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CYCLIC OLIGOMER CONSIDERATIONS IN THE SIZE EXCLUSION CHROMATOGRAPHY OF POLY(ETHYLENE TEREPHTHALATE)

L. MARTIN AND S. T. BALKE
Department of Chemical Engineering and Applied Chemistry
University of Toronto
Toronto, Ontario, Canada M5S 1A4

ABSTRACT

Room temperature Size Exclusion Chromatography (SEC) for poly(ethylene terephthalate) (PET) was developed using a mobile phase mixture of 5% hexafluoroisopropanol (HFIP) in methylene chloride ($MeCl_2$). Calibration was carried out with three different approaches, each time with and without considering the presence of cyclic oligomer in PET samples and standards. At typical concentrations of cyclic oligomer a calibration curve generated from a chromatogram truncated to eliminate the oligomer peak had its slope distorted such that it gave molecular weight average values inaccurate by up to 8%, whereas correcting for the oligomer explicitly resulted in average errors of about 1%. Although the effect of this small peak may be negligible for typical SEC applications involving repeated analysis of similar samples, it may alter the calibration curve significantly if not corrected.

INTRODUCTION

HFIP is an excellent room temperature solvent for PET but is very costly and somewhat hazardous. Its use has been reported as pure mobile phase [1], mixed with other solvents [2], and as a 2% mixture with chloroform [3], but most commonly as a 30% (or azeotropic) mixture with methylene chloride [4,5]. This paper reports the use of 5% HFIP with methylene chloride, a composition which lowers cost and health risk yet is still suitable for higher molecular weight and crystalline PET samples.

SEC chromatograms of PET often show a distinct low molecular weight peak which corresponds to cyclic oligomers, mainly trimer [3,4]. Although the true molecular weight of trimer represented by this peak is 576, the peak retention volume corresponds to molecular weights ranging from about 275 [4] to 2500 [6,7] on extrapolated PET calibration curves, depending upon the mobile phase used.

Interpretation of PET chromatograms containing the "trimer" peak requires consideration of three main aspects: (i) definition of the chromatogram to be interpreted, (ii) assignment of molecular weight averages corresponding to the defined chromatogram, and (iii) use of the chromatogram and molecular weight averages to obtain calibration curves. These three aspects are discussed in turn in the next section.

THEORY

Definition of the Chromatogram to be Interpreted

Figure 1 shows a typical PET chromatogram containing a "trimer" peak. This peak originates from the presence of 0.5-1.5% of cyclic oligomers which are mainly trimer [3,4],

$$(-CO-C_6H_4-CO-O-CH_2-CH_2-O-)_n\ ,\quad n = 2\text{-}9 \tag{1}$$

where n = 3 for trimer. As mentioned above, these species may not obey molecular weight calibration curves obtained for linear PET molecules. In this solvent system, the trimer peak elutes at a retention volume corresponding to a molecular weight of about 1000 to 2000.

The most common method of dealing with this problem is to avoid using the trimer peak in the interpretation. This is conventionally done by drawing the baseline to intersect the PET chromatogram in front of the trimer peak (Figure 2). In this work this "Truncation" approach is compared with two alternatives: allowing the trimer peak to remain in the interpreted chromatogram and mathematically subtracting the trimer peak. The latter alternative involved fitting the trimer peak with a spline fit.

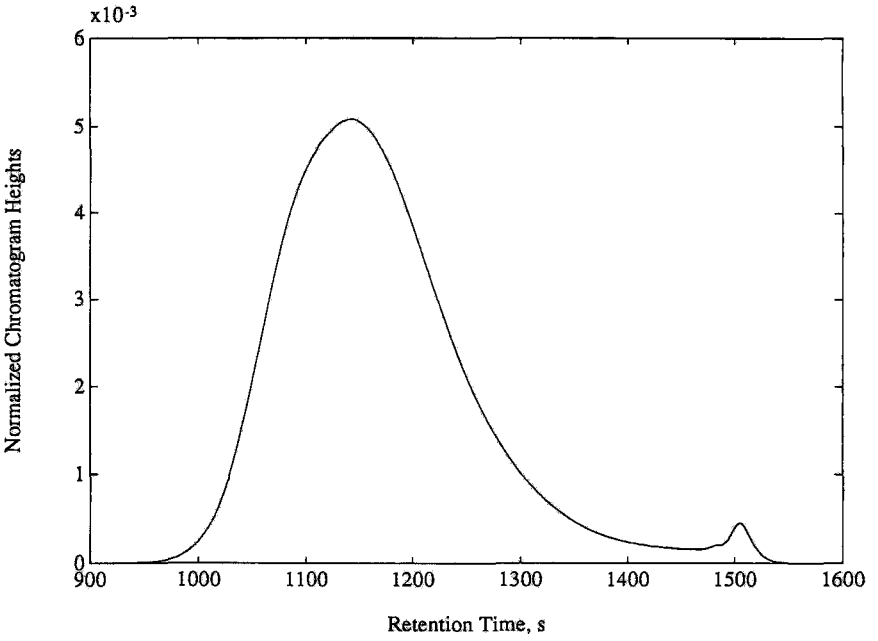

FIGURE 1 Typical chromatogram of PET showing "trimer" peak.

Assignment of Molecular Weight Averages to the Defined Chromatogram

Vendor values of M_n and M_w are generally based upon absolute methods such as light scattering, viscometry, and osmometry which reflect the presence of all molecules actually present in the sample. Thus, when the trimer peak is not included in the SEC interpretation, these values must be corrected for the absence of these species so that the averages correspond to the molecules represented by the defined chromatogram. Assuming the UV detector response factor is the same for all species, the fraction of the total area under the trimer peak and the fraction under the remainder of the chromatogram (the "linear PET" peak) provide an estimate of the weight fractions of cyclic oligomers (w_{trimer}) and polymer ($w_{polymer}$) respectively. Then, the vendor value of M_w, $M_{w,vendor}$ is:

$$M_{W,vendor} = w_{polymer} M_{W,polymer} + w_{trimer} M_{W,trimer} \quad (2)$$

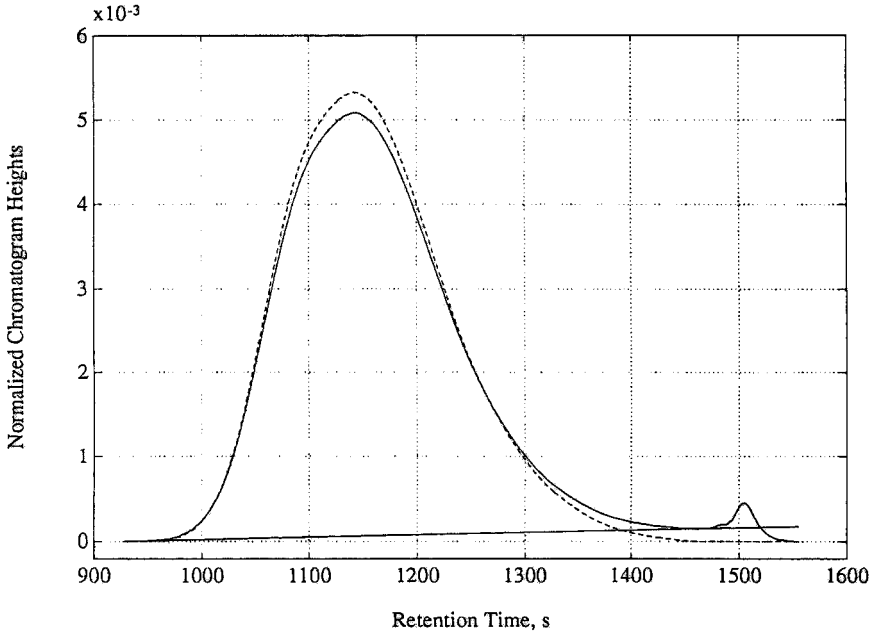

FIGURE 2 Example of truncation of "trimer" peak from chromatogram: original chromatogram and baseline (solid lines), truncated chromatogram (dashed line).

where $M_{w,polymer}$ is the weight average molecular weight of the molecules constituting the linear PET peak and $M_{w,trimer}$ is the weight average molecular weight of the cyclic oligomers responsible for the trimer peak.

