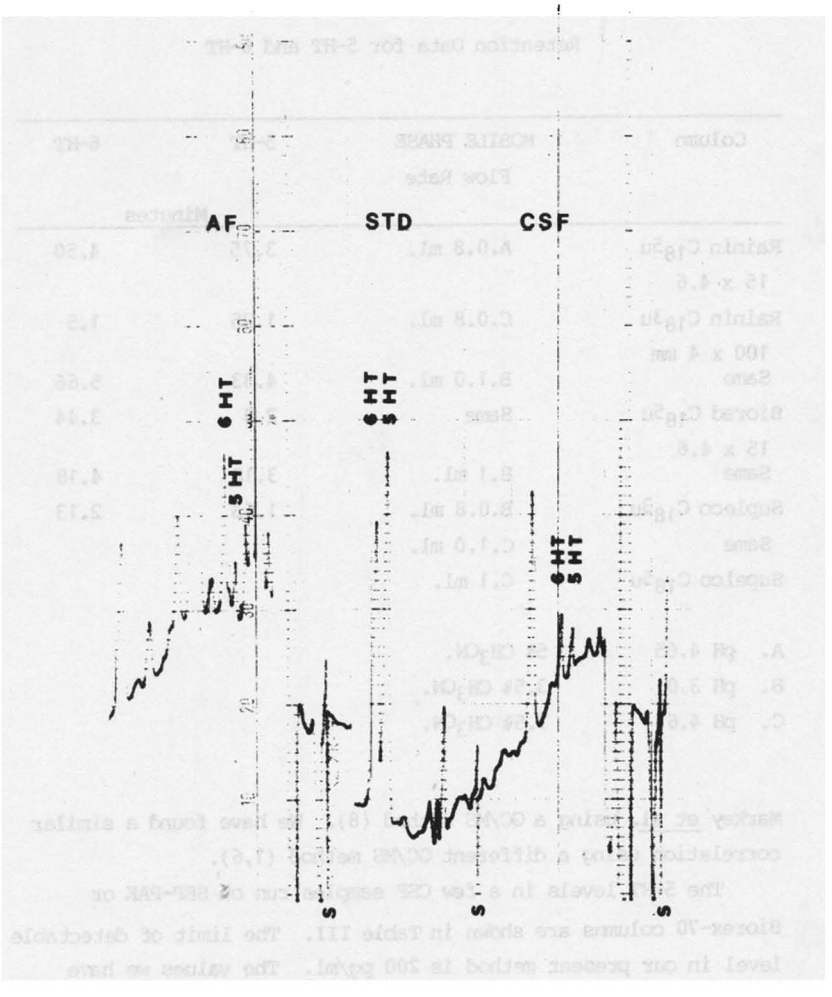



FIGURE 1B. Chromatgrams of cerebrospinal fluid (CSF) and amniotic fluid (AF) samples, 2 ml sample was used in each case with 10 ng of 6-HT for CSF and 25 ng for AF.

TABLE 1

Retention Data for 5-HT and 6-HT

| Column | MOBILE PHASE Flow Rate | 5-HT | 6-HT |
|---|---|---|---|
| | | Minutes | |
| Rainin $C_{18}$5u | A.0.8 ml. | 3.75 | 4.50 |
| 15 x 4.6 | | | |
| Rainin $C_{18}$3u | C.0.8 ml. | 1.25 | 1.5 |
| 100 x 4 mm | | | |
| Same | B.1.0 ml. | 4.33 | 5.66 |
| Biorad $C_{18}$5u | Same | 2.8 | 3.44 |
| 15 x 4.6 | | | |
| Same | B.1 ml. | 3.18 | 4.18 |
| Supleco $C_{18}$3u | B.0.8 ml. | 1.63 | 2.13 |
| Same | C.1.0 ml. | | |
| Supelco $C_{18}$5u | C.1 ml. | | |

A. pH 4.65 5% $CH_3CN$.
B. pH 3.0 3.5% $CH_3CN$.
C. pH 4.65 7.5% $CH_3CN$.

Markey et al. using a GC/MS method (8). We have found a similar correlation using a different GC/MS method (1,6).

