JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 7 NUMBER 4 1984

CODEN: JLCHD8 7(4) i-viii, 647-860 (1984)
ISSN: 0148-3919

ASI HPLC PACKINGS

GUARANTEED* REPLACEMENTS FOR ALL WATERS' uBONDAPAK™ PACKINGS

ASI offers a complete line of 10um, fully porous packings to compete with the Waters' uBondapak™ series. We believe we have duplicated or, in some cases improved the chemistry that has made uBondapak™ the most popular packings in the world.

ASI-packed columns are more stable and last longer than those packed by our competitors – we guarantee it! If you are not delighted with any ASI product you may return it for a prompt refund.

Why 10 micron, irregular particles?

SELECTIVITY

Enhanced selectivity is possible because irregular particles have more surface area than spherical particles. Selectivity is more important than efficiency. Selectivity is an inherent property of a particle; efficiency is not. Efficiency can change quickly and separations based on efficiency alone can be lost suddenly and without warning. Loss of selectivity happens gradually and predictably allowing for planned column replacement.

COLUMN LIFE

ASI believes that HPLC columns should last until the coating is used up or wears off. You cannot produce a stable packed bed using irregularly shaped particles of less than 10um. Excessive operating pressures cause premature column failures providing a poor value for your column dollar.

ASI PACKING	DIRECT REPLACEMENT FOR	PREPACKED		REPACKED	
		PART #	PRICE	PART #	PRICE
ASI C18					
3.9 x 15	uBondapak™ C18	101	$195.	RP-101	$150.
3.9 x 30		102	245.	RP-102	195.
7.8 x 30		103	550.	RP-103	450.
ASI PHENYL					
3.9 x 15	uBondapak™ Phenyl	201	$195.	RP-201	$150.
3.9 x 30	&	202	245.	RP-202	195.
7.8 x 30	Fatty Acid Analysis	203	550.	RP-203	450.
ASI SILICA					
3.9 x 15	uPorasil™	301	$180.	RP-301	$135.
3.9 x 30		302	220.	RP-302	170.
7.8 x 30		303	495.	RP-303	395.
ASI NH_2					
3.9 x 15	uBondapak™ NH_2	401	$195.	RP-401	$150.
3.9 x 30	&	402	245.	RP-402	195.
7.8 x 30	Energy Analysis	403	550.	RP-403	450.
ASI CYANO					
3.9 x 15	uBondapak™ CN	501	$195.	RP-501	$150.
3.9 x 30		502	245.	RP-502	195.
7.8 x 30		503	550.	RP-503	450.
ASI CARBOHYDRATE					
3.9 x 15	Carbohydrate	601	$195.	RP-601	$150.
3.9 x 30	Analysis	602	245.	RP-602	195.
7.8 x 30	Column	603	550.	RP-603	450.

asi ANALYTICAL SCIENCES INCORPORATED.
SUITE B-24, AIRPORT PARK • 1400 COLEMAN AVENUE • SANTA CLARA, CALIF. 95050 • (408) 779-0131
TELEX 176646

ASI

Circle Reader Service Card No. 101

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

April 1984

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

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

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

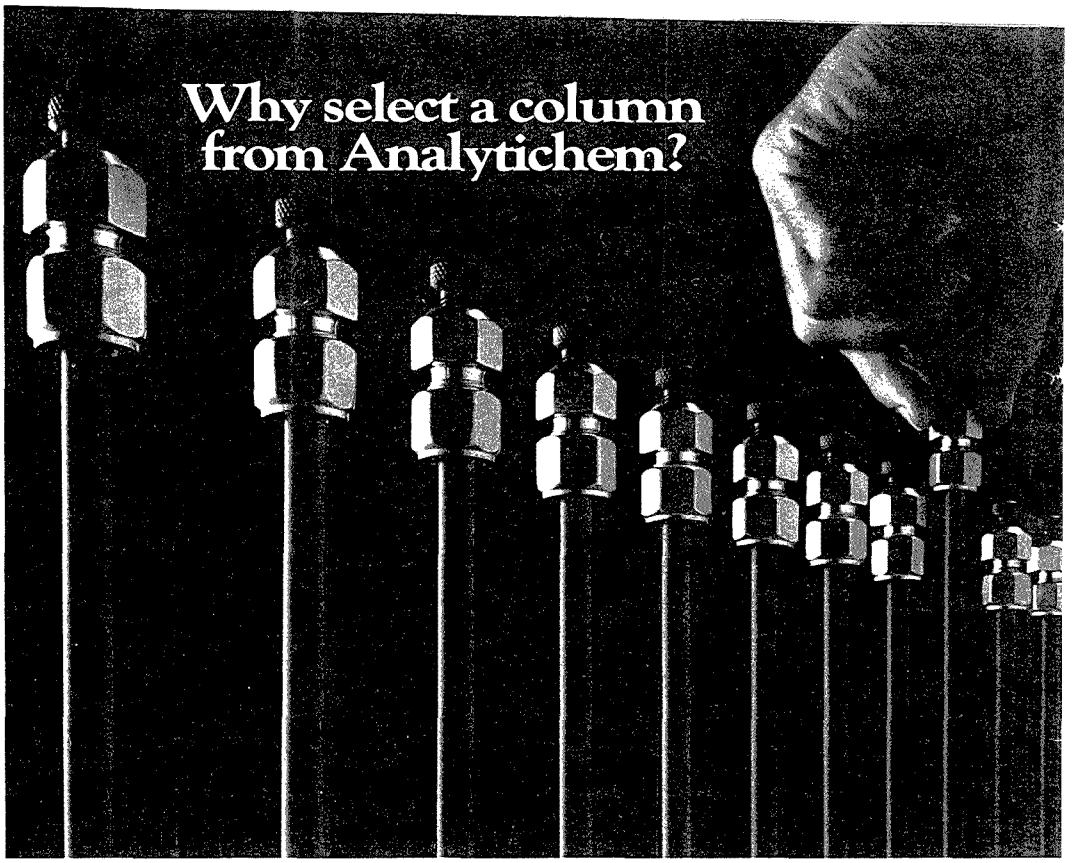
Manuscript Preparation and Submission. See the last page of this issue.

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

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

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

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



Because the greater the selection the greater the selectivity.

The new line of HPLC columns from Analytichem provides selectivity unequalled by anyone. The reason is simple. Analytichem offers a wider selection of phases than anyone.* Now you can choose the phase that is precisely suited to your particular application. These new columns, packed with our unique Sepralyte™ 5μm spherical media, set an unprecedented standard of chromatographic efficiency... regardless of the phase you select.

The performance of each new Analytichem column is fully guaranteed and backed by the industry's strongest customer service and technical support teams. Our technical advisors have the training and hands-on experience to assist you in solving virtually any separation problem. Next time you're considering HPLC columns, be selective. Call Analytichem. You'll find the columns you need and the service you deserve.

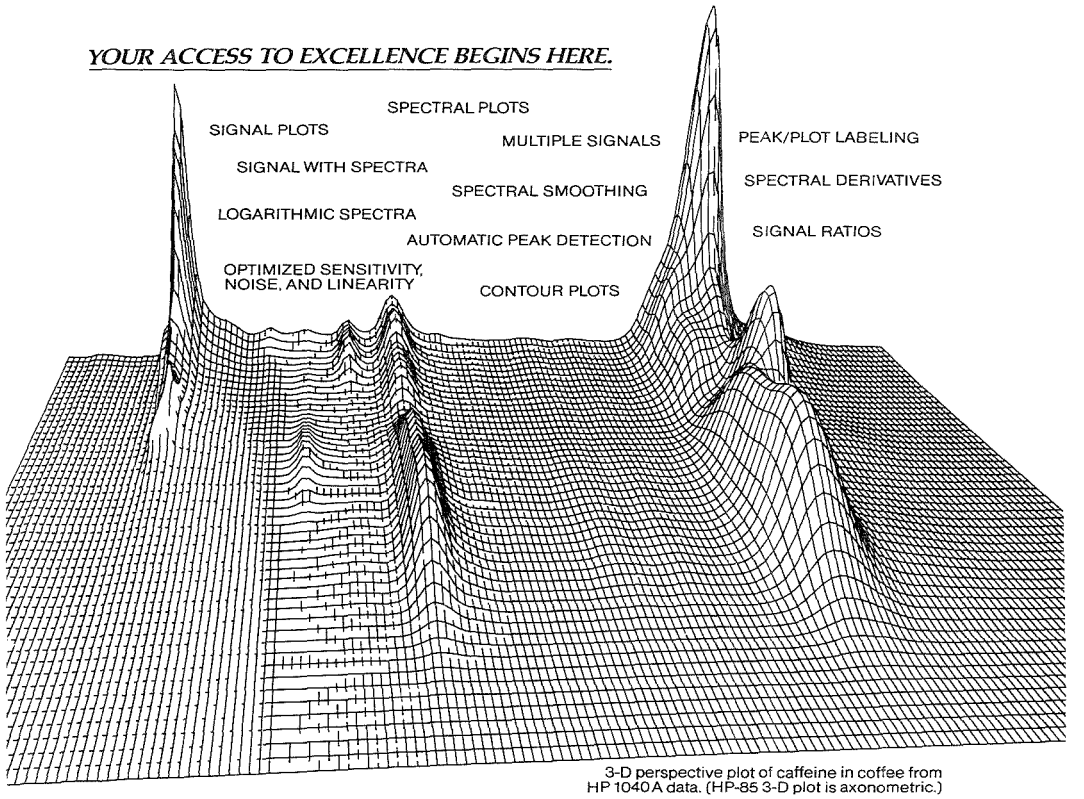
Analytichem International
24201 Frampton Ave., Harbor City,
CA 90710, USA., (800) 421-2825,
In California (213) 539-6490
TELEX 664832 ANACHEM HRBO

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 7, Number 4, 1984

CONTENTS

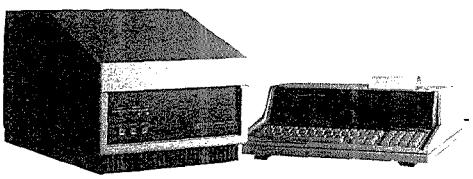
YOUR ACCESS TO EXCELLENCE BEGINS HERE.



3-D perspective plot of caffeine in coffee from HP 1040A data. (HP-85 3-D plot is axonometric.)

There's more behind this plot than meets the eye.

See the whole picture. Get our free HPLC applications brochure. Three-dimensional plotting is just one of the many useful presentations possible from the extensive data generated by the remarkable HP 1040 A HPLC Detection System. Behind the plot we've listed other analytical procedures and capabilities provided by the HP 1040 A. For the details, send the coupon for your free HPLC applications brochure today.



Yes, I want more information on The HP 1040 A HPLC Detection System.

NAME_____

ADDRESS_____

CITY_____ STATE_____ ZIP_____

COUNTRY_____

PHONE (_____)_____

Europe, North Africa and Middle East countries:
Hewlett-Packard Co.
Central Mailing Dept.
P.O. Box 529
1180 AM Amstelveen
The Netherlands

All other countries:
Hewlett-Packard Co.
Analytical Group
1820 Embarcadero Rd.
Palo Alto, CA 94303

HEWLETT PACKARD

AG04313-1

Circle Reader Service Card No. 116

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 647-660 (1984)

SOLUTE RETENTION IN COLUMN LIQUID CHROMATOGRAPHY. III. COMPUTER OPTIMIZATION OF MOBILE-PHASE COMPOSITIONS: PROGRAM WINDOW

R. J. Laub

Department of Chemistry
San Diego State University
San Diego, CA 92182

ABSTRACT

A program is described that calculates solute retentions (thence optimized conditions for their separation) from data acquired solely from chromatographic measurements. A pre-sorting loop identifies the relevant (window-diagram boundary) pairs of solutes within a user-defined value of the most-difficult separation factor, S_f. The program run time is consequently shortened by several factors over previously-used "brute-force" techniques wherein all possible pairs are considered at each value of the independent parameter(s) to be optimized. The required CPU space reserved for arrays is thereby also diminished. The program was written for an APPLE II Plus system; statements not compatible with other versions of BASIC are pointed out and discussed.

INTRODUCTION

The major drawback to chromatographic separations in general is that it is impossible at the present time to predict on an a priori basis the precise set of conditions which will effect resolution of the mixture at hand. As a result, a number of optimization strategies have been proferred over the years, these including SIMPLEX (1) and the Laub-Purnell window-diagram strategy (2,3). The former makes use of what amounts to an intelligent yet near-random search for the **single** optimum of the parameter

 0148-3919/84/0704-0647$3.50/0

of interest and is hence subject to local minima. In contrast, the latter defines pictorially the **global set** of optima; it is then left to the user to superpose additional local criteria (such as analysis time, cost, and so forth). The overall **practical** optimum is then determined simply by inspection of the global set.

A number of requests have been received for the global optimization algorithm presented in a recent technical report by Laub (4) and so, the computer program is presented here in detail.

THEORY

The independent variable most frequently employed in optimizing column liquid-chromatographic separations is the composition of the mobile phase. As a result, there have been formulated over the years a number of relations which purport to describe solute retentions as a function of mobile-phase solvent/additive ratio in terms of mole- , weight- , or volume-fraction or molar or molal concentration. The most successful of these is that by McCann, Purnell, and Wellington (5), followed by Madden, McCann, Purnell, and Wellington (6), as described in the previous two papers. They modified the relation first proposed by Scott and Kucera (7) such that all isotherm shapes common to lc could be represented. The result, for which no exception is known at this time, can be expressed in terms of raw retentions with a given column and fixed flow rate as:

$$\frac{1}{t_{R(M)}} = \phi_A \left\{ \frac{1}{t_{R(A)}} + \frac{b\phi_S}{1 + b'\phi_S} \right\} + \frac{\phi_S}{t_{R(S)}} \qquad (1)$$

where values of b and b' are derived from an analysis of the experimental data.

The fitted parameters have yet to be rationalized either from one solute to the next or from one solvent system to another. Nevertheless, the ability to represent generally (hence predict) the variation of solute retentions as a well-defined function of mobile-phase composition represents a very considerable advance since relative retentions (i.e.,

separations) can then be reduced also to a mathematical formulation. The two relevant equations are:

$$\alpha_{i/j} = \frac{t'_{R(M)\,i}}{t'_{R(M)\,j}} \tag{2}$$

$$S_f = \frac{2R_s}{N^{1/2}} = \frac{t_{R(M)\,i} - t_{R(M)\,j}}{t_{R(M)\,i} + t_{R(M)\,j}} \tag{3}$$

where $\alpha_{i/j}$ and S_f are referred to here as the alpha value and the separation factor, and where R_s and N are the resolution and number of theoretical plates. The latter expression, derived initially by Jones and Wellington (8), has some advantage in practice (see preceding paper) since t_R represents a **raw** retention time, i.e., uncorrected for column void space. Thus, the dead time t_A (or peak baseline or half-height widths) need not be determined. In addition, for R_s set to unity (4σ separation), the number of plates required N_{req} to effect a separation is calculable directly as $(2/S_f)^2$. In contrast, capacity factors or adjusted retention times (hence t_A) must be known in order to do so with values of alpha, where (9):

$$N_{eff} = 16 \left(\frac{\alpha}{\alpha - 1}\right)^2 \left(\frac{k' + 1}{k'}\right)^2 \tag{4}$$

and where N and N_{req} are related by:

$$N_{eff} = N \left(\frac{k'}{k' + 1}\right)^2 \tag{5}$$

Eqn. 3 is therefore used in what follows.

When the separation factors of the relevant pairs of solutes (see later) are plotted graphically against the independent parameter (here, mobile-phase composition), the result (window diagram) resembles a set of inverted and partially-overlapped triangles. A perpendicular dropped to the abscissa from the point of the tallest open region (window) formed by the intersection of the sides of two of these triangles (or one triangle with an ordinate) then specifies the optimum mobile-phase composition. A

horizontal line from the top of the window to the left-hand ordinate subsequently yields the most-difficult separation factor (all others are easier). The number of plates (hence the column efficiency) required to effect the separation can then be calculated. Reference back to eqn. 1 also provides the order of elution of the solutes at the chosen optimum (10).

PROGRAM DESCRIPTION

In the program that follows, it is assumed that the liquid-chromatographic separation of solutes is to be optimized in terms of mobile-phase composition in accordance with eqns. 1 and 3. Substitution of appropriate functions for other variables, such as $\log(t_{R(M)})$ against T^{-1}, could of course also be appended (11,12). For the sake of clarity, the program statements have not in many instances been concatenated where it would otherwise be possible (and even beneficial) to do so and, for the same reason, potential savings in execution time are sacrificed in favor of presentation of the logic in expanded form.

Data Input (Statements 1000–1200)

```
1000  REM  DATA INPUT— INPUT THE SOLVENT AND SOLUTE
      NAMES, AND THE RESPECTIVE RETENTIONS. THEN DISPLAY
      THESE VALUES.
1010  HOME : PR#0 : DIM N$(51), A(51), S(51), B1(51), B2(51), X(500),
      Y(500), M$(500)
1020  PRINT : PRINT : PRINT : PRINT : PRINT : PRINT
1030  PRINT "SOLVENT 'A' IS: ";
1040  INPUT A$
1050  PRINT : PRINT
1060  PRINT "SOLVENT 'S' IS: ";
1070  INPUT S$
1080  PRINT : PRINT
1090  PRINT "THE NUMBER OF SOLUTES (MAXIMUM OF 50) IS: ";
1100  INPUT N
1110  HOME
1120  PRINT : PRINT
1130  PRINT "ENTER THE RESPECTIVE SOLUTE NAMES AND
      RETENTIONS WITH SOLVENTS 'A' AND 'S' "
1140  PRINT : PRINT
1150  PRINT "SOLUTE NAME, TR(A), TR(S), B1, AND B2" : PRINT
```

```
116Ø   FOR I = Ø TO N - 1: INPUT N$(I), A(I), S(I), B1(I), B2(I): NEXT I
1165   HOME : PRINT : PRINT : PRINT : PRINT
117Ø   PRINT "THE LOWER MOBILE-PHASE COMPOSITION PERCENT
       TO BE CONSIDERED IS (WHOLE NUMBER) ";
1175   INPUT DL
118Ø   PRINT : PRINT "THE UPPER MOBILE-PHASE COMPOSITION
       PERCENT TO BE CONSIDERED IS (WHOLE NUMBER) ";
1185   INPUT DU
119Ø   PRINT : PRINT "THE MOBILE-PHASE COMPOSITION PERCENT
       INTERVAL TO BE CONSIDERED IS (WHOLE NUMBER; SMALLEST
       PERMISSIBLE IS 1%) ";
12ØØ   INPUT D
```

These statements first clear the screen (1Ø1Ø), dimension the variables, and then query the user for the names of the solvents and the number of solutes. The program then clears the screen again (111Ø) and asks for the names of the solutes, the respective retentions with solvents A and S, and the fitted values of b (B1) and b' (B2) (113Ø ff.). The data entry format is as shown, namely, SOLUTE NAME (comma), TR(A) (comma), TR(S) (comma), B1 (comma), B2, then <RETURN>. The program then asks for the mobile-phase composition range and interval (e.g., every 1%, every 5%, etc.) to be considered (117Ø-12ØØ); note that the lowest permitted interval, for reasons of memory conservation, is 1%.

Data Verification (Statements 121Ø–137Ø)

```
121Ø   PR#1
122Ø   PRINT : PRINT
123Ø   PRINT TAB(26); "*****RETENTION DATA*****"
124Ø   PRINT : PRINT
125Ø   PRINT TAB(5); "SOLVENT 'A' IS "; A$
126Ø   PRINT TAB(5); "SOLVENT 'S' IS "; S$
127Ø   PRINT : PRINT
128Ø   PRINT TAB(5); "SOLUTE"; TAB(2Ø); "TR(A)"; TAB(35); "TR(S)";
       TAB(52); "B1"; TAB(27); "B2"
129Ø   PRINT
13ØØ   FOR I = Ø TO N - 1
131Ø   PRINT TAB(5); LEFT$ (N$(I),1Ø); TAB(2Ø); A(I); TAB(35); S(I);
       TAB(5Ø); B1(I); TAB(55); B2(I): NEXT I
133Ø   PRINT : PRINT : HOME
134Ø   PRINT "MIXTURES OF 'A' WITH 'S' WILL BE CONSIDERED AT
       EVERY "; D; "% FROM 'A' = "; DL; " TO "; DU; "%."
135Ø   PRINT : PR#Ø: PRINT : PRINT : PRINT : PRINT : PRINT
136Ø   PRINT "FIRST, HOWEVER, THE RELEVANT PAIRS OF SOLUTES
       FOR CALCULATION OF THE WINDOW DIAGRAM WILL BE
       DETERMINED."
```

The solute and solvent data are printed out on the hard-copy device PR#1. The program uses a simple loop (13ØØ,131Ø) to do so after the title (123Ø) and column headings (128Ø) are printed. Note that the solute names are contained as strings in the array N$(I), and that the retentions with solvents A and S (named A$ and S$) are in the arrays A(I) and S(I), respectively.

Determination of Relevant Pairs of Solutes (Statements 15ØØ-198Ø)

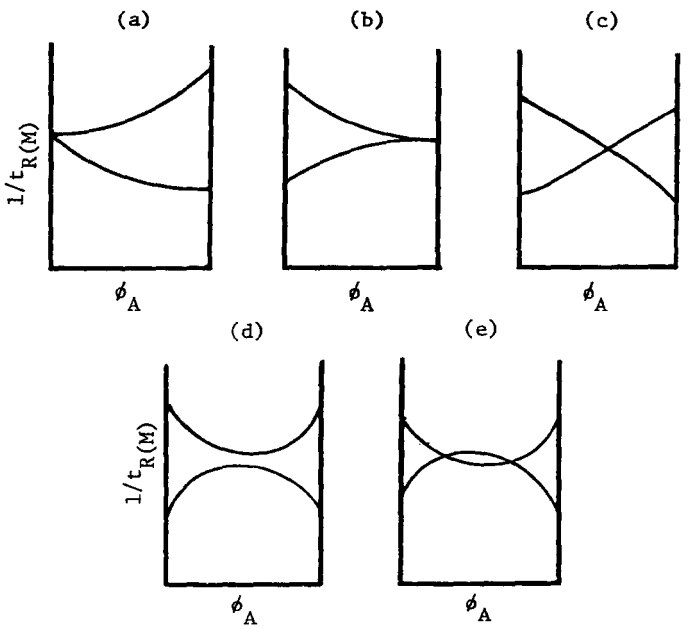
```
15ØØ   REM  THIS SECTION OF THE PROGRAM WILL DETERMINE THE
       RELEVANT PAIRS OF SOLUTES FOR CALCULATION OF THE
       WINDOW-DIAGRAM ARRAY.
151Ø   PRINT : PRINT : PRINT
152Ø   PRINT "ENTER THE UPPER LIMIT OF SEPARATION FACTOR
       (>Ø) TO BE CONSIDERED: ";
153Ø   INPUT MAX
154Ø   Z = Ø
155Ø   Z1 = Ø
156Ø   FOR J = Ø TO N - 2
158Ø   HOME : PRINT : PRINT : PRINT : PRINT : PRINT "THE NUMBER
       OF RELEVANT PAIRS": PRINT : PRINT : PRINT "FOUND SO FAR
       IS :";Z1
162Ø   FOR I = J + 1 TO N - 1
167Ø   LP = (A(I) - A(J))/(A(I) + A(J))
168Ø   IF (ABS(LP)) < MAX THEN GOTO 173Ø
169Ø   LQ = (S(I) - S(J))/(S(I) + S(J))
171Ø   IF (ABS(LQ)) > MAX THEN IF (LP/LQ) > Ø THEN GOTO 4ØØØ

       4ØØØ  FOR P = DL TO DU STEP D
       4Ø2Ø  COMP = P * Ø.Ø1
       4Ø3Ø  L1 = COMP * ((1/A(I)) + (B1(I) * (1 - COMP)/(1 + B2(I) * (1 -
             COMP)))) + (1 - COMP)/S(I)
       4Ø4Ø  L2 = COMP * ((1/A(J)) + (B1(J) * (1 - COMP)/(1 + B2(J) * (1 -
             COMP))) + (1 - COMP)/S(J)
       4Ø5Ø  SF = (L1 - L2)/(L1 + L2)
       4Ø6Ø  IF (ABS(SF)) > MAX THEN GOTO 4Ø8Ø
       4Ø7Ø  GOTO 173Ø
       4Ø8Ø  NEXT P
       4Ø9Ø  GOTO 185Ø


173Ø   Z1 = Z1 + 1
174Ø   HOME : PRINT : PRINT : PRINT : PRINT : PRINT "THE NUMBER
       OF RELEVANT PAIRS": PRINT : PRINT : PRINT "FOUND SO FAR
       IS :"; Z1: FOR PAUSE = 1 TO 1ØØ: NEXT PAUSE
175Ø   K = J
176Ø   FOR Z = Z TO (Z + 1)
177Ø   X(Z) = A(K)
```

```
1775    Y(Z) = S(K)
178Ø    M1(Z) = B1(K)
1785    M2(Z) = B2(K)
179Ø    M$(Z) = N$(K)
1795    K = I
18ØØ    NEXT Z
185Ø    NEXT I
19ØØ    NEXT J
19Ø5    IF Z1 = Ø THEN GOTO 33ØØ
191Ø    HOME : PR#1 : PRINT : PRINT
1915    PRINT TAB(26); "************************"
192Ø    PRINT : PRINT : PRINT TAB(5); "THE NUMBER OF RELEVANT
        PAIRS OF SOLUTES IS "; Z1; "."
193Ø    PRINT : PRINT
194Ø    PRINT TAB(5); "THE RELEVANT PAIRS ARE:" : PRINT
196Ø    FOR Z = Ø TO (Z1 * 2 - 1) STEP 2
197Ø    PRINT TAB(15); (LEFT$ (M$(Z),1Ø)); "/"; (LEFT$ (M$(Z + 1),1Ø))
198Ø    NEXT Z
```

Rather than calculating the separation factors for all pairs of solutes at all compositions, the program first determines the number and identity of pairs of solutes that have values of S_f less than the user-defined limit MAX at some point within the specified composition range of DL to DU% of A in (A + S). The task is straight-forward when the variation of solute retentions is known as a function of column composition. Five situations arise generally:



In situations (a) and (b), full overlap of the solutes occurs at one or the other of the ordinates. S_f is therefore Ø at each of these points. In the third case, (c), the order of elution of the solutes is reversed on passing from one extremum to the other. Hence, while S_f is greater (or less) than Ø at one ordinate, it will be less (or greater) than Ø at the other. Finally, situations (d) and (e) encompass those instances where the curves do not intersect at any or at more than one composition. These can be identified only by examination of the solute retentions at intermediate mobile-phase compositions.

In order to test for each of the above possibilities (hence identify the relevant pairs), the separation factors for each solute pair are calculated at each of the ordinates (154Ø-19ØØ) and, where necessary, at intermediate compositions (subroutine 4ØØØ-4Ø9Ø). First, however, and following a displayed message so indicating, the user is prompted to enter the upper limit of S_f which will be used to define what constitutes a relevant pair. Judicious choice of the limiting separation factor can lead to an enormous savings in the time of calculation of the window boundary, since whatever pairs are eliminated at this point will not be considered again. (An S_f of Ø.Ø2828 corresponds to a column of 5ØØØ plates and minimum resolution R_s of unity.) If **no** relevant pairs are found, the program branches at 19Ø5 to statement 33ØØ and displays a mesage so informing the user:

```
33ØØ   HOME : PRINT : PRINT : PRINT : PRINT : PRINT : PRINT "NO
       PAIRS FOUND— ALL COMPOSITIONS WILL PROVIDE GOOD
       RESOLUTION. WANT TO TRY A HIGHER VALUE OF SF (Y/N)?":
       INPUT ANS$
331Ø   IF ANS$ = "N" THEN GOTO 327Ø
332Ø   PRINT : PRINT : PRINT : GOTO 152Ø
```

The final task of this section of the program (191Ø-198Ø) gives a hard-copy print-out of the number and identity of the relevant pairs of solutes.

Calculation of the Window Boundary Array (Statements 3ØØØ-32ØØ)

```
3ØØØ   REM  THIS SECTION OF THE PROGRAM CALCULATES THE
       WINDOW DIAGRAM ARRAY, HERE, SF AS A FUNCTION OF
```

```
      MOBILE-PHASE COMPOSITION FOR LIQUID CHROMATOG-
      RAPHY.
3Ø1Ø  HOME : PR#Ø
3Ø2Ø  DIM Q$(1Ø1), R$(1Ø1), SFP(1Ø1)
3Ø4Ø  BSFP = Ø
3Ø45  FOR P = DL TO DU STEP D
3Ø5Ø  HOME : PRINT : PRINT : PRINT : PRINT : PRINT : PRINT
      : PRINT
3Ø6Ø  PRINT "THE COLUMN COMPOSITION CURRENTLY BEING":
      PRINT : PRINT "CONSIDERED IS "; P; "%"
3Ø7Ø  SFP(P) = MAX
3Ø75  COMP = P * Ø.Ø1
3Ø8Ø  Q$(P) = "(NONE)"
3Ø85  R$(P) = "(NONE)"
31ØØ  FOR Z = Ø TO (Z1 * 2 - 1) STEP 2
311Ø  L1 = COMP * ((1/X(Z)) + (M1(Z) * (1 - COMP)/(1 + M2(Z) * (1 -
      COMP)))) + (1 - COMP)/Y(Z)
3115  L2 = COMP * ((1/X(Z + 1)) + (M1(Z + 1) * (1 - COMP)/(1 + M2(Z + 1)
      * (1 - COMP)))) + (1 - COMP)/Y(Z + 1)
312Ø  SF = (L1 - L2)/(L1 + L2)
3125  IF (ABS(SF)) > SFP(P) THEN GOTO 317Ø
313Ø  SFP(P) = ABS(SF)
314Ø  Q$(P) = M$(Z): R$(P) = M$(Z + 1)
317Ø  NEXT Z
3175  IF SFP(P) < BSFP THEN GOTO 32ØØ
318Ø  BSFP = SFP(P)
3185  BA$ = Q$(P)
319Ø  BS$ = R$(P)
3195  OPT = P
32ØØ  NEXT P
```

Once the relevant pairs of solutes have been identified, separation factors for each are calculated in turn at each column composition and the lowest (most-difficult) is saved in the array subscripted as P. Thus, SFP(P) (313Ø) is the most-difficult (window-boundary) value of S_f at the column composition corresponding to P, while solutes Q$(P) and R$(P) (314Ø) are the names of the solutes. The **overall best** value of SFP(P), BSFP (318Ø), is updated on each pass through the outer loop, as are the names of the corresponding most-difficult solutes, BA$ (3185) and BS$ (319Ø). The **overall best** (optimum) column composition is also stored (3195) as OPT.

This section of the program is by far the slowest, the rate-limiting statements being 311Ø and 3115. To indicate that the computer is still working (and to time the program if desired), the composition currently being considered is displayed.

SFP(P), Q$(P), and R$(P) default (3Ø7Ø,3Ø8Ø,3Ø85) to the value of MAX and the string "(NONE)" if, at a given column composition, the separation factors of all relevant pairs of solutes exceed that of MAX (see later).

Data Output (Statements 32Ø5–329Ø)

```
32Ø5   PR#1
321Ø   HOME : PRINT : PRINT : PRINT TAB(5); "THE WINDOW-
       BOUNDARY DATA ARE:"
3215   PRINT : PRINT
322Ø   PRINT TAB(11); "SOLUTE"; TAB(36); "COL."; TAB(57); "SEPN."
3225   PRINT TAB(12); " PAIR"; TAB(36); "COMP."; TAB(16); "FACTOR"
323Ø   PRINT : PRINT
3235   FOR P = DL TO DU STEP D
324Ø   PRINT TAB(5); LEFT$ (Q$(P),1Ø); "/"; LEFT$ (R$(P),1Ø); TAB(37);
       P; TAB(54); (INT(1Ø ∧ 5 * (SFP(P)) + 0.02))/1Ø ∧ 5
3245   NEXT P
325Ø   HOME : PRINT : PRINT
3255   PRINT "THE BEST COLUMN COMPOSITION IS: "; OPT; "%."
326Ø   PRINT : PRINT "THE MOST-DIFFICULT SEPARATION FACTOR
       AT THIS COMPOSITION IS: "; BSFP; "."
3265   PRINT : PRINT "THE MOST DIFFICULT SOLUTES TO SEPARATE
       AT THIS COMPOSITION ARE: "; BA$; " FROM "; BS$; "."
327Ø   PR#Ø
3275   PRINT : PRINT : PRINT : PRINT : PRINT : PRINT : PRINT :
       PRINT : PRINT TAB(1Ø); "*****THAT'S ALL, FOLKS*****"
329Ø   END
```

A hard-copy print-out of the window-boundary array is accomplished by the loop, 3235-3245. For easier reading, the separation-factor data are truncated (324Ø) to five places. If at a given column composition the separation factors of all relevant pairs exceed the value of MAX, the solute-pair print-out is (NONE)/(NONE) and the separation factor printed is MAX. (A plot of the data in this composition region thus would show a flat top.) Also printed out (3255-3265) are the overall best column composition, the most-difficult S_f at this composition, and the associated (most-difficult) solute pair.

Generalization of the Algorithm

The program as written considers that inverse retentions vary in a non-linear fashion with mobile-phase composition. If the regression is in fact linear, eqn. 1 reduces to the trivial form:

$$\frac{1}{t_{R(M)}} = \phi_A \left(\frac{1}{t_{R(A)}}\right) + \phi_S \left(\frac{1}{t_{R(S)}}\right) \qquad (6)$$

That is, both b and b' are negligible. The program and data entry procedure need not be modified in this instance other than to enter $\emptyset$ when asked for values of B1 and B2.

The terms $(1/t_{R(i)})$ (i = A, S, or M) could of course also be used to represent ordinate data from some other function which may or may not be linear. For example, the (linear) diachoric solutions relation pertinent to retentions in gas chromatography is (13,14):

$$K^o_{R(M)} = \phi_A K^o_{R(A)} + \phi_S K^o_{R(S)} \qquad (7)$$

where $K^o_{R(i)}$ are solute liquid-gas partition coefficients with the stationary phases A, S, and M (= A + S). To utilize the program, in this instance for optimization of the stationary-phase composition, $\emptyset$ would be entered for B1 and B2, and $1/K^o_{R(i)}$ entered for "$t_{R(i)}$". Eqns. 6 and 7 would thereby be made equivalent. However, the value of "S_f" thence calculated would no longer be equal to $2R_s/N^{1/2}$ unless it were true that the sum of the raw retentions were much larger than twice the average of the column dead times:

$$\frac{K^o_{R(M)2} - K^o_{R(M)1}}{K^o_{R(M)2} + K^o_{R(M)1}} = \frac{t_{R(M)2} - t_{R(M)1}}{t_{R(M)2} + t_{R(M)1} - 2t_A} \qquad (8)$$

Fortunately, this can be expected to be the case more often than not in open-tubular column gc, and will certainly be true for conventional packed-column gas chromatography.

In contrast, suppose that for some reason or another a particular liquid-chromatographic system were represented by the relation (cf. eqn. 1 of preceding paper):

$$\log k'_{(M)} = \log k'_{(S)} - S \phi_A \tag{9}$$

where S is an empirical constant and where it is assumed that t_A can be determined unambiguously. In order to identify the appropriate quantities for "$t_{R(A)}$" and "$t_{R(S)}$", eqn. 6 is rearranged to the form:

$$\frac{1}{t_{R(M)}} = \frac{1}{t_{R(S)}} - \phi_A \left(\frac{1}{t_{R(S)}} - \frac{1}{t_{R(A)}} \right) \tag{10}$$

Comparison of coefficients hence yields the identities:

$$"t_{R(S)}" = \frac{1}{\log k'_{(S)}} \tag{11a}$$

$$"t_{R(A)}" = \frac{1}{\log k'_{(S)} - S} \tag{11b}$$

Entry of these values for "$t_{R(A)}$" and "$t_{R(S)}$" would then yield a separation factor defined by:

$$10^{S_f} + k'_{(M)1} k'_{(M)2} = \frac{k'_{(M)1}}{k'_{(M)2}} = \alpha_{1/2} \tag{12}$$

for which a program statement could easily be added to retrieve the correct window-diagram boundary, here, alpha as a function of mobile-phase composition.

In the cases considered above, it was assumed that the example relations were linear. If this were not so, the program could still be made to function with appropriate (fitted) values of B1 and B2 defined analogous to those of eqn. 1. It appears, therefore, that the algorithm is likely to be useful in virtually any situation in chromatography wherein retentions can be described as a function of the parameter(s) to be optimized.

Commands Indigenous to APPLE BASIC

The only three commands used here which may not be compatible with other versions of BASIC are PR#1, PR#Ø, and HOME. The first two of these specify the hard-copy printer and the display unit, respectively, while the third command causes the display to clear and the cursor to be positioned in the upper left-hand corner of the screen. These commands appear in the following statements:

Command	Statement Nos.
PR#1	121Ø, 191Ø, 32Ø5
PR#Ø	1Ø1Ø, 135Ø, 3Ø1Ø, 327Ø
HOME	1Ø1Ø, 111Ø, 1165, 191Ø, 3Ø1Ø, 3Ø5Ø, 321Ø, 325Ø, 33ØØ

There may also be difficulty with multiple TAB statements depending upon the printer employed (here, an Epson MX-7Ø). We have found that substitution of POKE (36,nn) for TAB (nn) solves this problem.

ACKNOWLEDGMENT

We gratefully acknowledge support of this work provided by the Department of Energy.

REFERENCES

1. For example, see Biles, W. E., and Swain, J. J., **Optimization and Industrial Experimentation,** Wiley-Interscience, New York, 198Ø.

2. Laub, R. J., and Purnell, J. H., **J. Chromatogr., 112,** 71, 1975.

3. See the review: Laub, R. J., in **Physical Methods in Modern Chemical Analysis,** Vol. **3,** T. Kuwana, Ed., Academic Press, New York, 1983, Ch. 4.

4. Laub, R. J., **Program WINDOW,** Technical Report DOE-ER-1Ø554-19 Rev. 1 to the Department of Energy; **Chemical Abstracts, 98,** 78732h,

1983. See also: Laub, R. J., Purnell, J. H., and Williams, P. S., **Anal. Chim. Acta, 95**, 135, 1977.

5. McCann, M., Purnell, J. H., and Wellington, C. A., in **Chromatography, Equilibria, and Kinetics**, Faraday Society Symposium No. 15, D. A. Young, Ed., The Royal Society of Chemistry, London, 198Ø, p. 82.

6. Madden, S. J., McCann, M., Purnell, J. H., and Wellington, C. A., paper presented at the 184th National Meeting of the American Chemical Society, Kansas City, Missouri, 1982; Madden, S. J., Ph.D. Thesis, University College of Swansea, Swansea, Wales, 1983.

7. Scott, R. P. W., and Kucera, P., **Anal. Chem., 45**, 749, 1973; **J. Chromatogr. Sci., 12**, 473, 1974; **13**, 337, 1975; **J. Chromatogr., 112**, 425, 1975; **122**, 35, 1976; **149**, 93, 1978; **171**, 37, 1979.

8. Jones, P., and Wellington, C. A., **J. Chromatogr., 213**, 357, 1981.

9. Purnell, J. H., **J. Chem. Soc.**, 1268, 196Ø.

1Ø. The window diagram strategy is of course completely general, and will therefore be useful in any situation wherein optimization of an independent (abscissa) system parameter is sought in terms of a dependent (ordinate) chromatographic, electrochemical, spectroscopic, or even purely mathematical variable: Laub, R. J., **Am. Lab., 13**(3), 47, 1981.

11. Maggs, R. J., **J. Chromatogr. Sci., 7**, 145, 1969.

12. Laub, R. J., and Purnell, J. H., **J. Chromatogr., 161**, 49, 1978.

13. Purnell, J. H., and Vargas de Andrade, J. M., **J. Am. Chem. Soc., 97**, 3585, 359Ø, 1975.

14. Laub, R. J., and Purnell, J. H., **J. Am. Chem. Soc., 98**, 3Ø, 35, 1976.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 661-674 (1984)

SEPARATION OF OLIGOSACCHARIDE ISOMERS CONTAINING ACETAMIDO AND NEUTRAL SUGARS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Elizabeth F. Hounsell[1], James M. Rideout[2]
Nicola J. Pickering[1], C.K. Lim[2].

[1]Applied Immunochemistry Research Group and
[2]Division of Clinical Cell Biology,
MRC Clinical Research Centre,
Harrow, Middlesex, HA1 3UJ.

ABSTRACT

High performance liquid chromatography (HPLC) has been investigated for the separation of the following reduced oligosaccharides containing neutral and acetamido sugars;
Galβ1-3GlcNAc-ol and Galβ1-4GlcNAc-ol,
Galβ1-3GlcNAcβ1-6Gal-ol,
Galβ1-4GlcNAcβ1-6Gal-ol,
Galβ1-4GlcNAcβ1-3Gal-ol,
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ol (LNT-ol),
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-ol (LNNT-ol),
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-ol,
Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-ol (LNFII-ol),
Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc-ol (LNFIII-ol).
These alditols were studied as standards for the separation of mixtures of reduced oligosaccharides obtained from glycoproteins.

A combination of several HPLC systems using normal and reverse phase column packings was required for separation of the isomers as follows. A novel chromatography system using acetylated alditols eluted from silica (Hypersil) with dichloromethane/hexane/isopropanol as mobile phase separated the disaccharides and the first trisaccharide from the next two. These last two trisac-

 0148-3919/84/0704-0661$3.50/0

charides could in turn be separated as non-acetylated alditols chromatographed on Hypersil eluted with aqueous acetonitrile containing 0.05% tetraethylenepentamine (TEPA) or on silica chemically bonded with aminopropyl groups (APS-Hypersil). The pentasaccharides were resolved as acetylated alditols chromatographed on reverse phase, octodecyl silica (ODS-Hypersil).

Isomeric separation of the tetrasaccharides was not achieved. However LNT-ol could be obtained essentially free of LNNT-ol by isolation of its di-N-acetylated product. The third tetrasaccharide studied was readily separated from the other tetrasaccharides and the pentasaccharide isomers on normal or reverse phase chromatography because of its greater acetamido/neutral sugar ratio. In general the varying ratios of acetamido/neutral sugars and their different glycosidic linkages conferred distinct but predictable chromatographic properties to the alditols on silica and reverse phase chromatography.

INTRODUCTION

An important requirement in the analysis of the detailed structure and function of the carbohydrate chains of glycoconjugates is the purification of oligosaccharide isomers. Multiple oligosaccharide isomers are present in secretions such as milk and are released from certain glycoproteins, for example those of mucin-type. Such oligosaccharide structures, although having very similar chemical properties, are, for example, recognised as distinct antigens by anti-carbohydrate antibodies (1).

High-performance liquid chromatography (HPLC) offers several advantages over paper, thin-layer (TLC) and column chromatographies in the speed and ease of preparative separation. Furthermore, many different types of HPLC column packings and a wide range of mobile phases are available. This flexibility can be exploited for isomer separation.

Although there have been several reports of the analytical separation of oligosaccharides by HPLC (2-9) only a limited number of isomers has been purified and in general the separation of reduced oligosaccharides has not been systematically investigated. We have compared several HPLC systems having different stationary and mobile phases and radioactivity or UV detection for the chromatography of oligosaccharide alditols and their acetylated derivatives. Di- to penta-saccharides which contain both acetamido and neutral sugars and vary only in the positions of the

glycosidic linkages were separated and a protocol was formulated for the purification of oligosaccharides of various sizes and ratios of acetamido and neutral sugars which would be obtained from glycoproteins.