Now, solving for $M_{w,polymer}$ and setting $M_{w,trimer}$ equal to three times the monomer molecular weight ($3 \cdot M_0$) we obtain:

$$M_{W,polymer} = \frac{M_{W,vendor} - w_{trimer} \cdot 3 \cdot M_0}{1 - w_{trimer}} \quad (3)$$

*Calibration Curve Determination

Narrow standards of PET are generally not available for calibration. Thus, broad molecular weight distributions displaying the usual trimer peak are used in calibration curve search methods. Three such methods are examined here: (i) search for a linear

calibration curve which, when applied to the chromatogram of the standard, yields the "true" M_n and M_w (the "Linear" calibration curve search method) [8]; (ii) using a polystyrene calibration curve in a search for two groups of Mark Houwink constants to derive a PET calibration curve which, when applied to the chromatogram of the standard, yields the "true" M_n and M_w (the "Mark Houwink Constants" search method) [9]; and (iii) calculation of a calibration curve from the chromatogram of a PET standard of known molecular weight distribution (the "MWD" calibration method). These methods are well known in the published literature [10].

With respect to the MWD calibration method, the molecular weight distribution used for standards was obtained from two sources [11,12]: vendor supplied molecular weight distributions obtained via SEC; and by use of the theoretically based "Flory distribution" for linear condensation polymers [13]. The Flory distribution is described by the following equation:

$$W_n = np^{n-1}(1-p)^2 \tag{4}$$

where W_n is the weight fraction of n-mers and p is the degree of polymerization, which may be calculated from molecular weight averages with the companion equations for these averages:

$$M_n = \frac{M_0}{(1-p)} \qquad M_w = M_0\frac{(1+p)}{(1-p)} \tag{5}$$

In this work the effect of all three of the above factors was of interest: definition of the chromatogram used, assignment of the molecular weight averages to the defined chromatogram, and calibration curve determination method. Table I shows the specific combinations of these factors examined.

EXPERIMENTAL

PET samples were dissolved in 30% HFIP/70% $MeCl_2$ at room temperature in less than one hour. Highly crystalline PET is more soluble in 30% HFIP than in pure HFIP [4]. The solutions were then diluted down to 5% HFIP, 1-2 mg polymer/mL, before injection into the 5% HFIP mobile phase. The addition of 1.0 g/L of tetraethylammonium chloride to the mobile phase and sample was used to eliminate the "polyelectrolyte effect", or agglomeration of polymer molecules [14] (apparent as a

Table 1
Information Used in Calibrations

Method	Standards	Chromatogram oligomer peak	Molecular Weight Information
Linear, Truncated	Std 1	Truncated	Vendor MW Averages
Linear, Included	Std 1	Included	Vendor MW Averages
Linear, Subtracted	Std 1	Subtracted	Corrected MW Averages
Mark Houwink Constants, Included	Std 1,3	Included	Vendor MW Averages
Mark Houwink Constants, Subtracted	Std 1,3	Subtracted	Corrected MW Averages
MWD, Truncated	Std 1	Truncated	Vendor MW Distribution
MWD, Subtracted	Std 1,3	Subtracted	Synthetic MW Distribution

high molecular weight prepeak). An internal standard was required for flowrate adjustment as the low boiling point of the solvent mixture tended to cause a non-reproducible flowrate [15]. In PET samples the cyclic oligomer served as an internal standard for correcting flowrate changes from run to run. Trichlorobenzene was used for polystyrene samples.

Detection by UV absorption at 254 nm with a Perkin-Elmer Tri-Det detector provided excellent chromatogram signal-to-noise ratio and baseline resolution. Jordi Gel Linear columns (Jordi Associates, Inc.) were stable in this solvent mixture for at least seven months. A Waters 510 pump and a Hewlett Packard Series 1050 autosampler completed the SEC equipment. Data was collected with an ADALab A/D conversion card, a PC-compatible computer, and in-house software.

To evaluate the sensitivity of the system to changes in the sample preparation and analysis technique, factorial design was used to plan experiments and analysis of variance was applied to the resulting molecular weight averages, peak area, and oligomer fraction w_{trimer}. Analysis of samples aged four days showed no significant differences, indicating that the polymer was stable in the solvent. High sample concentration combined with larger injection volume (ie. 2 mg/mL and 100 µL) affected the chromatograms, indicating a limit above which a concentration effect existed in the size exclusion separation. All experiments were performed with small enough total polymer injection to eliminate this effect.

Chromatograms of broad PET standards were defined three different ways with respect to cyclic oligomer peaks, as described earlier. With respect to the method of subtraction of the trimer peak, a spline fit of the peak shape was obtained from the highest molecular weight PET standard.

RESULTS AND DISCUSSION

Oligomer Content in PET Standards

Table 2 shows that the weight fraction of cyclic oligomer in the PET samples used ranged from 1.4% for Standard 1 to 0.5% for Standard 4. With this amount of trimer removed mathematically from the polymer, scarcely any effect on the vendor values of M_n or M_w was evident. Calculated values of degree of polymerization, p, obtained from Equation (5) using the $M_{w,polymer}$ values are also shown in Table 2, and are all above 0.99.

There appears to be a strong relationship between w_{trimer} and p. The weight fraction of cyclic oligomer decreased as the degree of polymerization of the sample increased. Figure 3 illustrates that equally good fits were obtained with linear, quadratic, or logarithmic equations.

In the published literature there are diverse results with respect to the correlation of w_{trimer} and p. Well-established theory predicts a positive correlation [16], though this has not been systematically confirmed for PET. There are numerous reports [4,17,18,19] that maintaining PET at a temperature between the glass transition and the melting point results in a decrease in w_{trimer}. If the conditions of the heat treatment permit solid-state polycondensation [20], large increases in molecular weight may arise, indicating a negative correlation between w_{trimer} and p. Keeping in mind that the PET standards of different molecular weight are prepared by solid-stating, and that an equilibrium distribution of cyclic oligomers may not be present without lengthy treatment [19], the negative correlation obtained in this work and elsewhere may be a non-equilibrium trend.

Table 2
PET Standards

Name	M_w, Vendor	w_{trimer} (mean, std.dev)	$M_{w,adj}$	p_{adj}	Comments
Std 1	M_n 21,000 M_w 39,000	1.427% ± .097	M_n 21,836 M_w 39,556	0.99033	American Polymer Standards 39K
Std 2	47,240	0.957% ± .035	47,691	0.99198	Eastman 7352, solid stated
Std 3	58,000	0.838%	58,485	0.99345	Eastman 9902, solid stated
Std 4	71,560	0.529%	71,937	0.99467	Eastman 10388, solid stated

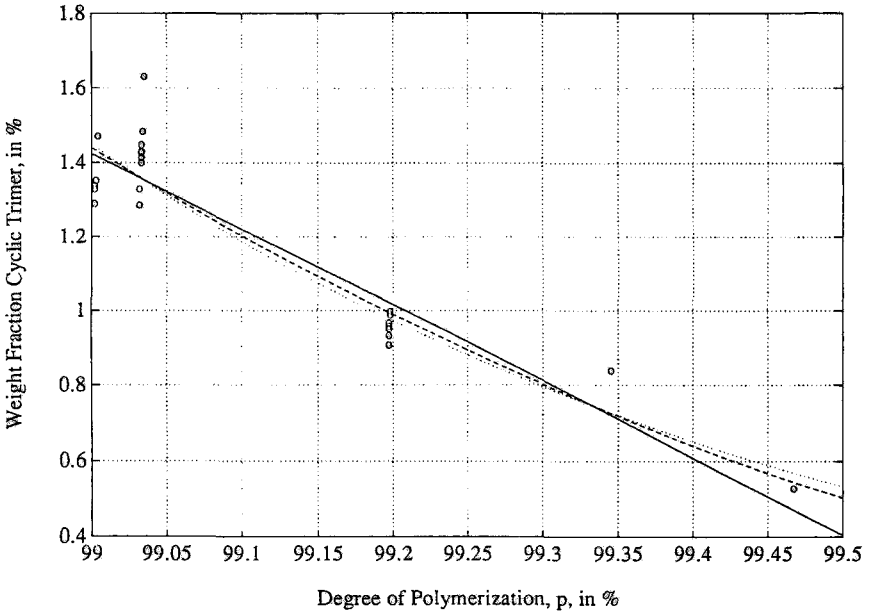

FIGURE 3 Correlation between w_{trimer} and p: linear fit (solid line), quadratic fit (dashed line), logarithmic fit (dotted line).

