JOURNAL OF LIQUID CHROMATOGRAPHY

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 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 per volume by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 6 (1983), containing fourteen numbers and two supplements, is $298.00 per volume (prepaid). The special discounted rate for individual professionals and students is $149.00* per volume. To secure this special rate, your order must be prepaid by personal check or may be charged to MasterCard or VISA. Add $36.80 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.

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

JOURNAL OF LIQUID CHROMATOGRAPHY

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

JOURNAL OF LIQUID CHROMATOGRAPHY

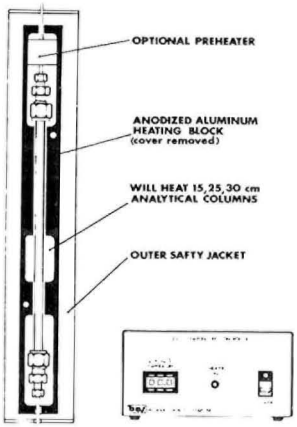
electrochemical detectors for LC & FIA

bas *BIOANALYTICAL SYSTEMS*
Pioneers in Electrochemical Detection

1205 KENT AVENUE ● W LAFAYETTE, IN 47906 ● (317) 463-2505 ● telex 276141

TEMPERATURE CONTROL FOR LC COLUMNS

Temperature control in liquid chromatography has generally been neglected. Improved resolution, efficiency, and precision often result from operation of LC columns at controlled temperatures above ambient.



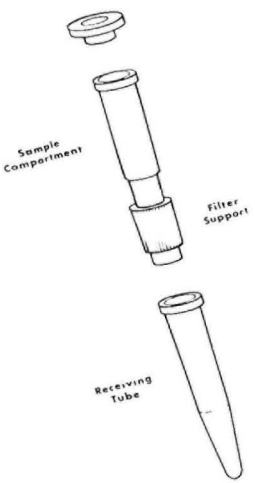
Bioanalytical Systems offers a low cost column heating system based on a metal block heater and solid state controller. The versatile controller can be used with other heating elements and probes for a variety of laboratory applications.

bas
BIOANALYTICAL SYSTEMS INC.

1205 Kent Avenue
West Lafayette, IN 47906
(317) 463-2505 telex 276141

bas

Centrifugal Microfilters

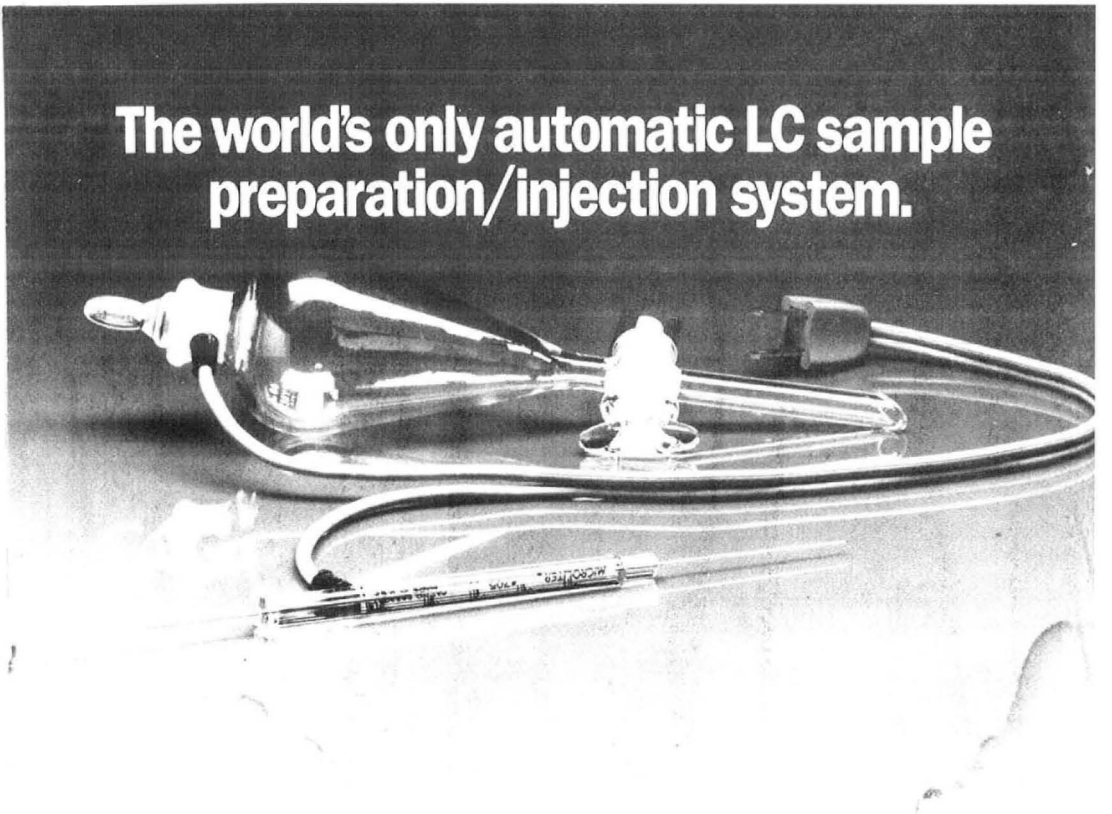


Bioanalytical Systems introduces a centrifugal microfilter for membrane filtration of small volumes using the force of a conventional bench top centrifuge. Ideal for LC sample preparation, extraction of TLC spots, and many other applications.

Please circle these numbers on the Reader Service card for further information: No. 106 for Electrochemical Detectors, No. 107 for Microfilters, No. 108 for Temperature Controllers

See us in booths 4013 and 4015 at the Pittsburgh Conference.

The world's only automatic LC sample preparation/injection system.



It's not that no one has tried. They have. But it took Analytichem to develop an automatic LC sample preparation/injection system that really works. It's called AASP. An acronym for Analytichem Automated Sample Preparation, AASP is a new on-line system that combines solid-phase sample preparation with syringeless injection. In one simple operation.

Samples are extracted by AASP sorbent cassettes that selectively isolate and concentrate the compounds of interest. The compounds are then quantitatively transferred directly onto the HPLC column by the AASP LC Sample Transfer Module. Automatically. The result is a degree of chemical isolation never before possible. Until AASP. Call Analytichem for full details at (800) 421-2825. In Calif. (213) 539-6490.

Analytichem International
24201 Frampton Ave.,
Harbor City, CA 90710, USA
Telex: 664832 ANACHEM HRBO

See the AASP System at the Pittsburgh Conference, Booths G19-G22

Circle Reader Service Card No. 110

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 6, Number 2, 1983

CONTENTS

Circle Reader Service Card No. 104

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 199-213 (1983)

A COMPARATIVE STUDY OF THE SEPARATION OF THE TRYPTIC PEPTIDES OF THE β-CHAIN OF NORMAL AND ABNORMAL HEMOGLOBINS BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

E. Minasian , R.S. Sharma[+], S.J. Leach ,
B. Grego*, and M.T.W. Hearn*[1]

Department of Biochemistry, University of Melbourne,
[+]Hematology Department, Queen Victoria Hospital,
*St. Vincent's School of Medical Research,
41 Victoria Parade,
FITZROY, VICTORIA 3065, AUSTRALIA.

ABSTRACT

The separation of the tryptic peptides of the human hemoglobin A β-chain by reversed phase high performance liquid chromatography under different elution conditions on several microparticulate alkylsilica supports is described. Similar methods have been used to separate the tryptic peptides of β-chain hemoglobin variants including HbC, HbE, and Hb(Kempsey). Selectivity differences which can be achieved under the different chromatographic conditions have been exploited to permit the assignment of all the anticipated peptide fragments derived from the tryptic digestion of these β-chain Hb-variants.

INTRODUCTION

A large variety of procedures is now available for the structural characterisation of human hemoglobin (Hb) variants. In many cases, the identification has been based on the analysis of tryptic peptides of the isolated Hb polypeptide chains. Until

---

[1] Author to whom correspondence should be sent.

 0148-3919/83/0602-0199$3.50/0

recently, the usual methods of analysis of these tryptic fragments included combinations of cation- and anion- exchange chromatography or alternatively combinations of two dimensional chromatography and electrophoresis on inert support matrices. With the advent of reversed-phase high performance liquid chromatography (RP-HPLC), procedures have now become available for the rapid, high resolution separation of complex peptide mixtures, including those generated by enzymatic digestion of protein samples (1). Several of these procedures have already attracted attention for the peptide mapping of hemoglobin variants. For example, RP-HPLC techniques have been used, in conjunction with amino acid analysis and/or sequence analysis, to distinguish single amino acid replacement differences between hemoglobin variants using acetonitrile-water-orthophosphoric acid elution systems (2-4). Alternative RP-HPLC micro- and semi-preparative methods for the separation of hemoglobin fragments have also been described based on the use of non-volatile as well as completely volatile eluents, including various hydro-organic solvent combinations containing such additives as trifluoroacetic acid, ammonium acetate, triethylammonium acetate or trifluoroacetate, sodium perchlorate-orthophosphoric acid, ammonium bicarbonate or pyridine - acetic acid buffers (1, 5-12).

Most RP-HPLC studies on homology mapping of hemoglobins have largely been devoted to the separation of the tryptic peptides of either intact hemoglobins or the isolated α-, β-, γ- and δ-chains under a single set of chromatographic conditions. However, with complex peptide mixtures, a single elution protocol is rarely sufficient to allow complete resolution, i.e., with $R_s = 1$ of all the components. As a consequence of renewed interest in resolution optimisation for peptides separated on alkylsilicas, attention has recently been directed to examination of the chromatographic selectivity differences known (5, 13, 14) to be induced by different organic solvents as well as the concentration and composition of the buffer components, the pH and associated mobile phase conditions. A knowledge of these effects is clearly needed, if the full potential of RP-HPLC is to be exploited as an analytical tool in the detection of different hemoglobinopathies. The present study was addressed to this issue using as model systems the tryptic peptides of the isolated β-chains of normal and several hemoglobin variants in conjunction with low and neutral pH elution systems separated on different microparticulate alkylsilicas.

EXPERIMENTAL

High performance liquid chromatography was performed on μBondapak - alkylphenyl or -C18 columns (Waters Assoc., Milford, Mass.) and on LiChrosorb RP-8 (E. Merck, Darmstadt, G.F.R.) columns. The chromatographs used were a Waters model 202/401, equipped with a M440 gradient module and a M450 variable wavelength UV detector, and microprocessor-controlled

Spectra-Physics SP 8000B (Spectra-Physics, Santa Clara, Calif.) equipped with a SP 840 variable wavelength UV detector. Eluents and solutions of peptides were filtered using 0.45um type HA membranes from Millipore Corp. (Bedford, Mass.).

The normal hemoglobin A as well as the variants were obtained from blood samples collected in the usual fashion with EDTA as an anticoagulant. The erythrocytes were washed with 0.8% saline and hemolysed at ca. 0 under hypotonic conditions. The cell debris was removed by centrifugation at 2000xg and the supernatent dialyzed at 4 against 3 changes of 50mM Tris-HCl, pH 8.6, buffer (500ml) overnight. The hemoglobins were isolated by anion-exchange chromatography on a DEAE-Sephadex A50 column (64.5 x 1.5 cm) equilibrated with 50mM Tris-HCl, pH 8.6 at a flow rate of 50ml/h. Elution of the non-bound components was achieved with 150ml of the equilibration buffer. The bound hemoglobin components were eluted with a two stage gradient, commencing with a linear gradient of 50mM Tris-HCl (pH 8.5, 150ml) to 50mM Tris-HCl (pH 7.5, 150ml) up to an elution volume of 200ml, followed by a further linear gradient from 50mM Tris-HCl (pH 7.5, 150ml) to 50mM Tris-HCl (pH 6.5, 150ml). Elution of the hemoglobins was monitored at 415nm. The polypeptide chains of the normal and variant hemoglobins were separated by the procedure of Clegg et al. (15).

The isolated β-chains were aminoethylated and digested with trypsin (DPCC-treated) according to established methods (7, 15, 16). The samples were freeze-dried and stored at -20° until required. Individual tryptic peptides of the β-chain of HbA were resolved, as well as variant peptides identified, using conventional two dimensional high voltage electrophoretic-chromatographic procedures (17) for use as comparative solutes in the RP-HPLC experiments. Distilled water was further purified by reverse-osmosis. Acetonitrile was obtained from Burdick and Jackson (Muskegon, Mich.) or Waters Assoc. Orthophosphoric acid was from May and Baker (Dagenham, U.K.), ammonium acetate and ammonia were both Analar grade from B.D.H. (Poole, U.K.) and acetic acid was from Merck.

The reversed-phase columns were conditioned to all new elution conditions for ca. 30 min. and subjected to at least two blank gradient elutions prior to use. In the present study, the following elution protocols were employed: (A), a 60 min. linear gradient from aqueous 15mM orthophosphoric acid to 50% acetonitrile - 50% water - 15mM orthophosphoric acid (16, 19), and (B) a 80 min. linear gradient from 10mM ammonium acetate, pH 6.0, to 40% acetonitrile - 60% water - 10mM ammonium acetate (6). A flow rate of 1.0 or 2.0 ml/min. was used as indicated in the text. Peptide samples were dissolved in the initial eluent of the gradient system immediately prior to use and centrifuged at 5,000xg for 2 min. in a Microfuge. Sample injections were made using Hamilton (Reno, Nev.) model 1010W syringes and ranged from

45-850µg. The bulk solvents and various mobile phases were prepared and degassed as described previously (4).

Recovered peptide fractions from the reversed-phase separations using the (A)-elution system were immediately adjusted to pH 7.0 with 15mM NaOH. These fractions, as well as appropriate fractions obtained with the (B)-elution system, were dried under nitrogen and hydrolysed in vacuo in 6M HCl containing 0.1% phenol at 110° for 24 hr. The hydrolysates were analysed on a Joel amino acid analyser or a Beckman 121MB analyser.

RESULTS AND DISCUSSION

Fig. 1a shows the chromatogram of the tryptic peptides of the aminoethylated normal HbA β-chain eluted under the pH 2.1

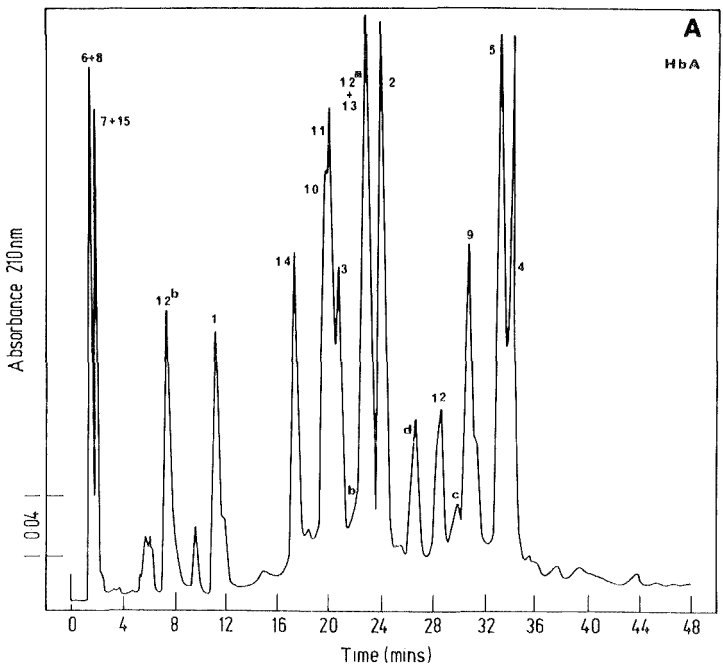


Figure 1(a). Separation of the peptides from the trypsin digestion of the aminoethylated normal human Hb β-chain by RP-HPLC. Chromatographic conditions: column, µBondapak - alkylphenyl; flow rate, 2 ml/min.; linear 60 min. gradient from aqueous 15mM orthophosphoric acid to 50% acetonitrile in aqueous 15mM orthophosphoric acid; sample loading, 810µg injected in a volume of 100µl.

phosphate mediated elution conditions from a μBondapak alkylphenyl column. As was noted in our earlier studies (1, 13, 18, 19) on the separation of tryptic and thermolysin peptides of a variety of globular proteins with aquo-acetonitrile gradients containing phosphate buffers at various molarities and pHs, these conditions permit excellent separation of complex peptide mixtures with little loss of resolution for sample loading up to ca. 500 nmoles on standard analytical (30 x 0.4 cm) reversed-phase columns. Following recovery of all the eluted peaks shown in Fig. 1a individual tryptic peptides were readily identified from their amino acid compositions. It was evident from these compositional data that all the expected major tryptic peptides had been recovered, and that most of the eluted peak zones contained only single peptide fragments. Peptides T-6 (Val^{60}Lys) and T-8 (Lys^{66}) coelute under these conditions as do T-7 (Ala^{62}His Gly Lys) and T-15 (Tyr^{145}His). The two peptides (T-12A and T-12B) corresponding to cleavage at the aminoethylated Cys^{112} were well resolved and a minor peak containing the intact peptide T-12 recovered. In

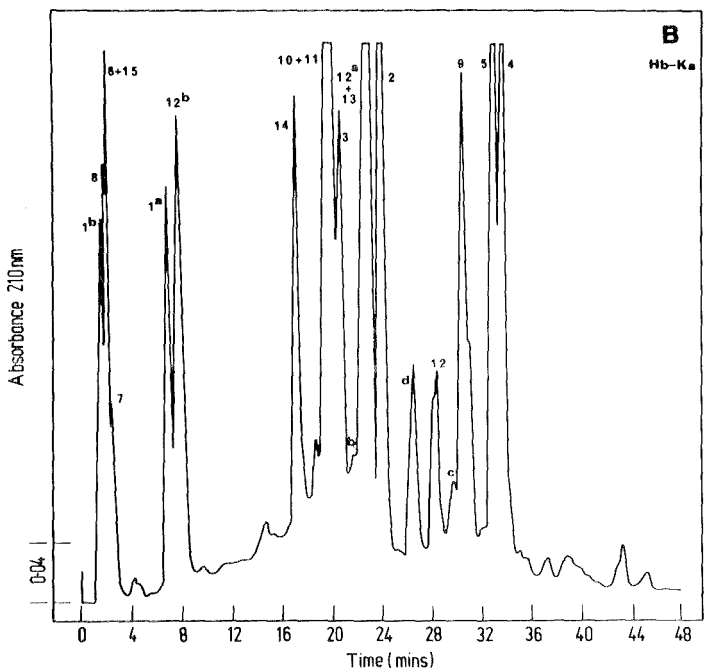


Figure 1(b). Separation of the peptides from the trypsin digestion of the aminoethylated abnormal human Hb(Ka) β-chain by RP-HPLC. Chromatographic conditions as in Fig. 1a; sample loading; 870μg injected in a volume of 100μl.

TABLE

Comparative elution orders for the tryptic peptides of normal Hb β-chain separated under different RP-HPLC conditions

Tryptic Peptide*	Residue †	Sequence**	Condition 1 Elution Order+	Condition 2 Elution Order§	Condition 3 Elution Order∫	Calculated Relative Hydrophobicity $\Sigma f_n \emptyset$	Calculated Relative Hydrophobicity $\Sigma X_n \emptyset\emptyset$
T-1	1-8	VHLTPEEK	6	5	5	4.35	2.21
T-2	9-17	SAVTALWGK	13	13	11	6.52	3.79
T-3	18-30	VNVDEVGGEALGR	10	7	6	4.59	4.25
T-4	31-40	LLVVYPWTQR	15	16	16	9.47	7.32
T-5	41-59	FFESFGDLSTPDAVMGNPK	16	15	14	11.78	16.85
T-6	60-61	VK	1	1	1	1.98	2.09
T-7	62-65	AHGK	3	3	3	0.82	-2.60
T-8	66	K	1	2	2	0.52	1.01
T-9	67-82	VLGAFSDGLAHLDNLK	14	14	14	11.35	15.45
T-10	83-95	GTFATLSELHCDK	8	11	10	7.40	6.71
T-11	96-104	LHVDPENFR	9	8	7	4.23	4.47
T-12A	105-112	LLGNVLVC	11	12	13	9.47	9.64
T-12B	113-120	VLAHHFGK	5	6	9	6.28	-1.04
T-13	121-132	EFTPPVQAAYQK	12	9	8	6.49	2.60
T-14	133-144	VVAGVANALAHK	7	10	12	4.81	1.68
T-15	145-146	YH	3	3	4	1.47	-0.28

* Peptides are numbered in order of their final position in the sequence.

† Amino acid sequence numbers.

** The one letter code for the amino acid as given by M.O. Dayhoff in Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Silver Spring, M.D., U.S.A.

+ Relative order of elution from a μBondapak-alkylphenyl column with elution system (A).

§ Relative order of elution from a μBondapak C_{18} column with elution system (B).

ſ Relative order of elution from a LiChrosorb RP-8 column with elution system (B).

Ø Calculated using the Σf_n (amino acid) values from ref. 31 with f_n (Glycine) = 0 and f_n (arginine) = -1.10 respectively. ØØ Calculated using the ΣX_n (amino acid) values from ref. 30

addition, the partial cleavage tryptic peptides corresponding to Lys^{66} Val ... Lys^{82} (T-8 + T-9) and Leu^{105} ... Lys^{132} (T-11 + T-12) were also present. Two peaks, both with amino acid compositions characteristic of peptide T-5 were obtained; the minor peak presumably corresponding to the oxidised Met^{55}-nonadecapeptide. Amino acid compositions of the remaining minor peaks indicated they contained peptides arising from residual chymotryptic cleavage, e.g., β-chain (131-132) or residual α-chain contamination, e.g., α(62-76) and α(77-90) of peptide α-T-9. The identification of even these minor peaks which occur regularly, but are not necessarily predictable in a sample emphasise again the excellent resolution which can be obtained with the current RP-HPLC methods compared to conventional procedures for Hb peptide mapping.

The RP-HPLC profile for the HbA- β-chain tryptic peptides eluted with a water-acetonitrile (0-40%) - 10mM ammonium acetate, pH 6.0, gradient on a LiChrosorb RP-8 column is shown in Fig. 2a. The peak assignments and relative elution order for the individual tryptic peptides recovered from this, and the corresponding μBondapak C18column separation are given in the Table. In both cases, amino acid compositional analyses revealed that the majority of recovered peak zones again contained only single tryptic fragments. Peptides T-6 (Val^{60} Lys), T-7 (Ala^{62} His Gly Lys) and T-8 (Lys^{66}) were only partially resolved on the LiChrosorb RP-8 support and coeluted with the μBondapak C18column. Superficially, the retention behaviour of the other tryptic peptides on the LiChrosorb RP-8 (5μm, spherical particle, ca. 14% carbon loading w/w, with nominal pore size of 6nm and surface area ca. 250 m^2/g) and the μBondapak C18 (10um, irregular particle, ca. 10% carbon loading w/w, with a nominal pore size of 8nm and surface area ca. 350 m^2/g) were similar under these pH 6.0 mobile phase conditions with many of the peptides following the same elution order. However, changes in relative selectivity, that is $\Delta\alpha/\alpha$, were evident for some peptides with the most noticeable elution order differences associated with the characteristic peptide cluster T-10, T-12, T-2 and T-14 which eluted over the intermediate concentration range of the organic solvent modifier (20-30% acetonitrile). Within this volume fraction range of the organic solvent modifier, relative selectivities of polypeptides on alkylsilicas are known to be responsive to stationary phase surface effects other than those due to the w/w percentage of the carbon loading, for example the presence of a more open siloxane-silanol network in the parent silica matrix which gives rise to different solvent extraction isotherms. Such subtle differences in stationary phase characteristics can be revealed from comparative plots of the logarithmic capacity factors for peptides/polypeptides versus the volume fraction of the organic solvent modifier (20).

Although the peptide mixture produced by the tryptic cleavage of the HbA β-chain contains peptides encompassing a wide range of

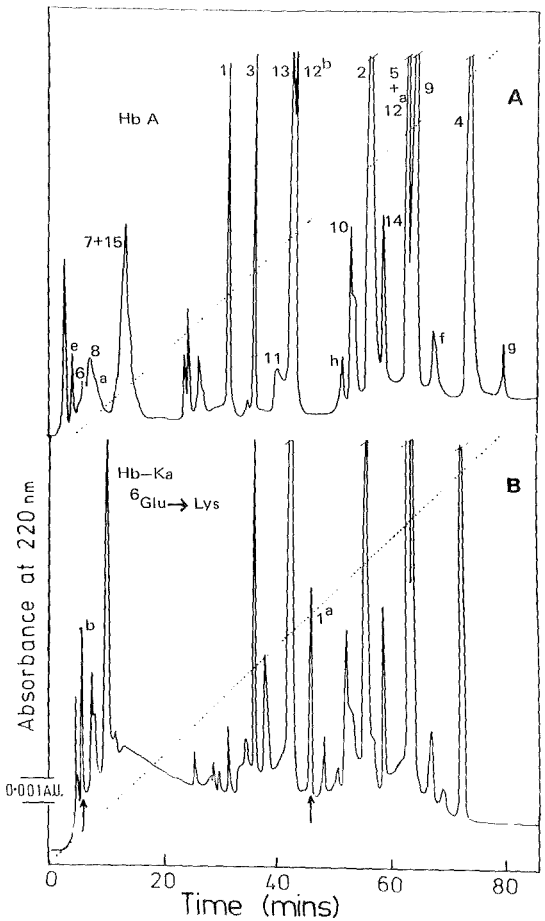


Figure 2. Separation of the peptides from the trypsin digestion of the aminoethylated normal and abnormal (Ka) human Hb β-chain by RP-HPLC. Chromatographic conditions: column, LiChrosorb RP-8; flow rate, 1ml/min.; linear 80 min. gradient from aqueous 10mM ammonium acetate, pH 6.0, to 40% acetonitrile in 10mM ammonium acetate, pH 6.0; sample loading, 100μg in a volume of 10μl.

hydrophobicities, on all three alkylsilica phases examined many of these peptides individually eluted within a narrow and characteristic ranges of organic modifier percentages irrespective whether the 10mM ammonium acetate, pH 6.0, or the 15mM orthophosphoric acid, pH 2.1, primary mobile phase conditions were employed, i.e., the retention of these peptides to a large extent was independent of secondary solution equilibria mediated by difference in the pH or buffer composition. Thus peptide T-2 (SAVTALWGK) eluted near to 25% acetonitrile under the two buffer and pH conditions irrespective of which stationary phase was selected. This type of retention behaviour has numerous precedents with peptides separated on alkylsilicas. The dominant role which the organic solvent exerts on the distribution equilibria for peptides separated under regular reversed-phase conditions (5, 20-23) is well recognised. Many peptides exhibit pronounced dependencies of their logarithmic capacity factors on the volume fraction of the organic solvent in the eluent. Where very steep dependencies exist, the retention behaviour of such peptides may become relatively unresponsive to secondary solution equilibrium effects due to changes in ionisation, solvation or buffer-ion complexation.

As expected, the smaller, more polar peptides were not strongly retained on the various reversed phases under the pH2.1 or the pH6.0 gradient elution conditions. With several Hb tryptic peptides, significant selectivity differences were noted presumably mediated by the different pH and/or buffer ion conditions. For example, peptides T-10 and T-14 elute much rapidly, and peptides T-3 and T-13 are retained longer, with the low pH, phosphate-based eluent when compared to the ammonium acetate conditions. Several studies (5, 24-26) have shown that relatively non-polar peptides (as is the case with T-10 and T-14) have augmented retentions on capped alkylsilica stationary phases with mobile phases near pH 7.0 but that the retention of these peptides is significantly decreased at lower pHs, e.g., pH 2.0-4.0 in the presence of phosphate, perchlorate or acetate ions. This situation is in contrast to peptides containing acidic amino acid residues at the C-terminus or endo-positions in the sequence (as is the case with T-3 and T-5) where retention times are generally smaller at high pHs, but progressively increase as the pH is lowered. It is unlikely that the changes seen in the retention behaviour for the above group of the Hb β-chain peptides under the two elution conditions have their origin solely due to differences in solute ionisation, but more likely reflect composite effects which give rise to variations in the effective molecular hydrophobic contact area of the peptide at the stationary phase interface (5). The observed differences nevertheless highlight the important role which secondary solution equilibria can play in modulation peptide selectivity on alkylsilicas. With tryptic fragmentation of proteins, the commonest occurrence of such pH-dependent selectivity changes on alkylsilicas can be anticipated with peptides containing several internal Asp and/or Glu residues

where potentially the side chain ionisation provides additional control for the optimisation of resolution of a complex peptide mixture.

In Fig. 1b, 2b and 3a are shown representative elution profiles for the tryptic peptides of the β-chains of the two variants Hb(Ka) and Hb(Ha) separated under the different chromatographic conditions. Comparison of the elution profiles for the tryptic peptides of the Hb(Ka) and normal β-chains indicated the presence of two new peaks, with the peak corresponding to peptide T-1 of the normal β-chain absent. Amino acid compositions of the appropriate fractions confirmed the substitution β^{6}- (Glu → Lys), thus identifying this variant as HbC. The peptide T-1A (Val^{1} ... Lys) of this HbC β-chain variant was well resolved from the other tryptic fragments under all the elution conditions examined. The dipeptide T-1B (Glu^{7} -Lys) resulting from the substitution, however, coeluted with peptide T-8 under the low pH phosphate conditions. The variant Hb(Ha) with an amino acid substitution in peptide T-3 was readily identified from the amino acid composition of the two peptides T-3A (Val^{18} ... Lys^{26}) and T-3B (Ala^{27} ... Arg^{30}) indicative of the substitution β^{26}(Glu → Lys) which is characteristic of the HbE variant. Using similar experimental methods, the variant Hb (Kempsey) was found to have a variant peptide T-11 which showed enhanced chromatographic retention. Subsequent analysis confirmed the β-chain amino acid substitution Asp^{99}→ Asn.

Because of the potential diversity in peptide structure, complete resolution of all the tryptic peptides generated by enzymatic cleavage of different hemoglobin variants (and, in general, of other proteins) is unlikely under a single RP-HPLC gradient elution condition. It should, however, be possible to achieve this goal by exploiting the selectivity differences which arise when two (or more) different elution conditions are employed sequentially. Such approaches have been applied to multidimensional separations of peptides using hydrophobic pairing ion systems (5, 28). The separation of partially resolved peptides can be achieved in several ways on reversed-phases. The simplest in terms of equipment requirements involves discrete rechromatography of the recovered zone using alternative elution systems, i.e., as exemplified by the characterisation of the HbC and HbE variants where peptides partially resolved by the phosphate based eluent could be separated by rechromatography using the ammonium acetate eluent under gradient (cf Fig. 1b, 2b) or isocratic conditions. Alternatively, on-line switching valves can be employed to redirect the appropriate portions of the column effluent directly onto a second coupled column pre-equilibrated to a second set of mobile phase conditions. Practical consideration for the use of coupled column strategies for the RP-HPLC separation of polar solutes, including peptides, have been discussed elsewhere (27, 28). Although the latter approach permits shorter overall analysis times for selected components, it

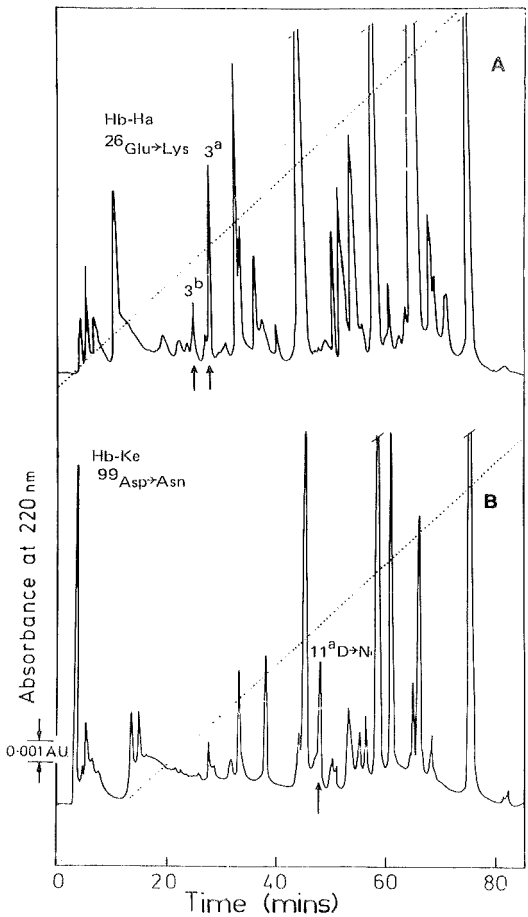


Figure 3. Comparison of the RP-HPLC profiles for the peptides from the tryptic digests of the aminoethylated β-chains of the Hb (Ha) and Hb (Kempsey) variants. Chromatographic conditions as in Fig. 2.

can suffer from reduced detection sensivity and elution behaviour perturbation for small polar peptides due to the effect of larger sample loading volumes.

In conclusion, this publication has further demonstrated that RP-HPLC peptide mapping of the tryptic digests of isolated aminoethylated Hb β-chain variants is a rapid, and extremely versatile technique. Single amino acid substitutions can be observed in the elution profile and readily confirmed by amino acid and/or sequence analysis. The combination of several gradient elution systems used with discrete or coupled column strategies should generally permit unequivocal resolution and assignment to be made for a specific abnormal peptide. Furthermore, Hb variants which do not involve changes in overall charge may now be systematically sought using these rapid RP-HPLC procedures which are becoming of increasing importance for the routine screening of hemoglobinopathies. One benefit of using elution systems of different pH and composition in combination is that advantage can be taken of pH-dependent selectivity changes exhibited peptides on alkylsilica supports. Clearly other combinations of ionic modifiers (e.g., TFA, ammonium bicarbonate) and pH conditions could be used in a similar manner to those employed in the present study to manipulate the resolution of peptide fragments generated in the characterisation of protein variants. The combined use of such alternative systems will be described elsewhere (29).

ACKNOWLEDGEMENTS

The authors are indebted to Miss Evelyn Parker for expert assistance with the numerous amino acid composition analyses. This work was supported in part by the National Health and Medical Research Council of Australia and the Australian Research Grants Committee.

REFERENCES

1. M.T.W. Hearn, J. Liquid Chromatogr., 3, 1255-1276, 1980.

2. M.T.W. Hearn and W.S. Hancock, Trends in Biochem. Sci., 4, 58-62, 1979.

3. M.T.W. Hearn and W.S. Hancock in Biological/Biochemical Applications of Liquid Chromatography (G.L. Hawk, ed.) Marcel Dekker, New York, 242-271, 1979.

4. C.A. Bishop, W.S. Hancock, S.O. Brennan, R.W. Carrell, and M.T.W. Hearn, J. Liquid Chromatogr. 4, 599-612, 1981.

5. M.T.W. Hearn, Adv. in Chromatogr., (J.C. Giddings, P.R. Brown, E. Grushka and J. Cazes) Marcel Dekker, New York, 20, 1-82, 1982.

6. W.A. Schroeder, J.B. Shelton, J.R. Shelton and D. Powars, J. Chromnatogr., 174, 385-392, 1979.

7. J.B. Wilson, H. Lam, P. Pravatmuang, and T.H.J. Huisman, J. Chromatogr., 179, 271-290, 1979.

8. G.J. Hughes, K. Winterhalter and K. Wilson, FEBS Lett., 108, 81-86, 1979.

9. P. Bohlen and G. Leeman, J. Chromatogr., 205, 65-75, 1981.

10. W.A. Mahoney and M.A. Hermodson, J. Biol. Chem. 255, 11199-11203, 1981.

11. T.H.J. Huisman, C. Altay, B. Webber, A.L. Reese, M.E. Graverley, K. Okonjo and J.B. Wilson, Blood, 57, 75-82, 1981.

12. J.B. Shelton, J.R. Shelton and W.A. Schroeder, J. Liquid Chromatogr., 4, 1381-1392, 1981.

13. M.T.W. Hearn, G. Grego and W.S. Hancock, J. Chromatogr., 185, 429-444, 1979.

14. S.J. Su, B. Grego and M.T.W. Hearn, J. Liquid Chromatogr., 4, 1547-1568, 1981.

15. J.B. Clegg, M.A. Naughton and D.J. Weatherall, J. Mol. Biol., 19, 91-96, 1966.

16. R.T. Jones, Cold Spring Harbour Symp. Quant. Biol., 29, 297-302, 1964.

17. T.H.J. Huisman and J.H.P. Jonxis, The Hemoglobinopathies: Techniques of Identification, Marcel Dekker, New York, 1977.

18. M.T.W. Hearn, B. Grego and C.S. Bishop, J. Liquid Chromatogr., 4, 1725-1744, 1981.

19. W.S. Hancock, C.A. Bishop, R.L. Prestidge and M.T.W. Hearn, Anal. Biochem., 89, 203-212, 1978.

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

21. K.E. Bij, Cs. Horvath, W.R. Melander and A. Nahum, J. Chromatogr., 203, 63-84, 1981.

22. M.T. Hearn and B. Grego, J. Chromatogr., 218, 497-507, 1981.

23. B. Grego and M.T.W Hearn, Chromatographia, 14, 589-592, 1981.

24. J.J. O'Hare and E.C. Nice, J. Chromatogr., 171, 209-226, 1979.

25. S. Terabe, R. Konaka and K. Inouye, J. Chromatogr., 172, 163-168, 1979.

26. K. Krummen and R.W. Frei, J. Chromatogr., 132, 27-32, 1977.

27. Y. Tapuhi, N. Miller and B.L. Karger, J. Chromatogr., 205, 325-337, 1981.

28. M.T.W Hearn in HPLC, Advances and Perspectives (Cs. Horvath, ed.) Academic Press, New York, in press, 1982.

29. B. Grego and M.T.W. Hearn manuscript in preparation.

30. S.J. Su, B. Grego, B. Niven and M.T.W Hearn, J. Liquid Chromatogr., 4, 1745-1764, 1981.

31. R.F. Rekker in the Hydrophobic Fragmental Constant, Elsevier, Amsterdam, pp. 301-324, 1977.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 215-228 (1983)

SEPARATION AND ASSIGNMENT OF THE TRYPTIC PEPTIDES OF HUMAN GROWTH HORMONE (hGH) AND THE 20K DALTON hGH VARIANT BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Milton T.W. Hearn*[1], Boris Grego* and George E. Chapman[+]

*St. Vincent's School of Medical Research
Victoria Parade
FITZROY, VICTORIA 3065, AUSTRALIA

[+]National Hormone Laboratory, Department of Biochemistry,
University of Auckland
Auckland, NEW ZEALAND

ABSTRACT

Reversed-phase high performance liquid chromatography (RP-HPLC) has been used to separate the peptides generated by tryptic cleavage of human growth hormone (hGH) and the 20K dalton human growth hormone variant. The total amino acid compositions of both these pituitary proteins has been accounted for on the basis of these chromatographic mapping procedures. Structural analyses of the peptides isolated from semi-preparative RP-HPLC separations has confirmed that the primary structure of the variant differs from that of the sequence of hGH by deletion of the amino acid residues 32-46.

INTRODUCTION

Two distinct forms of growth hormone occur in extracts of frozen human pituitary glands. In addition to the well characterised 22K dalton version of human growth hormone (22K-hGH), a variant of lower molecular weight can be detected (1, 2) in the extract by polyacrylamide gel electrophoresis in

---

[1] Author to whom correspondence should be addressed.
High performance liquid chromatography of amino acid, peptides, and proteins XXXVIII. For the previous paper in the series, see Ref. 23.

0148-3919/83/0602-0215$3.50/0

sodium dodecylsulphate. This variant, designated 20K-hGH, comprises 5-10% of the growth hormone content of the extract and occurs in various aggregated forms with the majority present as a heterologous dimer with the usual 22K dalton version of hGH. The 20K-hGH variant can be resolved from the 22K-hGH form by ion-exchange chromatography in the presence of 6M urea. Structural studies on the purified 20K dalton hGH by Lewis and co-workers (2, 3) and Chapman et. al. (4) have indicated that the amino sequence of the variant differs from that of the usual 22K dalton form by deletions of amino acid residues 32 to 46. This structural relationship raises the possibility that the deletion is a result of a variation in excision of an intervening gene sequence, a possibility in accord with the observation that an intron has been found (5, 6) in the pre-hGH gene immediately following the DNA sequence coding for residue 31. These findings have stimulated our interest in the mechanism of expression and regulations of the two growth hormone mRNA species as well as in the functional biological differences, including differences in the growth promoting and potentiating diabetogenic activities (7), of these two structurally related proteins. As part of these studies (8), we have utilised reversed-phase high performance liquid chromatography (RP-HPLC) to monitor the isolation and solution properties of these proteins. In this report, we describe the application of these techniques to the separation of the tryptic peptides of hGH and the identification of the aberrant peptides of the 20K dalton variant.

MATERIALS AND METHODS

Equipment

A Waters Assoc. (Milford, Mass.) model 224 liquid chromatograph equipped with gradient elution capability, a U6K sample injector and a model 450 variable wavelength detector were employed. Chromatographic separations were performed on μBondapak C18 columns (30 x 0.4 cm, 10μm) and Radial Pak A cartridges (10 x 0.8 cm). Sample injections were performed with Pressure Lok liquid syringes, series B110 from Precision Sampling (Baton Rouge, La.). The pH measurements were performed with a Radiometer PHM64 pH meter equipped with a combination glass electrode.

Chemicals and Reagents

Orthophosphoric acid and sodium dihydrogen phosphate were ARISTAR grade reagents obtained from BDH (Poole, U.K.), sodium heptanesulphate was obtained from Fluka, A.G. (Switzerland) and ammonium hydrogen carbonate from Ajax Chem. (Aus.). Human growth hormone was isolated from extracts of frozen human pituitaries by the method of Chapman et al. (4) based on the procedure of Lumley-Jones et al. (9). The 20K-hGH variant was obtained from the hGH-dimer pool by fractionation on DEAE-cellulose using a linear gradient of 65mM to 130mM ammonium bicarbonate in 6M

urea. Sigma type XI trypsin (DPCC treated) was used for all the digests. The trypsin was dissolved in 1mM HCl, 2mM Ca Cl_2 at a concentration of 1mg/ml.

AMINO ACID COMPOSITIONAL AND STRUCTURAL ANALYSES

Protein and peptide samples were hydrolysed in vacuo at 110° for 24 hours in 250ul of 6N HCl containing 0.1% phenol. Amino acid compositions were determined on a Jeol Analyser. N-terminal residues were determined by the method of Percy and Buchwald (10) and automated sequential analysis followed established procedures. Reduction and carboxyamidomethylation of the proteins was carried out essentially as described by Cresfield et al. (11). In brief, the hormone (20mg) was dissolved in freshly deionised 6M urea (1.5ml) and 1M Tris-HCl, pH 8.6, (300µl) added. The solution was flushed with nitrogen and β-mercaptoethanol (40µl) was added under a nitrogen barrier and incubated for 2 hours at room temperature. Iodoacetamide (102mg) dissolved in 100mM Tris-HCl, pH 8.6 (700µl) was added to the reaction mixture. Following a 15 min. reaction period, the mixture was chromatographed on a Sephadex G25 column (15 x 0.9cm) equilibrated with 10mM ammonium bicarbonate adjusted to pH 9.5 with ammonium hydroxide. The Cys-(Cm)-protein was recovered by lyophilisation. All enzymatic digests of the proteins and their Cys-(Cm)-derivatives were carried out at 37° using a substrate: trypsin ratio of 100:1. The proteins were dissolved in 50mM Tris-HCl, 2mM $CaCl_2$, pH 7.8, at a concentration of 10mg/ml. Reactions were quenched by the addition of 1/10 volume of a solution of 10mM PMSF/methanol, and the samples lyophilised. The one-letter code for the amino acids is used as described by Dayhoff (12).