EXPERIMENTAL

Materials and Reagents

The oligosaccharides Galβ1-3GlcNAc, Galβ1-4GlcNAc, Galβ1-3GlcNAcβ1-6Gal, Galβ1-4GlcNAcβ1-3Gal, Galβ1-4GlcNAcβ1-6Gal, and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc obtained by chemical synthesis (10,11) were provided by Professor S. David and Drs. C. Augé and A. Veyières, Orsay, France. The oligosaccharides Galβ1-3GlcNAcβ1-3Galβ1-4Glc, Galβ1-4GlcNAcβ1-3Galβ1-4Glc, Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc and Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc obtained from human milk were provided by Dr. W.M. Watkins of this Institute. Chitotriitol was prepared by the partial acid hydrolysis of chitin. Tritiated sodium borohydride (10 Ci/mmole) was obtained from Amersham International (Amersham, U.K.). Sodium borohydride, ammonium acetate, tetraethylenepentamine (TEPA), isopropanol, hexane and dichloromethane were AnalaR grade from BDH Chem. Ltd. (Poole, U.K.). Acetonitrile was 'S' grade from Rathburn Chem. Ltd. (Walkerburn, U.K.).

Derivatisation of Oligosaccharides

Oligosaccharides were reduced in 0.1 M borate buffer pH 9 containing 0.1 M sodium borohydride. After standing for 16 hr at 4°C the samples were adjusted to pH 4.5 with glacial acetic acid and evaporated with methanol (4 x 5 ml). Radioactively labelled oligosaccharides were obtained by prior treatment for 2 hr at 4°C with tritiated sodium borohydride (1mCi/100 nmole oligosaccharide) Samples for acetylation were dried overnight over phosphorus pentoxide and acetylated for 3 h at 100°C in 1:1 acetic anhydride/pyridine followed by evaporation and chloroform/water extraction. Non-acetylated samples were applied in water to a Bond Elut SCX cation exchange column (Analytichem Int.Inc., Harbor City, CA, USA) primed with methanol. The eluate and 3 x 0.5 ml water washings were collected and lyophilised before being dissolved in water for HPLC.

HPLC Apparatus

A Varian Associates (Walnut Creek, CA., U.S.A.) model 5000 liquid chromatograph was used with either a Varian UV-50 variable wavelength detector operating at 190-210 nm or a Berthold LB 503 HPLC radioactivity monitor employing a solid scintillant cell (130 µl) which would readily detect 10^5 cpm.

HPLC Columns and Mobile Phases

Hypersil (5µm spherical silica), APS-Hypersil (silica chemically bonded with aminopropyl groups) and ODS-Hypersil (octadecylsilica) were obtained from Shandon Southern Products (Runcorn, U.K.). The columns, 250x5mm, were eluted at a flow rate of 1 ml/min. The mobile phases for the APS-Hypersil and ODS-Hypersil were varying proportions of acetonitrile in either water adjusted to pH2.9 with hydrochloric acid, 15mM phosphate buffer pH 5.2 (ref 7) or 0.5 M ammonium acetate (pH 7.0). The mobile phases for the Hypersil column were either acetonitrile-water each containing 0.05% tetraethylenepentamine (TEPA; for oligosaccharide alditols) or isopropanol in 70:30 dichloromethane/hexane (for acetylated oligosaccharide alditols).

RESULTS AND DISCUSSION

HPLC of Oligosaccharide Alditols using Silica Eluted with Organic Solvents and Silica Modified with TEPA or Chemically Bonded with Aminopropyl Groups.

Previous studies using TLC have shown that native oligosaccharide alditol isomers not separable on silica could be resolved after acetylation (12-15). Figure 1 shows the equivalent separation by HPLC of acetylated di- and tri-saccharide alditols on a Hypersil column eluted isocratically with 5% isopropanol in 70:30 dichloromethane/hexane. The separation of Galβ1-3GlcNAc-ol from Galβ1-4GlcNAc-ol and of Galβ1-3GlcNAcβ1-6Gal-ol from Galβ1-4GlcNAcβ1-6Gal-ol and Galβ1-4GlcNAcβ1-3Gal-ol was achieved. The acetylated oligosaccharides containing the Galβ1-3GlcNAc linkage had a shorter retention time than those having a Galβ1-4GlcNAc linkage. These last two trisaccharides having a Galβ1-4GlcNAc linkage could not be separated as acetylated deriva-

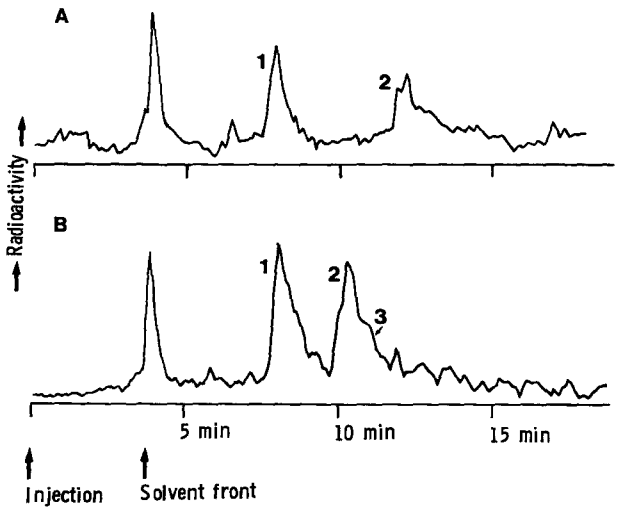


FIGURE 1 HPLC of acetylated di- and tri-saccharide alditol isomers. Column, Hypersil (250x5mm); eluent, 5% isopropanol in dichloromethane-hexane (70:30 v/v); flow rate, 1 ml/min; detector, radioactivity monitor.
A1 Galβ1-3GlcNAc-ol; A2 Galβ1-4GlcNAc-ol;
B1 Galβ1-3GlcNAcβ1-6Gal-ol;
B2 Galβ1-4GlcNAcβ1-6Gal-ol;
B3 Galβ1-4GlcNAcβ1-3Gal-ol.

tives on Hypersil using any of the conditions tested (20 min gradient of 4-14% isopropanol or isocratic elution with 2,3,4 and 5% isopropanol in 70:30 dichloromethane/hexane). However, as shown in Figure 2, they could be separated as non-acetylated derivatives on the Hypersil column eluted with aqueous acetonitrile containing TEPA. Thus a combination of HPLC systems using acetylated and non-acetylated derivatives was capable of purifying all three trisaccharide alditols.

The capacity factors (k') given in Table 1 show the differing retention behaviours of the acetylated and non-acetylated derivatives on silica chromatography. The non-acetylated alditols behaved similarly on the APS-Hypersil column and on the silica column modified with TEPA. In each case the trisaccharides having

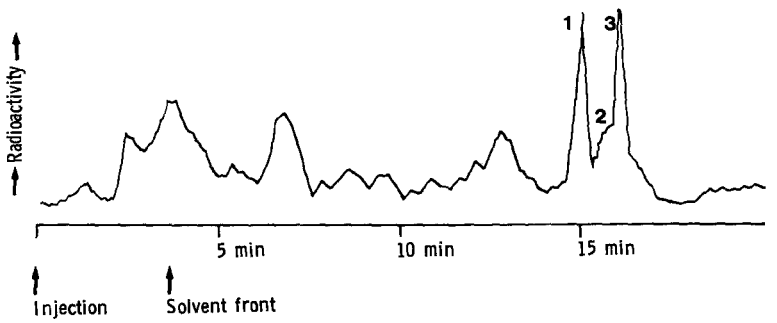


FIGURE 2 HPLC of tri-saccharide alditol isomers. Column, Hypersil (250x5 mm); eluent, 20 min linear gradient elution (75-55%) acetonitrile in water, both solvents containing 0.05% TEPA; flow rate, 1ml/min; detector, radioactivity monitor.
1, Galβ1-4GlcNAcβ1-3Gal-ol;
2, Galβ1-3GlcNAcβ1-6Gal-ol;
3, Galβ1-3GlcNAcβ1-6Gal-ol.

a GlcNAcβ1-6Gal-ol linkage had a longer retention time than that having a GlcNAcβ1-3Gal-ol linkage. A similar finding has been documented for non-reduced oligosaccharides chromatographed on silica columns bonded with amine groups (7,16). Besides the differing behaviour conferred by their linkages, oligosaccharides with a greater acetamido/neutral sugar ratio eluted slower as their acetylated derivatives and faster when underivatised. For example the fucosylated pentasaccharides were not retained on chromatography of their acetylated derivatives due to their low acetamido/neutral sugar ratio. Thus the order of elution of the oligosaccharides is not necessarily related to their size and an initial molecular sizing step is recommended prior to HPLC of mixtures of oligosaccharides.

Chromatography of acetylated LNT-ol gave a second peak with a k' of 0.77 using the Hypersil column and elution with 4-14% isopropanol in 70:30 dichloromethane/hexane (results not shown). This was presumed to be di-N-acetylated LNT-ol previously reported (3) to be formed on O-acetylation. It was partially converted on rechromatography to the peak eluting at k' 2.19 (Table 1) which

TABLE 1

Capacity Factors (k') of Oligosaccharide Alditols Chromatographed on Silica (Hypersil) and Silica Bonded With Aminopropyl (APS-Hypersil) or Octadecylsilyl (ODS-Hypersil) Groups.

Oligosaccharide Alditols	k'						
	Hypersil		APS-Hypersil	ODS-Hypersil			
	Acetylated	Non-acetylated		Acetylated	Non-acetylated		
	4-14% (20 min) $CH_3CH(OH)CH_3$ in CH_2Cl_2/ hexane 70:30	75-55% (20 min) CH_3CN in H_2O/TEPA pH 9.9	75-55% (20 min) CH_3CN in H_2O/HCl pH 2.9	30-60% (20 min) CH_3CN in NH_4OAc pH 7	100% H_2O pH 2.9 (HCl)	100% 15 mM phosphate pH 5.2	100% 0.5M NH_4OAc pH 7
GlcNAc-ol	2.42	1.78	1.24	1.75	0.94	0.25	0.00
Galβ1-3GlcNAc-ol	1.73	2.16	2.09	3.31	1.31	NT	NT
Galβ1-4GlcNAc-ol	2.16	2.03	2.01	3.34	1.35	NT	0.00
Galβ1-3GlcNAcβ1-6Gal-ol	2.17	3.57	2.84	5.08	0.97	0.27	NT
Galβ1-4GlcNAcβ1-6Gal-ol	2.30	3.68	2.98	5.12	1.10	0.37	NT
Galβ1-4GlcNAcβ1-3Gal-ol	2.35	3.41	2.53	4.97	1.00	0.35	NT
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ol	2.19	4.31	4.34	5.77	0.97	0.40	0.00
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-ol	2.22	4.30	4.32	5.92	0.93	0.36	NT
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-ol	NT	NT	2.42	4.30	1.27	NT	NT
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ol |1,4 Fucα	0.00	5.00*	5.07*	6.73	1.09	NT	NT
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-ol |1,3 Fucα	0.00	5.00*	5.07*	7.20	1.00	NT	NT

$k' = V_1 - V_o \div V_o$ where V_1 = elution volume of solute and V_o = elution volume of non-retained peak;
NT = not tested; ⊓ = separation of isomers. *Chromatographed as mixtures only and the isomers were not resolved.

was characterised as fully O-acetylated mono-N-acetylated LNT-ol by direct probe electron impact mass spectrometry (E.F. Hounsell and A.M. Lawson, unpublished results). LNNT-ol and the mono-, di- and tri-saccharides used in the present study did not appear to undergo di-N-acetylation as deduced by their chromatography as a single peak and mass spectrometry. Therefore, although none of the HPLC systems tested achieved an isomeric separation of the tetrasaccharides, LNT-ol could be separated from its isomer LNNT-ol by harvesting the di-N-acetylated product of LNT-ol.

Separation of the tetra- and penta-saccharide isomers and the two trisaccharides having the 1-6Gal-ol linkage could not be achieved on chromatography of their non-acetylated derivatives on either the Hypersil or APS-Hypersil column although several conditions were explored; 20 min gradients of 75-55%, 70-30%, 60-40% acetonitrile and isocratic elution with 65%, 70% and 75% acetonitrile in water (with both solvents containing 0.05% TEPA for chromatography on the Hypersil column). A comparison of the two types of column showed that the in situ loading of silica with TEPA resulted in a much more stable and reliable system compared to aminopropyl silica which has a shorter column life and may vary from batch to batch.

HPLC of Oligosaccharide Alditols on ODS-Hypersil

As shown in Table 1 the retention behaviours of the acetylated derivatives on an ODS-Hypersil column eluted with a gradient of acetonitrile in ammonium acetate resembled those of non-acetylated alditols on Hypersil and APS-Hypersil, but differed from acetylated derivatives chromatographed on Hypersil. However, complete separation of the trisaccharide isomers Galβ1-4GlcNAcβ1-6Gal-ol and Galβ1-4GlcNAcβ1-3Gal-ol could not be achieved on the ODS-Hypersil column although several conditions were explored (20 min gradients of 30-60% and 40-60% acetonitrile and isocratic elution with 50%, 45% and 40% acetonitrile in 0.5 M ammonium acetate).

Fig. 3A shows an example of the separation of acetylated oligosaccharide alditols that can be achieved by ODS-Hypersil.

The two isomers of lacto-N-fucopentaose (LNF), Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc-ol (LNFII) and Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-ol (LNFIII), in a preparation of LNFII obtained from human milk were completely resolved. Rechromatography of the peak eluting at 27 min gave the original peak and a peak which co-chromatographed with per-O-acetylated LNFII. After de-O-acetylation the two peaks were indistinguishable. These characteristics indicated that the peak was the di-N-acetylated product of LNFII.

Fig. 3B shows the chromatography of per-O-acetylated tetrasaccharide Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ol (LNT) and its di-N-acetylated product discussed above. Also shown in Fig. 3 is the chromatography of the acetylated tetrasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-ol and an unidentified peak also presumed to be its di-N-acetylated product.

Non-acetylated alditols were only retained on the ODS-Hypersil column when 100% aqueous eluent at pH 2.9 or pH 5.2 was used (Table I) and no separation of isomers was achieved. When ammonium acetate at pH 7 was used as the eluent none of the oligosaccharides tested were retained. The increased elution time at pH 3 of the disaccharides and the tetrasaccharide having a 1:1 acetamido/neutral sugar ratio (Table 1) and the further increased elution time of chitotriitol (GlcNAcβ1-4GlcNAcβ1-4GlcNAc-ol, k' 1.88; results not shown) suggest that reverse phase chromatography of non-acetylated alditols may be useful for the separation of larger oligosaccharide isomers containing a high acetamido/neutral sugar ratio, for example those obtained by base/borohydride degradation of mucin- type glycoproteins having a reduced-end N-acetylgalactosaminitol.

Recovery of Oligosaccharide Alditols by Preparative HPLC

Reduced oligosaccharides are used for preparative separation by HPLC for two main reasons. Firstly, HPLC usually results in the separation of the α and β anomers at the reducing end of oligosaccharides and therefore two peaks have to be collected and characterised for each oligosaccharide. This property has been

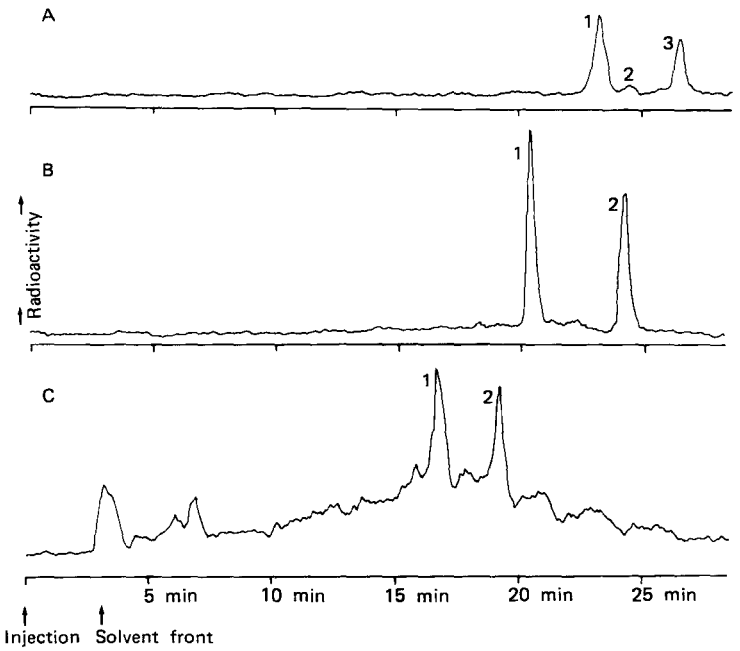


FIGURE 3 HPLC of acetylated tetra- and penta-saccharide alditols. Column, ODS-Hypersil (250x5mm); eluent, 20 min linear gradient elution (30%-60%) acetonitrile in 0.5 M ammonium acetate followed by isocratic elution at 60% acetonitrile for 10 min.
flow rate, 1ml/min; detector, radioactivity monitor.
A1, Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-ol (LNF II); A2, Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc-ol (LNF III); A3, Di-N-acetylated LNF II; B1, Galβ1-3GlcNAcβ1-3Galβ1-4Glc-ol (LNT); B2, Di-N-acetylated LNT; C1, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-ol; C2, presumed di-N-acetylated product of C1.

exploited to purify LNT from LNNT as one of the reducing-end anomers of LNT is separable from its second anomer and the two anomers of LNNT (9,17). With mixtures of unknown isomers a complex chromatographic profile would result and this would be a disadvantage. Secondly, unreduced oligosaccharides form Schiff bases with TEPA and the amino groups bonded to silica, thus decreasing their yields.

In addition, reduction renders oligosaccharides stable to base. This is a high yielding chemical reaction which is often carried out simultaneously with the release of oligosaccharides from glycoproteins.

HPLC of 1-10 mgs of reduced oligosaccharides on the 250x5mm columns used in the present studies resulted in yields of >80%. Recovery of acetylated derivatives was 70-80% and their overall yields were decreased further by the acetylation and de-acetylation reactions. Acetylation at 100°C results in complete O-acetylation, but also yields di-N-acetylated products. Acetylation at ambient temperature is reported not to give rise to di-N-acetylated products (3,8) however this may lead to only partial O-acetylation.

Removal of TEPA and the ions introduced from the de-O-acetylation reaction and by the use of buffers can be achieved in high yield by cation exchange chromatography and solvent evaporation.

Comparison of Methods of Detection in the HPLC of Oligosaccharides

Oligosaccharides containing N-acetyl, O-acetyl or CO_2H groups can be detected at 195-210 nm UV absorbance when acetonitrile 'S' grade and water are used as the eluents. In analytical studies 1 nmole of oligosaccharides was detected. UV monitoring is convenient although non-oligosaccharide peaks may also be detected.

A tritium group introduced on reduction enables more specific detection of the oligosaccharide alditols to be achieved although background peaks may appear on storing at 4°C or on freezing and thawing the labelled oligosaccharides (Figs. 1,2 and 3C). Routinely 10^5 cpm/nmole oligosaccharide were detected by a radioactivity monitor. The specific activity could be increased for more sensitive analytical studies or decreased for preparative separations. Monitoring of radioactivity, unlike detection using UV absorbance, enables the use of the column modifiers e.g. TEPA, buffer gradients and organic solvents other than acetonitrile 'S'.

CONCLUSIONS

HPLC of several oligosaccharides containing acetamido and neutral sugars has shown that more than one column system will usually be required in the purification of multiple oligosaccharide isomers. The different chromatographic properties afforded by hydroxyl, N-acetyl and O-acetyl groups on normal and reverse phase HPLC of native and acetylated derivatives will separate many isomers containing acetamido and neutral sugars. As this separation is never strictly by size an initial gel filtration step is also required. The following scheme is suggested for the preparative separation of oligosaccharide mixtures: (a) Biogel P4 column chromatography (18); (b) chromatography of reduced oligosaccharides on a reverse phase column eluted with buffer/ acetonitrile; (c) acetylation of the separated compounds, extraction into chloroform and removal of salt by washing with water, (d) chromatography of the acetylated derivatives on a reverse phase column using the conditions described in Table 1 followed by removal of ammonium acetate by chloroform/water extraction (e) chromatography of the acetylated derivatives on a silica column eluted with isopropanol/hexane/ dichloromethane; (f) de-O-acetylation followed by chromatography on a silica column eluted with aqueous acetonitrile/TEPA and (g) removal of TEPA and salt by cation exchange chromatography.

The reproducibility of the chromatographic systems described above enables structural information as to size and acetamido/ neutral sugar ratio to be gained from the retention behaviours of the oligosaccharides during purification.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. T. Feizi for her comments on this study and Mrs. M. Moriarty for preparation of the manuscript.

REFERENCES

1. Hounsell, E.F., Feizi, T., Gastrointestinal Mucins : Structures and Antigenicities of Their Carbohydrate Chains in Health and Disease. Medical Biology, 60, 227-236, 1982.

2. Wells, G.B., Lester, R.L., Rapid Separation of Acetylated Oligosaccharides by Reverse-Phase High-Pressure Liquid Chromatography. Anal. Biochem., 97, 184-190, 1979.

3. Ng Ying Kin, N.M.K., Wolfe, L.S., High-Performance Liquid Chromatographic Analysis of Oligosaccharides and Glycopeptides Accumulating in Lysosomal Storage Disorders. Anal. Biochem., 102, 213-219, 1980.

4. Boersma, A., Lamblin, G., Degand, P., Roussel, P., Separation of a Complex Mixture of Oligosaccharides by HPLC on Bonded-Primary Amine Packing using a Linear-Gradient Solvent System. Carbohyd. Research, 94, C7-C9, 1981.

5. Turco, S.J., Rapid Separation of High-Mannose-Type Oligosaccharides by High-Pressure Liquid Chromatography. Anal. Biochem., 118, 278-283, 1981.

6. Daniel, P.F., DeFeudis, D.F., Lott, I.T., McCluer, R.H., Quantitative Microanalysis of Oligosaccharides by High-Performance Liquid Chromatography. Carbohyd. Research, 97, 161-180, 1981.

7. Bergh, M.L.E., Koppen, P.L., Van den Eijnden, D.H., Arnarp, J. Lönngren, J. High Pressure Liquid Chromatography of Isomeric Oligosaccharides That Form Part of the Complex-Type Carbohydrate Chains of Glycoproteins. Carbohyd. Research, 117, 275-278, 1983.

8. Egge, H., Dell, A., von Nicolai, H. Fucose containing oligosaccharides from Human Milk. I. Separation and Identification of New Constituents. Arch. Biochem. Biophys., 224, 233-253, 1983.

9. Dua, V.K., Bush, C.A. Identification and Fractionation of Human Milk Oligosaccharides by Proton-Nuclear Magnetic Resonance Spectroscopy and Reverse-Phase High Performance Liquid Chromatography. Anal. Biochem., 133, 1-8, 1983.

10. Augé, C., David, S., Veyrières, A. The Molecular Basis of the Human Ii Blood Group System : A Contribution to the Problem from the Synthesis of I-active Oligosaccharides. Nouveau J. De Chimie, 3, 491-497, 1979.

11. Veyrières, A. Blood-Group Ii-Active Oligosaccharides. Synthesis of a Tetrasaccharide, a β(1-3) Dimer of N-Acetyl-lactosamine. J. Chem. Soc. Perkin 1., 1626-1629, 1981.

12. Hakomori, S., cited in Zopf, D.A., Tsai, C.-M. and Ginsburg, V., Antibodies Against Oligosaccharides Coupled to Proteins : Characterisation of Carbohydrate Specificity by Radioimmune-assay. Arch. Biochem. Biophys., 185, 61-71, 1978.

13. Hounsell, E.F., Wood, E., Feizi, T., The Separation of Galactose and N-Acetylglucosamine Containing Synthetic Oligosaccharides With and Without Blood Group I and i Activities, Proc. Vth Int. Sym. Glycoconjugates; Eds. Schaure R., Boer, P., Buddecke, E., Kramer, M.F., Vliegenthert, J.F.G., Weigandt, H., George Thieme Publishers, (1979), p. 122-123.

14. Hounsell, E.F., Gooi, H.C., Feizi, T., The Monoclonal Antibody Anti-SSEA-1 Discriminates Between Fucosylated Type 1 and Type 2 Blood Group Chains. FEBS Letts., 131, 279-282, 1981.

15. Hounsell, E.F., Lim, C.K., Rideout, J.H., Pickering, N.J., Feizi, T., High-Performance Liquid Chromatographic Separation of Oligosaccharide Isomers of the Lacto- and Lacto-N-neo series. Proc. VIIth Int. Symp. Glycoconjugates; Eds. Chester, A., Heinegård, D., Lundblad, A., and Svensson, S., Rahms i Lund, (1983), p. 153-154.

16. Blanken, W.H., Hooghwinkel, G.J.M., Van den Eijnden, D.H., Biosynthesis of Blood-Group I and i Substances : Specificity of Bovine Colostrum β-N-Acetyl-D-glucosaminide β1-4 Galactosyltransferase. Eur. J. Biochem., 127, 547-552, 1982.

17. Cheetham, N.W.H., Dube, V.E., Preparation of Lacto-N-Neo-Tetraose from Human Milk by High-Performance Liquid Chromatography. J. Chromatog., 262, 426-430, 1983.

18. Yamashita, K., Tachibana, Y., Kobata, A., Oligosaccharides of Human Milk : Structural Studies of two New Octasaccharides, Difucosyl Derivatives of Para-Lacto-N-Hexaose and Para-Lacto-N-Neohexaose. J. Biol. Chem., 252, 5408-5411, 1977.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 675-689 (1984)

EVALUATION OF A SIMPLE HPLC CORRELATION METHOD FOR THE ESTIMATION OF THE OCTANOL-WATER PARTITION COEFFICIENTS OF ORGANIC COMPOUNDS

Jerome E. Haky* and A. Michael Young
Warner-Lambert/Parke-Davis Pharmaceutical Research
Ann Arbor, Michigan 48105

ABSTRACT

A simple reverse-phase high performance liquid chromatographic method is evaluated for the estimation of octanol-water partition coefficients (log P) of organic compounds by correlation with their chromatographic capacity factors (k'). Using an unmodified commercial octadecylsilane column and a mobile phase consisting of methanol and an aqueous buffer, a linear relationship is established between the literature log P values of 68 compounds and the logarithms of their k' values. For the determination of the partition coefficients of unknowns, one of two sets of standards is used to calibrate the system, the choice being dependent on the hydrogen-bonding character of the compounds being evaluated. The overall method is shown to be rapid and widely adaptable and to give log P data which are comparable to results obtained by classical or other correlation methods.

INTRODUCTION

The octanol-water partition coefficient (commonly expressed as log P) is an important physical parameter which has been directly correlated with the biological activities of a wide variety of organic compounds (1). While there has recently been much effort to calculate this parameter on the basis of chemical

 0148-3919/84/0704-0675$3.50/0

structure alone (1-3), imperfections in this method and the need for reference values often still requires the experimental measurement of log P values. Experimental methods for this determination include the direct chromatographic (4) or spectroscopic (2) assay of compounds in an equilibrated octanol-water system, potentiometric titrations of compounds in a biphasic octanol-water mixture (5) and determinations based upon established correlations of log P values of compounds with their thin-layer (6) or column liquid chromatographic (7,8) behavior. Of these latter chromatographic methods, there has been considerable interest in the development and utilization of relationships between octanol-water partition coefficient values and reverse-phase high performance liquid chromatography (HPLC) capacity factors (k'). Such relationships are based upon the observed similarities in the hydrophobic partitioning processes occurring in an octanol-water mixture and in a reverse-phase HPLC system with an aqueous mobile phase.

Recently, there has been much work on the attempted improvement of correlations between log P and k' values by increasing the similarities between the octanol-water and reverse-phase HPLC partitioning systems. Such attempts have included the reduction of free silanol sites in the column by exhaustive silylation (9,10), the presaturation of the column with octanol (11,12) and the use of totally aqueous mobile phases (12). While many of these modifications have been somewhat successful at improving the

correlation between log P and k', they have achieved only limited applicability due to increases in the complexity of equipment and experimental time required for their implementation.

Recent advances in methods of stationary phase preparation have resulted in the commercial production of reverse-phase HPLC columns with high homogeneity and high levels of surface alkyl bonding. Based on our premise that the utilization of such a modern column should give a higher degree of correlation between k' and log P than that previously obtained, we have developed and evaluated a simple, rapid HPLC method for the determination of partition coefficients of organic compounds from their k' values, using an unmodified commercial reverse-phase column and a standard aqueous mobile phase.

EXPERIMENTAL

Materials: All solvents were glass distilled (Burdick and Jackson). The chemicals used were obtained from commercial sources (mostly from Aldrich Chemical Company) and were used without further purification.

Apparatus: The HPLC system consisted of an Altex high pressure pump, a Waters U6K injector and an octadecylsilane column (Alltech RP-18, 10 μm particle size, 250 mm x 4.6 mm i.d.). The system was fitted with a Waters Model 440 absorbance detector with an extended wavelength module operated at a fixed wavelength of 214 nm. Chromatographic data were recorded and processed on a Perkin-Elmer Sigma 10 data system.

Throughout this study, the mobile phase consisted of 55% methanol and 45% aqueous ammonium phosphate buffer (0.05M). The pH of this mobile phase (seven unless otherwise specified) was adjusted by the addition of phosphoric acid and/or ammonium hydroxide. The flow rate of the mobile phase was set at a constant 2 ml/minute.

Procedure: Generally, 10 µl of each sample as a solution in methanol or water (1 mg/ml) were injected, although larger amounts were occasionally injected for compounds with low detector responses. The chromatographic capacity factor, k', of each compound was calculated by the formula:

$$k' = \frac{t - t_o}{t_o}$$

where t is the compound's retention time and t_o is the retention time of an unretained substance, determined by injection of an aqueous solution of sodium nitrite. Logarithms are all expressed in base ten.

RESULTS AND DISCUSSION

In accord with the goal of developing a simple, easily adaptable method, the system used in the study consisted entirely of commercial equipment and a standard aqueous mobile phase. While the choice of the specific column was arbitrary, the column type, octadecylsilane (C-18), was chosen on the basis of the good correlations between log P and log k' which have been obtained with related columns in previous studies (8,13-15).

While the use of a totally aqueous mobile phase would maximize partitioning between the column and mobile phase on the basis of hydrophobicity, such an approach also results in unacceptably long retention times for compounds with high log P values. To reduce this problem, an organic modifier was added to the mobile phase. Methanol was chosen since it has been shown to interfere the least with hydrophobic partition mechanisms in reverse-phase HPLC among common organic solvents (15-17). Under the conditions of this study, a mobile phase consisting of 55% methanol and 45% aqueous phosphate buffer allowed compounds with log P values as high as 3.5 to be eluted in 30 minutes or less.

The relationship between octanol-water partition coefficients and HPLC retention behavior in this system was established by the determination of k' values of 68 compounds of widely varying functionality and structure type (See Table 1). Figure 1 shows a plot of the log P values of these compounds (obtained from the literature) versus the logarithm of their k' values obtained under the conditions of this experiment. Considering the wide range of hydrophobicities and functional groups in these compounds, the degree of correlation between log P and log k' ($r = .966$) clearly indicates a linear relationship between these two parameters, which allows a simple estimation of log P values of compounds from their k' values.

The use of any HPLC system for the evaluation of octanol-water partition coefficients by correlation requires calibration

TABLE 1

Experimental Capacity Factors and
Literature Octanol-Water Partition Coefficients†

Compound	Log k'	Log P	Compound	Log k'	Log P
Benzyl alcohol	.151	1.16	Ethyl propionate	.421	1.50
Cinnamic alcohol	.529	1.95	Ethyl acetate	.092	0.34
p-Nitrobenzyl alcohol	.138	1.26	Phenyl acetate	.480	1.49
Allyl Alcohol	-.353	0.17	Methyl benzoate	.790	2.18
Benzonitrile	.361	1.56	Ethyl benzoate	1.07	2.64
Phenylacetonitrile	.323	1.56	Methyl salicylate	.980	2.46
p-Tolunitrile	.643	1.95*	Benzyl acetate	.750	1.96
Cinnamonitrile	.620	1.96	Acetanilide	.104	1.16
2,4-Dimethylphenol	.742	2.30	Pthalimide	.007	1.15
2,6-Dimethylphenol	.703	2.36	Formanilide	.060	1.12
1-Naphthol	.826	2.71	Benzamide	-.261	0.65
p-Cresol	.429	1.94	Thiobenzamide	.073	1.49
p-Cyanophenol	.040	1.63	N-Methylaniline	.477	1.66
Catechol	-.216	0.86	N-Propylaniline	1.06	2.45
p-Methoxyphenol	.036	1.37	p-Toluidine	.314	1.39
Thymol	1.28	3.30	Quinoline	.588	2.03
Benzene	.827	2.01	Indole	.554	2.25
n-Propylbenzene	1.78	3.62	2,6-Lutidine	.435	1.68
Toluene	1.16	2.74	2-Acetylpyridine	.066	0.85
Naphthalene	1.43	3.37	Aniline	.022	0.90
m-Dibromobenzene	1.70	3.75	o-Ansidine	.204	1.23
o-Dibromobenzene	1.54	3.64	2-Picoline	.266	1.20
Biphenyl	1.77	4.06	Acridine	1.19	3.39
Phenanthrene	2.02	4.46	Skatole	.865	2.60
Bromobenzene	1.22	2.99	Acetophenone	.446	1.66
Chlorobenzene	1.14	2.49	Benzophenone	1.22	3.18
p-Xylene	1.48	3.15	Propiophenone	.751	2.20
o-Xylene	1.42	2.77	2-Hexanone	.291	1.38
m-Xylene	1.48	3.20	p-Quinone	-.235	0.20
Anisole	.803	2.08	Anthraquinone	1.40	3.48*
Phenyl n-propyl ether	1.44	3.18	2-Bromoaceto-phenone	.632	2.43
Diphenyl ether	1.73	4.21	Chloroform	.563	1.94
Phenetole	1.06	2.51	Dichloromethane	.270	1.25
Nitropropane	.089	0.69	Trichloroethylene	1.05	2.29

†Values from Reference 1, unless otherwise specified.
*Calculated value, based on the method in Reference 22.

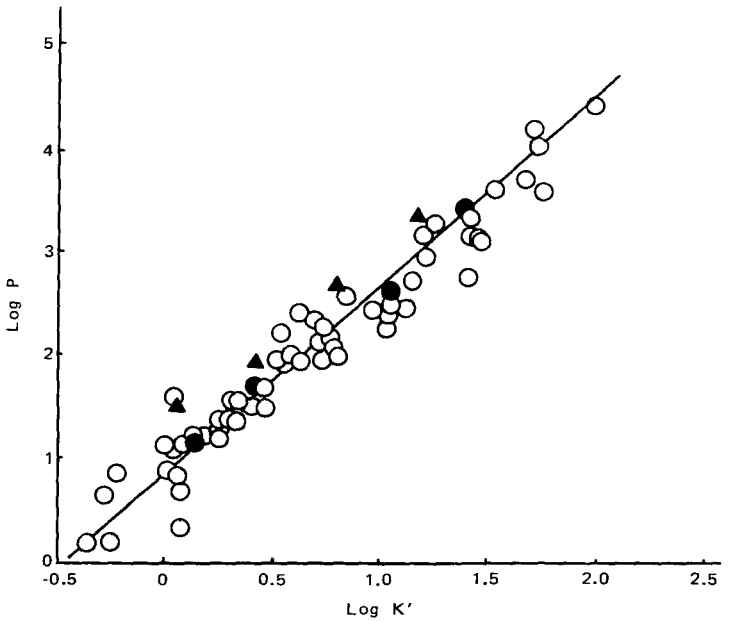


Figure 1. Log P vs Log k' for the 68 Compound Data Set.

● Non-phenolic Calibration Standards
▲ Phenolic Calibration Standards

of the system against standards with known log P values. While highest accuracy is ensured by the utilization of a large number of standards such as the 68 compounds described above, this is clearly impractical. For this reason, four compounds, benzyl alcohol, acetophenone, toluene, and naphthalene, were chosen as a "standard" calibration mixture for the evaluation of the log P's of unknowns using this method. The specific choice of these four compounds was based on four considerations:

1. They all have high UV detector responses.

2. The log P's span a range of 1.16 - 3.37 units, allowing for the calibration of the system over a wide range.
3. The compounds do not ionize over the usable pH range of the HPLC system (ca 2-8), and thus maintain their partitioning properties without regard to the pH of the mobile phase.
4. The partition coefficients of these compounds and their capacity factors are reasonably consistent with the correlation line established by the larger 68 compound data set (Figure 1).

The octanol-water partition coefficients of 25 compounds calculated from their k' values and the calibration curve established by the four "standard" compounds are listed in Table 2. With the exception of values determined for the phenols, all other log P values are in accord (± 0.2 log P units) with previously determined literature values.

Inaccuracies in log P values obtained by chromatographic correlation methods for phenolic compounds have been observed previously and have been attributed to a number of causes, including hydrogen bonding of such compounds to residual silanol sites on the reverse-phase column (9,12,18). While the extent of residual silanol sites in the column used in this study is unknown, the fact that such inaccuracies in the HPLC correlation method have previously occurred for phenolic compounds even with exhaustively silylated columns (9) is indicative of causes other

TABLE 2

Experimental and Literature Partition Coefficients

Compound	Log P_{exp}	Log P_{lit}	Difference
Cinnamic alcohol	1.78	1.95	-.17
p-Nitrobenzyl alcohol	1.13	1.26	-.13
Benzonitrile	1.50	1.56	-.06
Phenylacetonitrile	1.44	1.56	+.12
Cinnamonitrile	1.94	1.96	-.02
m-Xylene	3.37	3.20	+.17
m-Dibromobenzene	3.74	3.75	-.01
Phenanthrene	4.28	4.46	-.18
Anisole	2.24	2.08	+.16
Phenetole	2.68	2.51	+.17
Ethyl propionate	1.60	1.50	+.10
Methyl benzoate	2.22	2.18	+.04
Methyl salicylate	2.54	2.46	+.08
Acetanilide	1.16	1.07	+.09
Formanilide	1.00	1.12	-.12
N-methylaniline	1.70	1.66	+.04
p-Toluidine	1.42	1.39	+.03
Quinoline	1.88	2.03	-.15
2,6 Lutidine	1.63	1.68	-.05
Propiophenone	2.16	2.20	-.04
2-Hexanone	1.39	1.38	+.01
1-Naphthol	2.28	2.71	-.43
2,6-dimethylphenol	2.07	2.36	-.29
p-Cresol	1.62	1.94	-.32
Catechol	.53	.86	-.33

Log P_{exp} = Partition coefficient calculated from k' values and calibration curve established by benzyl alcohol, acetophenone, toluene, and naphthalene. Calibration equation: Log P = 1.67 Log k' + 0.90 (r = 0.997).
Log P_{lit} = Log P values from Reference 1.

than bonding to residual silanol sites. It is known that partitioning of a compound between water and octanol is governed not only by its hydrophobicity, but also by the degree to which the compound can hydrogen-bond to octanol itself (19). In their early studies, both Leo and Hansch (20,21) and Seiler (22) found that correlations between octanol-water and other solvent-water partition coefficients could be improved through separate consideration of these hydrogen-bonding effects. To a first approximation, an analogous treatment of the log P - log k' correlations in this study can be achieved by splitting the data into at least two subsets on the basis of the compounds' hydrogen-bonding character.

Table 3 lists the log P vs log k' linear regression parameters obtained for this HPLC system when the 68 tested compounds are considered altogether as well as split into one set containing phenolic compounds (strong hydrogen-bond donors) and one containing the rest. While the correlation coefficients of the split data sets are only slightly better than for the overall data set, the other regression parameters of the correlation lines for phenolic and non-phenolic compounds are significantly different from each other, suggesting different types of partitioning mechanisms for the two sets of compounds. Of particular significance is the large difference in the intercepts of the correlation lines (>0.4 log P units), which was also observed by Seiler (22) in correlations between the octanol-water

TABLE 3

Log P vs Log k' Linear Regression Parameters for the 68 Compound Data Set

Parameter	All Data	Phenols Only	All Except Phenols
Slope	1.65	1.53	1.69
Intercept	0.949	1.32	0.876
Correlation Coefficient	.966	.985	.973

and other solvent-water partition coefficients of strongly and weakly hydrogen-bonding compounds. Analogous to the results of those studies, the magnitude of the intercept of the log P - log k' correlation line appears to be directly related to the extent to which hydrogen bonding is involved in the partitioning of the compounds between octanol and water.

Demonstrated differences in partitioning mechanisms of strongly hydrogen-bonding and other compounds requires consideration of at least two sets of calibration standards in the use of the HPLC correlation system for the evaluation of log P values. The calibration standards used for low and non-hydrogen-bonding compounds were described earlier, and result in acceptably accurate values for these types of compounds. In accord with the requirements established for those standards, four phenols, p-methoxyphenol, p-cresol, 1-naphthol, and thymol, were chosen as standards for the evaluation of the log P's of strongly hydrogen-bonding compounds. Table 4 lists the

TABLE 4

Partition Coefficients Based on Nonhydrogen Bonding and Phenolic Calibration Curves

Compound	Log P_n	Log P_p	Log P_{lit}
2,4-Dimethylphenol	2.14	2.49	2.30
2,6-Dimethylphenol	2.67	2.43	2.36
p-Cyanophenol	.96	1.37	1.63
Catechol	.54	.97	.86
Salicylic acid	1.13	1.92	2.23 (2.00)*
p-Toluic acid	1.97	2.39	2.27 (2.26)*
Phenoxyacetic acid	.91	1.53	1.42
Benzoic acid	1.44	1.87	1.87 (1.78)*

Log P_n = Partition Coefficient based on calibration curve consisting of benzyl alcohol, acetophenone, toluene, and naphthalene.

Equation: Log P = 1.67 Log k' + 0.90 (r = 0.997).

Log P_p = Partition Coefficient based on calibration curve consisting of p-methoxyphenol, p-cresol, 1-napthol, and thymol.

Equation: Log P = 1.59 Log k' + 1.31 (r = 0.997).

Log P_{lit} = Partition Coefficient from Reference 1.

*Data from Reference 12.

calculated log P's of some strongly hydrogen-bonding compounds (phenols and carboxylic acids) based on each of the two sets of calibration standards. Ionization of the acids in the operating pH range of the HPLC system (ca pH 2-8) required the evaluation of the apparent partition coefficients of these

compounds (D) at three or four pH's, and then extrapolation of these values to the value (P) at zero ionization, in accord with the equation described by Unger et al (12):

$$D = P + K_a (-D/H)$$

Log P values obtained for both the phenols and the carboxylic acids based on the phenolic standards are clearly more in accord with the literature values than those based on the non-hydrogen-bonding standards, and are comparable to those obtained with a much more complicated correlation system utilizing an octanol-saturated column (12). The choice of the proper calibration system based upon the structure of the compound whose partition coefficient is to be evaluated is essential for highest accuracy of values obtained by this method.