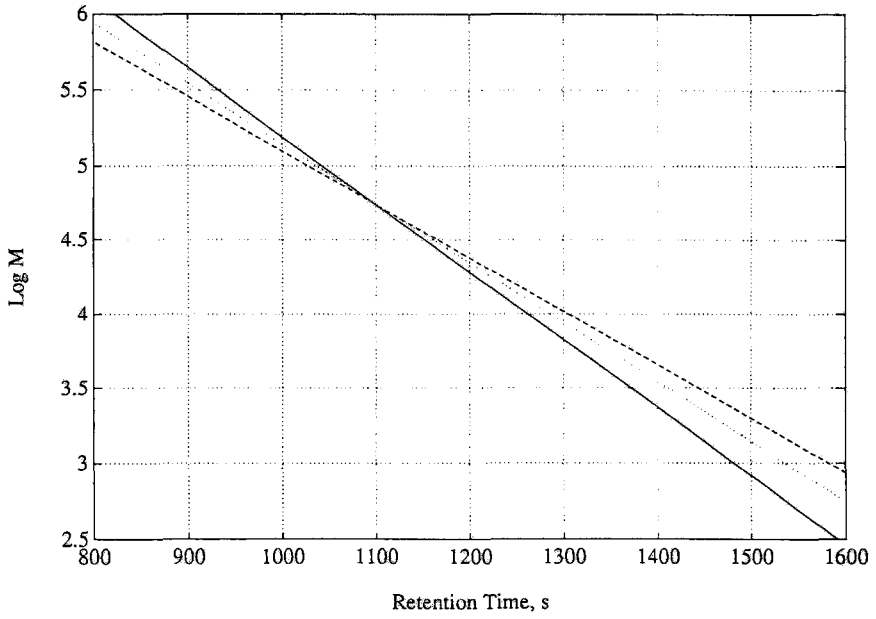

FIGURE 4 Calibration curves using Linear method: truncated trimer peak (solid line), included trimer peak (dashed line), subtracted trimer peak (dotted).

A possible reason for this trend involves annealing, which also occurs under heat-treatment conditions. If cyclic oligomers are not incorporated into the growing crystalline regions, they will be forced towards an equilibrium concentration with respect to amorphous fraction only [4]. The weighted average of w_{trimer} in the semi-crystalline sample will then decrease as crystallinity increases. Further thermal treatment can completely change the crystalline content much more quickly than the cyclic oligomer content, so this theory cannot be easily tested without preparing the samples from a common starting material.

Comparison of Calibration Curves and Molecular Weight Averages

The PET calibration curves obtained from the information summarized in Table 1 are plotted in Figures 4 to 6. Molecular weight averages calculated using each calibration curve are given in Table 3. For each method, the test chromatograms were defined in

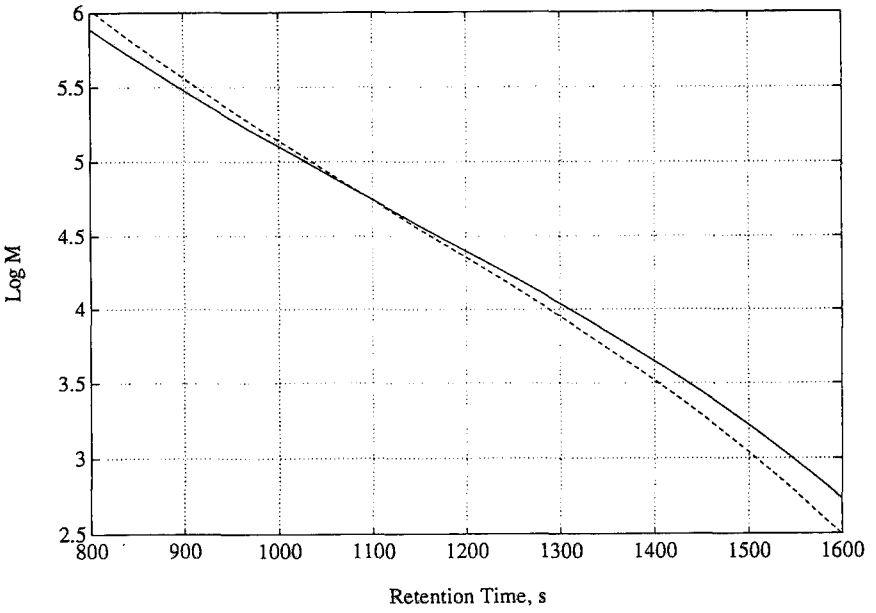

FIGURE 5 Calibration curves using Mark Houwink Constants method: included trimer peak (solid line), subtracted trimer peak (dashed line).

the same way as the calibration chromatograms: for instance, to test the Linear Truncated calibration curve, the chromatograms for Standards 2, 3 and 4 were also truncated. The main points evident in making these comparisons are:

i) The slopes of calibration curves increased with respect to trimer peak in the chromatogram in the order Included, Subtracted, and Truncated. As a result, the Linear Truncation calibration overestimated the M_w of the highest molecular weight test standard by over 8%, whereas the calibrations using Included chromatograms underestimated M_w. The calibrations using the Subtracted chromatograms were most accurate across the entire molecular weight range.

ii) Linear and Mark Houwink Constants methods gave very similar calibration curves when used with the same chromatograms and averages. The errors in calculated molecular weight averages likewise was very close, though the use of an

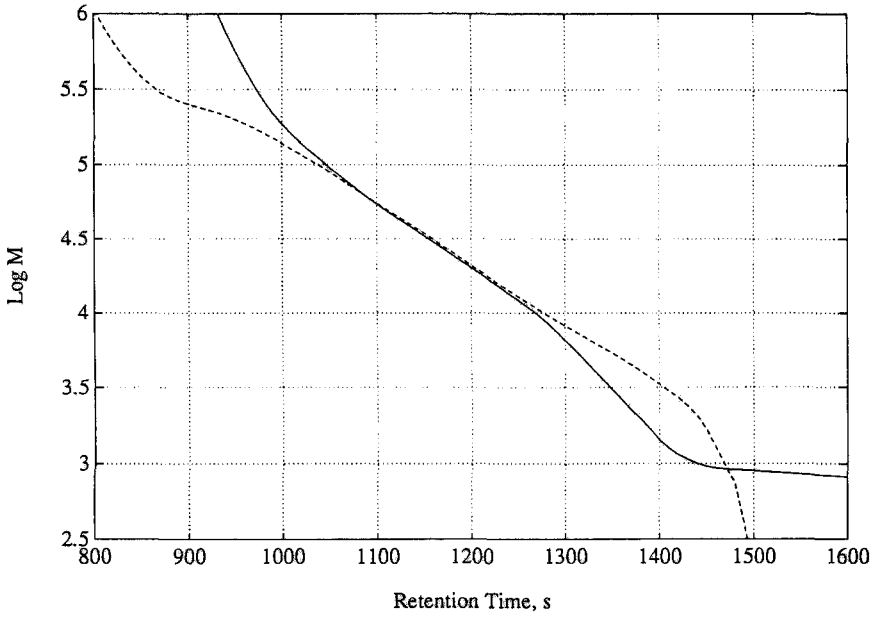

FIGURE 6 Calibration curves using Molecular Weight Distribution method: truncated trimer peak with vendor molecular weight distribution (solid line), subtracted trimer peak with Flory distribution (dashed line).

additional higher molecular weight standard for the Mark Houwink Constants method resulted in slightly smaller errors in high molecular weight test samples.

iii) The MWD method gave calibration curves which overlapped the others in the center, but deviated erratically at the tails. This is due to the intrinsic sensitivity of this method to the tails of both the chromatograms and the "true" molecular weight distributions. In addition, since the calibration data points were fitted with splines, extrapolation beyond the molecular weight range of the calibration standard(s) was very unreliable, and gave molecular weight averages in very large error.

iv) The molecular weight predicted for trimer, which is listed in the last column of Table 3, is higher than the true value with almost all calibration methods. It is highest for calibrations using Included trimer peaks and lowest for Truncated trimer peaks, in accordance with the trend of calibration slopes. For calibrations using Subtracted

Table 3
Comparison of Calibration Methods
Errors in Calculated Molecular Weight Averages [a]

Whole Polymer Sample

Standard and MW averages	Std 1		Std 2		Std 3		Std 4		MW predicted for trimer
Calibration	21000	39000	23737[b]	47240	29098[b]	58000	35866[b]	71560	576
Linear Truncated	***0.1%***	***0.2%***	4.8%	4.4%	-2.0%	1.6%	-0.7%	8.2%	782
Linear A	***0.0%***	***0.0%***	9.8%	0.2%	1.0%	-4.6%	-0.5%	-5.4%	1899
Mark Houwink A	***-0.6%***	***2.8%***	9.5%	2.4%	1.1%	***-2.4%***	-0.4%	-3.6%	1575
MWD A	***-2.5%***	***3.6%***	0.8%	11.2%	-5.2%	17.4%	-4.0%	86.2%	891

Linear Fraction

Standard and MW averages	Std 1		Std 2		Std 3		Std 4		MW predicted for trimer
Calibration	21836	39556	23961[c]	47691	29339[c]	58485	36054[c]	71937	576
Linear B	***0.0%***	***0.0%***	12.4%	2.5%	4.5%	-0.5%	1.3%	0.9%	1327
Mark Houwink B	***-0.1%***	***0.5%***	12.3%	2.3%	4.8%	***-0.4%***	0.7%	0.6%	1031
MWD B	***-12.6%***	***-1.2%***	-1.4%	1.6%	***-0.8%***	***-2.1%***	-17.0%	-1.5%	120

Notes: [a] Values in ***bold italics*** were used in obtaining calibration: represent goodness-of-fit of search
[b] Calculated from p and w_{trimer} in Table 2 using Equations (3) and (5)
[c] Calculated from p in Table 2 using Equation (5)

trimer peaks and corrected molecular weight averages, which would represent linear PET molecules only, the trimer peak elutes at a retention time corresponding to a molecular weight of about 1000 to 1300.