CHROMATOGRAPHIC CONDITIONS

All chromatograms were carried out at ambient temperature (ca. 18°). Bulk solvents and mobile phases were filtered through a 0.5um Millipore filter (Millipore Corp., Bedford, Mass.), and degassed by sonication. Flow rates were maintained between 1.0ml/min. and 4.0ml/min. Detection of peptides was at 210nm. All samples were dissolved in the mobile phase corresponding to the initial elution condition with 10-1500µg sample injected in volumes of 10-150µl. The elution fractions were collected manually immediately adjusted to pH7 with 15mM NaOH, the organic solvent partially removed under nitrogen and lyophilised. Analytical and semi-preparative separations were carried out using the standard stainless analytical columns (30 x 0.4 cm). Semi-preparative separations were also carried out using the Waters RCM module with the flexible walled Radial Pak A cartridges (10 x 0.8cm) and 100mM ammonium bicarbonate based eluents. Where peptide assignments or compositional ambiguities arose, recovered peptide fractions were rechromatographed using different elution protocols as described previously (13-15).

RESULTS AND DISCUSSION

The use of RP-HPLC for the structural mapping of polypeptides and proteins is now a well established technique (for recent reviews, see ref. 15, 16). Compared to conventional methods for analytical or micro-preparative separation of protein digests, RP-HPLC procedures offer numerous advantages including short analysis times, generally good sample recoveries and ready appraisal of the homogeneity of the eluted components. Because of the excellent reproducibility and resolution which can be achieved with these RP-HPLC procedures, they also permit direct assessment of the enzymatic digestion conditions including the optimal time course. The potential of these methods to map homologous proteins has been exploited previously in studies reported (17-20) from this laboratory including the identification of hemoglobin variants and phosphorylated forms of rat caseins. In view of the close structural relationship between hGH and the 20K dalton variant, we anticipated similar methods would simplify the separation and analysis of the peptides derived from the tryptic digestion of these two proteins.

In preliminary experiments, the progress of the tryptic digestion of hGH as a function of time was followed by analytical separations (10-150µg protein digest) on a µBondapak C_{18} column using a linear 60 min. gradient from 0.1% orthophosphoric acid to 50% acetonitrile - 50% water - 0.1% orthophosphoric acid (17) at a flow rate of 2ml/min. Representative chromatograms covering digestion times from 15 min. up to 6 hours are shown in Fig. 1. Under these chromatographic conditions 22K-hGH elutes with a retention time of 55.6 min. As is evident from Fig. 1, no significant changes in the elution profile of the native 22K-hGH were obtained with digestion periods longer than ca. 60 min. However, to ensure complete digestion of the 22K-hGH and the 20K dalton variant, a digestion time of 6 hours was employed in the comparative studies.

Comparison of the elution profiles (Fig. 2) obtained for the separation of the tryptic peptides of 22K-hGH (1500µg) and the 20K dalton variant (1200µg) clearly shows the close similarity between these two proteins. Compositional analyses of the various major peaks are provided in Tables I and II. The elution profile for the separation of the tryptic digest of hGH (1400µg) on the Radial Pak A column using a linear 3 hours gradient from aqueous 100mM ammonium bicarbonate to 50% acetonitrile - 50% water - 100mM ammonium bicarbonate is shown in Fig. 3. For clarity of presentation in Table II, the tryptic peptides of the 20K dalton variant are numbered accordingly to the order they would appear in the final sequence of the protein rather than by alignment with hGH itself. Inspection of the composition and chromatographic data reveals several salient features. Firstly, the sum of the amino acid compositions of the tryptic peptides 22K-hGH (T-1 to T-21) shown in Table I and of the tryptic peptides of the 20K dalton

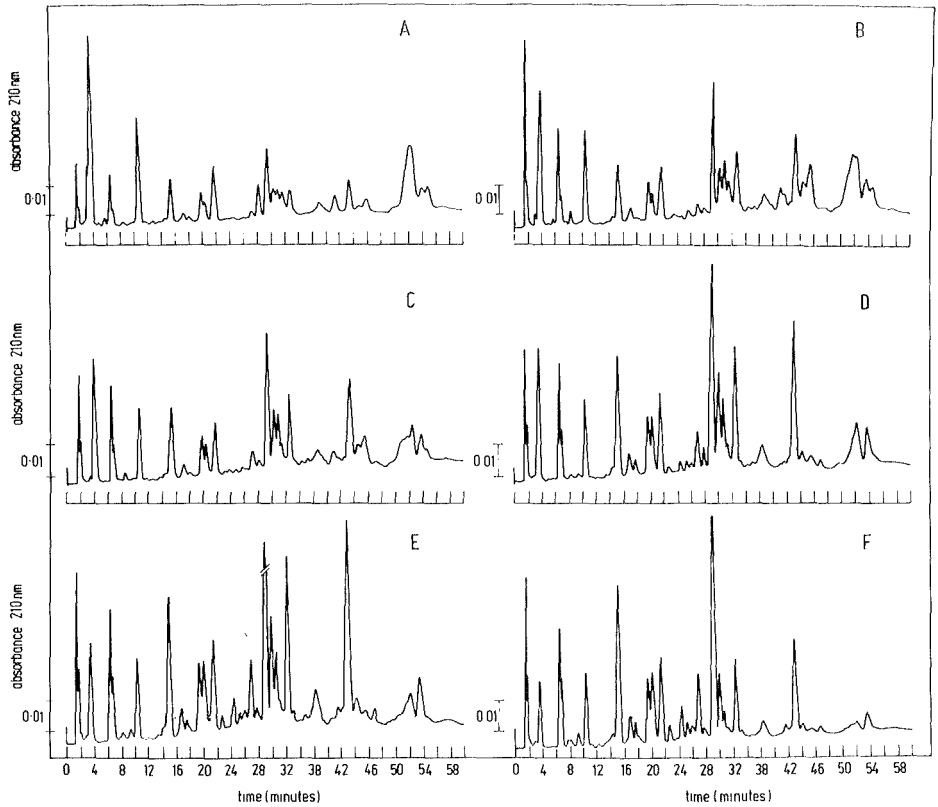


Figure 1. Time course of tryptic (DPCC-treated) digestion of the 22K dalton human growth hormone. All digestions were carried out in 50mM Tris-HCl, 2mM Ca Cl_2, pH 7.8 buffer at 37° using a protein: enzyme ratio of 100:1. Reactions were quenched at 15, 30, 60, 120, 240 and 360 min. by the addition of 10mM PMSF methanol and samples (ca. 150µg) lyophilised. Chromatographic conditions: column, µBondapak C_{18}; flow rate, 2ml/min.; mobile phase, 60 min. linear gradient from 0.1% orthophosphoric acid to 50% acetonitrile - 50% water - 0.1% orthophosphoric acid.

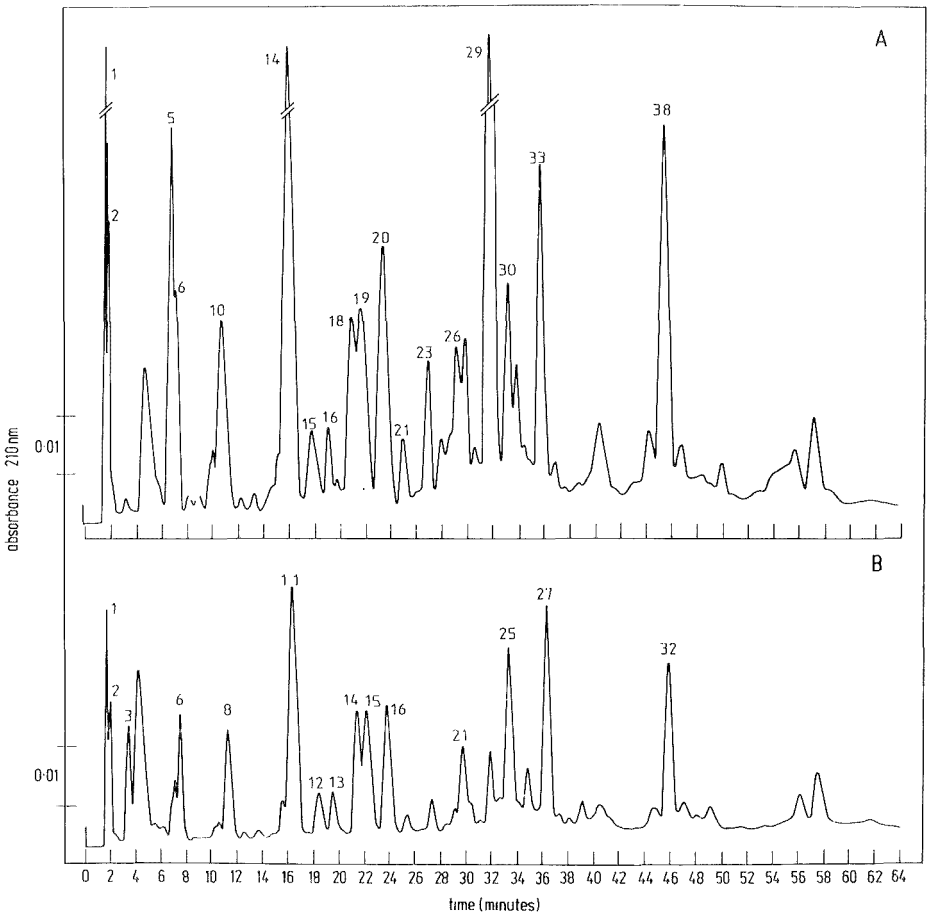


Figure 2. Semi-preparative reversed phase separations of (A) 22K-hGH digest (1500μg) and (B) 20K-hGH digest (1200μg). Chromatographic conditions as in Figure 1.

TABLE I

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES* OF INTACT hGH SEPARATED BY RP-HPLC

Amino Acid	T-1	T-2	T-3	T-4	T-5	T-6 + T-16∇	T-7	T-8	T-9	T-10	T-11
	1-8	9-16	17-19	20-38	39-41	42-64 + 159-167	65-70	71-77	78-94	95-115	116-127
Asx		2.0(2)		1.1(1)		3.0(3)		1.1(1)		3.8(4)	1.1(1)
Thr	0.9(1)			0.7(1)		2.3(2)	0.8(1)				1.2(1)
Ser	1.1(1)					4.6(5)		0.7(1)	1.6(2)	3.7(4)	
Glx				4.2(5)	2.1(2)	3.4(3)	3.6(4)	1.0(1)	2.8(3)		2.8(3)
Pro	1.8(2)			1.1(1)		2.7(3)			0.8(1)		
Gly						1.0(1)				0.9(1)	1.7(2)
Ala		1.0(1)	1.0(1)	1.8(2)						2.0(2)	
Cys						1.4(2)					
Val									1.1(1)	2.5(3)	
Met		0.9(1)									0.8(1)
Ile	0.9(1)			0.9(1)		1.1(1)			2.0(2)		0.8(1)
Leu	1.1(1)	2.0(2)		2.0(2)		4.1(4)		2.9(3)	4.8(5)	3.1(3)	2.0(2)
Tyr				1.8(2)		2.8(3)				1.8(2)	
Phe	0.8(1)	1.0(1)		1.8(2)		2.8(3)			1.2(1)	1.0(1)	
His			0.9(1)	1.0(1)							
Lys				0.9(1)	1.0(1)		1.1(1)			0.9(1)	
Arg	1.0(1)	1.0(1)	1.1(1)			1.8(2)		1.0(1)	1.0(1)		1.0(1)
Trp									a(1)		
Total Residues	8	16	19	38	41	73	79	86	103	124	136
Peak	20	18	1	29	1	33	2	15	38	30	26

* Peptides are numbered in order of their position in the final sequence.

+ Amino acid sequence numbers.

∇ Not present in the Cys(Cm)-hGH digest, but replaced by peptide T-6 (YSFLQNPQTSLCFSESIPTPSNR) and peptide T-16 (NYGLLYCFR).

The values in parentheses are the number of residues based on the sequence.

TABLE I (Continued)

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES* OF INTACT hGH SEPARATED BY RP-HPLC

Amino Acid	T-12	T-13	T-14	T-15	T-17	T-18	T-19	T-20 + T-21[K]	Total
	128-134	135-140	141-145	146-158	168	168-178	169-178	179-183 + 184-191	
Asx	1.1(1)			5.1(5)		1.6(2)	1.8(2)		20
Thr		0.6(1)	0.9(1)	1.1(1)		0.8(1)	0.8(1)		10
Ser	1.0(1)		1.0(1)	0.9(1)				1.9(2)	18
Glx	0.9(1)	1.1(1)	0.7(1)			1.2(1)	1.1(1)	2.1(2)	27
Pro	0.8(1)								8
Gly	1.0(1)	1.0(1)						1.8(2)	8
Ala				1.1(1)					7
Cys								0.5(2)	4
Val						0.9(1)	0.8(1)	1.7(2)	7
Met						0.6(1)	0.9(1)		3
Ile		0.9(1)						0.8(1)	8
Leu	1.1(1)			1.9(2)		1.1(1)	0.8(1)		26
Tyr			0.9(1)						8
Phe		1.0(1)		1.0(1)		0.9(1)	0.9(1)	1.0(1)	13
His				0.9(1)					3
Lys		0.9(1)	1.0(1)	1.0(1)	1.0(1)	2.0(2)	0.7(1)		9
Arg	1.1(1)					1.1(1)	1.0(1)	1.0(1)	11
Trp									1
Total Residues	143	149	154	167	168	178	178	191	191
Peak	6	10	5	14	1	16	19	14	

* Peptides are numbered in order of their position in the final sequence.
± Amino acid sequence numbers.
▽ Not present in the Cys(Cm)-hGH digest, but replaced by peptide T-6 (YSFLQNPQTSLCFSESIPTPSNR) and peptide T-16 (NYGLLYCFR).
κ Not present in the Cys(Cm)-hGH digest, but replaced by peptide T-20 (IVQCR) and peptide T-21 (SVEGSCGF).
a Not quantitated.
The values in parentheses are the number of residues based on the sequence.

TABLE II

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES* OF 20K DALTON hGH SEPARATED BY RP-HPLC

Amino Acid	T-1	T-2	T-3	T-4 +	T-14√	T-5	T-6	T-7	T-8	T-9	T-10	T-11
	1-8	9-16	17-19	20-49 +	144-152	50-55	56-62	63-79	80-100	101-112	113-119	120-125
Asx		2.0(2)		4.3(4)			1.1(1)		4.0(4)	0.8(1)	1.1(1)	
Thr	1.0(1)			2.6(3)		1.0(1)				1.0(1)		0.9(1)
Ser	1.0(1)			3.1(4)			0.8(1)	1.5(2)	2.8(4)		0.9(1)	
Glx				4.9(5)		4.0(4)	1.1(1)	3.0(3)		3.0(3)	1.1(1)	1.1(1)
Pro	2.0(2)			2.9(3)				0.8(1)			0.8(1)	
Gly				1.1(1)					1.2(1)	2.0(2)	1.0(1)	1.0(1)
Ala		1.0(1)	0.9(1)	1.1(1)					2.0(2)			
Cys				0.9(2)								
Val								1.1(1)	2.3(3)			
Met		0.9(1)								0.7(1)		
Ile	0.9(1)			1.2(1)				1.9(2)		0.9(1)		1.0(1)
Leu	1.2(1)	1.9(2)		4.8(5)			2.6(3)	4.7(5)	3.1(3)	2.1(2)	1.0(1)	
Tyr				2.6(3)					1.5(2)			
Phe	0.9(1)	1.2(1)		3.6(4)				1.2(1)	1.0(1)			1.0(1)
His			0.8(1)	0.8(1)								
Lys						1.0(1)			0.8(1)			0.9(1)
Arg	0.9(1)	0.9(1)	1.0(1)	1.9(2)			0.9(1)	1.0(1)		1.0(1)	1.0(1)	
Trp								a(1)				
Total Residues	8	16	19	68		74	81	98	119	131	138	144
Peak	16	14	1	27		2	12	32	25	21	6	8

* Peptides are numbered in order of their position in the final sequence.
\+ Amino acid sequence numbers
√ Not present in the Cys-(Cm)-20K-hGH digest, but replaced by peptide T4 (LHQLAFDTYQEFNPQTSLCFSESIPTPSNR) and peptide T-14 (NYGLLYCFR)
a Not quantitated.
The values in parentheses are the number of residues based on the sequence.

TABLE II (Continued)

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES* OF 20K DALTON hGH SEPARATED BY RP-HPLC

Amino Acid	T-12	T-13	T-15	T-16	T-17	T-18 + T-19[θ]	Total
	126-130	131-143	153	153-163	154-163	164-168 + 169-176	
Asx		4.7(5)		1.8(2)	1.3(2)		20
Thr	0.9(1)	1.1(1)		1.1(1)	0.9(1)		10
Ser	1.0(1)	0.9(1)				1.4(2)	17
Glx	0.9(1)			1.2(1)	1.1(1)	2.0(2)	22
Pro							7
Gly						1.8(2)	8
Ala		1.1(1)					6
Cys						0.9(2)	4
Val				1.1(1)	0.9(1)	1.7(2)	7
Met				0.7(1)	0.8(1)		3
Ile						1.0(1)	7
Leu		2.0(2)		1.2(1)	1.0(1)		25
Tyr	1.1(1)						6
Phe		0.9(1)		1.0(1)	1.0(1)	0.9(1)	12
His		0.9(1)					3
Lys	0.8(1)	1.0(1)	1.0(1)	2.1(2)	0.8(1)		7
Arg				0.9(1)	0.9(1)	1.1(1)	11
Trp							1
Total Residues	149	162	163	163	163	176	176
Peak	3	11	1	13	15	11	

* Peptides are numbered in order of their position in the final sequence.
+ Amino acid sequence numbers
√ Not present in the Cys-(Cm)-20K-hGH digest, but replaced by peptide T4 (LHQLAFDTYQEFNPQTSLCFSESIPTPSNR) and peptide T-14 (NYGLLYCFR)
θ Not present in the Cys-(Cm)-20K-hGH digest, but replaced by peptide T-18 (IVQCR) and peptide T-19 (SVEGSCGF).
a Not quantitated.
The values in parentheses are the number of residues based on the sequence.

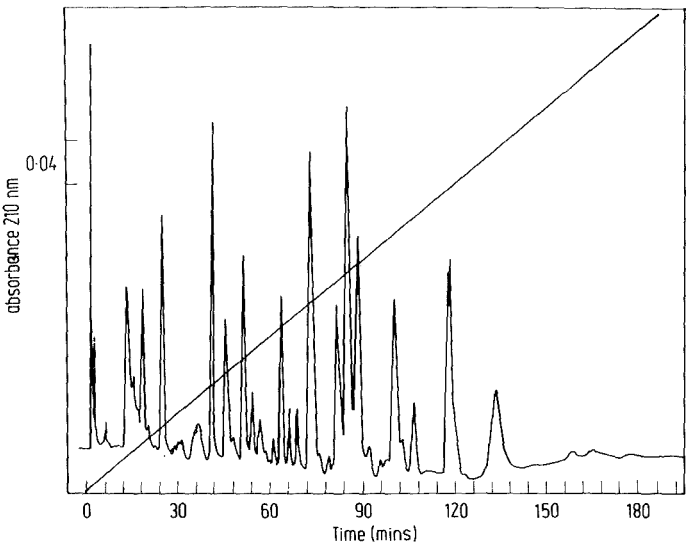


Figure 3. Separation of the tryptic peptides of human growth hormone (1400µg) on a Radial Pak A column at a flow rate of 1.5ml/min. using a 3-hour linear gradient generated from aqueous 100mM ammonium bicarbonate to 50% acetonitrile in 100mM ammonium bicarbonate.

hGH variant (T-1 to T-19) shown in Table II completely account for the intact 22K-hGH and 20K-hGH proteins respectively. Secondly, tryptic peptides T-1 to T-3, T-7 to T-13 and T-15 to T-21 of the 22K-hGH correspond in composition and retention characteristics to the tryptic peptides T-1 to T-3, T-5 to T-11 and T-13 to T-19 of the 20K dalton hGH variant. It is noteworthy that the tryptic peptides T-18 and T-19 of hGH (which correspond to the pair T-16 and T-17 of the hGH variant) are well resolved despite the single N-terminal lysine difference. These peptides, respectively (KDMDKVETFLR) and (DMDKVETFLR), correspond to residues 168-178 and 169-178 in the sequence of hGH. The relative elution order observed for these two peptides on the microparticulate octadecyl-silica with the low pH phosphate mediated elution condit ; used is in accord with the known (21, 22) influence of an ada onal N-terminal lysine residue on peptide selectivity. Thirdly, ident cal chromatographic profiles were obtained for the corresponding peptides obtained from the digests of the Cys-(Cm) - proteins. In these cases, only peak 1 was shown to contain more than one tryptic peptide (corresponding to T-3, T-5 and T-17 of 22K-hGH). Under isocratic conditions with aqueous 50mM sodium dihydrogen phosphate, these peptides were not retained on the

μBondapak C_{18} column, but they can be resolved using a pairing ion elution system such as 15mM sodium heptane sulphonate, pH 3.0, (7, 21, 22) thus allowing unequivocal assignment for T-3 (AHR), T-5 (EQK) and T-17 (K). In the digest of the 20K dalton variant the peptide EQK (22K-hGH-T-5) is absent.

The results presented above indicate that portion of peptide T-4, peptide T-5 and part of peptide T-6 of hGH are not found in the 20K dalton hGH variant. These three peptides correspond to residues 20-38, 39-41 and 42-64 of the hGH sequence. Automated sequence analysis of this region of interest contained in peptide T-4 of the 20K dalton variant has shown that the partial sequence is LHQLAFDTYQEFNPQTSLC .... which corresponds in position to residues 20-31 and 47-53 of 22K-hGH. These results are consistent with the conclusion made in earlier studies by Lewis et al. (3) and Chapman et al. (4) that the N- and C- terminal sequences of the 20K dalton hGH variant are identical to the 22K dalton hGH form, but differs internally due to a 15 residue deletion corresponding to residues 32-46 of hGH.

Peak 5 shown in Fig. 2a eluted with a retention time of 6.7 min. and contained tryptic peptide T-14 of 22K-hGH. The compositional analysis of this peptide (Thr (0.9), Ser (1.0), Glx (0.7), Tyr (0.9) and Lys (1.0)) was in accord with values expected for a peptide with sequence QTYSK. Although the same peptide was present in the elution profile of the tryptic digest of the 20K dalton variant, an additional peptide (peak 3, Fig. 2b) of the same amino and composition, but shorter retention time (3.6 min.) was also obtained. The decreased retention on the octadecylsilica support shown by this peptide is consistent with a Gln → Glu change. The most plausible explanation for this observation is that the 20K dalton hGH variant, as isolated by the ion exchange procedure, contains material with either Gln or Glu at position 126 of 20K-hGH (position 141 on the hGH sequence). The origin of this difference is currently under investigation.

In summary, the separation of the tryptic peptides of the 22K dalton hGH and the 20K dalton hGH variant by RP-HPLC is described. By comparison with the known sequence of hGH, it was evident that all of the anticipated peptides were recovered thus permitting the sequence relationship between the 22K dalton and the 20K dalton hGH versions to be confirmed.

ACKNOWLEDGEMENTS

These studies were supported in part by a grant from the National Health and Medical Research Council of Australia (to M.T.W.H.).

REFERENCES

1. R.N.P. Singh, B.K. Seavey, and U.J. Lewis, Endocrinol. Res. Commun., 1, 449-464, 1974.

2. U.J. Lewis, J.T. Dunn, L.F. Bonewald, B.K. Seavey and W.P. Vanderlaan, J. Biol. Chem., 253, 2679-2687, 1978.

3. U.J. Lewis, L.F. Bonewald and L.J. Lewis, Biochem. Biophys. Res. Commun., 92, 511-516, 1980.

4. G.E. Chapman, K.M. Rogers, T. Britain, R.A. Bradshaw, O.J. Bates, C. Turner, P.D. Cary and C. Crane-Robinson, J. Biol. Chem., 256, 2395-2401, 1981.

5. J.C. Fiddes, P.H. Seeburg, F.M. Denoto, R.A. Hallewell, J.D. Bacter and H.M. Goodman, Proc. Natl. Acad, Sci., 76, 4294-4298, 1979.

6. M. Wallis, Nature, 284, 512, 1980.

7. U.J. Lewis, R.N.P. Singh, G.F. Tutwiller, M.B. Sigel, E.F. Vanderlann and W.P. Vanderlaan, Rec. Prog. Horm. Res., 36, 477-509, 1980.

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

9. R. Lumley-Jones, G. Benker, P.R. Salacinski, T.D. Lloyd and P.J. Lowry, J. Endocr., 82, 77-86, 1979.

10. M.E. Percy and B.M. Buchwald, Anal. Biochem., 45, 60-67, 1972.

11. A.M. Crestifield, S. Moore and W.H. Stein, J. Biol. Chem., 238, 622-628, 1963.

12. M.O. Dayhoff in Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Silver Spring, Md., U.S.A., DZ, 1972.

13. M.T.W Hearn, B. Grego and W.S. Hancock, J. Chromatogr., 185, 429-444, 1979.

14. M.T.W. Hearn, B. Grego and C.A. Bishop, J. Liquid Chromatogr., (4) 1725-1744, 1981.

15. M.T.W. Hearn in 'Advance in Chromatography' (J.C. Giddings, P.R. Brown, E. Grushka and J. Cazes eds) Vol. 20, Chapter 2, Marcel Dekker, New York, 1982, 1-82.

16. M.T.W. Hearn, J. Liquid Chromatogr., 3, 1255-1276, 1980.

17. W.S. Hancock, C.A. Bishop, R.L. Prestidge, and M.T.W. Hearn, Anal. Biochem., 89, 203-212, 1978.

18. C.A. Bishop, W.S. Hancock, S.O. Brennan, R.W. Carrell and M.T.W. Hearn, J. Liquid Chromatogr., 4, 599-612, 1981.

19. A.A. Hobbs, B. Grego, M.G. Smith and M.T.W. Hearn, J. Liquid Chromatogr., 4, 651-659, 1981.

20. M.T.W. Hearn and W.S. Hancock, Trends in Biochem. Sci., 4, 58-61, 1979.

21. M.T.W. Hearn and B. Grego, J. Chromatogr., 203, 349-363, 1981.

22. M.T.W. Hearn, S.J. Su, and B. Grego, J. Liquid Chromatogr. 4, 1547-1567, 1981.

23. S.J. Su, B. Grego, B. Niven and M.T.W. Hearn, J. Liquid Chromatogr., 4, 1745-1764, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 229-248 (1983)

CHARACTERIZATION OF PROTEIN-PROTEIN AND PROTEIN-LIGAND INTERACTIONS BY HIGH PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

Kenneth C. Ingham, Thomas F. Busby, Donald H. Atha
and Hilda Forastieri
American Red Cross Plasma Derivatives Laboratory
9312 Old Georgetown Road
Bethesda, Maryland 20814

ABSTRACT

HPLC has been used in our laboratory to characterize a wide range of protein-protein and protein-ligand interactions. In a study of the dissociation and recombination of human chorionic gonadotropin subunits, HPLC provided a fast and sensitive method for directly observing the state of association of samples equilibrated under various conditions. The α subunit (15 Kd) was easily resolved from the β subunit (23 Kd) using a Toyo Soda type SW 3000 column (0.8 x 60 cm) eluting at 1 ml/min. The subunit was poorly resolved from the intact hormone (38 Kd) in agreement with results obtained using conventional exclusion media. In another study, the same column was used to assess the degree of aggregation of various protease inhibitors (antithrombin III (AT III), C1-inactivator and α_1-proteinase inhibitor) after heating, as part of an effort to determine conditions under which these potentially therapeutic proteins might withstand pasteurization to reduce the risk of transfusion hepatitis. The ability of AT III (65 Kd) to bind heparin (5-20 Kd) and thrombin (37 Kd) was also readily ascertained by HPLC. When native inhibitor was premixed with excess heparin, its elution shifted toward the void and became broader due to the polydispersity of the mucopolysaccharide. By contrast, formation of a complex with thrombin only slightly increased the rate of elution of AT III. Nevertheless, the extent of complex formation could be determined from the depletion of the much slower moving thrombin peak. The latter approach proved useful for characterizing thrombin after covalent attachment of fluorescent probes.

 0148-3919/83/0602-0229$3.50/0

INTRODUCTION

Our interest in HPLC was triggered by the advent of an exclusion column through which at least some of the proteins being studied in our laboratory would pass freely. The ability to ask simple short term questions regarding the state of association of a macromolecule and realizing the answer in only 20-30 min has had a definite impact on our program. Most of our experience is with systems involving strong complexes which are stable on the column. These include (1) protease inhibitors, which form covalent complexes with their target enzymes, and some of which interact with heparin, a mucopolysaccharide which binds strongly but reversibly, shifting the elution of the inhibitors to shorter times, (2) human chorionic gonadotropin (hCG) subunits which interact reversibly to form an active hormone with an association constant $> 10^8 M^{-1}$ at room temperature; and (3) heated proteins whose denaturation is sometimes detected by the formation of aggregates, or by loss of ability to interact with other macromolecules, either event being amenable to detection and characterization by HPLC . The latter application is part of a program to determine conditions which prevent denaturation so that potentially therapeutic human plasma proteins can be pasteurized to reduce the risk of transfusion hepatitis. The purpose of this report is to share our experience with high-performance size-exclusion chromatography so that others might appreciate the power of this tool. The results presented here

have been published in preliminary form as part of the abstracts of the International Symposium on HPLC of Proteins and Peptides, November, 1981, Washington D.C.

MATERIALS AND METHODS

Highly purified hCG and its subunits were obtained from Dr. Robert Canfield of Columbia University via the Center for Population Research, National Institute Child Health and Human Development, NIH. Antithrombin III and C1-Ina were gifts from Dr. Milan Wickerhauser of this institution. The purification and characterization of AT III has been described (1). C1-Ina was partially purified from plasma by ion exchange chromatography and further purified as described herein. Alpha$_1$-proteinase inhibitor was obtained from Dr. Charles Glazer of the Institutes of Medical Sciences, San Francisco. C1s was partially purified by a modification of the procedure of Bing et al., (2) and Taylor et al., (3). Thrombin was a gift of Dr. John Fenton, of the New York State Department of Health, Albany. Bovine Pancreatic Trypsin was purchased from Cal Biochem and used without further purification.

All chromatography experiments were conducted at room temperature using 0.8 x 60 cm exclusion columns donated by or purchased from Toyo Soda Manufacturing (TSK G3000SW). Unless otherwise stated, the eluting buffer was 0.02 M potassium phosphate pH 7.35, plus 0.15 M NaCl. Elution was controlled at 1

ml/min with a Waters M600A pump. Samples were injected with a Waters U6K injector and protein elution was monitored at 280 nm with a Waters Model 450 Variable Wavelength detector. Other details of the experimental conditions can be found in the figure legends and in the appropriate references given in the text.

RESULTS

Human Chorionic Gonadotropin

Human chorionic gonadotropin (hCG) is a glycoprotein comprised of two nonidentical subunits, α and β, held together by noncovalent bonds (4,5). The dissociated subunits are biologically inactive but activity can be recovered by combining the isolated subunits under physiological conditions (6). Studies of the kinetics of dissociation and recombination would be facilitated by the availability of a fast and sensitive method to assess the state of association of a given sample. The TSK G3000SW column has proven useful for this purpose and we have utilized HPLC along with that of fluorescence polarization, to study the reversible dissociation of hCG subunits at elevated temperatures and neutral pH (7).

Figure 1A illustrates the elution of several samples of hCG which were incubated in 0.01 M potassium phosphate pH 7, at various temperatures until equilibrium was established. Profile A refers to the intact native hormone which elutes as a single symmetrical peak, while profile B refers to a sample incubated

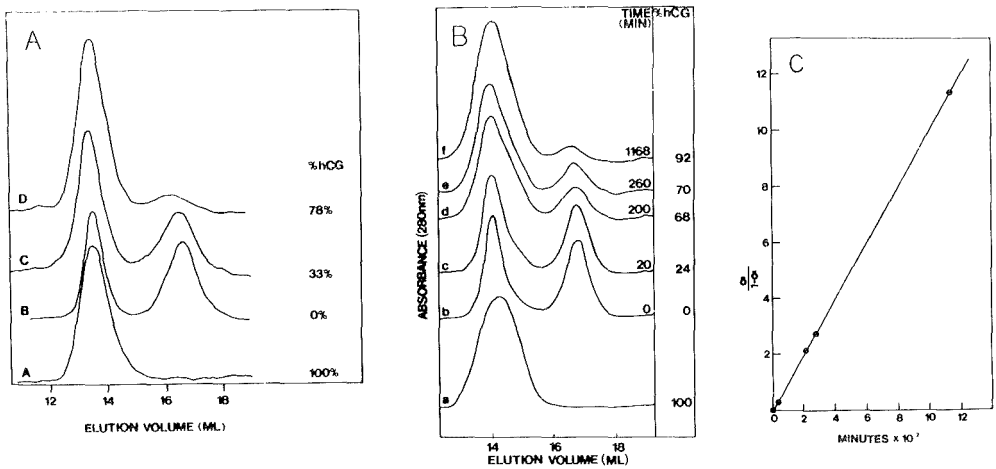


Figure 1 - Analysis of the state of association of human chorionic gonadotropin subunits using Toyo Soda exclusion column TSK G3000SW.

A) Determination of the extent of dissociation of hCG (54 M) following prolonged incubation at neutral pH at various temperatures a) 25°C (100% associated), b) 80°C (100% dissociated), c) 65°C (33% associated), d) dissociated subunits incubated at 37°C for 18 hrs (78% asssociated).

B) Analysis of the time couse for recombination of acid-dissociated hCG subunits (22 μM) a) intact native hormone, b) dissociated subunits obtained by incubation at pH 2.5 and 37°C for 1 hr, c-f) samples withdrawn at the indicated times after adjustment of pH to 7.

C) Second order kinetic plot of the recombination data shown in panel b. $k = 385 M^{-1} min^{-1}$.

at 80° C resulting in complete dissociation. The α subunit elutes several minutes later than either the intact hormone or the β subunit, the latter two being unresolved on this column. A similar lack of resolution between hCG and hCG-α is obtained by conventional chromatography using Sephadex G-100 (8). The rate of subunit recombination at room temperature under the conditions of elution in Fig. 1A is too slow to cause a significant change in the state of association during the time required for the analysis. The relative areas under the two peaks in profile B is consistent with the known extinction coefficients of the subunits. Profile C refers to a sample equilibrated at 65° C. Analysis of the areas indicates that 33% of the hormone is in the associated state, corresponding to a dissociation constant of 7.3×10^{-5}M. Using this approach it was possible to determine the dependence of the subunit dissociation constant on temperature (7). Profile D illustrates the reversibility of the reaction. This profile refers to a sample which was first equilibrated at 80° C causing complete dissociation and then held overnight at 37° resulting in 78% recombination; longer times are required for complete recombination under these conditions.

Figure 1B illustrates the time course for recombination of acid-dissociated hCG subunits in 0.01 M potassium phosphate pH 7 at 37° C. The intact hormone (profile A) was dissociated by incubating at pH 2.5 and 37° (profile B) and then neutralized. A sample withdrawn immediately after neutralization had the same

profile. Samples withdrawn at various times after neutralization showed increasing amounts of recombination (profiles C-F). A second order plot of the kinetic data obtained in this fashion is shown in Figure 1C. The rate constant obtained from the slope ($k = 385 M^{-1} min^{-1}$) is in good agreement with that obtained previously by spectral methods (9).

The lack of resolution between hCG and hCG-β on this column as well as on conventional exclusion columns could be due to an unfolding of the isolated β subunit resulting in a larger exclusion radius. However, an unfolded conformation seems unlikely in view of the high polarization of tyrosyl fluorescence (8). Studies with the homologous hormone, ovine lutropin, suggested that most of the unfolding which accompanies subunit dissociation occurs in the α subunit (10). An alternative explanation for the peculiar elution behavior would be the existence of a highly asymmetric but perhaps rigid conformation for hCG-β which combines with a flexible α subunit to form a more spherical complex whose exclusion radius is similar to that of isolated β. It is of interest that the elution peak for hCG-β is narrower than that of the intact hormone suggesting a lower diffusion coefficient for the former, consistent with an asymmetric conformation.

Antithrombin III

Antithrombin III (AT III) is a circulating protease inhibitor which inactivates thrombin and most serine proteases of

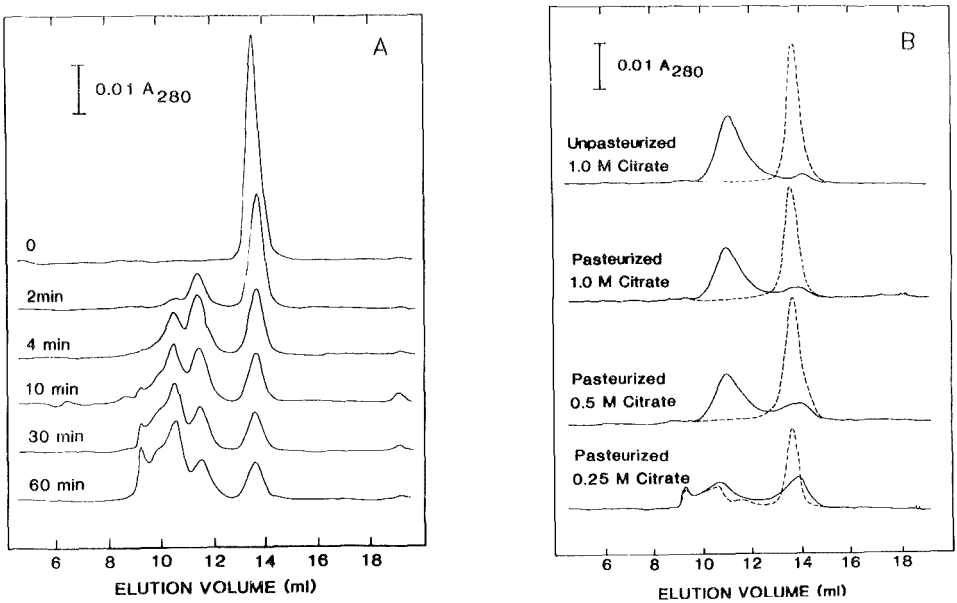


Figure 2 - A: Formation of high molecular weight aggregates of antithrombin III as a function of time at 60°C. AT III samples (2 mg/ml) were heated in 0.02 M potassium phosphate buffer, pH 7.35 containing 0.15 M NaCl, removed at indicated times, stored at 2°C and subsequently applied to the column. B: Measurements of heparin binding to samples of AT III in the presence of citrate. AT III samples (2 mg/ml) containing the indicated concentrations of sodium citrate were heated at 60°C in 0.02 M potassium phosphate buffer, pH 7.35, containing 0.15 M NaCl, removed after 10 hr, cooled to 2°C and subsequently applied to the HPLC. The heated and control samples were dialyzed against 0.02 M potassium phosphate buffer, pH 7.35, plus 0.15 M NaCl, mixed with heparin for a final concentration of 0.7 mg/ml AT III, 12 mg/ml heparin and applied to the column. (----), no heparin; (————), heparin added. Adapted from Busby et al., (18).

the blood coagulation system (11,12). It is of potential therapeutic interest for replacement therapy in patients with inherited or acquired deficiencies. A major risk associated with such therapy is transfusion hepatitis and one approach to reduce this risk is to determine conditions under which the protein can be pasteurized (for 10h at 60°) to inactivate the virus. In the case of AT III, the presence of 0.5 M citrate is sufficient to preserve most of the activity (13). In the absence of stabilizers, the inhibitor undergoes extensive aggregation and loses its ability to bind the mucopolysaccharide heparin and inhibit thrombin. All three of these events can be readily monitored by HPLC.

Figure 2A illustrates the time course of aggregation in a sample of AT III which was heated at 60° C. Higher molecular weight forms are detectable after 2 min and by 60 min very little monomer remains. The multiple peaks presumably correspond to different oligomers which are formed and can be resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (data not shown). This aggregation can be entirely prevented, even after 10h at 60° C, by addition of sodium citrate at concentrations ≥0.5 M. This is illustrated by the dashed profiles in Figure 2B where it can be seen that the samples pasteurized in 0.5 or 1.0 M citrate are indistinguishable from the unpasteurized control, whereas that pasteurized in 0.25 M citrate showed substantial aggregation.

The solid profiles in Figure 2B illustrate the manner in which HPLC can be used to assess the ability of AT III to bind heparin. Premixing with an excess of the mucopolysaccharide, which is essentially transparent at 280 nm, shifts the absorbance profile of unpasteurized AT III to an earlier elution time with a concomitant broadening of the peak. This broadening is presumably due to the polydisperse nature of the crude heparin used in these experiments (M_r = 5000-20000) Similar results are seen with the sample pasteurized in 1.0 M citrate whereas the sample pasteurized in 0.5 M citrate appeared to contain slightly more monomer which did not shift. The 0.25 M citrate sample contained appreciable amounts of higher molecular weight species even in the absence of heparin and addition of heparin caused only a partial depletion of the monomer peak. This approach obviously has considerable potential for characterizing the interaction of AT III with various heparin preparations and for rapid assessment of the ability of various modified antithrombins to interact with heparin.

HPLC has also been used to monitor the formation of complexes between AT III and one of its target enzymes, thrombin. This is part of an effort in our laboratory to prepare fluorescent-labeled derivatives of AT III and thrombin which retain their ability to form enzyme-inhibitor cmplexes in a time dependent reaction whose rate could be directly monitored by observing changes in fluorescence polarization. The elution

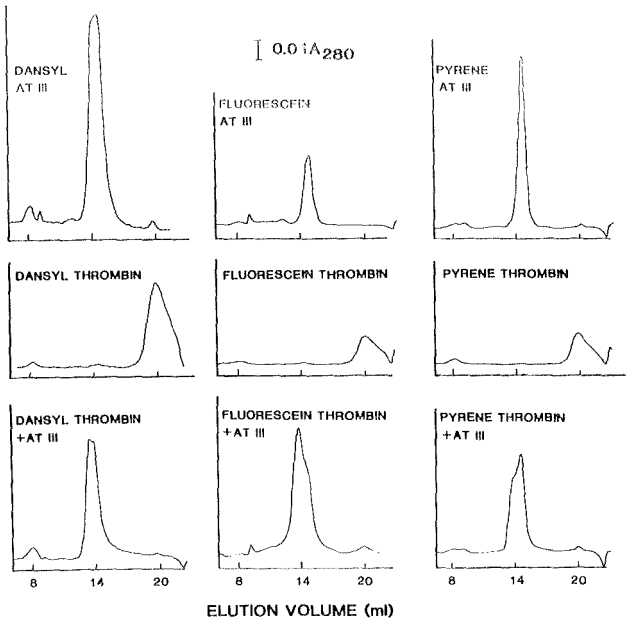


Figure 3 - High performance size exclusion chromatography of various fluorescent-labeled derivatives of thrombin, antithrombin, and their mixtures. Samples (25 µl) of thrombin (1 mg/ml) or AT III (1-2 mg/ml) were injected into the HPLC and eluted with 0.01 M K phosphate pH 7.35, 0.1 M NaCl. Complex formation was observed in mixtures containing labeled thrombin (0.5 mg/ml) and an excess of unlabeled AT III (1.0 mg/ml). These mixtures were incubated 15 minutes at 37°C prior to injection (50 µl).

profiles of several labeled derivatives of AT III and thrombin are shown in the upper and middle portion of Figure 3. Thrombin, which has a molecular weight of about 37,000, elutes close to the salt volume (22 ml) suggesting a tendency to interact with the column. When mixed with excess unlabeled AT III, the thrombin peak disappeared and was replaced by a new peak emerging slightly

ahead of AT III, as shown in the lower three panels of Figure 3. Thus, the presence of the labels did not interfere with the ability to form complexes.