CONCLUSIONS

In this study, a simple, rapidly adaptable HPLC method for the evaluation of octanol-water partition coefficients is described, and demonstrated to give values which are in accord with literature values for a wide variety of compounds. Indeed, the overall accuracy of the method may in fact be better than the data indicates, since the degree of accuracy of a number of literature values is unknown.

Since the method requires consideration of the degree of hydrogen-bonding character in the compound being evaluated, some prior knowledge of the structure of the compound is required for

highest accuracy. Another limitation of the method, of course, is the possibility of inaccuracy of a value obtained for a particular compound due to wide deviations from the overall correlations established for the general case. While splitting of compounds into further subsets and utilizing different mobile phases could increase the accuracy of the method, the data set is not large enough to draw any conclusions concerning this, and more importantly, further modification of the method may increase its complexity to an unreasonable level. In any event, the method described here is sufficiently accurate for the evaluations of the partition coefficients of compounds for correlations with their biological activity, and for evaluation of the relative solubilities of compounds in aqueous and organic media.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Horst Schneider for reviewing this manuscript, Ms. Emily Domonkos for assistance in the computer analysis of the data, and Ms. Carola Spurlock for valuable discussions.

REFERENCES

(1) Hansch, C. and Leo, A., Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley, New York, 1979.

(2) Rekker, R. R., The Hydrophobic Fragmental Constant, Elsevier, Amsterdam, 1977.

(3) Rekker, R. R. and deKort, H. M., Eur. J. Med. Chem., 14, 479, 1970.

(4) Nahum, A. and Horvath, C., J. Chromatogr., 192, 315, 1980.

(5) Seiler, P., Eur. J. Med. Chem., 9, 663, 1974.

(6) Tomlinson, E., J. Chromatogr., 113, 1, 1975.

(7) Haggerty, W. J., and Murnl, E. A., Res. Dev., 25, 30, 1974.

(8) Carlson, R. M., Carlson, R. E., and Kopperman, H. R., J. Chromatogr., 107, 219, 1975.

(9) McCall, J. M., J. Med. Chem., 18, 549, 1975.

(10) Brent, D. A., Sabatka, J. J., Minick, D. J., and Henry, D. W., J. Med. Chem., 26, 1014, 1983.

(11) Mirless, M. M., Moulton, S. J., Murphy, C. T., and Taylor, P. J., J. Med. Chem., 19, 615, 1976.

(12) Unger, S. H., Cook, J. R., and Hollenberg, J. S., J. Pharm. Sci., 67, 1364, 1978.

(13) McDuffie, B., Chemosphere, 10, 73, 1981.

(14) Veith, G. D., Austin, N. M., and Morris, R. T., Water Research, 13, 43, 1979.

(15) Braumann, T., Weber, G., and Grimme, L. H., J. Chromatogr., 261, 329, 1983.

(16) Karger, B. L., Gant, J. R., Hartkopf, A., and Weiner, P. H., J. Chromatogr., 128, 65, 1976.

(17) Tanaka, N., Goodell, H., and Karger, B. L., J. Chromatogr., 158, 233, 1978.

(18) Unger, S. H., and Chiang, G. H., J. Med. Chem., 24, 262, 1981.

(19) Davis, S. S., J. Pharm. Pharmacol., 25, 982, 1973.

(20) Leo, A., and Hansch, C., J. Org. Chem., 36, 1539, 1971.

(21) Leo, A., Hansch, C., and Elkins, D., Chem. Rev., 71, 525, 1971.

(22) Seiler, P., Eur. J. Med. Chem., 9, 473, 1974.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 691-707 (1984)

NEW PRINCIPLES OF ION-EXCHANGE TECHNIQUES SUITABLE TO SAMPLE PREPARATION AND GROUP SEPARATION OF NATURAL PRODUCTS PRIOR TO LIQUID CHROMATOGRAPHY

Birthe Bjerg, Ole Olsen, Kim Wedel Rasmussen and
Hilmer Sørensen, Chemistry Department,
Royal Veterinary and Agricultural University
40 Thorvaldsensvej, DK-1871 Copenhagen V, Denmark

ABSTRACT

A Gentle method of group separation of low molecular weight hydrophilic natural products is reported. The method is based on separation of the compounds according to their net charge at different pH values using different types of ion-exchange columns connected in series. Precolumns retaining interfering compounds are used in some cases. Elution of the compounds retained on the columns is performed by use of volatile eluents. The elution principle for two of the ion-exchangers in question is removal of the charges on the column materials while for the third column the positive net charge on the compounds retained is removed. Thereby, the total amount of ions retained on the different columns is released and eluted into small volumes, which after evaporation leaves the ions as well defined salts. The method is experimentally simple and efficient to separation of natural products into groups suitable to direct use in sensitive methods of analysis as e.g. high-performance liquid chromatography and gas chromatography. Combinations of these column chromatographic methods have been adpated for micro or semimicro determinations of naturally occurring compounds, e.g., aromatic choline esters, amines, amino acids and esters of phenolic carboxylic acids. The methods seem to be general practicable for group separation of low molecular weight hydrophilic compounds.

 0148-3919/84/0704-0691$3.50/0

INTRODUCTION

High-performance liquid chromatography (HPLC) and gas chromatography (GC) are methods of analysis which possess high resolution when used for well defined mixtures of low molecular weight compounds. Reversed-phase ion-pair HPLC has been described as a rapid and simple quantitative method of analysis for intact individual glucosinolates (1). Furthermore, HPLC appears to be an efficient supplement to GC and other chromatographic methods in studies of glucosinolates (2) and several other groups of natural products including amino acids and pyrimidines (3,4), phenolic carboxylic acids (5) as well as derivatives thereof such as neutral and acidic esters (6), amides (7) and choline esters (8). The easy access to these methods of analysis makes it desirable with group separation of the complex mixtures of natural products present in all extracts from living cells prior to chromatographic investigations and quantitative determinations. Otherwise, reliable interpretations of the results are complicated (2) and especially the HPLC column materials are rapidly contaminated and destroyed.

Anion-exchange chromatography based on a new elution principle has been described as a suitable method for the quantitative isolation of unstable acidic compounds such as glucosinolates (9,10). This ion-exchange principle has recently been adapted to a new cation-exchange chromatographic technique which allows quantitative isolation of phenolic choline esters under gentle conditions (11).

The present communication describes further developments of these techniques to comprise a semimicro-one-step group separation of different types of natural products using the columns connected in series.

The intention is not a review of the numerous papers which describes traditional ion-exchange methods. It is a

presentation of a group separation procedure based on a new way of using ion-exchange materials in special combinations and comprising new elution principles. Thereby, the problems with purification of unstable hydrophilic natural products as well as problems with unacceptable recoveries of especially aromatic compounds from the columns are reduced. Application of the new isolation procedure in combination with HPLC or other chromatographic methods allows efficient,simple and reproducible semimicrodeterminations of individual intact glucosinolates, phenolic choline esters and amines.The separation of amino acids into groups of basic, neutral and acidic amino acids as well as a convenient alternative separation method for investigation of other ionic and hydrophilic natural products are briefly described.

EXPERIMENTAL

Plant materials

Seeds of Sinapis alba L. cv. Trico (white mustard), of Brassica oleraceae cv. Ditmarsken and of Vicia faba L. were obtained from Trifolium Silo A/S, DK-2630 Tåstrup, Denmark. Seeds of Reseda luteola were collected from plants growing in their natural habitat at Faxe, Denmark.

Chemicals and materials

Tetraalkylammonium bromides and sodium heptanesulphonic acid monohydrate were obtained from Fluka (Bucks, Switzerland); all other reagents were of analytical grade from E. Merck (Darmstadt, G.F.R.). Only deionized water was used and the HPLC solvents were filtered under vacuum through a 0.5 µm Millipore FH type filter or 0.45 µm Millipore HA type filter and degassed before use. The column material CM-Sephadex C-25 was obtained from Pharmacia (Sweden); Dowex 50 w x 8 was obtained from Fluka (Bucks,

Switzerland); Servacel Ecteola 23 was obtained from Serva (Heidelberg, G.F.R.); Bondapak C_{18} 75 µm was obtained from Waters Associates (Roskilde, Denmark). Fintips 61 for microliter pipettes, range 200-1000 µl code 940 1070, Labsystem OY were obtained from Pulttitie 9 (Helsinki, Finland).

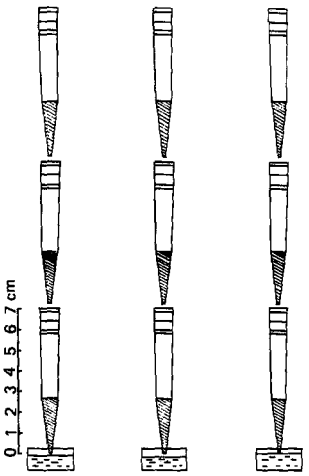
Crude extracts

Preparations of crude extracts were performed according to a previously described method (11) but slightly modified and adapted to the semimicro method now presented. The plant materials were homogenised three times in boiling methanol-water (7:3) with an Ultra Turrax homogeniser, using 3 x 12 ml to samples of 2 g lyophilised plant material, 3 x 8 ml to 0.5 g seed samples, 3 x 5 ml to 0.1 g samples. The homogenates were centrifuged (3000 x g, 10 min, 0°C) and the combined supernatants were concentrated to about 1 ml and centrifuged again at the same conditions. The supernatant was used as the crude extract.

Combined ion-exchange columns and group separations

The column materials were regenerated in relatively large columns as shown elsewhere (10). CM-Sephadex C-25 and Dowex 50 w x 8, 200-400 mesh were treated with 1 M HCl (10 x column volume) and washed to neutral pH with water. Ecteola-cellulose was treated with 2 M acetic acid (10 x column volume) and washed to neutral pH with water. Bondapak C_{18} was activated with methanol and washed with water prior to use.

The columns employed for the crude extracts from 2 g samples were pasteur pipettes with a small plug of glass wool as bottom and filled with the appropriate column materials to a height of 7 cm. In the case of extracts from 0.1 g and 0.5 g samples the columns were Fintips with a small plug of glass wool as bottom and filled to a height of 2-3 cm with the appropriate column materials (Fig. 1).



(A) COLUMNS: weakly acidic cation-exchangers.

$-OCH_2COO^-$, H^+

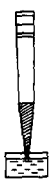
(B) COLUMNS: strongly acidic cation-exchangers.

$-SO_2O^-$, H^+

(C) COLUMNS: weakly basic anion-exchangers.

$-(OCH_2CH_2)_3{}^+NH$, AcO^-

H_2O effluents from the columns.

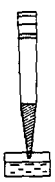


(A) COLUMN:

$-OCH_2COO^-$, B^+

B^+ = metal ions, alkaloids, amines, basic amino acids.

Elution of retained cations by 1 M CH_3COOH. The eluent protonates the functional groups of the ion-exchanger to $-OCH_2COOH$, thereby releasing the retained cations.

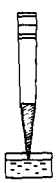


(B) COLUMN:

$-SO_2O^-$, $H_3{}^+N-\underset{\displaystyle R}{\overset{\displaystyle COOH}{\underset{|}{\overset{|}{C}}}}-H$

R = side chain in neutral and acidic amino acids

Elution of retained neutral and acidic amino acids by 1 M pyridine. The eluent deprotonates the carboxyl groups of the amino acids to $RCH^+NH_3COO^-$, thereby releasing the retained compounds.



(C) COLUMN:

$-(OCH_2CH_2)_3{}^+NH$, A^-

A^- = carboxylates, phosphates, sulphonates, sulphates.

Elution of retained anions by 1 M pyridine. The eluent deprotonates the functional groups of the ion-exchanger to $-(OCH_2CH_2)_3N$:, thereby releasing the retained anions

FIGURE 1. Columns fit up for group separation, distribution of different types of natural products on the columns after flushing with water and elution principles used for the three different types of ion-exchange columns.

The columns were fit up vertically on a glass plate with stick tape, and at least three columns were connected in series above each other. From top to bottom the columns were: (A) CM-Sephadex C-25 (H^+); (B) Dowex 50 w x 8, 200-400 mesh (H^+); (C) Ecteola-cellulose (AcO^-) (Fig. 1).

The crude extract was applied to the (A) column and the columns were allowed to drain. The tube which contained the crude extract was washed with water (3 x 0.5 ml). These solutions were also applied to the (A) column before washing with water commenced (approximately 10 x column volume). The effluent passing through the columns was collected. Thereafter, the columns were disconnected and the (A) column was eluted with 2 M acetic acid-methanol (1:1) whereas both the (B) and (C) columns were eluted with 1 M pyridine. If necessary, the eluates were concentrated to a volume appropriate for further investigations.

Chromatography

The liquid chromatograph used consisted of two Waters M-6000 A pumps, a Waters M-450 variable wavelength absorbance detector, a Waters M-720 system controler and a Rheodyn Model 7125 injection valve with a 20 µl loop. Chromatograms were recorded on a Kipp and Zonen Model BD-41 recorder. The experiments were performed on 120 x 4.6 mm or 250 x 4.6 mm I.D. columns (Knauer, Berlin, G.F.R.) packed by the dilute slurry technique. The experimental details used for HPLC analysis of the different types of compounds are described in the legend to the respective figures.

Details concerning paper chromatography (PC), thin layer chromatography (TLC), high voltage electrophoresis (HVE) and GC of choline esters, amines, amino acids, carboxylic acids, glucosinolates and carbohydrates have been described elsewhere (2,9-15.

RESULTS AND DISCUSSION

Consideration of the principles underlying the method of group separation is necessary for elaboration and use of the described technique (Fig. 1). Compounds with positive net charge at pH 3-5 are retained on the (A) column in the serie which is a weakly acidic cation-exchanger, e.g., choline esters, amines, alkaloids, basic amino acids, and metal ions. Compounds such as neutral and acidic amino acids, including the large group of naturally occurring glutamyl and aspartyl peptides (12), as well as some purine and pyrimidine derivatives (vide infra) do not have a positive net charge at the pH in the (A) column. In strongly acidic solutions, however, they are protonated and therefore retained on the (B) column which is a strongly acidic cation-exchanger. Compounds passing through the first two columns but with protolytic active groups which results in a negative net charge at pH 6-8 are retained on the (C) column in the serie which is a weakly basic anion-exchanger, e.g., carboxylates, phosphates, sulphonates and sulphates including glucosinolates. Compounds which do not obtain a net charge at the pH in any of the applied ion-exchange columns are flushed through the three columns with water if strong adsorptions to the materials in the columns are not involved. In such special cases a fourth Bondapak column has been used as a pre-column.

Elution of the ions retained on the (A), (B) and (C) columns is performed with weakly acidic and weakly basic volatile eluents, respectively, (Fig. 1) leaving the eluted compounds as salts after evaporation of the eluates to dryness. Thereby, volatile amines retained on the (A) column and volatile acids retained on the (C) column do not escape during evaporation of the eluents. The principles underlying the elution of

compounds from these two columns is removing of the charges on the column materials, as shown in Fig. 1, whereas elution of compounds from the (B) column, e.g. neutral and acidic amino acids, is obtained by removal of their positive net charge by use of 1M pyridine. This weak and volatile base is removed by evaporation leaving the amino acids as zwitterions. Elution of compounds retained on the pre-column is performed by use of methanol. These elution principles make it possible to obtain quantitative elution applying only some few ml of eluent, and excesses of eluents can be removed by evaporation at gentle conditions, e.g., by lyophilization.

The ion-exchange materials used in the (A)- and (C) columns have polymeric carbohydrates as supports which minimize unwanted adsorption due to hydrophobic interactions, which otherwise are a problem with other types of ion-exchange materials with e.g. polyvinylbenzene as support. Recoveries of aromatic choline esters from the small (A) columns (Fig. 1) using the described elution technique are in fact close to quantitative. Several types of low molecular weight aromatic and phenolic compounds without positive net charge are retained on the (B) column, most likely caused by adsorption or hydrophobic interaction. However, these types of compounds can be retained on the described Bondapak C 18 pre-column introduced in the serie of columns before the (B) column. Alternatively, the (B) column has been omitted from the serie allowing these compounds to pass through the (C) column, including the neutral amino acids; anions, including the acidic amino acids are in this case retained on the (C) column. Recoveries of vicine and the amino acids retained on the (B) column (vide infra) are close to 100% when the small columns and the described elution techniques are used, except for dopa as some oxidation of this compound occurs. Also

the elution technique used to release the compounds retained on the (C) column appeared to result in quantitative recoveries for the investigated compounds (vide infra).

Amino acids in the eluate from the (B) column are separated into groups of acidic and neutral amino acids, respectively, by renewed use of the (C) column. Neutral amino acids are flushed through this column with water whereas acidic amino acids are retained on the column and released again with pyridine as described above for the (C) column.

Separation and quantitative determination of the individual compounds in each group are then performed by selected methods of analysis, e.g. HPLC (vide infra), PC, TLC, HVE and/or amino acid analyser (13). Irrespective of choice of analytical method, the results obtained are much improved when the described method of group separation is included.

Figure 2 shows the chromatograms obtained by HPLC analysis of the total amine and choline ester fraction isolated from S. alba seeds (0.1 g). The eluate from the (A) column was dissolved in water (5 ml) and a sample of this solution (20 µl) was injected. The results reveal that reversed-phase ion-pair HPLC (8) in combination with the group separation method is an efficient analytical procedure for phenolic choline esters. It is possible to make some discriminations between the individual compounds by use of different detection wavelengths. Alkaline conditions are avoided during all isolation steps which is essential for quantitative isolation of these esters (11) as well as for other alkaline labile phenolic cations, e.g. anthocyanins, retained on the (A) column. The elution principle makes it furthermore to an efficient method of isolation of amines and basic amino acids.

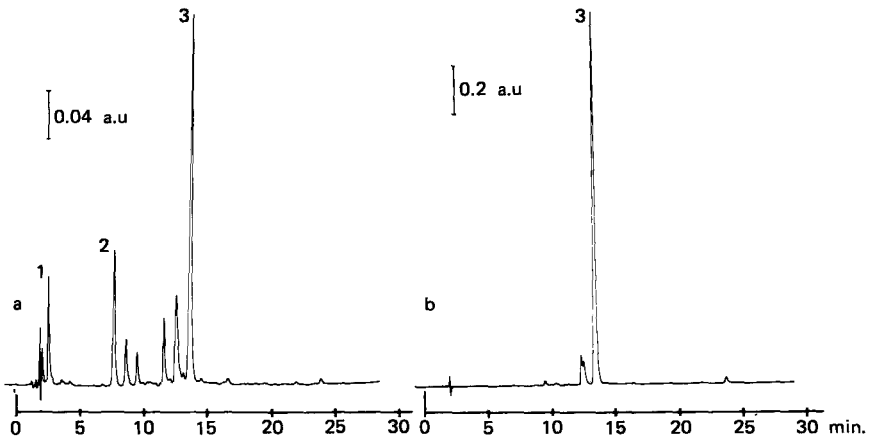


FIGURE 2. HPLC chromatograms of the eluate from column (A) obtained by group separation of the compounds in an extract from 0.1 g of seeds of Sinapis alba cv. Trico. Support: Nucleosil 5 C_8, 120 x 4.6 mm. Mobile phase: a linear gradient of solvent A - solvent B (20:80) to (70:30) for 30 min, flow 1.0 ml/min.
Solvent A: 0.02 M phosphate buffer, 0.02 M dibutylamine and 0.02 M sodium heptanesulfonic acid (pH 2.0) modified with 50% acetonitril. Solvent B: 0.01 M phosphate buffer, 0.01 M dibutylamine and 0.01 M sodium heptanesulfonic acid (pH 2.0). Recorder speed 5 mm/min. Detection wavelength; (a) 280 nm, (b) 313 nm. Peaks: 1 = 4-hydroxybenzylamine; 2 = 4-hydroxybenzoylcholine; 3 = 3,5-dimethoxy-4-hydroxy-cinnamoylcholine (sinapine) other peaks are unknown compounds discussed elsewhere (8,11).

Figure 3 shows the chromatogram from HPLC analysis of the (B) column eluate obtained by group separation of an extract from 0.5 g Reseda luteola seeds. The eluate was dissolved in water (5 ml) and a sample (20 µl) thereof was injected. Figure 3 comprises also a standard chromatogram of authentic reference compounds, including the acidic aromatic amino acids previously

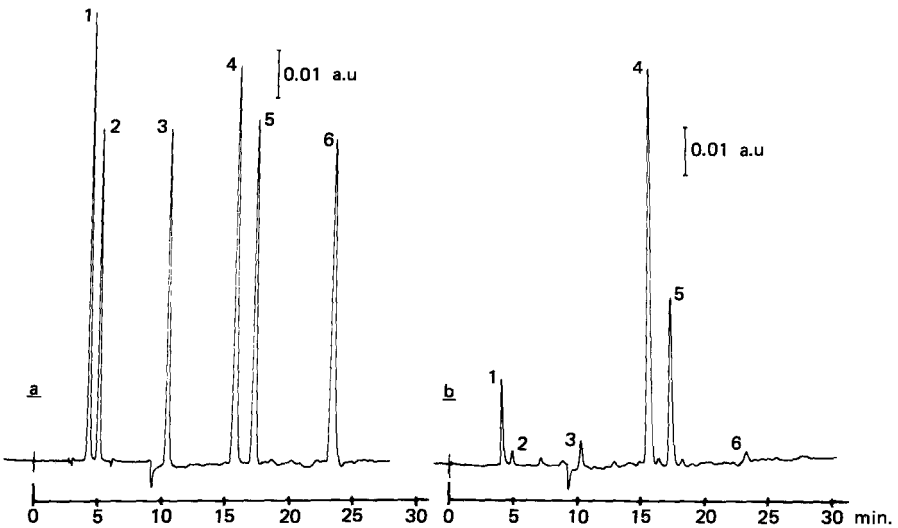


FIGURE 3. HPLC chromatograms of the eluate from column (B) obtained by group separation of the compounds in an extract from 0.5 g of seeds of Reseda luteola. Support: Nucleosil 5 C_{18} (250 x 4.6 mm). Mobile phase: a linear gradient of solvent B (0:100) to (50:50) for 25 min, flow 1.0 ml/min. Solvent A: 0.0125 M sodium formiate buffer (pH 3.5) modified with 25% acetonitrile. Solvent B: 0.01 M sodium formiate buffer (pH 3.5). Recorder speed 5 mm/min. Detection wavelength 280 nm. Peaks: 1 = 3-carboxy-4-hydroxyphenylglycine; 2 = 3-carboxyphenylglycine; 3 = tyrosine; 4 = 3-carboxytyrosine; 5 = 3-carboxyphenylalanine; 6 = tryptophane. Chromatogram a) is an artificial mixture of authentic reference compounds; chromatogram b) is the mixture of amino acids isolated from R. luteola.

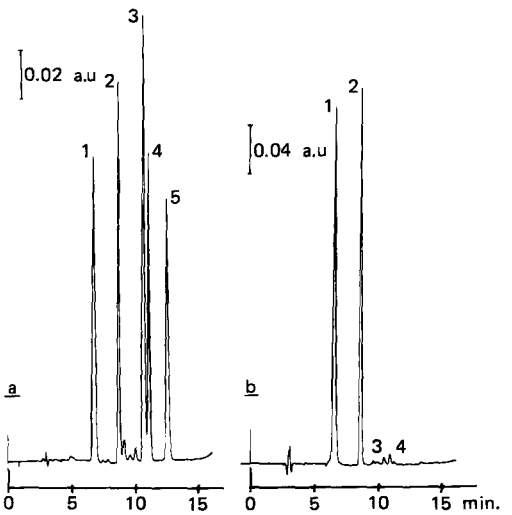


FIGURE 4. HPLC chromatograms of aromatic amino acids and pyrimidine glucosides isolated from Vicia faba; a) an artificial mixture of authentic reference compounds; b) an extract from V. faba seeds.
Support: Nucleosil 5 C_{18}, 250 x 4.6 mm. Mobile phase: a linear gradient of solvent A - solvent B (1:99) to (99:1) for 10 min maintaining this final conditions for additional 5 min, flow 1 ml/min.
Solvent A: 0.0125 M phosphate buffer (pH 2.0) modified with 25% methanol. Solvent B: 0.01 M phosphate buffer (pH 2.0). Recorder speed 5 mm/min. Detection wavelength 280 nm. Peaks: 1 = 2,6-diamino-5-(β-B-glucopyranosyloxy)-4-pyrimidinone (Vicine); 2 = 6-amino-2-hydroxy-5-(β-D-glucopyranosyloxy)-4-pyrimidinone (Convicine); 3 = 3,4-dihydroxyphenylalanine (Dopa); 4 = 4-hydroxy-3-(β-D-glucopyranosyloxy)phenylalanine (Dopa-glucoside); 5 = tyrosine.

isolated from R. luteola (16). Separation of these acidic amino acids by the described HPLC method of analysis is in accordance with their pKa_2 values and depends on careful adjustment of pH in the solvents used as mobile phase. Subsequent group separation of the compounds in the (B) column eluate by reuse of the (C) column, revealed that the neutral amino acids flushed through the column with water while the acidic amino acids were retained on the column and finally eluted therefrom with M pyridine. Slightly modified HPLC conditions appeared to be more efficient to separation of mixtures containing some other aromatic amino acids and pyrimidine derivatives (Fig. 4). Tyrosine, Dopa and Dopa-glucosid co-occur with vicine and convicine as quantitative dominating constituents of Vicia faba (17) and other legumes. Vicine and the amino acids are retained on the (B) column whereas convicine appears in the water effluent in accordance with pKa values for the aromatic amino groups. Owing to the high concentrations of vicine and convicine in V. faba seeds and the efficient UV-absorption of these compounds (17) it is possible to perform HPLC analysis direct on deproteinised extracts from these seeds without further purification (3,4) (Fig. 4). However, this is not the case when extracts with lower concentrations of these compounds and higher concentrations of interfering constituents are to be analysed e.g., investigations of other legumes, especially green parts, food and feed, contents in the digestive tracts of animals, urine and blood.

The eluate from the (C) column contains acidic compounds as carboxylic acids, glucosinolates and phosphates. When seed extracts from glucosinolate containing plants are investigated for individual intact glucosinolates (2) it is possible to use the eluate from the (C) column directly for HPLC analysis (1,10) without ad-

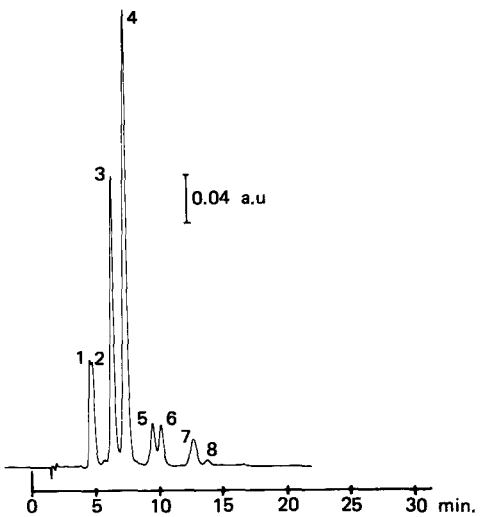


FIGURE 5. HPLC chromatogram of the eluate from column (C) obtained by group separation of the compounds in an extracts from 0.1 g of seeds of Brassica oleracea cv. Ditmarsken.
Support: Nucleosil 5 C_{18}, 120 x 4.6 mm. Mobile phase: 0.01 M phosphate buffer (pH 7.0) modified with 60% methanol containing 0.005 M tetraheptylammonium bromide, flow 1.0 ml/min. Recorder speed 5 mm/min. Detection wavelength 235 nm. Peaks: 1 = 3-methylsulfinylpropylglucosinolate; 2 = 4-methylsulfinylbutylglucosinolate; 3 = 2-hydroxybut-3-enylglucosinolate; 4 = allylglucosinolate; 5 = but-3-enylglucosinolate; 6 = 3-methylthiopropylglucosinolate; 7 = 4-methylthiobutylglucosinolate; 8 = pent-4-enylglucosinolate.

ditional purification (Fig. 5). However, to avoid serious problems caused by interfering compounds, pre-columns are needed prior to HPLC analysis of extracts obtained from blood and contents in the digestive tract of animals (18) as well as from seedlings and green parts of some plants with a particular low glucosinolate content e.g. double low rape varieties.

CONCLUSIONS

The combined column chromatography method now described is experimentally simple, fast, cheap and efficient for semimicro group separation of hydrophilic natural products. The principle implies separation of the compounds according to their net charges at different pH values using gentle conditions. Neutral to weakly acidic conditions are used throughout the isolation of compounds retained on the (A) column. This is especially important for the quantitative isolation of volatile amines and cations which are unstable in alkaline solutions e.g. choline esters, anthocyanins and several other phenolic compounds. Protons released from the cation-exchangers are immediately neutralised on the weakly alkaline anion-exchanger - the (C) column. Thereby, long time extreme pH values are avoided in the solutions containing compounds to be retained on this column. This is important for quantitative isolation of e.g. glucosinolates. The groups of compounds isolated are thus purified and obtained as well defined salts suitable to further qualitative and/or quantitative investigations of the individual compounds by use of sensitive methods of analysis. Problems caused by contamination of columns and detection systems in e.g. HPLC and GC instruments are efficiently reduced. Resolution and reliability in interpretation of results obtained by

these and other chromatographic methods are much improved. The method show off to advantage when large number of samples have to be examined for different types of hydrophilic low molecular weight constituents owing to the easy way of arranging several series of columns side by side on glass plates.

ACKNOWLEDGEMENTS

Support from the Danish Agricultural and Veterinary Research Council, from EEC and from DANIDA, the Danish Council on Development Research, is gratefully acknowledged.

REFERENCES

1. Helboe, P., Olsen, O. and Sørensen, H. Separation of glucosinolates by high-performance liquid chromatography, J. Chromatogr., 197, 199 1980.
2. Olsen, O. and Sørensen, H. Recent advances in the analysis of glucosinolates, J. Amer. Oil Chem. Soc., 58, 857 1981.
3. Marquardt, R.R. and Frolich, A.A. Rapid reversed-phase high-performance liquid chromatographic method for the quantitation of vicine, convicine and related compounds, J. Chromatogr., 208, 373 1981.
4. Quemener, B., Gueguen, J. and Mercier, C. Determination of vicine and convicine in faba beans by high pressure liquid chromatography, Cand. Inst. Food Sci. Technol. J., 15, 109 1982.
5. Roggendorf, E. and Spatz, R. Selectivity effects of ternary mobile phases, Chromatography Review 6, 10 1981.
6. Strack, D. Separation and quantification of basic hydroxycinnamic amides and hydroxycinnamic acids by reversed-phase high-performance liquid chromatography, Planta, 155, 31 1982.
7. Ponchet, M., Martin-Tanguy, J., Poupet, A., Marais, A. and Beck, D. Development of 1-O-sinapoyl-β-glucose: L-malate sinapoyltransferase activity in cotyledons of red radish (Raphanus sativus L. var sativus), J. Chromatogr., 240, 397 1982.

8. Clausen, S., Olsen, O. and Sørensen, H. Separation of aromatic choline esters by high-performance liquid chromatography, J. Chromatogr., 260, 193 1983.

9. Olsen, O. and Sørensen, H. Isolation of glucosinolates and the identification of o-(α-L-rhamnopyranosyloxy)benzylglucosinolate from Reseda odorata, Phytochemistry, 18, 1547 1979.

10. Sørensen, H. New methods of quantitative analysis of glucosinolates, in E.S. Bunting (Editor), Production and Utilization of Protein in Oilseed Crops, Martinus Nijhoff Publishers, London, 1981, p. 107.

11. Clausen, S. Olsen, O. and Sørensen, H. 4-Hydroxybenzoylcholine: a natural product present in Sinapis alba, Phytochemistry, 21, 917 1982.

12. Kasai, T. and Larsen, P.O. Chemistry and biochemistry of γ-glutamyl derivatives from plants including mushrooms (Basidiomycetes), Fortschr. Chem. Org. Naturst. 39, 173 1980.

13. Kristensen, E.P., Larsen, L.M., Olsen, O. and Sørensen, H. Synthesis and properties of hydroxylated and alkylated acidic amino acids, especially glutamic acids, Acta Chem. Scand., Ser. B 34, 397 1980.

14. Olsen, O., Rasmussen, K.W. and Sørensen, H. Glucosinolates in Sesamoides canescens and S. pygmaea: identification of 2-(α-L-arabinopyranosyloxy)-2-phenylethylglucosinolate, Phytochemistry, 20, 1857 1981.

15. Olsen, O. and Sørensen, H. Glucosinolates and amines in Reseda media, Phytochemistry, 19, 1783 1980.

16. Meier, L.K., Olsen, O. and Sørensen, H. Acidic amino acids in Reseda luteola, Phytochemistry, 20, 1857 1981.

17. Bjerg, B., Knudsen, J.C.N., Poulsen, M.H. and Sørensen, H. Quantitative estimation of favism releasing factors in Vicia faba seeds, Pulse Crops Newsletter, 1, 36 1981.

18. Bille, N., Eggum, B.O., Jacobsen, I., Olsen, O. and Sørensen, H. Antinutritional and toxic effects in rats of individual glucosinolates (± myrosinases) added to a standard diet. 1. effects on protein utilization and organ weights, Z. Tierphysiol. Tierernährg. u. Futtermittelkde., (1983) in press.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 709-730 (1984)

SELECTION OF THE MOBILE PHASE FOR ENANTIOMERIC RESOLUTION VIA CHIRAL STATIONARY PHASE COLUMNS

M. Zief, L. J. Crane and J. Horvath
Research Laboratory, J. T. Baker Chemical Company
Phillipsburg, NJ 08865

ABSTRACT

The optimization of enantiomeric resolution by mobile phase variation was studied with the chiral stationary phase derived from R-N-(3,5-dinitrobenzoyl)phenylglycine covalently coupled to 5 μm spherical 3-aminopropyl silica. Chromatography was routinely performed with mobile phase compositions having polarities as high as 2.5 without column deterioration. The relative strength of a solvent as a hydrogen acceptor was found to be an important basis for selection of the polar component in a binary mobile phase. The substitution of tert-butanol for 2-propanol or ethanol in an alcohol/hexane mixture, for example, afforded improved separation factors with several enantiomers. In addition, the need for a polar mobile phase such as 50/50 methylene chloride/hexane to minimize non-specific polar absorption of enantiomers has been demonstrated. Enhancement of specific chiral interactions and suppression of interfering reactions have been obtained with a number of clinically relevant derivatives as model compounds.

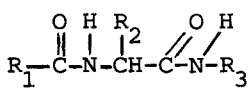
INTRODUCTION

The design of chiral stationary phases for the chromatographic separation of enantiomers has been a

 0148-3919/84/0704-0709$3.50/0

major focus of interest in a number of laboratories over the past several years. Originally the chiral stationary phases were developed for gas chromatographic separations. More recently, specific optically active phases have been used in the HPLC mode as well.

Although a variety of approaches has been used, one very important class of optically active phases in both the HPLC and GC mode has been derived from a chiral diamide functional group (I). This diamide group contains multiple hydrogen bond donor and acceptor groups[1]. In addition, restricted rotation around the amide bond affords a preferred face for interaction with one of a pair of enantiomeric solutes.



(I)

Some of the most effective optically active phases in GC studies are based upon naturally occurring amino acids such as S-valine, in which $R_2 = -CH(CH_3)_2$. Attachment to the solid support occurs either through the amine R_3 or the carboxyl R_1. The same approach is being used with considerable success in HPLC. In this case either R_1 or R_3 represents an attachment to silica via a bridging group; most commonly R_3 represents n-propyl silica. This structure is most often synthesized by treating the carboxyl group of the chiral amino acids with 3-aminopropyl silanized silica.

Perhaps the most extensive investigation of enantiomeric separations on HPLC chiral stationary phases of

this type has been conducted by Pirkle and his colleagues, who have developed several variations of (I) in which R_1 is 3,5-dinitrophenyl, R_2 is phenyl or isobutyl and R_3 is propyl-silanized silica[2]. These phases have thus been derived from α-R-phenylglycine or S-leucine, by bonding N-3,5-dinitrobenzoyl derivatives of these chiral amino acids to 3-aminopropyl silanized silica either covalently via an amide linkage or ionically via acid-base interactions. Both of these types of chiral stationary phases are now commercially available.

Although investigators differ somewhat in the details of interpreting bonding forces required for chiral resolution (recognition), it is generally agreed that differential binding of enantiomers results from differences in the summation of binding energies of hydrogen bond acceptor and donor groups, π bonding and steric interactions. Hydrogen bonding has long been recognized as a contributing interaction; Pirkle has more recently proposed, with his stationary phase, that dipole stacking plays an important role[3].

Whatever the specific types of interaction involved between the enantiomers and the chiral stationary phase, it is clear that any competing interaction may alter enantiomeric resolution. In GC, where the mobile phase is an inert carrier gas, the possibility of polar interaction with chiral and non-chiral groups is minimal. In HPLC, however, the mobile phase is a dynamic part of the system and must be recognized as a potential source of

polar functional groups capable of interacting with both the chiral stationary phase and with the enantiomeric solute. The approach of most investigators has been to mimic GC conditions by using a mobile phase that is as "inert" as possible (i.e., non-polar) while possessing sufficient solubilizing ability to move the enantiomer through the HPLC column. Most commonly, hexane with small amounts of isopropanol (3-10%) has been the preferred mobile phase. We have taken a somewhat different approach, basing our studies upon the proposition that the mobile phase for chiral chromatography can be utilized, as in other modes of HPLC, to optimize both enantiomeric resolution and overall chromatographic quality of any chiral separation. The results that we have obtained with a number of clinically relevant derivatives as model compounds are presented in this paper.

MATERIALS AND METHODS

Apparatus

The chromatography was performed with an Altex 110 high-pressure pump, a Gilson Model 111 UV-detector set at 254 nm and a Hewlett-Packard strip-chart recorder.

The HPLC column used was a BAKERBOND Chiral Phase™ DNBPG (covalent), from J. T. Baker Chemical Company, a standard 25 cm x 4.6 mm I.D. stainless steel HPLC column packed with a bonded phase of R-N-3,5-(dinitrobenzoyl)-phenylglycine covalently coupled to 5 μm spherical 3-aminopropyl silica.

Materials

The β-naphthamide of amphetamine and the 3,4-dimethyl-2(-2-naphthyl)-5-phenyloxazolidine were obtained from Dr. E. Wainer, Food and Drug Administration, Washington, DC. Dr. W. H. Pirkle, University of Illinois, supplied the 7-chloro-1,3-dihydro-3-benzyl-5-phenyl-1,4-benzodiazepin-2-one. The 1-methoxy-3(1[-2-nitroimidazole])-propanol-2 was furnished by Dr. J. L. Day, Florida A&M University. All the solvents were J. T. Baker HPLC grade.

Derivatives

Preparation of N, and N,O-α-naphthoyl derivatives was carried out by a modification of the procedure of Pirkle and Welch[3].

Propranolol Derivatives

Propranolol hydrochloride (0.1 g) and α-naphthoyl chloride (0.1 g) were added to 5 mL of methylene chloride in a 20-mL vial. To this mixture was added 3 mL of 5% aqueous sodium hydroxide solution. The vial was capped and shake vigorously for one minute. The aqueous layer was removed with a Pasteur pipet. The lower organic layer was washed once with 3% hydrochloric acid, then twice with distilled water, and was finally dried over anhydrous sodium sulfate. The filtered organic layer was directly injected onto the HPLC column.

DISCUSSION

Mobile phases for chiral columns are usually binary mixtures of solvents. For such mixtures the polarity

(P^1) is readily obtained from the following equation:

$$P^1 = \phi_A P_A + \phi_B P_B$$

Where O_A and O_B are the volume fractions of solvents A and B and P_A and P_b are the P^1 values of the pure solvents[4].

As can be seen in Table 1, the (0-10%) IPA-hexane mobile phases used most frequently in published studies of the R-N-3,5-dinitrobenzoylphenylglycine column range in polarity from 0.2-0.48. This low polarity enhances weak chiral interactions, as emphasized previously, but also has been considered necessary to prevent leaching of the ionically bound chiral phase from the column (leaching reported at a polarity of 0.805[5]). More recently, columns have become available with the chiral amino acid covalently bound to the silica support via an amide linkage. Chromatography with the covalent columns has been routinely performed with methylene chloride/ hexane mixtures at polarities as high as 2.5 with no column deterioration. Additionally, the covalent columns have been extensively washed with water (P^1 = 10.2) with no deterioration. Thus far, no combination of solutes and solvents routinely used in normal phase chromatography has altered the structure of the covalent stationary phase. Furthermore, strongly retained adsorbates can be removed from the column rapidly with methanol.

The increased stability of the covalent stationary phase has made it possible to investigate mobile phase

TABLE I

Polarity (P^1) of Binary Mobile Phases

Mobile Phase	Solvent A 2-Propanol, %	Solvent B Hexane, %	P^1
		100	0.1
1	5	95	0.29
2	10	90	0.48
3	20	80	0.85
4	30	70	1.24
5	50	50	2.00
6	100		3.9
	Methylene Chloride, %	Hexane, %	
7	50	50	1.60
8	70	30	2.20
9	80	20	2.50
10	100		3.1

composition as a variable in optimizing chiral chromatography. The need to explore mobile phase selectivity became apparent when simple variation of 2-propanol/hexane binary mixtures failed to resolve the N,O-α-naphthoyl derivatives of propranolol, even though resolution would be predicted based upon the present hypotheses for selective interaction.

Snyder has proposed that solute retention in liquid-solid chromatography on polar adsorbents can be explained by a comprehensive model based on solvent adsorption onto and subsequent displacement from sites on the adsorbent by molecules of solute[6]. According to this hypothesis, the relative ease of displacement of solvent on the adsorbent by solute will largely determine the retention time of the solute. If there are specific localized sites on the adsorbent, these will be most important in solvent adsorption for small increments of interactive solvents. Therefore in our system one should be able to compare solvent adsorption for interactive solvents in binary mixtures by measuring relative elution times for specific optical isomers at the same solvent polarity (same solvent strength). A decrease in elution time would signify that solvent molecules adsorbed onto active sites of the chiral phase are displaced with greater difficulty by solute molecules.

Snyder's hypothesis is general for polar adsorbents, including silica, alumina, and bonded phase sorbents such as amino alkyl, and includes less site specific solvent-adsorbent interactions as well. For chiral phase I where the π-acidic 3,5-dinitrophenyl group is R_1, the acidic amide hydrogen offers the possibility for specific interaction for basic solvents, i.e., solvents having hydrogen acceptor capabilities.

To see whether an interaction of this nature is important, we referred to the solvent selectivity triangle of Snyder[7] and selected for replacement of

2-propanol (a hydrogen donor-acceptor) in the 2-propanol/hexane mixture, ethyl ether (a hydrogen acceptor), chloroform (a pure hydrogen donor) and methylene chloride (a poor hydrogen donor or acceptor, but possessing a large dipole moment). In addition, tetrahydrofuran and ethyl acetate, intermediate between ethyl ether and methylene chloride were investigated.

Two compounds were chosen as model solutes for most of our studies; 2,2,2,-trifluoro-1-(-9-anthryl)ethanol, weakly retained by the column but well-resolved because of its clearly defined sites for specific interaction, including a strongly interactive alcohol moiety; and the α-naphthamide of 1-(α-naphthyl)ethylamine, quite strongly retained by the stationary phase by dipole-dipole interaction and π-π bonding.