The 5-HT levels in a few CSF samples run on SEP-PAK or Biorex-70 columns are shown in Table III. The limit of detectable level in our present method is 200 pg/ml. The values we have obtained are in the range reported in the literature using radioenzymeassay procedure or radioimmunoassay procedure (9). The reproducibility of the assay is established by replicate analysis (Table III). In three human amniotic fluid samples run on Biorex-70 column, 5-HT levels were 3.5, 2.8 and 3.2 ng/ml. The

TABLE 2

Serotonin Levels in Plasma, Serum and Platelet-rich Plasma Samples. ng/ml

| Sample | Biorex-70 | SEP-PAK | Direct Injection |
|---|---|---|---|
| Plasma (MAOI) | 728 | 730 | |
| Plasma (MAOI) | 550 | 500 | |
| Serum pooled (N=4) | 110±13 | | |
| PRP | 930 | 960 | 970 |
| PRP | 510 | 525 | 510 |
| PRP | 750 | 728 | 760 |
| PRP | 920 | | 900 |
| PRP | 650 | | 630 |

TABLE 3

Serotonin Levels in Human CSF

| Sample | 5HT(ng/ml) | Method |
|---|---|---|
| 1 | 0.81 | SEP-PAK $C_{18}$ |
| 2 | 1.25 | SEP-PAK $C_{18}$ |
| 3 | 0.33 | Biorex-70 |
| 4 | 0.5 | Biorex-70 |
| Pooled CSF (N=4) | 1.25±0.15 | SEP-PAK |

Standard Calibration Curve $r = 0.992$

chromatograms of plasma, CSF and amniotic fluid are shown in Figure 1.

## DISCUSSION

Several methods are reported in the literature for the quantitation of 5-HT and the relative merits of the methods have been discussed (10). Of these, the most specific method is the GC/MS method using $d_4$-5-HT as internal standard (6). More recently, the HPLC method with amperometric detection has been used for 5-HT determination in biological fluids (1-4). However, samples need cleanup, especially those with very low levels. Solvent extraction methods give very poor extraction efficiency (8). Methods to improve 5-HT recovery have been reported (8). Earlier methods using a combination of ion exchange procedure for sample cleanup and HPLC-EC for quantitation did not use an internal standard (2,3). We have not found any interference by any endogenous compound under the HPLC conditions used in this study. The method has been successfully applied to a variety of biological samples such as serum, platelets, cerebrospinal fluid and the results validated by GC/MS method. We have recently reported an elegant method for extraction of trace amines using $C_{18}$ SEP-PAK cartridges and have now extended it for the extraction of serotonin from plasma and CSF samples with the isomeric 6-HT as internal standard. This method is particularly useful for CSF samples where the levels are extremely low (1 ng/ml) and any organic solvent extraction will only yield extremely poor recoveries. This is the first report of the use of isomeric non-biological compound in LC-EC analysis. We have also demonstrated an alternate ion exchange procedure which gives comparable results and is less expensive. This is an improvement over the RIA method. We have validated the LC-EC results with platelets, or platelet-rich plasma using either SEP-PAK or ion exchange column procedure by comparing the results of direct

injection with 6-HT as internal standard and GC/MS procedure. This method is now in routine use in our laboratory for the assay of 5-HT in serum and CSF samples of different diagnostic groups of patients. In an earlier study, we suggested that measurement of platelet or plasma or serum serotonin would be a better indicator of treatment response with MAO inhibitors (tranylcypromine or phenelzine) (10). The HPLC-EC method described here which is readily accessible and less expensive could be used in clinical studies with MAO inhibitors.