The small difference between the elution of AT III and that of the AT III-thrombin complex could be due to retardation of the latter due to interaction of the thrombin moiety with the column or to conformational changes accompanying complex formation which result in a more compact structure. This behavior is reminiscent of that seen with the combination of gonadotropin subunits, as discussed above and is in contrast to the behavior seen with heparin which caused a substantial shift in the elution of AT III. The latter effect can be attributed to the formation of a heparin:AT III complex from which substantial portions of the polysaccharide chain protrude, producing a larger effective radius.

Alpha$_1$-Proteinase Inhibitor

Alpha$_1$-proteinase inhibitor (α_1-PI, also termed α_1-antitrypsin) is the most abundant protease inhibitor in plasma (14). Individuals with inherited deficiencies of this protein are predisposed to pulmonary emphysema and might benefit from replacement therapy with the purified protein (15). As with AT III, pasteurization to inactivate hepatitis virus results in loss of activity and extensive aggregation. Treatment for 10h at 60°C in the absence of stabilizers shifted all of the protein into

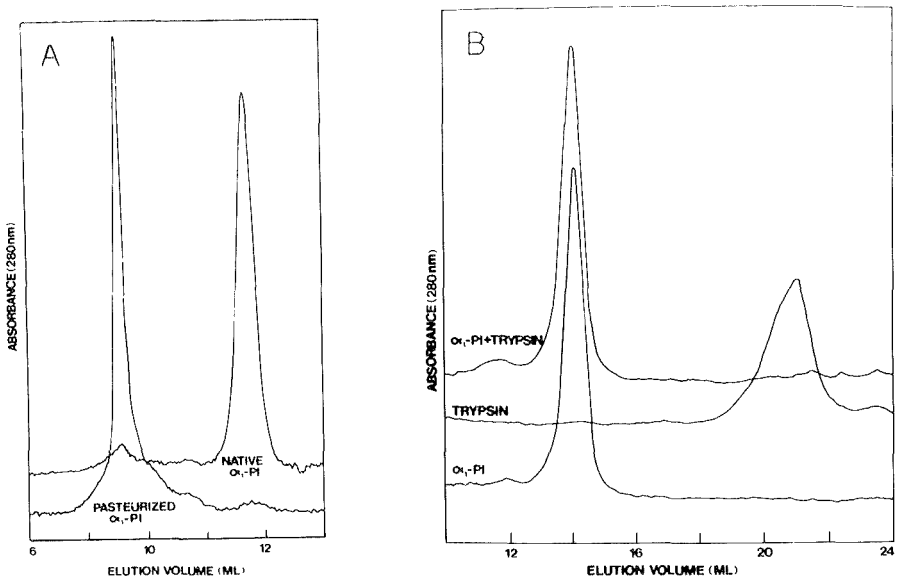


Figure 4 - A: Heat induced aggregation of α_1-proteinase inhibitor (α_1-PI). The protein (0.50 mg/ml) was "pasteurized" at 60°C for 10 hr in 0.02 M potassium phosphate buffer, pH 7.35 containing 0.15 M NaCl. B: complex formation between α_1-PI (0.56 mg/ml) and trypsin (1.0 mg/ml) incubated together for 10 min at 25°C. Note disappearance of trypsin peak without formation of new peak.

the void of the TSK-G3000SW column (Figure 4A) suggesting that the aggregation is even more extensive than with AT III.

Formation of a complex between α_1-PI and trypsin is illustrated in Figure 4B. The peak due to free trypsin near 21 ml disappears when the enzyme is premixed with excess α_1-PI. No new peak is apparent, suggesting that the enzyme-inhibitor complex elutes in the same position as the free inhibitor. The small peak eluting ahead of the main peak in the upper profile is

not large enough to account for more than a small fraction of the total amount of complex expected. This behavior is also similar to that seen with hCG/hCG-β and AT III/thrombin.

C1-Inactivator

C1-inactivator (C1-Ina) is another protease inhibitor involved in the regulation of the first component of the complement system. Partially purified preparations of this protein have been shown to be effective in the treatment of hereditary angioedema patients (16). HPLC has been useful in monitoring the heat-induced aggregation of C1-Ina as part of an effort to define conditions for pasteurization.

Figure 5A illustrates the manner in which HPLC was used for rapid purification of C1-Ina and one of its target enzymes, C1s, from crude concentrates of each protein. The upper two profiles represent the impure starting materials which gave multiple peaks. When antigen positive fractions were pooled and reapplied to the column, the two lower profiles were obtained indicating size homogeneity. A portion of each of the peak fractions was rechromatographed, separately, and after mixing, producing the profiles in Figure 5B. The upper profile of the mixture contained a new peak, well resolved from either constituent, presumably representing the enzyme-inhibitor complex. Residual material in the position of the individual components could be due to impurities of the same size as the components or to unreacted or inactive components.

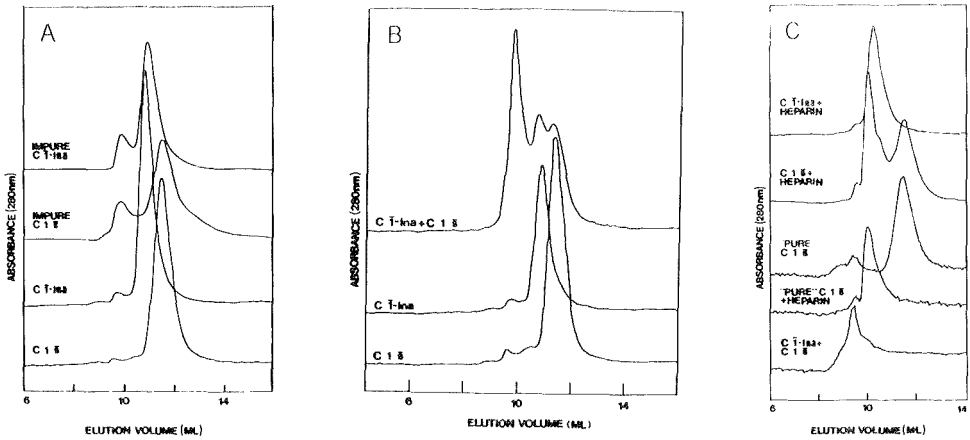


Figure 5 - Use of HPLC to further purify, characterize heparin binding, and demonstrate complex formation of C1s and C1-inactivator (C1-Ina). A: After injection of crude preparations of C1s and C1-Ina (top two profiles), and pooling the immunoreactive fractions, substantial purification of both proteins from larger molecular weight contaminants was achieved (bottom two profiles). B: Upon mixing the C1s and C1-Ina purified in 5A, followed by a 30 min incubation prior to injection into the HPLC, the formation of a complex could be demonstrated (top curve). C: When the purified C1s and C1-Ina from 5A were premixed with heparin before injection, the protein-heparin complexes eluted earlier than the proteins alone (compare top two curves with those in panel B). The profile of the C1s-heparin mixture (second from top) indicates the presence of a substantial amount of non-heparin binding protein contaminant which elutes in the same position as C1s alone. Further purification on heparin-Sepharose, gave material represented by the middle curve from which the non-heparin binding contaminant was eliminated, as shown by the second curve from bottom. A mixture of the purified C1s and C1-Ina, after prior incubation for 30 min, exhibited a single peak, presumably that of the enzyme-inhibitor complex (lower curve). The eluting buffer for these analyses was 0.033 M sodium citrate, 0.045 M NaCl, pH 7.

When C1-Ina was premixed with heparin, its elution shifted from 10.9 ml (Figure 5B) to about 10.3 ml (Figure 5C). When C1s was premixed with heparin, only part of the material shifted indicating the presence of impurities which fail to interact with heparin (Figure 5C). Therefore, the impure preparation of C1s was fractionated by affinity chromatography on heparin Sepharose. Gradient elution with NaCl produced a fraction which was more homogenous on HPLC and all of which was shifted toward the void when premixed with heparin (Figure 5C). When this material was mixed with C1-Ina (in the absence of heparin), a complex was formed which eluted earlier than either component alone with less evidence of residual material in the position of the individual components. The lower signal to noise ratio seen in the lower profiles of Figure 5C is due to the higher sensitivity range required on the samples which were now quite dilute because of the successive manipulations. These examples nicely illustrate the power of this tool in probing the purity and functional integrity of interacting proteins.

DISCUSSION

The molecular weights and elution properties of the various proteins mentioned in this report are summarized in Figure 6. The filled circles represent globular protein standards, identified in the legend, which were chromatographed sequentially on the same column on a single day. The open symbols represent

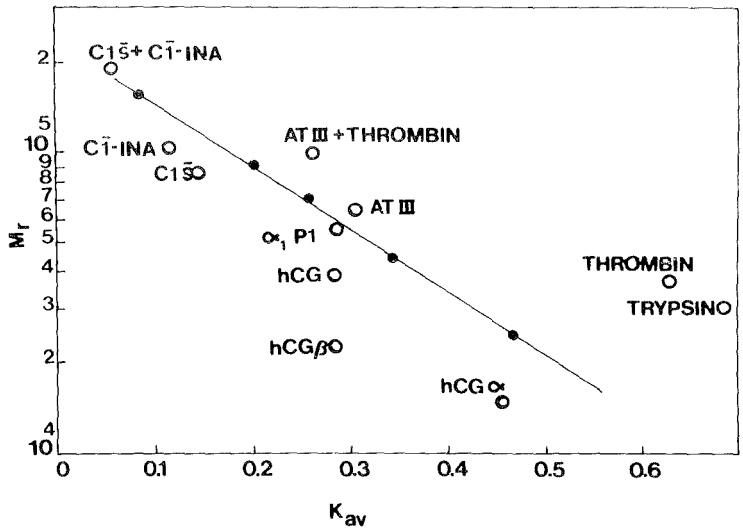


Figure 6 - Summary of the elution parameters for the various proteins used in these studies. The filled circles represent standard globular proteins in order of decreasing M_r: phosphorylase, aldolase, albumin, ovalbumin and chymotrypsinogen. K_{av} values were calculated as $(V_e - V_o)/(V_{total} - V_o)$.

data gathered over a period of two years on three different columns of identical size. Thrombin and trypsin are both basic proteins which may tend to interact with the column accounting for their late elution. The relatively early elution of some of the other proteins may be due to the presence of large amounts of carbohydrate in their structures, a property which also affects the elution from conventional exclusion media (17). In our experience, the value of this tool for a particular application can best be determined by a few preliminary experiments with the

proteins involved. It is difficult to predict a priori whether a given protein will be amenable to analysis. For example, human plasma fibronectin, a large (440 Kd) glycoprotein interacted so strongly with the column that it could not be eluted in the absence of denaturants. However, with 6 M urea in the buffer, the column was useful for observing the degradation of fibronectin into smaller fragments. The high expense of these columns discourages extensive exploration of the use of denaturants. This is unfortunate since exclusion chromatography under dissociating conditions could provide a fast and sensitive alternative to SDS-PAGE. These and other applications are certain to emerge as improved and less expensive column materials become available.

ACKNOWLEDGEMENTS

This report is contribution no. 553 from the American Red Cross Blood Services. The work was also supported by NIH Grant HL-21791 and RR 05737 and by Research Career Development Award to Kenneth C. Ingham (HL-00325). We are indebted to Toyo Soda Corporation for donation of a column which allowed this work to be initiated. Thanks also to Alicia R. Williams for typing this manuscript.

Present address of Donald H. Atha is Massachusetts Institute of Technology, Whitaker College of Health Sciences, Technology and Management, Cambridge, MA 02139.

ABBREVIATIONS

hCG, human chorionic gonadotropin; AT III, Antithrombin III; α_1-PI, α_1-proteinase inhibitor; C1-Ina, C1-inactivator or C1-inhibitor.

REFERENCES

1. Wickerhauser, M. and Williams, C., Isolation of Antithrombin III Without Interfering With Ethanol Fractionation System, Thromb. Haemost., 42, 168, (abstract 400), 1979.

2. Bing, D.H., Andrews, J.M., Morris, K.M., Cole, E. and Irish, V., Purification of Subcomponents C1q, C1$\bar{r}$ and C1$\bar{s}$ of the First Component of Complement From Cohn Fraction I by Affinity Chromatography, Prep. Biochem., 10, 269, 1980.

3. Taylor, P.A., Fink, S., Bing, D.H. and Painter, R.H., Further Studies on the Identification of the Subcomponents of the First Component of Complement After Affinity Chromatography of Human Serum on IgG-Sepharose, J. Immunol., 118, 1722, 1977.

4. Canfield, R.E., Morgan, F.J., Kammerman, S., Bell, J.J. and Agosto, G.M., Studies of Human Chorionic Gonadotropin, Recent Prog. Horm. Res., 27, 121, 1971.

5. Morgan, F.J. and Canfield, R.E., Nature of the Subunits of Human Chorionic Gonadotropin, Endocrinology, 88, 1045, 1971.

6. Swaminathan, N. and Bahl, O.P., Dissociation and Recombination of the Subunits of Human Chorionic Gonadotropin, Biochem. Biophys. Res. Commun., 40, 422, 1970.

7. Forastieri, H. and Ingham, K.C., Thermal Stability of Human Chorionic Gonadotropins: Reversible Dissociation of Subunits at Neutral pH, J. Biol. Chem., 257, 7976, 1982.

8. Forastieri, H. and Ingham, K.C., Acid- and Temperature-Induced Conformational Changes in Human Chorionic Gonadotropin and the Mechanism of Subunit Dissociation, Arch. Biochem. Biophys., 205, 104, 1980 .

9. Ingham, K.C., Weintraub, B.D. and Edelhoch, H., Kinetics of Recombination of the Subunits of Human Chorionic

Gonadotropin. Effect of Subunit Concentration, Biochemistry, 15, 1720, 1976.

10. Ingham, K.C. and Bolotin, C., Intrinsic and Extrinsic Fluorescence Probes of Subunit Interactions in Ovine Lutropin, Arch. Biochem. Biophys., 191, 134, 1978.

11. Rosenberg, R.D. and Damus, P.S., The Purification and Mechanism of Action of Human Antithrombin-Heparin Cofactor, J. Biol. Chem., 248, 6490, 1973.

12. Davie, E.W. and Hanahan, D.J., Blood Coagulation Proteins in The Plasma Proteins, Putnam, F.W., ed., 2nd Ed., Academic Press, New York, 1977, Vol. III, pp. 421.

13. Holleman, W.H., Coen, L.J., Capobianco, J.O. and Barlow, G.H., Isolation of Human Antithrombin-III by Affinity Chromatography on Heparin-Agarose, Thromb. Haemost., 38, 201, 1977.

14. Heimburger, N., Haupt, H. and Schwick, H.G., Proteinase Inhibitors of Human Plasma in Proceedings of the International Research Conference on Proteinase Inhibitors, Fritz, H. and Tschesche, H., eds., Berlin and New York, 1971, p.1.

15. Gadek, J.E., Klein, H.G., Holland, P.V. and Crystal, R.G., Replacement Therapy of Alpha 1-Antitrypsin Deficiency. Reversal of Protease-Antiprotease Imbalance Within the Alveolar Structures of PiZ Subjects, J. Clin. Invest., 68, 1158, 1981.

16. Gadek, J.E., Hosea, S.W., Gelfand, J.A., Santaella, M., Wickerhauser, M., Triantaphyllopoulos, D.C., and Frank, M.M., Replacement Therapy in Hereditary Angioedema. Successful Treatment of Acute Episodes of Angioedema with Partly Purified C1 Inhibitor, N. Engl. J. Med., 302, 542, 1980.

17. Ward, D.N. and Arnott, M.S., Gel Filtration of Proteins, With Particular Reference to the Glycoprotein, Luteinizing Hormone, Anal. Biochem., 12, 296, 1965.

18. Busby, T.F., Atha, D.H. and Ingham, K.C., Thermal Denaturation of Antithrombin III. Stabilization by Heparin and Lyotropic Anions, J. Biol. Chem., 256, 12140, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 249-258 (1983)

EXCLUSIVE USE OF HIGH PRESSURE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF THE COMPLETE AMINO ACID SEQUENCE OF THE 12K FRAGMENT OF AVIAN SARCOMA VIRUS STRUCTURAL PROTEIN p27

Ajit S. Bhown, J. Claude Bennett,
Thomas W. Cornelius and Eric Hunter
Department of Medicine, Division of Clinical Immunology and Rheumatology, and Department of Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294

ABSTRACT

Using exclusively high pressure liquid chromatography for the protein and peptide separation complete primary structure of the 12,000 molecular weight (12K) amino terminal (1-87 residues) fragment obtained by mild acid hydrolysis of p27 (Avian Sarcoma Virus structural protein) has been determined. The sequence was established by direct degradation of the native molecule and its (12K) peptides isolated by molecular exclusion and reverse phase HPLC.

 0148-3919/83/0602-0249$3.50/0

INTRODUCTION

Avian Sarcoma viruses (ASV) possess a major internal structural polypeptide (p27) of 27,000 daltons, which forms the inner capsid shell of the virion (1,2). This protein, p27, is highly conserved and carries group specific antigenic determinants, that characterize the avian sarcoma viruses (ASV). The relatedness of the conserved or interspecies antigenic determinants of a viral protein can be considered indicative of the taxonomy of the viruses (3,4). The present study was undertaken to delineate the total primary structure of p27 to help locate the conserved areas of amino acid sequences responsible for its antigenicity.

The study of protein structure presents unique problems that require innovative approaches. The limiting factor in achieving the total primary structure of a protein molecule is the availability in sufficient amounts of pure homogeneous sample. The classical methods of protein purification involving gravity fed open column chromatography suffer with numerous major drawbacks. These include sample losses and larger volumes not to mention the time of separation which frequently extends into days. HPLC has emerged as an excellent alternative to overcome these otherwise insurmountable problems of the protein chemist.

Earlier methods (5) of separation of p27 by gel filtration utilizing 6 M quanidine hydrochloride were time consuming and involved desalting which frequently resulted in significant losses. Ion exchange chromatography, a commonly used technique for peptide separation, is nearly always associated with low recoveries. In addition all of these techniques are time consuming. By utilizing selective methods of chemical and enzymatic cleavages followed by their size and reverse phase separation by HPLC, it is feasible to obtain structural

information on protein and peptides on small amounts of sample. Furthermore, the speed of separation, high yields and use of volatile buffer system makes the technique even more attractive for structural analysis.

p27, when subjected to mild acid catalyzed hydrolysis, generates two fragments with 12,000 (12K) and 15,000 (15K) molecular weights as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Earlier (6) we have reported the alignment of these two peptides in p27, in the present study, we report the determination of complete primary structure of the 12,000 daltons (12K) fragment (6) employing only HPLC and volatile buffer systems.

MATERIALS AND METHODS

Chemicals: Sequencer grade chemicals were purchased from Spinco Division, Beckman Instruments (Palo Alto, CA). Methanol (Omnisolv) for high pressure liquid chromatography was the product of MCB Manufacturing Chemicals Inc. (Cincinnati, OH). All other chemicals were of highest purity grade and were obtained from Pierce and/or Fisher.

Virus: Rous Sarcoma virus (RSV-PRC) was propagated in chick-helper factor-negative C/E chicken embryo cells as described earlier (6)

Purification of p27: The viral protein p27 was purified by high pressure liquid chromatograph (HPLC) from Rous Sarcoma virus (RSV-PRC) as described by Bhown et al. (7) except the sample was not reduced and alkylated.

Amino acid analysis: For amino acid analysis protein samples were hydrolyzed at 110°C with constant boiling HCl in sealed evacuated ampules for 24 hrs and analyzed on a Durrum D-500 analyzer.

Acid Digestion: Acid catalyzed hydrolysis of the single aspartylprolyl bond in p27 was performed as described by Bhown et al. (6).

Separation of the products of acid digestion: Acid cleaved fragments were separated by HPLC (Waters Associates, Milford, Mass.) equipped with two 6000A solvent delivery pumps, a U6K universal septumless injector, model 440 dual channel absorbance detector and four I-125 columns (Part #84601 Waters Associates) attached in series. A mixture of acetic acid: propanol: water (20:15:65) was employed as a mobile phase, at a flow rate of 0.2 ml/min. The effluent was monitored at 280 nm, the peaks were collected manually and lyophilized.

Cyanogen bromide digestion: The low molecular weight fragment (12K) was digested with 30 fold excess of cyanogen bromide in 70% formic acid for 4 hrs at room temperature. The digest was diluted with water and lyophilized.

Separation of the products of cyanogen bromide digestion: The products of CNBr digestion were fractionated by HPLC employing two I-60 columns (Part #85250 Waters Associate) in series and acetic acid: propanol: water (20:15:65) as mobile phase at 0.2 ml/min flow rate. Peak fractions were manually collected and lyophilized.

Acetylation: The 12K fragment was dissolved in 0.2 M N-ethylmorpholine acetate pH 9.0 and 10 fold molar excess of acetic anhydride over protein was added during constant vortexing. The mixture at this stage develops a cloudy appearance. The sample was lyophilized.

Trypsinization: The acetylated 12K fragment was solubilized in 1% ammonium-bi-carbonate and digested at 37°C for 4 hrs with TPCK trypsin (Worthington Biochemical Corp. Freehold, N.J.) solubilized in 1mM HCl, using an enzyme:protein ratio of 1:50. The trypsin digest was lyophilized and peptides fractionated on reverse phase HPLC.

Tryptic peptide isolation: Peptides resulting from trypsin digestion of acetylated 12K were chromatographed on a Waters C_{18} μBondapak column (0.46 x 25cm part #) as follows: Solvent "A" was 0.1% TFA and "B" 60% acetonitrile containing 0.1% of TFA. The column was developed with a 60 min linear gradient of 0% B to 100% B at a flow rate of 2.0 ml/min with a 10 min delay between start of the gradient and sample injection. The effluent was monitored at 206 nm in an LKB absorbance detector (Uvicord model #2138).

Amino acid sequence analysis: Sequential degradation of proteins and peptides was achieved on a modified Beckman 890C automated sequenator as described by Bhown et al. (8). Phenylthiohydantoin (PTH) derivatives of cleaved amino acids were identified and quantitated by HPLC as described earlier (8).

RESULTS AND DISCUSSION

Separation and amino acid sequence of 12K: In order to obtain large peptides which could be easily, separated by molecular exclusion HPLC and sequenced p27 was subjected to mild acid catalyzed hydrolysis as reported earlier (6). This chemical cleavage yields two major fragments with 12,000 (12K) and 15,000 (15K) molecular weight. Separation of these products (80-90% yield) was carried out by gel permeation HPLC (Figure 1). The amino terminal amino acid sequence analyses of these fragments confirmed that the 12K fragment orginated from the amino terminus of the parent molecule (6).

Amino terminal sequence: Quantitative recovery (>90% yield) of p27 by gel permeation HPLC (7) have permitted assignment of 36 residues from the amino terminus as shown in figure 4.

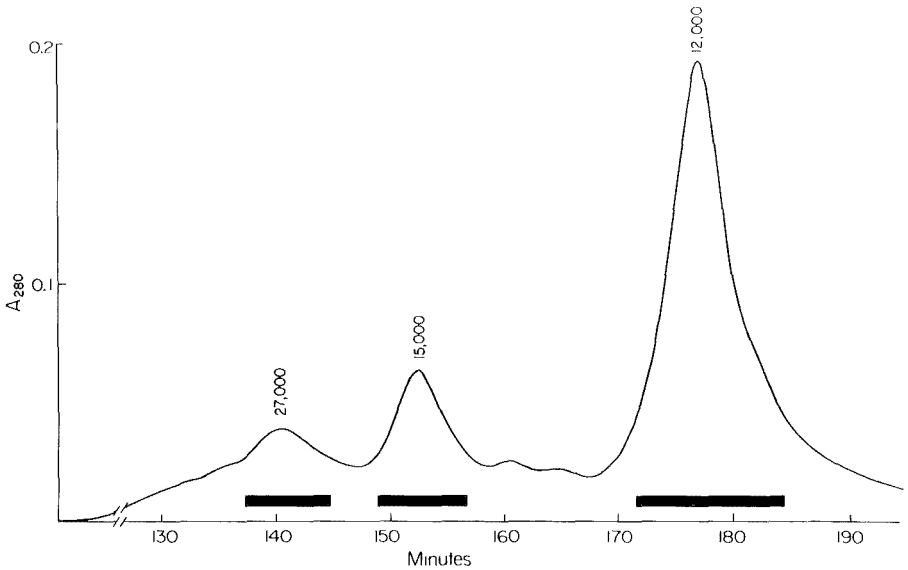


Figure 1. Molecular exclusion HPLC pattern of the products of acid catalyzed hydrolysis of p27. Horizontal bars indicate the peak area pooled. For details, see text.

Separation of the products of cyanogen bromide digest of the 12K fragment: Amino acid analysis (not shown) of this fragment (12K) revealed the presence of 4 methionines. Cyanogen bromide digestion of 12K fragment was attempted next which produced three major peptides. These peptides were separated on a low molecular weight exclusion column (I-60) on HPLC. Separation of the peptides is shown in Figure 2. Amino acid sequence analysis of second and third fraction, (in order of elution) helped in establishing the total primary structure of the 12K fragment.

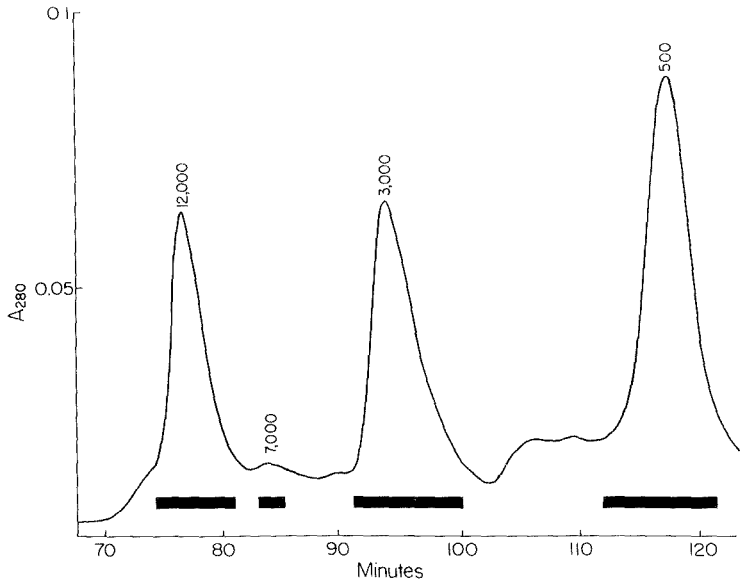


Figure 2. Gel filtration of cyanogen bromide digest of the 12K fragment on I-60 columns attached in tandem. Horizontal bars indicate the peak area pooled. For details, see text.

Separation of tryptic peptides: In order to restrict the trypsin cleavage sites lysine residues of the 12K fragment were irreversibly blocked with acetic anhydride. The separation of tryptic peptides was achieved on reverse phase HPLC and is shown in Figure 3. Tryptic peptides nine and fourteen on amino acid sequence analysis provided the necessary data to complete the structural studies of this fragment.

Amino acid sequence analysis: Peptides (2-4 nmols) obtained by cyangen bromide and trypsin digest were separated by HPLC and sequenced wih an average repetitive yield of 95%. The results are summarized in figure 4.

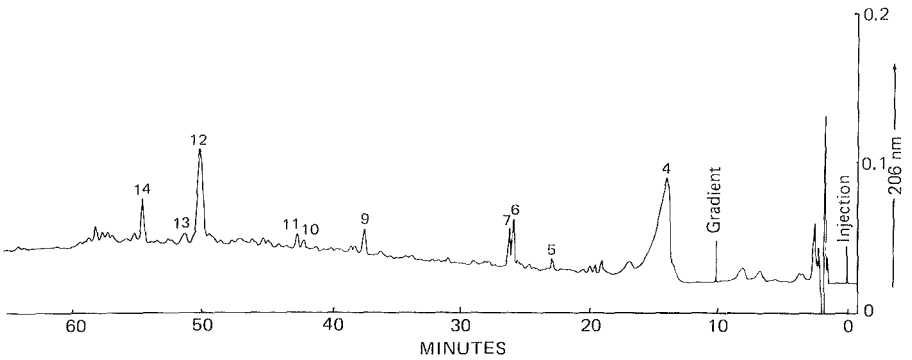


Figure 3. Reverse phase HPLC of tryptic peptides of the acetylated 12K fragment.

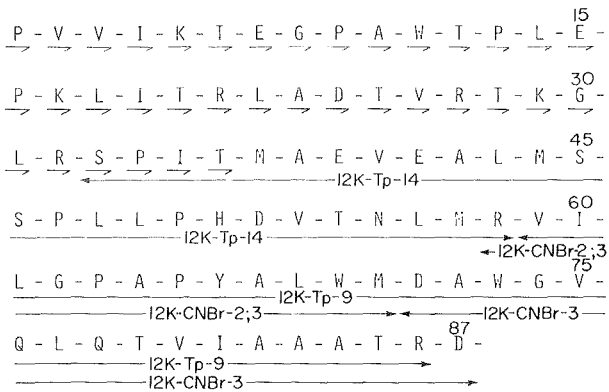


Figure 4. Amino acid sequence of the 12K fragment.

Our HPLC techniques have allowed us to obtain p27 in highly purified form and in high yields that have prompted us to undertake detailed studies of this molecule. The presence of a single aspartylprolyl bond, susceptible to dilute acid almost in the center of the molecule between residues 87-88 simplified the inital approach to sequence analysis. By employing both gel permeation HPLC and reverse phase HPLC we have successfully isolated the necessary cleavage fragments to complete the total primary structure of a 12,000 molecular weight fragment of p27, thus establishing the amino acid sequence of residues 1-87 (Fig. 4). This is in total agreement with that predicted from DNA sequence studies (D. Schwartz, Harvard University, personal communication).

ACKNOWLEDGEMENTS

The authors wish to thank Thomas Cornelius, Jane Ellen Robbins, Karen Buttler, Lisa Kallman and Edgar Hill for their expert assistance, and to Brenda Gosnell and Melissa Ham for typing the manuscript. This work was supported by grants AM 03555, AM 20614, CA-13148 and CA 29884.

REFERENCES

1. Fleissner, E. and Tress, E. (1973) J. Virol. 12:1612-1615.

2. Sarma, P.S., Turner, H.C. and Huebner, R.J. (1964) Virology 23:313-321.

3. Gilden, R.V., Oroszlan, S., and Hatanaka, M. In, Viruses, Evolution and Cancer, Editor E. Kurstak and K. Maramorosch, Academic Press, Inc. New York, 1973, p. 235.

4. Temin, H.M. (1974) Ann. Rev. Genet. 8, 155.

5. Green, R.W., and Bolognesi, D.P. (1974) Anal. Biochem. 57:108-117.

6. Bhown, A.S., Bennett, J.C. and Hunter, E. (1980) J. Biol. Chem. 255:6962-6965.

7. Bhown, A.S., Bennett, J.C., Mole, J.E., and Hunter, E. (1981) Anal. Biochem. 112:128-134.

8. Bhown, A.S., Mole, J.E. and Bennett, J.C. (1981) Anal. Biochem. 110:355-359.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 259-269 (1983)

A GRAPHIC REPRESENTATION OF BINARY MOBILE PHASE OPTIMIZATION IN REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Haleem J. Issaq*, Gary M. Muschik and George M. Janini**
Chemical Carcinogenesis Program
NCI-Frederick Cancer Research Facility
Frederick, MD 21701

ABSTRACT

A method for mobile phase selection for optimal separation in reversed-phase high pressure liquid chromatography is presented. The system is based on a plot of solute retention time versus binary mobile phase composition. A total of five data points are required. The method is simple and does not require a computer for data analysis.

INTRODUCTION

The selection of the mobile phase in high pressure liquid chromatography (HPLC) and thin layer chromatography (TLC) is by far the most critical step for a successful sample separation. The mobile phase not only determines the separation of the components in a mixture, but it also affects resolution and controls selectivity and the time of analysis.

Until recently the mobile phase was selected by trial and error based on the properties of the solute and the stationary phase. Systematic approaches

---

This work was supported by Contract No. NO1-CO-75380, with the National Cancer Institute, NIH, Bethesda, MD 20014.

* Author to whom correspondence should be addressed.

**Present address, Univ. of Kuwait, Chemistry Dept., Kuwait.

0148-3919/83/0602-0259$3.50/0

to the selection of the mobile phase that will give optimum separation using normal and reversed phase TLC plates (1) and HPLC columns (1-4) have been published. The methods are based on statistical calculations, whereby peak pair resolution is plotted versus mobile phase composition. The resulting overlapping resolution mapping (ORM) plots indicate areas of maximum pair resolution. The union of the peak pair ORM plots will give the region where all resolutions are above a level predetermined by the analyst. Combinations of three pure, or mixed, solvents, and ten chromatographic runs with mobile phases of different solvent proportions are required to generate the experimental data base necessary for the subsequent statistical analysis.

The (ORM) approach works extremely well when three organic modifiers plus base solvent are necessary to achieve optimum resolution of all components of a complex mixture. It has been shown to be of wider application than the chromatographic optimization function (COF) method (2). The ORM approach can optimize resolution when only two organic modifiers and a base solvent are used. Belinky (5) used two organic modifiers and water to achieve separations in reversed phase HPLC. His system required 17 data points to achieve an optimum mobile phase. A simpler approach (6) with two organic modifiers was used which required only 10 data points. A computer program (1) is used to select the mobile phase which will give optimum resolution of the components in a mixture.

Recently (7), another approach to solvent optimization was published based on the linear relationship between log K' and log mole fraction of the solvent. This approach is not as sound or general as the statistical approaches discussed earlier.

In this study, a more practical approach to mobile phase optimization with two organic modifiers is presented. Only five chromatographic runs are required for the data base, and the subsequent mathematical treatment of the data is much less involved. The method is based on the window diagram technique which was originally developed by Laub and Purnell (8-11) for the optimization of separations in gas-liquid chromatography. Recently, a review of the window diagram application to GC, electrochemistry and spectroscopy was published (12). The technique has previously been used for the optimization of resolution of

hydrophilic compounds with variation of the pH of the mobile phase in liquid chromatography (4,13). Contrary to the conclusion of Glajch et al (2) who dismissed the window diagram technique, the method is not limited to linear retention behavior nor to two-component solvent systems. Peak crossovers are also easily handled. In this work the method is successfully applied to the optimization of separation of a five-component mixture in reversed phase HPLC with two organic modifiers and water base solvents.

EXPERIMENTAL

Materials: Solvents were glass distilled (Burdick and Jackson). The chemicals were analytical grade (Aldrich Chemical Co.) and used without further purification.

Apparatus: A modular HPLC system consisting of Laboratory Data Control (LDC) constametric I and II Pumps attached to an LDS Gradient Master, a Chromatronix dual-channel uv absorbance detector, a Rheodyne injector, and a strip-chart recorder operated at 0.2 in/min was used.

The RP-18 reversed phase column was 250 mm x 4.6 mm prepacked with 10 μm particle size materials (Waters Associates). 10 μl samples were injected. Experiments were run at room temperature using a mobile phase flow rate of 1 ml/min. Retention times, peak widths (W) and resolutions (R_S) were determined with a 3352A Laboratory Data System (Hewlett-Packard) linked through a Hewlett-Packard 1865 A/D converter to the UV detector output of the liquid chromatograph. The output from the data system was recorded on a 9866A thermal line printer (Hewlett-Packard).

Separation Strategy:

The selection of the initial solvents (A and B) is based on the properties of the solute mixture and the stationary phase (normal, reversed phase or ion exchange). For reversed phase, the three most widely used solvents are acetonitrile/water, methanol/water and tetrahydrofuran/water. The initial ratios of organic/water selected are approximately 70-75% methanol/water, 60-65% acetonitrile/water and 40-50% tetrahydrofuran/water. The strategy for selecting

two of these three is illustrated below. For simplicity, assume that a five component mixture is to be separated. The sample is first injected where 60% acetonitrile/water is the mobile phase. Should four peaks be obtained, standards are used to identify the two coeluting peaks. Only these two are then reinjected and eluted using a different solvent, for example, 50% tetrahydrofuran/water. Should two components be separated, then different mobile phase compositions are prepared using 60% acetonitrile as solvent A and 50% THF as solvent B. Should 50% THF/H_2O fail to separate the pair coeluting the percentage of THF is adjusted or another mobile phase is selected. This approach is simple and time saving because the analyst has only to separate the pair not resolved. Also, the identification of two components is simpler than identifying all components in a mixture.

This separation strategy was used to separate anthraquinone, 2-methylanthraquinone, 2-ethylanthraquinone, naphthalene and biphenyl. The sample solution was chromatographed with 60% AN/H_2O. Only three peaks were observed. Anthraquinone and naphthalene coeluted, as did 2-methylnaphthalene and biphenyl. However, both solute pairs were separated with 40% THF/H_2O. This demonstrates that each of the four pairs had been resolved in at least one of the initial solvents.

After selecting the initial solvents and the proportions of each in the three solvent combinations, the retention times data base is generated by recording the retention time of each solute in each of the different solvent combinations.

RESULTS AND DISCUSSION

The window diagram technique as presented by Laub and Purnell (8-11) is a graphical method for representing retention data. It was originally developed for the optimization of separations with respect to the binary stationary phase composition in gas-liquid chromatography. We have used the method to optimize separations in HPLC with respect to the mobile phase composition using two organic modifiers and a water-base solvent.

Table I shows the composition of solvents used and the retention times for each of the five solutes with each different mobile phase. The retention data

TABLE I: RETENTION TIMES FOR EACH OF THE SOLUTES AT EACH DIFFERENT MOBILE PHASE COMPOSITION

Mobile Phase Compostion						Retention Time, Min.				
			Actual Component Percentage							
Solvent	% A(a)	% B(b)	AN	THF	H_2O	Anthra-quinone	2-Methylanthra-quinone	Naphthalene	2-Ethylanthra-quinone	Biphenyl
1	100	0	60	0	40	7.23	8.98	7.23	10.94	8.98
2	75	25	45	10	45	8.26	10.30	9.16	13.27	11.77
3	50	50	30	20	50	9.57	11.98	11.30	15.82	15.13
4	25	75	15	30	55	11.65	14.75	14.67	19.98	20.38
5	0	100	0	40	60	16.44	21.20	22.41	30.19	34.03

(a) Represent 60% Acetonitrile (AN)/Water

(b) Represent 40% Tetrahydrofuran (THF)/Water

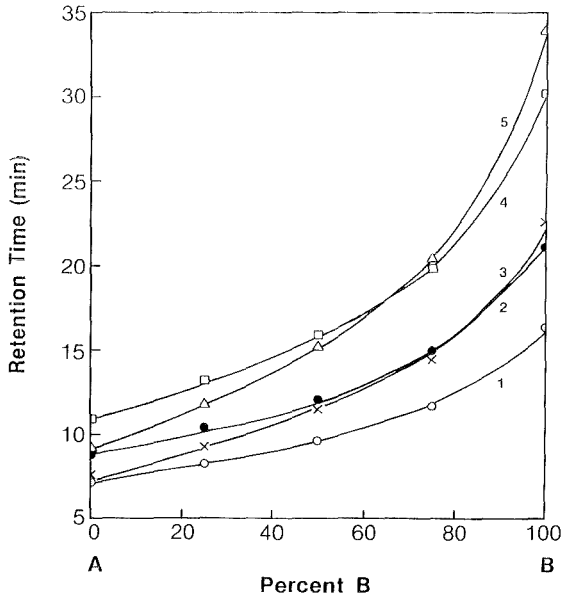


Figure 1: Retention time vs mobile phase composition for five solutes.

Solvents: A = 60% acetonitrile/water

B = 40% tetrahydrofuran/water

Solutes: 1 = anthraquinone;

2 = 2-methylanthraquinone;

3 = naphthalene;

4 = 2-ethylanthraquinone;

5 = biphenyl.

as a function of mobile phase composition was fit to a polynomial of the fourth order by least squares analysis.

Figure 1 shows plots of the calculated retention times for each solute as a function of mobile phase composition. The experimental points are also indicated. Note that, in contrast to gas-chromatography, the plots are not linear. This is due to the complicated nature of solute-mobile phase, solute-stationary phase and mobile phase-stationary phase interactions (14).

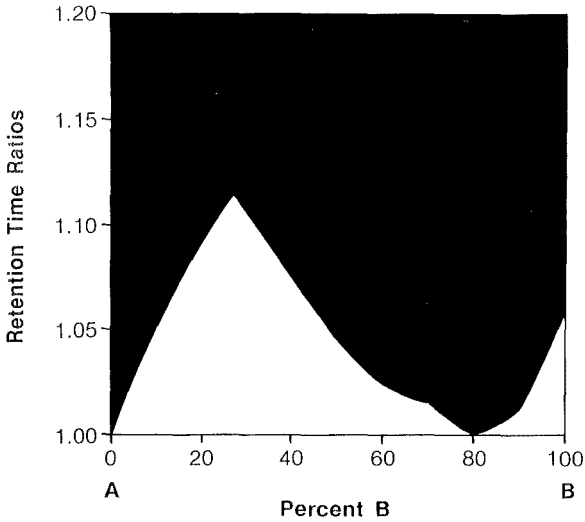


Figure 2: Window diagram for all ten pairs of five solutes, based on retention data as in Figure 1.

Figure 2 is a window diagram showing plots of retention time ratios versus mobile phase composition for all ten pairs of the five solutes. The region of retention time ratio values that are higher than the minimum found at each mobile phase composition is shaded. Note that when the relative retention is calculated to be less than unity (peak crossover) the reciprocal is taken such that the ratio is always greater than, or equal to, unity. The tops of the windows represent the mobile phase composition giving the best separation for the least separated pair. Two windows are seen in Figure 2, one at 27% B with a minimum retention ratio = 1.1, and a considerably smaller window (poorer separation) at 100% B. Thus the optimum mobile-phase composition for this particular separation is predicted to be 27% B (10.8% THF/43.8% AN/44.4%H_2O), which does, in practice, give base line separation of the components of the mixture (Figure 5).

The theoretical measure of separation of a solute pair in chromatographic techniques is the relative retention (α), which in HPLC is defined as the ratio

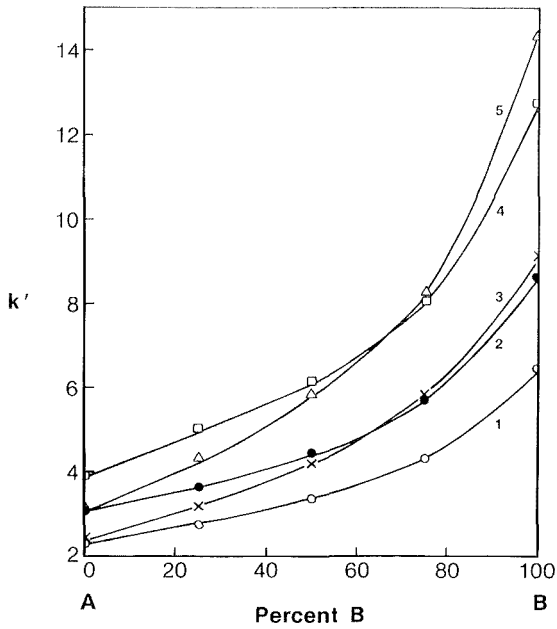


Figure 3: k' vs mobile phase composition for five solutes. Symbols for solvents and solutes as in Figure 1.