The data in Table II showing relative elution times support the concept that the relative strength of the solvent as a hydrogen acceptor is an important basis for solvent adsorption. With solute A and a mobile phase polarity of 0.48, relative elution times place solvent adsorption onto the chiral phase in the following order: 2-propanol > tetrahydrofuran > ethyl acetate > ethyl ether > methylene chloride > chloroform. These data agree with the hydrogen accepting ability reported by Taft, et al, who listed hydrogen-acceptor constants (pK_{HB}) of 1.26 for tetrahydrofuran, 1.08 for ethyl acetate, and 0.98 for ethyl ether[8]. N-Butylamine (pK_{HB} = 2.11) is so strong an acceptor that it may form a tertiary complex with the solute on the stationary phase and therefore retard elution.

TABLE II

Evaluation of Mobile Phases for Enantiomeric Resolution

Solute A: 2,2,2,-trifluoro-1(-9-anthryl)ethanol

Mobile Phase	P^1	t_1	t_2	α	k_1^1
5/95 n-butylamine/hexane	0.30	solute retained beyond 20 minutes			
10/90 +-butanol/hexane	0.50	3.8	5.1	1.62	1.23
10/90 2-propanol/hexane	0.48	3.6	4.6	1.56	1.0
9/91 ethanol/hexane	0.48	3.8	4.5	1.33	1.23
10/90 tetrahydrofuran/hexane	0.49	6.1	8.1	1.47	2.38
9/91 ethyl acetate/hexane	0.49	8.5	11.9	1.50	3.72
14/86 ethyl ether/hexane	0.48	12.0	17.0	1.49	6.06
40/60 methylene chloride/hexane	1.30	15.6	21.7	1.44	7.66
50/50 methylene chloride/hexane	1.60	6.0	8.0	1.48	2.33

70/30 methylene chloride/hexane	2.20	3.5	4.1	1.42	0.94
60/40 chloroform/hexane	2.50	5.0	6.1	1.33	1.94

Solute B: α-naphthamide of 1-(α-naphthyl)ethylamine

30/70 2-propanol/hexane	1.24	8.7	13.8	1.73	4.12
40/60 methylene chloride/hexane	1.30	16.8	23.7	1.46	8.88
50/50 methylene chloride/hexane	1.60	10.0	13.9	1.48	4.88
70/30 methylene chloride/hexane	2.20	5.1	6.6	1.44	2.0

Conditions: 25 cm x 4.6 mm covalent chiral column.

Flow Rate: 2 mL/min.

2-Propanol and other alcohols are uniquely effective solvents as mobile phase additives with respect to solute A, which is itself an alcohol. As the 2-propanol content in the mobile phase increases, displacement of the solvent by solute A becomes more and more difficult. It appears that the hydroxyl group of solute A competes directly with the hydroxyl group of 2-propanol for specific sites on the stationary phase. The first isomer of solute A elutes at 3.6 minutes with 10/90 2-propanol/hexane, at 2.6 minutes with a 20/80 ratio and 2.3 minutes with a 30/70 ratio, the effect diminishing as adsorption sites are saturated.

When methylene chloride is substituted for 2-propanol in the mobile phase, the solute can then displace the adsorbed solvent readily. Removal of the solute from the chiral stationary phase then requires a polarity of 2.2 in order to attain the k_1^1 value (approximately 1.0) observed with 10/90 2-propanol/hexane ($P^1 = 0.48$). When chloroform replaces methylene chloride in the mobile phase, removal of the solute demands a mobile phase of still higher polarity. These data support the hypothesis that a hydrogen donor solvent is most easily displaced from the chiral stationary phase.

The structure of solute B would predict adsorptive interactions of a far less site specific, geometrically constrained nature, and indeed, though 2-propanol is still a more effective solvent than methylene chloride, its unique effectiveness in minimizing retention times is

muted. Whereas the elution times t_1 for solute A differ by a factor of 6.8 for 30/70 2-propanol/hexane and 40/60 methylene chloride/hexane, they differ by only a factor of 1.9 for solute B. Here the high solvent strength of the methylene chloride binary mobile phase becomes an important factor.

Variation of Enantiomeric Separation, α, with the Mobile Phase

Chromatographic separation of enantiomers on a chiral stationary phase depends solely upon the differential ability of the two optical isomers to form very specific, transient, geometrically constrained complexes with a small number of adjacent sites on the stationary phase. For this reason, although retention times change as the composition (and therefore polarity) of a particular binary mixture changes, the separation factor stays relatively constant (see Figure 1). Most of the "site specific" interactions previously discussed as retention time determinants affect both enantiomers relatively equally; i.e., they are not chiral in nature.

The separation factor does increase, however, as the tendency of the active solvent to interact specifically with the chiral moiety on the stationary phase increases. Thus as Table II shows, tertiary butanol and 2-propanol give higher α values for solute A than do the other solvents capable of acting as hydrogen acceptors, which as a group have higher α values than does the single hydrogen donor solvent chloroform. The same trend is indicated for solute B.

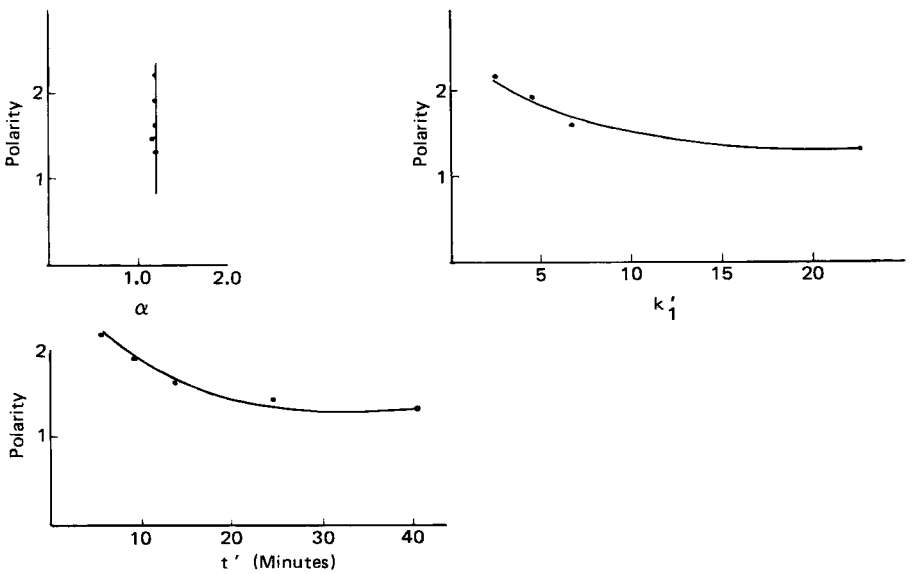


Figure 1

Behavior Of Propranolol ∝ - Napththamide In Methylene Chloride/Hexane Mobile Phases Flow Rate: 2 ml/min.

Of the three alcohols tested with solute A, t-butanol gives the highest α value. As the alcohol increases in bulk, both enantiomers are able to displace solvent molecules more readily. The more tightly bound isomer, the S-form, introduces more desolvation of the stationary phase than the R-form, resulting in an increase in α due primarily to an increase in the retention time of the more tightly bound enantiomer.

The bulkiness of the solvent molecule seems to be an important factor contributing to larger α values with ethers also. Of course, ethers are primarily hydrogen

acceptors whereas alcohols are able to donate hydrogen bonds as well. If bulkiness is important, one would predict that the combination of t-butyl methyl ether/-hexane as a mobile phase should not only have lower retention times because of its greater basicity than a corresponding ethyl ether/hexane mixture of equal polarity, but also have a higher α value. Indeed, as Table III shows, when solute A was chromatographed in a mobile phase of a 17/83 t-butyl methyl ether/hexane mixture (P = 0.49), the retention times were approximately half those for an equivalent ethyl ether/hexane mixture, and the α value approached the value obtained with 2-propanol as the interactive solvent. The highest α value (2.12) was obtained by accident, when solute A dissolved in 50/50 2-propanol/ hexane was chromatographed in 17/83 t-butyl methyl ether/hexane. The retention time of the first enantiomer was sharply reduced, reflecting the transient effect of the small amount of 2-propanol in the system, while the retention time of the second enantiomer was nearly the same as in the mobile phase alone.

Application of Mobile Phase Selectivity to Enhance Enantiomeric Resolution

Our working hypotheses in solvent selection were tested with a variety of enantiomeric pairs in an attempt to optimize resolution. Table IV lists a number of such compounds. Compound C is a β-naphthyl oxazolidine derivative of ephedrine, originally suggested by Wainer (9) as a means to achieve chromatographic enantiomeric

TABLE III

Evaluation of t-Butyl Methyl Ether/Hexane Mobile Phase

Solute A: 2,2,2,-trifluoro-1-(9-anthryl)ethanol

Mobile Phase	P^1	t_1	t_2	α	k_1^1
[a]17/83 t-butyl methyl ether/hexane	0.49	6.3	8.8	1.54	2.7
[b]17/83 t-butyl methyl ether/hexane	0.49	4.8	8.3	2.12	1.82
14/86 ethyl ether/hexane	0.48	12.0	17.0	1.49	6.06

(a) Injection of solute dissolved in the mobile phase.

(b) Injection of solute dissolved in 50/50 2-propanol/hexane.

resolution of this biologically important compound. This derivative is particularly interesting because of its low retention on the chiral phase chromatographed in the traditional 5/95 2-propanol/hexane mobile phase. When this solvent (P = 0.29) was used in our laboratory, the k' value was 0.73; α was 1.07. When the mobile phase was changed to 14/86 diethyl ether hexane (P = 0.48) retention was prolonged (k' = 2.2) and better resolution of the peaks was observed.

Propranolol is another hydroxy amine that can form several derivatives. Condensation with α-naphthoyl chloride gives the N and the N,O-substituted naphthamides (I) containing two and three naphthoyl residues, respectively. With 2-propanol/hexane mobile phases, the N-naphthamides are easily resolved. In this binary solvent mixture, however, the N,O-derivatives, are retained inordinately long at low polarity (P = 0.29) and elute together with no separation at high polarity (P = 1.24). When the polarity is reduced to 0.86, separation occurs, but retention times are still inconveniently long. At polarities sufficiently high to give convenient retention times, adsorption of 2-propanol on the chiral phase obviates resolution of the bulky N,O-derivatives. Simple replacement of 2-propanol with methylene chloride solves the problem. Table IV shows that at a polarity of 2.2 the separation is complete within 12 minutes.

Compound H is a good example of the need for a polar mobile phase selected to minimize non-specific polar adsorption of the enantiomers onto the stationary phase.

TABLE IV

Separation of Racemic Mixtures on a Covalent R-N-(3,5-Dinitrobenzoyl)phenyl Glycine Chiral Stationary Phase[1]

Solute	Chemical Name	Mobile Phase [2]	t_1	t_2	k_1^1	α	t_3	t_4	α[5]
C	3,4-dimethyl-2(β-naphthyl)-5-phenyloxazolidine	1	10.9	11.5	0.73	1.07			
D	7-chloro-1,3-dihydro-3-benzyl-5-phenyl-2H-1,4-benzodiazepin-2-one	1	8.0	19.0	3.44	2.77			
E	β-naphthamide of amphetamine	1	17.8	19.2	9.47	1.09			
F	α-naphthamide of 1(α-naphthyl)ethylamine	4	8.7	13.8	4.1	1.73			
G	1-(α-naphthyl)ethylamine	4	13.3	14.3	6.8	1.09			

H	1-methoxy-3(2-nitro-imidazole)propanol-2-α-naphthoate	8	6.9	8.5	3.06	1.31			
I	N and N,O-α-naphthoyl derivatives of propranolol	2	>25						
		3	9.4	10.3	4.5	1.12	15	16	1.08
		4	7.5	8.3	3.4	1.14	11	11	1.00
		5	4.5	5.1	1.6	1.20	7.5	7.5	1.00
		7	13.8	16.0	6.6	1.18	25.92	29.0	1.11
		8	5.8	6.4	2.4	1.15	10.51	11.6	1.13
		9(3)	4.0	4.0					
		10(4)	1.7	1.7					

(1) Flow rate of mobile phase: 0.5 mL/min with compound C; 2 mL/min with other ocmpounds.
(2) See Table I.
(3) No resolution.
(4) Solute appears at solvent front.
(5) Values of t_3, t_4 and α for N,O-α-naphthoyl derivatives of propranolol.

The nitroimidazole group may bind to the stationary phase and prolong elution of the enantiomers. At a relatively high polarity (2.2), however, methylene chloride provides excellent resolution within 9 minutes. Resolution disappears if 2-propanol is a component of the mobile phase.

Compound G is particularly interesting because it contains an amine group as part of the chiral center. Traditionally underivatized amines have not been chromatographed on this type of chiral column because the relatively non-polar mobile phases used failed to elute such compounds from the column. To solve this problem the hydrogen donor properties of 2-propanol are necessary for complexing with the amine group and thus reducing the attachment of the free amine to the chiral phase. At a solvent ratio of 30/70 2-propanol/hexane, the enantiomers resolve with an α value of 1.09. In this case derivatization is desirable for increasing resolution as well as decreasing the relatively strong binding of the original NH_2 group. Compound F elutes in the same mobile phase with an α value of 1.73; k' decreases from 6.8 to 4.1. Derivatization is not always feasible if compounds must be recovered but it is frequently a simple way to dramatically enhance resolution.

Compound E (amphetamine) is closely related to F inasmuch as both compounds are naphthamides of 1-substituted ethyl amines. The 1-naphthyl substituent in G is replaced by the benzyl group in amphetamine. Since the π-basicity of the benzyl group is lower than that of naphthyl, weaker interaction of E with the 3,5-dinitro-

benzoyl fragment of the stationary phase is expected[3]. For optimum resolution, therefore, compound E should be exposed to a mobile phase of lower polarity than that (1.24) used with F. Even at the low polarity of 0.29, however, the separation factor for E was reduced to 1.09.

Finally, it is encouraging to include in this listing of challenging compounds a pharmaceutically interesting molecule (of the benzodiazepam family), compound D, that resolves in the classical mobile phase with an impressive α value (2.77) and reasonable elution times. This compound contains a substituted amide as part of a seven-membered ring system. The large α value can be attributed to the destabilization of the weaker diastereomeric chiral amino acid solute complex by the benzyl group.

SUMMARY

An increasing number of studies are being published on enantiomeric separation via chiral stationary phases in LC. The two approaches previously reported to increase the applicability of this technique include derivatization of the enantiomers to enhance resolution or increase elutability, and alteration of the chiral moiety on the stationary phase. We have presented a third approach, manipulation of the mobile phase, on a DNBPG chiral column, to enhance specific chiral interactions while minimizing interfering or non-productive interactions. This approach should assist in optimizing chromatography for any enantiomeric pair intrinsically capable of resolution on a DNBPG chiral column.

REFERENCES

1. Feibush, B., Chem. Commun., 544 (1971). Beitler, U. and Feibush, B., J. Chromatogr., 123, 149 (1976).

2. Pirkle, W. H., Finn, j. M., Schreiner, J. L. and Hamper, B. C., J. Am. Chem. Soc., 103, 3964 (1981); Pirkle, W. H., House, D. W. and Finn, J. M., J. Chromatogr., 192, 143 (1980). Pirkle, W. H. and Welch, C. J., Tet. Lett., in press.

3. Pirkle, W. H., Welch, C. J. and Hyun, M. H., J. Org. Chem., in press.

4. Snyder, L. R., and Kirkland, J. J., Introduction to Modern Liquid Chromatography, Second Edition, J. Wiley & Sons, Inc., New York, 1979, p. 260.

5. Weems, H. B. and Yang, S. K., Analyt. Biochemistry, 125, 156 (1982).

6. Snyder, L. R., J. Chromatogr., 255, 3 (1983).

7. Snyder, L. R., J. Chromatogr., 92, 223 (1974).

8. Taft, R. W., Gurka, D., Joris, L., von R. Schleyer, P. and Rakshys, J. W., J. A.m. Chem. Soc. 91, 4801 (1969).

9. Wainer, I. W., Doyle, T. D., Hamidzadeh, Z. and Aldridge, M., J. Chromatogr., 261, 123 (1983).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 731-741 (1984)

THE APPLICATION OF HPLC CHIRAL STATIONARY PHASES TO PHARMACEUTICAL ANALYSIS: THE RESOLUTION OF SOME TROPIC ACID DERIVATIVES

Irving W. Wainer*, Thomas D. Doyle and Christopher D. Breder
Division of Drug Chemistry
Food and Drug Administration
Washington, DC 20204

ABSTRACT

A number of amide and ester derivatives of tropic acid were chromatographed by using a commercially available covalently bonded HPLC chiral stationary phase, (R)-N-(3,5-dinitrobenzoyl)-phenylglycine. The amide derivatives, including pharmacologically important tropicamide, were resolved on this column, but the ester derivatives, including atropine, were not.

INTRODUCTION

Tropic acid, dl-α-(hydroxymethyl)benzeneacetic acid, is a vital component of a number of pharmacologically important molecules. Atropine (dl-hyoscyamine), for example, is the 3-tropanol ester of racemic tropic acid. The pharmacologically active isomer, l-hyoscyamine, is the 3-tropanol ester of l-tropic acid. Because of the pharmacological difference between d- and l-hyoscyamine, there has been a great deal of

 0148-3919/84/0704-0731$3.50/0

interest in the development of an assay for the enantiomeric purity of atropine, which, in fact, is an assay to quantitate the d- and l-tropic acid moieties.

A number of researchers have reported the preparative resolution of tropic acid and atropine. Fodor and Csepreghy (1), for example, resolved tropic acid by the fractional crystallization of the diastereoisomeric D-(-)- and L-(+)-threo-1-(p-nitrophenyl)-2-amino-1,3-propanediol salts. Werner and Miltenberger (2) resolved tropic acid by using camphor-D-sulfonic acid. Although these approaches are successful on a preparative scale, they are not applicable to the analysis of pharmaceutical preparations or biological samples.

Landen and Caine (3) approached an analytical assay for atropine through the synthesis of diastereoisomeric urethane derivatives. They were able to form the diastereoisomers, but were unable to separate them via HPLC. To date, a survey of the literature shows no quantitative method available for the stereochemical determination of dl-tropic acid or dl-hyoscyamine.

The development and commercial introduction of HPLC chiral stationary phases (CSPs) such as the one described by Pirkle et al. (4), (R)-N-(3,5-dinitrobenzoyl)phenylglycine, offer a new approach to the solution of this problem. Pirkle et al. (5) and Wainer and Doyle (6) have shown that this CSP is capable of resolving the enantiomeric amides of α-methylarylacetic acids.

This paper reports the investigation of the applicability of this CSP to the resolution of some tropic acid amide and ester derivatives.

MATERIALS

Apparatus

The chromatography was performed with a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 8000 liquid chromatograph equipped with an SP 8000 data system, a Spectra-Physics Model 8310 UV-visible detector set at 254 nm, and a temperature-controlled column compartment.

The column was a stainless steel, J.T. Baker-packed Pirkle covalent (R)-N-(3,5-dinitrobenzoyl)phenylglycine column (25 cm x 4.6 mm I.D.) with a silica packing of 5-μm spherical particles which were bonded through α-aminopropyl groups to the CSP.

Reagents

Atropine, dl-tropic acid, acetyl chloride, acetic anhydride, thionyl chloride, 1-naphthalenemethylamine (1-NAMA) and 1-naphthalenemethanol (1-NAMOL) were purchased from Aldrich (Milwaukee, WI, U.S.A.). dl-Tropicamide was a reference standard obtained from U.S. Pharmacopeial Convention, Inc. (Rockville, MD, U.S.A.). All HPLC organic solvents were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). The

remaining chemicals were reagent grade and were used as purchased.

METHODS (7)

Synthesis of Acetyltropic Acid (8)

Acetyl chloride (0.28 mole) was added at room temperature to 0.24 mole of tropic acid. The mixture was stirred until a clear liquid formed and then for an additional 5 min. The excess acetyl chloride was removed under a stream of nitrogen, and the resulting viscous oil was cooled until it produced a white crystalline solid.

Synthesis of Acetyltropyl Chloride (8)

Thionyl chloride (0.006 mole) was added to 0.005 mole of acetyltropic acid, and the mixture was stirred at 30°C until the evolution of gas ceased. The excess thionyl chloride was removed under vacuum, yielding a viscous oil which was used without purification.

The acid chloride was also synthesized by using oxalyl chloride (6). Oxalyl chloride (12.5 ml) was added to 0.001 mole of acetyltropic acid, and the mixture was heated at 60°C for 15 min. The excess oxalyl chloride was evaporated under a stream of nitrogen and the resulting viscous oil was used directly.

Synthesis of Acetyltropic Acid Naphthalenemethylamide

Acetyltropyl chloride was synthesized by starting with 0.001 mole of acetyltropic acid and following the oxalyl chloride procedure described above. Chloroform (15 ml) followed by 1-NAMA (0.006 mole) was added to the acid chloride and the solution was stirred overnight. The solution was then washed successively with two portions of 4N HCl and one portion of H_2O. The chloroform layer was collected and dried over anhydrous sodium sulfate. Evaporation of the solvent yielded a colorless crystalline solid which was characterized by IR and NMR analysis.

Synthesis of Tropic Acid Naphthalenemethylamide

Acetyltropic acid naphthalenemethylamide synthesized above was refluxed on a steam bath for 1 h with 3N HCl. After the mixture was allowed to cool, the pH was adjusted to 9 with ammonium hydroxide and the mixture was extracted with chloroform. The chloroform layer was collected, dried over sodium sulfate and evaporated, yielding a colorless solid. The solid was recrystallized from ethyl acetate/hexane and characterized by using IR and NMR.

Synthesis of Acetyltropic Acid Naphthalenemethylester

Acetyltropyl chloride was synthesized by starting with 0.002 mole of acetyltropic acid and using the method involving thionyl

chloride described above. The resulting oil was dissolved in 25 ml of chloroform and 0.003 mole of 1-NAMOL was added. The resulting solution was stirred overnight. The chloroform was washed successively with three portions of 4N HCl, with one portion of H_2O, and finally with a saturated sodium bicarbonate solution. The chloroform layer was collected and dried over sodium sulfate and the chloroform was evaporated. The resulting viscous oil was characterized by IR and NMR analysis.

Synthesis of Acetyltropicamide

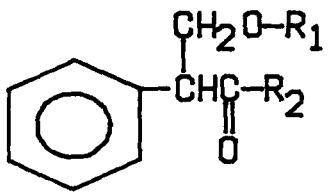
Acetic anhydride (0.008 mole) was added to 0.007 mole of tropicamide and the mixture was heated until clear. The resulting solution was mixed with chloroform and the chloroform layer was washed with 0.1N NaOH. The chloroform layer was collected, dried over sodium sulfate and evaporated to yield a colorless solid which was characterized by IR and NMR analysis.

Chromatographic Conditions

The compounds were chromatographed by using mobile phases of hexane and isopropanol mixed in various proportions (Table 1). The flow rate was 2 ml/min and the column temperature was 20°C.

TABLE 1

Chromatographic Results



Compound	R_1	R_2	k_1'[a]	α	R_s	Mobile Phase[b]
1 (amide)	acetyl	1-NAMA[c]	13.0	1.13	1.29	90:10
2 (amide)	H	1-NAMA	6.5	1.11	0.89	90:10
3 (ester)	acetyl	1-NAMOL[d]	5.3	1.00	0.00	95:5
4 (amide)	H	NENPMA[e]	15.3	1.03	0.39	95:5
5 (amide)	acetyl	NENPMA	12.9	1.08	0.57	95:5
6 (ester)	H	3-tropanol	33.6	1.00	0.00	85:15

[a]Capacity factor of first eluted enantiomer.
[b]The mobile phase was a mixture of hexane:isopropanol; the flow rate was 2 ml/min and the column temperature was 20°C.
[c]1-Naphthalenemethylamine.
[d]1-Naphthalenemethanol.
[e]*N*-Ethyl-*N*-(4-pyridinylmethyl)amine.

RESULTS

Attempts to synthesize the acid chloride of tropic acid directly were unsuccessful; it was necessary to first convert tropic acid to the *O*-acetyl derivative. Acetyl tropic acid was easily converted to the acid chloride by using either thionyl chloride or oxalyl chloride; the desired amide or ester

derivative was then readily obtained by the addition of the appropriate amine or alcohol.

The enantiomeric amides formed from dl-acetyltropyl chloride and l-NAMA, compound 1, Table 1, were resolved by the CSP; the separation factor (α) = 1.13. To determine the effect of the O-acetyl function on this separation, the acetyl moiety was removed by acid hydrolysis (8) and the resulting product, compound 2, was chromatographed. There was essentially no change in the resolution of the two compounds; α = 1.13 vs 1.11 for 1 and 2, respectively. However, there was a decrease in the resolution factor (R_s) when the acetyl group was removed, i.e., 1.29 vs 0.89 for 1 and 2, respectively.

The enantiomeric esters formed from dl-acetyl tropic acid chloride and l-NAMOL, compound 3, were not resolved by the CSP under chromatographic conditions similar to those which resolved the amides. Atropine, 6, the tropine ester of dl-tropic acid, was also not resolved by the CSP.

The anticholinergic agent tropicamide, compound 4, which is used in ophthalmic preparations, was resolved directly on the CSP without derivatization; α = 1.03. This compound is the N-ethyl-N-(4-pyridinylmethyl)amide of dl-tropic acid. To determine the effect of an O-acetyl group on this resolution, tropicamide was derivatized by using acetic anhydride. There was a slight increase in the resolution of the resulting compound, 5; α = 1.08 vs 1.03, for 5 and 4, respectively; there was also an increase in R_s, 0.57 vs 0.39.

DISCUSSION

Pirkle et al. (5,9) have suggested that the chiral recognition mechanism for amides on this CSP involves the formation of a CSP-solute complex which is dependent upon a dipole-dipole interaction between the 3,5-dinitrobenzoylamide moiety on the CSP and the amide moiety on the solute. The steric environment at the chiral center determines the stability of the complex, and, thus, the resulting resolution and order of enantiomeric elution.

Work in this laboratory on the resolution of α-methylaryl-acetic acids (6) supports this postulate. It was found that the amide derivatives of the compounds studied were resolved on the CSP, whereas corresponding ester derivatives were not. This difference was explained on the basis of the difference in dipole moments between amides and esters and the resulting difference in the strength of the interaction between the CSP and the solute, which, in turn, affects the ability of the CSP to differentiate between the enantiomers.

The results of this study are consistent with the proposed chiral recognition mechanism. The slight difference in resolution between the amides of tropic acid, 2 and 4, and the amides of O-acetyltropic acid, 1 and 5, seems to indicate that neither hydrogen bonding involving the hydroxyl hydrogen in the unacetylated molecule nor hydrogen bonding involving the carbonyl function in the acetylated derivative plays a key role

in the formation of the CSP-solute complex. However, since these groups are part of the steric environment surrounding the chiral center, they probably play a role in the chiral recognition process once the complex is formed.

On the other hand, the fact that the amides are resolved and the esters, 3 and 6, are not, suggests that the dipole strength of the carbonyl derivative of tropic acid is a major factor in the formation of the CSP-solute complex. The amide-amide interaction appears to lead to the formation of a strong CSP-solute complex which promotes the chiral recognition process. The amide-ester dipole interaction, however, produces a weaker complex; compare, for example, the capacity factors of amide 1 and ester 3. Therefore, there is no effective discrimination between the ester enantiomers.

In light of the proposed chiral recognition mechanism, it is not surprising that atropine was not resolved on this CSP. The solution to this analytical problem perhaps awaits the development of alternative CSPs that are effective in the resolution of esters as a class.

REFERENCES

1. Fodor, G. and Csepreghy, G., Egyesult Gyogyszer es Tapszergyar, Austrian Patent 222,814, Aug. 10 (1962); Chem. Abstr., 57, 15016d.

2. Werner, G. and Miltenberger, K., Zur Trennung der optischen Antipoden von Homatropin und Atropin; Synthese von L(+)- und D(-)-Homatropin-sulfat, Justus Liebigs Ann. Chem., 631, 163 (1960).

3. Landen, W.O. and Caine, D.S., Preparation of Diastereomeric Urethane Derivatives of Atropine and l-Hyoscyamine Using (-)-1-Phenylethylisocyanate, J. Pharm. Sci., 68, 1039 (1979).

4. Pirkle, W.H., Finn, J.M., Schreiner, J.L. and Hamper, B.C., A Widely Useful Chiral Stationary Phase for the High-Performance Liquid Chromatography Separation of Enantiomers, J. Am. Chem. Soc., 103, 3964 (1981).

5. Pirkle, W.H., Finn, J.M., Hamper, B.C., Schreiner, J. and Pribish, J.R., A Useful and Conveniently Accessible Chiral Stationary Phase for the Liquid Chromatographic Separation of Enantiomers, in Eliel, E.L. and Otsuka, S., eds., ACS Symposium Series, No. 185, Asymmetric Reactions and Processes in Chemistry, Am. Chem. Soc., Washington, DC, U.S.A. (1982), pp. 245-260.

6. Wainer, I.W. and Doyle, T.D., Application of High-Performance Liquid Chromatographic Chiral Stationary Phases to Pharmaceutical Analysis: Structural and Conformational Effects in the Direct Enantiomeric Resolution of α-Methylarylacetic Acid Anti-Inflammatory Agents, J. Chromatogr., submitted.

7. The compounds synthesized in this work were unambiguously identified by IR and NMR analysis and will be fully characterized elsewhere.

8. Rey-Bellet, G., Tropic Acid N-(ß-picolyl)-N-lower-alkenylamides, U.S. Patent 2,677,689, May 4 (1954); Chem. Abstr., 50, 1089g.

9. Pirkle, W.H., private communication, manuscript in preparation.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 743-752 (1984)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 5-FLUOROCYTOSINE IN HUMAN PLASMA.

S. Bouquet, S. Quehen, A.M. Brisson, Ph. Courtois, J.B. Fourtillan.
Laboratoire de Pharmacocinétique - Centre Hospitalier Universitaire
Jean-Bernard - 86021 Poitiers Cedex France.

ABSTRACT

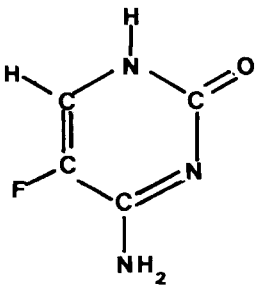
A high performance liquid chromatographic method has been developed for the determination of an antifungal drug : 5-Fluorocytosine (5-FC), in human plasma, using reversed-phase technique.

The rapid method involved single ethyl acetate extraction, in the presence of an internal standard (5-Fluorouracil). The eluent mixture was a pH 4.8 acetate buffer. A wavelength of 280 nm was used to monitor 5-FC and the internal standard.The limit of sensitivity of the assay was 0.6 µg/ml with a precision of $\pm$ 8 %. This method is used to quantitative 5-Fluorocytosine in human plasma from renal failure patients, with satisfactory accuracy and precision. Endogenous substances and a variety of drugs concomitantly used in (5-FC) therapy did not interfere with the assay.

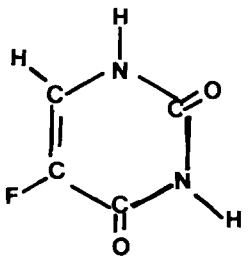
INTRODUCTION

5-Fluorocytosine (5-FC), (4-Amino, 5-fluoro, 2-oxo, 1,2 dihydropyrimidine), is a derivative, with antifungal properties in several systemic mycotic infections including cryptococcal meningitidis, visceral candidiasis, torulopsis, chromomycosis (1, 2, 9).

 0148-3919/84/0704-0743$3.50/0



- 1 -
5-Fluorocytosine
(4-amino-5 fluoro-2 oxo-1-2 dihydropyrimidine)



- 2 -
5-Fluorouracil
(5-fluoro-2-4-dioxopyrimidique)

About 90 % of 5-Fluorocytosine is excreted unaltered in the urine (4, 7, 8, 12, 14, 16). The measurement of plasma 5-Fluorocytosine allows the dosage to be adjusted in patients suffering pathological conditions, e.g., when renal function is compromised by renal disease or from chronic drug administration. The determination of antifungal agents in biological fluids is often performed by microbiological assay procedures (11, 15). These assay techniques are long and not very specific, especially when the patients involved have received multiple antimicrobial agents.

A gas liquid chromatographic (G.L.C.) assay has been described (10). This method is not very rapid and has the disadvantage of low recovery (35 %).

Recently, two high performance liquid chromatographic (H.P.L.C.) procedures, with the use of thermostated cation exchange columns and direct injections of plasma onto column (3) deproteinization of plasma before assay (6), have been developed.

We report the development of a reversed-phase H.P.L.C. assay requiring only simple extraction with ethyl acetate, and using a structurally related agent (5-Fluorouracil) as the internal standard.

This method is rapid, selective and reproducible and has been used for plasma samples obtained following pharmacokinetic in renal failure patients.

METHODS

The procedure involves the addition of 5-Fluorouracil (5-FU) as the internal standard. After addition of 1.0 ml phosphate buffer (pH 7.0), plasma samples are extracted using ethyl acetate.

After evaporation of the organic solvent, the residue is dissolved in the mobile phase and the drug is analysed isocratically by reversed-phase liquid chromatography with pH 4.8 acetate buffer as eluant. The effluent is monitored by U.V. detection at 280 nm.

Apparatus

The H.P.L.C. system consists of a Model 6000-A solvent delivery system and a Model 710-A WISP sample injector (Waters Associates, Inc., Milford, Mass, 01757, U.S.A.).

A model 440 absorbance detector (280 nm wavelength) (Waters Associates, Inc., Milford., Mass, 01757, U.S.A.) was used at a sensitivity of 0.01 absorbance units full scale (a.u.f.s.) for plasma samples. The chromatograms were recorded on an Omniscribe (Houston Instruments, Gistel, Belgium).

The mobile phase : acetate buffer (pH 4.8) and methanol (99: 1, v/v) was filtered through a 0.45 µm membrane filter type GS-cellulose ester (Millipore Corp., Bedford, Mass, 01730) and carried through an octadecylsilane µ-Bondapak C_{18} column (30 cm x 3.9 mm, particle size 10 µm, Waters Assoc.) at 1 ml/min. and ambient temperature.

Under these conditions, 5-Fluorocytosine and the internal standard (5-FU) were eluted with retention times of 5.4 and 6.5 min ., respectively, as illustrated in Figure 1.

Chemical and reagents :

5-Fluorocytosine (5-FC) and 5-Fluorouracil (5-FU) were both supplied by Roche Laboratories (Neuilly - France). Water was doubly distilled and filtered through a 0.22 µm (type GS-cellulose ester) Filter (Millipore, Corp., Bedford, Mass, 01730).

Standard stock solutions of 5-FC and internal standard were prepared by dissolving solid standards in bidistilled water at concentrations of 400 µg/ml and 100 µg/ml respectively and could be stored at 4°C for two weeks in amber glass containers. Standard concentrations of 5-Fluorocytosine in plasma ranging from 0.625 to 20 µg/ml were made by appropriate dilution of the stock solution.

- All organic solvents (methanol, ethyl acetate) were H.P.L.C. grade (Carlo Erba, Milan, Italy).
- Acetic acid (Art. 63, Merck, Darmstadt G.F.R.).
- Sodium acetate (R.P. Prolabo, France).
- Phosphate buffer, pH 7.0, was prepared by dissolving 7.72 g of disodium hydrogen phosphate. 2 H_2O (Merck) and 3.18 g of potassium dihydrogen-phosphate in 1000 ml of bidistilled water.

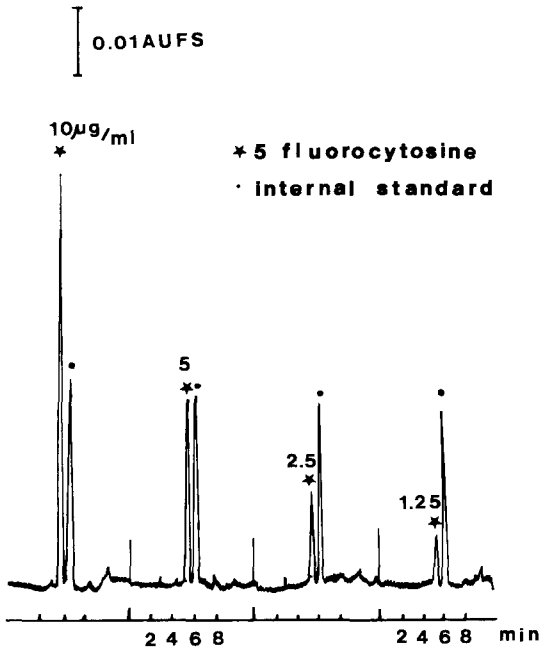


Figure 1 : Chromatogram of 5-Fluorocytosine.

- Acetate buffer (pH 4.8) was prepared by mixing 10 ml of a 0.2 M acetic with 15 ml of 0.33 M sodium acetate and 975.0 ml of bi-distilled water.

Extraction procedure :

In a 10 ml screw-capped tube, one milliliter of plasma (sample to be assayed or standard) was supplemented with 50 µl of an aqueous solution (5 µg/ml) of the internal standard and 1.0 ml of pH 7 phosphate buffer, then homogenized by slow rotation.

The drug was extracted with 6.0 ml of ethyl acetate by shaking mechanically for 10 min. The two phases were separated by centrifugation for 15 min. at 3500 rpm. An aliquot of the upper organic layer was transferred to another clean glass tube and evapored to dryness under a stream of nitrogen at 40°C.

The dry residue was redissolved into 200 µl of mobile phase and an aliquot of 20 µl was injected into the H.P.L.C. system.

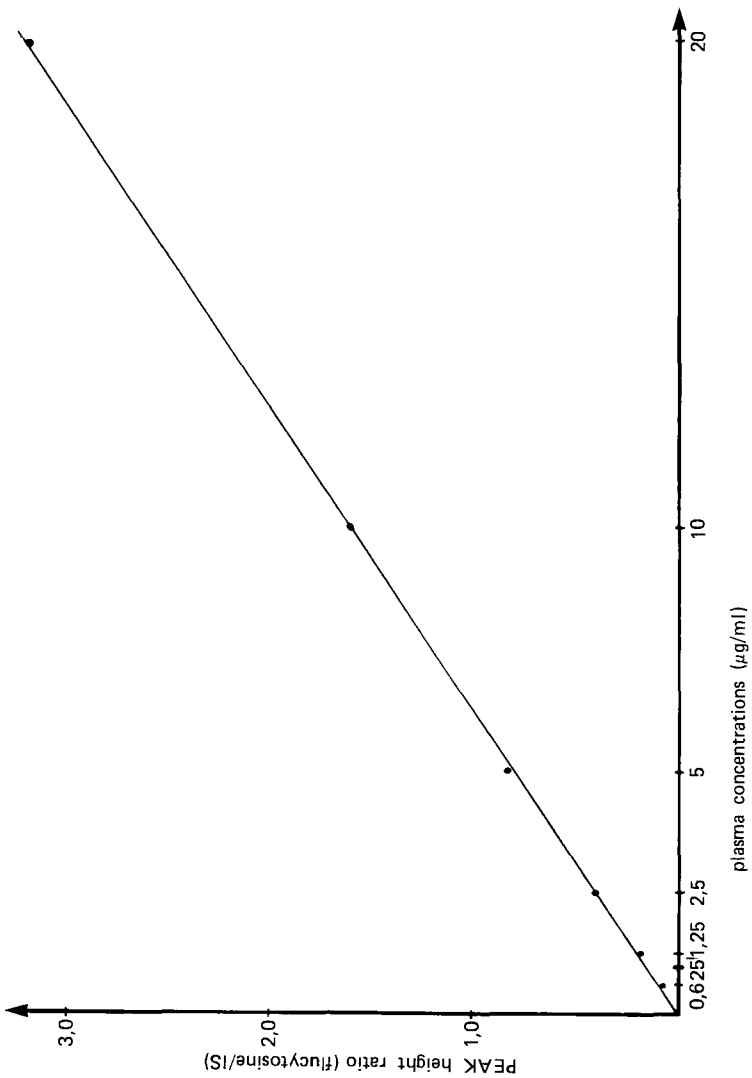


Figure 2 : Linear regression analysis of 5-Fluorocytosine.

Table I : INTRA-ASSAY PRECISION 5-FLUOROCYTOSINE IN HUMAN PLASMA.

Spiked concentration (µg/ml)	Measured concentration (n = 10) Mean ± s.d. (µg/ml)	C.V. (%)
1.0	0.94 ± 0.07	8.1
20.0	20.1 ± 0.80	4.1

Calibration :

A standard concentration curve (Fig. 2) was obtained by adding 5-Fluorocytosine at concentrations of 0.625, 1.25, 2.5, 5.0, 10, 20 µg/ml in control plasma under the same experimental conditions as used for sample analysis.

RESULTS AND DISCUSSION

We found a linear correlation between the concentration of 5-FC and the ratio of peak heights : 5-FC/i.s, in the range 0.625 to 20 µg/ml.

Each point of Figure 2 represents an average of three determinations. The line drawn is the least-squares regression line of equation.

$y = 0.1608\ x - 0.00696$ (x = 0.625 to 20 µg/ml, $n = 6$, $r = 0.9998$).

The intra-assay precision for 5-FC was assessed by repeated analysis on fresh drug-free human specimens spiked with known concentrations of 5-FC. As shown in Table I, within-day precision of the method, the coefficient of variation was 4.1. and 8.1 for 20.0 and 1.0 µg/ml respectively. The inter-assay precision, evaluated by analysing spiked plasma samples on different days over one week ($n = 5$), was found to be ± 5.8 % for samples to concentration 10 µg/ml.

Figure 4 illustrates the chromatographic profile of a human plasma from a patient with renal failure receiving daily infusion administration 1.25 g of 5-FC (15 th hour sample after end of infusion). The stability of samples was tested from spiked human plasma. The samples were stored deep-frozen. Frozen samples remained stable for at least two months.

As can be seen from Figure 3, no plasma constituent peak extracted from the blank interferes with that of 5-Fluorocytosine and i.s., which is well below the detection limit.

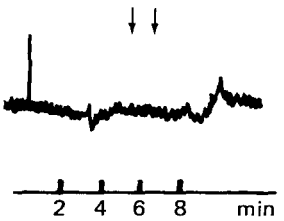


Figure 3 : Chromatograph of human drug free plasma extract.

The limit of detection of this method was 0.6 µg/ml, allowing a signal-to-noise ratio of 3, when 1.0 ml of plasma was used (Figure 5).

The sensitivity of this H.P.L.C. procedure is thus found to be much higher than obtained previously (1.0 µg/ml), using a cation-exchange chromatographic system (3,6).

The buffer and extraction solvent chosen here were, however, found to lead to a minimum of interference in the analysis. The pH and the type of eluent are important when analyzing plasma samples. Using acetate buffer at pH of 4.8, no endogenous peak is detected.

No interfering peak were observed in the plasma of patients receiving 5-Fluorocytosine in combination with drugs such as : amphotericin B, miconazole, carbenicillin, ticarcillin, piperacillin, penicillin G, ampicillin, amoxicillin, cefadroxyl, cefazolin, cefotaxim, moxalactam, cefoxitin, vidarabin.

The life time of the column appears to be very good, as it is still in excellent condition after 6 months use.