## REFERENCES

1. Narasimhachari, N., Boadle-Biber, M.C. and Friedel, R.O., Res. Comm. Chem. Path. Pharmacol., 37, 413, 1982.

2. Koch, D.D. and Kissinger, P.T., Anal. Chem., 52. 1980.

3. Sasa, S., Blank, C.L., Wenke, D.C. and Sczupak, C.A., Clin. Chem., 24, 1978.

4. Kitts, C.D., Breebe, G.R. and Mailman, R.B., J. Chromatogr., 225, 347, 1981.

5. Narasimhachari, N., Biochem. Biophys. Res. Comm., 56, 36, 1974.

6. Beck, C.O., Wiesel, F. and Sedvall, G., J. Chromatogr., 134, 404, 1977.

7. Narasimhachari, N., J. Chromatogr., 225, 189, 1981.

8. Markey, S.P., Colburn, R.W. and Johanessan, J.N., Biomed. Mass Spec., 8, 301, 1981.

9. Voldby, B., Engbeck, F. and Enevoldsen, E.M., Stroke, 13, 184, 1982.

10. R. P. Maickel, Serotonin in Health and Disease (Essman, W.B., Ed.), SP Medical and Scientific Books, New York 1 1, 1978.

11. Narasimhachari, N., Chang, S. and Davis, J. M., Soc. Biol. Psychiatry Abs., 35. 52, 1980.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2691-2692 (1984)

## LC NEWS

CHROMATOGRAPHY REFERENCE GUIDE contains many clear illustrations and sample chromatograms to help one select the right product for specific separation needs. It includes easy-to-read charts. Whatman, Inc., JLC/84/13, 9 Bridewell Place, Clifton, NJ, 07014, USA.

BIOCHEMISTRY AND BIOLOGY LAB AUTOMATION USING ROBOTICS includes binding assays, e.g., RIA, EIA, ELISA and EMIT; kinetic and endpoint enzyme assays; and chemical assays, e.g., protein assays and analytical chemical techniques including spectroscopy and chromatography. Zymark, Inc., JLC/84/13, Zymark Center, Hopkinton, MA, 01748, USA.

HPLC COLUMN KITS contain selections of columns to solve methods development problems. They are available in 100mm, 150mm, and 250mm lengths and are made with polished 4.6mm i.d. tubing and inverse compression fittings. Shandon Southern Instruments, JLC/84/13, 515 Broad Street, Drawer 43, Sewickley, PA, 15143, USA.

REVERSE PHASE COLUMN FOR HPLC contains five micron spherical c18 bonded silica particles, endcapped, and packed into a precision polished tube. Burdick & Jackson Laboratories, JLC/84/13, 1953 S. Harvey Street, Muskegon, MI, 49442, USA.

MICRODISPLACEMENT PUMP/HPLC GRADIENT PROGRAMMING SYSTEM features flow range from .01 ml to 10 ml per minute with choice of 19 gradient forms, hold/run/reset, gradient duration up to 999 minutes. Applied Chromatography Systems, Inc., JLC/84/13, Suite 125, 315 S. Allen Street, State College, PA, 16801, USA.

pH GRADIENT CONTROLLER/PROGRAMMER uses a discontinuous, non-linear approach for feedback control, which is ideally suited to pH control, both batch and continuous. Varying of plug-in range resistor (potentiometer) permits stepwise or continuous programming of pH. Luft Instruments, Inc., JLC/84/13, Old Winter Street, Lincoln, MA, 01773, USA.

IMPROVED HPLC SEPARATIONS with ion pair reagents for ionic solutes without the use of ion exchange columns. Ion pair reagents modify the mobile phase to effectively attenuate or enhance solute retention, improve peak symmetry and control selectivity. Both

cationic and anionic species are available, as well as perfluoroalkanoic acids and triethylamine. Pierce Chemical Co., JLC/84/13, P. O. Box 117, Rockford, IL, 61105, USA.

POLYACRYLAMIDE RIGID GEL FOR SEC/GPC may be operated at high pressures and undergoes minimum swelling and shrinkage when transferred between polar eluents. Applications include separations of polysaccharides, polyphenols, and synthetic aqueous polymers. Polymer Laboratories, JLC/84/13, Essex Road, Church Stretton, Shropshire, SY6 6AX, UK.