In contrast to the small effect of correcting the molecular weight averages for w_{trimer}, definition of the chromatogram used had a very significant effect on the SEC calibration curve obtained. The reasons are evident in the moment analysis plots [10] for M_n shown in Figure 7. In a moment analysis plot, the areas under the plot across a specific retention time range reflects the importance of chromatogram heights in that range to the calculation of a molecular weight average. The small changes in the extreme low molecular weight tail were magnified in the moment W/M(t) which is used in the calculation of M_n. The portion of the chromatogram after 1450 s contributed less than 0.05% to the calculation of M_n for the Truncated chromatogram, 4% with trimer subtracted, and an inflated 18% with trimer included. The calibration searches "found" calibration curves which were pivoted as required to obtain the same

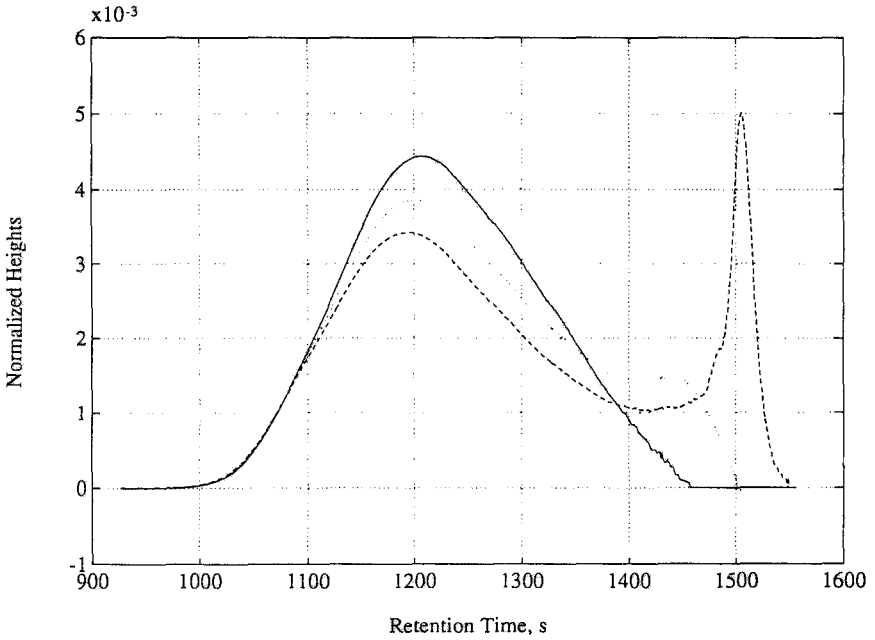

FIGURE 7 Moment analysis plots for M_n: trimer peak truncated (solid), trimer peak included (dashed), trimer peak subtracted (dotted).

molecular weight average with these differing moment distributions. Moment analysis plots for M_w were almost identical for all three chromatograms.

Note that "known" M_n values for Standards 2, 3, and 4 were estimated using the Flory distribution and the oligomer weight fraction. This would appear to be a poor estimate for Standard 2, since all calibrations except the two Direct methods gave M_n about 10% to 12% higher. Standard 2 was the lowest molecular weight standard obtained from Eastman, and had been solid-stated [20] the least: perhaps it was not at an equilibrium distribution as described by Flory.

CONCLUSIONS

A solvent system consisting of 5% hexafluoroisopropanol in methylene chloride was demonstrated for the room temperature size exclusion chromatography of

poly(ethylene terephthalate). It is a suitable alternative to much more expensive and/or hazardous solvent systems, and samples dissolve at room temperature. Experiments in a factorial design showed that the effect of sample preparation on the chromatograms was not significant, indicating that the polymer is stable in the solvent system, though care must be taken to avoid concentration effects.

The practice of truncating PET chromatograms to eliminate the cyclic oligomer peak led to calibration curves which give M_w values too high by up to 8%. Since the cyclic oligomers do not elute at retention times corresponding to their true molecular weights on a linear polymer calibration, they must be properly removed from both the chromatograms and molecular weight averages used to construct calibration curves.

Of the methods used in this work, a Universal Calibration type of method using oligomer-corrected data from multiple PET standards of differing molecular weights gave the most accurate calibration curve. A Linear Calibration using one corrected standard was almost as good. In order to avoid using both the Universal Calibration assumption and narrow PS standards, the Linear Calibration may be preferred, especially if it is modified to use more than one standard. The method of Direct Calibration, using a known molecular weight distribution for the standard, was unreliable beyond the range of molecular weight in the calibration standards as well as being too dependent on accurate tails of the chromatograms, which are difficult to obtain.

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REFERENCES

1. H. Jordi, Jordi Associates, Inc., Bellingham, MA., telephone conversation
2. S. Berkowitz, *J. Appl. Poly. Sci.*, 29, 4353 (1984).
3. K. Weisskopf, *J. Poly. Sci., Part A*, 26, 1919 (1988).
4. J.R. Overton and H.L. Browning, Jr., Size Exclusion Chromatography, ACS Symposium Series #245, 219, 1984.
5. R.L. Miller, R.V. Brooks, and J.E. Briddell, *Poly. Eng. Sci.*, 30(1), 59 (1990).
6. G. Caflisch, Eastman Kodak, Tennessee, telephone conversation.
7. J. Armonas, American Polymer Standards Corp., Mentor, Ohio, telephone conversation.
8. F.C. Frank, I.M. Ward and T. Williams, *J. Polym. Sci., Part A-2*, 6, 1357 (1968).
9. S. Mori, *Anal. Chem.*, 53, 1813 (1981).
10. S.T. Balke, Quantitative Column Liquid Chromatography, a survey of chemometric methods, Elsevier Science Publishing, New York NY (1984).
11. M.J.R. Cantow, R.S. Porter and F.J. Johnson, *J. Polym. Sci., Part A*, 5, 1391 (1967).
12. A.R. Weiss and E. Cohn-Ginsberg, *J. Polym. Sci., Part A-2*, 8, 148 (1970).
13. P.J. Flory, Principles of Polymer Chemistry, Chapter VIII, Cornell University Press, Ithica, N.Y., 1953.
14. E.E. Drott, Liquid Chromatography of Polymers and Related Materials, ed. N.J. Cazes, Chromatographic Science Series, V.8, Marcel Dekker, N.Y., 1976.
15. B.L. Neff and J.R. Overton, *J. Liq. Chrom.*, 7(8), 1537 (1984).
16. H. Jacobson and W.H. Stockmayer, *J. Chem. Phys.*, 18(12), 1600 (1950).
17. S.A. Jabarin and D.C. Balduff, *J. Liq. Chrom.*, 5(10), 1825 (1982).
18. A.L. Cimecioglu, S.H. Zeronian, K.W. Alger, M.J. Collins, and G.C. East, *J. Appl. Polym. Sci.*, 32(4), 4719-33 (1986).
19. D.R. Cooper and J.A. Semlyen, *Polymer*, 14, 185 (1973).
20. L.-C. Hsu, *J. Macromol. Sci.-Phys.*, B1(4), 801 (1967).