The G.L.C. assay (10) requires a total clean up at least 2 h, due to a multitude of diverse extraction, reextraction and centrifugation steps. The H.P.L.C. method described here involves a single extraction procedure. A.D. Blair et al (3) reported a greater precision and speed of the H.P.L.C. method comparatively with the microbiological assay (11, 15). However, we have not compared the present H.P.L.C. assay to the microbiological method. 5-Fluorouracil (5-FU) was retained as internal standard, because 5-Fluorocytosine was reported not to be metabolized to 5-Fluorouracil in mammalian tissues (5).

In conclusion, the H.P.L.C. procedure proposed here is a rapid, sensitive and reproducible technique for the determination of 5-FC.

The sensitivity and rapidity are better than that reported previously with microbiological assay (11, 15) and earlier H.P.L.C. methods (3,6) using an ion-exchanged column with heating.

The chromatograms are interference-free of normal components of fresh human plasma as well as from drugs simultaneously administered. We

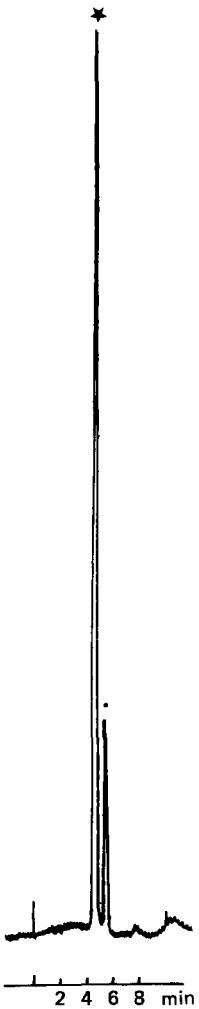


Figure 4 : Chromatogram of plasma extract from a patient receiving 1.25 g of 5-Fluorocytosine.
(15 th hour sample after end of infusion).

loop: 25 µl, S=0.01 a.u.f.s.
✱ 5-Fluorocytosine .(i.s)
21.6 µg/ml of plasma

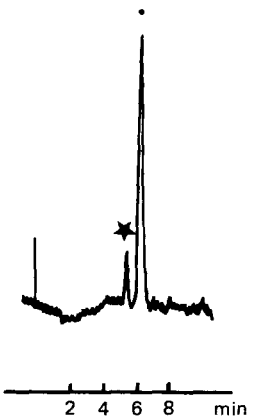


Figure 5 : Chromatogram from a spiked plasma sample (0.6 µg/ml) showing the detection limit of the method.

loop: 20 µl, S=0.01 a.u.f.s.
✱5-Fluorocytosine .(i.s.)

have found that this H.P.L.C. procedure is particulary valuable where rapid determination of 5-FC has been necessary, such as in patients with renal failure.

Acknowledgement

The authors express their grateful thanks to M.C. Garandeau and M.P. Deshoulieres.

REFERENCES

(1) J.E. Bennett, "Medical intelligence-drug therapy-chemotherapy of systemic mycoses", N. Engl. J. Med., **3** : 95, (1973).

(2) J.E. Bennet, "Flucytosine", Ann. Intern. Med., **86** : 319, (1977).

(3) A.D. Blair, A.W. Forrey, B.T. Meijsen, R.E. Cutler, "Assay of flucytosine and furosemide by high-pressure liquid chromatography", J. Pharm. Sci., **64**, 1334, (1975).

(4) E.R. Block, J.E. Bennett, "Pharmacological studies with 5-Fluorocytosine", Antimicrob. Ag. Chemother., **1** : 476, (1972).

(5) S. Chaube, M.L. Murphy, "The teratogenic effects of 5-Fluorocytosine in the rat", Cancer Res., **29**, 554, (1969).

(6) R.B. Diasio, M.E. Wilburn, S. Shadomy, A. Espinel-Ingroff, "Rapid determination of serum 5-Fluorocytosine levels by high-performance liquid chromatography", Antimicrob. Ag. Chemother., **13**, 500, (1978).

(7) E. Drouhet, P. Babinet, J.P. Chapusot, D. Kleinknecht, "5-Fluorocytosine in the treatment of candidiasis with acute renal insufficiency. Its kinetics during haemodialysis and peritoneal dialysis", Biomed, **19**, 408, (1973).

(8) E. Drouhet, J.C. Borderon, E. Borderon, P. Boulard, "Evolution des concentrations sériques de 5-Fluorocytosine chez les prématurés", Bull. Doc. Franç. Mycol. Med., **3**, 37, (1974).

(9) R.J. Fass, R.L. Perkins, "5-Fluorocytosine in the treatment of cryptococcal and candida mycoses", Ann. Intern. med., **74**, 535, (1971).

(10) S.A. Harding, G.F. Johnson, H.M. Solomon, "Gas-chromatographic determination of 5-Fluorocytosine in human serum", Clin. Chem., **22**, 772, (1976).

(11) R.L. Kaspar, D.J. Drutz, "Rapid simple biossay for 5-Fluorocytosine in the presence of amphotericin B", Antimicrob. Ag. Chemother., **7**, 462, (1975).

(12) B.A. Koechlin, F. Rubio, S. Palmer, T. Gabriel, R. Duschinsky, The metabolism of 5-Fluorocytosine-2-^{14}C and of cytosine ^{14}C in the rat and the disposition of 5-Fluorocytosine-2-^{14}C in man", Biochem. Pharmacol., **15**, 435, (1966).

(13) J.S. Remington, "Toxicity of 5-Fluorocytosine", Ann. Intern. Med., **76**, 830, (1972).

(14) J. Schönebeck, A. Polak, M. Fernex, H.J. Scholer, "Pharmacokinetic studies on the antimycotic agent 5-Fluorocytosine in individuals with normal and impaired kidney function", Chemother., **18**, 321, (1973).

(15) S. Shadomy, "Technique for bioassaying body fluids for antifungal agents" p. 143-146, In L.D. Haley and P.G. Standard (Ed), Laboratory methods in medical mycology, 3nd ed. U.S. Department of Health, education and welfare, Public Health Service, Atlanta, Ga.

(16) D.N. Wade, G. Sudlow, "The kinetics of 5-Fluorocytosine elimination in Man", Aust. N.Z.J. Med., **2**, 153, (1972).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 753-763 (1984)

DETERMINATION OF NITRATE AND NITRITE IONS IN HUMAN PLASMA BY ION EXCHANGE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

J. Osterloh and D. Goldfield
Northern California Occupational Health Center
San Francisco General Hospital Medical Center
1001 Potrero Avenue
San Francisco, California 94110

ABSTRACT

Acetonitrile precipitation of plasma samples followed by injection of supernatant onto a reverse phase precolumn coupled to an anion exchange column allowed ultraviolet detection (214 nm) of eluting nitrate and nitrite ions. Sensitivity in plasma is about 0.01 mM for both ions and linearity is excellent from 0.02 to 1.0 mM. Nitrite accuracy assessed by diazotization coupling was good. Reproducibility studies demonstrated within-run coefficients of variation of < 4%. Interferences were few. Random endogenous serum nitrate concentrations (0.03-0.12 mM) were determined. Serum nitrite and nitrate concentrations were measured in a patient following an overdosage of isobutyl nitrite. The method is applicable for nitrite/nitrate studies in plasma at these concentrations.

INTRODUCTION

Many methods for measuring nitrite and nitrate are available (1). Few are applicable to measuring these analytes in biologic material (2). Modified Griess reactions with and without reduction of nitrate to nitrite are commonly employed (3,4).

 0148-3919/84/0704-0753$3.50/0

Variability is introduced for nitrate quantitation by the reduction process through interferences and blanking procedures (5). However, the diazotization coupling reaction for NO_2^- alone performs well. Nitration assays followed by gas chromatography may suffer from poor recoveries or use of dangerous chemicals (2,6). Preparatory cleanup with anion exchange columns may be incorporated into any of the methods. This requires larger samples for preconcentration and may introduce some variation while reducing background and increasing sensitivity (7). Anion exchange pretreatment has been used successfully prior to chemiluminescence determination on urine samples for NO_2^- and NO_3^- (8). Neither nitrate nor nitrite were measured directly and although quite sensitive for nitrite, the procedure is long and special apparatus is required.

Measurement of nitrate and nitrite by high pressure chromatographic anion exchange or ion pair technique offers a more direct approach (9-13) at least in water, waste waters, and brine. These techniques have not been applied to serum samples possibly due to interferences from many other anions (approximately 150 mM total plasma anions). In rare environmental situations sample preconcentration is required for determinations below 0.02 mM (1 ppm) by these techniques. However, low ppm sensitivity is adequate for nitrate in human serum as will be demonstrated. Suppression of chemical and electronic backgrounds is normally necessary in other methods, but is not required of the liquid

chromatographic method presented herein because of the unique combination of sample preparation, precolumn-column pairing, mobile phase, and detection techniques used. This method was developed for use in studying nitrite-nitrate interconversion in human blood.

EXPERIMENTAL

High pressure liquid chromatographic conditions

A Beckman (Berkeley, CA, USA) Model 110A high pressure pump and Model 210 injector equipped with 20 or 100 µl sample loops for injection and elution of a Whatman (Clifton, NJ, USA) precolumn (2.1 mm I.D. x 60 mm) packed with 35 µ CO:PELL ODS (C-18 pellicular) coupled to a polystyrenedivinylbenzene-based strong anion exchange column (4.1 mm I.D. x 250 mm) from Wescan Instruments (Santa Clara, CA, USA) were used. Detection was by ultraviolet absorption at 214 nm using a Beckman Model 160 detector at 0.1-0.5 AUFS per 10 mV output. Final mobile phase composition was 50 mM NaH_2PO_4, 3mM NaCl, and 4 mM acetic acid in water (final pH = 3.95). Reverse osmosis/deionized water was used and all mobile phases were filtered with Ultipor NR nylon-66, 0.22 µ inert filters (Woburn, MA, USA) under 5 mm Hg vacuum. Flow rate was 4.0 ml/min.

Sample and standards preparation

Serum or plasma preparation was by acetonitrile precipitation of proteins. Chromatographic grade acetonitrile (400 µl) and

serum (200 µl) were vortexed in 1.5 ml Sarstedt (Princeton, NJ, USA) capped centrifuge tubes and then centrifuged at 15,000 r.p.m. on a Brinkmann (Westbury, NY, USA) Model 5412 centrifuge for 2 min. Exactly 20 or 50 µl of the supernatant was injected. A sample injection loop improves reproducibility. Peak heights of sample nitrite and nitrate peaks were compared with standard curve peak heights for quantitation.

Other preparatory cleanup procedures were attempted including dilution, methanol precipitation, ultrafiltration, and bonded anion exchange resins. Background interferences were problems with all but the exchange resin techniques and acetonitrile precipitation. Exchange resins provided clean baselines and the opportunity for approximately a twofold preconcentration of the sample. However, it was determined that a separate column type was required for each nitrate or nitrite and variation in recovery was difficult to control. Analytichem (Harbor City, CA, USA) Bond-Elute primary amine-bonded column could be used for nitrite and Bond-Elute quarternary amine column could be used for nitrate. Elution of nitrate required strong molarity salt solutions that interfered with subsequent ion chromatography. Nitrate was not retained on the weaker anion exchange column. Reproducibility and recovery varied on these columns probably because of the many competing anions already present in serum. Acetonitrile precipitation was easy and was expected to introduce little variation and have adequate recovery.

Standards were made in banked plasma, serum, and water using sodium nitrate and sodium nitrite at 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 1.0, 2.0, and 3.0 mM. Nitrate and nitrite are stable for several weeks in dilute aqueous solutions that are kept cold and dark. Nitrite plasma standards lose 5% of initial concentrations by 3 hr at room temperature. Fresh plasma standards should be made daily. Linearity, reproducibility, background specificity, nitrite accuracy by diazo-coupling assay (14) and interference studies were performed. Interferences were tested using analytic grade chemicals dissolved in water and mixed with acetonitrile as described earlier. Random assayed serum samples were frozen (-15°C); i.e., clinical specimens and one sample from a patient who had ingested isobutyl nitrite.

RESULTS

Table 1 shows the various experimental mobile phases and nitrate and nitrite retention times. For all the listed mobile phases, capacitances and resolutions were adequate. Peak efficiency was poor (tailing) with phosphate buffers alone. Addition of sodium chloride improves peak efficiency alone or in combination with phosphate buffers, but sodium chloride has high background absorbance. Therefore, sodium chloride and phosphate buffer was optimized to give the least background absorbance but adequate peak efficiency. Addition of acetic acid effected elution of interfering anions (probably organic) after the nitrate

TABLE 1

Effect of various mobile phase compositions on retention time of nitrite and nitrate ions

Mobile phase composition (mM)			Retention time (min)*		
NaH_2PO_4	NaCl	CH_3COO^-	NO_2^-	NO_3^-	Comment
50	0	0	6.5	10.0	Tailing peaks
60	8	0	6.5	9.8	Endogenous interference at NO_2^-
100	8	0	4.8	7.8	Endogenous interference at NO_2^-
110	20	0	3.5	4.8	Endogenous interference at NO_2^-
120	3	0	4.0	5.5	Endogenous interference at NO_2^-
50	3	0	5.8	8.5	Endogenous interference at NO_2^-
35	3	0	6.8	10.5	Minor interference at NO_2^-
35	3	4	5.0	7.5	No interference
65	3	2	4.0	6.0	No interference
60	3	3	4.0	6.0	No interference

* Flow rate = 3.0 ml/min.

and nitrite peaks. Sample chromatograms of nitrate and nitrite in plasma are shown in Figure 1. Retention times of nitrite and nitrate ion were 3.2 and 4.6 min (4.0 ml/min), respectively. Because of the acetonitrile precipitation, the majority of proteins are not introduced onto the column. The reverse phase (C-18)

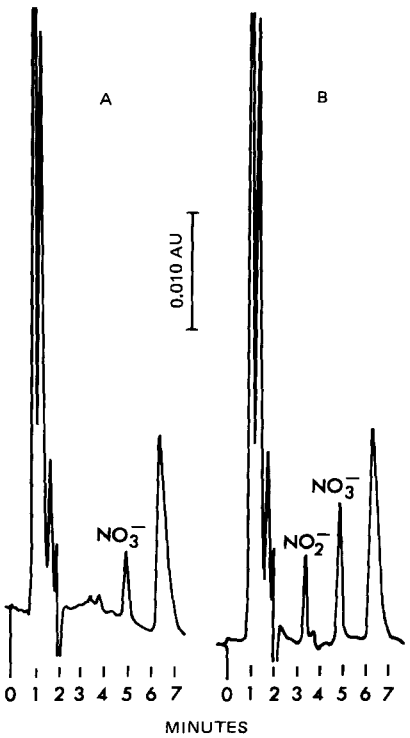


Figure 1. Chromatographic tracings: A, blank plasma with 0.05 mM endogenous nitrate; B, a plasma standard with 0.10 mM nitrite and nitrate added.

precolumn is effective in further removing organic substances. Five millimolar concentrations of fluoride, chloride, phosphates, and sulfates are transparent at 214 nm UV. Bromide (3.2 min) and iodide (9.2 min) absorb at millimolar concentrations, but usually are not present in sufficient quantities. Chloride in serum (100 mM) precipitates produces a peak at 1.5 min.

Previous ion exchange techniques have used conductivity detectors and elution with an organic anion such as phthalate (11-13). High phthalate absorbance prohibits measurement of several other anions with ultraviolet absorbance detection. With conductivity detectors high concentrations of serum anions (chloride 100 mM, bicarbonate 20 mM) can overwhelm the detector relative to the lower concentrations of nitrite and nitrate. In a preliminary attempt on a similar anion-ion exchange high pressure liquid chromatographic system with a conductivity detector, neither supernatants of acetonitrile-precipitated serum nor high molarity salt eluates from preparatory anion exchange columns could be analyzed because of this problem. Therefore, in the described system, the wavelength of ultraviolet detection, phosphate rather than phthalate buffer, sample preparation and the reverse phase precolumn improved the assay specificity and allowed measurement of nitrate and nitrite in serum.

Linearity studies demonstrated regression to zero in aqueous standards and demonstrated a positive bias of 0.04 mM for nitrate and 0.004 mM for nitrite in plasma. The nitrate bias is due to endogenous nitrate in plasma. The nitrite bias is due to unknown interferences that prohibited sensitivity to < 0.01 mM in serum. Sensitivity for nitrite and nitrate in water was 0.005 mM. Sensitivity for nitrate in plasma was 0.01 mM above the endogenous nitrate value. Linearity was good over the fiftyfold range tested with aqueous and plasma regression coefficients of 0.9995

and 0.9996, respectively, for nitrite and 0.9996 and 0.9993, respectively, for nitrate. When plasma samples were compared with aqueous samples at the same concentrations, the recovery was 97% for nitrate and 82.8% for nitrite. Because sample manipulation is minimal, nitrite losses must be due to protein binding or chemical reaction in plasma. Even though biases can be measured and subtracted using aqueous standards, plasma standards must be used to account for recovery because the method cannot be conveniently standardized internally with another anion. Internal standardization is difficult because other suitable anions cannot be detected, interfere, or compete with the anion exchange process. Accuracy of nitrite determinations by high pressure liquid chromatography was compared with the diazotization-coupling reaction method. Actual plasma samples were used when nitrite concentrations were changing with time (2.73 mM to 0.2 mM) and both analyses on each sample were performed within 10 min of each other. The correlation coefficient, slope, and intercept between two methods were 0.9983, 0.821, and -0.003 (y = high pressure liquid chromatography method), respectively. Although the correlation was quite good between the two methods with no constant bias (intercept), the proportionate bias (slope) of 0.821 indicates lesser recovery by the high pressure liquid chromatography method and is consistent with the recovery study mentioned earlier. Within-run reproducibility (n = 5) was excellent at concentrations of 0.10 mM for both nitrate and

nitrite showing within-run coefficients of variation of 2.6% and 3.3%, respectively. This is probably a result of minimal preparatory sample manipulation.

Five random normal sera were tested for nitrate and nitrite. Nitrate concentrations ranged from 0.03-0.12 mM (mean = 0.06) and nitrite concentrations were not detectable. In a case of ingested isobutyl nitrite overdose, serum taken several hours after the overdose showed a nitrite concentration of 0.14 mM and nitrate of 1.08 mM. The assay presented is simple, reproducible, linear, and accurate. Single serum samples can be assayed in 8 min. Such a procedure is useful for rapid analysis of nitrite and nitrate during their transformation in human plasma.

ACKNOWLEDGMENTS

This work was supported in part by the Academic Senate Committee on Research, University of California, San Francisco.

REFERENCES

1. Standard Methods - for the Examination of Water and Wastewater (15th Ed.). American Public Health Association, 367-383, 1980.

2. Radomski, J.L., Palmiri, C. and Hearn, W.L., Toxicol. Appl. Pharmacol. 45, 62-68, 1978.

3. Tannenbaum, S.R., Fett, D., Young, V.R., Land, P.D. and Bruce, W.R., Science 200, 1487-1489, 1978.

4. Sen, N.P. and Donaldson, B., J. Assoc. Off. Anal. Chem., 61, 1389-1394, 1978.

5. Witter, J.P. and Balish, E. Appl. Environ. Microbiol. 38, 861-869, 1979.

6. Glover, D.J. and Hoffsommer, J.C., J. Chromatogr. 94, 334-337, 1974.

7. Saul, R.L., Kabir, S.H., Cohen, Z, Bruce, W.R. and Archer, M.C., Cancer Res. 41, 2280-2283, 1981.

8. Cox, R.D., Frank, C.W., Nikolaisen, L.D. and Caputo, R.E., Anal. Chem. 54, 253-256, 1982.

9. Iskandarani, Z. and Pietrzyk, D.J., Anal. Chem. 54, 2601-2603, 1982.

10. Skelly, N.E., Anal. Chem. 54, 712-715, 1982.

11. Small, H., Stevens, T.S. and Bauman, W.C., Anal. Chem. 47, 1801-1803, 1975.

12. Wetzel, R.A., Anderson, C.L., Schleicher, H. and Crook, G.D., Anal. Chem. 51, 1532-1535, 1979.

13. Buchholz, A.E., Verplough, C.I. and Smith, J.L., J. Chromatogr. Sci. 20, 499-501, 1982.

14. Ignarro, L.J., Lippton, H., Edwards, J.C., Baricos, W.H., Hyman, A.L., Kadowitz, P.J. and Gruetter, C.A., J. Pharmacol. Exp. Ther. 218, 739-749, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 765-778 (1984)

HPLC METHOD FOR THE EVALUATION OF BLOOD ACETALDEHYDE WITHOUT ETHANOL INTERFERENCE

C. Pezzoli, M. Galli-Kienle*, C. Di Padova**
and G. Stramentinoli
BioResearch Co., Research Laboratories, Liscate (Milan)
*Department of Medical Chemistry and Biochemistry
University of Milan
**3rd Medical Clinic, School of Medicine
University of Milan
Milan, Italy

ABSTRACT

The evaluation of acetaldehyde blood levels is important in view of possible toxic effects in the acute and chronic alcohol intoxication. Artefactual formation of acetaldehyde and its binding to erythrocyte components are the main problems that scientists have faced with in the measurement of acetaldehyde blood levels. The results reported herein show that addition of butyraldehyde as internal standard to the blood immediately after withdrawal allows to obviate these inconveniences. Aldehydes converted into their 2,4-dinitrophenylhydrazones are then analyzed by HPLC. The mean value of acetaldehyde blood concentration measured by this method in 15 healthy subjects was 12.2 $\pm$ 1.3 μM. The increase of acetaldehyde concentration in rabbits after ethanol infusion is also shown.

INTRODUCTION

Acetaldehyde is found in traces in the organism as the product of reactions occurring in the intermedi-

 0148-3919/84/0704-0765$3.50/0

ary metabolism (1). Significant concentrations are only found after ethanol ingestion.

Due to its chemical reactivity, acetaldehyde can be involved in manifestations of acute and chronic alcohol intoxication: so, it has been associated with the pathogenesis of alcoholic liver disease (2) and ethanol induced bone-marrow toxicity (3) and it was considered a basis of alcohol addiction (4). The research on acetaldehyde toxic effects has been prevented by problems connected with its determination in biological samples. To this regard, several studies were carried out concerning the measurement of acetaldehyde blood levels; the results obtained are discussed in details in a review article (5). Essentially, acetaldehyde concentration in blood can be either underestimated or overestimated: low values may be the result of an interaction of aldehyde with the erythrocyte proteins, as shown in rats for the binding with hemoglobin (6), or they may derive from a rapid metabolism of the compound possibly catalyzed by enzymes (7,8). To obviate this inconvenience, advice has been given (5) for a blood deproteinization to be performed within few seconds after blood withdrawal.However, just in the course of denaturation an artefactual acetaldehyde formation due to oxidation of the ethanol present in blood, has been observed, resulting in overestimation of blood content (9,10).

Some methods were proposed to obviate artefactual acetaldehyde formation; the use of a rapid denaturation by perchloric acid in order to avoid ethanol biological

oxidation has been shown to be most reliable (5): in this case, reference curves should be prepared using control blood ethanol (9,10). This method however does not allow to know blood acetaldehyde basal concentration but only its increasing induced by alcohol consumption.

A rapid separation of plasma from blood can be carried out and followed by plasma deproteinization in which ethanol oxidation is no longer active. Plasma separation can be performed after addition to the blood of semicarbazide which traps acetaldehyde and avoids its binding to erythrocyte proteins (11,12). However, under the suggested conditions, the reagent seems not or hardly react with erythrocyte bound acetaldehyde.

It has been recently observed that if extration is carried out by an organic solvent on the whole blood added with 2,4-dinitrophenylhydrazine, high levels of acetaldehyde are found (13). In the present work we describe a method for the evaluation of acetaldehyde blood levels by HPLC after formation of its 2,4-dinitrophenylhydrazone in analogy with Thomas et al. (13). Addition to blood of butyric aldehyde as internal standard allows to obtain results corrected both for interaction of the aldehyde group of acetaldehyde with amino groups, and for formation and extraction of the derivative compound. Alcohol addition to blood does not modify the obtained results. The mean value of acetaldehyde blood levels measured by this method in 15 control subjects was 12.2 $\pm$ 1.3 μM (SEM).

MATERIALS AND METHODS

Acetic and butyric aldehydes were purchased from Merck (Darmstadt, FRG); 2,4-dinitrophenylhydrazine was from C. Erba (Milan, Italy); isooctane was from Fluka (Buchs, Switzerland) and CH_3CN used in HPLC analysis from Merck; 2,4-dinitrophenylhydrazones (2,4-DNP) of acetaldehyde, acetone, propionaldehyde and butyraldehyde to be used as reference standards were prepared by the usual procedure (14). HPLC conditions were similar to those described by Selim (15). A stainless steel μ-Bondapack C_{18} (Waters Assoc., Milford, Mass.) 10 μ, 3.9 mm x 30 cm, reverse phase column was used. The derivatives were eluted from the column in a Waters 6000A solvent delivery system with 3 ml/min flow of CH_3CN:H_2O (50:50, v:v). A UV detector (Waters, Mod.450) was set at 336 nm for the detection of the aldehyde derivatives. Under these conditions, retention times of the 2,4-DNP of acetaldehyde, acetone and butyraldehyde were 3'40", 4'50" and 7'40", respectively. Generally, butyraldehyde (100 nmol) in isopropanol (10 μl) was added as internal standard to 2 ml of heparinized blood, immediately after withdrawal. Plasma obtained by centrifugation at 4°C was treated with 3 M perchloric acid (0.7 ml), 2,4-dinitrophenylhydrazine in 6 N HCl (2.3 pmol, 100 μl), and 3 M sodium acetate (1.7 ml). Centrifugation at 15000 x g for 15 min gave a supernatant which was extracted by shaking with 2 ml of isooctane for 20 min. The organic phase was separated and the solvent evaporated to dryness. The residue was then dissolved in CH_3CN:H_2O (50:50, v:v) and analyzed

by HPLC as described above. Blood used for measurement of acetaldehyde basal levels and for calibration curves was collected from healthy volunteers who had not consumed alcohol for at least 24 hrs, and it was immediately transferred to ice cold tubes.

The same procedure was used for blood collection from male New Zealand albino rabbits (2.5-2.8 kg b.w.) before and after treatment with 1.5 g/kg of ethanol. The dose was given by a 15-min infusion of a 50% aqueous solution at 0.7 ml/min into the ear vein.

Calibration curves were prepared adding butyraldehyde in isopropanol (100 nmol, 10 µl), and acetaldehyde as aqueous solution (10, 16, 20, 40 and 100 nmol) to blood aliquots (2 ml). The samples were then processed as described above for the measurement of blood levels. A curve was also prepared by mixing the 2,4-DNP of butyraldehyde (100 nmol) with 10, 16, 20, 40 and 80 nmol of the 2,4-DNP of acetaldehyde.

Levels of acetaldehyde in blood were calculated from the ratio of the peak areas of acetaldehyde and butyraldehyde 2,4-DNP on the basis of the calibration curve obtained in blood (Fig. 1). In some cases the level was calculated from the intercept of the calibration curve obtained using aliquots of the same blood as that of the sample.

RESULTS

Fig. 1 shows the results obtained form the analysis of a typical calibration curve prepared by adding a constant amount of butyraldehyde and increasing amounts of

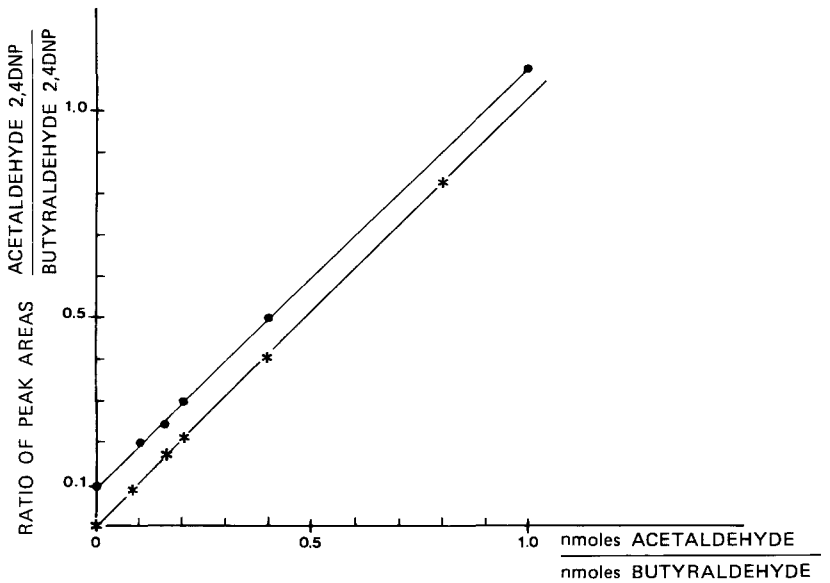


FIGURE 1

Linearity of the evaluation of blood acetaldehyde. Points represent results obtained in the analysis of duplicate samples: ●A constant amount of butyraldehyde (100 nmol) and increasing amounts of acetaldehyde were added to 2 ml of blood to obtain the ratios shown in the abscissa. Samples were then processed as described in the text to obtain 2,4-DNPs which were then analyzed by HPLC. *A constant amount of authentic butyraldehyde 2,4-DNP (100 nmol) and increasing amount of authentic 2,4-DNP of acetaldehyde were mixed to obtain the shown ratios. The compounds were dissolved in isooctane and analyzed by HPLC as described in the text.
Lines obtained by regression analysis were:
$y = 1.00(\pm 0.01)\ x + 0.1(\pm 0.005)$ for blood analysis
$y = 1.03(\pm 0.01)\ x + 0.002(\pm 0.005)$ for standard 2,4-DNP.
The intercept on the axis was significantly different from zero (t test) in the curve of blood analysis (●), whereas the line passed through zero in the analysis of authentic 2,4-DNP (*).
Comparison of the two lines (16) showed that they are parallel.

acetaldehyde to human blood. When the ratio between nmoles of acetaldehyde and butyraldehyde added to the blood was plotted against the ratio between the peak areas of acetaldehyde and butyraldehyde 2,4-DNP, a line was obtained with a slope that did not significantly differ from that obtained when mixtures of the 2,4-DNP of acetaldehyde and butyraldehyde were directly analyzed by HPLC (Fig. 1). This ensured that if interaction of acetaldehyde with erythrocytes occurs, it is active to the same extent as with the butyraldehyde used as internal standard. While the line obtained from analysis of authentic 2,4-DNP passed through zero (Fig. 1), from the intercept observed in the analysis of the curve in blood a basal concentration of 5.0 $\pm$ 0.25 µM acetaldehyde was calculated. This value did not significantly differ from that obtained in the triplicate analysis of the same blood to which only butyraldehyde had been added as internal standard (5.0 $\pm$ 0.40 µM). The comparison of peak areas of the 2,4-DNP in the blood extracts with those in the authentic standards allowed to calculate a recovery from the blood of 31 $\pm$ 6.6% (mean $\pm$ SEM in 7 samples) for acetaldehyde at all tested concentrations and of 27 $\pm$ 3.2% for butyraldehyde. The results of analysis of the samples where 2,4-DNPs of acetaldehyde were obtained from aqueous solutions of the two aldehydes showed that the yield of the derivative formation and of its extraction was 44.3 $\pm$ 5.6% and 47 $\pm$ 2.7% for acetaldehyde and butyraldehyde, respectively. The lower recovery from blood seems to confirm that reaction occurs between the aldehydes and blood components. Basal blood levels of acet-

TABLE 1

Blood Acetaldehyde Basal Levels in Healthy Subjects

Sex	Subject	Age	Acetaldehyde µM	
			A	B
F	C.G.	30	11.2	-
	D.B.	20	9.5	-
	C.P.	33	9.1	-
	E.M.	24	6.8	-
	C.S.	26	8.6	9.2
	G.I.	60	19.7	21.6
	P.V.	35	8.6	9.0
	M.G.	33	13.2	11.2
M	R.S.	28	8.3	-
	A.R.	33	9.5	-
	G.M.	29	9.7	-
	F.B.	25	10.2	-
	L.B.	28	19.4	19.4
	M.P.	30	17.5	16.8
	A.F.	77	21.7	20.5

A = Values obtained from the analysis of duplicate aliquots of the same blood to which only butyraldehyde had been added.

B = Values extrapolated from the standard curve obtained by addition of a constant amount of butyraldehyde and increasing amounts of acetaldehyde to blood aliquots.

aldehyde in 15 healthy subjects who had not consumed alcohol for at least 48 hrs are reported in Table 1. In no case a difference was observed between the level calculated from the intercept of the curve prepared for each blood sample as described in Fig. 1 and that deriving from the duplicate analysis of the same blood to which no acetaldehyde had been added.

Table 2 shows the results obtained from human blood mixed with ethanol. Direct blood denaturation induced an acetaldehyde level increase as already de-

TABLE 2

Acetaldehyde Levels in Human Blood containing Ethanol

Ethanol added to blood (μmol)	Acetaldehyde (nmol)	
	Blood denaturation	Plasma denaturation
0	108	104
30	255	104
60	305	103
120	480	114

Aliquots of blood taken from a healthy subject who had not consumed alcohol for at least 48 hrs were mixed with the shown amounts of ethanol. Denaturation was carried out with perchloric acid added either directly to the blood or to plasma after its separation. Butyraldehyde as internal standard was always added to blood before any other treatment. Results represent the mean of duplicate analyses.

scribed (9,10), whereas by the procedure here reported no influence of ethanol was observed in the evaluation of aldehyde levels.

Fig. 2 shows the blood levels of both ethanol measured as described by Bücker and Redetzky (17) and acetaldehyde determined using the method reported here in rabbits treated with 1.5 g/kg ethanol. At the end of the 15-min infusion, both alcohol and acetaldehyde levels were significantly higher than basal levels. Ethanol levels then decreased as shown in Fig. 2, where as a slight increase was observed for acetaldehyde. This further demonstrated that no aldehyde was artefactually produced from ethanol.

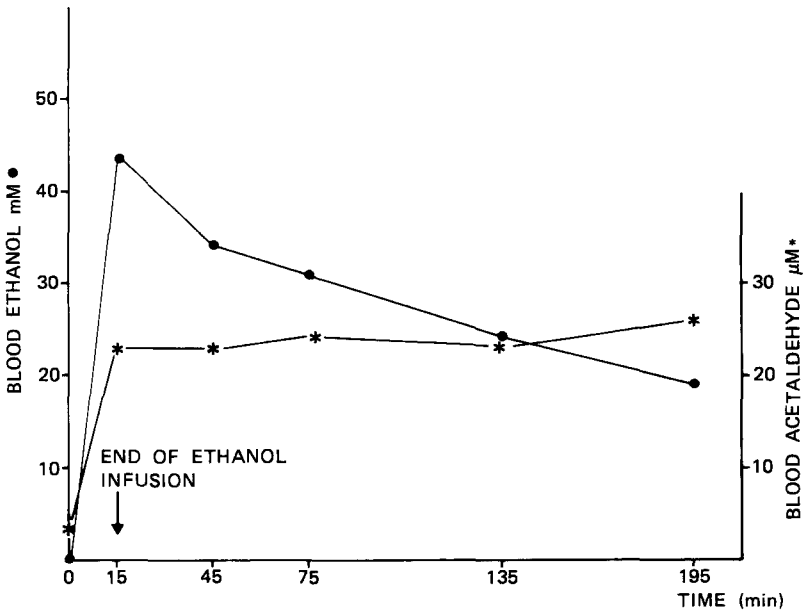


FIGURE 2

Ethanol concentration in blood was determined by the method of Bücker et al. (17). Points represent the mean of ethanol and acetaldehyde levels in two rabbits before (t_0) and after a 15-min infusion with 1.5 g/kg of ethanol. Assays were made in duplicate.

DISCUSSION

The method here reported for evaluation of acetaldehyde blood levels is based on the transformation of the compound into its 2,4-DNP and on the derivative analysis by HPLC. In this regard, it does not differ from the method briefly described by Thomas et al. (13). These Authors actually suggest the addition of the reactive 2,4-dinitrophenylhydrazine directly into the blood, followed by extraction with a solvent they do

not specify. The determination by HPLC is made after addition to the extract of the 2,4-DNP of propionic aldehyde. We modified both internal standard and general procedure for the extraction. Choice of butyraldehyde as internal reference instead of propionaldehyde has been made because the 2,4-DNP of the latter showed a retention time very similar to that of the 2,4-DNP of acetone, under the analysis conditions reported in the present study and under others preliminarly experimented. As acetone resulted to be always present as a contaminant in the assayed extracts, it was not possible to calculate the peak area of the propionic aldehyde derivative correctly. The longer retention time of butyric aldehyde derivative allowed instead a correct evaluation of the peak area. Moreover, in our experimentation, the addition of solvents directly into blood as suggested by Thomas et al. (13) generated gels from which the organic phase was hardly separable. There was, therefore, the necessity for a denaturation with perchloric acid but if made directly in blood when it contained alcohol one could have noted the production of artefactual acetaldehyde in a quantity depending on alcohol concentration. This did not occur when denaturation was made after plasma separation.

On the other hand, since recovery of the 2,4-DNP of the acetaldehyde added to blood was constantly lower than that obtained by addition of the acetaldehyde to water, the compound disappearance may be reasonably due to interaction with the erythrocyte proteins (6). Assuming that the interaction is not specific for acet-

aldehyde but depends only on the aldehyde group reactivity, we added butyric aldehyde as internal reference directly to the blood. The recovery of the derivative of this aldehyde corresponds to that obtained for acetaldehyde at all the tested concentrations; hence the linear response reported in our results. A good reproducibility was found; indeed, the acetaldehyde concentration values observed in two aliquots of the same blood specimen did not differ from one another more than 10% for all the examined blood samples.

The results of the analysis of basal levels in control subjects showed a concentration of 12.2 $\pm$ 1.3 µM. This value is remarkably higher than those obtained with the method implying addition of semicarbazide to blood (11,12). This may be explained by the fact that at least part of blood acetaldehyde remains bound to macromolecules even in the presence of excess semicarbazide. Demonstration of this is also given by the partial recovery of 2,4-DNP both of acetic and butyric aldehydes added to blood.

Our values, however, are lower than that found by Thomas et al. (13) in control subjects, which was 60 $\pm$ 18 µM. Yet, such a difference might depend on special dietary conditions of the different ethnic groups to which subjects belong.

Acknowledgements: we wish to thank Mr. A. Romanò for his tecnical assistance.

REFERENCES

1. von Wartburg, J.P., Biological Aspects of Alcohol:

Remarks to the Sessions on the Biochemistry of Alcohol, Ann.NY Acad.Sci., 273, 146, 1976.

2. Lieber, C.S., Alcohol, Protein Metabolism and Liver Injury, Gastroenterology, 79, 373, 1980.

3. Meagher, R.C., Sieber, F. and Spivak, J.L., Suppression of Hematopoietic-Progenitor-Cell Proliferation by Ethanol and Acetaldehyde, N.Engl.J.Med., 307, 845, 1982.

4. Myers, R.D., Tetrahydroisoquinolines in the Brain: The Basis of an Animal Model of Addiction, Alcoholism Clin.Exp.Res., 2, 145, 1978.

5. Eriksson, C.J.P., Problems and Pitfalls in Acetaldehyde Determination, Alcoholism Clin.Exp.Res., 4, 22, 1980.

6. Stevens, V.J., Fantl, W.J., Newman, C.B., Sims,R.V., Cerami, A. and Peterson, C.M., Acetaldehyde Adducts with Haemoglobin, J.Clin.Invest., 67, 361, 1981.

7. Inone, K., Ohbora, Y.and Yamasawa, K., Metabolism of Acetaldehyde by Human Erythrocytes, Life Sci., 23, 179, 1978.

8. Pietruszko, R. and Vallari, R.C., Aldehyde Dehydrogenase in Human Blood, FEBS Lett., 92, 89, 1978.

9. Stowell, A.R., Greenway, R.M. and Batt, R.D., Stability of Acetaldehyde in Human Blood Samples, Biochem.Med., 20, 167, 1978.

10. Eriksson, C.J.P., Mizoi, Y. and Fukunaga, T., The Determination of Acetaldehyde in Human Blood by the Perchloric Acid Precipitation Method: The Characterization and Elimination of Artefactual Acetaldehyde Formation, Anal.Biochem., 125, 259, 1982.

11. Stowell, A.R., An Improved Method for the Determination of Acetaldehyde in Human Blood with Minimal Ethanol Interference, Clin.Chim.Acta, 98, 201, 1979.

12. Stowell, A.R., Lindros, K.O. and Salaspuro, M.P., Breath and Blood Acetaldehyde Concentrations and their Correlation during Normal and Calcium Carbimide-Modified Ethanol Oxidation in Man, Biochem.Pharmacol., 29, 783, 1980.

13. Thomas, M., Lim, C.K. and Peters, T.J., Assaying Acetaldehyde in Biological Fluids, Lancet II, 530, 1981.

14. Vogel, A.I., Practical Organic Chemistry, Longmans Green, London, 1967, p. 1066.

15. Selim, S., Separation and Quantitative Determination of Traces of Carbonyl Compounds as their 2,4-dinitrophenylhydrazones by High-Pressure Liquid Chromatography, J.Chromatogr., 136, 271, 1977.

16. Diem, K. and Lentner, C., In Tables Scientifiques, Ciba Geigy Ed. Septième Edition, Bâle, 1973, p. 181.

17. Bücker, T. and Redetzky, H., Eine spezifische photometrische Bestimmung von Äthylalkohol auf fermentativem Wege, Klin.Woschr., 29, 615, 1951.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 779-791 (1984)

A BIDIMENSIONAL HPLC SYSTEM FOR DIRECT DETERMINATION OF THEOPHYLLINE IN SERUM

H. Ong and S. Marleau
Faculty of Pharmacy, University of Montreal
P.O. Box 6128, Montreal, Québec, Canada

ABSTRACT

A bidimensional HPLC system combining steric gel exclusion and reverse phase ODS columns for determination of theophylline in serum is reported. Factors involved in the development of the method and its performance are discussed. This technique is a practical alternative for the determination of theophylline levels in serum without any clean up procedure before chromatography.

INTRODUCTION

Theophylline, commonly used in the treatment of asthma, and in the apnea of the premature infants, is characterized by a large interindividual variation of its clearance. For monitoring of this drug, numerous assays have been reported; a review of these techniques has been done by Berthou et al (1). At present, enzyme immuno assays and HPLC are the prevailing methods. Some advantages for the latter technique would be the possible simultaneous measurement of theophylline and its metabolites. Most of them resemble each other and differ only in the composition of the mobile phase, pH, proportion of the organic solvent,

 0148-3919/84/0704-0779$3.50/0

presence of tetrabutylammonium sulfate as ion pair, on choice of the internal standard. These procedures require a deproteinization step by acetonitrile (2,3), trichloracetic acid (4) or ammonium sulfate (5,6) followed by the injection of the supernatant onto the chromatograph or by extraction of the drug by organic solvents (5,6,7,8,9,10,11). Additional steps such as centrifugation and solvent evaporation are necessary after the drug extraction.

We propose a bidimensional chromatographic system combining a steric exclusion and a reverse phase ODS columns for quantitation of the theophylline by direct injection of serum samples without requiring any clean up procedure.

MATERIAL AND METHODS

Solvents and Chemicals

Acetonitrile HPLC grade is purchased from Fisher Scientific (Montreal, Canada). All other reagent grade chemicals are obtained from Baker (Canlab, Montreal, Canada). Theophylline is purchased from Sigma (St-Louis, Mo, USA)., 1-methylxanthine and 3-methylxanthine from ICN pharmaceuticals (NY, USA), 3-methyluric acid and 1,3-dimethyluric acid from Adams chemicals (Round Lake, Il, USA). Stock solutions of theophylline of 1 mg/ml are prepared in methanol; theophylline metabolites, 5 mg/ml, are prepared in 0.05M phosphate buffer pH 7.4.