LC CAPABILITY BOOK contains 56 pages of illustrations and information including "Choosing the System", that helps potential users determine the most appropriate equipment for their needs. Pye Unicam, Ltd., JLC/84/13, York Street, Cambridge CB1 2PX, UK.

RESOLUTION OF EVEN HIDDEN COMPOUNDS is achieved through use of two user-selected wavelengths to analyze compounds of like or similar chemical structure. Overlapping peaks are resolved without changing analysis conditions or resorting to exotic and expensive detection methods. Micromeritics Instrument Co., JLC/84/13, 5680 Goshen Springs Road, Norcross, GA, 30093, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2693-2696 (1984)

# LC CALENDAR

## 1984

OCTOBER 28 - NOVEMBER 1: 2nd International Congress on Computers in Science, Washington, DC. Contact: S. R. Heller, EPA, PM-218, Washington, DC, 20460, USA.

OCTOBER 28 - NOVEMBER 2: 98th Annual AOAC Inteernational Meeting - Centennial Meeting, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, USA.

NOVEMBER 13 - 16: 23rd Eastern Analytical Symposium, New York Penta Hotel, New York City. Contact: S. D. Klein, Merck & Co., P. O. Box 2000/R801-106, Rahway, NJ, 07065, USA.

NOVEMBER 19-24: Expoquimia: International Chemical Forum, Barcelona, Spain. Contact: Expoquimia, Feria de Barcelona, Barcelona, Spain.

DECEMBER 10-12: "TLC/HPTLC-84: Expanding Horizons in TLC," Sheraton-University City, Philadelphia, PA. Contact: J. C. Touchstone, University of Pennsylvania, Dept. OB-GYN, 3400 Spruce e Street, Philadelphia, PA.

DECEMBER 10-12: Fourth International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baltimore, MD. Contact: Shirley E. Schlessinger, Symposium Manager, 400 East Randolph, Chicago, IL, USA.

DECEMBER 16-21: International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, sponsored by the Chemical Inst. of Canada, Chemical Soc. of Japan, and the American Chem. Soc. Contact: PAC CHEM '84, International Activities Office, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

## 1985

FEBRUARY 10-15: Symposium on the Interface Between Theory and Experiment, Canberra, Australia. Contact: Leo Radom, Research

School of Chemistry, Australian National University, Canberra, ACT 2601, Australia.

FEBRUARY 11-14: Polymer 85, Int'l Symposium on Characterization and Analysis of Polymers, Monash University, Melbourne, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Polymer 85, RACI, 191 Royal Parade, Parkville Victoria 3052, Australia.

FEBRUARY 25 - MARCH 1: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, LA. Contact: Linda Briggs, 437 Donald Road, Pittsburg, PA, 15235, USA.

MARCH 23-24: Conference on Creativity & Science, Honolulu, Hawaii. Contact: D. DeLuca, Scientists and Humanities Conf., Winward Community College, University of Hawaii, 45-720 Keaahala Rd., Kaneohe, Hawaii, 96744, USA.

APrIL 8 - 11: 10th Annual AOAC Spring Training Worshop, Sheraton Hotel, Dallas, Texas. Contact: M. V. Gibson, USFDA, 332 Bryan, Dallas, TX, 75204, USA.

APRIL 15 - 17: Second International Symposium on Instrumental TLC, Wurzburg, West Germany. Contact: H. M. Stahr, 1636 College Veterinary Medicine, Iowa State University, Ames, IA, 50011, USA., or Prof. S. Ebel, Institute for Pharmacies, A. M. Hubland, D-8700 Wuerzburg, West Germany.

APRIL 15-18: Materials Research Society Spring Meeting, San Francisco, CA. Contact: Susan Kalso, Xerox Palo Alto Res. Center, 3333 Coyote Hill Road, Palo Alto, CA, 94304, USA.

APRIL 28 - MAY 3: 189th National ACS Meeting, Miami Beach. Contact: A. T. qinstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

APRIL 29 - MAY 2: Symposium on Analytical Metods in Forensic Chemistry & Toxicology, Miami, Florida; in conjunction with 189th ACS Nat'l Meeting. Contact: Dr. M. H. Ho, Dept. of Chem., University of Alabama, Birmingham, AL, 35294, USA.