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REVERSED-PHASE CHROMATOGRAPHIC SEPARATION OF BENZO[a]PYRENE METABOLITES WITH β-CYCLODEXTRIN AS A MOBILE PHASE MODIFIER

M. ROZBEH AND R. J. HURTUBISE
Department of Chemistry
University of Wyoming
Laramie, Wyoming 82070

ABSTRACT

β-Cyclodextrin (β-CD) was investigated as a mobile phase modifier for the separation of fourteen metabolites of benzo[a]pyrene. A wide range of β-CD concentrations was employed, and the chromatographic properties of four different classes of metabolites were compared in methanol-water, acetonitrile-water, and methanol-acetonitrile-water mobile phases with and without β-CD. The chromatographic bands for all the metabolites sharpened with β-CD in the mobile phase. The resolution for four classes of metabolites improved with β-CD in the binary and ternary mobile phases. The monohydroxyl-benzo[a]pyrenes used in this study were structurally similar and difficult to separate with methanol-water. However, with β-CD in the mobile phase, overall improvement in the separation of the monohydroxyl-benzo[a]pyrenes was achieved. Substantial improvement was obtained for the separation of 6-hydroxyl-benzo[a]pyrene from 12-hydroxyl-benzo[a]pyrene with β-CD in methanol-water and for 2-hydroxyl-benzo[a]pyrene from 9-hydroxyl-benzo[a]pyrene with β-CD in the acetonitrile-water.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) can be formed by combustion reactions or high-temperature processes involving carbonaceous materials such as

coal, coal tar, pitch, asphalt, and oil (1). The potential carcinogenic effect of PAH is the basic reason for concern about the levels of these compounds in the working environment (2). Some PAH have been shown to cause cancer in animals, and they are suspected to be human carcinogens as well (1-3). Of the PAH, benzo[a]pyrene (B[a]P) has been studied the most (4-6). The profile of the metabolites of B[a]P has been largely worked out by using high-performance liquid chromatography (HPLC) (7-15). For example, Selkirk et al. (9-11) employed HPLC for the isolation and separation of metabolic isomers of B[a]P. Zaleski and co-workers (8) used HPLC to compare the metabolic profiles of B[a]P and B[a]P-t-7,8-dihydrodiol in freshly isolated hepatocyte from mirror carp. However, the separation of some of the structural isomers and closely related B[a]P metabolites has been difficult to achieve by chromatography (14).

The use of CD in liquid chromatography as a mobile phase modifier has been shown to be effective in the separation of several classes of isomers (16,17). Cyclodextrins are a series of macrocyclic oligosaccharides produced by the action of *Bacillus macerans amylase* on starch and contain from 6 to 12 α-1,4 linked D-glucose units (18). The most widely used CD consist of six, seven, and eight glucose monomers arranged in a torus shape and are designated as α-, β-, and γ-CD, respectively (19). The coupling of the glucose moieties gives the CD molecule a rigid, conical molecular structure with a hollow interior. The interior of the cavity of a cyclodextrin molecule is composed of two rings of C-H groups with a ring of glucosidic oxygen in between, allowing the rings to be hydrophobic in nature (20). The internal diameters of the cavities of α-, β-, and γ-CD are

approximately 5.7, 7.8, and 9.5 Å, respectively, and the depths are roughly 7.8 Å (20). The cavities enables the CD to trap guest molecules in their interior, resulting in the formation of inclusion or "host-guest" complexes (16,21-23). The stability of an inclusion complex depends on the size of CD cavity and on intermolecular forces such as hydrogen bonding, van der Waals attraction, and hydrophobic interaction. These macrocyclic carbohydrate molecules can be discriminating in their inclusion complexing tendencies toward different structural, positional, or stereoisomeric molecules.

β-Cyclodextrin has been used as a mobile phase modifier in the separation of structural isomers (16,24-25). For example, Mohseni and Hurtubise (16) investigated the effects of β-CD on the separation of several structural isomers and concluded that the addition of β-CD to the mobile phase in HPLC resulted in dramatic improvements in the separation of structurally similar hydroxyl aromatics. Woodberry et al. (21) investigated the effects of β- and γ-CD bonded-phase columns on the separation of two of the metabolites of benzo[a]pyrene isomers. Their results suggested that HPLC methodology with CD as a stationary phase or as a mobile phase additive could be used to separate B[a]P-t-7,8-dihydrodiol from its 9,10-isomer.

We previously reported a systematic method for determining optimal mobile phases for the separation of a complex mixture of B[a]P metabolites (26). That work was based on the window diagram approach (27-29), and the solubility parameter optimization method (30). A mixture of fourteen B[a]P metabolites was separated by us using optimization procedures developed for binary and ternary mobile phases (26). Also, the retention characteristics of the fourteen different

metabolites of B[a]P were investigated using methanol-water, acetonitrile-water, and methanol-acetonitrile-water mobile phases (26).

The purpose of this work was to assess the effects of β-CD as a mobile phase modifier for separating B[a]P metabolites and the effects of β-CD on the compound-class separation of the metabolites. α-Cyclodextrin was not investigated in this work because its cavity size is too small for the metabolites, and γ-CD was not investigated in this work because of its expense.

EXPERIMENTAL SECTION

Apparatus

The liquid chromatographic system consisted of a Waters Model 6000A pump (Waters Associates, Milford, MA) connected to a U6K injector, a dual-channel ultraviolet detector model 440 detector set at 254 nm and a dual-channel 5.0 V recorder (Linear Instruments Co. Concord, CA). A model FIAtron temperature controller (Oconomowoc, WI) was employed to keep the temperature of the column constant at 25 ± 0.1° C. The column employed was a 5-μm BakerBond C_{18} (250 mm x 4.6 mm i.d.) purchased from J. T. Baker (Pillipsburg, NJ).

Reagents

The benzo[a]pyrene (B[a]P) metabolites were purchased from the National Cancer Institute (NCI) repository at Midwest Research Institute (MRI, Kansas City, MO). The β-CD samples were obtained from Aldrich (Milwaukee, WI). Methanol and water were HPLC grade and were purchased from J. T. Baker Inc. (Pillipsburg, NJ). Acetonitrile was HPLC grade and was obtained from Fisher Scientific (Fair Lawn, NJ).

Procedures

The organic modifiers and water were prefiltered through a Millipore type HA 0.45 μm filter. An accurately weighed amount of β-CD, which was vacuum dried at 75° C for 8 hr, was dissolved in prefiltered water. Then, the appropriate amount of organic modifier was added to the β-CD solution. After complete dissolution at room temperature, the remaining amount of mobile phase was added to the volumetric flask to bring the volume to 1000 mL. Solutions of 0.1 mg/mL for individual metabolites and 0.01 mg/mL for the mixture of fourteen standards were prepared in methanol or acetonitrile, depending on the mobile phase composition. To prevent decomposition of the metabolites, the standard solutions were stored under nitrogen gas at -15° C and in the dark. The column void volume was obtained by injecting a methanol or acetonitrile solution of potassium nitrite.

Mobile Phases and β-CD Concentrations

Optimum mobile phases from a previous investigation (26) and other binary mobile phases were employed in this study to determine the effects of β-CD on the retention characteristics of the fourteen metabolites. The methanol:water (MeOH:H_2O, v:v) solvents used for the metabolites were, 55:45 for a mixture of four tetrols, and 65:35, 70:30 and 81.75:18.25 for a mixture of fourteen metabolites. The maximum analytical concentrations of β-CD in MeOH:H_2O mobile phases of 55:45, 65:35, 70:30 and 81.75:18.25 were 5.0, 4.6, 3.5 and 2.0 mM, respectively.

The acetonitrile:water (ACN:H_2O, v:v) composition used for the separation of the fourteen metabolites were 60:40 and 65:35. The largest analytical concentration of β-CD in ACN:H_2O solvents were for 60:40, 3.0 mM, and for 65:35, 2.0 mM. The methanol:acetonitrile :water (MeOH:ACN:H_2O) composition was 17:50:33 (v:v:v). The maximum amount of β-CD that could be dissolved in this ternary mobile phase was 2.0 mM.

RESULTS AND DISCUSSION

Effects of the Percentage of Methanol and β-Cyclodextrin Concentration on the Retention Characteristics of the Metabolites of Benzo[a]pyrene

Previously, we used mobile phase optimization methods to determine an optimum binary mobile phase for the metabolites (26). In this work, several different concentrations of β-CD were investigated with the optimum binary mobile phase, MeOH:H_2O (81.75:18.25). This optimum mobile phase had a high percentage of methanol, therefore the maximum analytical concentration that could be dissolved in this mobile phase was 2.0 mM β-CD. With this concentration, the overall retention times for the mixture of fourteen metabolites were reduced and the bands were sharper. Also, the order of separation of the metabolites was the same as with the mobile phase without β-CD. However, as discussed below, better separation of the metabolites was obtained with β-CD when the percentage methanol was lower than in the optimum binary mobile phase.