Chromatographic Instrumentation and Conditions

The bidimensional HPLC system consists of 2 parts: (Fig 1) the steric gel exclusion part consists of a protein column (I-60

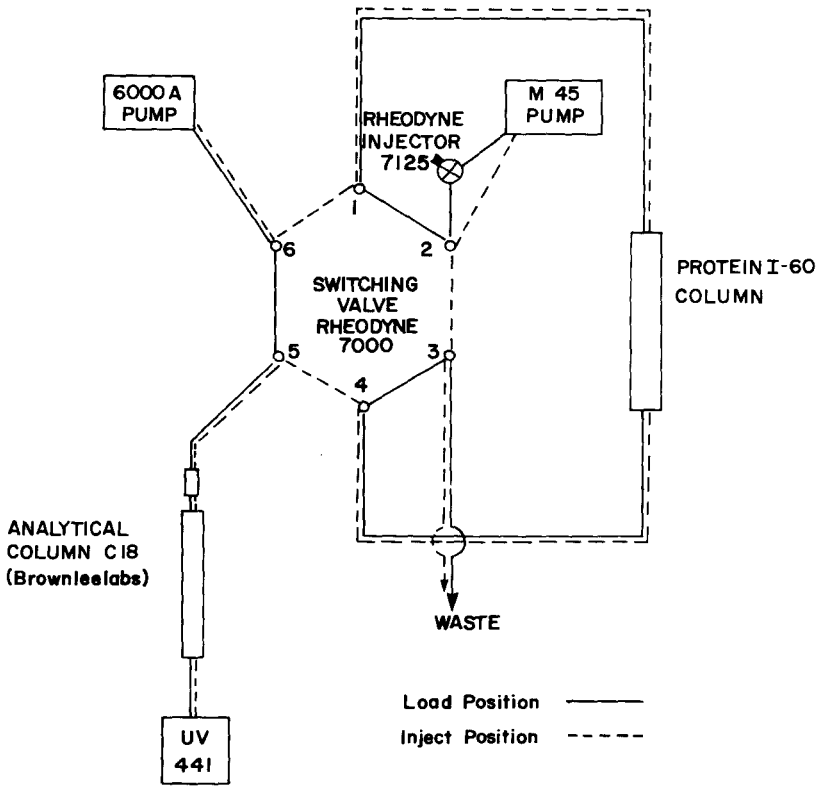


Fig 1 = Diagram of the bidimensional chromatographic system.

type 7.8 mm x 300 mm) with a precolumn filled with the guard column support (Cat. No. 85290), connected to an M-45 pump (all from Waters Assoc., Milford, MA, USA) and a injection valve (Model 7125, Rheodyne, Berkeley, CA, USA) with a 100 µl loop. The mobile phase used for the steric gel exclusion is a 0.02 M triethylamine acetate buffer pH 7.4 at a flow rate of 1 ml/min. The analytical part consists of an ODS reversed stationary phase column (Spherisorb 5 µm 250 x 3 mm, Brownlee, Santa Clara, CA, USA)

protected by an MPLC guard column (Brownlee). The mobile phase used is a 0.01 M acetate buffer (pH 4) - acetonitrile (90:10 v/v) pumped by an 6000A pump (Waters Assoc., Milford MA, USA) at a flow rate of 1 ml/min. The two parts are linked by a switching valve (Model 7000, Rheodyne, Berkeley, CA, USA).

The detection system consists of a fixed wavelength detector (model 441, Waters Assoc., Milford, MA, USA) operated at 280 nm and a recorder (Model 561, Hitachi, Japan).

Procedure

The standard curve is prepared by adding theophylline 1, 2, 4, 8, 16, 32 µg/ml to drug free serum samples. Three aliquots are used at each concentration. These are assayed as described below. For analysis of serum samples, a 100 µℓ aliquot is loaded in the injection valve. The switching valve, Rheodyne 7000, is set at load position (fig 1); nine minutes after the injection of the sample in the steric gel exclusion part, the swithching valve is then set to inject position for 2 minutes permitting the transfer of theophylline to the analytical column. The switching valve is reset to load position for the reequilibration of the protein column by the triethylamine buffer for 10 minutes before the injection of the next serum sample, while chromatographic separation is carried out on the analytical column.

Quantitation

All measurements are done by peak heights. Recovery is estimated by comparing the peak heights of the standard curve in

serum with that obtained on chromatographing the stock theophylline solution on the ODS column. The slope and intercept of the standard curve are obtained by linear regression of peak height on concentration ($Y = Ax + B$).

Method Validation

A kinetic profile of theophylline is established on a rabbit (new Zealand, 15 kg) following an IV dose of 10 mg/kg. Blood samples (2 ml) are collected from the ear vein at 0, 6, 15, 60, 240 and 360 minutes after the dose. Serum is then separated and transferred. Aliquots are used for theophylline determination following the Soldin and Hill method (12) and by the bidimensional on line HPLC proposed.

RESULTS AND DISCUSSION

Chromatography

The elution diagram of serum proteins, theophylline and its metabolites from I-60 protein column is monitored by collecting 1 ml fractions and reading the absorbance at 280 mn (Fig 2). The elution volume of serum proteins is 5.5 ml which corresponds to the void volume of this column. The exclusion of the protein column used is 20 000 allowing the elimination of the proteins from the sample injected.

Despite the fact that steric exclusion chromatography separates on the basis of molecular size, we observe different elution volumes for methyluric acid derivatives, theophylline and its

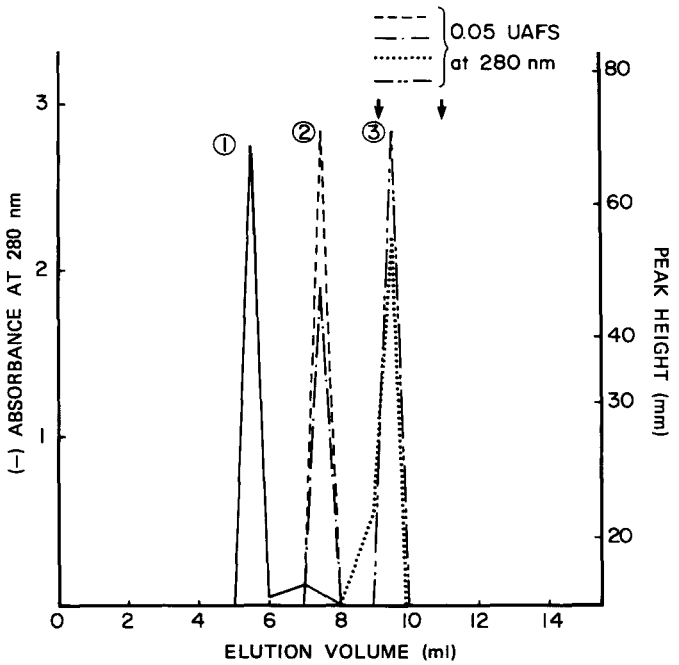


Fig 2 = Elution profile of (1) serum proteins (100 µℓ) (——) (2) 1-methyluric acid (1 µg) (---), 1,3-dimethyluric acid (1 µg) (—·—·) (3) 3-methylxanthine (1 µg) (....) and theophylline (2 µg) (—·—··)from the I-60 protein column. (↓↓) Elution volume transferred to the analytical column.

basic metabolites. The calculated Kav is 0.224 for 1-methyluric acid and 1,3-dimethyluric acid and 0.454 for theophylline and 3-methylxanthine metabolite; ($Kav = \frac{Ve - Vo}{Vt - Vo}$, Ve = elution volume of the compound, Vo and Vt are the void volume and the total bed volume respectively). Thus some interaction may occur between the column packing material and the compounds tested which could explain the difference in the Kav obtained for theophylline and its metabolites. This interaction affects also the recovery.

TABLE 1

Percentage of Theophylline Recovery Following the Gel Exclusion Chromatography

Mobile Phase Tested	Percentage of Recovery (Mean ± Standard Error) (n, Number of Assays)
1. 0.2 M phosphate buffer pH 7.4	76.33% ± 0.27 (n = 9)
2. 0.5 M phosphate buffer pH 7.4	82.60% ± 1.45 (n = 8)
3. 0.02 M triethylamine acetate buffer pH 7.4	95.15% ± 0.42 (n = 18)

Several mobile phases for the gel exclusion system have been tested and the results reported in Table 1.

As shown in Table 1, the concentrations tested are from 1 to 32 µg/ml of theophylline. Higher recovery is obtained by increasing the ionic strenght of the mobile phase as observed with the 0.5 M phosphate buffer. But high variations in the recovery are noted. The triethylamine acetate buffer allows the best and constant recoveries for all the concentrations tested. The use of serum samples for theophylline measurement is required for the protein column long life. The fibrinogen in the plasma hamper rapidly the resolution of I-60 column; furthermore it prevents the injection of plasma volumes exceeding 20 µℓ. The elution profile of the I-60 column is controlled routinely after the analysis of a hundred samples; any modification could be corrected by the replacement of the precolumn support.

Chromatograms from the dual column system are shown in Fig 3. Good resolution is obtained due to the reconcentration on the

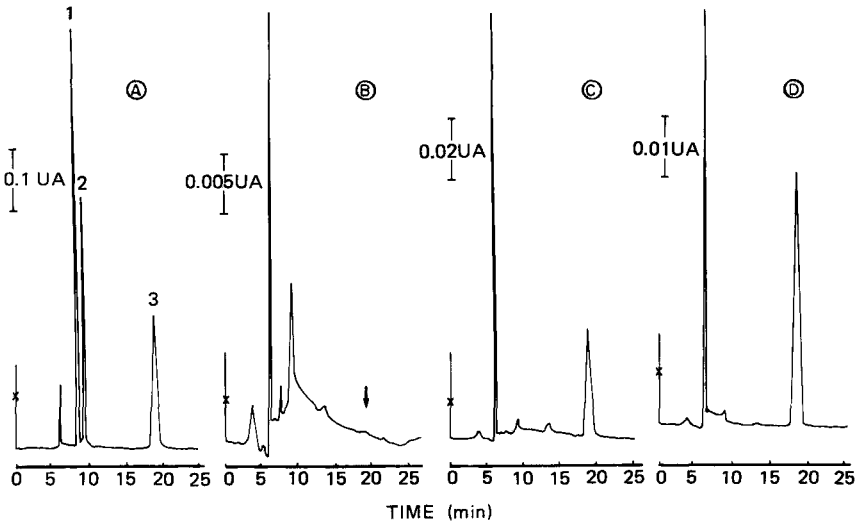


Fig 3 = Chromatography of theophylline and its metabolites. (A) chromatograms of aqueous standards. Peaks: 1, 3-methylxanthine (5 µg); 2, 1-methylxanthine (5 µg); 3, theophylline (5 µg). (B) serum blank. (C) blank serum spiked with 8 µg/ml theophylline. (D) rabbit 1-h serum sample following an IV dose of 10 mg/kg theophylline (estimated concentration 9.8 µg/ml).

analytical column of the 2 ml eluate from the I-60 column. Basic metabolites which coelute with theophylline in the gel exclusion system are well separated on the analytical ODS column (Fig 3 A). No interference peak is observed at the retention time of 18.6 minutes corresponding to that of theophylline.

Standard Curves

Using the method described above, standard curves are constructed for serum at concentrations of 1, 2, 4, 8, 16 and 32 µg/ml. The linearity of the standard curve for serum with 3 determinations at each concentration is excellent ($R^2 > 0.99$ n = 18) and a least

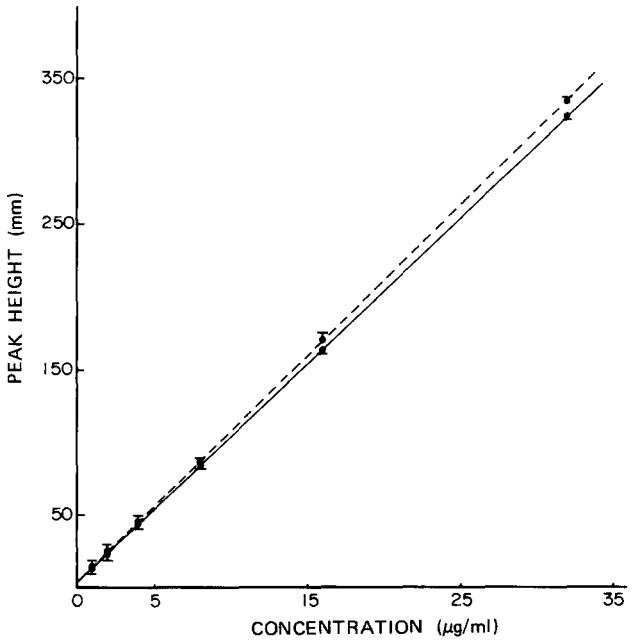


Fig 4 = Standard curves of theophylline (mean ± standard error) obtained from blank serum spiked with 1, 2, 4, 8, 16 and 32 µg/ml theophylline following the bidimensional system chromatography (----) and from standard solutions of theophylline in phosphate buffer (0.05M pH 7.4) by direct injection on the analytical column (——).

squares linear regression of peak height (mm) on concentration (µg) gives a slope of 10.12, an intercept of 0.57 and a mean coefficient of variation of 1.30%. The recoveries for the concentrations from 1 to 32 µg/ml of theophylline in serum are shown by figure 4. The mean recovery is 95.1%. It appears that the binding of theophylline to serum proteins is very weak resulting a complete dissociation in the triethylamine buffer during the gel exclusion chromatography. Because of the good recovery and the use of constant volume injection loop, the use of an internal

TABLE 2

Accuracy of the Method

Theoretical concentration µg/ml	n	$\bar{\Delta}$	$\bar{\Delta}$%	SE	RSD%
15	5	0.011	0.07	0.479	0.30
7.5	5	0.008	0.11	0.438	0.56

n = number of samples
$\bar{\Delta}$ = absolute error (mean)
$\bar{\Delta}$% = relative error (mean)
SE = standard error of the mean
RSD = relative standard deviation

standard is found unnecessary. The mean interday coefficient variation is 1.36%. It is calculated following the Rodbard program for the calculation of within and between assay variance (13). The accuracy of the method is tested on spiked serum samples is shown in table 2.

Method Validation

Separate aliquots of the serum of a rabbit treated with 10 mg/kg theophylline and collected at different times are used for determination of theophylline using the Soldin and Hill method and the multidimensional on line chromatography system described. The results obtained are presented in table 3. They show excellent agreement. ($Y = 1.096\ x - 0.577$; R^2: 0.989).

In conclusion, the combination of gel exclusion - reversed phase chromatography could be valuable alternative for theophylline determination in serum without any clean up procedure, advantageous compared to that with direct injection of plasma on

TABLE 3

Kinetic Profile of Theophylline After an IV Dose of 10 mg/kg

Time of blood collection after the IV dose (min)	Theophylline concentration (µg/ml)	
	Soldin and Hill Method	Bidimensional Chromatography
0	-	-
6	14.08	15.43
15	11.46	11.63
60	9.99	9.80
240	5.15	5.43
360	3.92	3.73

reversed phase column. The latter technique, proposed by some authors (14), is limited to few microliters of samples which is a factor limiting the sensitivity and presents high risk of protein precipitation on the analytical column. The bi-dimensional system developed could be fully automated, reducing the technician cost. It would be attractive for the direct simultaneous measurement of theophylline and its metabolites.

ACKNOWLEDGEMENTS

The authors wish to thank Waters Associates, Canada for supporting this work and Ms G. Déziel for her skilled technical assistance. S. Marleau was recipient of a summer undergraduate research scholarship from Medical Council Research of Canada.

REFERENCES

(1) Berthou, F., Riche, C., Alix, D., Dreano, Y. and Curunet, M., Critique de la spécificité des méthodes de dosage de la théophylline, Conséquences thérapeutiques, Thérapie 37, 53 (1982).

(2) Dresse, Al. E., Delapierre-Gabriel, D., Measurement of theophylline, mexiletine and a new cephalosporin in plasma by LC, Chromatogr. Sci. 20, 161 (1982).

(3) Frutkoff, I.W., Kidroni, G., and Menezel, J., Monitoring of serum theophylline by high-performance liquid chromatography without interference by various coadministered drugs, Jsr J Med Sci 18, 639 (1982).

(4) Valia, K.H., Hartman, C.A., Kucharczyk, N. and Sofia, R.D., Simultaneous determination of dyphylline and theophylline in human plasma by high-performance liquid chromatography, Journal of Chromatography 221, 170 (1980).

(5) Van Aerde, P., Moerman, E., Van Severen, R., Braeckman, P., Determination of plasma theophylline by straight-phase high-performance liquid chromatography: elimination of interfering caffeine metabolites, Journal of Chromatography 222, 467 (1980).

(6) Sommadossi, J.P., Aubert, C., Cano, J.P., Durand, A. and Viala, A., Determination of theophylline in plasma by high performance liquid chromatography, Journal of liquid chromatography 4, 97 (1981).

(7) Weidner, N., Dietzler, D.N., Ladenson, J.H., Kessler, G., Larson, L., Smith, C.H., James, T., McDonald, J.M., A clinically applicable high-pressure liquid chromatographic method far measurement of serum theophylline, with detailed evaluation of interferences, Am J Clin Pathol 73, 79 (1980).

(8) Broussard, L.A., Stearns, F.M., and Tulley, R., Theophylline determination by "high-pressure" liquid chromatography, Clin. Chem. 27, 193 (1981).

(9) Kabra, P.M. and Marton, J.L., Liquid-chromatographic analysis for serum theophylline in less than 70 seconds, Clin. Chem. 27, 687 (1982).

(10) Naish, J.P., Cooke, M. and Chambers, R.E., Rapid assay for theophylline in clinical samples by reversed phase high performance liquid chromatography, Journal of Chromatography 163, 363 (1979).

(11) Bengt, K., and Anders, H., Simultaneous determination of acetaminophen, theophylline and salicylate in serum by high-performance liquid chromatography , Journal of chromatography 229, 492 (1982).

(12) Soldin, S.J., Hill, J.G., A rapid micromethod for measuring theophylline in serum by reversed phase high performance liquid chromatography, Clin Biochem, 10, 1774 (1977).

(13) Rodbard, D., Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clin. Chem. 20, 1255 (1974).

(14) Mano, B.R., Mano, J.E. and Hilmann, B.C. A direct injection HPLC procedure for the quantitation of theophylline in blood and saliva, J. Anal. Toxicol. 3, 81 (1979).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 793-808 (1984)

LC-EC OF ENDORPHINS

L. H. Fleming and N. C. Reynolds, Jr.
Department of Neurology
University of Wisconsin Medical School
Milwaukee Clinical Campus
Mount Sinai Medical Center
P. O. Box 342
Milwaukee, Wisconsin 53201

ABSTRACT

Reversed phase liquid chromatography with electrochemical detection (LC-EC) was used to separate a series of endorphin standards. Chromatographic conditions were manipulated so that methionine- and leucine-enkephalin were clearly resolved from other endorphins of similar hydrophobicity using an isocratic mobile phase. The most significant factors affecting endorphin retention were the concentration and type of organic modifier in the isocratic mobile phase. Hydrodynamic voltammograms were performed for methionine- and leucine-enkephalin to assess their electroactivity. Both enkephalins were oxidized with a glassy carbon electrode only at high potentials ($\geq$+.90V vs Ag/AgCl). The effect of these high potentials on the sensitivity of electrochemical detection of endorphins was evaluated.

INTRODUCTION

The discovery of neuropeptides with opiate-like activity focused considerable interest on the isolation, characterization, and localization of these molecules. This research effort has attempted to elucidate the roles of endorphins in normal and disease states. Physiologic studies have implicated endogenous opiates in stress (1) and pain (2), memory and reward behavior (3), psychosis (1) and petit mal seizures (4). At the molecular level, the endorphins may act as neuromodulators (5,6) or in some cases, as neurotransmitters (5-7).

 0148-3919/84/0704-0793$3.50/0

To evaluate endorphin activities in physiological fluids and tissues, highly specific and sensitive techniques are required. Radioimmunoassay (RIA) has been used to quantify endorphins such as methionine-(ME) and leucine-(LE) enkephalin (8,9) but has been limited for other endorphins by the lack of highly specific antibodies (10,11). The cross-reactivity of the monoclonal antibody to β-endorphin (BE) that was prepared by Herz et al (12) demonstrates the difficulty in achieving highly specific antibodies for RIA of closely-related endorphins. In an effort to improve the specificity of endorphin assay, high performance liquid chromatography (LC) has been used to separate and identify endorphins (10,11,13-15). Most LC techniques use reversed phase chromatography and detect endorphin standards by far UV absorbance (14, 16). However, quantification of endorphins in brain samples is usually performed by collecting chromatographic fractions which are then analyzed by RIA for each endorphin of interest (10,11,17). Although these procedures are time-consuming, expensive and introduce additional experimental variables, they are necessary because the concentration of endorphins in most physiologic samples is below the limits of detection by far UV spectroscopy.

An electrochemical detector (EC), which can measure compounds on the basis of their electroactivity, has been used with LC for the quantification of catechol- and indol-amines in the 10^{-11} gram range from small biological samples (18,19). The electroactivity of ME and LE was originally demonstrated by Meek et al (20) in their study of enkephalin catabolism. Although they showed that ME and LE could be detected electrochemically in 10^{-8} gram quantities, they did not develop LC-EC as an assay technique for endorphins. Using differential pulse voltammetry, Bennett et al (21) reported the electroactivity of other neuropeptides, such as vasopressin and somatostatin, and some amino acids (tyrosine, tryptophan and cysteine). In this report, we have developed conditions for the separation and detection of a series of endorphins standards using LC-EC. A preliminary report of this work has been presented (22).

MATERIALS AND METHODS

ME, LE, [D-Ala^2]-leucine enkephalinamide (LEA), human β-endorphin and glycylglycine were purchased from Sigma Chemical Company, St. Louis, Mo. Dynorphin 1-6 and 1-17, pro-enkephalin, β-neo-endorphin, α-neo-endorphin 1-8 and 1-10, and α-endorphin were obtained from Bachem, Torrance, CA. Reagents included HPLC grade potassium dihydrogen phosphate, ammonium acetate, phosphoric acid (85%), methanol, tetrahydrofuran (THF) and acetonitrile (Fisher Scientific, Pittsburgh, PA). Ultrex acetic acid was from J. T. Baker Co., Phillipsburg, N.J. Water for LC mobile phases was prepared by adding activated charcoal (Sigma) to fresh glass distilled water. After standing overnight, the water was filtered through a 0.2μm or 0.45μm Nylon-66 filter (Rainin Instrument Co., Inc; Woburn, MA) and degassed.

The chromatographic system consisted of Model 6000A pumps (Waters Associates, Milford, MA) and a Model U6K sample injector (Waters Associates). The reversed phase columns included a 5μm Biophase ODS (250 x 4.6mm, Bioanalytical Systems, West Lafayette, IN) protected by a C_{18}/Corasil guard column (Waters), a 5μm Ultrasphere-Octyl (150 x 4.6mm, Altex Scientific, Inc., Berkeley, CA) and a 10μm μBondapak (300 x 3.9mm, Waters). A 10μm Aquapore RP-300 column (250 x 4.6mm, Brownlee Labs, Santa Clara, CA) and a 5μm Sepralyte Octyl column (250 x 4.6mm, Analytichem International, Harbor City, CA) were protected by a Soft Seal guard column (Applied Science Laboratories, Inc., State College, PA) packed with a 10μm Ultrapack-Octyl (Altex). An LC-4 or LC-4B amperometric detector with a TL-5 glassy carbon electrode (Bioanalytical Systems) was set at a potential of +1.05V referenced to a Ag/AgCl electrode unless indicated otherwise. A Topaz Line 2 power conditioner (Topaz Electronics Div., San Diego, CA) was used with the LC-4 and LC-4B detector for all experiments. Only isocratic mobile phases were used and their compositions are described in the figure legends. All mobile phases were filtered through a 0.2μm or 0.45μm Nylon-66 filter and degassed before use. Steel solvent reservoir filters were omitted from mobile phase reservoirs. Where indicated,

the mobile phase reservoir was suspended in a circulating water bath at 27°C. A flow rate of 1.0 ml/min was used for all separations.

Endorphin standards were dissolved in a 30mM KH_2PO_4-27.5µM glycylglycine, pH 2.3 (adjusted with 85% phosphoric acid). Aliquots were lyophilized and stored dessicated at -20°C. Prior to chromatography, the endorphin standards were dissolved in an appropriate volume of 25mM KH_2PO_4-27.5µM glycylglycine, pH 2.3 and stored for approximately one month at -20°C.

RESULTS

Various chromatographic parameters were manipulated in an effort to optimize ME and LE resolution. These parameters included various analytical reversed phase columns and mobile phase components. Factors which affected the performance and sensitivity of the electrochemical detector were also examined.

Chromatographic Conditions

1. Columns

The reversed phase columns described above were used to separate ME and LE from other endorphin standards. A representative chromatogram is shown in Figure 1. The longest endorphin retention times were obtained with the 5µm Ultrasphere-Octyl and the 5µm Biophase ODS columns. Intermediate retention times were obtained with the 5µm Sepralyte Octyl. The least retention of the endorphins was found with the 10µm µBondapak and the 10µm Aquapore RP-300; there was no significant difference in the chromatographic behavior of the endorphins on either column.

2. Mobile Phase

The effect of changes in the mobile phase composition on the chromatographic behavior of endorphins was investigated. Components of the mobile phase that were examined included buffers, pH, organic modifiers and ion-pairing reagents.

A. Buffer. Several buffers were used for the chromatography of ME, LEA and LE including ammonium acetate, potassium phosphate, sodium phosphate and triethylamine phosphate. Each of these buffers could be used for endorphin chromatography with appropriate adjust-

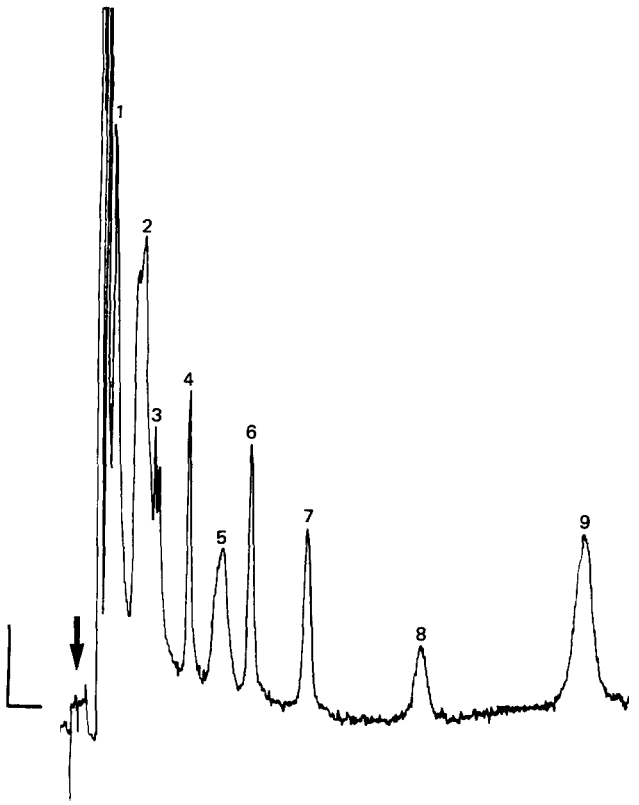


FIGURE 1

Isocratic elution of endorphin standards. Column = Aquapore RP-300; Mobile phase = 128 ml [50mM KH_2PO_4 -55μM glycylglycine, pH 2.3 (adjusted with 85% phosphoric acid) containing 29% acetonitrile] diluted to 250 ml with water; Mobile phase temperature = 27°C; Flow Rate = 1.0ml/min.; Applied Potential = +1.05V vs Ag/AgCl; Reference Bars: Ordinate = 0.2nA and Abscissa = 15 min. Peak 1 = 10ng α-neo-endorphin 1-8, 2 = 10ng α-neo-endorphin 1-10, 3 = 10ng dynorphin 1-6, 4 = 6.6ng ME, 5 = 10ng β-neo-endorphin, 6 = 7.34ng LEA, 7 = 6.6ng LE, 8 = 25ng α-endorphin, 9 = 20ng pro-enkephalin.

ments in concentration. The use of a formic acid - pyridine buffer in the mobile phase was unacceptable because it caused a substantial decrease in the sensitivity of the working electrode.

The effect of other salts on endorphin retention was examined by adding potassium chloride or potassium perchlorate to the phosphate buffer-acetonitrile mobile phase. In general, increases in the potassium chloride concentration (0.25mM to 2.5mM, final concentrations) decreased the retention times of ME and LE. Increases in potassium perchlorate concentrations (0.25mM to 2.5mM, final concentrations) had little effect on endorphin retention times.

B. pH. Mobile phases ranging in pH from 2.0 to 7.5 were used to chromatograph ME and LE. Although a decrease in pH caused an increase in ME and LE retention, small changes in pH did not substantially alter the chromatographic behavior of ME or LE. A pH of 2.3 was chosen for the phosphate buffer-acetonitrile mobile phase because retention of the early eluting endorphins (α-neo-endorphin 1-8 and 1-10) was improved at low pH.

C. Organic Modifier. Methanol, acetonitrile, tetrahydrofuran and propanol were used as organic modifiers in the isocratic mobile phase. For each organic modifier, the endorphins could be chromatographed only within a limited concentration range. Organic modifier concentrations above or below this range caused neither retention nor elution of the endorphins. (i.e. using the Aquapore 300 column, the K' of β-neo-endorphin when using 10% acetonitrile in the mobile phase was > 20 but with 14.8% CH_3CN, the K' was reduced to 6.4). This effect was independent of the type of column or buffer used for endorphin chromatography.

It was not possible to substitute one organic modifier for another solely by maintaining an equivalent polarity of the aqueous-organic mobile phase. Table 1 shows that the accuracy of K' predictions for ME and LE from the calculated polarity of the mobile phase was dependent on the type of organic modifier. A comparison of calculated K' ratios vs K' ratios obtained experimentally with aqueous-acetonitrile, aqueous-methanol and aqueous-acetonitrile-tetrahydrofuran mobile phases indicates that ME and LE are not eluted

TABLE 1

Comparison of Experimentally Determined (Exp.) K' Ratios to Calculated (Calc.) K' Ratios

Solvent Polarities P_2' ; P_1' (Organic Modifier)	Calc. K' Ratios*	ME Exp. K' Ratios	ME % Calc./Exp.	LE Exp. K' Ratios	LE % Calc./Exp.
9.408 : 9.32 (CH_3CN only)	1.11	1.78	62	1.62	68.5
9.32 ; 9.14 (CH_3CN ; CH_3CN-THF)	1.23	1.17	105	1.04	118
9.14 ; 9.052 (CH_3CN-THF only)	1.11	1.19	93	1.20	92.5
9.052 ; 8.67 (CH_3CN-THF ; MeOH)	1.55	0.32	484	0.26	596
9.408 ; 8.67 (CH_3CN ; MeOH)	2.34	0.86	272	0.58	403

Column = 5µm Biophase ODS; Mobile phase = 10mM ammonium acetate, pH 4.25 (adjusted with glacial acetic acid) + varying amounts of organic modifiers to obtain the P' values indicated above. CH_3CN = acetonitrile; THF = tetrahydrofuran; MeOH = methanol.

K' ratios* calculated by the formula $\frac{K_2'}{K_1'} = 10^{(P_2' - P_1')\div 2}$ (23)

only on the basis of polarity considerations. Aqueous-acetonitrile mobile phases were more efficient in decreasing the K' of ME and LE than other aqueous-organic mobile phases of equivalent polarities.

Another significant factor for the resolution of ME and LE from other endorphins was the total salt concentration in the mobile phase versus the concentration of the organic modifier. Small changes in salt concentration below an optimal level caused a change in peak shape even if the concentration of organic modifier was sufficient to maintain an equivalent K'. The two endorphins most affected were β-neo-endorphin and α-nec-endorphin 1-10.

In Figure 1, the α-neo-endorphin 1-10 peak is split. A sharp single peak was obtained by increasing the final KH_2PO_4 concentration to 30mM in the mobile phase. Figure 2 shows the effect of small changes in the KH_2PO_4 and acetonitrile concentrations on the elution of ME and β-neo-endorphin. The peak shape of ME was essentially unchanged while the peak shape of β-neo-endorphin broadened.

D. Ion-pairing Reagents. Data obtained with trifluoroacetic acid and nonylamine indicated that the retention times of both ME and LE were similarly decreased with increasing ion-pairing reagent concentrations in the mobile phase. Increases in tetrabutylammonium hydrogen sulfate concentration caused small increases in the retention of ME and LE.

After examining the effect of various mobile phase components on endorphin chromatography, the composition of the isocratic mobile phase was adjusted so that the ME and LE peaks were clearly resolved from the other endorphin standards using the Aquapore RP-300 column. As shown in Figure 1, a mobile phase consisting of 128ml [50mM KH_2PO_4-55μM glycylglycine, pH 2.3 (adjusted with 85% phosphoric acid) + 29% CH_3CN] diluted to 250ml with water, was warmed to 27°C in a circulating water bath and used for endorphin chromatography. This buffer was suitable as a mobile phase for endorphin chromatography using each of the reversed phase columns described above by making small adjustments in the phosphate or acetonitrile concentrations. Therefore, this mobile phase was used for most of these studies.

Glycylglycine was added to the mobile phase to prevent absorption of the endorphins to residual silanols on the column packing material (10). There was no change in the shape of the endorphin peaks with an increase in glycylglycine concentration in the mobile phase. The order of endorphin elution was α-neo-endorphin 1-8 followed by α-neo-endorphin 1-10, dynorphin 1-6, ME, β-neo-endorphin, LEA, LE, α-endorphin, and pro-enkephalin (Table 2). BE and dynorphin 1-17 was not eluted from the column with this mobile phase. The order of endorphin elution was different from that predicted by the summation of the retention coefficients of component amino acids as determined by Wilson et al (15) and Meek and Rossetti (25) (Table 2).

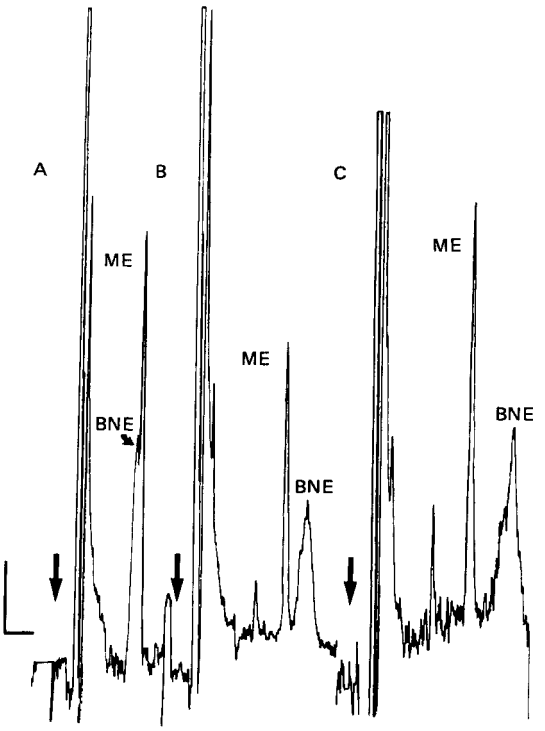


FIGURE 2

Effect of changes in mobile phase on ME and β-neo-endorphin (BNE) elution. Column = Aquapore RP-300; Oxidation Potential = +1.05V vs Ag/AgCl; Flow Rate = 1.0ml/min; Reference Bars: Ordinate = 0.1nA; Abscissa = 4 min.; arrow denotes injection.
A = 128 ml of 52mM KH_2PO_4-55μM glycylglycine, pH 2.3 containing 32% CH_3CN diluted to 250 ml with H_2O.
B = 128 ml of 50mM KH_2PO_4-55μM glycylglycine, pH 2.3 containing 29% CH_3CN diluted to 250 ml with H_2O.
C = 128 ml of 47mM KH_2PO_4-55μM glycylglycine, pH 2.3 containing 31% CH_3CN diluted to 250 ml with H_2O.

Electrochemistry

1. Hydrodynamic Voltammograms

Hydrodynamic voltammograms for ME, LE and LEA were performed with the mobile phase described in Figure 1. The curves in Figure 3 indicate that ME, LE and LEA are oxidized by the glassy carbon working electrode at potentials above +.90V referenced to Ag/AgCl. The

TABLE 2

Order of Endorphin Elution

Endorphin	This study	Meek & Rossetti (25)	Wilson et al (15)
α-neo-endorphin 1-8	1	4	6
α-neo-endorphin 1-10	2	5	4
dynorphin 1-6	3	2	2
ME	4	1	1
β-neo-endorphin	5	9	7
LEA	6	8	-
LE	7	3	3
α-endorphin	8	7	5
pro-enkephalin	9	6	8

The order of endorphin elution observed in this study was compared with the order calculated by the summation of the retention coefficients (25,15) of the amino acids contained in each endorphin. In this study, endorphins were chromatographed with either the μBondapak or Aquapore RP-300 reversed phase columns at a flow rate of 1.0 ml/min with an isocratic mobile phase consisting of 128 ml of [50mM KH_2PO_4 -55μM glycylglycine, pH 2.3 (adjusted with 85% phosphoric acid) containing 29% acetonitrile] diluted to 250 ml with water. The mobile phase reservoir was suspended in a circulating water bath at 27°C.

plateau region of the curve occurs at approximately +1.09V for LE and LEA. For ME, however, the peak height is still increasing with increasing oxidation potentials. Other endorphins, α-neo-endorphin 1-8 and 1-10, dynorphin 1-6, β-neo-endorphin, α-endorphin and pro-enkephalin were also electroactive at applied potentials above +.90V. A potential of +1.05V was chosen for these studies as a compromise between increasing peak height and increasing noise. (A chelator such as ethylenediamine-tetraacetic acid was not used in the mobile phase due to its oxidation at these potentials).

2. Standard Curves

The detector response was linear from 1 ng to 20 ng of ME, LE or LEA (the concentration range used for these studies).

3. Sensitivity

The most significant factor affecting the sensitivity of electrochemical detection of endorphins was the age of the working electrode.

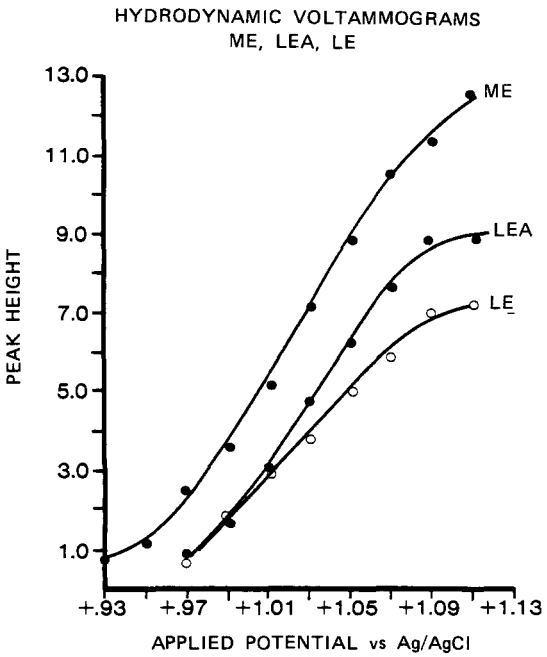


FIGURE 3

Chromatographic conditions as in Figure 1. (Peak height in centimeters; Applied potential in volts; Sensitivity = 2nA/V).

With a new TL-5 glassy carbon electrode (Bioanalytical Systems) approximately 300 picograms of ME (signal to noise = 3; 1.7nA/ng) were detected. The detection limit for LE using the same electrode was approximately 600 picograms. The sensitivity of the electrode gradually decreased with continued use at high potentials (>+1.0V). Repolishing the electrode restored some of the sensitivity but not to its original level. Another factor influencing the sensitivity of EC detection was the composition and pH of the mobile phase. Both ME and LE oxidized more readily in a mobile phase consisting of 10mM ammonium acetate (adjusted to pH 4.25 with glacial acetic acid) containing methanol with an optimal potential of +.99V referenced to Ag/AgCl. The use of a lower potential decreased the background current and baseline noise causing a small increase in sensitivity.

The chromatographic resolution of the endorphins, however, was improved when the phosphate-acetonitrile mobile phase was used.

Other factors aiding EC sensitivity included several minor modifications to the LC system. The mobile phase reservoir was suspended in a circulating water bath at a constant temperature (27°C) to minimize baseline shift. Stainless steel solvent reservoir filters were omitted from all mobile phase reservoirs to eliminate baseline noise due to oxidation of metallic contaminants (personal communication, Dr. Michael Joseph, MRC Clinical Research Centre, U.K.) A Topaz power conditioner was used to protect the electrochemical detector from aberrant power line fluctuations.

DISCUSSION

Several previous studies of endorphin chromatography have used gradient elution to separate and investigate the behavior of a series of endorphin standards on reversed phase columns (10, 14, 15, 24, 25). This study investigated the use of an isocratic mobile phase for the elution of endorphins. Using LC-EC, various chromatographic conditions were manipulated to separate ME and LE from other endorphins of similar hydrophobicity. The chromatographic behavior of the endorphins in response to certain changes in the composition of the isocratic mobile phase was different from the behavior that has been reported for gradient elutions (15, 25, 26). Alterations in the organic modifier and total salt concentration affected endorphin resolution dramatically, either by a change in K' or by a change in peak shape. The role of the total salt concentration in the mobile phase observed in this study for the isocratic resolution of endorphins, however, is in agreement with the results obtained with gradient elution (26). The most important factors for endorphin retention were the concentration and type of organic modifier in the mobile phase. As reported for ACTH-related peptides (26), the peptides used in this study were efficiently chromatographed only within a limited concentration range for each organic modifier. In addition, endorphin retention could not be predicted solely on the basis of polarity considerations when substituting different organic modifiers in the mobile phase (Table 1). Like somatostatin (27), selectivity was increased when acetonitrile was used as the organic

modifier instead of methanol. This is in contrast to what has been observed using gradient elution (26). Reversals in retention order were reported with the use of some organic modifiers but, for the most part, the retention times of the polypeptides were shifted according to the polarity of the solvent (26).

Recently, the elution characteristics of an extensive series of peptides have been investigated in an effort to correlate peptide retention with amino acid composition (15, 25). In both reports, large numbers of peptides (n=95-100) were used to determine the hydrophobicity or retention coefficients for each amino acid. An examination of predicted and actual retention times of peptides using gradient elution, showed a high degree of correlation (Meek and Rossetti (25), correlation coefficient = 0.98; Wilson et al (15) correlation coefficient = 0.83). However, this study demonstrates that these retention coefficients can not be used to predict the order of endorphin elution when using isocratic mobile phases (Table 2). The observation of differences in the chromatographic behavior of endorphins when comparing gradient elution and isocratic elution was not unexpected since some of the endorphins are particularly affected by changes in the mobile phase composition.