MAY 13 - 15: Infant Formula Conference, Sheraton Hotel, Virginia Beach, VA. Contact: Dr. James Tanner, USFDA-Hff-266, 200 C Street, SW, Washington, DC, 20204, USA.

MAY 19: Middle Atlantic Regional ACS Meeting, Sponsored by ACS Monmouth County Section. Contact: M. Parker, Dept. of Chem., Monmouth College, West Long Branch, NJ, USA.

JUNE 9-15: ACHEMA 85, Frankfurt, West Germany. Contact: DECHEMA, Organization ACHEMA, P.O.Box 97 01 46, D-6000 Frankfurt, 97, West Germany.

JUNE 24 - 28: 59th Colloid & Surface Science Symposium, Clarkson College of Technology, Potsdam, NY. Contact: J. P. Kratohvil, Institute of Colloid & Surface Science, Clarkson College of Technology, Potsdam, NY, 13676, USA.

JULY 1-5: Ninth International Symposium on Column Liquid Chromatography, sponsored by the Chromatography Discussion Group and by the Royal Society of Chemistry's Chromatography & Electrophoresis Group, Edinburgh, Scotland. Contact: Prof. J. H. Knox, 9th ISCLC Secretariat, 26 Albany Street, Edinburgh, EH1 3QH, Great Britain.

JULY 4: 4th International Flavor Conference, Greece. Contact: C. J. Mussinan, IFF R&D, 1515 Highway 36, Union Beach, NJ, 07735, USA.

SEPTEMBER 1-6: 6th International Symposium on Bioaffinity Chromatography & Related Techniques, Prague, Czechoslovakia. Contact: Dr. J. Turkova, Institute of Organic Chemistry & Biochemistry CSAV, Flemingovo n. 2, CS-166 10 Prague 6, Czechoslovakia.

SEPTEMBER 8-13: 190th National ACS Meeting, Chicago. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

SEPTEMBER 29 - OCTOBER 4: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: T. Rains, NBS, Center for Analytical Chemistry, Chemistry B-222, Washington, DC, 20234, USA.

OCTOBER 27 - 30: 99th Annual AOAC Meeting, Shoreham Hotel, Washington, DC. Contact:Margaret Ridgell, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, USA.

## 1986

APRIL 6-11: 191st National Am. Chem. Soc. Mtng., Atlantic City, NJ. Contact: A. T. Winstead, ACS, 1155 16th Streeet, NW, Washington, DC, 20036, USA.

SEPTEMBER 7-12: 192nd National Am. Chem. Soc. Mtng., Anaheim, Calif. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

SEPTEMBER 21-26: XVIth International Symposium on Chromatography, Paris, France. Contact: G.A.M.S., 88,Boulevard Malesherbes, F-75008 Paris, France.

OCTOBER 12 - 16: 100th Annual AOAC International Meeting, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, USA.

1987

APRIL 5-10: 193rd National Am. Chem. Soc. Mtng., Denver, Colo. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

AUGUST 30 - SEPTEMBER 4: 194th National Am. Chem. Soc. Mtng., New Orleans, LA. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

1988

JUNE 5 - 11: 3rd Chemical Congress of the North Americanmn Continent, Toronto, Ont., Canada. Contact: A. T. Winstead, ACS, 1155 Sixteenth St, NW, Washington, DC, 20036, USA.

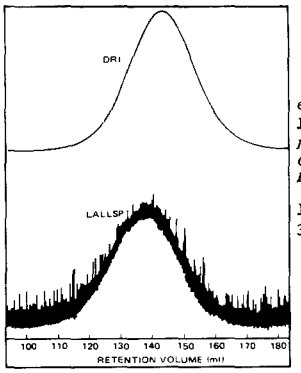
SEPTEMBER 25 - 30: 196th ACS National Meeting, Los Angeles, CA. Contact: A. T. Winstead, ACS, 1155 Sixteenth Street, NW, Washington, DC, 20036, USA.