Our earlier investigation with different percentages of methanol as an organic modifier indicated that the two most difficult pairs of metabolites to separate were 6-OH-B[a]P and 12-OH-B[a]P, and 2-OH-B[a]P and 9-OH-B[a]P

(26). Also, in this work, lower percentages of methanol and β-CD gave better separation of the metabolites than higher percentages of methanol. Therefore, smaller percentages of methanol with a wide range of β-CD concentrations were emphasized in this work. The capacity factors of the fourteen metabolites of B[a]P with different concentrations of β-CD in MeOH:H_2O (70:30, 65:35 and 55:45) were obtained. The retention properties of the metabolites were compared in MeOH:H_2O with and without β-CD and with other binary and ternary mobile phases as well. Criteria such as, the decrease in the capacity factors, class separation, band sharpening and increased resolution between the most difficult peak pairs of isomers were compared with and without β-CD. It was found that the retention properties of the monohydroxylated B[a]P were affected the most by increasing the β-CD concentration in MeOH:H_2O (70:30 and 65:35). For example, the k′ value of 6-OH-B[a]P changed from 19.4 with no β-CD in the mobile phase to 17.22 with 3.5 mM β-CD in MeOH:H_2O (70:30) (Table 1). The k′ value of 12-OH-B[a]P was reduced from 19.22 without β-CD to 16.4 with the addition of 3.5 mM β-CD in MeOH:H_2O (70:30). With MeOH:H_2O (70:30) and β-CD small changes in k′ values of tetrols were obtained (Table 1). Benzo[a]pyrene-t-7,8-dihydrodiol and the diones showed relatively larger changes in their k′ values with MeOH:H_2O (70:30) and β-CD. Also, because of the small retention times for tetrols and B[a]P-t-9,10-dihydrodiol, there was overlap between these two classes of metabolites when using the MeOH:H_2O (70:30) and β-CD mobile phase. Therefore, a good separation for the four tetrols and B[a]P-t-9,10-dihydrodiol was not obtained in a mixture of fourteen metabolites with this mobile phase. As seen

in Table 1, there was a decrease in retention of all of the metabolites with β-CD in the mobile phase.

The retention characteristics of fourteen metabolites of B[a]P with MeOH:H_2O (65:35) over a wide range of β-CD concentrations were also studied. Table 2 lists the capacity factors of the fourteen metabolites in MeOH:H_2O (65:35) with different amounts of β-CD. The k′ values were rather large for the hydroxyl aromatics with this mobile phase compared to MeOH:H_2O (70:30). However, a relatively large decrease in the k′ values of the monohydroxyl-B[a]P metabolites was obtained with increasing β-CD concentration in MeOH:H_2O (65:35). For example, the k′ values for 3-OH-B[a]P in the absence of β-CD was 42.86, whereas with 4.6 mM β-CD present, it was reduced to 32.11. This was mainly due to the higher concentrations of β-CD that were achieved with MeOH:H_2O (65:35) mobile phase compared to MeOH:H_2O (70:30) (see Table 1 and Table 2).

Separation of a Complex Mixture of the Metabolites

Comparison of the chromatograms in Figures 1a and 1b for a mixture of the fourteen metabolites with MeOH:H_2O (65:35) indicates, band sharpening, a decrease in the retention times, and the separation of 6-OH-B[a]P and 12-OH-B[a]P isomers with 4.0 mM of β-CD as a mobile phase modifier. However, significant improvement in the separation of B[a]P-1,6-dione and B[a]P-3,6-dione was not obtained with β-CD. Three classes of metabolites were separated, namely, monohydroxyl-B[a]P, diones, and dihydrodiols (Figure 1b). Nevertheless, the chromatographic bands overlapped for B[a]P-t-9,10-dihydrodiol and the tetrols with and without β-CD (Figures 1a and 1b).

TABLE 1
The k'-Values of the Metabolites of Benzo[a]pyrene for Methanol-Water (70:30) with Different Concentrations (mM) of β-CD

	β-CD (mM)					
Solute[a]	0.0	1.0	2.0	2.5	3.0	3.5
1	0.81	0.8	0.78	0.78	0.76	0.76
2	0.98	0.94	0.91	0.91	0.85	0.84
3	1.02	1.03	1.04	1.05	1.0	0.85
4	1.36	1.35	1.35	1.35	1.3	1.22
5	1.27	1.25	1.22	1.22	1.17	0.83
6	5.56	5.02	4.26	4.26	4.15	3.93
7	9.51	9.15	8.15	8.11	7.93	7.27
8	11.56	10.91	10.37	10.46	8.9	9.75
9	19.22	19.15	18.11	17.44	17.10	16.40
10	18.76	17.92	16.9	16.5	16.82	16.77
11	19.0	18.3	17.3	16.6	16.4	16.0
12	22.72	21.0	20.6	20.0	19.3	18.3
13	24.02	23.2	22.42	22.42	22.28	22.02
14	19.40	19.1	18.55	18.30	18.01	17.22

[a] 1. Benzo[a]pyrene-r-7,t-8,9,c-10-tetrahydrotetrol (I-1)
2. Benzo[a]pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol (II-1)
3. Benzo[a]pyrene-r-7,t-8,9,10-tetrahydrotetrol (I-2)
4. Benzo[a]pyrene-r-7,t-8,c-9,10-tetrahydrotetrol (II-2)
5. Benzo[a]pyrene-trans-9,10-dihydrodiol
6. Benzo[a]pyrene-trans-7,8-dihydrodiol
7. Benzo[a]pyrene-1,6-dione
8. Benzo[a]pyrene-3,6-dione
9. 12-Hydroxybenzo[a]pyrene
10. 9-Hydroxybenzo[a]pyrene
11. 2-Hydroxybenzo[a]pyrene
12. 7-Hydroxybenzo[a]pyrene
13. 3-Hydroxybenzo[a]pyrene
14. 6-Hydroxybenzo[a]pyrene

The structures of the compounds are given in Ref. 26.

TABLE 2
k'-Values Obtained for Metabolites of Benzo[a]pyrene with Methanol-Water (65:35 v/v) with Different Concentrations (mM) of β-CD

	β-CD (mM)					
Solute[a]	0.0	2.0	3.0	4.0	4.5	4.6
1	1.11	1.1	1.07	0.98	0.96	1.00
2	1.31	1.32	1.31	1.17	1.15	1.15
3	1.57	1.55	1.51	1.35	1.34	1.34
4	2.07	1.98	1.97	1.82	1.8	1.8
5	1.84	1.8	1.8	1.73	1.72	1.71
6	7.27	7.1	6.95	6.51	6.28	6.24
7	13.3	12.96	12.86	12.42	12.33	12.28
8	17.04	16.95	16.55	15.88	15.57	14.91
9	33.04	32.5	30.35	26.42	24.86	24.15
10	31.54	31.05	29.66	25.84	24.51	24.2
11	31.72	31.0	29.75	25.17	24.2	23.89
12	39.8	38.98	37.88	31.93	30.02	30.03
13	42.86	40.05	39.97	33.57	32.37	32.11
14	33.72	32.1	31.84	27.45	25.6	25.4

[a] See the footnote in Table 1 for the names of the compounds.

The structures of the compounds are given in Ref. 26.

Comparison of the results between the two mobile phases, MeOH:H_2O (65:35) and MeOH:H_2O (70:30), indicated similar results for the compound class separation with different concentrations of β-CD, except the k' values were shorter with the MeOH:H_2O (70:30). However, with MeOH:H_2O (65:35) and 4.0 mM of β-CD, the four tetrols and B[a]P-t-9,10-dihydrodiol were partially separated and 6-OH-B[a]P was separated from 12-OH-B[a]P. In contrast, with MeOH:H_2O