The specific aim of this study was to use LC-EC to separate and detect a series of endorphin standards. Therefore, various chromatographic parameters were investigated to develop conditions for the isocratic elution of endorphins. A simple phosphate buffer - acetonitrile isocratic mobile phase was used to separate endorphins on C_8 or C_{18} reversed phase columns. The oxidation characteristics of both ME and LE were examined by hydrodynamic voltammetry and the electroactivity of several other endorphins (α-neo-endorphin 1-8 and 1-10, dynorphin 1-6, β-neo-endorphin, α-endorphin and pro-enkephalin) was demonstrated. These data indicate that the specificity and and sensitivity of LC-EC may provide an efficient and inexpensive alternative to RIA for the assay of endorphins. Application of this technique to the study of brain samples is currently in progress.

ACKNOWLEDGMENTS

The authors wish to thank Sharon A. Neuman for typing this manuscript and Diane M. Pergande for technical assistance.

REFERENCES

1. Beddell, C. R., Lowe, L. A., and Wilkinson, S., Endogenous opioid peptides - The enkephalins and endorphins, Prog. Med. Chem.,17, 2, 1980.

2. Panerai, A. E., Martini, A., DeRosa A., Salerno, F., Di Giulio, A. M., and Mantegazza, P., Regulatory Peptides: From Molecular Biology to Function, Costa E. and Trabucchi, M., eds., Raven Press, New York, 1982, p. 139.

3. DeWied, D. and Jolles, J., Neuropeptides derived from pro-opiocortin; Behavioral, physiological, and neurochemical effects, Physiol. Reviews, 62, 976, 1982.

4. Dingledine, R., Possible mechanisms of enkephalin action on hippocampal CA1 pyramidal neurons, J. Neurosci., 1, 1022, 1981.

5. Miller, R. J., Peptides as neurotransmitters: Focus on the enkephalins and endorphins, Pharmac. Ther., 12, 73, 1981.

6. Nathanson, J. A., Neurosecretion and Brain Peptides, Martin, J. B., Reichlin, S., Bick, K. L., eds., Raven Press, New York, 1981, p. 599.

7. Sheehy, M., Schacter, M., Marsden, C. D., Parkes, J. D., Research Progress in Parkinson's Disease, Rose, F. C. and Capildeo, R., eds., Pilman Medical, Kent, 1981, p. 165.

8. Akil, H., Watson, S. J., Berger, P. A., Barchas, J. D., The Endorphins, (Adv. Biochem. Psychopharm., vol. 18), Costa, E., and Trabucchi, M., eds., Raven Press, New York, 1978, p. 125.

9. Przewlocki, R., Höllt, V., Kleber, G., Gramsch, C. H., Haarmann, I., and Herz, A., Long-term morphine treatment decreases endorphin levels in rat brain and pituitary, Br. Res., 174, 357, 1979.

10. Loeber, J. G., Verhoef, J., High-pressure liquid chromatography and radioimmunoassay for the specific and quantitative determination of endorphins and related peptides, Methods Enzymol., 73, 261, 1981.

11. Morris, H. R., Etienne, A. T., Dell, A., and Albuquerque, R., A rapid and specific method for the high resolution, purification and characterization of neuropeptides, J. Neurochem., 34, 574, 1980.

12. Herz, A., Gramsch, C., Höllt, V., Meo, T., Riethmuller, G., Characterization of a monoclonal β-endorphin antibody recognizing the N-terminus of opioid peptides, Life Sci., 31, 1721, 1982.

13. Currie, B. L., Chang, J.-K., Cooley, R., High performance liquid chromatography of enkephalin and endorphin peptide analogs, J. Liq. Chromatogr., 3, 513, 1980.

14. Davis, T. P., Schoemaker, H., Chen, A., Yamamura, H., High performance liquid chromatography of pharmacologically active amines and peptides in biological materials, Life Sci., 30, 971, 1982.

15. Wilson, K. J., Honegger, A., Stotzel, R. P., Hughes G. J., The behavior of peptides on reverse-phase supports during high-pressure liquid chromatography, Biochem. J., 199, 31, 1981.

16. Mousa, S., Mullet, D., Couri, D., Sensitive and specific high performance liquid chromatographic method for methionine and leucine enkephalins, Life Sci., 29, 61, 1981.

17. McDermott, J. R., Smith, A. I., Biggins, J. A., Chyad Al-Noaemi, M. and Edwardson, J. A., Characterization and determination of neuropeptides by high-performance liquid chromatography and radioimmunoassay, J. Chromatogr., 222, 371, 1981.

18. Kissinger, P. T., Bruntlett, C. S., and Shoup, R. E., Neurochemical applications of liquid chromatography with electrochemical detection, Life Sci., 28, 455, 1981.

19. Warsh, J. J., Chiu, A., Godse, D. D., Simultaneous determination of norepinephrine, dopamine and serotonin in rat brain regions by ion-pair liquid chromatography on octyl silane columns and amperometric detection, J. Chromatogr., 228, 131, 1982.

20. Meek, J. L., Yang, H. Y. T., Costa E., Enkephalin catabolism in vitro and in vivo, Neuropharmac., 16, 151, 1977.

21. Bennett, G. W., Brazell, M. P., Marsden, C. A., Electrochemistry of neuropeptides: A possible method for assay and in vivo detection, Life Sci., 29, 1001, 1981.

22. Fleming, L. H., Reynolds, N. C., and Stein, E. A., LCEC of opiate peptides: Separation and quantification from rat brain, Third Int. Symposium on LCEC and Voltammetry, 52, 1982.

23. Snyder, L. R. and Kirkland, J. J., Introduction to Modern Liquid Chromatrophy, Second edition, Wiley, New York, 1979.

24. Meek, J. L., Prediction of peptide retention times in high pressure liquid chromatography on the basis of amino acid composition, Proc. Natl. Acad. Sci. USA., 77, 1632, 1980.

25. Meek, J. L., Rosetti, Z. L., Factors affecting retention and resolution of peptides in high-performance liquid chromatography, J. Chromatogr., 211, 15, 1981.

26. O'Hare, M. J. and Nice, E. C., Hydrophobic high-performance liquid chromatography of hormonal polypeptides and proteins on alkyl-silane-bonded silica, J. Chromatogr., 171, 209, 1979.

27. Abrahamsson, M., and Grőningsson, K., High-performance liquid chromatography of the tetradecapeptide somatostatin, J. Liq. Chromatogr., 3, 495, 1980.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 809-821 (1984)

A COMBINED HPLC-VIS SPECTROPHOTOMETRIC METHOD FOR THE IDENTIFICATION OF COSMETIC DYES

J.W.M. Wegener and H.J.M. Grünbauer*
Department of Organic Chemistry
Vrije Universiteit
De Boelelaan 1083
1081 HV Amsterdam
The Netherlands

R.J. Fordham and W. Karcher
Commission of the European Communities
DG XII, Joint Research Centre
Petten Establishment
1755 ZG Petten
The Netherlands

ABSTRACT

Ion-pair reversed phase HPLC was observed to give very good separations of 20 representative cosmetic dyes whilst numerical analysis of VIS spectra provided an efficient additional means of identification when similar retention times for different dyes were encountered. The results strongly suggest that a combination of HPLC and rapid scanning VIS spectrophotometry should be very promising, especially when on-line computing facilities are available.

INTRODUCTION

As part of its programme to harmonise the European Community's legislation on cosmetics the Council of the EC has approved a list of organic dyes for use in cosmetic products (1).

* Present address : Physical Pharmacy Group, Department of Pharmacy, University of Amsterdam, Plantage Muidergracht 24, 1018 TV Amsterdam, The Netherlands.

 0148-3919/84/0704-0809$3.50/0

This decision and the evident need to verify adherence to the Directive led to interest in a rapid and efficient HPLC method for identification purposes. Several reports have already been published in the closely related field of food-dye analysis (2-9). Although this work has been of value to our study, its results are only partially applicable to cosmetic dyes since the vast majority of permitted food dyes are hydrophylic anionic molecules. The behaviour of other types of dyes (non-ionic and basic) under similar HPLC conditions still remained to be clarified.

Market indications suggest that around 50 organic dyestuffs are in current use in cosmetics in the European Community whilst 256 organic colouring agents are permitted by the Directive. These numbers contrast sharply with the fact that at most 29 dyes have hitherto been studied simultaneously (6). Moreover, we estimate from published compilations of retention times (6) and chromatograms (2-4) that constituents in mixtures of at most 10 different colorants can be identified by chromatography alone. The use of a secondary means of identification therefore seems inevitable. We have chosen an approach in which HPLC is coupled with VIS spectrophotometry; results obtained with a representative collection of 21 cosmetic dyes are presented in this paper.

MATERIALS AND METHODS

Chromatograph

The liquid chromatograph comprised two Waters model 6000 A pumps connected to a Valco loop injection valve and a Waters model 660 Solvent Programmer.
The detector was a Perkin-Elmer LC 55 S Spectrophotometer with a 10 mm optical pathlength flow cell.

Column

CP SpherC18 (Chrompack); 25 cm x 4.6 mm i.d.

Pairing Ion

Waters PIC^{TM} Reagent A containing $(Bu_4N)_3PO_4$ diluted with methanol according to the manufacturers specification.

Elution

45 minutes linear gradient from 50 to 100% methanol (containing PIC^{TM} Reagent A) in water at a flow rate of 1 ml/min.

Colorants

Throughout this paper, dyes are referred to by their CI reference numbers taken from the Colour Index (10).

sulfonic acids : 13065, 14700, 15510, 15585, 15630, 15850, 15985, 16035, 19140, 42051, 42090.

carboxylic acids : 45350, 45370, 45380, 45396, 45410.

basic : 45170.

non-ionic : 12075, 12085, 26100, 61565.

Sample preparation

50-100 mg of a cosmetic sample was dissolved in 2 ml of a solution of H_3PO_4 (5% v/v) in dimethylformamide (DMF). A few ml hexane was added to extract any fatty material. This extraction procedure was repeated five times. The extraction step was ommitted for non-fatty samples, e.g. powders. If the combined hexane fractions were coloured, two back-extractions with 2 ml DMF-H_3PO_4 were carried out and the DMF layers added to the DMF extract. The DMF - H_3PO_4 solution was diluted with a methanolic PIC^{TM} Reagent A solution (until a suitable extinction was reached) and was subsequently chromatographed.

Spectrophotometer

All visible spectra were recorded using an Aminco DW2a spectrophotometer equipped with a semi-automatic device for recording absorption readings at 5 nm intervals between 350 and 750 nm.

Spectrophotometric identification

In order to distinguish between colorants, with similar or identical retention times (e.g. CI 14700 and CI 15850, see Fig. 1), a generally applicable regression method has been employed. This method is based on the Lambert-Beer law :

$$\frac{A_i}{A_i^o} = \frac{c_i}{c_i^o} \qquad \ldots \quad (1a)$$

or

$$A_i = \frac{c_i}{c_i^o} \cdot A_i^o \qquad \ldots \quad (1b)$$

where the absorbance at wavelength λ_i of an "unknown" colorant solution and its reference sample are represented by A_i and A_i^o, respectively. The corresponding concentrations are denoted by c_i and c_i^o.

In the example cited, an unresolved HPLC fraction containing either CI 14700 or CI 15850 was collected and its VIS spectrum between 350 and 750 nm was recorded digitally, as described above. The validity of the Lambert-Beer law was verified at all wavelengths λ_i by computing a linear regression equation of the form:

$$y = ax + b \qquad \ldots \quad (2)$$

where the spectrum of the fraction is denoted by y and that of the reference spectrum of either CI 14700 or CI 15850 by x (11). It

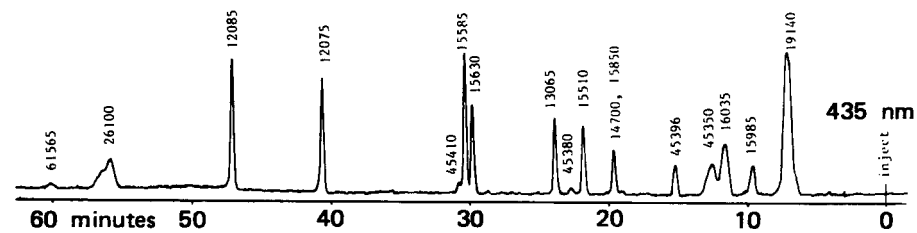
follows from equation 1b that, if the Lambert-Beer law holds, the slope a must be equal to the ratio of concentrations and the intercept b must be zero (11). The identification of the best fitting dye was based in part on the idea that calculated intercepts should be smaller than experimental error and also, in part, on the closeness of fit of the regression, as judged by several statistical test parameters (12). The parameters chosen were : Fisher's variance ratio and Student's t-test, both of which should be as high as possible; the correlation coefficient, which should be close to 1; and the standard error of estimate, which should be as small as possible.

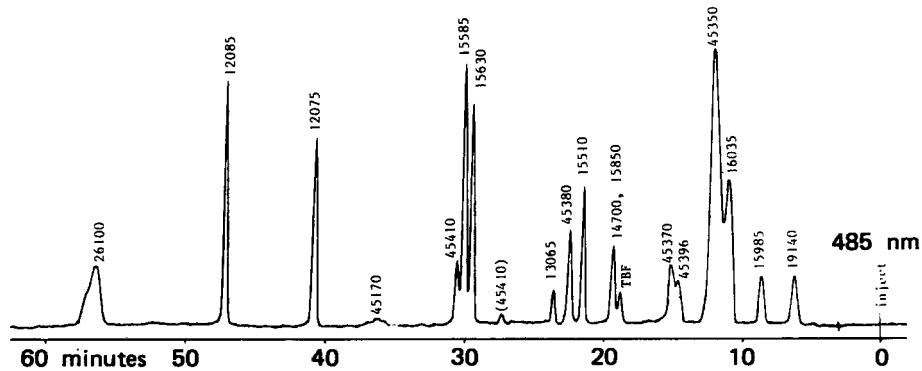
RESULTS AND DISCUSSION

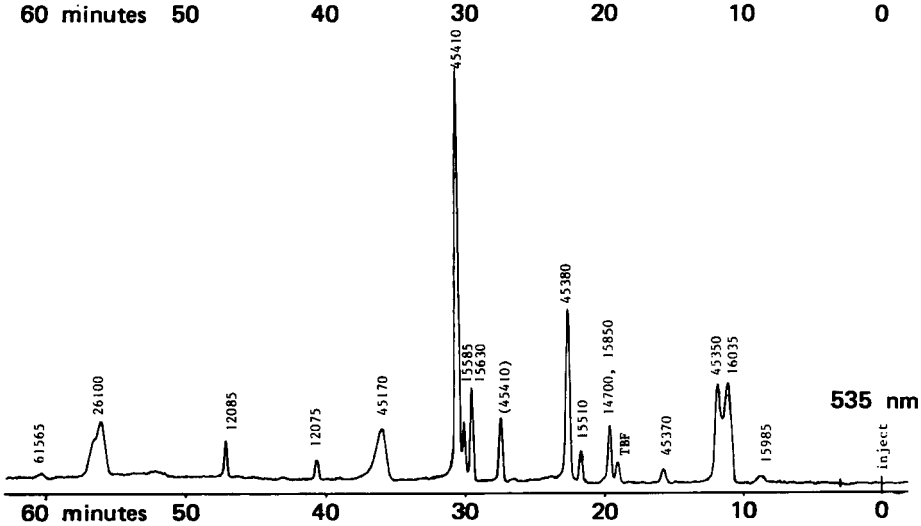
Resolution of a Mixture of 21 Colorants

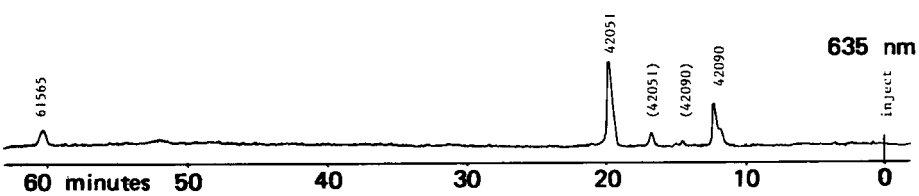
Figure 1 shows chromatograms of a mixture containing all 21 colorants recorded at the following wavelengths : 435, 485, 535 and 635 nm. The general order of elution by chemical classes is : acid colorants, basic colorant(s) and finally, non-ionic colorants. This sequence is somewhat surprising as far as the behaviour of the basic colorant, CI 45170, is concerned. It was apparent in preliminary studies that this colorant was not retarded at all when unbuffered cetrimide was used as the pairing ion. In the present study, the behaviour of CI 45170 can perhaps be explained by postulating the formation of ion pairs with the phosphate buffer ions.

Two colorants were found to have identical retention times. These colorants, CI 14700 and CI 15850, also exhibit very similar spectra (see Fig. 3) : a comparison using correlation analysis (12) resulted in a correlation coefficient of 0.936 between the spectra of both colorants dissolved in methanolic PIC^{TM} Reagent A solution. This value is rather close to the limit of 1, at which


61565
26100
12085
12075
45410
15585
15630
13065
45380
15510
14700, 15850
45396
45350
16035
15985
19140
inject
435 nm
60 minutes 50 40 30 20 10 0


26100
12085
12075
45170
45410
15585
15630
(45410)
13065
45380
15510
14700, 15850
TBF
45370
45396
45350
16035
15985
19140
inject
485 nm
60 minutes 50 40 30 20 10 0


61565
26100
12085
12075
45170
45410
15585
15630
(45410)
45380
15510
14700, 15850
TBF
45370
45350
16035
15985
inject
535 nm
60 minutes 50 40 30 20 10 0


61565
42051
(42051)
(42090)
42090
inject
635 nm
60 minutes 50 40 30 20 10 0

the spectra would have been identical. The problem of distinguishing between CI 14700 and CI 15850 therefore represents a sharp test of the power of the spectrophotometric method.

Chromatograms of individual colorant samples (not shown here) clearly demonstrated the presence of coloured impurities in samples of the following colorants : CI 42051, CI 42090, CI 45370, CI 45380 and CI 45410. The nature of these impurities is unknown except in CI 45370 (dibromofluorescein) and CI 45380 (tetrabromofluorescein or eosine) where tribromofluorescein (TBF) has been positively identified (7). In Figures 1 and 2, secondary peaks associated with a particular colorant are denoted by means of the CI number in parentheses with exception of tribromofluorescein, which is marked TBF.

HPLC of extracts of cosmetics

Eight different cosmetic samples supposedly containing from 1 to 4 organic colorants have been investigated. Figure 2 gives a typical chromatogram of a lipstick extract, recorded at 430, 485, 535 and 635 nm. It is obvious that at least six dyes must be present: CI 12085, CI 15850 (confirmed by its spectrum, see below), CI 45370, CI 45380, CI 45410 and TBF. Subsequent inspection of the disclosed formulation indeed showed this interpretation to be correct although it should be noticed that, according to the manufacturer, both TBF and CI 45380 should be considered as impurities of CI 45370. The small peak at about 7 minutes was assigned to an impurity of CI 45410.

Similar successful results were obtained for extracts from samples of 4 other lipsticks, a skin lotion, an after sun cream

FIGURE 1. Chromatograms of a mixture of 21 cosmetic colorants separated by ion pair reversed phase HPLC. Conditions : aqueous methanol (containing PIC™ Reagent A) 50% to 100% methanol linear gradient in 45 min. at 1 ml/min. flow rate on CP Spher C18. Detection at 435 (top), 485, 535 and 635 nm (bottom).

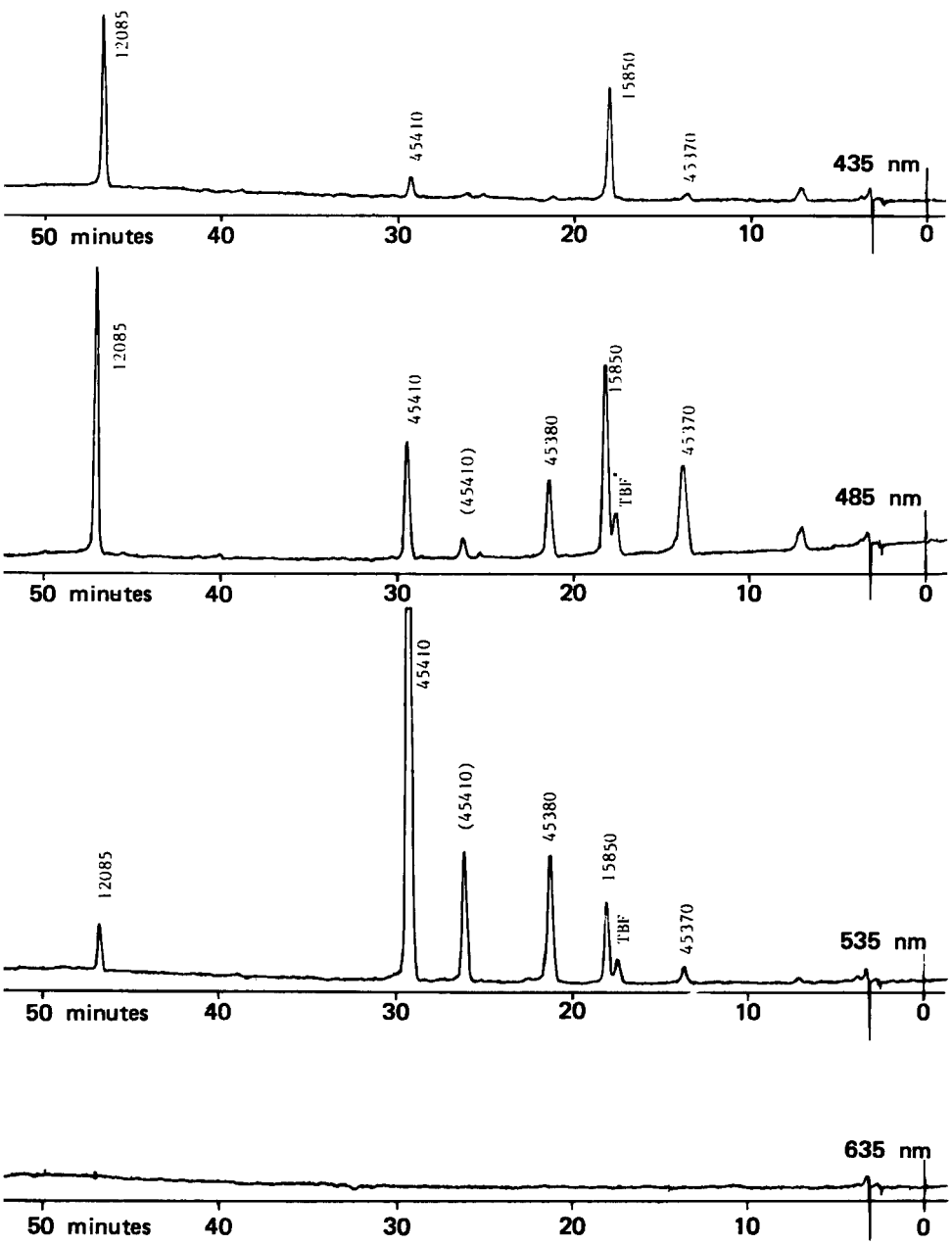


FIGURE 2. Chromatograms of an extract from a lipstick. Experimental conditions as in Fig. 1.

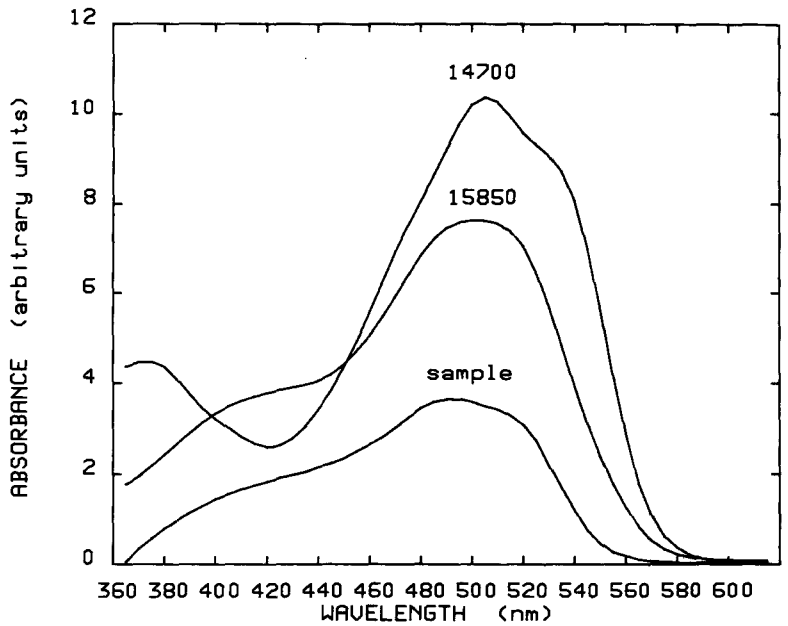


FIGURE 3: Visible absorption spectra of two colorants with identical retention characteristics in the chromatographic system used together with the spectrum of a fraction taken from the chromatograph of an unknown colorant separated from a lipstick (see text).

and a foam bath. In all cases, the right combination of colorants was correctly identified. When either CI 14700 or CI 15850 was indicated in the chromatogram, the spectrophotometric identification procedure was followed.

Spectrophotometric identification

The spectrum of the CI 14700/CI 15850 fraction of the lipstick extract of Figure 2 is shown in Figure 3, together with reference spectra of both dyes, recorded under similar conditions. Figure 3 shows clearly the similarities between the spectra which

TABLE 1

Multiple Regression Analysis of a HPLC Fraction from a Lipstick.

Regression	F	R	S	t
y versus CI 14700	174	0.845	0.007	13.2
y versus CI 15850	1280	0.974	0.003	35.8

Key: y = VIS spectrum of the HPLC fraction
F = F test for significance of fit
R = correlation coefficient
S = standard error of estimate of regression
t = Student's t-test

were confirmed by the regression analysis described earlier. Evidently, it will be very difficult to distinguish the dyes even when the full spectra are used instead of absorbance ratios at two different wavelengths.

The regression analysis of the spectra of Figure 3 is summarized in Table 1. Calculated intercepts are not shown but in both cases were smaller than the standard error of estimate. As may be seen from R in Table 1, CI 14700 can account for a fairly large part (about 70%) of the unknown spectrum, but CI 15850 leads to better results : all statistical tests are significantly better and almost 95% of the unknown spectrum is explained. This result is very satisfactory, especially when one realises that the absorbance of the unknown spectrum was very weak (a peak maximum of 0.04 absorption units). The additional 5% can be attributed to noise and CI 15850 alone is confirmed as being present in the sample.

CONCLUSIONS

1 Chromatographic conditions permitting the positive identification by a relatively simple procedure of at least 20 representative cosmetic dyes have been developed.

2 A numerical method using VIS spectra and (stepwise) multiple regression analysis is available to identify colorants with similar or identical retention time.

3 Application of both methods to representative cosmetic samples yielded satisfactory qualitative determinations of the colorant content.

As an overall conclusion, ion-pair reversed phase HPLC in combination with VIS spectrophotometric detection should be considered a very promising approach to the general problem of the identification of cosmetic dyes. In our opinion, an efficient approach to this problem could be the use of a linear photodiode array spectrophotometer coupled directly to the chromatograph. Such an arrangement, connected to an on-line computer, would permit routine identifications requiring in principle only one injection per sample in place of the four injections described here. In addition the need to collect fractions could be avoided.

ACKNOWLEDGEMENTS

This work was carried out under contract to the Commission of the European Communities (Directorate General XI - Protection of the Environment, Consumer and Nuclear Safety). Two of the authors (JWMW and HJMG) are grateful for the technical facilities and help provided by the Joint Research Centre of the European Communities, Petten Establishment and also for useful discussions with Mr. B. Le Goff. Thanks are also due to Prof. Dr. E. Tomlinson and Dr. H. van Rooy of the University of Amsterdam for advice and guidance on ion-pair

chromatography and to Dr. C. Werkhoven of the Free University at Amsterdam for assistance in the packing of columns. We also thank Prof. Dr. R.F. Rekker and Dr. T. Bultsma of the Free University at Amsterdam for the use of their Aminco DW 2a spectrophotometer.

REFERENCES

1. Council Directive of 27 July 1976 on the Approximation of the Laws of the Member States relating to Cosmetic Products (76/768/EEC), Official Journal N° L 262, 27.9.1976, p. 169-200; modified by the Amendment Directive of 17 May 1982 (82/268/EEC), OJ N° L 167, 15.6.1982, p. 1-32.

2. Lawrence J.F., Lancaster F.E. and Conacher H.B.S., Separation and Detection of Synthetic Food Colors by ion-pair High Performance Liquid Chromatography, J. Chrom., 218, 168, 1981.

3. Puttemans M.L., Dryon L. and Massart D.L., Ion-pair High Performance Liquid Chromatography of Synthetic Water-Soluble Acid Dyes, J. Assoc. Off. Anal. Chem., 64, 1, 1981.

4. Noda A. and Nishiki S., Separation of Synthetic Organic Food Colors by High Speed Liquid Chromatography, J. Food Hyg. Soc. Jap., 18, 321, 1977.

5. Boley N.P., Bunton N.G., Crosby N.T., Johnson A.E., Roper P. and Somers L., Determination of Synthetic Colours in Foods Using High-Performance Liquid Chromatography, Analyst, 105, 589, 1980.

6. Chudy J., Crosby N.T. and Patel I., Separation of Synthetic Food Dyes Using High Performance Liquid Chromatography, J. Chrom., 154, 306, 1978.

7. Peeples II W.A. and Heitz J.R., The Purification of Xanthene Dyes by Reverse Phase High Performance Liquid Chromatography, J. Liq. Chrom., 4, 51, 1981.

8. Martin G.E., Tenenbaum M., Alfonso F. and Dyer R.H., High Pressure Liquid and Thin Layer Chromatography of Synthetic Acid Fast Dyes in Alcoholic Products, J. Assoc. Off. Anal. Chem., 61, 908, 1978.

9. Gonnet C., Marichy M. and Naghizadeh A., Ion-pair Thin Layer Chromatography. Applications to the Analysis of Water-Soluble Food Dyes and Possibility of Data Transfer TLC-HPLC, Analusis, 8, 243, 1980.

10. Colour Index, 3rd Edition, Society of Dyers and Colourists, Bradford, 1971.

11. Grünbauer H.J.M., Bultsma T., Bijloo G.J. and Rekker R.F., Determination of Erythrocyte Membrane-Buffer Partition Coefficients by Numerical Analysis of Multicomponent Spectra, J. Pharm. Pharmacol, 31, 452, 1979.

12. Draper N.R. and Smith H., Applied Regression Analysis, Wiley NY, 1966.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 823-837 (1984)

A REVERSE PHASE HPLC ASSAY FOR THE DETERMINATION OF CALCIUM PANTOTHENATE UTILIZING COLUMN SWITCHING

Thomas J. Franks, John D. Stodola
The Upjohn Company
Product Control 7821-259-2
Kalamazoo, Michigan 49001

ABSTRACT

A reverse phase HPLC assay utilizing column switching has been developed and validated for the determination of calcium pantothenate (CP) in several multivitamin tablet formulations. The reverse phase system utilizes a DuPont Zorbax C-8 analytical column, an automatically switched and backflushed Brownlee RP-18 guard column for the elimination of a highly retained excipient peak, 88:12 0.25M phosphate buffer:MeOH mobile phase, and 214 nm detection. Sample preparation and the switched column chromatography cycle each require approximately 15 minutes. A spiked recovery study showed linearity over the 50-150% of theory concentration range. Average recovery was 99.7%. Assay precision studies yielded sample RSD's ranging from 0.8 to 2.3%. Results obtained by this method are comparable to those obtained by the USP method.

INTRODUCTION

Calcium pantothenate (CP) is the calcium salt of vitamin B_3. It is a component of a variety of multivitamin formulations. The USP microbiological assay for CP (1) requires a lengthy, labor intensive sample preparation, a 16 to 24 hour incubation and the measurement of turbidity of samples and standards. The time consuming nature of the USP assay provided the incentive to develop an HPLC assay capable of giving equivalent results. We

0148-3919/84/0704-0823$3.50/0

utilized column switching to minimize chromatography run time and a blender to simplify sample preparation.

Technical notes published by Hewlett-Packard (2) and DuPont (3) had demonstrated that it is possible to separate CP from other vitamins by HPLC. Quantitation of components in a tablet formulation often requires the masking or removal of interfering materials. Column switching has been utilized to remove interfering materials from chromatographic systems by a number of researchers (4-9). The development and validation of a sensitive, selective, reproducible column switching HPLC assay for CP in Upjohn multivitamin tablets is the subject of this report.

EXPERIMENTAL

Instrumentation

The HPLC system included the following components; a Varian 5060 programmable ternary chromatograph interfaced to a Varian 8055 autosampler with programmable 110V AC external events, a Valco AH60 six port injector, a Rheodyne 7000 six port valve with air actuator, an Altex 110A HPLC pump, a 110V AC event controlled Humphrey solenoid valve and AC outlet device, an LDC Spectromonitor III variable wavelength detector, and a Sargent-Welch UKR single pen recorder. Chromatographic traces were integrated on an in-house PDP 11/40 based computer system.

Reagents

Methanol (Burdick and Jackson), double distilled water, reagent grade NaH_2PO_4 and phosphoric acid were used to prepare the mobile phase. Reagent grade (99+%) adipic acid (Aldrich) was used as the internal standard. Tablet excipient materials were obtained in-house from production stocks. A representative tablet placebo was prepared by mixing appropriate quantities of excipients.

Backflush Flow Rate:	0.7 ml/min
Detector:	214 nm at 0.05 AUFS
Injection Volume:	10 μl
Internal Standard:	1 g adipic acid/liter in 25% MeOH:75% water solution. Each sample preparation requires 250 ml

Reference Standard

Solution: Accurately weigh approximately 13.2 g USP Reference Standard CP and transfer to a 50 ml volumetric flask. Dissolve in internal standard solution and dilute to volume.

Valve Switching - Backflush Sequence:

Time 0:00	Sample injected, guard column in line with analytical column, backflush pump off.
Time 4:00	Guard column switched off line, backflush pump turned on.
Time 16:00	Guard column switched in line with analytical column, backflush pump off.
Time 17:00	Load autosampler for next injection.

Sample Preparation

Accurately measure 250 ml of internal standard solution and transfer to a Waring blender. Weigh accurately a number of tablets equivalent to 55 to 80 mg of CP and add to the blender. Cover the blender and homogenize at low speed for about 5 minutes. Transfer about 25 ml of the resulting slurry to a centrifuge tube and centrifuge at 2000 rpm for 5 minutes. Filter through a 0.45 μm membrane filter. Inject the filtrate.

HPLC System Survey

A variety of HPLC column/mobile phase combinations were examined to establish conditions under which CP was resolved from

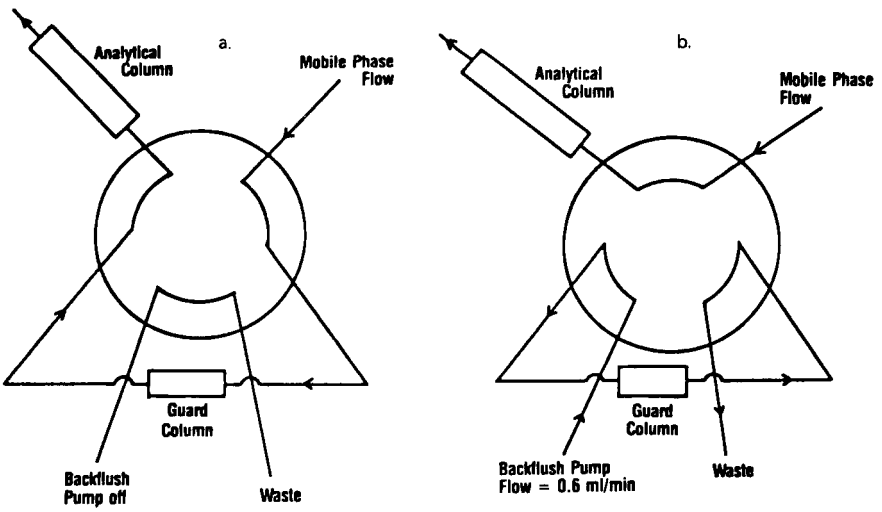


Figure 1. Valve configurations for column switching system.
a) When a sample is injected the mobile phase flows through the guard and analytical columns.
b) After 4 minutes CP and the internal standard have eluted from the guard column onto the analytical column while the highly retained excipient remains within the guard column. The system switches the guard column off line and simultaneously activates a backflush pump to remove the highly retained excipient from the system.

excipient materials (Table 1). An internal standard was chosen from a group of polar organic molecules which were soluble in the mobile phase. The specificity of the system was checked by examining chromatograms of placebo preparations. The retention behavior of CP, adipic acid and excipients was examined on the Brownlee guard column to establish the correct timing for valve position selection and control of the backflush pump.

HPLC Assay Validation

Validation experiments included a spiked placebo recovery/linearity study spanning approximately 50% to 150% of

Table 1

HPLC Systems Investigated

Column	Mobile Phase	Comments
DuPont Zorbax NH_2	pH 3.8 0.005M KH_2PO_4:ACN (20:80)	Resolves CP, ascorbic acid and thiamine; problems with ruggedness
DuPont Zorbax CN	pH 3.8 0.005M KH_2PO_4:ACN (20:80)	CP poorly retained even with weak mobile phase
DuPont Zorbax TMS	pH 3.5 0.25M NaH_2PO_4:MeOH (95:5)	CP unresolved
DuPont Zorbax C-8	pH 3.5 0.25M NaH_2PO_4:MeOH (88:12)	Resolves CP
DuPont Zorbax ODS	pH 3.5 0.25M NaH_2PO_4:MeOH (88:12)	Resolves CP
Waters μBondapak C-18	pH 3.5 0.25M NaH_2PO_4:MeOH (88:12)	Resolves CP
Regis Workhorse ODS	pH 3.5 0.25M NaH_2PO_4:MeOH (88:12)	Resolves CP

tablet potency. The final concentrations in the sample preparation in this study were 0.12 to 0.46 mg CP/ml. Multiple assays of a variety of lots of five products were performed over two days to examine precision. Results obtained by the HPLC assay were compared to those obtained by the USP assay (1).

Recommended HPLC Conditions

Analytical Column:	DuPont Zorbax C-8 25.0 cm x 4.6 mm i.d.
Mobile Phase/Backflush:	1000 ml 0.25M phosphate buffer (pH 3.5) mixed with 135 ml methanol. Filter and degas before using.
Guard Column:	Brownlee MPLC 3 cm cartridge column packed with RP-18 Spheri-5. See Figure 1

for mounting configuration on the Rheodyne 7000 valve

Analytical Flow Rate: 1.5 ml/min

RESULTS AND DISCUSSION

HPLC System Survey

Results of the survey of HPLC column/mobile phase combinations are summarized in Table 1. Chromatography on the Zorbax-NH_2 column was sufficient to resolve CP and ascorbic acid, but the retention and resolution of these compounds decreased drastically over a period of 6-8 hours. Flushing of this column with 0.1 M (NH_4) H_2PO_4 only temporarily returned resolution and retention. The Zorbax-CN and Zorbax-TMS columns did not adequately retain or resolve CP from excipients under the conditions studied.

The octyl and octadecyl modified silica columns surveyed were all capable of resolving CP from excipients under the conditions studied. The Zorbax-C8 column provided slightly better resolution of CP from niacinamide and the adipic acid internal standard and is therefore the column of choice. Tables 2-4 summarize the retention and resolution of niacinamide, CP and adipic acid on the reversed phase columns as MeOH concentration, buffer concentration and pH of the mobile phase were varied. The retention of CP and adipic acid increased with decreasing MeOH, decreasing pH and increasing buffer concentration. The retention of niacinamide increased with decreasing MeOH, increasing pH and increasing buffer concentration. This data suggests some limiting mobile phase conditions. Our desire to keep the chromatography run time to about 17 minutes sets the lower limit on MeOH concentration to about 10% on Zorbax-C8, 12% on Zorbax-ODS and 8% on Waters C-18 μBondapak columns. Mobile phase pH must be maintained at pH 3.5 or less to obtain good resolution of CP and niacinamide within 16 minutes. Adjustment of the buffer concentration has relatively little effect on the chromatography between 0.05 and 0.4M. A representative sample chromatogram is shown in Figure 2.

Table 2

The Effect of Percent Methanol on Retention and Resolution

% MeOH	T_{rn} (min)	t_{rcp} (min)	t_{rad} (min)	R_1	R_2
DuPont Zorbax C_8 25 cm					
14	4.7	6.1	9.5	1.8	4.9
12	5.9	9.3	14.0	4.0	5.9
10	6.6	11.8	17.2	5.5	5.7
8	7.7	15.9	22.1	7.8	5.0
6	9.3	21.7	28.5	9.5	4.4
DuPont Zorbax ODS 25 cm					
15	4.2	6.1	9.5	3.4	4.7
13	4.5	7.3	11.3	5.1	6.7
12	5.9	11.4	16.6	5.0	6.1
11	6.1	13.0	18.8	6.9	5.8
10	6.8	14.6	20.6	6.5	5.2
9	7.5	16.7	23.0	6.8	5.0
7	8.7	22.6	29.6	9.0	4.1
Waters μBondapak C-18 30 cm					
14	5.7	7.4	10.5	2.3	4.8
12	5.9	8.4	11.7	3.6	5.1
10	6.6	9.8	13.7	4.6	5.6
8	7.1	11.7	16.2	6.6	5.6
6	7.9	14.4	19.3	6.5	4.7

where: t_{rn} is the retention time of niacinamide

t_{rcp} is the retention time of calcium pantothenate

t_{rad} is the retention time of adipic acid

R_1 is the resolution between niacinamide and CP calculated by:

$$\frac{2\,(t_{rcp}-t_{rn})}{W_{cp}+W_n}$$

R_2 is the resolution between CP and adipic acid calculated by:

$$\frac{2\,(t_{rad}-t_{rn})}{W_{ad}+W_{cp}}$$

Table 3

The Effect of pH on Retention and Resolution

pH	t_{rn} (min)[a]	t_{rcp} (min)[a]	t_{rad} (min)[a]	R_1[a]	R_2[a]
DuPont Zorbax C_8 25 cm					
4.3	9.2	9.2	11.5	0	--
4.1	8.2	9.6	13.1	1.6	5.4
3.8	7.4	10.8	15.0	3.6	4.9
3.5	6.4	11.5	16.5	5.1	4.8
3.4	5.6	11.6	17.0	6.3	5.4
3.1	4.9	11.6	17.1	7.9	5.5
3.0	4.5	11.8	17.6	7.3	5.8
DuPont Zorbax ODS 25 cm					
4.2	10.8	7.9	10.8	CP eluted before niacinamide	
4.0	9.9	8.5	11.7	CP eluted before niacinamide	
3.8	9.2	9.5	13.5	0	--
3.5	7.9	10.5	15.0	2.6	5.0
3.4	7.0	10.8	15.7	3.4	4.9
3.2	6.5	11.1	16.1	4.1	5.0
3.0	5.6	11.2	16.2	5.9	5.3
Waters μBondapak C-18 30 cm					
3.5	6.8	10.5	14.9	3.7	4.0
3.4	6.3	10.6	15.2	4.3	3.8
3.2	6.1	10.8	15.6	4.3	3.7
3.1	5.6	10.8	15.6	4.8	3.7
3.0	5.0	10.8	15.8	5.0	3.7

[a]See Table 2 for definitions

Placebo chromatograms exhibited no interfering peaks near the CP or adipic acid peaks (Figure 3). A highly retained excipient peak was observed to elute approximately 2 hours after the injection of the excipients, however, and this peak interfered with subsequent chromatograms as shown in Figure 4. Attempts to remove this interfering excipient by modifying the extraction conditions of the sample preparation were not successful.