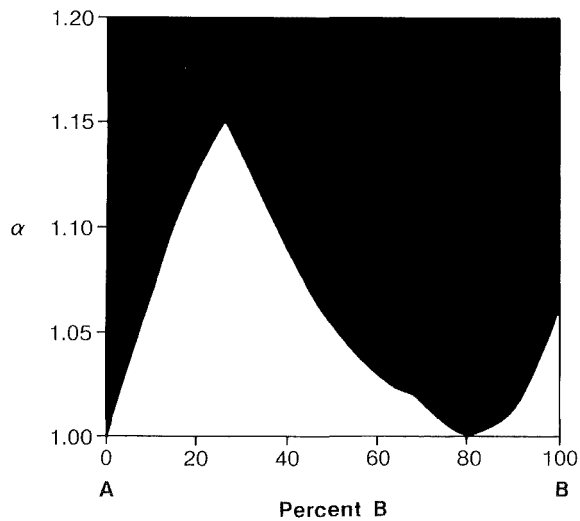


Figure 4: Window diagram for all ten pairs of five solutes, based on k' data as in Figure 3.

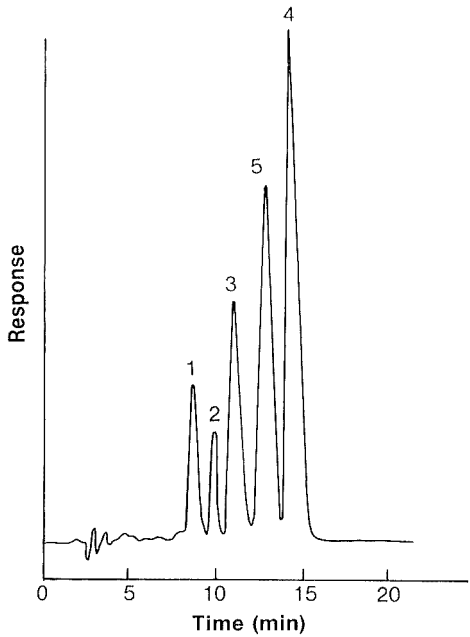


Figure 5: Chromatogram of the five solutes at optimum mobile phase composition (43.8% acetonitrile, 10.8% tetrahydrofuran, 44.4% H_2O) as determined from Figure 1.

of the capacity factor (k') of the more retained to the less retained solute. We calculated k' for all solutes at all solvent compositions by correcting for the column dead-volume. The k' data was treated in a similar way to the retention data of Table 1. Figure 3 shows plots of the calculated k' for each solute as a function of mobile phase composition, and Figure 4 gives the window diagram. Figures 1 and 3 and Figures 2 and 4 are strikingly similar. The optimum mobile-phase composition obtained from the larger window of Figure 4 is exactly the same as that obtained from Figure 2. However, here we can obtain the minimum value of α (1.15 at 27%B). Using this value we can calculate the minimum number of plates ($N_{req.}$) for separation according to Purnell's equation (15). In this instance, and assuming a capacity factor of five, $N_{req.}$ is calculated to be ap-

proximately 2350 plates. Note that accurate measurment of the column "dead volume" in HPLC is a difficult problem with no easy solution (16). Any optimization techniques dependent on k' data suffer from the unavailability of accurate methods for the determination of column dead volume. The window diagram method presented here does not require the accurate determination of k'. As demonstrated earlier the optimum solvent composition can be determined from raw retention time data. When retention time is plotted against mobile phase composition, it can be seen that a total of five runs (Figure 1) will give the mobile phase that will separate all the components of the mixture. It is clear that this approach requires no computer evaluation and is simpler than others (1-4) when two organic modifiers and a base solvent are used.

ACKNOWLEDGEMENT

"By acceptance of this article, the publisher or recipient acknowledges the right of the U.S. Government to retain a nonexclusive, royalty-free license in and to any copyright covering the article."

REFERENCES

1. Issaq, H.J., Klose, J.R., McNitt, K.L., Haky, J.E. and Muschik, G.M., J. Liquid Chromatogr. 4, 2091 (1981).
2. Glajch, J.L., Kirkland, J.J. and Squire, K.M., J. Chromatogr. 199, 57 (1980).
3. Glajch, F.L., Kirkland, J.J. and Snyder, L.R., J. Chromatogr. 238, 269 (1982).
4. Sachok, R., Kong, R.C. and Deming, S.N., J. Chromatogr. 199, 317 (1980).
5. Belinky, B.R., Analytical Technology and Occupational Health Chemistry, ACS Symposium Series, Volume 220 pp 149-168, American Chemical Society, Washington, D.C., 1980.
6. Issaq, H.J., Drug Metabolism Group Meeting,Plymouth, PA, May 1982.
7. Hara, S., Kunihiro, K., Yamaguchi, H. and Soczewinski, E., J. Chromatogr. 239, 687 (1982).
8. Laub, R.J. and Purnell, J.H., J. Chromatogr. 112, 71 (1975).
9. Laub, R.J. and Purnell, J.H., Anal. Chem., 48, 799 (1976).

10. Laub, R.J. and Purnell, J.H., Anal. Chem., 48, 1720 (1976).

11. Laub, R.J., Purnell, J.H. and Williams, P.S., J. Chromatogr., 134, 249 (1977).

12. Laub, R.J., American Lab., 13 (3), 47 (1981).

13. Deming, S.N. and Turoff, M.L.H., Anal. Chem., 50, 547 (1978).

14. Snyder, L.R. and Kirkland, J.J., "Indtrouction to Modern Liquid Chromatography", Wiley, New York, 2nd ed. 1979, Chap. 6.

15. Purnell, J.H., J. Chem. Soc., 1268 (1960).

16. Grushka, E., Colin, H. and Guiochon, G., J. Liquid Chromatogr. 5, 1397 (1982).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 271-280 (1983)

ELUTION BEHAVIOR OF ORGANIC ACIDS IN DONNAN EXCLUSION CHROMATOGRAPHY

Yuji Tokunaga, Hirohiko Waki and Shigeru Ohashi

Department of Chemistry, Faculty of Science,
Kyushu University
Hakozaki, Higashiku, Fukuoka, 812 JAPAN

ABSTRACT

The elution behavior of organic acids has been elucidated using a dextran gel-type cation-exchanger column with an eluent of 1.00 M tetramethylammonium chloride. It was found that Donnan exclusion chromatography is applicable to organic acids as well as inorganic ones. Some types of separation methods were performed by the use of the separation mode whose mechanism was confirmed by Donnan exclusion chromatography.

INTRODUCTION

Although the separation of organic acids by means of ion exclusion chromatography has been described by many investigators (1-4), only a qualitative explanation has been given to their elution behavior.

In Donnan exclusion chromatography based on electrostatic repulsion from an ion-exchanger phase, if one uses a concentrated electrolyte as an eluent, the elution behavior of a given ion is explained quantitatively by the Donnan membrane equilibrium equation. Its elution position from a column is dependent upon its charge. The treatment of this principle has been established for inorganic acids, mainly phosphorus oxoacids (5-8). In

 0148-3919/83/0602-0271$3.50/0

the present paper, the elution behavior and the separation of organic acids such as aliphatic, aromatic and aminopolycarboxylic acids are described. The investigation of the separation mechanism is emphasized here rather than the separation itself, because many separations of organic compounds have been done more efficiently by other types of chromatography.

EXPERIMENTAL

It is neccesary that an ion-exchanger contains a large volume of a non-adsorption region (distribution coefficient is 0 to 1) for Donnan exclusion chromatography. A cross-linked dextran gel-type cation exchanger, SP-Sephadex C-25, is swollen in electrolyte solution and suitable for this purpose. The column was packed with SP-Sephadex C-25 (15 mm I.D. x 92.5 cm). Tetramethylammonium chloride solution (1.00 M) was used as an eluent, because its cation does not interact appreciably with a sample anion. Then, 1 ml of the sample solution, dissolved in the eluent, was applied to the top of the column. Flow rate was maintained at 0.5 ml/min. The effluent was introduced into a flow cell of a variable-wavelength spectrophotometer (LDC Model SpectroMonitor III) for aromatic compounds and cobalt-aminopolycarboxylate complexes, or collected by a fraction collector into fractions of 1 ml for other samples. Aromatic compounds in effluents were detected at 275 nm wavelength and cobalt-aminopolycarboxylate complexes at 515 nm wavelength. Aliphatic carboxylic acids were determined as their hydroxamic acids formed by reaction with dicyclohexylcarbodiimide (9).

The calculation of the distribution coefficient is the same as that previously described in detail (5). The distribution coefficient, K_D, is defined as

$$K_D = \frac{V_e - V_0}{V_D} \quad (1)$$

where V_e is the elution volume, V_D the net volume of the exchanger phase except of a skeleton and V_0 the interstitial volume. In case of an equilibrium mixture with different charge states such as polybasic acids, H_nL, the distribution coefficient, D, can be written as

$$D = K_{D,L}\,\alpha_L + K_{D,HL}\,\alpha_{HL} + K_{D,H_2L}\,\alpha_{H_2L} + \cdots \quad (2)$$

where K_{D,H_jL} is the characteristic distribution coefficient of component H_jL, and α_{H_jL} is its mole fraction.

RESULTS AND DISCUSSION

In Donnan exclusion chromatography, the principal separation factor is an electrostatic repulsion between fixed groups of an ion exchanger and the co-charged sample ions. The elution volume of a given sample ion can be elucidated from its charge. When another factor, such as adsorption or steric exclusion, acts together with ionic exclusion, the elution position should shift to a larger volume (adsorption) or to a smaller volume (steric exclusion) than that predicted from ionic exclusion only. The shift, therefore, indicates the presence of another factor. If the separation mechanism is confirmed, separation would be improved, surely, by using these factors properly. The elution behavior of organic acids on a cation-exchanger column was estimated first and represented as the distribution coefficient indicating mechanism. From these results, some types of separations were accomplished.

Distribution coefficient, K_D or D.

The distribution coefficients of organic acids, except for adsorbed compounds stated later, were obtained by eqns. (1) and (2) [TABLE 1]. Fig. 1 illustrates the plots for the distribution coefficients, K_D, of the elution standards of crotonaldehyde (neutral), phosphinate (monovalent), phosphonate (divalent) and trimetaphosphate (trivalent), and the distribution coefficients, D, of orthophosphate at different pH as an equilibrium mixture with different charge states. When the distribution coefficients of organic acids are plotted against the (mean) charge calculated from the

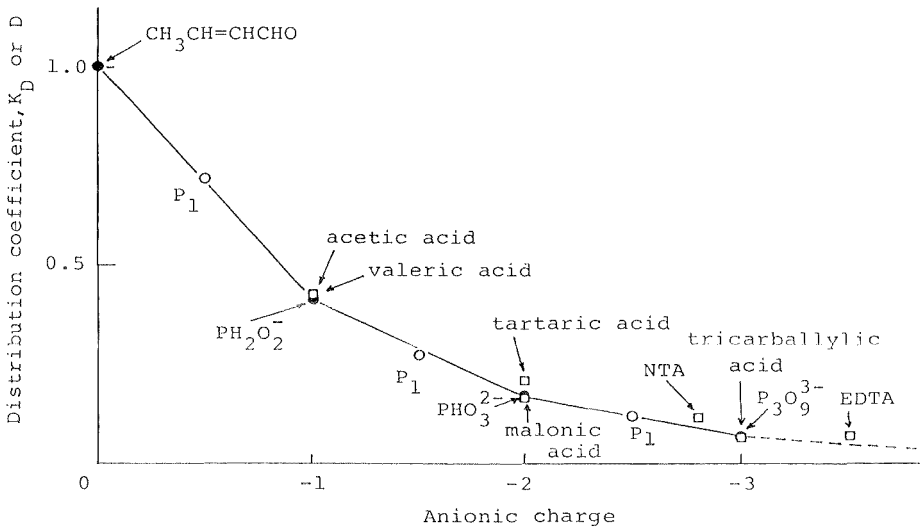


FIGURE 1

The relationship between distribution coefficient and anionic charge. ●; elution standard, ○; orthophosphate (P_1) at different pH, □; organic acid. The broken line between -3 and -4 is calculated from eqns. (1) and (2).

TABLE 1
Distribution Coefficient, K_D or D.

Species	K_D or D	Species	K_D or D
Acetic acid	0.426	Oxalic acid	0.213
Valeric acid	0.426	Tricarballylic acid	0.070
Malonic acid	0.156	NTA	0.116
Tartaric acid	0.207	EDTA	0.076

dissociation constant, these values are in fair agreement with those of phosphorus oxoacids. This means that Donnan exclusion chromatography can be applied generally to organic acids as well as inorganic acids and gives a quantitative explanation for their elution behavior.

The deviation of NTA and EDTA from the line of Fig. 1 may be due to the dispersion of charge on a large molecule.

Separation of aliphatic carboxylic acids.

It has been mentioned in previous papers (5,8) that the mutual separation of mono-, di- and trivalent anions of phosphorus oxoacids is completely accomplished. To make sure that Donnan exclusion chromatography is applicable to organic compounds, some mixtures of aliphatic carboxylic acids were separated on the cation-exchanger column. Fig. 2 shows the separation of acetic, tartaric and tricarballylic acids as the completely dissociated samples at pH 10 of mono-, di- and trivalent anions, respectively. Each elution volume can be interpreted quantitatively and its distribution coefficient agrees with the corresponding standards of phosphorus oxoacids.

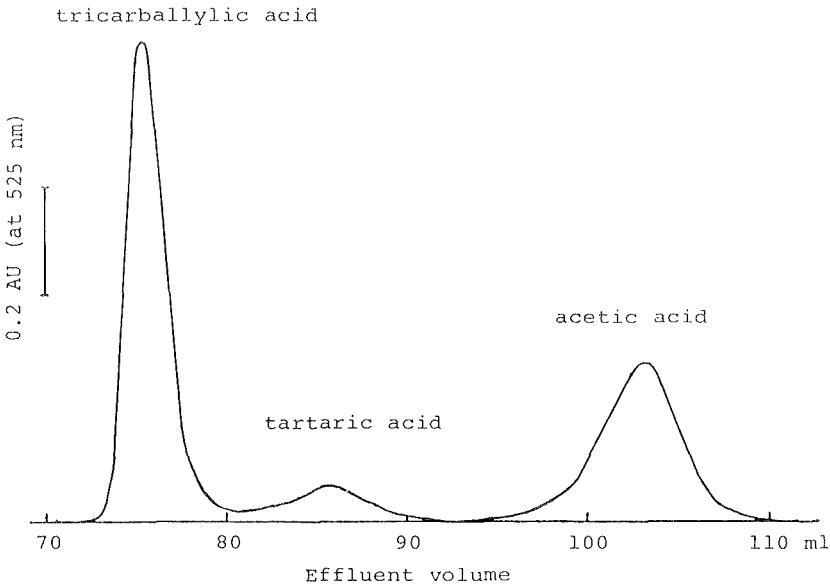


FIGURE 2

Separation of aliphatic carboxylic acids. Column; SP-Sephadex C-25 (Me_4N^+ form), 15 mm I.D. x 92.5 cm. Eluent; 1.00 M Me_4NCl (pH 10). Sample; 0.1 M $(Me_4N)_3C_6H_5O_6$, 0.1 M $Na_2C_4H_4O_6 \cdot 2H_2O$ and $NaC_2H_3O_2 \cdot 3H_2O$.

Separation of aromatic compounds.

The separation of phthalic acid, benzoic acid, salicylic acid and phenol is shown in Fig. 3. The charge states of these sample anions in the eluent at pH 10 are -2 for phthalic acid, -1 for benzoic acid and salicylic acid and *ca.* -0.5 for phenol, respectively. Although the elution volume depends upon its charge, anions with phenol group were eluted at a later position than predicted. It is possible that salicylic acid and phenol tend to be adsorbed due to the interaction between a phenol group and a dextran gel matrix. In

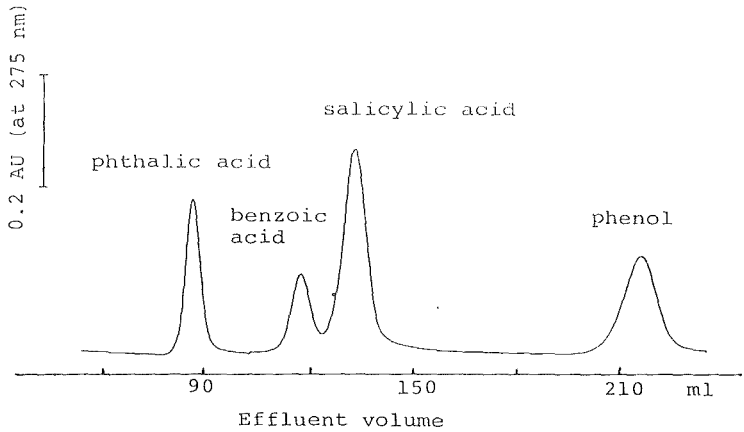


FIGURE 3

Separation of aromatic anions. Column; SP-Sephadex C-25 (Me_4N^+ form), 15 mm I.D. x 92.5 cm. Eluent; 1.00 M Me_4NCl (pH 10). Sample; each 4 x 10^{-3} M of $C_6H_4(COOK)(COOH)$, $C_6H_4(OH)COONa$, C_6H_5COONa and C_6H_5OH.

this case, the separation became more favorable by combined factors of ionic exclusion and adsorption.

Separation of highly-charged anions by formation of cobalt (II) complex.

A direct separation of highly-charged anions seems to be difficult because the difference of the ditribution coefficients decreases with the increase of each ionic charge on anions from the theoretical point of view. It may be considered that the anionic charge depression by complex formation with metal cation leads to better separation.

The elution curves for a mixture of cobalt(II) complex of IDA (iminodiacetic acid), NTA (nitrilotriacetic acid) and EDTA (ethylenediaminotetraacetic

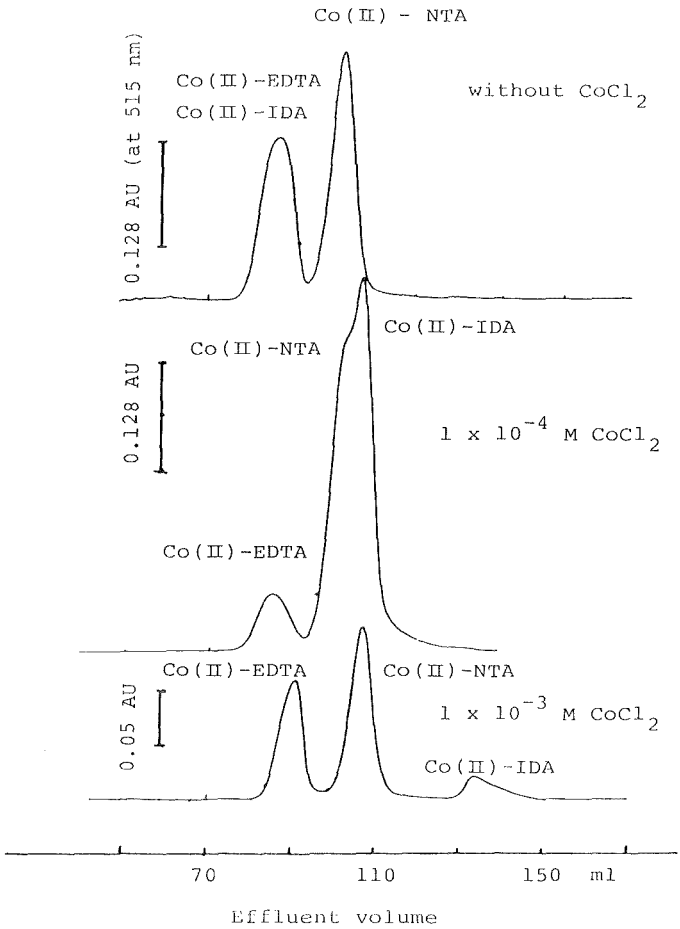


FIGURE 4

Separation of aminopolycarboxylic acid anions by Co(II) complex formation. Column; SP-Sephadex C-25, 15 mm I.D. x 92.5 cm. Eluent; 1.00 M Me_4NCl and (a) without $CoCl_2$, (b) 1×10^{-4} M $CoCl_2$ or (c) 1×10^{-3} M $CoCl_2$ (pH 6.0). Sample; each 0.1 M of Co(II)-IDA, Co(II)-NTA and Co(II)-EDTA.

acid) are shown in Fig. 4, with eluents containing cobalt(II) chloride at different concentrations (pH 6). The elution volumes of highly stable EDTA and NTA complexes are constant whether cobalt(II) ions are added to the eluent or not, whereas the elution position of the less stable IDA complex shifts gradually to a larger volume according as cobalt(II) ion concentration increases in the eluent. This indicates that cobalt(II) complexes of EDTA and NTA are completely formed but that of IDA is partially formed in the eluent at lower concentration of cobalt(II) chloride. These complexes were separated from each other with the eluent containing 1×10^{-3} M cobalt(II) chloride.

From the aspect of resolution, if adsorptive or steric exclusion effects are present, they often are present in a direction and magnitude that enhances the separation.

ACKNOWLEDGMENTS

This work was partially supported by a Grant-in-Aid for Scientific Research Nos. 516804 and 56104004 from the Ministry of Education, Science and Culture (JAPAN).

REFERENCES

1. Wheaton, R. M. and Bauman, W. C., Ind. Eng. Chem., 45, 228(1953).
2. Harlow, G. A. and Morman, D. H., Anal. Chem., 36, 2438(1964).
3. Thompson, J. F. and Morris, C. J., Arch. Biochem. Biophys., 82, 380(1959).
4. Buchanan, D. L. and Markiw, R. T., Anal. Chem., 32, 1400(1960).
5. Waki, H. and Tokunaga, Y., J. Chromatogr., 201, 259 (1980).
6. Waki, H., Bunseki, 72, 885(1980).

7. Tokunaga, Y., Waki, H. and Ohashi, S., J. Liq. Chromatogr., in press.

8. Waki, H. and Tokunaga, Y., J. Liq. Chromatogr., 5, 105(1982).

9. Kasai, Y., Tanimura, T. and Tamura, Z., Anal. Chem., 47, 34(1975).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 281-289 (1983)

ISOLATION OF GLYCOALKALOIDS WITH THE CHROMATOTRON* AND THEIR DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Irving R. Hunter** and Erich Heftmann

Western Regional Research Center, Agricultural Research Service

U.S. Department of Agriculture, Berkeley, California 94710

ABSTRACT

An extract of Solanum laciniatum leaves was fractionated with the Chromatotron. The glycoalkaloid fraction thus obtained was analyzed by reversed-phase HPLC. The load capacity of the Chromatotron with a 2-mm layer thickness is 600 mg (as determined with cholesterol) and the recovery of solasonine from the plant extract (as determined by HPLC) is 93-96%. The HPLC method permits the detection of as little as 1.5 µg solasonine or 3.5 µg solamargine with a linear detector response up to 375 µg for the former and 250 µg for the latter.

INTRODUCTION

In an earlier publication (1) we have described the analysis of the steroidal alkaloid in the fruits of Solanum khasianum by a combination of the Chromatofuge*** and HPLC. In plants, solasodine and other steroidal alkaloids occur in the form of glycosides. Two such glycoalkaloids occurring in S. laciniatum leaves are solasonine (solasodine + L-rhamnose

*Centrifugally accelerated radial thin-layer chromatograph manufactured by Harrison Research, 840 Moana Court, Palo Alto, CA 94306.

**To whom correspondence should be addressed.

***Centrifugal Chromatograph manufactured by NSA Hitachi Scientific Instruments, Mountain View, CA 94043.

Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

 0148-3919/83/0602-0281$3.50/0

+ D-glucose + D-galactose) and solamargine (solasodine + L-rhamnose + L-rhamnose + D-glucose). In connection with a project in Israel, we have developed a method for the isolation and determination of the solasodine glycosides.

For this work we have chosen to test the Chromatotron*, an instrument previously used by Hostettmann et al. (2) for the isolation of ginsenosides from ginseng. Quantitative analysis by HPLC has previously been applied to the solanidine glycosides by Bushway et al. (3-5). Our quantitative assay of the solasodine glycosides is based on a method by Crabbe and Fryer (6).

METHODS

Extraction

Freeze dried and powdered S. laciniatum leaves, weighing 22.2 g, were extracted for 24 hours in a Soxhlet extractor with 250 ml of 5% acetic acid in methanol. The extract was filtered and the residue on the filter paper was washed with methanol. The combined filtrates, which contained 6.87 g of dissolved solids, were diluted to 300 ml with methanol. A 100-ml aliquot of this extract was evaporated to a volume of less than 10 ml under reduced pressure, brought to pH 7.5 with conc. ammonium hydroxide, and filtered. The clear, dark-green solution was diluted to 25 ml with methanol-ethanol (9:1).

Chromatotron

The rotor of the Chromatotron is a frosted glass disk, ca 4 mm thick and 24 cm in diameter, with a 7-mm round hole in the center. The adsorbent layer, 2 mm in thickness, used for this work was prepared by slurrying a mixture of 50 g Silica Gel HF-254+366 Type 60-Merck (E.M. Laboratories, Scientific Products, McGaw Park, IL 60085) and 4 g $CaSO_4$ 1/2 H_2O in 50 ml of water. The slurry was added to the plate while the latter was spinning at 33 rpm on a converted record player. A polyethylene collar, made from a

conical wastebasket, prevented the slurry from running over the edge of the plate. The silica gel was then allowed to set for 5 hrs at room temperature and for 3 hrs at ca 80°C. The plate was scraped with the rotary scraping tool and finally with the finishing tool supplied by the manufacturer.

The thin-layer disk was mounted in the Chromatotron and, while it was spinning without the cover plate, a 5-ml aliquot of the extract (one-fifth of the sample) was applied, dropwise, to the inner edge of the adsorbent layer, followed by two 1-ml ethanol rinses. The cover plate was then installed and the chromatogram was developed with 250 ml ethanol at a rate of 2 ml/min, delivered by a single-piston reciprocating pump (Model 110, Altex-Beckman, Berkeley, CA 94710). The effluent from the Chromatotron was passed into a sump, from which it was pumped by another Altex Model 110 pump through a variable-wavelength detector (Model 155-10, Altex-Beckman), which was set at 210 nm and connected to an Omniscribe recorder (Houston Instrument, Austin, TX 78753), equipped with an event marker.

The sump was constructed from the barrel of a 3-ml hypodermic syringe with the hub pointing downward. The level of the liquid entering the barrel from the top was kept at 1 ml and controlled by means of a capacitance sensor (Thermocap relay, Niagara Electron Laboratories, Andover, NY 14806). The relay was connected to a clip, attached to the outside of the barrel at the desired level, and a grounded stainless-steel rod was placed inside the barrel. The relay activated the sump pump which was connected to the hub whenever the liquid level went past the top of the clip and shut it off when the liquid level fell below the clip. The effluent from the detector was collected in 10-ml portions in a fraction collector (LKB Model 7000, LKB Instruments, Rockville, MD 20852).

On the basis of our experiments with this system we devised the simple method of developing the chromatogram with 250 ml of ethanol. The eluate was collected and concentrated under reduced pressure to a volume of less

than 10 ml. A precipitate which formed was filtered off, and the filtrate and ethanol washes were made up to a volume of 10 ml with ethanol.

HPLC

The HPLC system consisted of an Altex Model 110 pump, connected through a sample injection valve with a 100- µl loop volume (Model 7125, Rheodyne, Cotati, CA 94928) to a Brownlee guard column (Rheodyne) and a prepacked stainless-steel column, 300 x 4.6 mm ID, both containing a 5- µm reversed-phase packing of silica gel-bonded octadecylsilane (IBM Instruments, Danbury, CT 06810). The detector was a Hitachi variable-wavelength spectrometer equipped with a flowcell having 10-mm pathlength and a 20- µl capacity, which was set at 210 nm and 0.1 AUFS. Signals from the detector were fed to a recorder, set at 10 mV.

The eluent was a mixture of acetonitrile:0.01 M Tris buffer (9:1), delivered to the column at a flowrate of 0.5 ml/min. Microgram quantities of solasonine and solamargine in methanol:ethanol (9:1) solution were injected and the detector response was determined. The quantitative assay was based on peak area measurements.

RESULTS AND DISCUSSION

Capacity and Efficiency of the Chromatotron

Most chromatographic analyses of natural extracts require a preparative "clean-up" step. The Chromatotron and the chromatofuge are both convenient instruments for purifying extracts either for isolation purposes or prior to quantitative analysis. In both instruments the solvent flow is accelerated by centrifugal force and the zones are sharpened during migration. However, they differ in load capacity.

The load capacity of the 2-mm layer of silica gel was determined with cholesterol as the sample and hexane:2-propanol (9:1) as the eluent. The maximum quantity of cholesterol giving a clearly defined zone without appreciable tailing was 600 mg. This is about the same as the load capacity determined for a 3-mm high layer of silica gel in the chromatofuge (1).

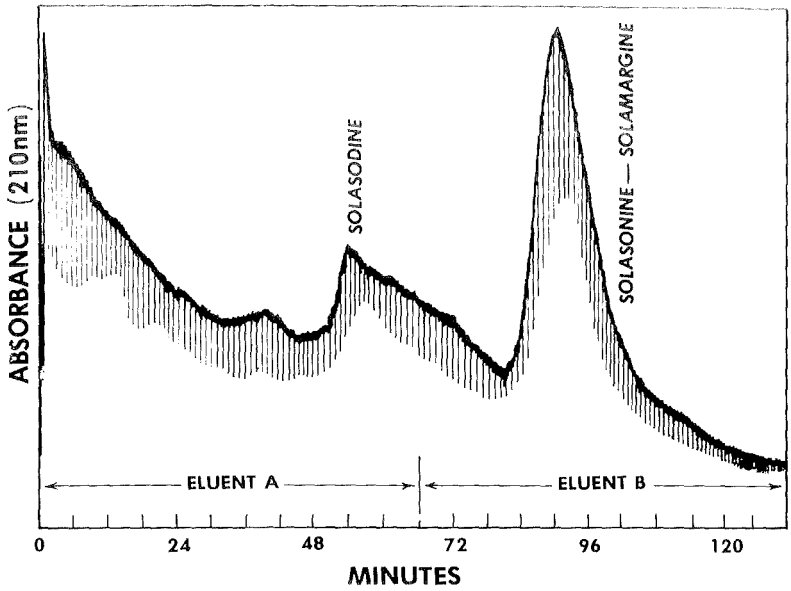


Fig. 1. Separation of solasodine from the glycoalkaloids, solasonine and solamargine, by the Chromatotron. A mixture of 7.9 mg solasonine, 1.4 mg solamargine, and 5 mg solasodine was applied to a 2-mm layer of silica gel. Eluent A, hexane:acetone:ethanol (18:1:1); Eluent B, ethanol. Coupling of the Chromatotron with a UV detector produced the tracing (see text).

Thus, the Chromatotron is equal to the chromatofuge in load capacity for approximately equal layer thickness. However, the layer thickness of the Chromatotron is limited to 4 mm by the cohesiveness of the binder, whereas the chromatofuge can be packed to 10 mm in height.

Fig. 1 illustrates the performance of the Chromatotron. It is a recorder tracing of an experiment in which 7.9 mg solasonine, 1.4 mg solamargine, and 5 mg solasodine were introduced into the Chromatotron, coupled with the UV detector. By elution with hexane:acetone:ethanol (18:1:1) for 66 min solasodine was recovered (peak at 54 min) and by elution

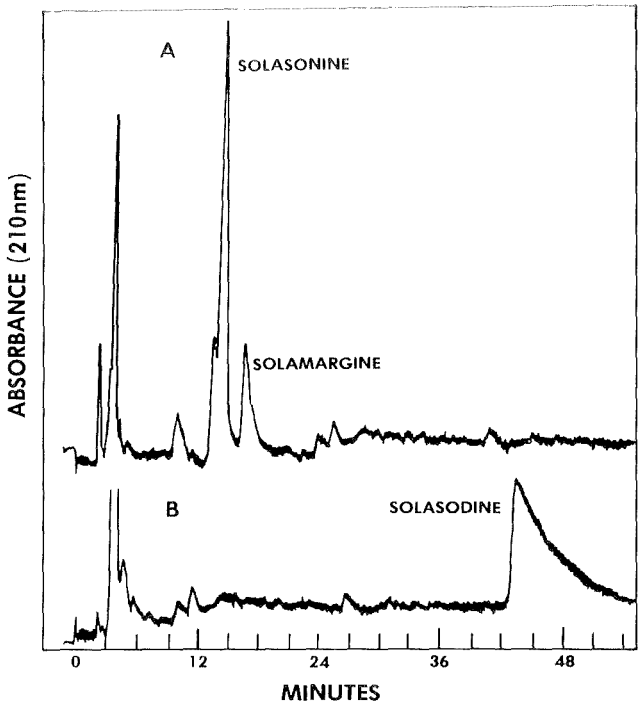


Fig. 2. HPLC of solasodine and its glycosides. Column, 300 x 4.6 mm ID, prepacked with silica ODS (5 µm); eluent, acetonitrile:0.01 M Tris (9:1); flowrate, 1 ml/min; pressure, 50 psi; UV detector at 210 nm, range 0.1; recorder speed 3 min/cm, span 10 mV.

A. Chromatogram of 10 µl S. laciniatum leaf extract (see text).

B. Chromatogram of the same extract after hydrolysis.

with ethanol solasonine and solamargine were obtained as a mixture (peak at 90 min). The vertical lines in the tracing are due to the intermittent operation of the pump delivering eluent to the detector.

Thus, while the glycoalkaloids were not separated from each other, they were clearly separated from the aglycone. An even simpler scheme suffices for separating the glycoalkaloids from miscellaneous contaminants in the plant extract (see Fig. 2A). Elution of the Chromatotron with ethanol furnishes material suitable for HPLC analysis in a shorter time than use of the chromatofuge (1).

Efficiency of HPLC

The acetonitrile:0.01 M Tris (9:1) eluent produces base-line separation of the two solasodine glycosides, solasonine (retention time 9.5 min) and solamargine (retention time 11.2 min) (6). Fig. 2 represents two chromatograms, produced under identical conditions. A illustrates the separation of the two glycoalkaloids in 10 µl (one-thousandth) of the eluate from the Chromatotron, while B shows the result of hydrolyzing an equivalent amount of the eluate as a test of identity.

Hydrolysis was carried out by a modification of the method of Crabbe and Fryer (7). The reaction was carried out in a 15-ml pear-shaped, stoppered flask, kept in a water-bath at 70°C for 3 hrs. A 200- µl aliquot of the eluate from the Chromatotron was treated with 3 ml of a mixture of methanol:water:conc. hydrochloric acid (80:9:7). After hydrolysis, the mixture was evaporated almost to dryness below 40°C under reduced pressure. The pH was adjusted to 7.5 with ammonium hydroxide and, after the addition of 400 µl dichloromethane with agitation, the dichloromethane layer was analyzed by HPLC.

Accuracy of the Chromatotron-HPLC Combination

Calibration curves for solasonine and solamargine were prepared by analyzing microgram quantities of the glycoalkaloids by HPLC. The plot of peak area vs. amount was linear for solasonine up to 375 µg and for solamargine

up to 250 µg. The slope of the calibration line (y = area in cm^2, x = amount in µg) for the former was 0.198 and for the latter it was 0.148. The minimum detectable quantity (signal/noise = 2) was 1.5 µg for solasonine and 3.5 µg for solamargine.

Recovery experiments were carried out by adding 5.1 mg, 5.6 mg, and 10.2 mg of pure solasonine to 5-ml aliquots of S. laciniatum extract whose solasonine and solamargine content had previously been determined and processing each sample as described under Methods. HPLC analysis of the Chromatotron eluates showed recoveries of 93%, 94%, and 96%, respectively, of the added solasonine. The experiments were repeated with the addition of 583 mg, 590 mg, and 594 mg of cholesterol with recoveries of 93%, 94%, and 95%, respectively, of the added cholesterol.

Solasonine and solamargine were identified in S. laciniatum by comparison of their retention times to known standards and by hydrolysis to solasodine. It was calculated that the dry leaves contained 2.2% of solasonine and 1.3% of solamargine. The combination of Chromatotron and HPLC thus permits the purification and analysis of these glkycoalkaloids in a natural extract.

ACKNOWLEDGEMENTS

The authors thank Dr. Zohara Yaniv of the Volcani Institute in Bet-Dagan, Israel, for gifts of reference substances and S. laciniatum leaf powder

REFERENCES

1. Nes, W. D., Heftmann, E., Hunter, I. R. and Walden, M. K., J. Liquid Chromatogr., 3, 1687, 1980.
2. Hostettmann, K., Hostettmann-Kaldas, M. and Sticher, O., J. Chromatogr., 202, 154, 1980.
3. Bushway, R. J., Barden, E. S., Bushway, A. W. and Bushway, A. A., J. Chromatogr., 178, 533, 1979.

4. Bushway, R. J., Barden, E. S., Wilson, A. M. and Bushway, A. A., J. Food Sci., 45, 1088, 1980.

5. Bushway, R. J. and Storch, R. H., J. Liquid Chromatogr., 5, 731, 1982.

6. Crabbe, P. G. and Fryer, C., J. Chromatogr., 187, 87, 1980.

7. Crabbe, P. G. and Fryer, C., Chem. Eng. Commun., 4, 135, 1980.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 291-299 (1983)

ANALYSIS OF INSECT HORMONES BY MEANS OF A RADIAL COMPRESSION SEPARATION SYSTEM

Ernest S. Chang
Bodega Marine Laboratory
P.O. Box 247, Bodega Bay, CA 94923
and
Department of Animal Science
University of California, Davis, CA 95616

ABSTRACT

The use of a radial compression separation system for the analysis of insect hormones is described. By simple isocratic elution, both steroid molting hormones and terpenoid juvenile hormones are rapidly separated. The system is used to analyze the metabolism of juvenile hormone by an established cell line of Drosophila melanogaster.

INTRODUCTION

Two major groups of non-peptide hormones influence the development of insects: ecdysteroids and juvenile hormones (JH) (Fig. 1). The ecdysteroids, polyhydroxy steroids that initiate molting, are present in both insects and crustaceans (1,2). The juvenile hormones are homosequiterpenoids that mediate the qualitative nature of the molting process. Juvenile hormones have been found conclusively so far only in insects. The separation techniques for these two groups of hormones include low-pressure column, thin-layer (TLC), gas-liquid (GLC), and high-performance

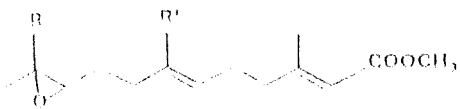
 0148-3919/83/0602-0291$3.50/0

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1725 (1983)

ERRATUM

E. S. Chang, J. Liq. Chrom., 6(2), 291-299 (1983)
"Analysis of Insect Hormones By Means of a Radial Compression Separation System."

Figure 1 in this paper was incorrect. The correct structures for Figure 1 are:



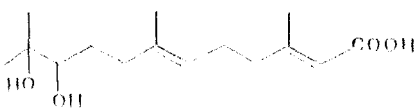
JHI I: R: Et, R': Et

JHI II: R: Et, R': Me

JHI III: R: Me, R': Me

JHI III acid

JHI III diol



JHI III acid diol

liquid chromatography (HPLC) (3,4). Radially compressed columns enable even more rapid separations of these compounds than have previously been reported.

MATERIALS AND METHODS

The juvenile hormones (JH I, methyl (2E,6E,10Z)-(10R,11S)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate; JH II, methyl (2E,6E,10Z)-(10R,11S)-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate; and JH III, methyl (2E,6E)-10-11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate) were obtained from Calbiochem. The metabolites of JH III (the JH III acid, JH III diol, and JH III acid-diol) were prepared according to previously published procedures (5,6). Radiolabeled JH III (10-^{3}H) was obtained from New England Nuclear (sp. act. 11 Ci/mmol). Ecdysone (2β,3β,14α,22R, 25-pentahydroxy-5β-cholest-7-en-6-one) was obtained from Simes and 20-hydroxyecdysone (2β,3β,14α,20R,22R, 25-hexahydroxy-5β-cholest-7-en-6-one) from Rohto Pharmaceutical Co. Hormones and metabolites were purified by TLC or HPLC if necessary.

Analyses were performed with a Waters system, consisting of a M-45 solvent delivery system, U6K injector, 440 UV detector set to 254 nm, RCM-100 radial compression module, and a C_8 Radial-Pak cartridge (0.5 i.d. x 10 cm). Data were analyzed with a reporting integrator (3390A, Hewlett-Packard). The solvents, reagent grade water (Milli-Q, Millipore Corp.) and methanol (Nanograde, Mallinkrodt), were filtered and degassed by means of extensive stirring prior to use. The ecdysteroids were eluted with 50% methanol at a flow rate of 1.0 ml per min, the JH homologs with 75% methanol at 2.0 ml per min, and the JH metabolites with 60% methanol at 1.5 ml per min.

The Drosophila melanogaster Kc cells were cultured as described previously (7). To examine the metabolism of ^{3}H-JH III by the Kc cells, approximately 10^{8} cells were washed in TMK buffer

(10 mM Tris, 5 mM $MgCl_2$, 150 mM KCl, pH 7.4) and resuspended in 1 ml of TMK buffer, containing 1 μCi of the labeled hormone. The cells were incubated at 25° for 30 min with gentle agitation. The cells were then pelleted and the supernatant was extracted with 3 x 2 ml ethyl acetate. The organic phases were combined and concentrated. The residue was resuspended in 1 ml of methanol, filtered (0.22 μm, Fluoropore membrane, Millipore Corp.) and concentrated to 50 μl prior to injection.

RESULTS

Figure 2 is a chromatogram of ecdysone and 20-hydroxyecdysone. Preliminary data indicate that other ecdysteroids can also be separated by this system.

The separation of the three principal homologs of JH is shown in Figure 3. Reduction in the side-chain length from ethyl to methyl results in an increase in the polarity of the molecule, reflected in a decreased elution volume.

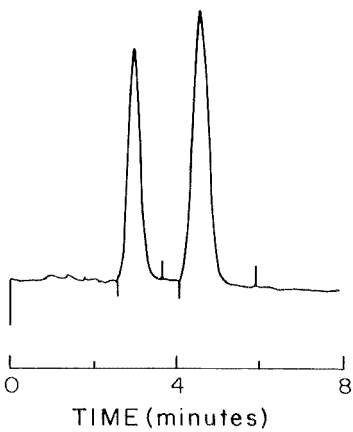


FIGURE 2. Chromatogram of 20-hydroxyecdysone (2.68 min, 88 pmol) and ecdysone (4.23 min, 138 pmol). Solvent, 50% aq. methanol; flow-rate, 1.0 ml/min.

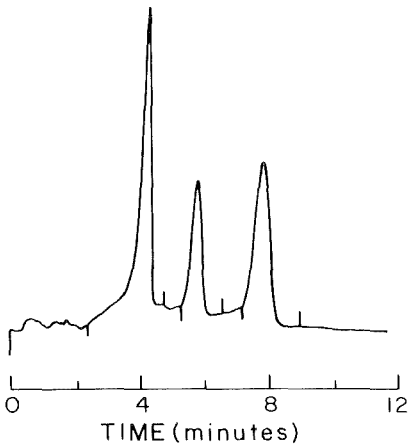


FIGURE 3. Chromatogram of JH III (3.90 min, 1.0 nmol), JH II (5.44 min, 0.4 nmol), and JH I (7.38 min, 0.7 nmol). Solvent, 75% aq. methanol; flow-rate, 2 ml/min.