The Journal of Liquid Chromatography will publish announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

*Providing state-of-the-art understanding of . . .*

# Steric Exclusion Liquid Chromatography of Polymers

(Chromatographic Science Series, Volume 25)




edited by
**JOSEF JANČA**
*Institute of Analytical Chemistry*
*Czechoslovak Academy of Sciences*
*Brno, Czechoslovakia*

January, 1984
352 pages, illustrated

*". . . Dr. Janča has succeeded in bringing together several of the most innovative giants in this field. Each has contributed significantly. In this monograph, we are able to look over their shoulders to see where future advances will lie."*
**–Dr. JACK CAZES**
Fairfield, Connecticut *(from the Foreword)*

WITH ITS UNSURPASSED capacity to determine a polymer's molecular weight distribution and other properties, it is easy to recognize the vast importance of steric exclusion chromatography– *AND the value of this unparalleled single-source reference!*

Certainly, no other work can match *Steric Exclusion Liquid Chromatography of Polymers* for its up-to-date, fully detailed coverage–provided by leading international experts–with features that include

- in-depth examination of separation mechanisms and secondary interactions and their correlation with the structure and behavior of macromolecules
- refinements in calibration and data evaluation techniques
- complete information on solvent and column packing, correction of zone dispersion, and experimental variables
- practical applications involving molecular weight distribution, polymer branching, chemical composition, chain growth and degradation mechanisms, and others

Complete with illustrative experimental data, this state-of-the-art volume leads the way to future innovations for a wide range of scientists. Furthermore, this volume serves as an excellent reference for a number of graduate-level chemistry courses.

***Readership:*** Liquid Chromatographers; Analytical, Polymer, Organic, and Industrial Chemists; Physicists; Polymer Technologists; Polymer Processors; and Graduate Chemistry Students.

**CONTENTS**



**ISBN: 0-8247-7065-X**

**MARCEL DEKKER, INC.**
**270 MADISON AVENUE, NEW YORK, N.Y. 10016**
**(212) 696-9000**

# INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

*Journal of Liquid Chromatography* is a bimonthly publication in the English language for the rapid communication of liquid chromatographic research.

**Directions for Submission**

One typewritten manuscript suitable for direct reproduction, carefully inserted in a folder, and two (2) copies of the manuscript must be submitted. Since all contributions are reproduced by direct photography of the manuscripts, the typing and format instructions must be strictly adhered to. Noncompliance will result in return of the manuscript to the authors and delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards and securely bound before mailing.

Manuscripts should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

**Reprints**

Owing to the short production time for articles in this journal, it is essential to indicate the number of reprints required upon notification of acceptance of the manuscript. Reprints are available in quantities of 100 and multiples thereof. For orders of 100 or more reprints, twenty (20) free copies are provided. A reprint order form and price list will be sent to the author with the notification of acceptance of the manuscript.

**Format of Manuscript**

1. The general format of the manuscript should be as follows: title of article; names and addresses of authors; abstract; and text discussion.

2. Title and Authors: The entire title should be in capital letters and centered on the width of the typing area at least 2 inches (5.1 cm) from the top of the page. This should be followed by three lines of space and then by the names and addresses of the authors in the following way (also centered):

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN SURFACE WATERS BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakra Way
Salt Lake City, Utah 84108

3. Abstract: Three lines below the addresses, the title ABSTRACT should be typed (capitalized and centered on the page). This should be followed by a single-spaced, concise, abstract comprising less than 10% of the length of the text of the article. Allow three lines of space below the abstract before beginning the article itself.

4. Text Discussion: Whenever possible, the text discussion should be divided into such major sections as INTRODUCTION, MATERIALS, METHODS, RESULTS, DISCUSSION, ACKNOWLEDGMENTS, and REFERENCES. These major headings should be separated from the text by two lines of space above and one line of space below. Each heading should be in capital letters, centered, and underlined. Secondary headings, if any, should be flush with the left margin, underscored, and have the first letter of all main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. Paragraphs should be indented five (5) typewriter spaces.