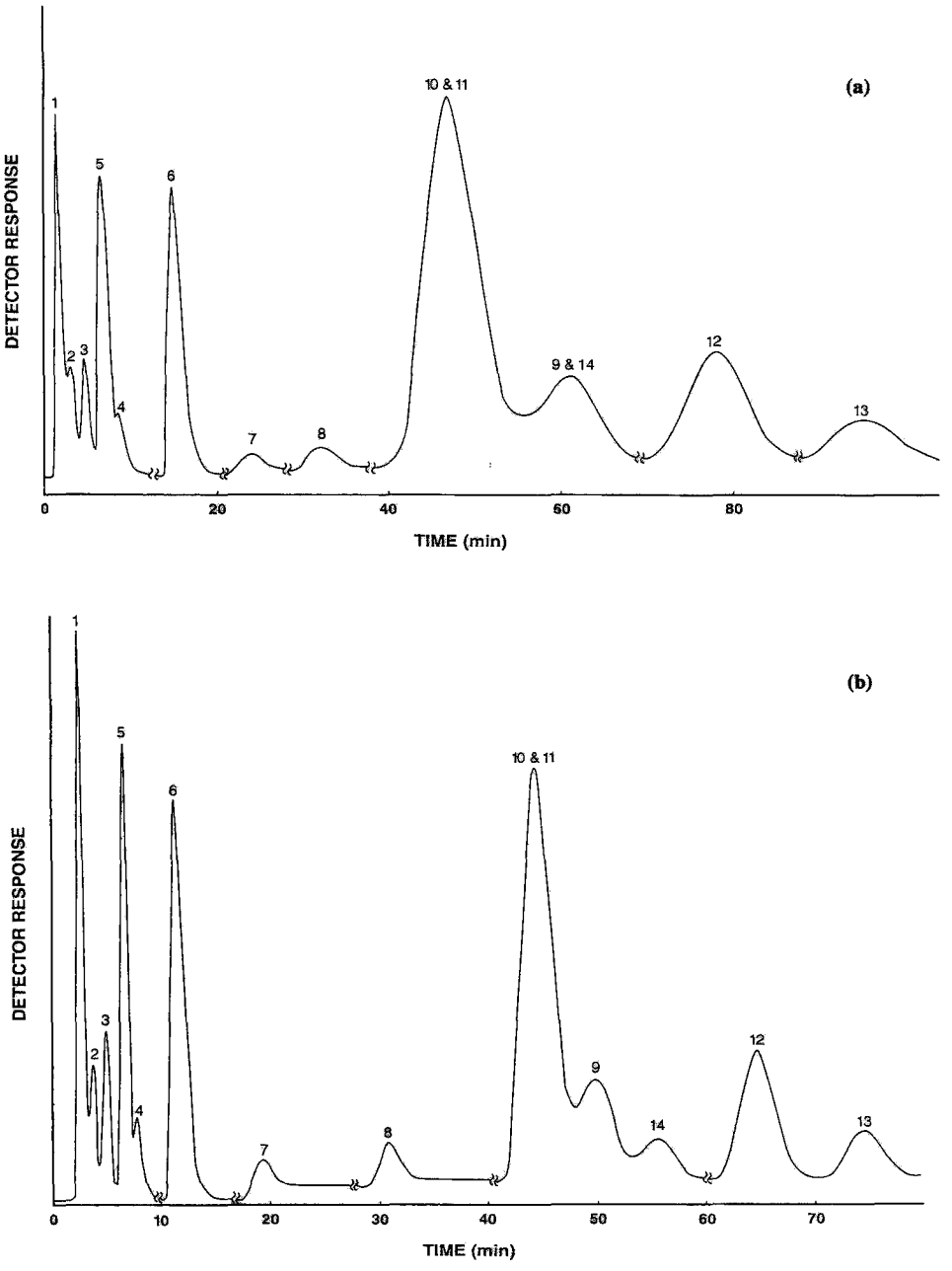

FIGURE 1. Chromatograms of the fourteen metabolites of benzo[a]-pyrene obtained with MeOH:H_2O (65:35) (a), and MeOH:H_2O (65:35) with 4.0 mM β-CD (b) on a 5.0 μm C_{18} column. The names of the compounds are given in the footnote of Table 1.

(70:30) and 3.5 mM β-CD, the four tetrols and B[a]P-t-9,10-dihydrodiol overlapped and 6-OH-B[a]P was partially separated from 12-OH-B[a]P. Also, there was no improvement in the resolution of 2-OH-B[a]P and 9-OH-B[a]P with either mobile phases. An increase in the concentration of β-CD in both MeOH:H_2O (70:30) and (65:35) only affected the retention characteristics of tetrols and dihydrodiols slightly because of the small k′ values for these two classes of metabolites. In spite of the relatively large retention times with MeOH:H_2O (65:35) and 4.0 mM β-CD, a good overall separation of thirteen compounds from the mixture of fourteen metabolites was obtained (Figure 1b). Thus, MeOH:H_2O (65:35) with β-CD was a more useful mobile phase than MeOH:H_2O (70:30) for the separation of a mixture of the metabolites.

Dependence of Capacity Factors of Tetrols on β-CD Concentration

The detection of tetrols in human body fluids and lower animals indicates the presence of B[a]P-DNA adducts. Thus, the study of tetrols is very important in cancer research. To examine the effects of different concentrations of β-CD on the chromatographic properties of the four tetrols, MeOH:H_2O (55:45) mobile phases with and without β-CD were investigated. The k′ values of the tetrols for different analytical concentrations of β-CD (0.0, 2.0, 3.0, 4.0 and 5.0 mM) were obtained. The MeOH:H_2O (55:45) mobile phase was used because the tetrols showed a much greater change in their retention times compared to MeOH:H_2O mobile phases with compositions of 81.75:18.25, 70:30, and 65:35. The k′ values for the monohydroxyl metabolites with MeOH:H_2O (55:45) were very large, and thus this mobile phase was not useful for the separation of the fourteen

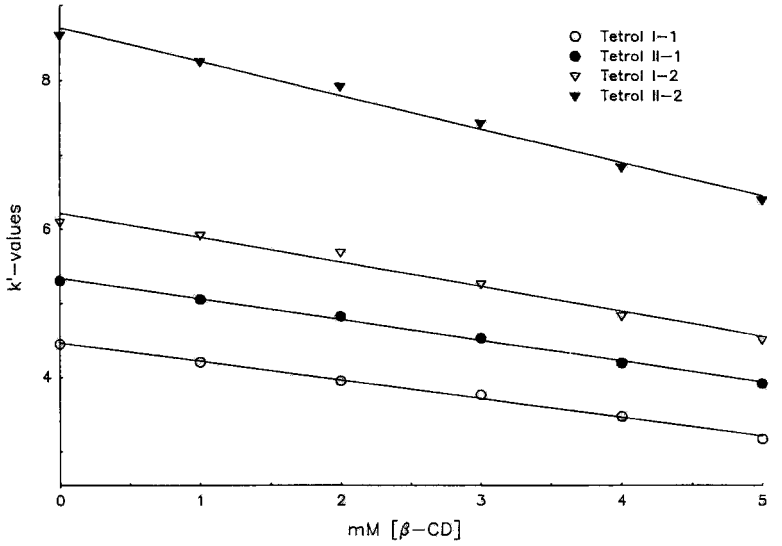

FIGURE 2. Plots of k′ values versus β-CD concentration for the four tetrols in MeOH:H_2O (55:45 v/v) at 25° C.

metabolites. Figure 2 shows the effects of the β-CD concentration on the retention of the four stereoisomers of the tetrols. As indicated in the Figure, a linear relationship was obtained between k′ and β-CD concentration for each of the tetrols. It is important to compare the changes in the capacity factors of the four tetrols with MeOH:H_2O (55:45) in the presence of β-CD. For example, benzo[a]pyrene-r-7,t-8,c-9,10-tetrahydrotetrol (tetrol II-2) showed a larger reduction in its k′ values with an increasing β-CD concentration compare to the other three tetrols (Table 3). The decrease in the capacity factors of tetrols with β-CD in MeOH:H_2O (55:45) resulted in the band sharpening, reduction in band width and a better separation of tetrols, which was due to their ability to form

TABLE 3
k'-Values of Tetrols with Methanol-Water (55:45 v/v) with Different Concentrations (mM) of β-CD

	β-CD (mM)					
Solutes[a]	0.0	1.0	2.0	3.0	4.0	5.0
1 (I-1)	4.45	4.2	3.95	3.76	3.47	3.17
2(II-1)	5.3	5.05	4.82	4.53	4.19	3.92
3 (I-2)	6.1	5.92	5.69	5.26	4.83	4.52
4(II-2)	8.6	8.25	7.93	7.44	6.85	6.41

[a] See the footnote in Table 1 for the names of the tetrols.

The structures of the tetrols are given in ref. 26.

inclusion complexes with β-CD. The best mobile phase investigated for the separation of tetrols was MeOH:H_2O (55:45) with 5.0 mM of β-CD.

Effects of the Composition of Acetonitrile and β-Cyclodextrin Concentration on the Retention of B[a]P Metabolites

In the absence of cyclodextrin, the retention of the fourteen B[a]P metabolites decreased as the acetonitrile (ACN) percentage increased in the mobile phase. At about ACN:H_2O (55:45), the k' values of the monohydroxyl-B[a]P were approximately 20, and the k' values became substantially greater at lower percentages of acetonitrile (26). At a percentage of 75% ACN, or greater, a white complex with small concentrations of β-CD formed. Thus, these ACN:H_2O mobile phases could not be used with β-CD. To examine the effects of β-CD and ACN on the retention characteristics of the metabolites, lower percentages of ACN were investigated (60:40 and 65:35).

With ACN:H_2O (60:40) four classes of metabolites, namely, tetrols, dihydrodiols, diones, and monohydroxyl-B[a]P, were separated with or without β-CD as a mobile phase additive. An increase in the concentration of β-CD didn't show a significant affect on the k'-values of tetrols and dihydrodiols. These results are related to the low retention times for these two classes of metabolites. The overall separation of the mixture of fourteen metabolites with ACN:H_2O (60:40) and β-CD was essentially the same as with ACN:H_2O (65:35) and β-CD.