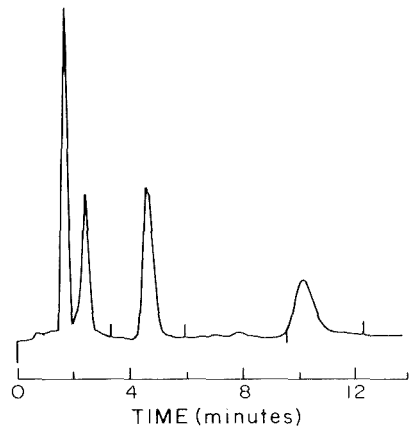


FIGURE 4. Chromatogram of JH metabolites: JH III acid-diol (1.56 min, 0.46 nmol), JH III acid (2.27 min, 0.33 nmol), JH III diol (4.41 min, 0.48 nmol), and JH III (9.60 min, 0.37 nmol). Solvent, 60% aq. methanol; flow-rate, 1.5 ml/min.

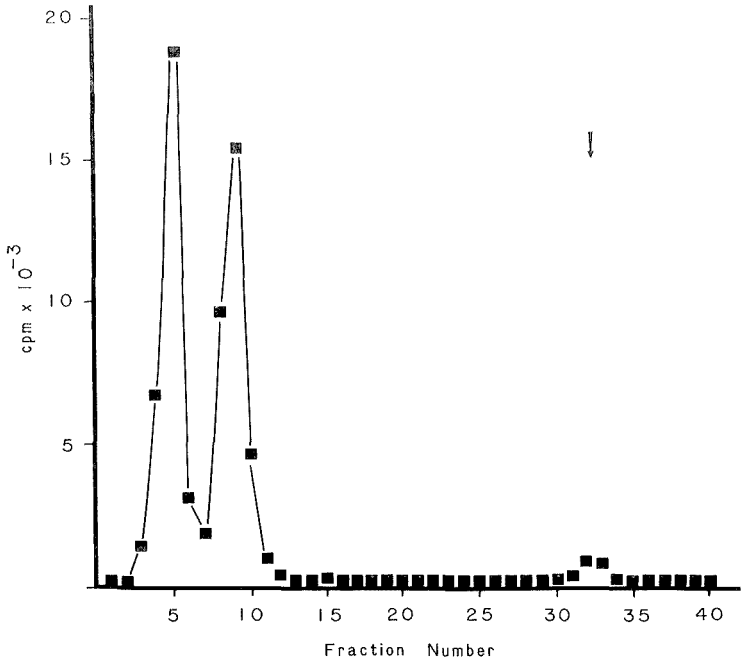


FIGURE 5. Radiochromatogram following HPLC analysis of ^{3}H-JH III, incubated with Drosophila Kc cells. Solvent, 60% aq. methanol; flow-rate, 1.5 ml/min, fractions, 0.45 ml/0.3 min. Arrow, elution of authentic JH III.

Figure 4 is a chromatogram of the predominant biological inactivation products of JH. The JH acid is formed in biological systems by the action of an esterase, the JH diol by hydrolysis of the diol by an epoxide hydrolase, and the JH acid-diol by the sequential action of both of these two enzymes. Depending on the enzyme system, either the acid or the diol may be the preferred intermediate in the acid-diol formation.

In order to determine whether the above separation system could be used to analyze metabolites from a biological system, ^{3}H-JH III was added to an established cell line of Drosophila

melanogaster. These cells have especially high levels of the catabolic enzymes (8). As seen in Fig. 5, almost all of the radiolabeled hormone was metabolized to the acid and the acid-diol. By addition of an internal JH III standard, it was ascertained that the biological extract did not alter the elution volume of the authentic hormone.

DISCUSSION

Because the two groups of insect developmental hormones, ecdysteroids and juvenile hormones, interact in a complex manner (1), it is important to consider the effects and titers of both classes of hormones in insect systems. HPLC has been used for such studies. Lafont et al. (9) have applied reversed-phase HPLC for the separation of complex mixtures of ecdysteroids and their metabolites. The conditions reported in the present paper, though perhaps not as well suited for complex mixtures of ecdysteroids, provide a more rapid method for the analysis of ecdysone and 20-hydroxyecdysone, the two molting hormones most frequently encountered in biological systems (10,11). This separation system should be suitable for the routine determination of the ecdysone to 20-hydroxyecdysone ratios in arthropod blood (12).

Rapid separations by reversed-phase HPLC have also been developed for JH (13), primarily as a purification step prior to mass spectrometry. The advantages of the conditions reported in the present publication are speed, use of the same solvents for all separations, and relatively low cost of the radially-compressed columns.

Although more extensive purification of biological samples is required for direct quantitation of these hormones by UV absorption, the separations described are adequate for hormone analysis by an indirect method, such as radioimmunoassay (14,15) or scintillation spectrometry. The separation of radiolabeled JH

metabolites described in this report indicates that no additional purification is necessary for HPLC analysis following extraction.

ACKNOWLEDGMENTS

Portions of this research were generously supported by a grant (CD-12) from the American Cancer Society.

REFERENCES

1. Granger, N.A., and Bollenbacher, W.E., Metamorphosis, A Problem in Developmental Biology, Gilbert, L.I., and Frieden, E., eds., 2nd edition, Plenum Press, New York, 1981, p. 105.

2. Gilbert, L.I., and Goodman, W., Metamorphosis, A Problem in Developmental Biology, Gilbert, L.I., and Frieden, E., eds., 2nd edition, Plenum Press, New York, 1981, p. 139.

3. Morgan, E.D., and Poole, C.F., The extraction and determination of ecdysones in arthropods, Advances In Insect Physiol., 12, 17, 1976.

4. Schooley, D.A., Analytical Biochemistry of Insects, Turner, R.B., ed., Elsevier, New York, 1977, p. 241.

5. Slade, M., and Zibitt, C., Insect Juvenile Hormones: Chemistry and Action, Menn, J.B., and Beroza, M., eds., Academic Press, New York, 1972, p. 155.

6. Goodman, W.G., and Goodman, H.L., Juvenile Hormone Biochemistry Action, Agonism and Antagonism, Pratt, G.E., and Brooks, G.T., eds., Elsevier/North-Holland, New York, 1981, p. 365.

7. Chang, E.S., Coudron, T.A., Bruce, M.J., Sage, B.A., O'Connor, J.D., and Law, J.H., Juvenile hormone-binding protein from the cytosol of Drosophila Kc cells, Proc. Nat. Acad. Sci. U.S.A., 77, 4657, 1980.

8. O'Connor, J.D., and Chang, E.S., Metamorphosis, A Problem in Developmental Biology, Gilbert, L.I. and Frieden, E., eds., 2nd edition, Plenum Press, New York, 1981, p. 241.

9. Lafont, R., Somme-Martin, G., Mauchamp, B., Maume, B.F., and Delbecque, J.-P., Progress in Ecdysone Research, Hoffmann, J.A., ed., Elsevier/North-Holland, New York, 1980, p. 45.

10. Chang, E.S., Sage, B.A., and O'Connor, J.D., The qualitative and quantitative determinations of ecdysones in tissues of the crab, Pachygrapsus crassipes, following molt induction, Gen. Comp. Endocrinol., 30, 21, 1976.

11. Bollenbacher, W., Vedeckis, W., Gilbert, L.I., and O'Connor, J.D., Ecdysone titers and prothoracic gland activity during the larval-pupal development of Manduca sexta, Develop. Biol., 44, 46, 1975.

12. Chang, E.S., and O'Connor, J.D., In vitro secretion and hydroxylation of α-ecdysone as a function of the crustacean molt cycle, Gen. Comp. Endocrinol., 36, 151, 1978.

13. Mauchamp, B., Lafont, R., and Krien, P., Juvenile Hormone Biochemistry Action, Agonism, and Antagonism, Pratt, G.E., and Brooks, G.T., eds., Elsevier/North-Holland, New York, 1981, p. 21.

14. Chang, E.S., and O'Connor, J.D., Methods of Hormone Radioimmunoassay, Jaffe, B.M., and Behrman, H.R., eds., 2nd edition, Academic Press, New York, 1978, p. 797.

15. Baehr, J.C., Caruelle, J.P., Porcheron, P., and Cassier, P., Juvenile Hormone Biochemistry Action, Agonism, and Antagonism, Pratt, G.E., and Brooks, G.T., eds., Elsevier/North Holland, New York, 1981, p. 47.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 301-310 (1983)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION IN HUMAN PLASMA OF A ANTICONVULSANT BENZODIAZEPINE : CLONAZEPAM

S. BOUQUET, P. AUCOUTURIER, A.M. BRISSON, Ph. COURTOIS and J.B. FOURTILLAN
Département de Pharmacocinétique
Centre Hospitalier Universitaire JEAN-BERNARD
86021 POITIERS - CEDEX - FRANCE

ABSTRACT

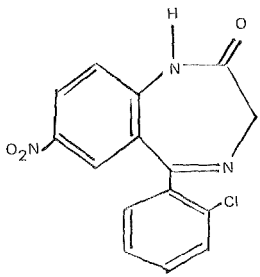
A rapid and specific high-pressure liquid chromatography method for determination of clonazepam in human plasma is described.

The analysis is linear for concentrations ranging from 5 to 100 ng. ml^{-1} plasma for clonazepam.

The method is applicable to quantitation of clonazepam in human plasma of subjects receiving 0.05 at 0.20 mg.kg^{-1} orally, with satisfactory accuracy and precision.

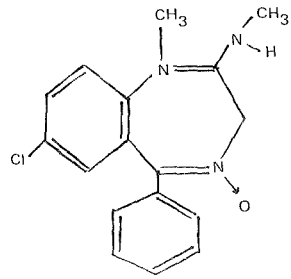
INTRODUCTION

In recent years, the use of clonazepam (5 ortho-chlorophenyl, 7 nitro, 2,3-dihydro, 1-4 benzodiazepine 2-one) has been developed as a treatment for convulsions (8 , 12).



CLONAZEPAM :

(5-ortho-chlorophenyl 7-nitro 2,3-dihydro 1,4-benzodiazepine 2-one)



CHLORDIAZEPOXIDE : (Internal standard)

(7-chloro-2 methylamino-5 phenyl-3H-1,4 benzodiazepine-4-oxide).

0148-3919/83/0602-0301$3.50/0

Recently, a few authors have advised the prescription of anticonvulsant drug monotherapy (2, 10, 12).

Meanwhile, it is difficult to understand why an identical drug dosage may exert a toxic effect in one patient and a therapeutic, or no reponse in another patient. Numerous clinical studies (2, 8, 9, 13) have adequately demonstrated the importance of total plasma concentrations of clonazepam in relation to its efficacy. Several analytical methods have been proposed for benzodiazepine determination in biological fluids : gas liquid chromatography (GLC) (4, 5) and high performance liquid chromatography (HPLC) (1, 3, 11, 13).

The GLC methods require somewhat lengthy clean-up procedures and, in some cases, derivatization or acid hydrolysis to the more volatile benzophenones (4, 5, 7).

High-performance liquid chromatography (HPLC) involves relatively simple extraction, no derivatization and U.V. detection to give high sensitivity, good stability, and linearity over wide concentration ranges.

This paper demonstrates the use of HPLC in a reverse phase mode to separate clonazepam from endogenous compounds, in human plasma samples.

An internal standardization technique is employed, using a structurally related benzodiazepine (chlordiazepoxide) as the internal standard.

METHODS

The procedure involves the addition of chlordiazepoxide as the internal standard. After addition of 200 µl 0.5 N NaOH ; samples are extracted using ether.

After evaporation of the organic solvant, the residue is dissolved in mobile phase and the benzodiazepine is analysed isocratically by reverse phase high-pressure liquid chromatography with 40 % (v/v) acetonitrile in distilled water as eluant. The effluent is monitored by U.V. detection at 254 nm.

Apparatus

The chromatographic determinations were performed with a Waters Associates Liquid Chromatograph Model No 440-03773, equipped with a model 440 absorbance detector (254 nm wavelength), a U_6K injector, a flow pump Model 6000 A (Waters Associates Inc. Milford, Mass. 01757, and a 10 mV recorder Omniscribe (Houston Instruments, Gistel, Belgium).An octadecylsilane µ-Bondapack C_{18} column 3.9 mm i.d.X 30 cm long (Waters Associates) was used under ambient conditions, for the separation. The isocratic mobile phase consisted of acetonitrile/bi-distilled water (40/60, v/v). The solution was filtered through 0.22 µm pore membrane filter type GS-ester of celluose (Millipore Corp., Bedford, Mass. 01730) and the flow rate was 1.5 $ml.mn^{-1}$. Under these conditions, clonazepam and the internal standard (chlordiazepoxide) were eluted with retention times of 8.3 and 9.2 min., respectively, as illustrated in Figure 1.

Reagents and drugs :

Clonazepam and chlordiazepoxide, pharmaceutical grade, were obtained from Roche Laboratories (Neuilly - France). The water was double-distilled and filtered through a 0,22 µ (type GS - ester of cellulose) filter (Millipore, Corp., Bedford, Mass. 01730).

Methanolic stock solutions of clonazepam and chlordiazepoxide (the internal standard) were prepared at a concentration of 100 µg/ml and could be stored at 4°C during a week, in the dark. For the determination of low concentrations, extraction solvent : diethyl ether, n-hexane and acetonitrile of high purity (Chrom AR Nanograde, Byk-Mallinckrodt, Wessel, GFR) were used.

U.V. grade methanol and sodium hydroxyde 30 % type RP, were purchased from (Prolabo, Paris, France) and (Carlo Erba, Milan, Italy), respectively.

Extraction procedure :

Into a screw-stoppered test tube, put one ml of plasma ; add 50 µl of aqueous chlordiazepoxide solution (10 $\mu g.ml^{-1}$), adjust to pH 9.5 with 0.5 N sodium hydroxyde (about 0,200 ml for 1 ml plasma), homogenize by slow rotation.

The drug was extracted with 5 ml of ethyl-ether by shaking mecanically for 10 min and centrifuged for 5 mn at 3000 rpm. An aliquot of the upper organic layer was transferred to another test-tube.

Re-extract the sample, proceeding as before. Combine the ethereal extracts and evaporate to dryness under dry nitrogen at 35°C.

Take up the residue with 200 µl of 0.2 M HCl, add 200 µl of n-hexane, homogenize for 30 sec. on a vortex mixer and centrifuge at 3000 rpm for 3 min. Remove and discard the upper hexanic phase (containing the lipids extracted from the plasma). An aliquot of 100 µl aqueous phase was injected into the HPLC system

RESULTS AND DISCUSSION

We found a linear correlation between the concentration of clonazepam and the ratio of peak heights : clonazepam : internal standard, in the range between 12.5 and 100 $ng.ml^{-1}$ of the plasma samples. (Figure 1 and 1 Bis) R = 0.99. Addition of an internal standard to the plasma prior to extraction allows quantitative measurements.

No interfering peak with the retention time of Clonazepam was present in extracts of "blank" control plasma (Figure 2).

For lower concentrations, it is advisable to start with 2 ml of plasma and work at 0.005 A.U.F.S. sensitivity. Under these conditions, the limit of detection of clonazepam was approximately 10 $ng.ml^{-1}$ of plasma.

Figure 3 illustrates the chromatographic profile of a human plasma extract, from a patient receiving daily oral administration 4 mg clonazepam. The known metabolites of clonazepam in which amino and acetamido substituents

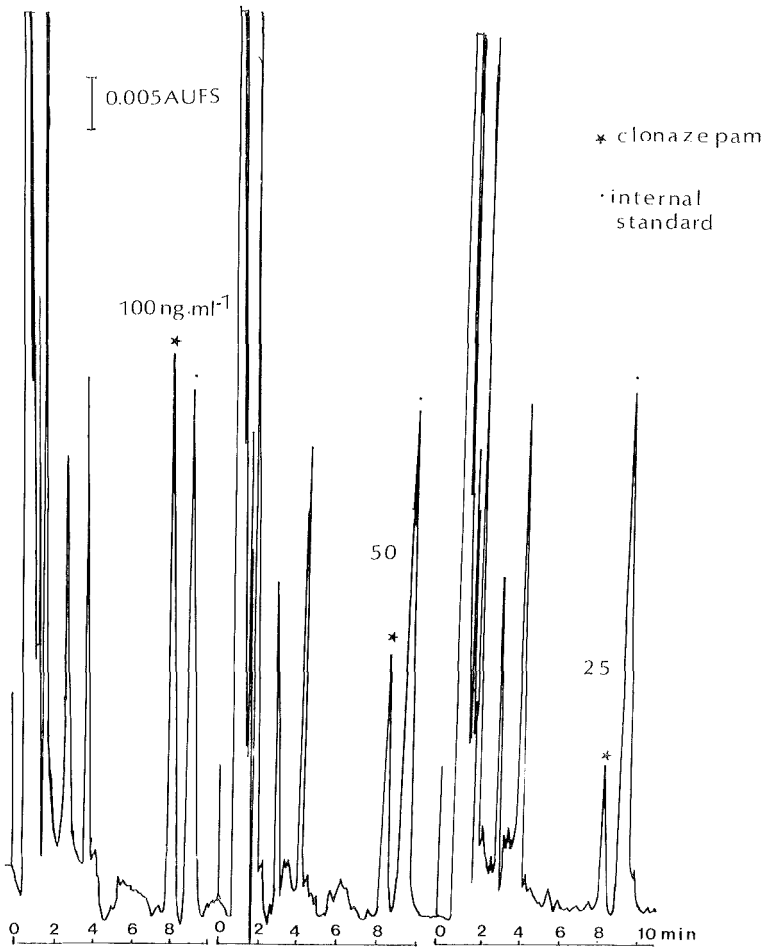


FIGURE 1 : CHROMATOGRAM OF CLONAZEPAM

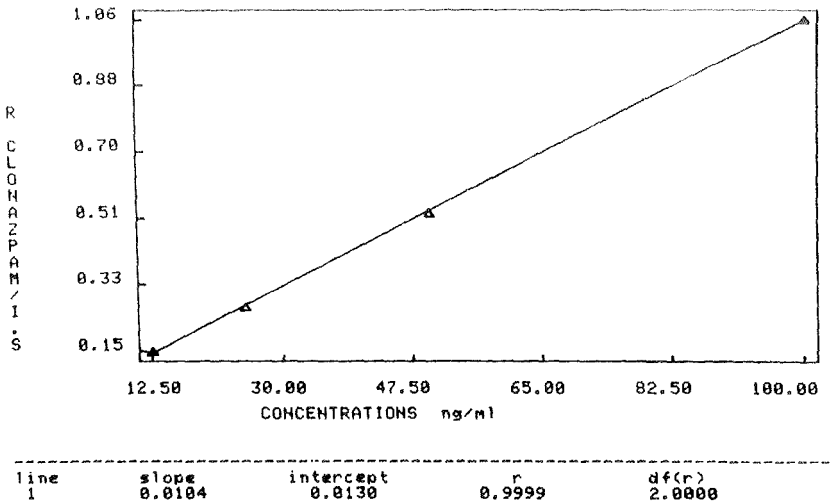


FIGURE 1 BIS : REGRESSION ANALYSIS CLONAZEPAM

are present on the drug molecule, should not interfere with the analysis of clonazepam (Figure 3).

Figure 4 : the separation of a test mixture of benzodiazepines (2, 3, 4, 5, 6, 7) and carbamazepine (1), showing the good selectively of the phase system for clonazepam (3).

In this work, flunitrazepam (5) and desmethyldiazepam (7) were found interfere with chlordiazepoxide (4) and clobazam (6), respectively.

Nevertheless, the peaks given by oxazepam (2) and carbamazepine (1) were not completely separated.

Reproducibility of the extraction procedure was determined by extracting a plasma sample containing 50 $ng.ml^{-1}$ clonazepam daily over a 10 day period, with the following results (n = 10, CV = 3.6 %).

Another standardization sample containing 200 $ng.ml^{-1}$ in water was directly injected daily, producting the following values (n = 10, CV = 4.5 %).

Steady-state plasma values for individual clonazepam are published (4, 6), obtained by other techniques, from adult chronic patients undergoing continuous treatment (4 to 8 mg daily). We chose values similar to these (I.e 20 to 60 $ng.ml^{-1}$). No appreciable loss in resolution was observed during the chromatography of more than 200 plasma sample extracts.

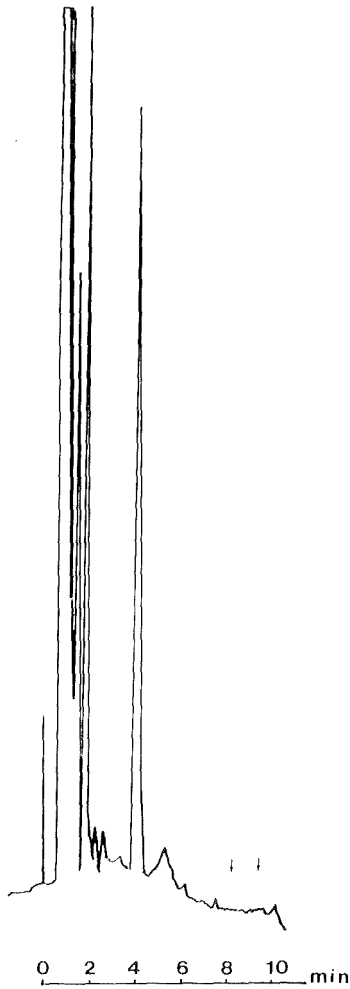


FIGURE 2 : A BLANK PLASMA EXTRACT

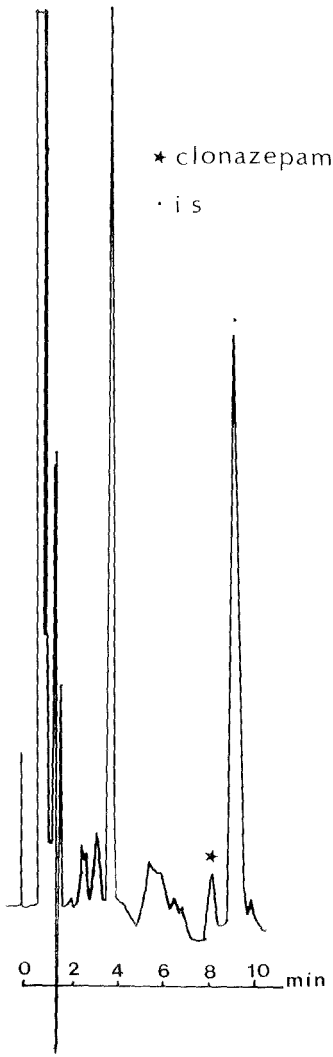


FIGURE 3 : CHROMATOGRAM OF A HUMAN PLASMA

(* 21 $ng.ml^{-1}$)

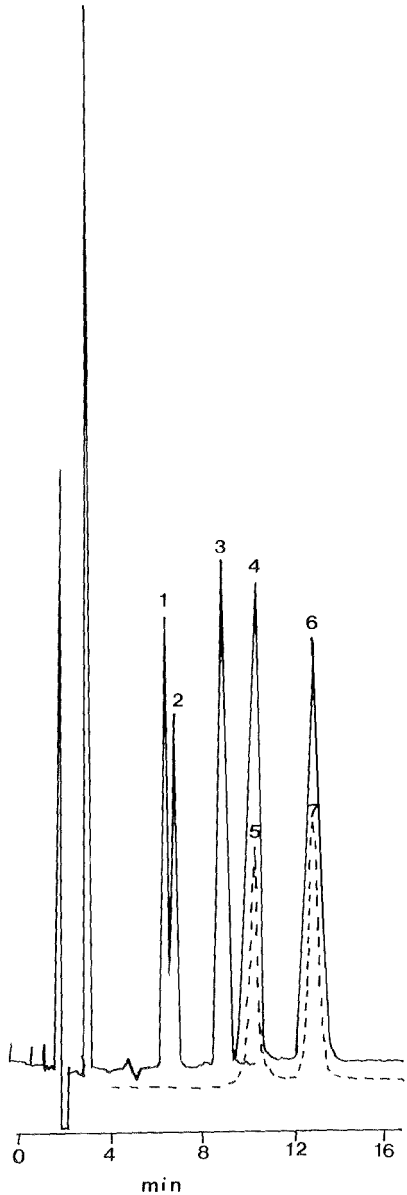


FIGURE 4

In conclusion, we consider that reverse-phase HPLC is the most suitable method of analysis for determining clonazepam in human plasma because of its specificity, sensitivity, simplicity and speed of execution.

REFERENCES

1. R.F. Adams, F.L. Vandemark, "Simultaneous high pressure liquid chromatographic determination of some anticonvulsants in serum", Clin. Chem., 22, 1, 25 (1976)

2. A. Baruzzi, G.P. Cabrini, V.A. Gernam-Sironi and P.L. Morselli, "Anticonvulsant plasma levels monitoring in epileptic patients undergoing stereo EEG in Gardner Thorpe (eds),"Clinical Pharmacology of Antiepileptic Drugs", vol. II, Pitman Medical, publisher Kent, (1977), p. 197.

3. R.R. Brodie, L.F. Chasseaud and T. Taylor, "High-performance liquid chromatographic determination of benzodiazepines in human plasma", J. Chrom., 150 : 361, (1978).

4. J.P. Cano, J. Guintrand, C. Aubert, A. Viala, "Determination of flunitrazepam, desmesthylflunitrazepam and clonazepam in plasma by gas liquid chromatography with an internal standard", Arzneimittel Forsch, 27 : 2, 338, (1977).

5. J.A. De Silva, I. Berkersky, C.V. Puglisi, M. Brooks, R.E. Weinfeld, "Determination of 1,4-benzodiazepines and diazepin-2-ones in blood by electron capture gas-liquid chromatography", Anal. Chem., 48 : 1, 10, (1976).

6. E. Eschenhof, "The pharmacokinetics of clonazepam", Arzneimittel Forsch, 23 : 390, (1973).

7. A. Frigerio, K.M. Baker and G. Belvedere, "Gas chromatographic degradation of several drugs and their metabolites", Anal. Chem., 45 : 1846, (1973).

8. J. W. Lance, M. Antony, "Sodium valproate and clonazepam in the treatment of intractable epilepsy", Arch. Neurol., 34 : 14, (1977).

9. P.L. Morselli, "Clinical significance of monitoring plasma levels of benzodiazepine tranquilisers and antiepileptic drugs", Neuro-Psychopharmacology, edited by P. Deniker, C. Radouco-Thomas, A. Villeneuve, Pergamon Press Oxford & New York (1978), p. 877.

10. E.H. Reynolds, S.D. Shorvon S.D., "Medical treatment of epilepsy : monotherapy versus polytherapy, New Press Med., 10 : 3717, (1981).

FIGURE 4 : SEPARATION OF A TEST MIXTURE OF DIFFERENT BENZODIAZEPINES AND CARBAMAZEPINE.
COLUMN : μ BONDAPACK C_{18} 3.9 MM I.D. x 30 cm L
ELUENT : ACETONITRILE - BI-DISTILLED WATER (40 : 60 v/v)
FLOW RATE : 1.5 $ml.mm^{-1}$.
PEAKS : 1 = CARBAMAZEPINE , 2 = OXAZEPAM, 3 = CLONAZEPAM,
4 = CHLORDIAZEPOXIDE, 5 = FLUNITRAZEPAM,
6 = CLOBAZAM, 7 = DESMETHYL-DIAZEPAM.

11. G.C. Skeller, J. Meier, B.I. Knight., B. Whiting, "The application of HPLC to the determination of some 1-4 benzodiazepines and their metabolites in plasma", Brit. Clin. Pharmacol., 5 : 483, (1978).

12. E.A. SWINYARD and A.W. Castellion, "Anticonvulsant properties of some benzodiazepines", J. Pharmacol. Exp. Ther., 151 : 369, (1966).

13. U.R. TJADEN, M.T.H.A. Meeles, C.P. Thys and M. Vanderkaay, "Determination of some benzodiazepines and metabolites in serum, urine and saliva by high-performance liquid chromatography", J. Chrom., 181 : 227, (1980).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 311-323 (1983)

THE ANALYSIS OF ENANTIOMERIC AND DIASTEREOISOMERIC MIXTURES OF EPHEDRINE AND PSEUDOEPHEDRINE USING REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF NICKEL DITHIOCARBAMATE COMPLEXES

G.K.C.Low*, P.R.Haddad* and A.M.Duffield†
Department of Analytical Chemistry (*) and Biomedical Mass Spectrometry Unit (†), University of New South Wales, P.O. Box 1 Kensington New South Wales 2033 Australia

ABSTRACT

Following their conversion to dithiocarbamate ligands and thence to nickel complexes, enantiomeric mixtures of ephedrine or pseudoephedrine may be separated and quantitated by reversed-phase High Performance Liquid Chromatography (HPLC) using ternary solvent mixtures. The solvent used to dissolve the complexes prior to injection was found to have a significant effect on the separation. In a similar manner, a mixture of the diastereoisomers ephedrine and pseudoephedrine was separated and quantitatively analysed using a binary solvent as the mobile phase. This separation was achieved both with prior formation of the nickel complexes and also with on-column formation using nickel(II) ions in the mobile phase. The analysis of diastereoisomeric contaminants in pharmaceutical products and raw materials containing ephedrine or pseudoephedrine is illustrated.

INTRODUCTION

Dithiocarbamate complexes of nickel can readily undergo ligand exchange reactions to produce ternary, or mixed-ligand, complexes (1), according to the following equilibrium:

$$Ni(L_1)_2 + Ni(L_2)_2 \rightleftarrows NiL_1L_2$$

 0148-3919/830602-0311$3.50/0

L_1 and L_2 are bidentate dithiocarbamate ligands, $Ni(L_1)_2$ and $Ni(L_2)_2$ are binary complexes and NiL_1L_2 is a ternary complex. If this reaction mixture is analysed by normal-phase High Performance Liquid Chromatography (HPLC), three peaks are observed, with the ternary complex peak lying between the two binary complex peaks. When L_1 and L_2 are enantiomers, then the two binary complexes co-elute as a single peak, prior to the ternary complex peak.

Moriyasu and Hashimoto (2,3) have utilised these observations for the precise quantitation of enantiomeric impurities in amines, after conversion of the amines to dithiocarbamate ligands by reaction with carbon disulphide under alkaline conditions, and subsequent formation of nickel complexes. The HPLC separation of the binary and ternary nickel complexes was achieved using a water deactivated silica column, however this method has the serious disadvantage that long equilibration times are required to give reproducible results (4).

In this paper, we describe the use of reversed-phase HPLC for the analysis of enantiomeric and diastereoisomeric impurities in amines, using binary and ternary solvent mixtures. This approach is discussed both for the separation of nickel complexes formed prior to injection, and also for the separation of dithiocarbamate ligands using mobile phases containing nickel ions. In the latter method, dithiocarbamate binary and ternary nickel complexes are formed on the column. This procedure reduces the number of manipulative steps in the analysis. The reversed-phase HPLC approach provides the basis of a rapid, sensitive analytical method for the screening of enantiomeric and structurally related contaminants in pharmaceutical products containing amines such as ephedrine and pseudoephedrine. Such an analysis has hitherto proved very difficult (5). A brief survey of the application of the proposed method to the analysis of some pharmaceutical products and raw materials is also presented.

EXPERIMENTAL

Standards and Reagents

(i) Carbon disulphide-chloroform solution. Carbon disulphide (A.R.Grade, AJAX) was freshly distilled in all glass apparatus and made up to 1% v/v solution with redistilled chloroform.

(ii) Nickel-ammonia solution. 50 ml of 1% w/v of $NiCl_2 6H_2O$ in water was made up to 100 ml with 35% ammonia solution (ARISTAR, BDH).

(iii) Aromatic amine solutions. (±) ephedrine hydrochloride, (-) pseudoephedrine hydrochloride, and (+) pseudoephedrine hydrochloride were obtained from Sigma Chemical Company (USA). (-) ephedrine was obtained from Fluka (Switzerland). These materials were shown to be free from contaminants by GCMS analysis and by compositional data from microanalysis. Test solutions containing approximately 1.0 mg/ml were accurately made up in methanol.

(iv) Tablets and raw materials containing ephedrine and pseudoephedrine. Raw materials containing pseudoephedrine and ephedrine were donated by various pharmaceutical manufacturers in Australia. Single ingredient tablets with declared potencies of 15, 30 or 60 mg of amine were purchased over the counter.

Synthesis of Dithiocarbamate Complexes

To 1.0 ml of aromatic amine solution, 2 ml of nickel-ammonia solution were added and the mixture extracted with carbon disulphide-chloroform (5 ml; 1% v/v). The chloroform layer was then washed with distilled water (3 x 2 ml) and dried over anhydrous sodium sulphate. The filtered chloroform layer was evaporated to dryness under a steady stream of nitrogen to remove excess carbon disulphide which may produce extraneous chromatographic peaks, and made up to an appropriate volume in methanol or acetonitrile. The nickel complex prepared in this way was shown

to give only one chromatographic peak by HPLC. The identity of each complex was confirmed by Desorption Chemical Ionisation Mass Spectrometry (6).

Analysis of Ephedrine and Pseudoephedrine in Pharmaceutical Formulations

Twenty randomly selected tablets from a single batch were crushed and an accurately weighed portion of powder equivalent to the average weight of a single tablet was taken and dissolved in water in a volumetric flask with the aid of an ultrasonic bath. The sample was diluted to a suitable volume to give a concentration of 1 mg/ml, after which the solution was filtered and an aliquot equivalent to 10 mg of drug was made alkaline with 2M NaOH and extracted with 20 ml of chloroform. The organic layer was passed through a column of anhydrous sodium sulphate and 10 ml was evaporated to dryness under a stream of nitrogen. The nickel complex was then formed by the method described in the previous section. The final solution was made up to a concentration of 0.1 mg/ml in methanol and 5-10 µl of the solution was injected with the HPLC detector set at 0.2 AUFS.

For the raw materials, appropriate dilutions were made to give the concentration described above and the analysis performed using the same procedure as for the tablets.

Quantitations were made using chromatographic peak heights and all assays were performed in triplicate. For most samples, two determinations at two different sensitivity levels were required: the first was performed at the most sensitive attenuation position of the detector to establish whether any contaminant was present, after which a less sensitive attenuation was used for the determination of the active ingredient in the sample.

HPLC Instrumentation and Procedure

The liquid chromatograph consisted of Waters Associates (Milford Ma) Model M6000 solvent pump, Model U6K injector, Model M440 UV

detector and QD 15 Hitachi Recorder. The column used was a 15 cm x 4.6 mm ID Ultrasphere (Altex Scientific Inc.,Berkeley,Ca) C_{18} column, with a mean particle diameter of 5 μm. The detector was operated at 313 nm with a sensitivity setting of 0.2 AUFS and all separations were carried out at 20°C using a mobile phase flow rate of 1.5 ml min^{-1}.

Methanol and triethylamine were of Analytical Grade and were distilled in all glass apparatus. Acetonitrile (HPLC Grade) was purchased from Waters Associates. The exact ingredients of the mobile phases used are given in the captions to the figures. Mobile phases were aspirated through 0.7μm glass microfibre paper filters (GF/F Whatman), degassed in an ultrasonic bath and allowed to equilibrate to ambient temperature before used.

RESULTS AND DISCUSSION

Analysis of Mixtures of Enantiomers

A mixture containing both enantiomers of ephedrine or pseudoephedrine was quantitatively analysed by conversion of these amines to dithiocarbamate ligands and thence to nickel complexes, with subsequent separation using reversed-phase HPLC. Typical chromatograms are shown in Fig. 1; these separations are similar to that previously reported for normal-phase HPLC using water deactivated silica columns (2,3). In each chromatogram, the symmetrical binary complexes derived from each enantiomer coeluted as the first peak, which was separated from the peak due to the ternary complex containing ligands derived from both enantiomers. To simplify reference to these complexes, they will be identified as follows: $Ni[CS_2:(+)eph]_2$ is the complex containing two ligand molecules, both of which are dithiocarbamate ligands derived from (+) ephedrine; similarly $Ni[CS_2:(+)eph][CS_2:(-)eph]$ is the ternary complex containing dithiocarbamate ligands derived from (+) ephedrine and (-) ephedrine. Pseudoephedrine will be abbreviated to pse.

Separation of the binary and ternary nickel dithiocarbamate complexes shown in Fig. 1 was achieved using a ternary solvent

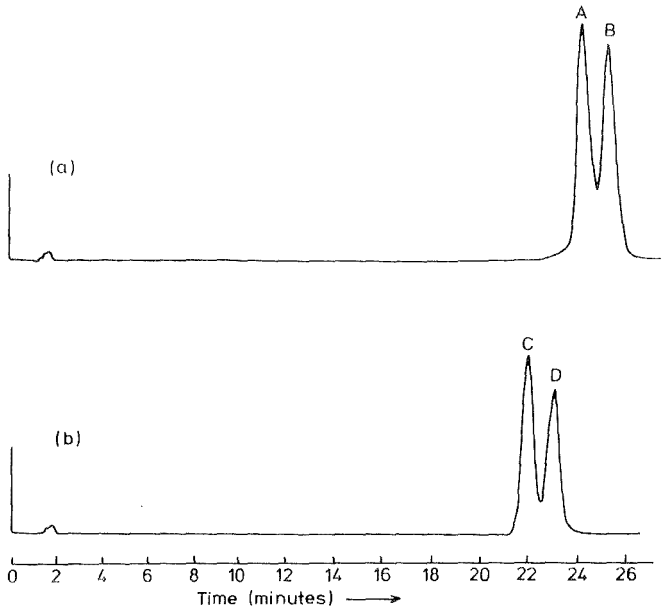


FIGURE 1. Analysis of a mixture of enantiomers of ephedrine [Fig. 1(a)] or pseudoephedrine [Fig. 1(b)].

Mobile Phase: 0.2% (v/v) triethylamine in 25:40:35 (v/v) $CH_3OH:CH_3CN:H_2O$. Flow rate 1.5 ml/min.

Peak Identities: See text for key to abbreviations A, unresolved $Ni[CS_2:(+)eph]_2$ and $Ni[CS_2:(-)eph]_2$; B, $Ni[CS_2:(+)eph][CS_2:(-)eph]$; C, unresolved $Ni[CS_2:(+)pse]_2$ and $Ni[CS_2:(-)pse]_2$; D, $Ni[CS_2:(+)pse][CS_2:(-)pse]$.

system. The seven experiment optimisation procedure of Glajch and co-workers (7) was applied to determine the composition of a suitable isocratic solvent system. A mobile phase containing 25:40:35 (v/v) methanol:acetonitrile:water was found to give optimum resolution, and a small amount of triethylamine was added to further improve the separation, because our previous experiences (6) have indicated that this solvent has a highly selective interaction with the dithiocarbamate complexes under study.

The solvent used to dissolve the complexes prior to injection was found to have a significant influence on the separation achieved by reversed-phase HPLC. When chloroform was used, poor separation and diffuse peak shape resulted; on the other hand, methanol and acetonitrile gave better peak shape and resolution, with the latter solvent giving optimum results. This effect was probably due to a change in the composition of the adsorbed layer of organic modifier (from the mobile phase) on the stationary phase surface. Adsorption of organic modifiers onto reversed-phase columns has been reported previously (8) and it has been proposed that this adsorbed layer can be partially displaced by solute or other molecules (9). In the present case, it is likely that injection of a chloroform solution caused a change in the adsorbed layer of methanol and acetonitrile (from the mobile phase), thereby influencing the ability of the column to resolve the closely related nickel complexes.

The formula proposed by Moriyasu and Hashimoto (2,3) for calculation of the composition of an enantiomeric mixture of amines using normal phase HPLC is equally applicable to reversed-phase HPLC. The reversed-phase method however has the advantage that no lengthy column equilibration time was required for reproducible results, as was the case for the normal-phase method. We have found excellent agreement for analyses of racemic mixtures using both methods.

Analysis of Diastereoisomeric Mixtures

A mixture containing two diastereoisomers, such as ephedrine and pseudoephedrine, can also be separated by reversed-phase HPLC using binary solvent mixtures as the mobile phase. Such a separation is shown in Fig. 2, where the three peaks in the chromatogram can be assigned to the two binary complexes $Ni[CS_2:(-)eph]_2$ and $Ni[CS_2:(-)pse]_2$ and to the ternary complex $Ni[CS_2:(-)eph][CS_2:(-)pse]$. Again, this chromatogram is similar to that obtainable with normal-phase HPLC (2,3). The relative peak heights in Fig. 2 may be used to quantitatively

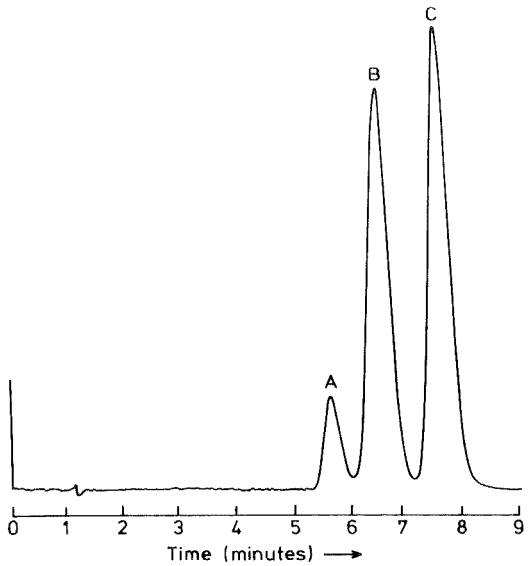


FIGURE 2. Analysis of a mixture of the diastereoisomers (-) ephedrine and (-) pseudoephedrine by prior formation of their nickel dithiocarbamate complexes.

Mobile Phase: 70:30 (v/v) $CH_3OH:H_2O$, flow rate 1.5 ml/min.

Peak Identities: See text for key to abbreviations
A, $Ni[CS_2:(-)pse]_2$; B, $Ni[CS_2:(-)pse][CS_2:(-)eph]$;
C, $Ni[CS_2:(-)eph]_2$.

determine the composition of the original mixture of diastereoisomers. Table 1 compares the results obtained using this method with those obtained using GCMS analysis (6) of a series of standard mixtures of ephedrine and pseudoephedrine.

The accuracy of the reversed phase HPLC method was somewhat poorer than the GCMS method and best results were obtained with mixtures containing a minor percentage of one diastereoisomer. This situation is likely to occur in the analysis of diastereoisomeric contaminants in pharmaceutical samples.

TABLE 1

Analysis of Standard Mixtures of the Diastereoisomers Eephedrine and Pseudoephedrine by the Proposed Reversed-Phase HPLC Method and by GCMS

% Ephedrine in standard diastereoisomeric mixtures		
Actual (%)	by HPLC* (%)	by GCMS (%)
100	100	101
82.3	87.4	83.0
62.6	69.0	64.4
41.2	38.0	41.6
21.0	22.6	22.0
0.0	0.0	0.5

* Results shown are the average of two runs. For the HPLC method, the maximum range obtained is 2.5% and for GCMS, 1.2%. The HPLC results were calculated using the following formulae applied to chromatograms similar to that given in Fig. 2

If peak 3 > peak 1, then

$$\% \text{ Ephedrine} = 100[0.5 + \sqrt{0.5(0.5 - H_2/\sum_{i=1}^{3} Hi)}]$$

If peak 3 < peak 1, then

$$\% \text{ Ephedrine} = 100[0.5 - \sqrt{0.5(0.5 - H_2/\sum_{i=1}^{3} Hi)}]$$

where H is the height of respective nickel complex peak.

A brief survey was conducted for the presence of diastereoisomeric contamination in ephedrine and pseudoephedrine tablet formulations and pharmaceutical raw materials. A binary mixture of solvents was used for the mobile phase, therefore the resultant chromatogram did not provide information on the presence of optical isomers in the active ingredient of the formulation. A ternary solvent mobile phase would be necessary to elucidate this information (see preceding section). The results are given in Table 2.