6. Acknowledgment of collaboration, sources of research funds, and address changes for an author should be listed in a separate section at the end of the paper.

7. References (including footnotes) in the text will be numbered consecutively by numbers in parentheses. All references (and footnotes) should then be aggregated in sequence at the end of the communication. No footnotes should be shown at the bottom of pages. The reference list follows immediately after the text. The word REFERENCES should be capitalized and centered above the reference list. It should be noted that all reference lists should contain initials and names of all authors; *et al.* will not be used in reference lists. Abbreviations of journal titles and styles of reference lists will follow the American Chemical Society's Chemical Abstracts List of Periodicals. References should be typed single-spaced with one line space between each reference.

8. Each page of manuscript should be numbered lightly at the bottom of the sheet with a light blue pencil.

9. Only standard symbols and nomenclature approved by the International Union of Pure and Applied Chemistry should be used.

10. Any material that cannot be typed, such as Greek letters, script letters, and structural formulae, should be drawn carefully in black India ink (do not use blue ink).

**Typing Instructions**

1. The manuscript must be typewritten on good quality white bond paper measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). Do not use Corrasible bond or its equivalent. The typing area of the article opening page, including the title, should be 5½ inches wide by 7 inches deep (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches deep (14 cm x 21.6 cm).

2. In general, the chapter title and the abstract, as well as the tables and references, are typed single-spaced. All other text discussion should be typed 1½-line spaced, if available, or double-spaced. Prestige elite characters (12 per inch) are recommended, if available.

3. It is essential to use black typewriter ribbon (carbon film is preferred) in good condition so that a clean, clear impression of the letters is obtained. Erasure marks, smudges, creases, etc., may result in return of the manuscript to the authors for retyping.

4. Tables should be typed as part of the text but in such a way as to separate them from the text by a three-line space at both top and bottom of each table. Tables should be inserted in the text as close to the point of reference as possible, but authors must make sure that one table does not run over to the next page, that is, no table may exceed one page. The word TABLE (capitalized and followed by an Arabic number) should precede the table and be centered on the page. The table title should have the first letters of all main words in capitals. Titles should be typed single-spaced. Use the full width of the type page for the table title.

5. Drawings, graphs, and other numbered figures should be professionally drawn in black India ink (do not use blue ink) on separate sheets of white paper and placed at the end of text. Figures should not be placed within the body of the text. They should be sized to fit within the width and/or height of the type page, including any legend, label, or number associated with them. Photographs should be glossy prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn. Captions for the pictures should be typed single-spaced on a separate sheet, along the full width of the type page, and preceded by the word FIGURE and a number in arabic numerals. All figures and lettering must be of a size to remain legible after a 20% reduction from original size. Figure numbers, name of senior author, and arrow indicating "top" should be written in light blue pencil on the back or typed on a gummed label, which should be attached to the back of the illustration. Indicate approximate placement of the illustrations in the text by a marginal note in light blue pencil.

6. The reference list should be typed single-spaced although separated from one another by an extra line of space. Use Chemical Abstract abbreviations for journal titles. References to journal articles should include (1) the last name of all author(s) to any one paper, followed by their initials, (2) article title, (3) journal, (4) volume number (underlined), (5) first page, and (6) year, in that order. Books should be cited similarly and include (1) author, surname, first and middle initials, (2) title of book, (3) editor of book (if applicable), (4) edition of book (if any), (5) publisher, (6) city of publication, (7) year of publication, and (8) page reference (if applicable). E.g., Journals: Craig, L. C. and Konigsber, W., Use of Catechol Oxygenase and Determination of Catechol, Chromatogr., 10, 421, 1963. Books: Albertsson, P. A., Partition of Cell Particles and Macromolecules, Wiley, New York, 1960. Article in a Book: Walter, H., Proceedings of the Protides of Biological Fluids, XVth Colloquim, Pteeters., H., eds., Elsevier, Amsterdam, 1968, p. 367.