Table 4

The Effect of Buffer Concentration on Retention and Resolution

Conc. NaH_2PO_4 (M)	t_{rn} (min)[a]	t_{rcp} (min)[a]	t_{rad} (min)[a]	R_1[a]	R_2[a]
DuPont Zorbax C-8 25 cm					
0.05	6.7	10.1	15.8	3.2	6.3
0.10	7.0	10.5	15.9	3.9	6.4
0.20	6.3	10.4	15.6	4.3	5.8
0.25	6.5	11.3	16.8	4.6	6.1
0.30	6.3	11.2	16.2	5.8	5.9
0.40	6.8	11.1	15.9	6.2	5.0
DuPont Zorbax ODS 25 cm					
0.15	7.0	8.7	13.1	1.8	4.9
0.20	6.8	8.7	13.0	2.1	5.7
0.25	6.5	8.8	13.1	2.3	4.8
0.30	7.8	12.9	17.9	4.6	4.2
0.40	7.9	13.6	18.8	5.2	4.3
Waters μBondapak C-18 30 cm					
0.15	5.1	9.0	13.2	4.9	4.2
0.20	4.9	9.0	13.3	6.0	4.3
0.25	4.9	9.2	13.3	5.0	4.1
0.30	4.8	10.7	15.4	6.2	3.8
0.35	5.5	10.1	14.5	4.2	3.5

[a]See Table 2 for definitions.

The use of a guard column, switching valve, and backflush configuration to trap highly retained excipients has been reported previously (9). We decided to use a commercially available guard column to remove the need to pack our own. See Figure 1 and the Experimental section for the configuration and conditions. CP, niacinamide and adipic acid eluted within 4 minutes from the Brownlee guard column and the highly retained excipient eluted in about 25 minutes. Switching the guard column off line at 4 minutes trapped the excipient peak about one-sixth of the way into the guard column. Backflushing the guard column for 12 minutes at

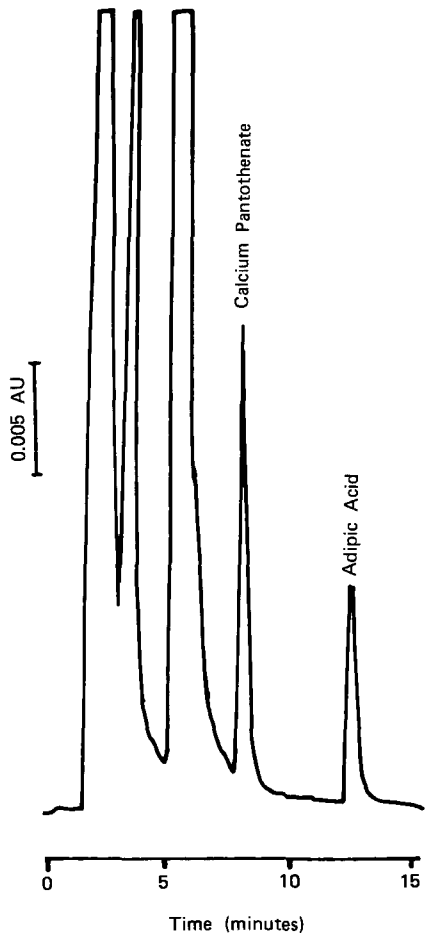


Figure 2. Typical chromatogram of a multivitamin tablet formulation. Eluted with phosphate buffer:methanol (88:12) at 1.5 ml/min, detection at 214 nm.

0.7 ml/min flushed this excipient peak out with 2.4 ml more mobile phase than was pumped forward in the loading phase, i.e. 6 ml pumped forward and 8.4 ml backflushed. The excess flush was used to assure the guard column was thoroughly cleaned. The use of the programmable event controls on the Varian 5000 pump to turn the backflush pump on and off reduced mobile phase consumption.

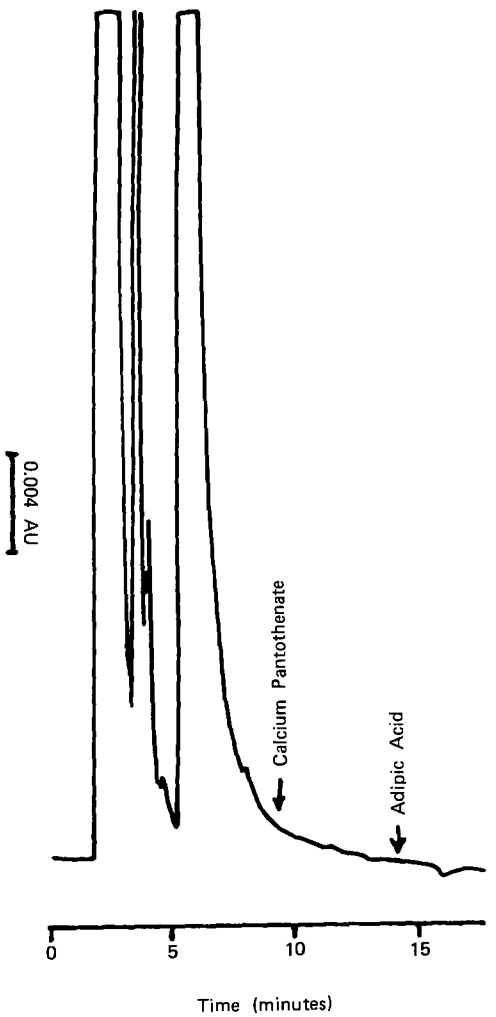


Figure 3. Placebo chromatogram.

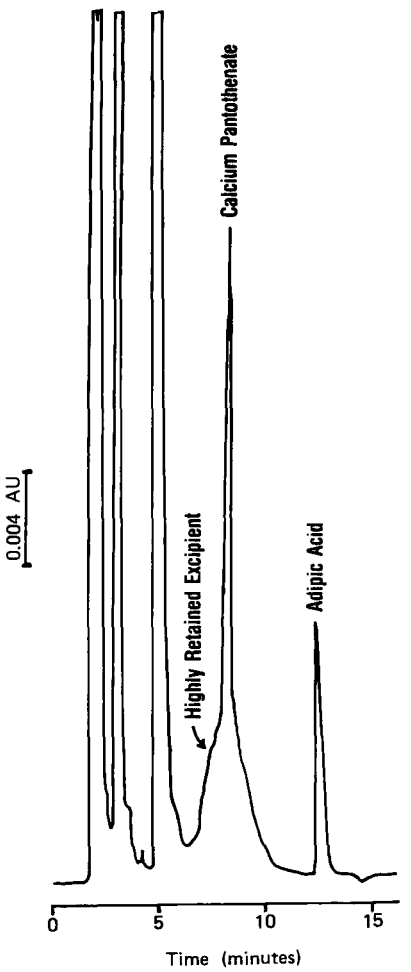


Figure 4. Chromatogram showing the highly retained excipient eluting 2 hours after injection and interfering with a later sample.

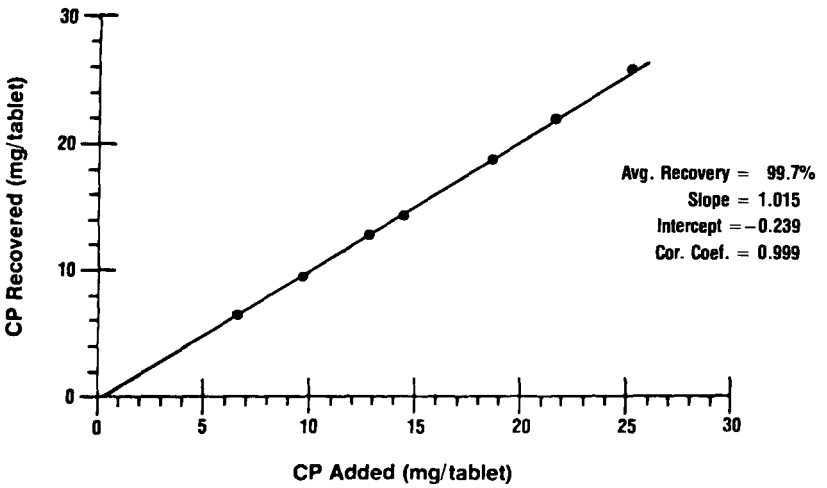


Figure 5. Universal placebo spiked recovery results, concentration found vs. concentration added using peak heights.

HPLC Assay Validation

The plot of the data from the spiked placebo recovery study is shown in Figure 5. Peak height calculations were used to obtain the amount recovered because peak height results were more precise than peak area results. No significant bias or deviation from linearity was observed over the range of concentrations studied. Average recovery was 99.7%.

The results of the precision study are summarized in Table 5. Pooled RSD's for two runs of triplicate assays of two lots each of five products ranged from 0.83% to 2.32%. The RSD for six injections of a single sample preparation was 1.58%. The RSD of the standard factors for 6 to 8 injections of duplicate reference standard preparations ranged from 0.52% to 0.93%. This data indicates that the method is precise.

The CP potencies obtained by the USP method (1) and the HPLC method were in good agreement. It must be noted that the HPLC

Table 5

Precision Study Results

Product	Pooled RSD
A	1.68%
B	1.42%
C	0.83%
D	2.32%
E	1.35%

assay is not specific for the biologically active d-isomer of CP, but rather measures total CP content of the tablets. A method specific for the d-isomer of CP, such as the microbiological assay, is performed on bulk drug CP before production of the tablets to insure the active form is present.

SUMMARY

The HPLC assay described provides a rapid, accurate, precise method for the determination of CP in multivitamin tablet formulations. This HPLC assay gives results which are comparable to those generated by the USP microbiological procedure in a substantially shorter period of time. The use of a column switching arrangement shortens chromatography run time and the use of a blender simplifies sample preparation.

ACKNOWLEDGEMENTS

The authors wish to thank F. Sabia for construction of the switching valve/backflush pump interface box and A. Fewless for his suggestions and help on the validation studies. This work was presented at the 1983 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy Paper 540.

REFERENCES

1. USP XX, Biological Tests-Calcium Pantothenate Assay <91> pp 889-890.

2. R. Schuster, "HPLC of Pharmaceutical Products-Vitamins", Hewlett-Packard Application Note AN 232-6, Sept. 1978.

3. "Multi-Vitamin Analysis Using High Performance Liquid Chromatography", DuPont Liquid Chromatography Report E-25475.

4. "Rapid HPLC Sample Preparation: Extraction and Cleanup Using a Loop-Column in the 7125 Injector", Rheodyne Inc. Technical Note 2, January, 1980.

5. Kreutzman, D.J., Silink, M., "Celite Multi-column Chromatography for the Simultaneous Separation of Progesterone, Deoxycorticosterone and 17α-hydroxyprogesterone from Small Plasma or Tissue Samples", J. Chromatog., 228, 95 (1982).

6. Oka, K., Dobashi, Y., Ohkuma T., Hara S., "Liquid Column Switching Extraction and Chromatography for Programmed Flow Preparation", J. Chromatog., 217, 387 (1981).

7. Alfredson, T.V., "HPLC Column Switching Techniques for Rapid Hydrocarbon Group-type Separations", J. Chromatogr., 218, 715 (1981).

8. Ogan, K., Katz, E., "Analysis of Complex Samples by Coupled-Column Chromatography", Anal. Chem., 54, 169 (1982).

9. Miller, R.L., Ogan, K., Poille, F. "Automated Column Switching in Liquid Chromatography", Am. Lab, 7, 52 (1981).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 839-854 (1984)

FLUOROGENIC LABELLING OF CARBONYLCOMPOUNDS WITH 7-HYDRAZINO-4-NITROBENZO-2-OXA-1,3-DIAZOLE (NBD-H)

G.Gübitz and R.Wintersteiger
Institute for Pharmaceutical Chemistry
University of Graz
A-8010 Graz, Austria

R.W.Frei
Department of Chemistry
Free University of Amsterdam
1081 HV Amsterdam
The Netherlands

ABSTRACT

A method for the prechromatographic fluorescence derivatization of carbonyl compounds with 7-hydrazino-4-nitrobenzo-2-oxa-1,3-diazole (NBD-H) is presented. The separation and quantitation of the hydrazones is carried out by TLC and HPLC on silica gel and RP-materials. Detection limits obtained for benzaldehyde by TLC with fluorodensitometric evaluation are 5 ng/spot and by HPLC with fluorescence detection 200 pg.

INTRODUCTION

Carbonyl compounds are widely occuring in the environment. The determination of traces of carbonyls in air and water is of great importance. Various aldehydes and ketones are also constituents of food aromas. Furthermore, a great number of pharmaceuticals contain carbonyl groups.

 0148-3919/84/0704-0839$3.50/0

The low extinction coefficients of most of the carbonyl compounds however, do not permit a sensitive detection Therefore, several authors applied derivatization methods to enhance the detectability.

2,4-Dinitrophenylhydrazine was described as a UV-derivatization reagent for HPLC-separations of aldehydes by various authors (1-6). Dansyl hydrazine, a fluorescence reagent, was used for TLC- as well as HPLC-separations of various carbonyl compounds (7), sugars (8,9) and ketosteroids (1o-14). Degradation products from the reagent however, interfere often with the quantitation of some compounds.

An alternative to this reagent is 7-hydrazino-4-nitrobenzo-2-oxa-1,3-diazole (NBD-hydrazine, NBD-H), which was proposed by Lawrence and Frei (7) as a potential fluorescence reagent for carbonyl compounds. NBD-hydrazine was prepared by treatment of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) with hydrazine. NBD-Cl (15-2O) as well as NBD-F (2O-22) have been used as fluorescence labelling reagents for amines and amino acids.

This work deals with the application of NBD-hydrazine to the pre-chromatographic derivatization of various carbonyl compounds for TLC and HPLC with fluorescence detection.

EXPERIMENTAL

Apparatus:

Perkin-Elmer Spectrofluorimeter MPF 44 with TLC-attachement and M 56 recorder.
Camag Nanomat (Muttens, Switzerland).
Perkin-Elmer Liquid Chromatograph Series 2, in combination with the Perkin-Elmer Spectrofluorimeter attached with a Hellma flow-through cell, 20 µl, or a Perkin-Elmer UV-detector LC 15.

Chemicals and materials:

7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)
(Serva, Heidelberg, FRG)
Hydrazine suprapure (Merk, Darmstadt, FRG)
Methanol, chloroform uvasol (Merck, Darmstadt, FRG)
Benzene UVasol for fluorescence spectroscopy (Merck, Darmstadt, FRG)
Precoated silica 60 - F_{254} TLC-plates 20 x 20 cm, washed twice with methanol-chloroform 3:1 prior to use.
HPTLC-RP-8 plates F_{254} 10 x 20 cm, (Merck, Darmstadt, FRG)
2 µl Microcaps, (Drummond Scientific)
200 nl Platin-Iridium capillaries (Antech, Bad Dürkheim, FRG)
Hibar LiChrosorb, 10 µ, 25 x 0.46 cm columns

Hibar LiChrosorb RP 8, 10 µ, 25 x 0.46 cm columns (Merck,Darmstadt, FRG).

Synthesis of NBD-hydrazine:

10 mg NBD-Cl are dissolved in 5 ml chloroform. After addition of 5 ml of a 1% hydrazine solution (0.2 ml 24% hydrazine suprapure solution in 5 ml methanol UVasol), the reaction mixture is allowed to stand in the dark at room temp. for one hour under nitrogen. The precipitated product is washed with benzene and dried at room temp. The product is to be stored in a vessel, which has been gased with nitrogen in the refrigerator.

Derivatization procedure for carbonyl compounds:

10 µl of a methanolic sample solution, containing not more than 100 nmole of carbonyl compounds are treated in conical vials with 10 - 50 µl of a freshly prepared solution of NBD-H in methanol-water (3:1), corresponding to a 5 fold molar excess, for 30 min (ketones for 2 hours) at 50°C.

To minimize the formation of fluorescent byproducts, the reaction is carried out under nitrogen in the dark.

After cooling 100 µl of water are added and the derivatives are extracted with 100 µl benzene. After

centrifugation an aliquot of the benzene layer is used for the TLC or HPLC separation.

TLC:

2 µl of the benzene layer are transfered with microcaps to silica gel plates. For the separation on HPTLC-RP-8 plates, 200 nl are applicated by Pt-Ir-capillaries.

Solvent systems:

I : Benzene-methanol (95:5)	for silica gel plates, migration distance: 10 cm
II : Benzene-ethylacetate (85:15)	
III: Methanol-water (80:20)	for RP-8 plates, migration distance: 4 cm
IV : Methanol-water (70:30)	
V : Acetonitril-water (80:20)	

The quantitation is carried out by fluorodensitometry at an excitation of 470 and an emission between 530 and 570 nm.

HPLC:

20 µl of the benzene layer are injected. As a mobile phase on the silica gel column benzene-chloroform 95:5 was used, for the RP-8 column acetonitril-water 50:50.

Detection is carried out either with a fluorescence detector or an UV-detector at 254 nm.

RESULTS AND DISCUSSION

Reaction:

The scheme of the reaction of carbonyl compounds with NBD-H is given in Fig. 1. Ketones show a considerably lower reactivity in comparison with aldehydes. With benzaldehyde, for example, the reaction is complete in about 10 minutes, acetone requires a reaction time of more than 1 hour. Fig. 2 shows the kinetics of the reaction of benzaldehyde and acetone with NBD-H.

The reaction yield was determined by comparison of the results of the reaction on analytical scale with the isolated and purified derivatives and was found to be 99% for benzaldehyde. To avoid the formation of fluorescent degradation products of the reagent, the reaction temperature should not exceed 50°C. The use of a higher temperature to reduce the reaction time leads to the formation of fluorescent byproducts. The same occurs, when acid catalysts are used. To minimize the reagent blank, it is advantageous to use a freshly prepared reagent.

Spectra

The excitation and emission spectra of benzaldehyde, butyraldehyde and acetone are shown in Fig. 3.

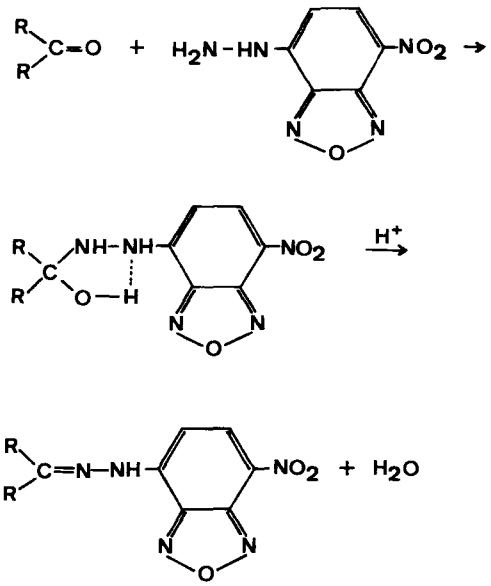


FIGURE 1: Reaction of carbonyl compounds with NBD-H.

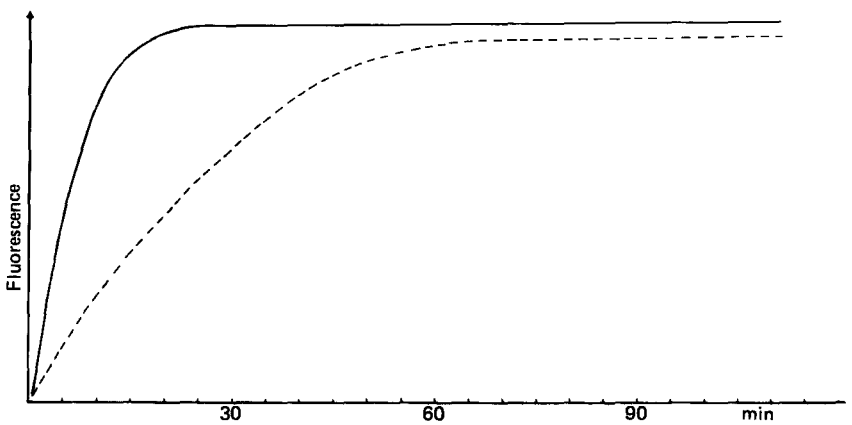


FIGURE 2: Kinetics of the reaction of benzaldehyde ——— and acetone ---- with NBD-H.

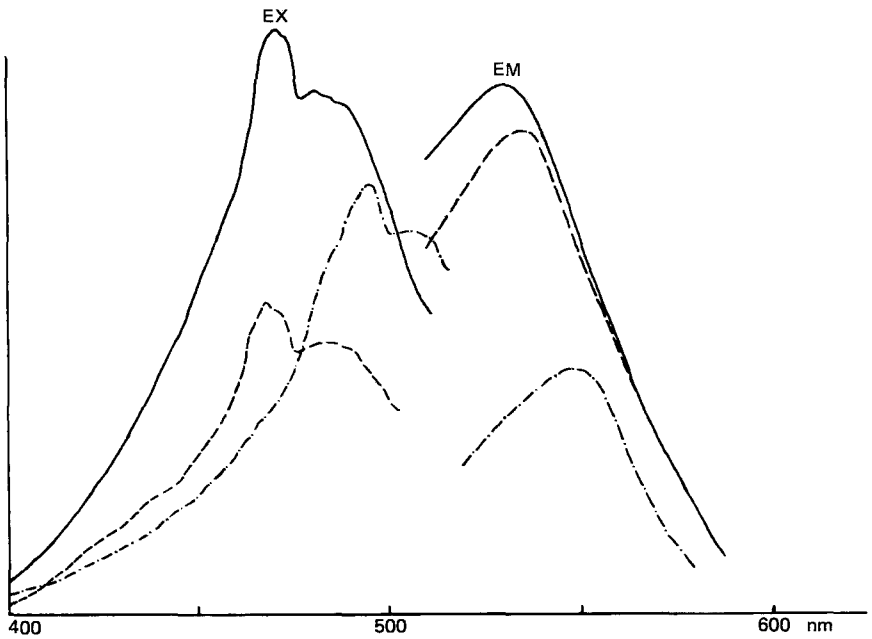


FIGURE 3: Excitation and emission spectra of the NBD-hydrazones of benzaldehyde -·-·-·, butyraldehyde ----- and acetone ———, recorded on a TLC-plate.

The emission maxima of the diverse carbonyl compounds range between 530 and 570 nm. The colors of the spots are yellow to purple. (Table 1)

The fluorescence intensity in solution depends greatly on the polarity of the solvent. In apolar solvents a high fluorescence is observed, in polar solvents like water or methanol an intensification of the color and a bathochromic shift occurs and the fluorescence is reduced such that visible detection becomes more sensitive than fluorescence detection.

TABLE 1. Excitation and emission maxima and visual appearance of the spots of some NBD-hydrazones.

Compound	Ex	Em	vis	Color
Butyraldehyde	467	535	450	yellow
Benzaldehyde	470,495	555	490	orange
Anisaldehyde	471	570	550	purple
Vanilline	471	570	550	purple
Acetone	470	530	450	yellow
Acetophenone	470	535	480	orange
Propiophenone	470	535	480	orange
Cyclopentanone	470	535	480	yellow

Separation of the NBD-hydrazones by TLC and HPLC:

The separation of the NBD-hydrazones of aldehydes were carried out on silica gel sheets as well as on HPTLC-RP-8 plates. A better separation and a higher sensitivity was obtained with the reversed phase systems. In Table 2 and 3 the Rf-values of the derivatives of various aldehydes and ketones, respectively, are given.

The quantitative evaluation was carried out by fluorodensitometry. Fig. 4 shows a scan of mixtures of the hydrazones of some aldehydes and ketones.

TABLE 2: Rf-values of NBD-hydrazones of aldehydes:

Compound	Rf x 100	
	Solv.I (silica gel)	Solv. IV (RP-8)
Formaldehyde	26	18
Butyraldehyde	40	24
Crotonaldehyde	37	26
Valeraldehyde	44	16
Benzaldehyde	44	15
Cinnamylaldehyde	44	13
Anisaldehyde	39	18
Aminobenzaldehyde	17	36
4-Dimethylaminobenzaldehyde	39	5
4-Hydroxybenzaldehyde	9	33
Salicylaldehyde	6	35
Vanilline	14	31
3-Nitrobenzaldehyde	27	25
Pyridin-2-aldehyde	26	25

As preliminary experiments have shown, this derivatization method is also applicable to the HPLC-separation of carbonyl compounds. The separation of some aromatic aldehydes was carried out on a silica gel column. (Fig. 5) For aliphatic aldehydes a RP-8 column

TABLE 3: Rf-values of NBD-hydrazones of various ketones:

Compound	Rf x 100		
	Solv.II (silica gel)	Solv.III (RP-8)	Solv.V (RP-8)
Acetone	21	66	81
Acetophenone	49	44	65
Propiophenone	-	47	65
Methylphenylketone	4	74	92
Isopropylmethylketone	-	56	65
Isobutylmethylketone	-	51	59
Diisopropylketone	-	47	65
Benzalacetone	39	43	52
Cyclohexanone	33	50	65
Cyclopentanone	38	56	71
Cycloheptanone	26	56	48
Indan-1,3-dione	7	63	67
1,4-Napthoquinone	11	64	57
Vitamine K_1	6	63	47
Prednisolone	-	34	46
Haloperidole	-	0	77
Methadone	-	60	72
Ketobemidone	-	58	70
Hydrocodone	-	55	68

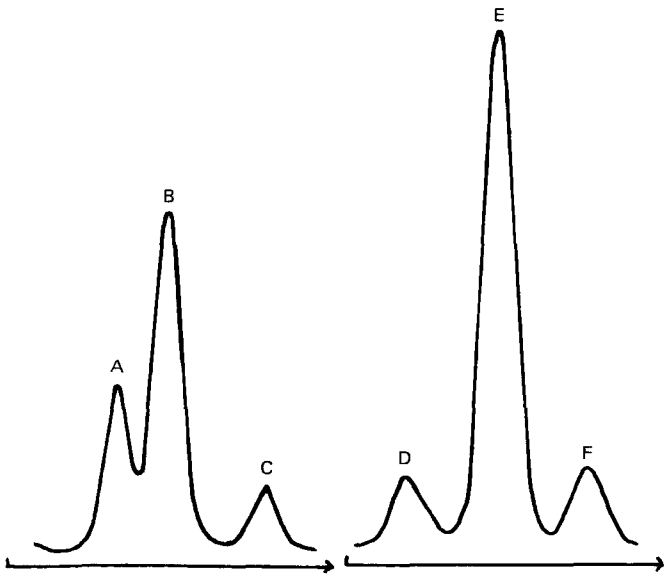


FIGURE 4: Scan of a TLC of the NBD-hydrazones of A: cinnamylaldehyde, B: nitrobenzaldehyde, C: vanilline, D: acetophenone, E: cyclopentanone, F: acetone. HPTLC-RP-8 plates, Solvent: methanol-water (70:30) Ex: 470 nm/Em: 530 nm.

showed a better selectivity, however, the high polarity of the usual eluents quenches the fluorescence considerably. Therefore an UV-detection was used in this case, with a resulting loss of sensitivity. Fig. 6 shows a separation of 4 aliphatic aldehydes on a RP-8 column.

Quantitation:

Calibration curves were prepared for various carbonyl compounds both for TLC and HPLC. The curves

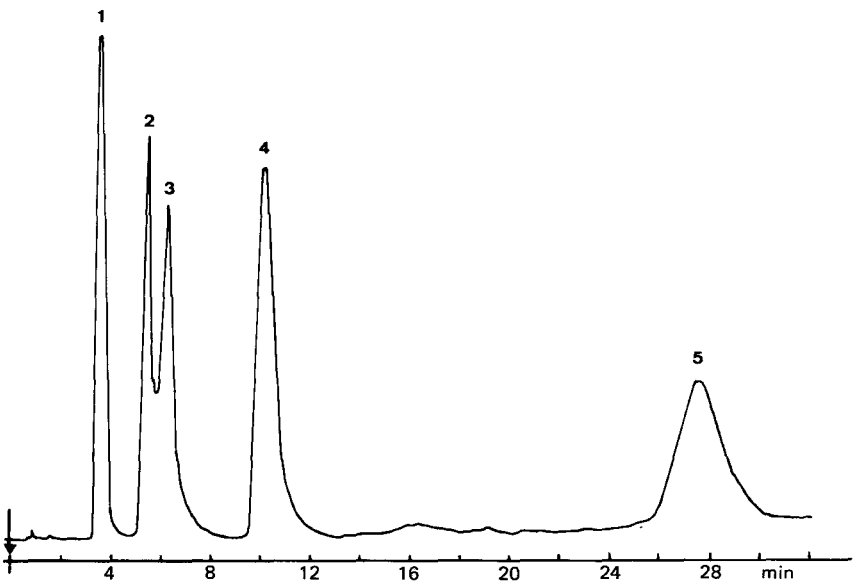


FIGURE 5: Separation of the NBD-hydrazones of aromatic aldehydes by HPLC. 1: Benzaldehyde, 2: cinnamylaldehyde, 3: anisaldehyde, 4: vanilline. Column: Silica gel, 25 x 0.46 cm. Mobile phase: Benzene-chloroform (95:5). Flow: 2 ml/min. Detection: Fluorescence, Ex: 470/Em: 560 nm.

are linear over a range of more than one decade. The correlation coefficients were between 0.996 and 0.999.

The relative standard deviation determined for benzaldehyde (50 ng/spot) by the TLC-method was 2.5% (n=8) and 3.8% for 5 ng, determined by HPLC.

The fluorescence detection limits obtained by TLC at a signal to noise ratio of 3:1 are 2 ng/spot for benzaldehyde and 10 ng for acetone. For vitamine K_1 the detection limit is 2 ng.

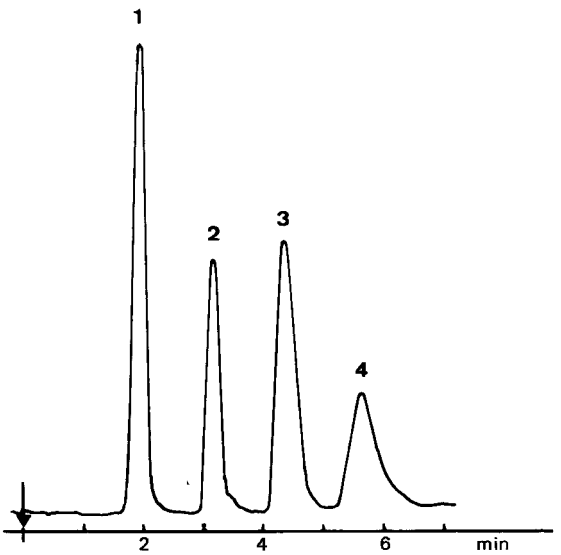


FIGURE 6: Separation of the NBD-hydrazones of aliphatic aldehydes by HPLC. 1: Propionaldehyde, 2: butyraldehyde, 3: valeraldehyde, 4: capronaldehyde. Column: RP-8, 25 x 0.46 cm. Mobile phase: acetonitril-water (50:50). Flow: 1 ml/min. Detection: UV 254 nm.

With HPLC the fluorescence detection limit for benzaldehyde was 200 pg. This could be improved by further optimization of the experimental conditions. The detection limits with UV-detection at 254 nm were 5-10 ng. It should be noted, that the sensitivity depends greatly on the purity of the reagent and the used solvents.

The optimization of the method with the goal of a sensitive determination of drugs and other compounds of interest containing carbonylgroups by HPLC is under way.

REFERENCES

1. Kikta, E.J. and Grushka, E., Anal.Chem., 48, 1098, 1976.
2. Selim, S., J.Chromatogr., 136, 271, 1977.
3. Demko, P., J.Chromatogr., 179, 361, 1979.
4. Vigh, G., Varga-Puchony, Z., Hlavay, J., Petro-Turcza, M. and Szarföldi-Szalma, I., J.Chromatogr., 193, 432, 1980.
5. Fung, K. and Grosjean, D., Anal.Chem., 53, 168, 1981.
6. Reindl, B. and Stan, H.J., J.Chromatogr., 235, 481, 1982.
7. Frei, R.W. und Lawrence, J.F., J.Chromatogr., 83, 321, 1973.
8. Avigad, G., J.Chromatogr., 139, 343, 1977.
9. Takeda, M., Maeda, M. und Tsuji, A., J.Chromatogr., 244, 347, 1982.
10. Chayen, R., Dvir, R., Gould, S. und Harrel, A., Anal.Biochem., 42, 283, 1971.
11. Graef, V., Z.Klin.Chem.u.Klin.Biochem., 8, 320, 1970.
12. Kawasaki, T., Maeda, M. und Tsuji, A., J. Chromatogr., 163, 143, 1979.
13. Kawasaki, T., Maeda, M. und Tsuji, A., J. Chromatogr., 226, 1, 1981.
14. Wintersteiger, R. und Gamse, E., Proc.of the Symp. on the Analysis of Steroids, Eger, Hungary, 453, 1981.
15. Lawrence, J.F. and Frei, R.W., Anal.Chem., 44, 2046, 1972.
16. Klimesch, H.-J. and Stadler, J.Chromatogr., 90, 141, 1974.
17. Wolfram, J.H., Feinberg, J.I., Doerr, R.C. and Fiddler, W., J.Chromatogr., 132, 37, 1977.

18. Ahnoff, M., Grundavik, I., Arfwidsson, A., Fonselius, J. and Persson, B., Anal.Chem., 53, 484, 1981.

19. Roth, M., Clin.Chim.Acta, 83, 273, 1978.

20. Imai, K. and Watanabe, Y., Anal.Chim.Acta, 130, 377, 1981.

21. Watanabe, Y. and Imai, K., Anal.Biochem., 116, 471, 1981.

22. Watanabe, Y. and Imai, K., J.Chromatogr., 239, 723, 1982.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 855-856 (1984)

LC NEWS

POLYACRYLAMIDE SOFT GEL FOR SEC/GPC may be operated at high pressures and undergoes minimum swelling and shrinkage when transferred between polar eluents. Uses include separations of polysaccharides, polyphenols, synthetic aqueous polymers, and biopolymers. Polymer Laboratories, JLC/84/4, Essex Road, Church Stretton, Stropshire, SY6 6AX, UK.

AUTOSAMPLER FOR HpLC offers repeatable and accurate sampling, high throughput, and total automation for up to 102 samples. Nine programs may be stored and positive sample identification is provided. Beckman Instruments, Inc., JLC/84/4, Altex Scientific Div., 1716 East 4th Street, Berkeley, CA, 94710, USA.

MICRO HPLC PUMP can be used at low flow rates. Two essentially pulse-free flows are combined in a micromixing system to produce linear gradients from 1,000 to 1 microliters/min. Syringe pumps are used with automatic refill after each run. Brownlee Laboratories, JLC/84/4, 2045 Martin Avenue, Suite 204, Santa Clara, CA, 95050, USA.

GPC+, FIRMWARE-BASED PROGRAM, calculates analytical results in gel permeation chromatography. It resides on a 8K chip enables the calculation of calibration curves via point-to-point, quadratic, or cubic fits of the data. Statistical data are presented to aid in selecting the best fit. Spectra-Physics Corp., JLC/84/4, 3333 North First Street, San Jose, CA, 95134, USA.

GPC/SEC SCOUT COLUMN is available in a highly cross-linked 10 micron divinylbenzene gel, which permits the use of many organic solvents, including DMF. It can be used for molecular weight distributions of polymers from 500 to 4 million. IBM Instruments, Inc., JLC/84/4, P. O. Box 332, Danbury, CT, 06810, USA.

CUSTOM HPLC PHASES AND PACKINGS SERVICE is available. One can specify particle size, shape, and pore diameter as well as structure of the desired stationary phase. ES Industries, Inc., JLC/84/4, 8 S. Maple Avenue, Marlton, NJ, 08053, USA.

ELECTROCHEMICAL DETECTOR FOR HPLC combines a large electrode surface area with low internal volume (1.5 microliter) for enhanced

sensitivity and compatibility with microbore requirements. The solid state reference electrode is an inytegral part of the flow cell and requires no regular maintenance. LKB Instruments, Inc., JLC/84/4, 9319 Gaither Rd., Gaithersburg, MD, 20877, USA.

INTELLIGENT VALVE POSITIONER consists of a valve driver assembly including valve, synchronous motor and encoder, plus a controller unit which uses a standard RS232 interface. A valve may be moved to any position, at any time, in any direction. Hamilton Co., JLC/84/4, P. O. 10030, Reno, NV, 89520, USA.

GLASS CARTRIDGE COLUMNS FOR HPLC withstand up to 200 bars pressure. Advantages offered include low solvent consumption, inertness, visibility of the packed bed, and smoothness of the internal wall. Chromapack Nederland BV, JLC/84/4, P. O. Box 3, NL4330 AA Middelburg, The Netherlands.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 857-860 (1984)

LC CALENDAR

1984

MARCH: "Basic GC School," a 3-day course (date to be announced), sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

MARCH 20: "New Developments in HPLC of Water Soluble Macromolecules," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL: "HPLC of Water Soluble Polymers," a 2-day course (date to be announced) sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL 8-13: National ACS Meeting, St. Louis, MO. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

APRIL 15-17: Short Courses: "LCEC" taught by Dr. R. Shoup, BAS, Inc.; "Derivatization & Sample Preparation" taught by Dr. C. Poole, Wayne State University - sponsored by the Minnesota Chromatography Forum; held at the Minneapolis Auditorium & Convention Hall. Contact: Meeting Management, 1421 E. Wayzata Blvd., Wayzata, MN, 55391, USA.

APRIL 17: "New Developments in TLC," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL 29 - MAY 3: Analytical Applications of Supercritical Fluids - Supercritical Fluid Technology Symposium, at the meeting of the AOCS, Dallas, TX. Contact: Dr. J. W. King, CPC Internat'l, Moffett Tech Center, Argo, IL, 60501, USA.

MAY 17: Symposium on Therapeutic Drug Monitoring & Toxicology for the 80's: Clinical & Instrumental Perspectives, Farmington, CT, sponsored by the UConn Medical School & AAAC Connecticut Valley

Chapter. Contact: Dr. Steven H. Wong, Dept. of Lab. Med., UConn Medical School, Farmington, CT, 06032, USA.

MAY 20 - 26: 8th Intl. Symposium on Column Liquid Chromatography, New York Statler Hotel, New York City. Contact: Prof. Cs. Horvath, Yale University, Dept. of Chem. Eng., P. O. Box 2159, Yale Stn., New Haven, CT, 06520, USA.

JUNE 3-5: International Symposium on LCEC and Voltammetry, Indianapolis Hyatt Regency Hotel, Indianapolis, IN. Contact: The 1984 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

JUNE 10-14: 14th Northeast Regional ACS Meeting, sponsored by the Western Connecticut and New Haven Sections, at Fairfield University, Fairfield, CT. Contact:D. L. Swanson, American Cyanamid Co., Stamford, CT, USA.

JUNE 18-20: Second International Conference on Chromatography & Mass Spectrometry in Biomedical Sciences, sponsored by the Italian Group for Mass Spectrometry in Biochemistry & Medicine, Milan, Italy. Contact: Dr. A. Frigerio, via Eustachi 36, I-20129 Milan, Italy, or Dr. H. Milon, P. O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland.

JUNE 18-21: Symposium on Liquid Chromatography in the Biological Sciences, Ronneby, Sweden, sponsored by The Swedish Academy of Pharmaceutical Sciences. Contact: Swedish Academy of Pharmaceutical Sciences, P. O. Box 1136, S-111 81 Stockholm, Sweden.

AUGUST 26-31: National ACS Meeting, Philadelphia, PA. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

SEPTEMBER 10-14: Advances in Liquid Chromatography, including the 4th Annual American-Eastern European Symposium on LC and the Int'l Symposium on TLC with Special Emphasis on Overpressured Layer Chromatography, sponsored by the Hungarian Academy of Sciences' Chromatography Committee & Biological Research Center and the Hungarian Chemical Society, in Szeged, Hungary. Contact: Dr. H. Kalasz, Dept. of Pharmacology, Semmelweis University of Medicine, P.O.Box 370, H-1445 Budapest, Hungary, or Dr. E. Tyihak, Research Inst. for Plant Protection, P.O.Box 102, H-1525 Budapest, Hungary.

SEPTEMBER 20 - 22: Labcon Central 83, 3rd Annual Lab Instrument & Equipment Conference & Exhibition, O'Hare Exposition Center, Rosemont, IL. Contact: Tower Conference Mgmt. Co., 143 N. Hale Street, Wheaton, IL, 60187, USA.

OCTOBER 1-5: 15th Int'l. Sympos. on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, D-6000 Frankfurt Main, West Germany.

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

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

1985

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

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

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

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

1986

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

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

1987

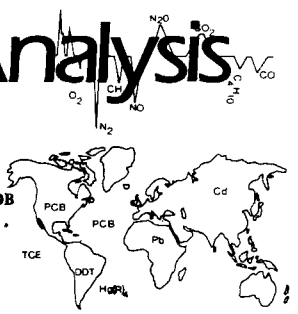
APRIL 5-10: 193rd National Am. Chem. Soc. Mtng., Denver, Colo. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

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

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

nproving your ability to conduct environmental analyses . . .

Chromatographic Analysis of the Environment



Second Edition, Revised and Expanded

edited by **ROBERT L. GROB**
Villanova University
Villanova, Pennsylvania

September, 1983
736 pages, illustrated
$95.00
(Price is 20% higher outside the U.S. and Canada)

Praise for the First Edition from both sides of the Atlantic . . .

". . . the editor, contributors and the publisher have combined to produce a splendid and valuable compendium; it should be made readily accessible to all those who are concerned with environmental monitoring."
—Alan T. Bull, *International Biodeterioration Bulletin*

"The unique organization of the book allows the reader to locate and examine information in his area of interest with a minimum of difficulty. . . ."
—Joseph H. Caro, *Soil Science*

"Professor Grob and his team of coauthors set out to provide a comprehensive work of chromatographic techniques of practical value to consultants, engineers and chemists'. . . . the book certainly provides a full and reasonably critical perspective of the practical applications of chromatography to environmental problems. . . ."
—H. Egan, *Chemistry in Britain*

". . . since it is a compendium prepared by 'users for users' it has real value to microbiologists who frequently need advice on analytical methods for traces of organic and inorganic compounds. . . ."
—D. Perlman, *ASM News*

Completely revised, expanded, and updated, this instructive work authoritatively examines the latest chromatographic techniques used to determine toxic substances in the atmosphere, water, waste effluents, and soil. With this comprehensive volume, environmental scientists have a single-source guide to the selection and performance of the most effective chromatographic analyses.

Organized for easy reference, *Chromatographic Analysis of the Environment:*

- **offers essential background material covering the theory and practice of chromatography**
- **provides a new chapter examining sample preparation techniques—an essential topic in analysis**
- **presents numerous time-saving references and a detailed subject index**
- **expands coverage of ion exchange and paper chromatography techniques—approaches undergoing a rebirth of interest**

With its concentration on environmental considerations, this important reference serves as a unique resource for environmental scientists, analytical chemists, chromatographers, and government environmental protection officials. In addition, the logical format of *Chromatographic Analysis of the Environment* makes it an ideal text for graduate-level courses and professional seminars examining topics in environmental analysis.

CONTENTS

ISBN: 0-8247-1803-8

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

INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

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

Directions for Submission

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

Manuscripts should be mailed to the Editor:

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

Reprints

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

Format of Manuscript

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

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

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

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

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

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

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

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

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

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

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

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

Typing Instructions

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

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

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

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

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

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