It is noteworthy that no interference in the assay method was detected for the sugars and binding compounds present in the tablet formulations. Only peaks corresponding to the active ingredient and the diastereoisomeric contaminant (if present)

TABLE 2

Analysis of Diastereoisomeric Contaminants in Pharmaceutical Formulations and Raw Materials Containing Ephedrine or Pseudoephedrine.

Results are given as percentage ± standard deviation. Each value represents the mean of three individual assay results. The results are calculated with respect to the labelled content of the drug in each tablet; raw products are assumed to be 100% pure. Each standard deviation is estimated from the range of three results.

Sample	Ingredient Type	% Contaminant	% Active Ingredient
A	Ephedrine	n.d.	99.8 ± 4.2
B*	Ephedrine	n.d.	98.9 ± 3.6
C	Ephedrine	n.d.	92.2 ± 4.3
D*	Ephedrine	6.0 ± 0.05	95.9 ± 1.2
E	Ephedrine	n.d.	96.9 ± 2.8
F*	Ephedrine	n.d.	102.0 ± 2.4
G	Ephedrine	3.9 ± 0.04	95.0 ± 1.2
H	Pseudoephedrine	n.d.	98.0 ± 3.2
I	Pseudoephedrine	n.d.	99.7 ± 1.6
J*	Pseudoephedrine	n.d.	101.6 ± 2.4
K*	Pseudoephedrine	n.d.	99.4 ± 1.3

n.d. indicates negligible detection
* indicates that the sample is a raw product

were observed, since the derivatisation procedure used was specific for primary and secondary amines. The sensitivity of this method, with which analyses at the sub µg/ml level presented no difficulty, compared favourably with that given by Barkan and co-workers (5) for their method. Linear calibration plots were obtained with ephedrine and pseudoephedrine for injected amounts of solute in the range 100 to 500 ng, with correlation coefficients better than 0.95.

Mobile Phases Containing Ni^{2+} Ions

One of the advantages of the use of reversed-phase HPLC for the analysis of metal complexes is that the separation system can

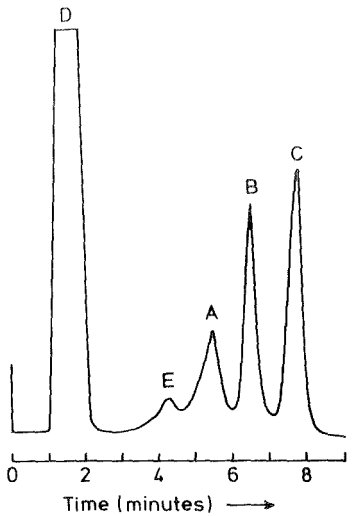


FIGURE 3. Analysis of a mixture of the diastereoisomers (-) ephedrine and (-) pseudoephedrine using on-column formation of nickel dithiocarbamate complexes.

Mobile Phase: 70:30 (v/v) CH_3OH:0.2% (w/v) aqueous $NiCl_2.6H_2O$. Flow rate 1.5 ml/min.

Peak Identities: A,B,C as for Fig. 2; D, excess carbon disulphide; E, solvent impurity.

be readily modified by addition of a metal ion to the mobile phase. When Ni^{2+} was added to the mobile phase and a mixture of diastereoisomeric dithiocarbamate ligands (derived from ephedrine and pseudoephedrine) was injected, the chromatogram shown in Fig. 3 resulted. The large first peak was due to excess carbon disulphide remaining after derivatisation of the amine durgs, and a small peak due to solvent impurity was also observed. The remaining peaks were assigned to binary and ternary nickel complexes formed during the migration of the dithiocarbamate ligands through the reversed-phase column.

This method of separation was more simple than the use of prior formation of nickel complexes and forms the basis of a rapid

and sensitive method for the detection of diastereoisomeric contaminants in primary and secondary amines. It is noteworthy that the relative peak heights obtained with the in-situ complex formation method were somewhat dependent on the mobile phase flow rate, indicating that differences in lability probably exist between the different complexes. This variation in relative peak heights was not observed when the nickel complexes were formed prior to injection.

CONCLUSIONS

Enantiomeric and diastereoisomeric amines may be separated and quantitated after their conversion to nickel dithiocarbamate complexes, using reversed-phase HPLC. This method gives similar results to those obtained using the previously reported normal-phase method, however the former procedure does not require extensive column equilibration times for reproducible results. In addition, the reversed-phase method can be modified to allow in-situ formation of the nickel complexes, thereby eliminating some of the manipulative steps. The proposed method was successfully applied to the analysis of diastereoisomeric contaminants in pharmaceutical products and raw materials containing ephedrine or pseudoephedrine.

REFERENCES

1. Liska, O., Guichon, G. and Colin, H., J.Chromatogr., 171, 145, 1979.

2. Moriyasu, M. and Hashimoto, Y., Chem.Lett., 117, 1980.

3. Moriyasu, M. and Hashimoto, Y., Chem.Lett., 761, 1980.

4. Engelhardt, H., J.Chromatogr.Sci., 15, 380, 1977.

5. Barkan,S., Weber, J.D. and Smith, E., J.Chromatogr., 219, 81, 1981.

6. Low, G.K.C., Haddad, P.R. and Duffield, A.M., Chromatographia, submitted for publication.

7. Glajch, J.C., Kirkland, J.J., Squire, K.M. and Minor, J.M., J.Chromatogr., 199, 57, 1980.

8. Tilly-Melin, A., Askemark, Y., Wahlund, K.G. and Schill, G., Anal.Chem., 51, 976, 1979.

9. McCormick, R.M. and Karger, B.L., J.Chromatogr., 199, 259, 1980.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 325-342 (1983)

SHALE OIL SEPARATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

William A. Dark
Waters Associates
34 Maple Street
Milford, MA 01757

ABSTRACT

In the characterization of syncrudes from alternate fossil fuel sources, there is a need for the rapid separation into hydrocarbon groups. Analytical separations where microgram quantities of sample can be used and preparative separations on multigram scale so that additional characterization and testing can be carried out.

Using samples of shale oil from Utah and Thailand, HPLC techniques are shown that accomplish these aims. The use of a mixed set of normal phase analytical columns for the automated separation into saturates, neutral aromatics by the number of rings and polar aromatics. Separation of multigram quantities of shale oil into major hydrocarbon groups, saturates, neutral aromatics, and polar aromatics is done in under 10 minutes.

INTRODUCTION

The development of transportation fuels and lubricants from highly heterogeneous fossil fuels represents many engineering challenges and requires rapid characterization techniques. There is a need for two types of characterization capabilities; one utilized by laboratory personnel for a complete evaluation of syncrude products, the second a rapid technique used by engineering personnel in the Pilot Plant. High Performance Liquid Chromatography (HPLC) of today can meet these needs. In its analytical mode, HPLC instrumentation can be automated to the point where

0148-3919/83/0602-0325$3.50/0

little operator involvement is necessary. In its preparative mode, multigram separations can be done routinely.

EXPERIMENTAL

Two samples of shale oil were used in these evaluations. The analytical separations were done on a tailored dual detector, Waters LC. The modifications were the use of a Rheodyne (Cotati, CA) fixed loop injector and a Rheodyne 6 port electropneumatic valve used to change the flow direction through the column. Detectors were UV at 254nm and a differential refractometer in series.

The mobile phase, HPLC grade n-Hexane, was maintained at 2.0 ml/min. The column was a 3.9mm by 30cm with a NH_2 modified support (ENERGY ANALYSIS COLUMN). Additional characterization were carried out where a high surface area silica gel (μPORASIL) column was used in series.

The preparative separations were carried out on a Waters PrepLC™ System 500A. The column used for the multigram separations in the preparative system are 57mm by 30cm. The support is a NH_2 modified silica. With columns of this diameter, sample loads of up to 8 grams have been used.

RESULTS

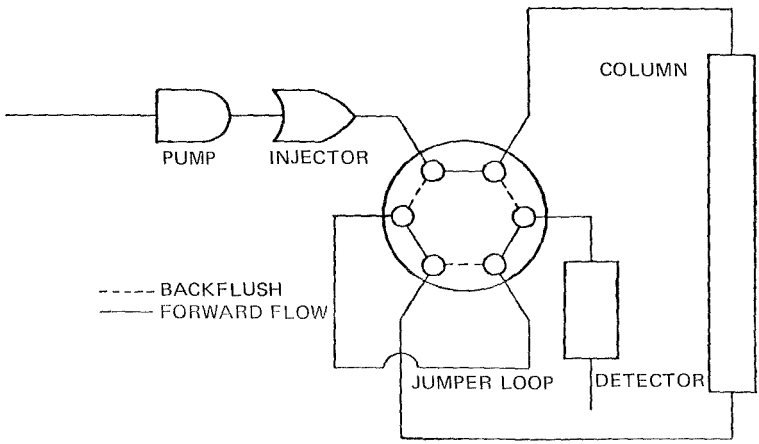
Analytical Separations

The separation of shale oil into saturates, neutral aromatics, and polar aromatics can be done across the 3.9mm ENERGY ANALYSIS COLUMN in 24 minutes using n-Hexane at 2.0 ml/min.

Approximately 0.5 grams of sample weighed to the nearest 0.1 milligram is dissolved in 20.0 ml of n-Hexane. The sample is filtered across a 0.5 micron membrane filter to remove any insolubles. These insolubles are generally classified as asphaltenes. The filtrate is loaded into the 10 microliter loop of the injector and then placed on the head of the column.

In this separation scheme, the saturates elute first. The saturate peak will contain all the normal, iso-, and cyclo-paraffins.

The olefins, normal and cyclo will also elute in this peak. Then the neutral aromatics elute. The selectivity of the column is such that all of the alkanes and alkenes, at least through cholestane, a 26 carbon saturate, will elute before benzene or an alkyl substituted benzene. The elution order of the neutral aromatics is by the number of condensed rings. When the neutral aromatics have eluted from the column, the mobile phase flow is reversed through the column. The polar aromatics will elute in a single peak. The selectivity of the column packing is such that thiophene, pyrrole, pyridine, and other heteroatom containing aromatics will elute in this envelope (Table I). A flow diagram of the six-port valve used for this column flow reversal is shown below.



This technique of column backflushing was first reported by Sautoni (1).

This technique of flow reversal through the column keeps the chromatographic equipment simple, uses a single solvent, and a single column. With an automatic injector, the system

TABLE I

Retention (k') of a variety of hydrocarbons using n-Hexane at 2.0 ml/min. across a 3.9mm NH_2 modified amine support.

hexadecane	0.10
dodecane	0.10
heptadecane	0.10
1-heptadecene	0.11
1-octene	0.12
1-octadecene	0.12
cycloheptane	0.10
pristane	0.10
cholestane	0.10
benzene	0.16
n-buytlbenzene	0.14
toluene	0.15
n-decylbenzene	0.13
mestylene	0.13
phenylundecane	0.13
biPhenyl	0.41
naphthalene	0.37
2-methylnaphthalene	0.35
2,3-dimethylnaphthalene	0.36
2,3,5-trimethylnaphthalene	0.36
acenaphthalene	0.40
acenphthyene	0.60
triphenylene	1.85
anthracene	0.83
phenanthrene	0.84
1-methylphenanthrene	0.81
fluoranthene	1.15
chrysene	2.88
benzo(a)pyrene	3.78

TABLE I (CONT'D)

ortho-cresol	11.16
phenol	11.00
2,2'bipyridine	11.23
pyridine	11.00
thiophene	13.00

The column is backflushed at k' of 5.0 and the analysis is completed at k' of 14.5.

can easily be automated. The chromatographic system uses two detectors in series, UV at 254nm is first followed by a differential refractometer. The saturated hydrocarbons are detected by the differential refractometer while those compounds with aromatic modiety are detected with greater sensitivity by UV. On the integrating recorder, the response of the UV is Pen 1 while that of the refractometer is on Pen 2. The offset of the pens gives the visual appearance that the saturates elute in the middle of the aromatic envelope, see Figures 1 and 2.

This approach was utilized for the compositional characterization of the shale oils from the two different sources (Figures 1 & 2). The hydrocarbon group composition of the two shale oils were determined to be:

	UTAH	THAILAND
Saturates	18.96 wt%	56.30 wt%
Neutral Aromatics	57.63	16.30
Polar Aromatics	23.40	27.50

The technique for quantitation utilized the approach that has been employed for crude oil (2).

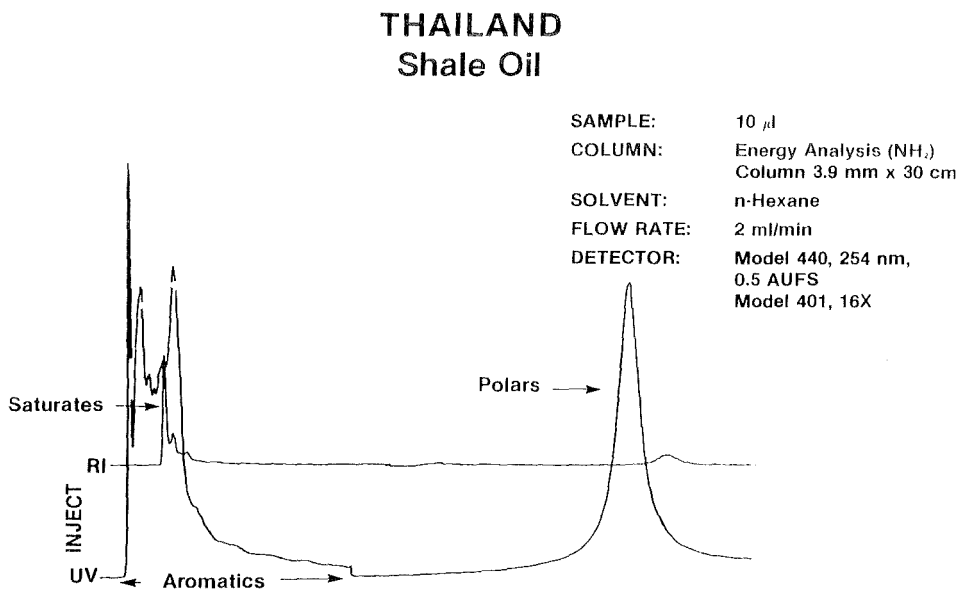


Figure 1: Thailand Shale Oil -- Hydrocarbon Group Separation.

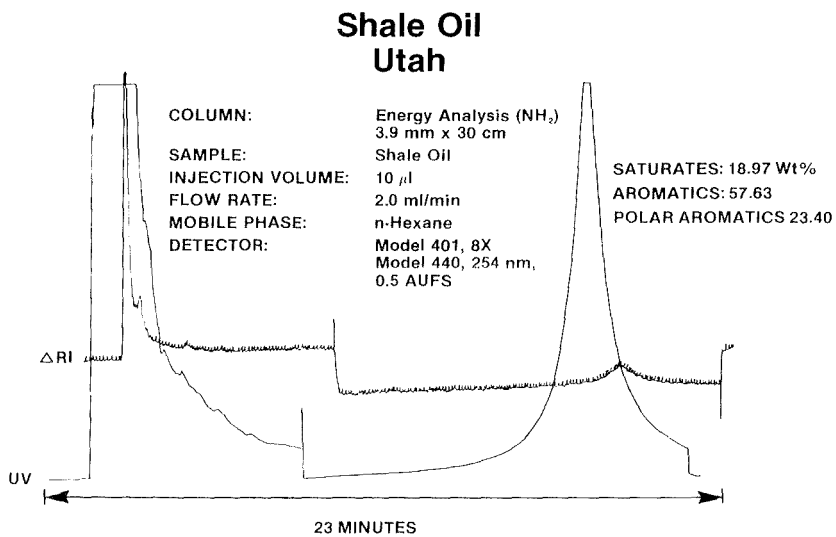


Figure 2: Utah Shale Oil -- Hydrocarbon Group Separation.

Preparative Separations

The analytical separation was scaled up so that multigram sample loads could be separated. These larger separations were done on the PrepLC™ System 500A. The column is 57mm by 30cm. The packing is a NH_2 modified silica, the same as the 3.9mm analytical column. Other than a larger diameter, the particle size of the preparative packing is a nominal 40 microns while that used in the analytical column is 10 microns. This preparative column is 200 times larger in cross-sectional area than the 3.9mm analytical column. Sample loads can be increased proportional to the cross-sectional area. If the same solvent is used at the same linear velocity, equivalent separations will be obtained in the same time frame. This translates to sample loads of up to 8 grams at a flow rate of 400 ml/min.

The preparative column is equilibrated with n-Hexane at 400 ml/min. at a pressure drop of 5 bars. The sample, up to 8 grams, is dissolved in 35-40 ml of n-Hexane. This volume is injected onto the head of the column following the manufacturer's recommendations. The saturates are collected (Figure 3 saturate collection is between #1 and #2), the aromatics are collected between #2 and #3. Instead of reversing the flow through the column, the mobile phase was stepped to dichloromethane. The polar aromatics elute with the solvent front. It is not that the flow cannot be reversed through this larger column, but by changing the mobile phase a reduction in overall solvent usage is realized. In the analytical separation, where solvent consumption is small, simplicity and automation are of prime concern. In the preparative separation, flowing at 400 ml/min. solvent consumption is of concern. Thus, the step change is solvent. The total analysis time is 6.5 minutes. After the polar aromatics have eluted from the column, it is re-equilibrated with n-Hexane, total time between injections is 10 minutes.

The fractions were recovered by evaporating the solvent to dryness. Any sample component that has a partial vapor pressure

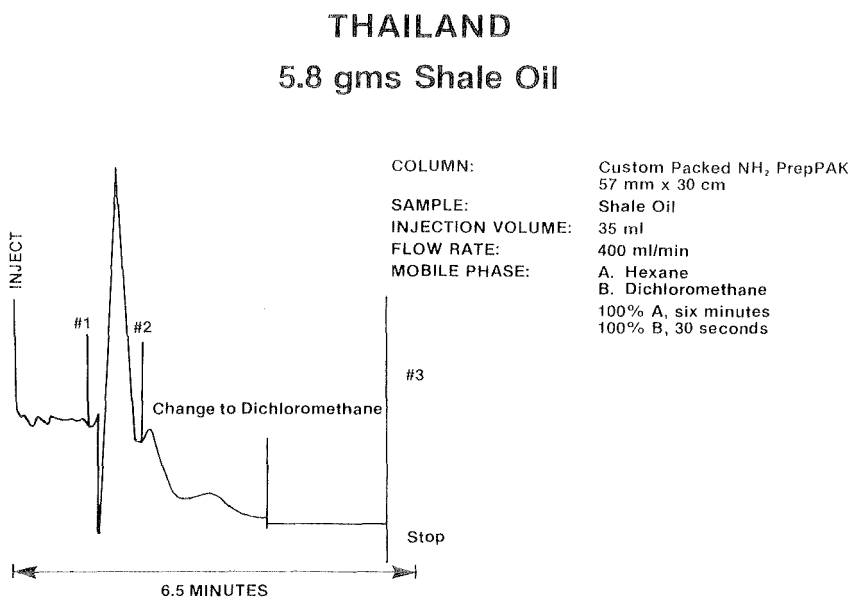


Figure 3: Thailand Shale Oil -- Preparative Hydrocarbon Group Separation.

similar to that of the solvent will be lost. After the gravimetric quantitation of the recovered fractions, they were analyzed on the 3.9mm by 30cm ENERGY ANALYSIS COLUMN (Figures 4-6). The recovered saturate fraction (Figure 4) shows response from the UV detector. This response may be due to carryover of neutral aromatics or from the olefins present in this fraction. The neutral aromatic fraction (Figure 5) shows the presence of polar aromatics. This is not due to poor chromatographic fractionation, but these polar aromatics are more soluble in the neutral aromatics than in the n-Hexane at the concentration applied to the preparative column. At the higher dilution level used in this analytical run, separation is achieved. The polar aromatic fraction is shown in Figure 6. There is sufficient material in each of these fractions so that additional characterization can be done.

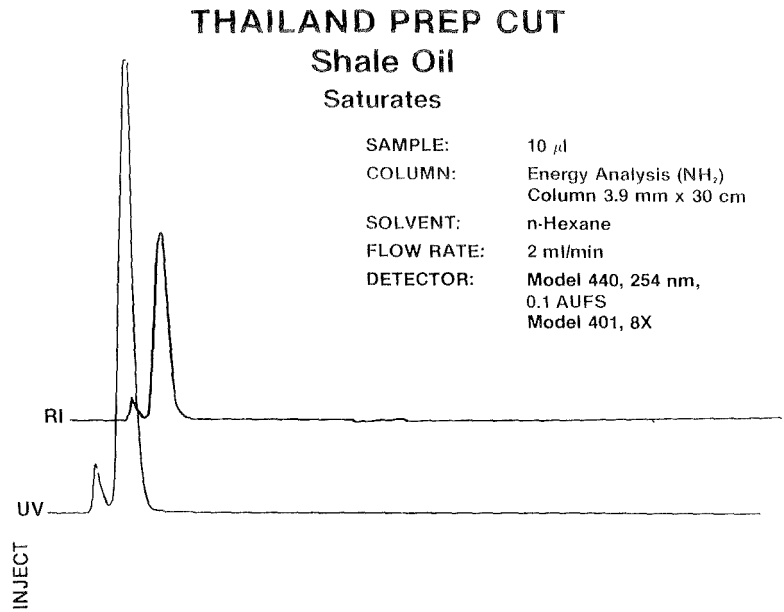


Figure 4: Thailand Shale Oil -- Saturate Fraction From Preparative Separation Analyzed Under Analytical Conditions.

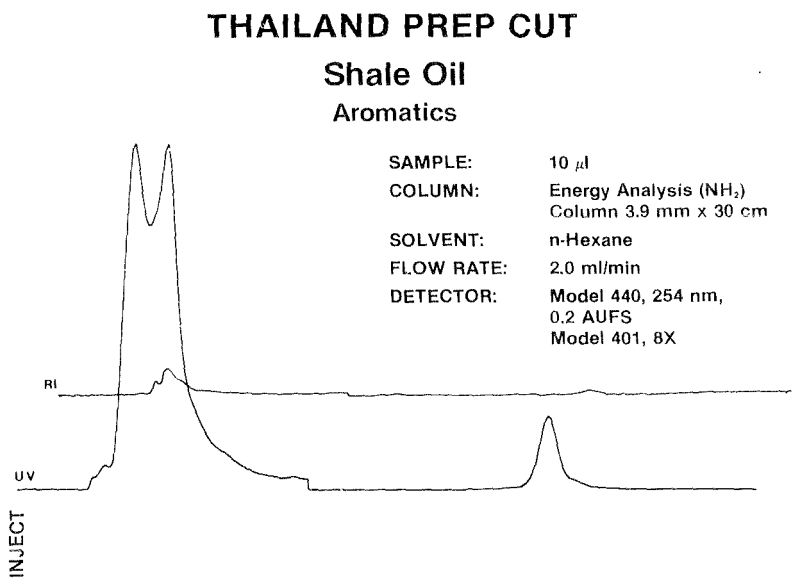


Figure 5: Thailand Shale Oil -- Aromatic Fraction from Preparative Separation Analyzed Under Analytical Conditions.

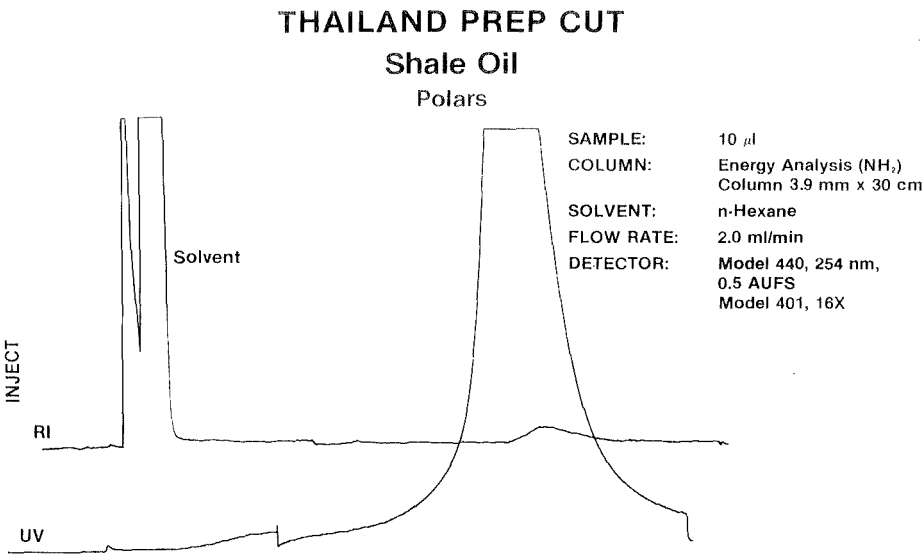


Figure 6: Thailand Shale Oil -- Polar Aromatic Fraction from Preparative Separation Analyzed Under Analytical Conditions.

Extended Separation Capabilities

The separation of saturates, neutral aromatics, and polar aromatics on the amine modified support can be carried one step further. By adding a 3.9mm by 30cm high surface area silica gel column in series with the amine column, the separation will be expanded. With these two different packings in series, the separation will now yield saturates, monoaromatics, diaromatics, three-ring aromatics, three plus ring aromatics, and the columns are backflushed to elute the polar aromatics. The backflush of the columns must be timed such that none of the polar aromatics reach the silica gel column. These heteratomic aromatics have shown very strong retention on silica. Therefore, the columns must be oriented so that the sample is injected onto the amine modified support.

The two shale oils were evaluated by this expanded separation scheme. The chromatograms, Figures 7 and 8, show the difference

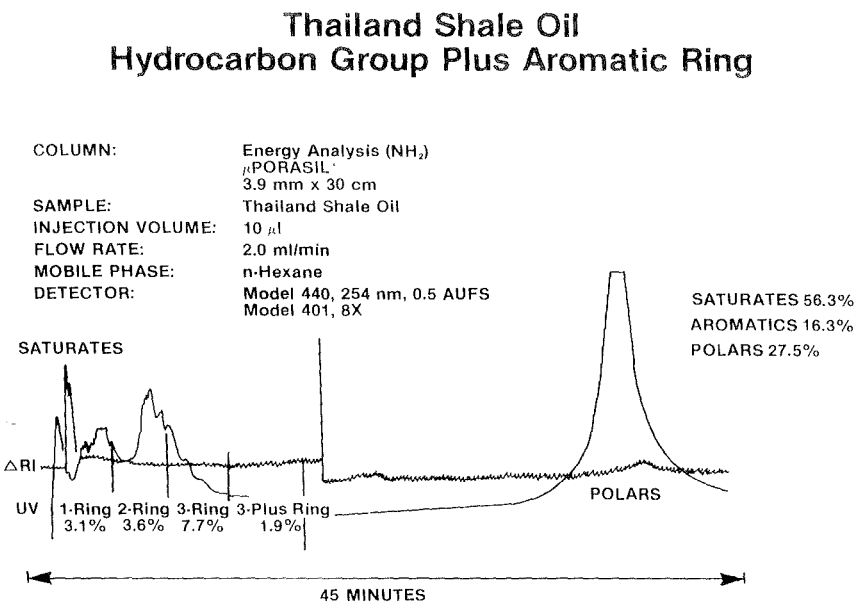


Figure 7: Thailand Shale Oil -- Hydrocarbon Group Separation with Separation of the Neutral Aromatics by Rings.

in the distribution of the neutral aromatics. The cut points of the various rings was determined by running a standard mixture of benzene, naphthalene, phenanthrene, chrysene, and perylene. The UV detector is Pen 1 and the refractometer is Pen 2 offset by 13.5mm. This gives the visual effect of the saturates eluting in the middle of the one-ring aromatics.

	UTAH Shale Oil	THAILAND Shale Oil
One Ring	7.3	3.1%
Two Ring	6.7	3.6%
Three Ring	29.0	7.7%
Three + Ring	14.7	1.9%

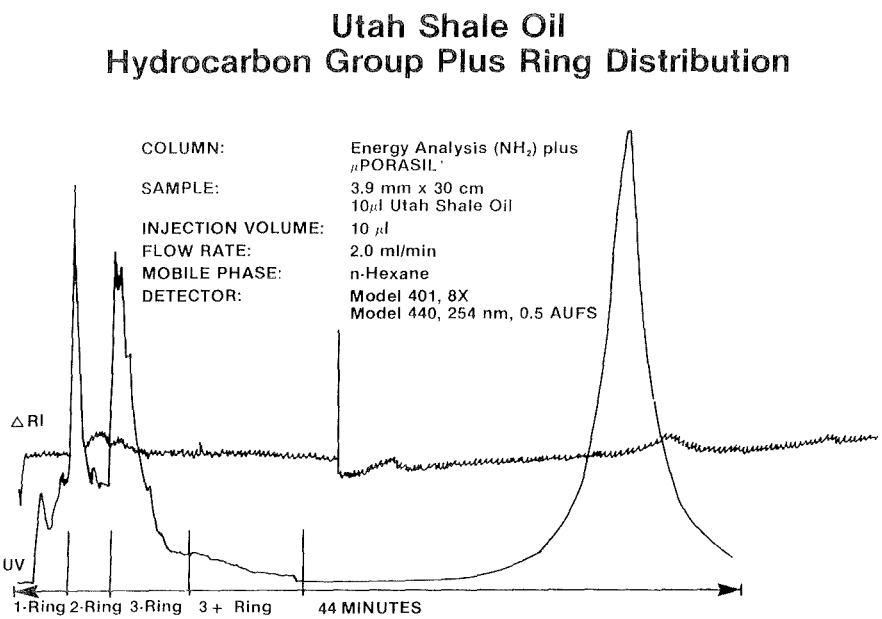


Figure 8: Utah Shale Oil -- Hydrocarbon Group Separation with Separation of the Neutral Aromatics by Rings.

Extended Preparative Capabilities

The extended analytical separation was scaled up to the multi-gram preparative separation.

Two preparative columns each 57mm by 30cm were used in series. The first column is the NH_2 modified packing while the second is a high surface area silica gel. The mobile phase is n-Hexane at 400. ml/min. as in the previous separation. The same sample work-up is used. The separation sequence should be saturates, mono-aromatics, diaromatics, three-ring aromatics, three plus ring aromatics, and then the polar aromatics. The saturates and neutral aromatics are eluted with n-Hexane while the polar aromatics will be eluted by stepping the solvent to dichloro-methane. However, the silica column must be removed from the flow stream before the polar aromatics are eluted from the NH_2

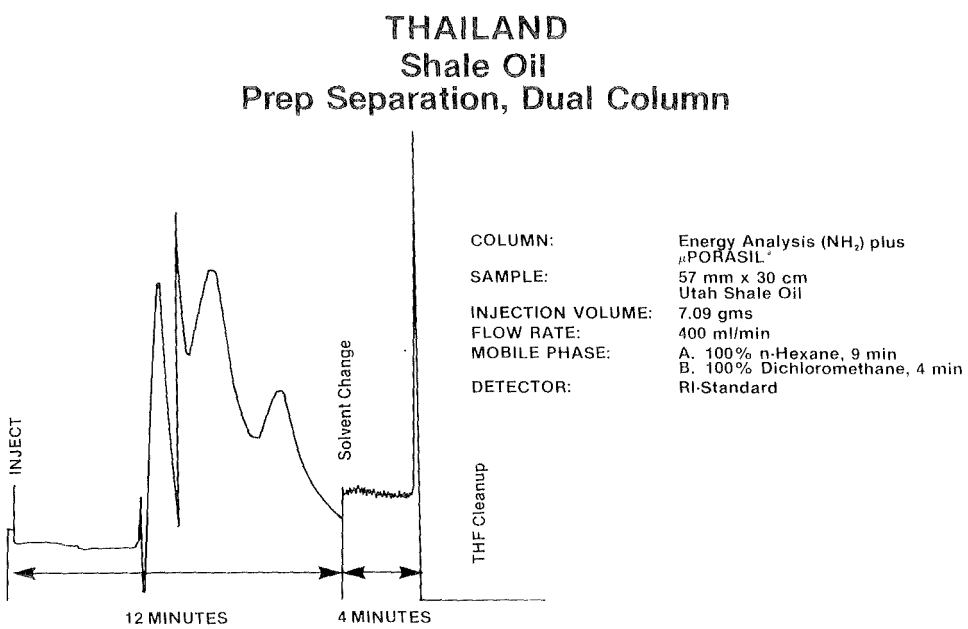


Figure 9: Thailand Shale Oil -- Preparative Separation by Hydrocarbon Group with Neutral Aromatics Separated by Rings.

packing. The polar aromatics have extremely large k's across silica with non-polar solvents. Using polar solvents to elute these polar aromatics from the silica column will lead to deactivation of the silica packing.

After the polar aromatics have been eluted from the NH_2 column, it is re-equilibrated with n-Hexane and then the two columns are placed in series. The total analysis time is 20 minutes for the separation and equilibration of the columns.

The preparative separation of the Thailand shale oil using this dual column approach is shown in Figure 9. The saturates are in the first peak, each of the other peaks were collected as neutral aromatics with increasing number of rings. The k' of cut points was predetermined by running a mixture of benzene,

napthalene, phenanthrene, chrysene, and perylene. After the neutral aromatics eluted from the columns, the silica column was removed and the solvent stepped to dichloromethane and the polar aromatics eluted.

The fractions were recovered by evaporating the solvents to dryness, with quantitation again being gravimetric. The fractions were then redissolved in n-Hexane and rechromatographed on the 3.9mm dual column analytical set-up described above (Figures 10-15). An examination of these chromatograms shows that the first fraction, saturates, contains little if any UV response and a k' that coincides with standards. The neutral aromatics fractions show peaks with increasing k' in later eluting fractions. The last eluting neutral aromatic fraction and the polar aromatic fraction show cross contamination, again, this is due to solubility at loads used in the preparative separation.

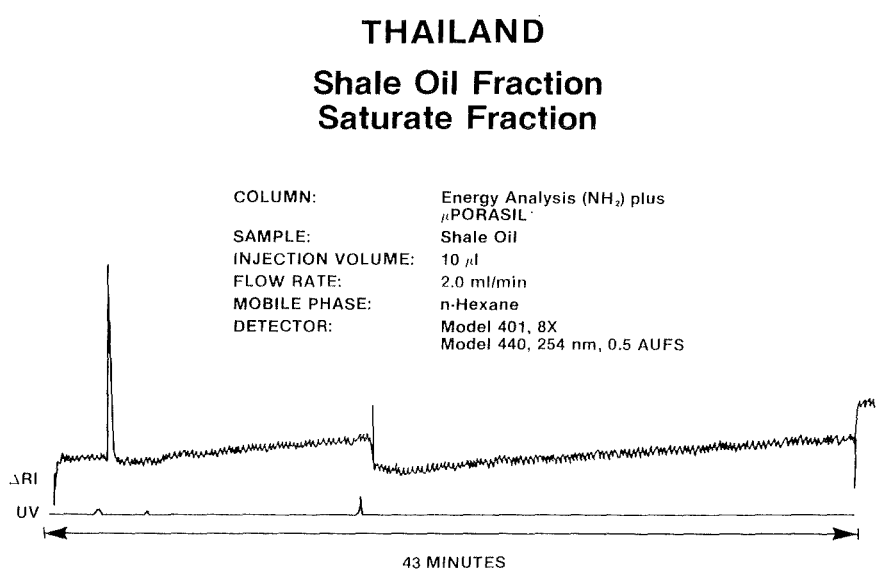


Figure 10: Thailand Shale Oil -- Saturate Fraction From Dual Column Preparative Separation Analyzed Under Analytical Conditions.

THAILAND
Shale Oil Fraction
Cut #2

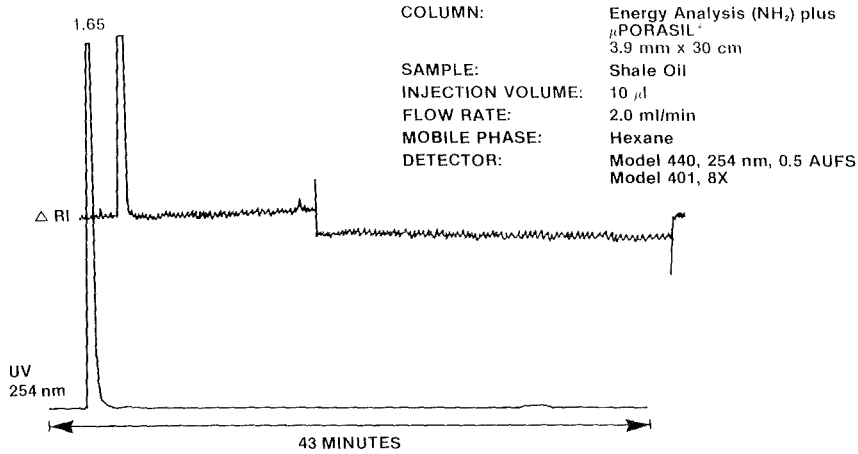


Figure 11: Thailand Shale Oil -- Cut #2 from Dual Column Preparative Separation Analyzed Under Analytical Conditions.

Thailand Shale Oil
Dual Column Prep Cut #3

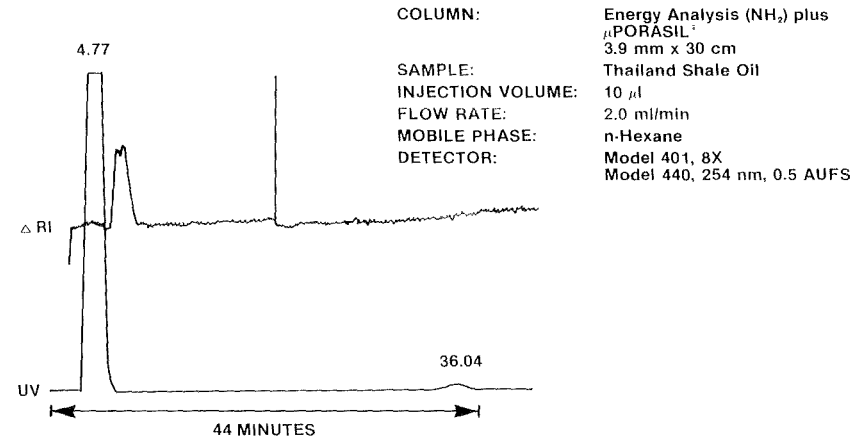


Figure 12: Thailand Shale Oil -- Cut #3 from Dual Column Preparative Separation Analyzed Under Analytical Conditions.

Thailand Shale Oil
Dual Column Prep Cut #4

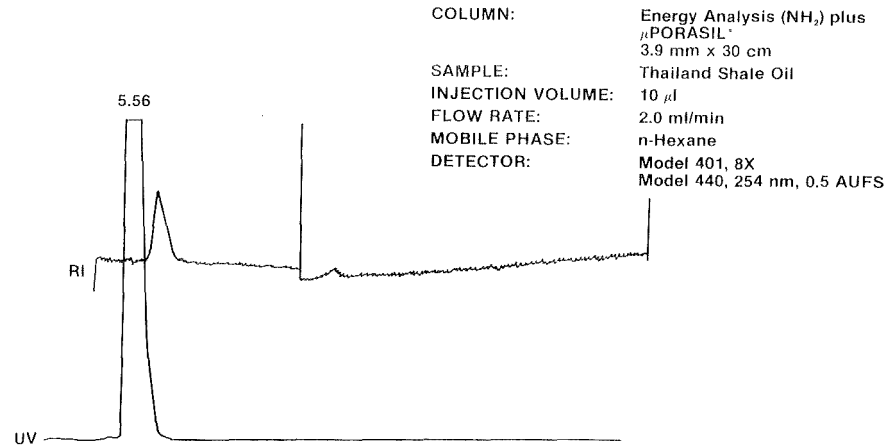


Figure 13: Thailand Shale Oil -- Cut #4 from Dual Column Preparative Separation Analyzed Under Analytical Conditions.

Thailand Shale Oil
Dual Column Prep Cut #5

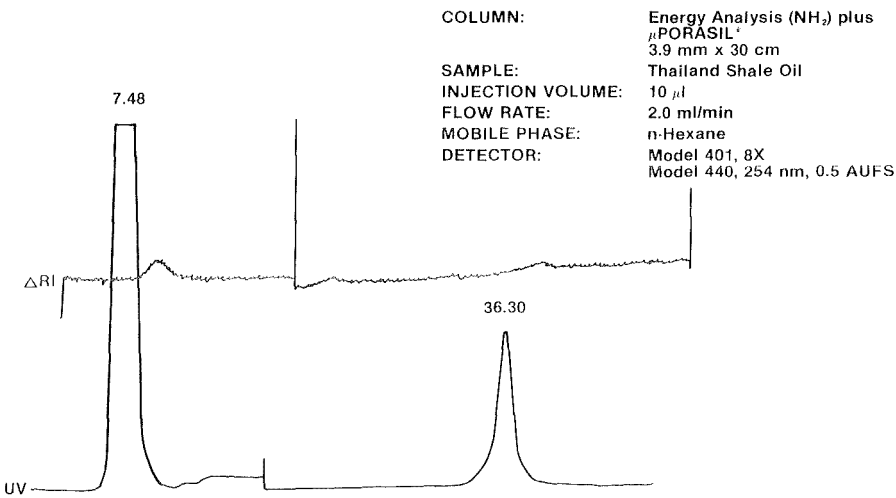


Figure 14: Thailand Shale Oil -- Cut #5 from Dual Column Preparative Separation Analyzed Under Analytical Conditions.

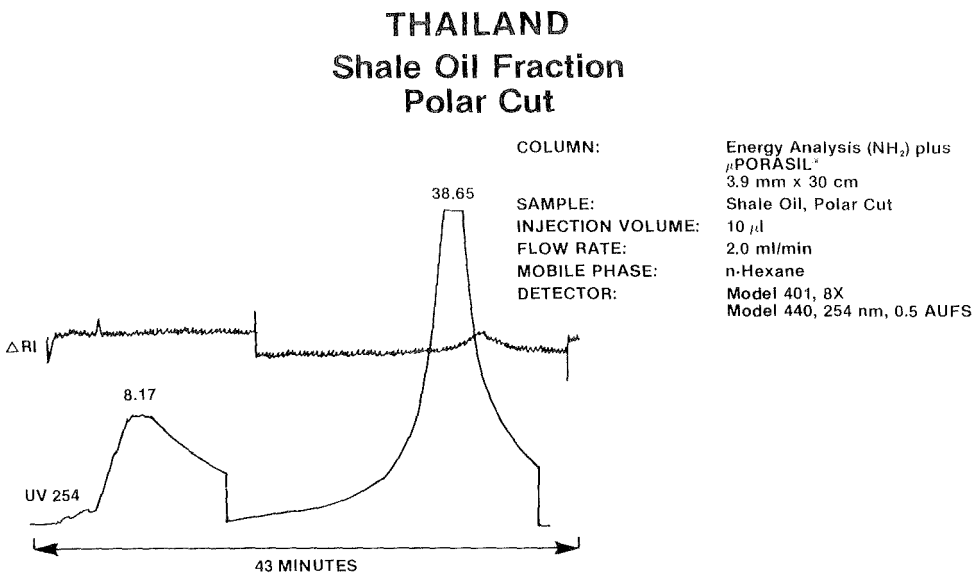


Figure 15: Thailand Shale Oil -- Polar Aromatic Cut from Dual Column Preparative Separation Analyzed Under Analytical Conditions.

A comparison of quantitation from the three separation schemes using the shale oil from Thailand is shown below. Two pooled injections across a single 57mm NH_2 column, three pooled injection across the dual 57mm columns and then the results from a single NH_2 column, 3.9mm, where detector responses were used are compared.

	ANALYTICAL RUN	NH2 13.43 gm	NH2-SILICA 20.62 gm
Saturates	18.97 wt.%	---	---
Aromatics	57.63	51.8 wt.%	(53.6) wt.%
1 Ring			43.7
2 Ring			5.6
3 Ring			3.0
3+ Ring			1.3
Polars	23.40	26.3	21.1
Recovery		78.0%	75.4%

Conclusions

High Performance Liquid Chromatography offers a new dimension in the separations of alternate fuels. Both preparative and fully automatable analytical systems can shorten the time required for separations. Tailored systems can provide Pilot Plant personnel with detailed and timely compositional data, shortening the decision time interval between sampling and results.

REFERENCES

(I) Sautoni, J.C. and Swab, R.E., Rapid Hydrocarbon Group-Type Analysis by High Performance Liquid Chromatography, J. CHROM. SCI., 13, 361 (1975).

(II) Dark, W.A., Crude Oil Hydrocarbon Separation Quantitation, J. LIQUID CHROM., in Press (1982).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 343-352 (1983)

DEGRADATION CHARACTERISTICS OF TWO TETRAHYDROISOQUINOLINES AT ROOM AND BODY TEMPERATURES: HPLC DETERMINATION WITH ELECTROCHEMICAL DETECTION

R.D. Myers, J.L. Garrison and E.C. Critcher
Center for Alcohol Studies
and
Departments of Psychiatry and Pharmacology
University of North Carolina School of Medicine
Chapel Hill, North Carolina 27514

ABSTRACT

Reversed-phase high-pressure liquid chromatography (HPLC) was utilized to determine the stability of tetrahydropapaveroline (THP) and salsolinol at two ambient temperatures and over varying time intervals of up to 27 hr. Although ascorbate, an antioxidant, was shown to retard the temporally contingent degradation of THP at both 22°C and 37°C, the breakdown of the tetrahydroisoquinoline product was more pronounced at 37°C. Salsolinol was virtually stable under all conditions. The formation of detectable by-products of THP was demonstrated by the presence of secondary peaks in the THP-water assay which were strikingly absent in the THP-ascorbate aliquots. Finally, the HPLC profiles of five THP samples obtained from four different sources revealed the presence of similar secondary peaks which varied considerably in shape and peak height from one sample to another. The implications of this lack of uniformity of THP for pharmacological studies of addictive processes is discussed.

INTRODUCTION

Certain of the amine-aldehyde condensation products, such as a tetrahydroisoquinoline (TIQ), exert potent pharmacological effects on the central nervous system (1). The catecholamine-aldehyde products can possess an addictive liability as evidenced

 0148-3919/83/0602-0343$3.50/0

from their pharmacological effects after direct administration into the brain (2, 3). Given systemically, they often exert opiate-like effects and analgesia (4).

Because of the relatively short half-life of the dopamine-dopaldehyde metabolite, tetrahydropapaveroline (THP) (5), a number of questions arise in terms of the potential instability of this class of compounds under various experimental conditions. Of paramount importance is the issue of whether a TIQ degrades at a more rapid rate at body temperature of ≃ 37°C than when the compound is maintained at a laboratory temperature of 22°C. Another question centers on the nature of the formation of secondary by-products during the breakdown of the alkaloid. Finally, does an antioxidant such as ascorbate, which also serves to lower pH, also alter the characteristics of the degradation of a TIQ amine-aldehyde product?

In the present experiments, HPLC procedures with electrochemical detection (6) were utilized to examine the rate of degradation of THP and salsolinol over time at two conditions of ambient temperature. The effect of the presence of ascorbate in the test solutions was also tested.

MATERIALS AND METHODS

Instrumentation

The HPLC system was comprised of a single pump (Altex Model 110, Solvent Metering Pump), with a pulse damper (Bioanalytical Systems [BAS]), and a syringe loading sample injector (Rheodyne Model 7120). A C_{18} reversed-phase column (3.9 mm i.d, x 300 mm μBondapak, Waters) protected by a pre-column filter (Rheodyne) was fitted in the system. A glassy carbon electrochemical cell, TL-8A thin layer transducer (BAS), was coupled with a model LC4 amperometric detector (BAS). The electrode potential was set at +0.70 V using a silver-silver chloride electrode as a reference. Detector sensitivity was set at 10 nA/V. A strip chart recorder (Fisher Recordall Series 5000) connected in parallel with a

plotting integrator (Hewlett Packard Model 3390A) completed the system.

Mobile Phases

The mobile phase for the THP assay consisted of 0.15 M acetic acid, 14% v/v methanol, and 0.25 mM heptanesulfonic acid sodium salt (HSA) used as an ion-pairing agent. For salsolinol, the mobile phase contained 0.15 M acetic acid and 2.5% v/v acetonitrile. The pH of both phases was adjusted to 3.0-3.2 as necessary with dilute sodium hydroxide. Each mobile phase was passed under vacuum through a double filter (0.3 µm Gelman A/E glass fiber filter and 2-3 µm Whatman #5 filter) and degassed by sonication. A flow rate of 1.5 ml/min was maintained in both assays.

Sample Preparation

The THP and salsolinol assays were divided into two parts which were subjected to the same conditions. One sample line was dissolved in dilute ascorbic acid (0.1 mg/ml), an anti-oxidizing agent, while the other was dissolved in glass distilled water. All samples were readily solubilized with sonication. An initial sample concentration was chosen to maximize peak height while remaining on-scale on the recorder to allow accurate quantitation. After the column had been conditioned with mobile phase and the detector activated for 20-30 min, samples were injected onto the column in 10 µl volumes delivered from a 50 µl Hamilton syringe flushed repeatedly with water. The concentrations used consistently for the degradation analysis of THP and salsolinol were 50 ng/10 µl and 40 ng/10 µl, respectively.

HPLC Separation

Once properly diluted, the freshly prepared samples were injected directly onto the HPLC column. After three aliquots of each compound were dissolved in distilled water or ascorbic acid, they were kept at room temperature, placed in a water bath maintained at 37°C or kept in a -20°C freezer. Aliquots of THP kept at a room temperature of 22°C or at 37°C were tested at 2-4, 10-

12, or 24-27 hrs following the initial assay. Aliquots of salsolinol kept at room temperature were tested at 4 and 24 hrs, whereas water bath samples held at 37°C were injected after 1, 4 and 24 hrs. Samples of both THP and salsolinol kept at -20°C were tested after a 24 hr interval. For the analysis of different samples of THP obtained from different sources, a freshly prepared quantity of 1.0 µg/10.0 µl THP was injected onto the column without any intervening period of time.

Glassware used in preparation of samples was washed and rinsed, sonicated in chromic acid, then rinsed five times in deionized water and again in distilled water. After the glassware was drained, it was subsequently baked in an oven for two hrs at 185°C. Syringes were also flushed with dilute chromic acid cleaning solution at the end of each day. The chromatograph injector port was flushed with 800 µl distilled water prior to sample injections. These precautions were taken to reduce the possibility of contamination of the original samples in an actual injection.

Compounds were weighed accurately to 10^{-3} mg using a Cahn 21 Automatic Electrobalance. Dilutions were made in ratios no greater than 1:9 with graduated pipets.

Reagents

L-ascorbic acid, sodium hydroxide and methanol were obtained from Fisher Scientific, the latter two compounds being certified as HPLC grade. Glacial acetic acid was purchased from Mallinckrodt and the 1-Heptanesulfonic acid sodium salt was obtained from Eastman Kodak. Samples of THP were kindly provided by Hoffmann-LaRoche (RO6-1673), R. Deitrich of the University of Colorado, Z. Amit of Concordia University and Burroughs Wellcome. Salsolinol was obtained from Aldrich Chemical Company.

RESULTS

The marked degradation of THP in water as determined by the HPLC with EC detection is shown in Fig. 1A. The solvent peak,

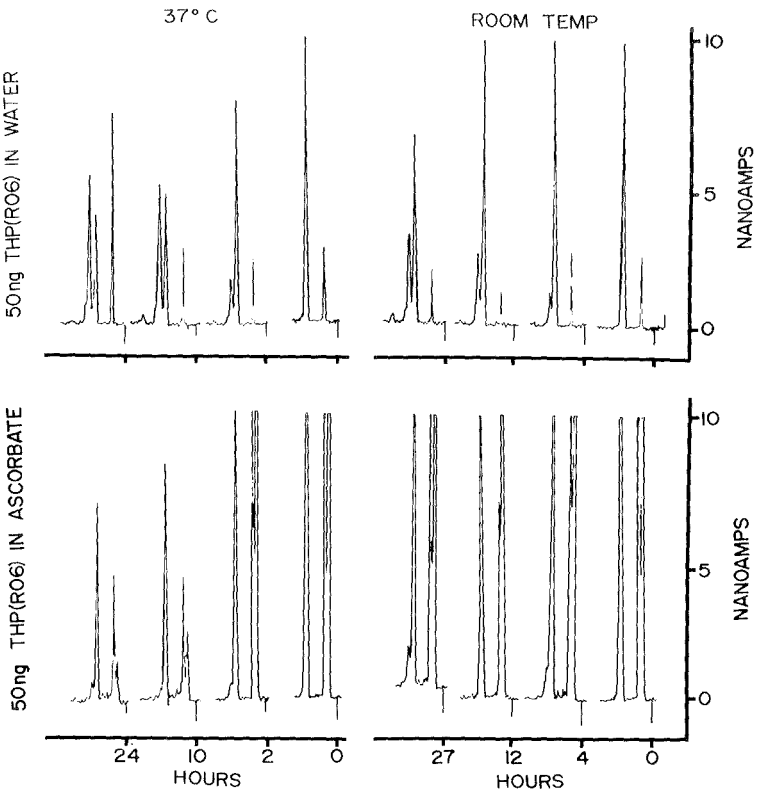


FIGURE 1: Degradation of THP in (A) water and (B) ascorbate at room temperature and at 37°C during 27 and 24 hrs, respectively.

with t_R = 3.1 min, was followed by the THP peak, t_R = 8.2 min. A second peak with t_R = 9.8 min became evident after four hours at both the ambient temperatures of 22°C and 37°C, and this peak increased over the duration of this experiment. As the second peak increased in size, a concomitant decline in the height of the actual THP peak also occurred. Though this trend was apparent both at room temperature and at 37°C, the higher ambient temperature caused a far greater rate of THP's degradation with

increase in this isoquinoline's by-products during the comparable period of time.

Figure 1B illustrates the decomposition of THP which was dissolved in ascorbic acid (0.1 mg/ml). The ascorbate peak itself was eluted at 2.0 min and the THP peak again followed thereafter at 8.2 min. Thus, the retention time of THP was unaffected by the presence of ascorbate. Further, in the test solution no clear-cut degradation of THP in the ascorbate medium occurred at a room temperature of 22°C. A small secondary peak was observed at four hours and at 27 hours, but the THP peak appeared to be unaffected. However, at 37°C evidence of degradation became apparent 10 hrs following preparation of the sample. A slight shoulder was registered after two hrs and stayed relatively constant throughout the remainder of the assay period. In contrast to the secondary peaks which dominated the THP chromatograms shown in Fig. 1A, no evidence of additional by-products was noted. A slight degradation of the ascorbate peak was observed at room temperature; however, its decomposition after 10 hrs in a 37°C environment was very pronounced.

In the second phase of this study, we obtained chromatograms of five different THP samples in order to compare their chemical profiles. The results of this analysis are shown in Fig. 2. The solvent eluted at 2.0 min followed by the primary THP peak at 8.0 min. Although each of the samples possessed four secondary peaks following the primary THP peak, large variations in the profile of these peaks as well as overall peak height were noted. The sample obtained from Deitrich exhibited the greatest amount of secondary activity whereas that supplied by Amit showed only minimal after-peaks.

Table 1 presents a composite comparison of the average percent degradation of THP and salsolinol under the various test conditions. Each calculated value is based on two-four replications of the assay for the given test condition. As shown in the Table, salsolinol retained its stability even at the 37°C temperature and after a 24-hr period.

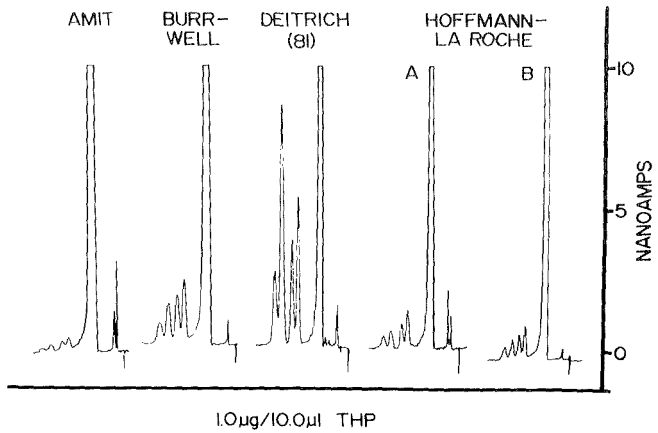


FIGURE 2: Profiles of five freshly prepared THP samples in a 1 µg quantity: two samples from Hoffmann-LaRoche (RO6 1673) and one sample each from Deitrich, Amit and Burroughs-Wellcome (Burr-Well) as described in text.

TABLE 1

Percent Degradation Over Time of Tetrahydropapaveroline and Salsolinol at 37°C and 22°C in H_2O and Ascorbate Vehicles

THP			Salsolinol		
37°C			37°C		
Elapsed Time	H_2O	Asc	Elapsed Time	H_2O	Asc
2 hrs	22.5	5.6	1 hr	0.0	0.0
10 hrs	46.8	30.	4 hrs	1.5	0.0
24 hrs	78.3	48.7	24 hrs	3.2	0.0
22°C			22°C		
Elapsed Time	H_2O	Asc	Elapsed Time	H_2O	Asc
4 hrs	4.0	0.8			
12 hrs	16.2	13.1	4 hrs	0.0	0.0
27 hrs	45.0	26.1	24 hrs	2.8	0.0

DISCUSSION

TIQs are potent pharmacological agents which can cause analgesia (4) and a variety of physiological effects on pulse and respiratory rates, blood pressure and smooth muscle tension (1,7). More recently, certain of the TIQs have been implicated in the symptoms of physical dependence on alcohol (8) as well as the development of abnormal preference for alcohol (3). Recently, salsolinol has been found in CSF and brain substance of the human alcoholic (9) as well as rat treated with alcohol (10). Because of the potential clinical importance of these substances, it is necessary that the physico-chemical properties and stability of TIQs under laboratory conditions are understood if they are to be studied experimentally.

In our experiments, we found that temperature is a critical factor in the stability of THP in solution. Noticeable degradation with by-product formation occurs at 37°C in a sample unprotected by an anti-oxidant. Therefore, it is suggested that a mini-pump embedded under the skin of an experimental animal cannot be appropriately used for the chronic administration of this TIQ compound. Solutions that are used for repeated injections could be maintained at room temperature, but they necessarily would have to be changed every 24, if not 12 hrs, to ensure the stability of THP.

Further, our results indicate that ascorbate does help to protect the compounds from degradation at both ambient and body temperatures. The use of ascorbate or a similar agent which prevents decomposition of the alkaloids is essential to maximize stability. Even though ascorbate does not alter the retention time or HPLC profile of THP's activity, certain questions must be raised, however, concerning the pharmacological effects of ascorbate itself. For example, does ascorbic acid influence the pharmacological activity of a TIQ or does it exert its own pharmacological action on addictive behavior?

The differences observed among various samples of THP available to the investigators imply that a "standard" THP does not really exist. The secondary peaks presumably represent degradative by-products which in themselves could possibly alter or augment any of the pharmacological effects of THP. In any case, this difference in sample purity would lead to an explanation of the discrepancies observed in pharmacological studies of these compounds (11,12). Although some of the secondary peaks could be due to O-methylated products of THP, as identified by Meyerson et al. (13), to what extent each of these substances may be active biologically is not presently known.

ACKNOWLEDGEMENTS

This research was supported in part by the North Carolina Alcoholism Research Authority Grant #8102 and National Institute on Alcohol Abuse and Alcoholism Grant #AA 04200-02. E.C.C. was a Visiting Research Fellow from the University of Tennessee. The authors thank Dr. G. Frye for his generous advice and the staff of Bioanalytical Systems Inc. for kind assistance with HPLC technology.

REFERENCES

1. Myers, R.D., Alcohol Tolerance and Dependence, Rigter, H. and Crabbe, J., eds., Elsevier/North-Holland Biomedical Press, The Netherlands, 1980, p. 337.

2. Duncan, C. and Deitrich, R.A., A Critical Evaluation of Tetrahydroisoquinoline Induced Ethanol Preference in Rats, Pharmac. Biochem. Behav., 13, 265, 1980.

3. Myers, R.D., Tetrahydroisoquinolines in the Brain: The Basis of an Animal Model of Alcoholism, Alcohol Clin. Exptl. Res., 2, 145, 1978.

4. Marshall, A. and Hirst M., Potentiation of Ethanol Narcosis by Dopamine- and L-dopa- Based Isoquinolines, Experientia, 32, 201, 1976.

5. Melchior, C.L., Mueller, A. and Deitrich, R.A., Half-Life of Tetrahydropapaveroline and Salsolinol Following Injection Into the Cerebral Ventricle of Rats, Fed. Proc., 37, 420, 1978.

6. Kenyhercz, T.M. and Kissinger, P.T., High-Performance Liquid Chromatographic Assay of Isoquinoline Alkaloid Formation from Reaction of Biogenic Amines and Aldehydes, J. Pharm. Sci., 67, 112, 1978.

7. Hjort, A.M., DeBeer, E.J. and Fassett, D.W. Some Tetrahydroisoquinolines. I. Their Relative Toxicology and Symptomatology, J. Pharmacol. Exp. Therap., 62, 165, 1938.

8. Blum, K., Briggs, A., Elston, S., Hirst, M., Hamilton, M. and Verebey, K., Alcohol Tolerance and Dependence, Rigter, H. and Crabbe, J., eds., Elsevier, The Netherlands, 1980, p. 371.

9. Borg, A., Kvande, H., Magnusson, E. and Sjöquist, B., Salsolinol and Salsoline in Cerebrospinal Lumbar Fluid of Alcoholic Patients, Acta Psychiat. Scan., 286, 171, 1980

10. Sjöquist, B., Lilequist, S. and Jörgen, E., Increased Salsolinol Levels in Rat Striatum and Limbic Forebrain Following Chronic Ethanol Treatment, J. Neurochem., 39, in press, 1982.

11. Myers, R.D. Melchior, C. and Swartzwelder, H.S., Amine-Aldehyde Metabolites and Alcoholism: Fact, Myth or Uncertainty. Substance and Alco. Actions/Abuse, 1, 223, 1980.

12. Brown, Z.W., Amit, Z. and Smith, B. Biological Effects of Alcohol, Begleiter, H., ed., Plenum Press, New York and London, 1980, p. 103.

13. Meyerson,L.R., Cashaw, J.L., McMurtrey, K.D. and Davis, V.E., Stereoselective Enzymatic O-Methylation of Tetrahydropapaveroline and Tetrahydroxyberbine Alkaloids, Biochem. Pharmac., 28, 1745, 1979.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 353-365 (1983)

SIMULTANEOUS DETERMINATION OF WARFARIN, SULPHAQUINOXALINE AND FENITROTHION IN WHEAT-BASED RODENTICIDE BAITS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

R.L. Perez

Roche-Maag Limited
Bankstown 2200
N.S.W. Australia

ABSTRACT

The simultaneous determination of warfarin, sulphaquinoxaline and fenitrothion in wheat-based rodenticides is achieved by extracting the three components from the bait with dimethylformamide followed by an isocratic, high-pressure liquid chromatographic separation using a reverse-phase RP-8 column and 0.005 M pentane sulphonic acid in methanol:water (60:40) as eluent. The three components are detected at 280 nm after separation. Recoveries in the concentration range investigated were fenitrothion 97.2%, warfarin 97.8% and sulphaquinoxaline 96.9%.

INTRODUCTION

Commercial grain baits containing warfarin, (3-[acetonyl-benzyl]-4-hydroxycoumarin) and sulphaquinoxaline (2-[p-aminobenzenesulphonamido] quinoxaline) are frequently used for the control of

0148-3919/83/0602-0353$3.50/0

rodent infestations. The addition of fenitrothion (O,O-dimethyl O-[3-methyl-4-nitrophonyl] phosphorothioate) helps to control insects that attack and infest the bait.

Several methods have been published for the determination of warfarin including thin layer chromatography (TLC) followed by ultra violet (UV) detection at 305 nm (1), electron capture gas chromatography (2), and extraction with 1% pyrophosphate solution followed by UV determination at 308 nm (3). In addition several high-pressure liquid chromatographic (HPLC) methods have also appeared (3,4,5.). The determination of sulphaquinoxaline in various matrices has also received considerable attention. Published methods include diazotization followed by coupling in the presence of zirconium and measurement of the coloured complex at 550 nm (6), underivatised UV determination at 350 nm (7,8), and HPLC (5).

Methods used for the determination of fenitrothion include infra-red analysis (9), gas chromatography (10, 11, 12) and HPLC (13, 14, 15).

Although the article by Trujillo (5) describes the determination of both warfarin and sulphaquinoxaline by HPLC, no information was found for the simultaneous determination of the three components

warfarin, sulphaquinoxaline and fenitrothion. In addition Trujillo's article refers to the analysis of rodenticide concentrates and not to the baits themselves.

The need for a quick and specific method for the determination of all three components in rodenticide baits led to the HPLC method described here, which involves extraction of the bait with dimethylformamide (DMF) followed by HPLC on an RP-8 reverse-phase column using methanol:water (60:40) containing pentane sulphonic acid, and detection at 280 nm.

EXPERIMENTAL

Apparatus

A Waters Model 6000A pump, U6K injector and Model 450 variable wavelength UV detector (Waters Associates, Sydney, Australia) were used. The column used was a Brownlee Laboratories RP-8, (10μ), 25 cm x 4.5 mm (i.d.) reverse-phase column (Activon Scientific Services, Granville, Australia). The detector was coupled to a Curken 250-1 recorder (Varian Pty. Ltd., Sydney, Australia) and injections were made with a Hamilton 25μl syringe.

Reagents and Standards

Fenitrothion 99.5% and sulphaquinoxaline 99.9% (Cooper Australia Ltd.).

Warfarin 99.0% (Chemoswed A.B., Sweden).
Methanol HPLC grade (Burdick and Jackson, from Alltech Associates, Sydney Australia).
A 0.25M solution of 1-pentanesulphonic acid in glacial acetic acid (from Waters Associates, Sydney, Australia).

Mobile Phase

The mobile phase was prepared by adding one vial of the 1-pentanesulphonic acid solution to 400 ml of distilled water and making to 1000 ml with HPLC grade methanol. The solution was then degassed by vacuum.

Preparation of Standard

A stock solution was prepared by dissolving 0.0126 g of fenitrothion, 0.4620 g warfarin and 0.2217 g of sulphaquinoxaline in 100 ml DMF. A 10 ml aliquot of this stock solution was transferred to a 100 ml volumetric flask and made to volume with DMF. This final analytical standard contained 0.00126% (12.6 ppm) fenitrothion, 0.04620% (462 ppm) warfarin and 0.02217% (221.7 ppm) sulphaquinoxaline.

Preparation of Baits

Using a mixture of 60% wheat and 40% cornflour, three samples of bait were prepared containing fenitrothion, warfarin and sulphaquinoxaline in the concentrations shown in Table I.

TABLE 1

Rodent Bait Samples Prepared and Analysed

Component (ppm) in Wheat/Cornflour Mix

Sample	Fenitrothion	Warfarin	Sulphaquinoxaline
1	10	440	200
2	12	460	220
3	14	480	240

Extraction Procedure

Approximately 20.0 g of each bait was accurately weighed and transferred to a 500 ml stoppered conical flask. After addition of approximately 150 ml of DMF the flask was stoppered and shaken for 1 hour by means of a mechanical shaker. At the end of 1 hour the contents of the flask were filtered into a 200 ml volumetric flask, the residue washed several times with small portions of DMF and the washings added to the flask. The solution was then made to 200 ml with DMF. A portion of the solution was then filtered through a 5 μm teflon filter by means of a syringe filter. This procedure was performed in duplicate for each sample.

The same extraction procedure was used on a single sample of untreated wheat/cornflour mixture.

Chromatography

With a flow rate of 2 ml/min, the detector set at 280 nm and 0.1 AUFS and the recorder at 0.5 cm/min, duplicate 25 µl injections including the blank were made and the average peak height of each duplicate determined. By comparing the average height of each sample injection with that of the standard, the amount of warfarin, sulphaquinoxaline and fenitrothion in the original bait was calculated.

Results and Discussion

There are no interfering co-extractives when DMF is used to extract fenitrothion, warfarin and sulphaquinoxaline from wheat/cornflour based rodent baits, as can be seen from the chromatogram of the blank extract. (Fig. 1). The chromatogram of the standard solution of fenitrothion, warfarin and sulphaquinoxaline (Feg. 2) shows that, using the chromatographic conditions described, good separation of the three components is achieved within 9 minutes. As shown in Table 2, the average recovery of the three components in the concentration range investigated is fenitrothion 97.2%, warfarin 97.8% and sulphaquinoxaline 96.9%. Fig. 3 is the resulting chromatogram of a bait manufactured under actual "production conditions" which has been extracted and chromatographed as described.

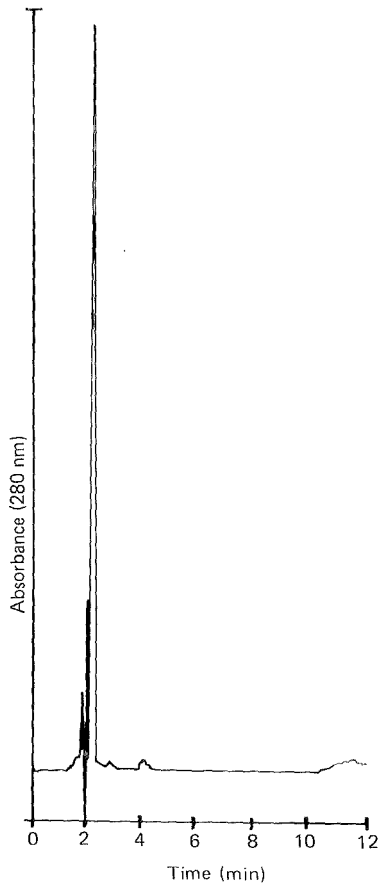


FIGURE 1. Chromatogram of untreated wheat/cornflour mixture, 25 ul injection (blank). Extraction and chromatographic conditions as described under Experimental.

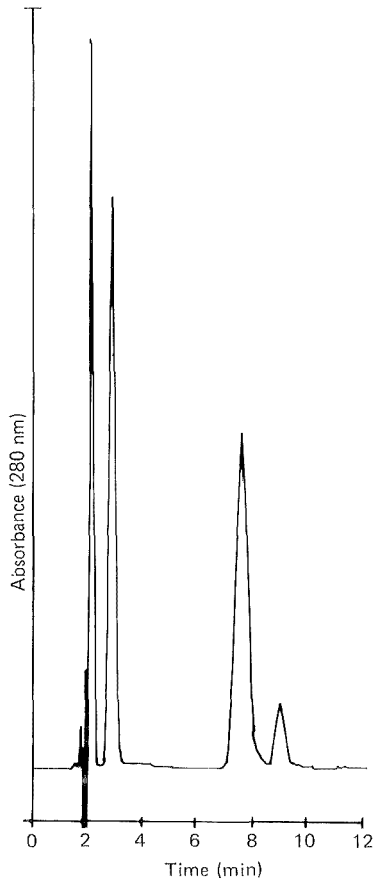


FIGURE 2. Chromatogram of standard analytical working solution. A, sulphaquinoxaline (221 ppm); B, warfarin (462 ppm); C, fenitrothion (12.6 ppm). Injection volume, 25 ul. Retention times: sulphaquinoxaline, 2.5 min; warfarin, 7.7 min; fenitrothion, 9.0 min. Chromatographic conditions as described in Experimental section.

TABLE 2

Recovery of Fenitrothion, Warfarin and Sulphaquinoxaline from Wheat/Cornflour Rodent Baits

Fenitrothion			Warfarin			Sulphaquinoxaline		
Added (ppm)	Found[(a)] (ppm)	Recovery %	Added (ppm)	Found (ppm)	Recovery %	Added (ppm)	Found (ppm)	Recovery %
10.0	9.8	98.0	440	431	98.0	200	191	95.5
10.0	9.6	96.0	440	432	98.2	200	195	97.5
12.0	11.8	98.3	460	451	98.0	220	213	96.8
12.0	11.3	94.2	460	449	97.6	220	213	96.8
14.0	13.9	99.3	480	470	97.9	240	236	98.3
14.0	13.6	97.1	480	466	97.1	240	231	96.3
Mean Recovery: 97.2%			Mean Recovery: 97.8%			Mean Recovery: 96.9%		

(a) As determined from average of two injections.

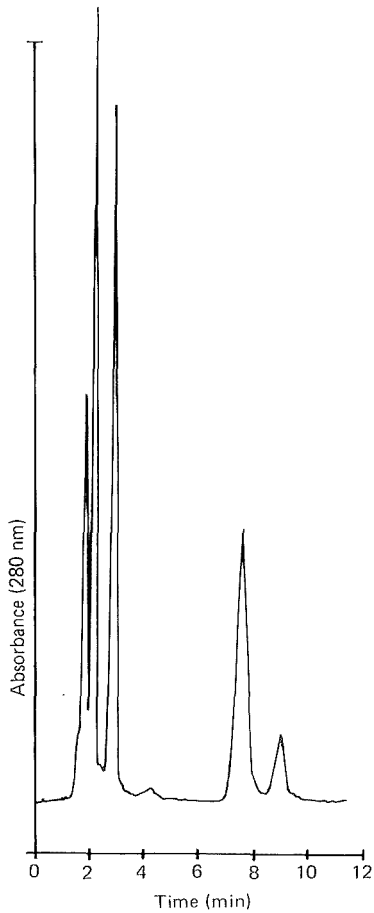


FIGURE 3. Typical chromatogram of extract of bait manufactured under actual "Production Conditions". Extraction and chromatographic conditions as described in Experimental section.

Chromatographic separation of the three components is based on a combination of ion-pairing and ion-suppression. The pentanesulphonic acid solution, as purchased, is buffered at pH 3.5 under which conditions sulphaquinoxaline, being a weak base, forms an ion-pair with pentanesulphonic acid and the ionisation of the weak acid warfarin is suppressed. Fenitrothion is essentially non-polar at pH 3.5. By means of this technique the retention and separation of the three compounds is achieved by reversed-phase chromatography.

CONCLUSION

The method described here for the extraction and analysis of rodent baits based on a wheat/cornflour mixture containing fenitrothion, warfarin and sulphaquinoxaline is rapid, reproducible and accurate with an average recovery of 97.2% fenitrothion, 97.8% warfarin and 96.9% sulphaquinoxaline. The extraction procedure described does not extract any component from the wheat/cornflour mixture that may interfere with the determination of the three components of interest.

REFERENCES

1. Fishwick, F.B. and Taylor, A., The Determination of Warfarin in Animal Relicta, Analyst, 92, 192, 1967.

2. Fessman, J., Quantitation of non-halogenated compounds by electron-capture gas chromatography, J. Chromatogr, 1980, 184(3); Chromatogr Ref, 24(3), 313.

3. "EPA" Manual of Chemical Methods for Pesticides and Devices." Assoc. Off. Anal. Chem, Bontoyal W.R., Chairman, Edit, Comm., Washington, D.C. 1976.

4. Billings, T.J., Hanks, A.R. and Colvin, B.M., Determination of Warfarin by High Performance Liquid Chromatography, J. Assoc. Off. Anal. Chem., 59, 1104, 1976.

5. Trujillo W.A., Determination of Warfarin and Sulfaquinoxaline in Rodenticide Concentrates by HPLC, J. Liq. Chromatogr, 3(8), 1219, 1980.

6. Szalkowski, C.R., Determination of sulfaquinoxaline in Feeds: Collaborative Study, J. Assoc. Off. Anal. Chem., 56(3), 758, 1973.

7. Cieri, U.R., Determination of Sulfaquinoxaline, Sulfathiazole, Sulfamerazine and Sulfamethazine in Feed Concentrates or Premixes. J. Assoc. Off. Anal. Chem., 56(6), 1475, 1973.

8. Cieri, U.R., Collaborative Study of the Determination of Sulfaquinoxaline, Sulfathiazole, Sulfamerazine, and Sulfamethazine in Feed Concentrates and Premixes. J. Assoc. Off. Anal. Chem., 57(2), 345, 1974.

9. Delves, R.B., and Williams, V.P., The Analysis of the Organophosphorus Pesticide Fenitrothion, by an Infrared Method, Analyst, 91, 779, 1966.

10. Desmarchelier, J., Bengston, M., Connell, M., Minett, W., Moore, B., Phillips, M., Snelson, J., Sticka, R., and Tucker, K., A Collaborative Study of Residues on Wheat of Chlorpyrifos-methyl, Fenitrothion, Malathion, Methacrifos and Perimiphos-methyl, Pestic. Sci. 8, 473, 1977.

11. Coburn, J.A., and Chan, A.S.Y., Confirmation of Pesticide Residue Identity. VIII Organophosphorus Pesticides. J. Assoc. Off. Anal. Chem., 57(6), 1272, 1974.

12. Grift, N., and Lockhart, W.L., Gas-Liquid Chromatographic Determination of Fenitrothion in Fish, Water and Sediment. J. Assoc. Off. Anal. Chem., 57(6), 1282, 1974.

13. Marshall, W.D., and Greenhalgh, R., Determination of Impurities in Technical Fenitrothion by Liquid Chromatography. Pestic. Sci. 5, 781, 1974.

14. Cochrane, W.P., Lanouette, M., Greenhalgh, R., Quantitation of the Contaminants in Technical Fenitrothion by Reverse Phase High Pressure Liquid Chromatography. J. Assoc. Off. Anal. Chem., 62(6), 1222, 1979.

15. Perez, R.L., Determination of Fenitrothion, Bioresmethrin and Piperonyl Butoxide in Aerosol Concentrates by High Performance Liquid Chromatography. J. Chromatogr., in press.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 367-373 (1983)

A SIMPLE AND RAPID METHOD FOR THE DETERMINATION OF 2-ACETYLAMINOFLUORENE IN LABORATORY DIETS

E. G. Perkins* and D. J. Hendren
Department of Food Science
University of Illinois
1208 West Pennsylvania
Urbana, Illinois 61801

and

C. R. Truex and W. J. Visek†
Clinical Nutrition and Metabolism
University of Illinois College of Medicine
Urbana, Illinois 61801

ABSTRACT

A simple and rapid method for the determination of 2-AAF in animal feeds was developed using high performance liquid chromatography. The column employed was an octadecyl bonded silica support with 80% methanol as mobile phase. Fluorene was added as an internal standard. The method is applicable for concentrations of 250-1200 μg AAF/kg diet.

INTRODUCTION

The carcinogen 2-acetylaminofluorene (2-AAF) has been used extensively to induce cancer in laboratory animals. Its physical properties have been discussed (1) and various methodologies

*Author to whom correspondence should be addressed.

†Dr. Visek is also a faculty member of the Department of Food Science.

0148-3919/83/0602-0367$3.50/0

developed for its analysis by gas liquid chromatography and fluorometry have been reviewed (2). West and Oiler (3) have also published a method for determining 2-AAF in laboratory animal chow by reverse phase liquid chromatography. We are describing here a method for determining 2-AAF in semi-purified diets by high pressure liquid chromatography (HPLC). The method requires no preliminary clean-up and utilizes fluorene as an internal standard.

MATERIALS

Chemicals

2-AAF and fluorene were obtained from Aldrich Chemical Co. (Milwaukee, WI). The acetonitrile and methanol used as solvents were obtained from Burdick and Jackson (Philadelphia, PA) and were glass distilled.

Synthetic Diets

The semipurified diets containing added 2-AAF and control diets were prepared commercially according to the AIN-76 reference standard (4) (Bio Serv Inc., Frenchtown, NJ). They contained 10 or 40% of isolated soybean protein or vitamin-free casein, 5% corn oil, 5% cellulose, 3.5% minerals, 1% vitamins, 0.3% dl-Methionine and 0.2% choline bitartrate. The balance of the mixture contained sucrose and cornstarch in a ratio of 1:3. The protein content was varied at the expense of the carbohydrate. Several batches of diet were also prepared in the laboratory, spiked with 2-AAF in ethanolic solution, dried, thoroughly mixed, and assayed to determine recovery of the chemical.

Analytical Procedure

Five grams of diet were weighed into a 100 ml round bottom flask and 50 ml of acetonitrile containing the internal standard, fluorene, at a concentration of 300 μg/ml, were added via a pipet dispenser. The flask was then stoppered and shaken mechanically

for 30 minutes at about 100 cycles/min. After the solids were allowed to settle, 10.0 ml of extract were transferred to a 3-inch glass funnel lined with a 12.5 cm Whatman filter paper. The filtrates, collected in 22 ml glass scintillation vials, were analyzed as described below.

Instrumentation

The analytical system consisted of a Tracor model 995 isochromatographic pump (Tracir Avi, Austin, TX) and a Rheodyne loop injector equipped with a 20 µl loop (Rheodyne, Berkeley, CA) and U.V. detection at 285 nm. The data were recorded by a Hewlett-Packard model 3380 electronic integrator, which was programmed to record results directly in ppm when compared to an internal standard. A Whatman PXS-10-25, ODS-2 reverse phase octadecyl bonded column with an attached C-18, 37-50 µ bonded pre-column (4" x 1/8") were used for separation. The mobile phase employed was 80% methanol:20% water at 1 ml per min flow rate.

RESULTS AND DISCUSSION

Complex matrices like diets and tissues often contain components which are extracted and interfere with subsequent analyses. The extracts prepared as described here did not appear to contain interfering components in the area of the chromatogram of present interest. The chromatogram of a control diet extract showed no peaks. Subsequent chromatograms of diet extracts with added internal standard and 2-AAF are shown in Figure 1. Fluorene, a degradation product of 2-AAF, and chosen for the internal standard (1), was well-separated from 2-AAF. Its presence in diet extracts prepared after diets were stored as long as 90 days could not be demonstrated. This is to be expected since the formation of fluorene from 2-AAF requires hydrolysis of the amide followed by deamination. Conditions favoring these reactions would not be expected in feed stored at 4°C. Though oxidative and hydroxylated products might be expected, none were found.

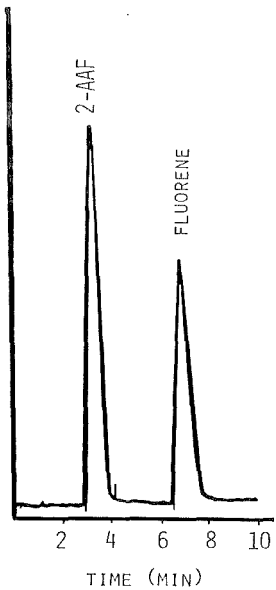


FIGURE 1. Chromatogram of diet extract containing 2-AAF and fluorene, an internal standard. Correlation: 25 cm x 4.2 m O.D.S. column (Whatman, 10 μ, Nutley, NJ). Mobile phase: 80% methanol:H_2O at 1 ml/min. Detection method: 285 nM.

The data shown in Table 1 indicate satisfactory overall recovery of 2-AAF from semi-purified diets to which the carcinogen and internal standard had been added in the laboratory. The difference in carcinogen content from that expected was slightly greater than one percent.

The data presented in Table 2 indicate a considerable difference both in the error between replicate samples and the percentage deviation from the amount presumed to be present in the diet mixture. The 54 samples analyzed to obtain these data were all obtained from diets provided by the same commercial supplier. They demonstrate the importance of analyzing dietary formulations before feeding of toxic or carcinogenic compounds which depend upon the amount consumed for their biological effects. The

TABLE 1

Recovery of Added 2-AAF from Control Diets

% Protein	Concentration of 2-AAF Added μg/g	n*	Concentration of 2-AAF Found μg/g ± σ	% Recovery
10	250	6	257.2 ± 4.5	102.9 ± 1.3
	333	6	344.1 ± 4.5	103.4 ± 1.3
	666	6	669.7 ± 13.7	100.6 ± 2.1
	1000	6	1005.9 ± 6.2	100.6 ± 0.6
40	333	6	348.3 ± 4.4	104.6 ± 1.3
	500	6	524.0 ± 4.9	104.8 ± 1.0
	666	6	695.9 ± 7.3	104.5 ± 1.1
	700	6	710.5 ± 7.5	101.5 ± 1.1
	1000	6	985.4 ± 9.4	98.5 ± 0.9

*Each sample result was the average of duplicate injections into the HPLC.

TABLE 2

Determination of 2-AAF in Commercially Prepared Diets

Diet Number	% Protein	Concentration of 2-AAF Added μg/g	n	Concentration of 2-AAF Found μg/g ± σ	% Recovery
1*†	10	333	6	306.4 ± 17.5	92.0 ± 4.3
		666	6	576.2 ± 20.4	86.5 ± 3.0
		1000	6	973.4 ± 47.6	97.3 ± 4.8
	40	333	6	332.7 ± 58.1	99.9 ± 17.5
		666	6	614.7 ± 11.7	92.3 ± 1.8
		1000	6	935.3 ± 53.0	93.5 ± 5.3
2*†‡	10	333	3	340.6 ± 6.5	102.3 ± 1.8
		666	3	671.1 ± 32.0	100.8 ± 4.8
		1000	3	990.4 ± 57.0	99.0 ± 5.7
	40	333	3	294.4 ± 8.4	88.4 ± 2.6
		666	3	641.0 ± 28.8	96.2 ± 4.3
		1000	3	951.6 ± 8.8	95.2 ± 0.9

*Diets supplied at two different time intervals and from different batches.
†Determined by duplicate injection of diet extract into the HPLC.
‡One injection of diet extract into the HPLC.

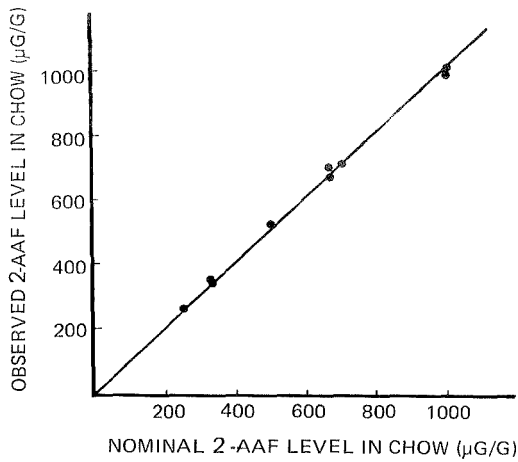


FIGURE 2. Relationship between observed and nominal 2-AAF level in chow.

linearity of the analytical method developed is illustrated in Figure 2. Reliable determinations were made for concentrations ranging from 250 μg to 1200 μg/kg of 2-AAF in the diet. The currently used analytical column has shown no signs of deterioration after analyses of over 500 diet extracts. It is necessary, however, to employ a short precolumn packed with 37-50 μ octadecyl stationary phase, which should be replaced after 200-225 analyses.

ACKNOWLEDGEMENT

This work was supported in part by the Illinois Agricultural Experiment Station and Contract NO1-CP-75899 from the U.S. National Cancer Institute.

REFERENCES

1. Bowman, M. C. and King, J. R., Analysis of 2-Acetylaminofluorene: Residues in Laboratory Chow and Microbiological Media, Biochem. Med., 9, 390, 1974.

2. Bowman, M. C., Carcinogens and Related Substances: Analytical Chemistry for Toxicological Research, Marcel Dekker, Inc., New York, 1979, pp. 60-75.

3. West, R. W. and Oiler, W. L., Analysis of 2-Acetylamino-fluorene in Laboratory Animal Chow by Reverse Phase Liquid Chromatography, J. Liquid Chrom., 1, 181, 1978.

4. American Institute of Nutrition, Ad Hoc Committee on Standards for Nutritional Studies Report of the Committee, J. Nutr. 107, 1340, 1977.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 375-381 (1983)

LIQUID CHROMATOGRAPHIC DETERMINATION OF EXCRETION PATTERNS OF URINARY PHENOLIC COMPOUNDS

Yoshihisa Yamaguchi

College of Biomedical Technology and Nursing
Biochemical Analysis
Osaka University
1-1 Machikaneyamacho
Toyonaka, Osaka , Japan

ABSTRACT

Excretion patterns of urinary phenolic compounds were determined by means of chromatography with Sephadex G-10, with 4-aminoantipyrine for the detection of phenolic compounds.

Derivatization for phenolic compounds is based on a coupling reaction with 4-aminoantipyrine in the presence of sodium metaperiodate. The reaction is complete within a few minutes and, thus, provides a simple detection method.

Excretion patters of samples with normal subjects and patients of catecholamine-producing tumor were determined and the results were compared.

From the data obtained, this method is shown to be useful as a screening test of some catecholamine-producing tumors.

INTRODUCTION

Numerous techniques have been developed for detection of catecholamine metabolites in urine, such as colorimetric methods preceded by solvent extraction, a gas liquid chromatographic method, gas chromatograph-

 0148-3919/83/0602-0375$3.50/0

ic - mass spectrometry and high performance liquid chromatography (1-4).

For spot tests and some semiquantitative methods for urinary catecholamine metabolites, a coupling reaction with diazotized p-nitroaniline is widely used. On the other hand, determination of urinary total phenolic compounds with use of 4-aminoantipyrine has been described previously by Yamaguchi (5).

Recently, high performance liquid chromatography, with use of Sephadex G-10 for isolation of urinary catecholamine metabolites, has been reported (4).

In this paper, a new method for determination of urinary phenolic compounds excretion patterns is described and proposed as a diagnostic aid in patients with suspected cases of pheochromocytoma and neuroblastoma.

MATERIALS AND METHODS

All compounds used for this study were purchased from Sigma Chemical Co., St. Louis, Mo. 63178.

Preparation of reagent:

Carbonate-bicarbonate buffer, 0.05 mol/L, pH 10.1. Dissolve 3.18 g of anhydrous sodium carbonate plus 1.68 g of sodium bicarbonate in water and dilute to 1 liter.

Solution A. Dissolve 90 mg of 4-aminoantipyrine in 200 ml of carbonate -bicarbonate buffer.

Solution B. Dissolve 2.6 g of boric acid and 0.4 g of sodium metaperiodate in water and dilute to 200 ml.

Preparation of urine sample:

A 24-h urine specimen is collected and aliquot of the 24-h urine is centrifuged for 3 min at 2500 g. One ml of the supernatant of urine is directly applied to the Sephadex G-10 column (1 x 30 cm).

Sephadex gel chromatography:

Sephadex G-10 is swollen by heating a suspension of the particles in acetate buffer (0.05 M, pH 5.0) for 4 h at 90 °C under constant stirring. The fines are removed by several decantations and the resultant slurry is poured directly into the column. The column is then washed for 3 h with acetate buffer solution. After application of sample, chromatographic separation is performed with acetate buffer (0.05 M, pH 5.0). Each fraction of effluent contains 1.3 ml; 10 fractions are run within 30 min, and 40 fractions are collected.

Procedure for a detection of phenolic compounds:

To 0.5 ml of each chromatographic effluent is added 0.6 ml of solution A and shaken well; then 0.6 ml of solution B is added. Absorbance at 500 nm is read against the first fraction of effluent.

Total excretion value of urinary phenolic compounds is determined by the method previously reported (5).

Preparation of diazo reagent:

a) 100 mg of p-nitroaniline and 2 ml of conc. HCl are dissolved in 98 ml water.
b) 200 mg of Na_2NO_2 is dissolved in 100 ml of water.
c) 10 g of Na_2CO_3 is dissolved in 100 ml of water.
Mix the reagent of a, b and c at a ratio of 1:1:2 immediately before use.

Color development of phenolic compounds by diazo reagent :

To 0.5 ml of each effluent fraction, 1 ml of diazo reagent is added, and then allowed to stand for 30 min at room temperature. Measure absorbance at 540 nm.

RESULTS

Specificity of the reaction with 4-aminoantipyrine:

Table 1 shows the specificity of reaction with various biological phenolic compounds. Precision and analytical recovery have already described in previous paper (5).

Excretion patters of urinary phenolic compounds

Chromatograms of standard compounds and of samples of normal subjects and patients of catecholamine-producing tumor are shown in Figure 1. Chromatograms of some other patients are also shown in Figure 2, in which samples from patients with diabetes mellitus and hypertension are analyzed.

TABLE 1.

Specificity of the reaction

Compounds tested	Absorbance (20 µg/tube)
Vanilmandelic acid	0.330
Normetanephrine	0.145
Epinephrine	0.138
3,4-Dihydroxyphenylalanine	0.100
Octopamine	0.095
Homovanillic acid	0.077
Noradrenaline	0.057
Dopamine	0.050
p-Hydroxyphenylacetic acid	0.000
Tyramine	0.000
Vanillin	0.000
Salicylic acid	0.000
Phenylalanine	0.000
Xanthurenic acid	0.580
Thyroxine	0.225
Guaiacol	0.290
Catechol	0.146
Serotonin	0.035

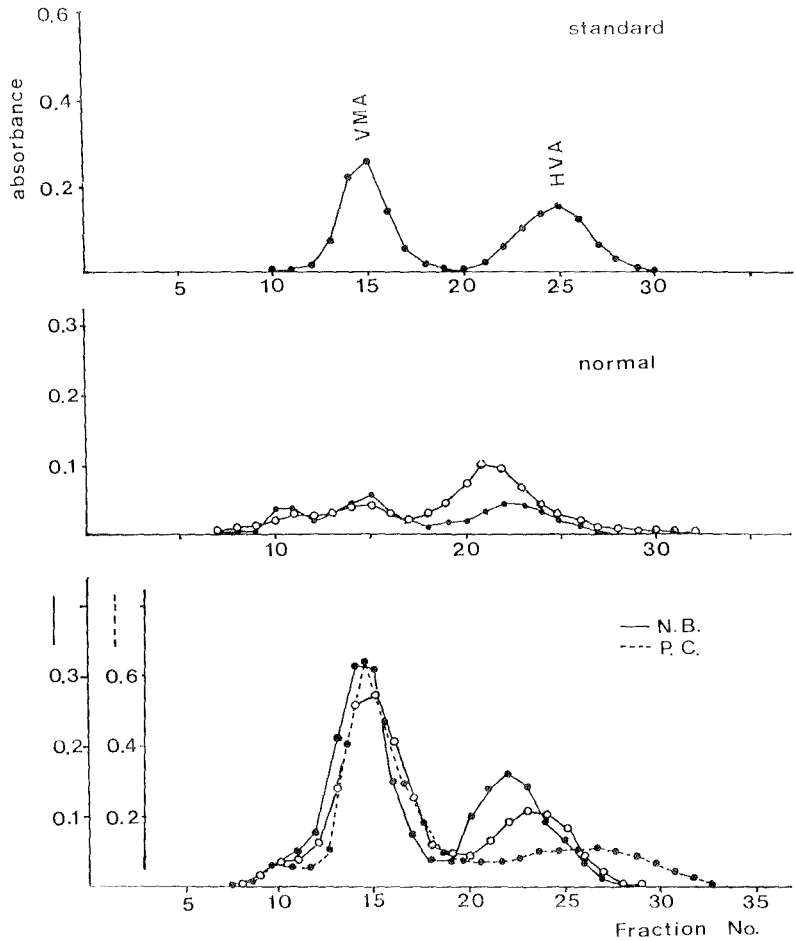


FIGURE 1. Excretion patterns of urinary phenolic compounds; chromatograms of standards, normal subjects and some patients of catecholamine producing tumor (N.B., Neuroblastoma, P.C., Pheochromocytoma)

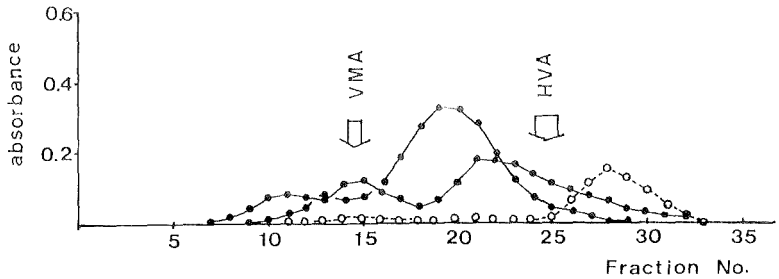


FIGURE 2. Excretion patterns of urinary phenolic compounds: ●——●, diabetes mellitus, and o---o, hypertension.

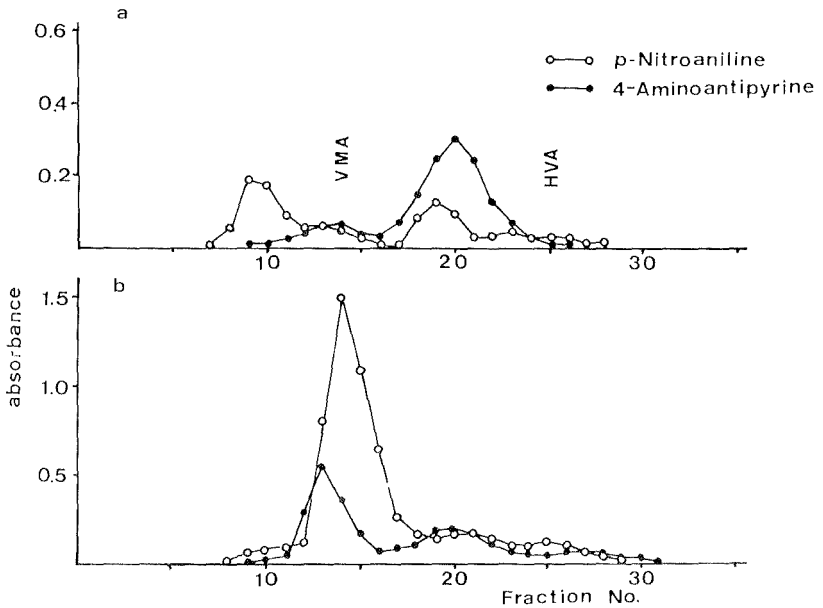


FIGURE 3. Comparison between the present method and the diazo reaction method. ●——●, present method and o——o, diazo reaction method. a) normal subject, and b) pheochromocytoma.

DISCUSSION

Comparison of excretion patterns of urinary phenolic compounds with diazo reaction method was also performed and the chromatograms were also shown in Figure 3, in which diazotization with p-nitroaniline showed high sensitivity and specificity for VMA (vanilmandelic acid) fraction but not for HVA (homovanillic acid) fraction.

Chromatograms between two methods are comparable; so the detection method using 4-aminoantipyrine can also applied to the diagnosis of catecholamine-producing tumors as shown in Figure 1.

The major advantage of this method is in its procedural simplicity and in the stability of reagents. Thus, this procedure provides new method that is useful for diagnostic purposes.

REFERENCES

1. J.J. Pisano, J.R. Crout and D. Abraham, Determination of 3-methoxy-4-hydroxymandelic acid in urine. Clin. Chim. Acta, 7,285 (1962)

2. G.G. Guilbault, P.J. Brignac and M. Juneau, New substrates for the fluorometric determination of oxidative enzymes. , Anal. Chem., 40,1256 (1968).

3. M.A. Brewster, D.H. Berry and M. Monarty, Urinary 3-methoxy-4-hydroxyphenyl acetic acid (HVA) and 3-methoxy-4-hydroxymandelic acid (VMA). , Clin. Chem. , 23, 2247 (1977).

4. N. Fornstedt, High-performance liquid chromatographic determination of homovanilic acid in urine using Sephadex G-10 for isolation., J.Chromatogr., 225, 440 (1981).

5. Y. Yamaguchi and C. Hayashi, Determination of urinary total phenolic compounds with use of 4-aminoantipyrine. , Clin. Chem., 23 , 2151 (1977).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 383-394 (1983)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF UROPORPHYRIN ISOMERS

James M. Rideout, Dennis J. Wright and C.K. Lim
Division of Clinical Chemistry, MRC Clinical Research Centre,
Harrow, Middlesex HA1 3UJ, U.K.

ABSTRACT

The separation of uroporphyrin I and III isomers by reversed-phase high performance liquid chromatography on ODS-Hypersil with ammonium acetate buffer - acetonitrile solvent systems is described. The effects of buffer concentration, pH, organic modifier proportion and different organic modifiers on the resolution are studied. The optimum conditions for the separation were 12-13% acetonitrile in 1M ammonium acetate buffer pH 5.10-5.20. The method also separated uroporphyrin I and III from the II isomers but the resolution of uroporphyrin III and IV isomers was not achieved.

INTRODUCTION

Uroporphyrinogen III is the universal precursor of chlorophylls, haem and vitamin B_{12}. It is formed initially by the condensation of four molecules of porphobilinogen to the unstable intermediate preuroporphyrinogen catalysed by the enzyme porphobilinogen deaminase. In the absence of this enzyme, preuroporphyrinogen is spontaneously rearranged into uroporphyrinogen I (1,2).

Much effort had been devoted to the development of separation methods for uroporphyrin I and III isomers (Figure 1), the two naturally occurring uroporphyrins. The separation is

 0148-3919/83/0602-0383$3.50/0

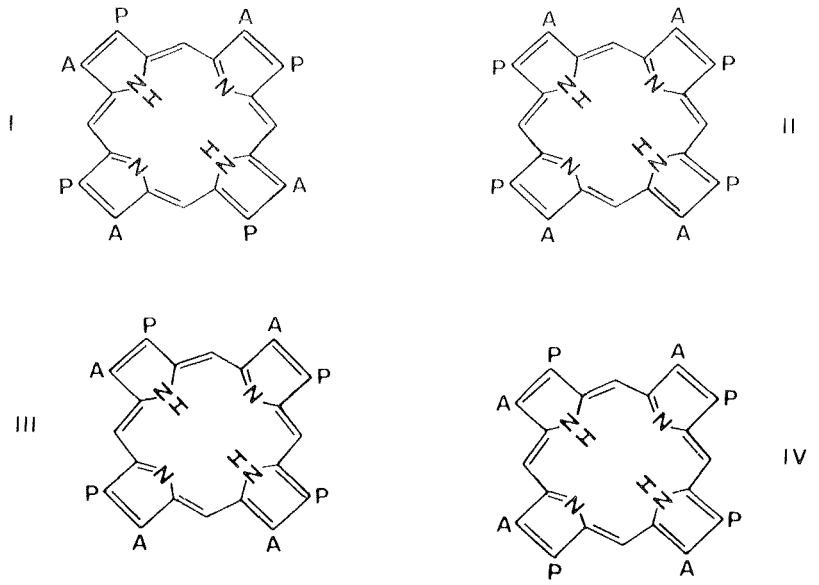


Figure 1 Structures of uroporphyrin I, II, III and IV isomers. A = CH_2COOH; P = $-CH_2-CH_2-COOH$.

important for the diagnosis of porphyrias and for developing an assay for uroporphyrinogen III cosynthetase where the quantitative separation of the I and III isomers is essential.

High performance liquid chromatography (HPLC) has been used to separate uroporphyrin I and III isomers as their octamethyl esters. Bommer et al. (3) separated the isomers after a five peak-recycle on two 30 cm silica columns in series. Nordlov et al. (4) modified the system and achieved separation without peak recycling but unfortunately the method required several hours of equilibration and a 3-hour separation. The reproducibility of such a system is doubtful. Walker et al. (5) and Jackson et al. (6) reduced the analysis time considerably but complete separation was not achieved. There has been hitherto only one paper describing the separation of uroporphyrin I and III isomers

as free acids (7) but no detailed study of their retention behaviour was presented.

The separation of the porphyrins as free acids is advantageous as the complicated and tedious extraction and derivatisation steps are avoided. We report here a rapid, highly effective and reproducible reversed-phase HPLC system for the separation of uroporphyrin I and III isomers as their free octacarboxylic acids. The retention behaviour of the isomers on ODS-Hypersil (5 μm spherical silica chemically bonded with octadecyl silyl groups) with buffered ammonium acetate-acetonitrile as the mobile phase is studied in detail. The separation of uroporphyrin II and IV isomers (Figure 1) is also described.

The practical applications of the method are demonstrated by the separation of the isomers in the urine of a patient with congenital porphyria and in the incubation mixture used in the determination of uroporphyrinogen III cosynthetase.

EXPERIMENTAL

Materials and Reagents

Porphobilinogen, uroporphyrin I and uroporphyrin III octamethyl ester were obtained from Sigma London Ltd. (Poole, U.K.). A statistical mixture of uroporphyrin I, II, III and IV was prepared by heating porphobilinogen in HCl (8). Ammonium acetate, glacial acetic acid and tetrahydrofuran were AnalaR grade from BDH Chem. Ltd. (Poole, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn Chem. Ltd. (Walkerburn, U.K.).

HPLC Apparatus

A Pye Unicam (Cambridge, U.K.) LC3-XP solvent delivery system was used with a Perkin-Elmer (Beaconsfield, U.K.) LS-3

fluorescence detector. The excitation and emission wavelengths were 406 nm and 619 nm respectively. A Rheodyne 7125 injector fitted with a 100 μl sample loop was used for injection.

HPLC Column and Mobile Phases

A 25 cm x 5 mm ODS-Hypersil (Shandon Southern Products, Runcorn, U.K.) reversed-phase column was used for the analysis. The mobile phases were acetonitrile (12-15%) in 0.25, 0.5, 0.75, 1.0 and 1.5M ammonium acetate buffer (pH 4.0-7.0). The pH was adjusted with acetic acid. Other eluents such as methanol-1M ammonium acetate pH 5.15 (16.6 : 83.4 $^{v}/v$), tetrahydrofuran-1M ammonium acetate buffer pH 5.15 (10.2 : 89.8 $^{v}/v$) and tetrahydrofuran-methanol-ammonium acetate buffer pH 5.15 (5.1 : 8.3 : 8.6 by vol.) were also used. The flow rate was 1 ml/min throughout.

RESULTS AND DISCUSSION

The Effect of Buffer Concentration on Retention and Resolution

The molar concentration of ammonium acetate buffer in the mobile phase significantly affected the retention and resolution of uroporphyrin I and III isomers. The variation of the capacity ratio (k') with the buffer concentration is shown in Figure 2. The optimum buffer concentration was 1M. At below 0.5M, excessive retention and peak broadening resulted while at above 1.5M, rapid elution with loss of resolution was observed. This observation suggested that ammonium acetate competes effectively with the solutes for extraction onto the stationary phase and is perhaps also a good masking agent for the residual silanol groups on the silica of the reversed-phase packing. It is therefore important that a high concentration of ammonium acetate solution (0.75-1M) is used to ensure good resolution and a short separation time.

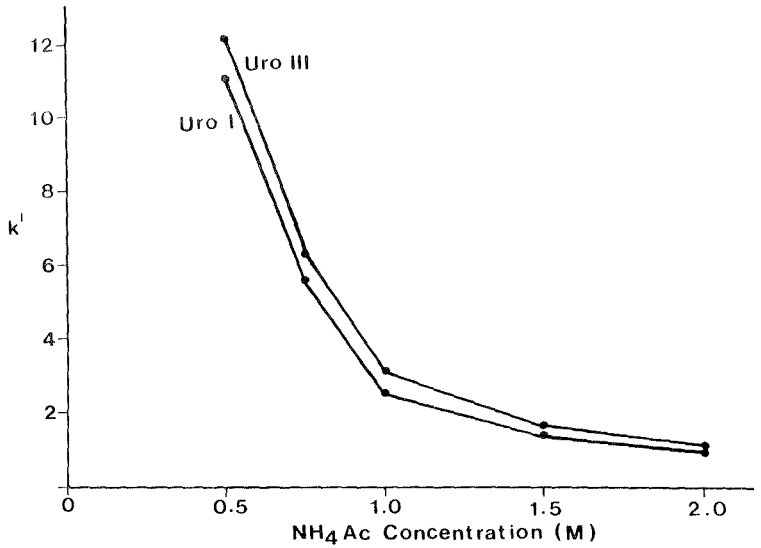


Figure 2 Variation of the capacity ratios (k') of uroporphyrin I and III with the molar concentration of ammonium acetate buffer in the eluent. The mobile phase was maintained at pH 5.15 and contained 13% acetonitrile.

The Effect of pH on Retention and Resolution

The retention and resolution of the porphyrins was greatly influenced by the pH of the ammonium acetate buffer used. Increasing the pH decreased the k' values (Figure 3) with loss of resolution. Figure 3 clearly shows that the usable pH range is narrow, to achieve rapid and effective separation the pH should be carefully adjusted to 5.0-5.2.

Acetonitrile Concentration Effect

The effect of acetonitrile concentration on the k' values of the porphyrin isomers (Figure 4) is that expected for reversed-phase chromatography. The k' values decreased with

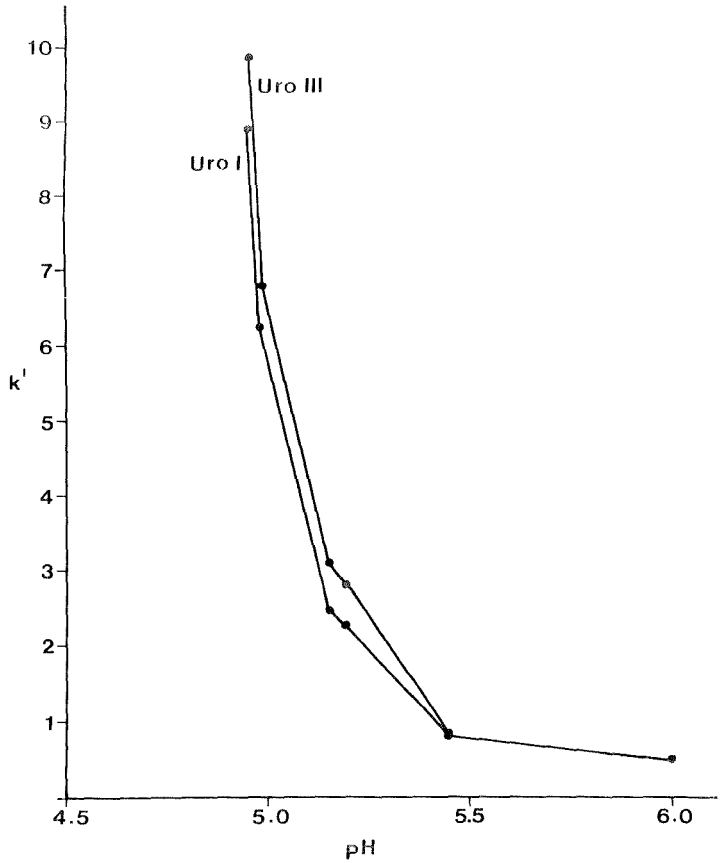


Figure 3 The effect of pH on the capacity ratios (k') of uroporphyrin I and III. The eluent was 13% acetonitrile in 1M ammonium acetate buffer.

increasing acetonitrile content in the mobile phase. The optimum concentration of acetonitrile was found to be 12-13%.

Organic Modifier Specificity

The solvent system described here is organic modifier specific. Replacing 13% acetonitrile with methanol or

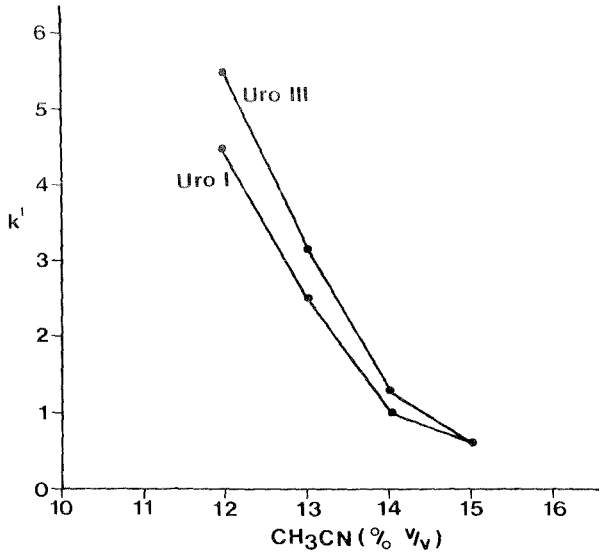


Figure 4 Relationship between capacity ratios (k') of uroporphyrin I and III and acetonitrile content in the mobile phase. The eluent was maintained at 1M ammonium acetate buffer pH 5.15.

TABLE 1

Capacity ratio (k') of uroporphyrin I and III isomers in mobile phases of equal polarity. Solvent A is 1M ammonium acetate-acetic acid pH 5.15. Column is ODS-Hypersil (2.5 cm x 5 mm).

Mobile phase B in A	k'	
B	URO I	URO III
13% CH_3CN	2.50	3.14
16.6 CH_3OH	> 20	> 20
10.2% THF	0.43	0.43
8.3% CH_3OH + 5.1% THF	2.30	2.64

tetrahydrofuran to give equal mobile phase polarity resulted in no elution and no retention respectively (Table 1).

A ternary mobile phase system of tetrahydrofuran-methanol-1M ammonium acetate buffer pH 5.15 (5.1 : 8.3 : 86.6 by vol.) which is equivalent to 13% acetonitrile in 1M ammonium acetate buffer pH 5.15 in terms of mobile phase polarity, did separate the isomers but the resolution was not as good.

The Optimum Solvent System for the Separation of Uroporphyrin I and III Isomers

From the results obtained it becomes obvious that for the fast and effective separation of the isomers on a 25 cm x 5 mm I.D. ODS-Hypersil column the recommended mobile phase is 13% acetonitrile in 1M ammonium acetate buffer at pH 5.15. A typical separation is shown in Figure 5.

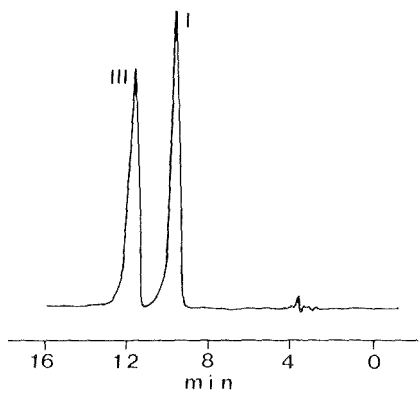


Figure 5 Separation of uroporphyrin I and III isomers. Column, ODS-Hypersil (25 cm x 5 mm); mobile phase 13% acetonitrile in 1M ammonium acetate buffer pH 5.15; flow rate, 1 ml/min., detector, fluorescence excitation 406 nm, emission 619 nm.

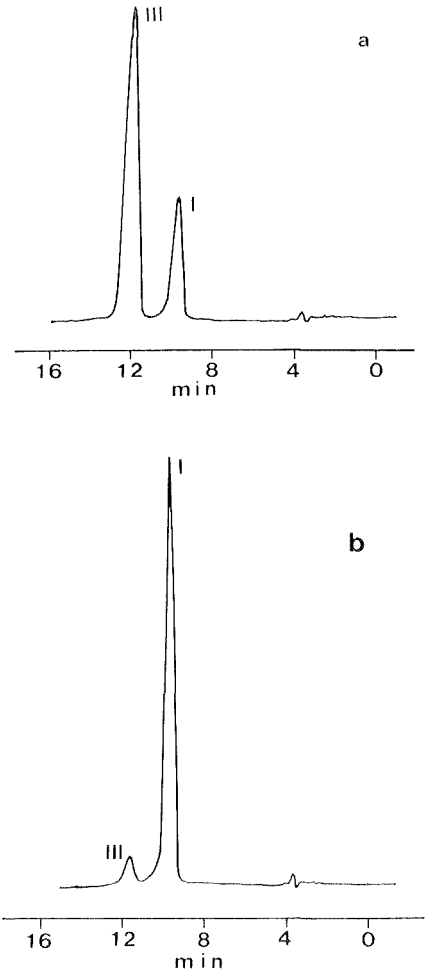


Figure 6 Separation of uroporphyrin I and III isomers in (a) incubation mixture for the determination of uroporphyrinogen III cosynthetase, (b) congenital porphyric urine. HPLC conditions as in Figure 5.

Applications of the Separation

The most important application is in the development of a rapid and specific assay for the enzyme uroporphyrinogen III cosynthetase. Our preliminary results clearly demonstrate this possibility and are shown in Figure 6(a), the separation of uroporphyrin III from the I isomer in the incubation mixture used for the determination of uroporphyrinogen III cosynthetase in haemolysed red blood cells. Figure 6(b) shows another practical application, the identification of uroporphyrin I in the urine of a patient with congenital porphyria. The separation and identification of the isomers is important for the diagnosis of this condition in order to differentiate it from other forms of porphyria.

The Separation of Uroporphyrin I, II, and III + IV Isomers

A mixture of four uroporphyrin isomers prepared by heating porphobilinogen in acid was resolved into 3 peaks as shown in Figure 7. The system could not separate uroporphyrin III from the IV isomers but uroporphyrin II was easily resolved. The separation of the I and III isomers from the II and IV isomers is unimportant, however, as the II and IV isomers are not naturally occurring compounds and therefore no attempt was made to improve the separation. The proportion of the four isomers (12.9% I, 12.0% II, 75.1% III + IV) correlates well with the statistical proportion of 12.5% I, 12.5% II, 50% III and 25% IV isomer (8).

Reproducibility of the Mobile Phase System

One of the major problems with the separation of uroporphyrin isomers as octamethyl esters by adsorption chromatography on silica is poor reproducibility. This is often due to the various degree of hydration and purity of the organic

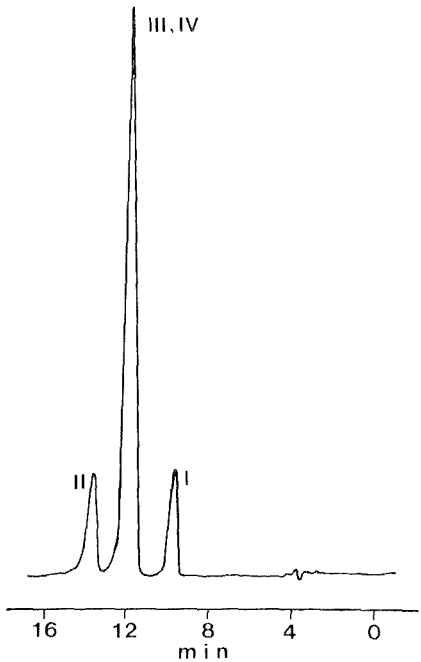


Figure 7 Separation of uroporphyrin isomers prepared by heating porphobilinogen in acid.

solvents used. Reversed-phase chromatography with buffer-controlled mobile phases is much less likely to suffer from such a variation. The reproducibility of the present system has been thoroughly tested. Several hundred analyses have been performed on the same column and on different columns packed with the same stationary phase. The results are highly reproducible. Any deterioration of column performance can often be cured by replacing the top 2 mm of the column packing.

CONCLUSION

The factors affecting the separation of uroporphyrin I and III isomers by reversed-phase chromatography have been studied

in detail. It is concluded that rapid and effective separation of the isomers required a mobile phase of 12-13% acetonitrile in 0.75-1.25M ammonium acetate buffer pH 5.0-5.2.

REFERENCES

1. Burton, G., Nördlov, H., Hosozawa, S., Matsumoto, H., Jordan, P.M., Fagerness, P.E., Pryde, L.M. and Scott, A.I., J. Amer. Chem. Soc., 101, 3114, 1979.

2. Battersby, A.R., Fookes, C.J.R., Matcham, G.W. and McDonald, E., Nature (London), 285, 17, 1980.

3. Boomer, J.C., Burnham, B.F., Coulson, R.E. and Dolphin, D., Anal. Biochem., 95, 444, 1977.

4. Nördlov, H., Jordan, P.M., Burton, G. and Scott A.I., J. Chromatogr., 190, 221, 1980.

5. Walker, I.C., Gilbert, M.T. and Stubbs, K., J. Chromatogr., 202, 491, 1980.

6. Jackson, A.H., Rao, K.R.N. and Smith, G., Biochem. J., 203, 515, 1982.

7. Wayne, A.W., Straight, R.C., Wales, E.E. and Englert, E., J. High Resolut. Chromatogr. Chromatogr. Commun., 2, 621, 1979.

8. Mauzerall, D., J. Amer. Chem. Soc., 82, 2601, 1960.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 395-396 (1983)

LC NEWS

AUTOMATED CHROMATOGRAPHY SYSTEM combines an LC with a computer with high resolution graphics. It features a pulse free pump, ternary gradient capability, and a variety of detectors, and can control up to four liquid chromatographs. Programs can be stored on disk for future use. IBM Instruments, Inc., JLC/83/2, P.O.Box 332, Danbury, CT, 06810, USA.

SHORT COURSE IN PARTICLE TECHNOLOGY is an introduction for scientists and engineers concerned with evaluating materials properties. Topics include influence of particle size and range, importance of surface area, effect of pores, and density. Micromeritics Instrument Corp., JLC/83/2, 5680 Goshen Springs Rd., Norcross, GA, 30093, USA.

AUTOMATED PREPARATIVE LC features interactive CRT for precise sample collection and repetitive injection. It can be used to isolate compounds from dilute solutions or to remove minor impurities from a concentrated sample. Waters Associates, Inc , JLC/83/2, 34 Maple Street, Milford, MA, 01757, USA.

HIGH PERFORMANCE AND RESOLUTION are offered in new lc columns, with plate counts up to 30,000 to 40,000 plates per meter, very close to the theoretical limit for 10 micron particles. Hamilton Co., JLC/83/2, P.O.Box 10030, Reno, NV, 89510, USA

PROGRAMMABLE WAVELENGTH DETECTOR is microprocessor controlled. It permits selection of any number of wavelengths in one-nanometer increments from 190 to 370 nm for optimal detection of all components. Utilizes keyboard entry that may be changed at any point and as often as desired. Varian Instrument Group, JLC/83/2, 2700 Mitchell Drive, Walnut Creek, CA, 94598, USA.

APPLICATIONS DEVELOPMENT KIT permits application of solid phase technology in the development of new sample preparation methods. One can experiment with new methods or solve existing problems with the phases included in the kit. Included are octadecyl, phenyl, cyanopropyl, aminopropyl, benzenesulfonic acid, quaternary amine, diol, and unbonded silica. Analytichem Internat'l, Inc., JLC/83/2, 24201 Frampton Avenue, Harbor City, CA, 90710, USA.

0148-3919/83/0602-0395$3.50/0

POST-COLUMN REACTOR can be used to determine metals. It features a pneumatic pump, mixing tee, and a packed bed reactor in a self-contained unit. Dionex, JLC/83/2, 1228 Titan Way, Sunnyvale, CA, 94086, USA.

DUAL ELECTRODES FOR LC/EC capable of handling applications in single, dual-series, and dual-parallel modes. The dual parallel mode permits ratioing for identification of chromatographic peaks and also enhances selectivity and saves time. Dual-series assays are possible for reversible redox couples and, in many cases, can enhance both selectivity and detection limits. Bioanalytical Systems, Inc., JLC/83/2, 111 Lorene Place, West Lafayette, IN, 47906, USA.

GEL FILTRATION COLUMNS are in widespread use for the separation of enzymes, proteins, polysaccharides, nucleic acids, water-soluble polymers and oligomers. A wide range of pore sizes accomodates a broad range of molecular weights. Kratos Analytical Instruments, JLC/83/2, 170 Williams Drive, Ramsey, NJ, 07446, USA.

HPLC OF CNBr CLEAVAGE FRAGMENTS of a bacterial toxin "parent" protein have been successfully separated with a Wide-Pore Octadecyl C-18 column. The 5 major fragments and several intermediates resulting from cyanogen bromide treatment were well resolved in less than 20 minutes. J. T. Baker Research Products, JLC/83/2, 222 Red School Lane, Phillipsburg, NJ, 08865, USA.

CHROMATOGRAPHY DATA SYSTEM FOR APPLE II fits into an empty slot of the Apple and receives analog signals from the chromatograph's recorder output and converts it to digital with 12-bit precision up to 20 times/sec. Signals are smoothed, then peaks identified and integrated. Chromatogram is displayed on the CRT in real time using the high resolution graphics mode. Analytical Computers, JLC/83/2, P. O. Box 285, Elmhurst, IL, 60126, USA.

CHANNELLED HPTLC PLATES make possible HPTLC without need for special spotting apparatus or spotting techniques. They offer a majr advantage when large volumes of sample must be applied. They are divided into 0.8 cm wide silica gel strips separated by 2 mm clear glass strips. This prevents bleed or cross contamination. Whatman, Inc., JLC/83/2, 9 Bridewell Place, Clifton, NJ, 07014.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 397-398 (1983)

THE EASTERN ANALYTICAL SYMPOSIUM

The 21st Eastern Analytical Symposium (EAS), which was held in New York City, November 17-19, was an impressive meeting. It started as a regional meeting, and is becoming a national one with an international flair. The organizers' objective has been to make EAS the scientific meeting for analytical chemists. If the recent symposium is any indication, the organizers' aim has been fulfilled. The symposium, which included discussions, and an instrument show of every major analytical technique, was organized into two main disciplines of analytical chemistry: separation (chromatography) and identification (spectroscopy).

Unlike other meetings, where papers are submitted, the EAS consists exclusively of invited symposia, organized and chaired by eminent scientists who are experts in their fields. Each session (36 were presented this year) consisted of four to five lectures of from 35-45 minutes each allowing speakers the opportunity to adequately present their material, and the audience to actively discuss it.

Among this year's sessions were all aspects of chromatography (GC, TLC, HPLC, and GPC) and spectroscopy, (atomic absorption and emission, NMR, IR, UV-Vis, fluorescence and lasers in spectroscopy). Also, symposia dealing with radioimmunoassay, computers and laboratory automation, and the role of analytical chemistry in pharmaceutical, environmental, forensic, precious metals and polymer analysis were presented.

0148-3919/83/0602-0397$3.50/0

"What we try to do", says Dr. Issaq, this year's co-chairman and next year's program chairman, "is to give the analytical chemist who attends the EAS a state-of-the-art view of analytical chemistry discussed by top scientists in the US and abroad." For example, the two MS sessions, which were arranged and chaired by Dr. Gross of Nebraska, included the most recent advances presented by Biemann, McLafferty, Fenselau, Cooks, Field, Hunt, and Rinehart. These are the leading mass spectroscopists not only in the US but throughout the world. In chromatography, the list of speakers included Karger, Horvath, Lochmuller, Snyder, Engelhardt, Schomberg, Brown, Regnier, Majors, Armstrong, Scott, Knox, Issaq, Stein, Laub, Bertsch, Deming and many others who presented excellent papers. The same was true in most of the other sessions.

Having invited symposia rather than contributed papers permits the organizers to present the analytical chemist with the best and the most up-to-date analytical techniques. This year, a budget of almost $40,000 was used to bring many of the speakers to New York.

We at The Journal of Liquid Chromatography wish the EAS organizers continued success in offering the analytical chemist a wide range of topics at the highest scientific level.

Dr. Jack Cazes
Editor
December 6, 1982

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 399-400 (1983)

LC CALENDAR

1983

MARCH 7-12: Pittsburgh Conference on Anal. Chem. & Applied Spectroscopy, Convention Hall, Atlantic City, NJ, USA. Contact: Mrs. Linda Briggs, Program Secretary, 437 Donald Rd., Pittsburgh, PA, 15235, USA.

MARCH 20-25: National Amer. Chem. Soc. Meeting, Seattle, WA, USA. Contact: A. T. Winstead, Amer. Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

APRIL 18-21: Fundamentals of Chromatographic Analysis, Kent State University, Kent, Ohio. Contact: University Conference Bureau, 211-A Kent Student Center, Kent State University, Kent, OH, 44242, USA.

MAY 2-6: VIIth International Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University. Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JUNE 1-3: The Budapest Chromatography Conference, Budapest, Hungary. Contact: Dr. T. Devenyi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary or Dr. H. Issaq, Frederick Cancer Research Facility, P.O.Box B, Frederick, MD, 21701, USA.

JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece. Contact: Dr. S. S. Kazeniac, Campbell Inst. for Food Research, Campbell Place, Camden, NJ, 08101, USA.

JULY 17-23: SAC 1983 International Conference and Exhibition on

0148-3919/83/0602-0399$3.50/0

Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V OBV, United Kingdom.

AUGUST 29 - SEPTEMBER 2: 4th Danube Symposium on Chromatography & 7th I'nt'l. Sympos. on Advances & Applications of Chromatography in Indudtry, Bratislava, Czech. Contact: Dr. J. Remen, Anal. Sect., Czech. Scientific & Techn. Soc., Slovnaft, 823 00 Bratislava, Czechoslovakia

1984

OCTOBER 1-5: 15th International Symposium on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

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

INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

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

Directions for Submission

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

Manuscripts should be mailed to the Editor:

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

Reprints

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

Format of Manuscript

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

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

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

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

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

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

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

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

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

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

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

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

Typing Instructions

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

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

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

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

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

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

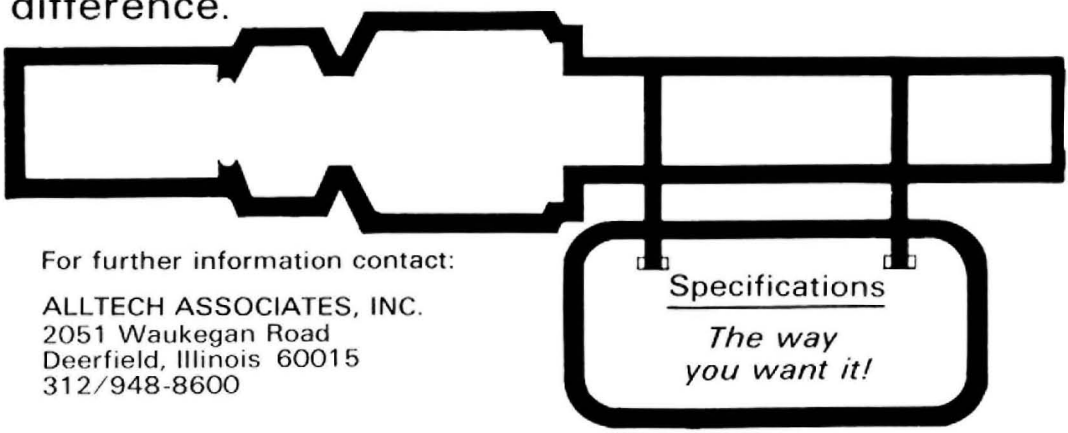
Custom packing HPLC columns has become our specialty. Any length, several ID's (including 3.2mm) and almost any commercially available packing material may be specified. We'll supply the column others won't.

With each column, you will receive the original test chromatogram plus a vial of the test mixture. Our advanced technology and computer testing is your assurance of a quality product.

When custom packing and testing is your special concern, we make the difference.

Each one is our special concern

CUSTOM PACKED HPLC COLUMNS



For further information contact:

ALLTECH ASSOCIATES, INC.
2051 Waukegan Road
Deerfield, Illinois 60015
312/948-8600

ALLTECH ASSOCIATES

See us in booths 77 - 79 at the Pittsburgh Conference.

Circle Reader Service Card No. 102