Generally, retention times decreased and the bands were sharper with β-CD in ACN:H_2O (65:35) mobile phase compared to ACN:H_2O (65:35) or MeOH:H_2O (65:35). Also, 2-OH-B[a]P and 9-OH-B[a]P were separated with ACN:H_2O (65:35) and β-CD compared to MeOH:H_2O (65:35) and β-CD (compare Figure 1b and Figure 3). This pair of isomers was one of the most difficult pairs to separate with MeOH:H_2O mobile phases. Evidence for sharpening of the bands is shown in Figure 3 for these two hydroxyl aromatics with β-CD present in ACN:H_2O (65:35) compare to ACN:H_2O (65:35). An earlier investigation with the fourteen metabolites (26) indicated that the ACN:H_2O (65:35) was capable of separating at least thirteen metabolites from a mixture of fourteen metabolites. Similar results were obtained by addition of β-CD to the mobile phase. Figure 3 shows the advantages of using β-CD as a mobile phase additive in ACN:H_2O (65:35). Comparison between this chromatogram and chromatogram obtained with ACN:H_2O (65:35) (26) indicated: a) decrease in the overall retention times, b) sharper bands in the chromatogram (Figure 3), and c) improvement in the compound-class separation.

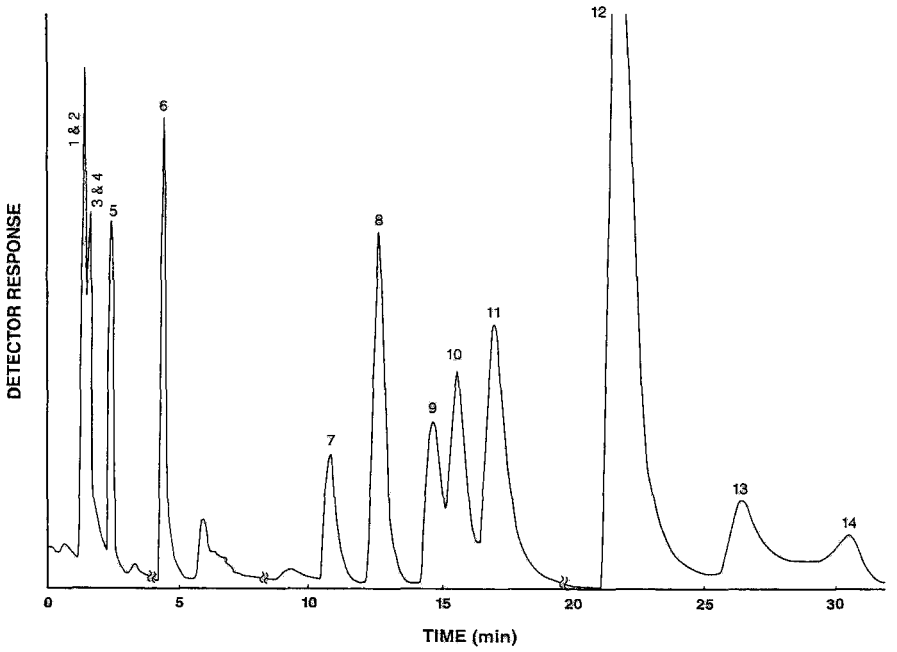

FIGURE 3. Chromatogram of fourteen metabolites of benzo[a]pyrene obtained with ACN:H_2O (65:35) with 1.5 mM β-CD at 25° C. The names of the compounds are given in the footnote of Table 1.

Comparison of MeOH:H_2O and ACN:H_2O

Comparison of Figure 3 and Figure 1b, shows that 2-OH-B[a]P and 9-OH-B[a]P can be separated with ACN:H_2O (65:35) and β-CD. However, with MeOH:H_2O (65:35) and 4.0 mM of β-CD the four tetrols were separated somewhat better than with ACN:H_2O (65:35) and β-CD (compare Figures 1b and Figure 3). In addition, Figure 1b shows the effectiveness of β-CD in the separation of structural isomers, 6-OH-B[a]P and 12-OH-B[a]P. These two isomers could not be separated with just methanol-water (Figure 1a). Also, MeOH:H_2O (65:35) with 4.0 mM of β-CD is capable of separating at least twelve

of the metabolites (Figure 1b). In contrast, ten of the metabolites were separated with ACN:H_2O (65:35) and 1.5 mM of β-CD (Figure 3). Larger k′ values for the six monohydroxyl-B[a]P metabolites were obtained with MeOH:H_2O (65:35) and β-CD (Table 2) compared to ACN:H_2O (65:35) (Figure 3). Generally, ACN:H_2O (65:35) with 1.5 mM β-CD was a better mobile phase for the separation of this class of metabolites. However, to obtain a better separation with a good baseline resolution of the fourteen metabolites MeOH:H_2O (65:35) with 4.0 mM of β-CD is preferable.

Effects of a Ternary Mobile Phases and β-CD Concentration on the Capacity Factors of the Metabolites

The retention characteristics of the fourteen metabolites of benzo[a]pyrene with different analytical concentrations of β-CD (0.0, 1.0, 1.5, 1.7 and 2.0 mM) and MeOH:ACN:H_2O (17:50:33 v/v/v) were obtained, because it was shown earlier that this ternary mobile phase was effective in separating the fourteen metabolites (26). With this mobile phase, the k′ values for the tetrols and dihydrodiols didn't change significantly with increasing β-CD concentration. For example, the k′ value of benzo[a]pyrene-trans-7,8-dihydrodiol decreased from 2.26 with no β-CD to 2.01 with 2.0 mM β-CD. The small changes in the k′ values of the dihydrodiols indicated a weak guest-host interaction between these metabolites and β-CD. The diones had relatively large k′ values with β-CD in the ternary mobile phase. An increase in the concentration of β-CD to 2.0 mM reduced the k′ values of both diones about 1.5-fold. The k′ values for the monohydroxylated compounds also decreased with an increase in the β-CD concentration. For

example, the k′ values of 12-OH-B[a]P and 9-OH-B[a]P were about 9.8 and 10, respectively, with the ternary mobile phase (26). With 2.0 mM β-CD, the k′-values for these two metabolites decreased to 6.4 and 6.9, respectively, resulting in band sharpening and shorter retention times and separation of these two compounds from each other. Thus, an increase in the concentration of β-CD to 2.0 mM resulted in the reasonable elution times $1<k'<15$ and band sharpening for the metabolites of benzo[a]pyrene.

CONCLUSIONS

The addition of β-CD to the mobile phases in this investigation resulted in a decrease in the capacity factors, band sharpening, and compound-class separation. Without β-CD in methanol-water mobile phases, two pairs of monohydroxyl isomers (6-OH-B[a]P and 12-OH-B[a]P, and 2-OH-B[a]P and 9-OH-B[a]P) could not be separated. However, with β-CD in MeOH:H_2O (65:35), 6-OH-B[a]P and 12-OH-B[a]P were separated. MeOH:H_2O (55:45) with 5.0 mM β-CD was a better mobile phase than either the ACN:H_2O or the ternary mobile phases with β-CD for the separation of the four tetrols. Using ACN:H_2O (65:35) and 2.0 mM β-CD, the monohydroxyl metabolites were separated with a shorter retention time compared to the MeOH:H_2O (65:35) with 4.0 mM β-CD. Also, 2-OH-B[a]P was separated from 9-OH-B[a]P with ACN:H_2O (65:35) and 2.0 mM of β-CD. These two metabolites were the most difficult pairs to separate with different concentrations of β-CD in the MeOH:H_2O mobile phases. Four classes of metabolites namely, tetrols, dihydrodiols, diones, and monohydroxyl-B[a]P were separated with ACN:H_2O (65:35) and β-CD. Addition of β-CD to the ternary

mobile phase MeOH:ACN:H_2O (17:50:33) improved the separation of 9-OH-B[a]P and 12-OH-B[a]P, because of a stronger interaction between β-CD and 12-OH-B[a]P (26). The elution order of the fourteen different metabolites of B[a]P didn't change by addition of β-CD to the binary or ternary mobile phases. However, the overall separation of the metabolites improved significantly by addition of β-CD to methanol-water compared to acetonitrile-water or ternary with β-CD (Figure 1b and Figure 3), especially in the region of the tetrols and B[a]P-t-9,10-dihydrodiol. The disadvantage of MeOH:H_2O (65:35) with β-CD was the relatively large k' values for the monohydroxyl compounds.

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REFERENCES

1. A. H. Conney, Pharmacol. Rev., 19: 317-366 (1967).

2. G. Becher, A. Bjorseth Handbook of Polycyclic Aromatic Hydrocarbons, A. Bjorseth and T. Ramdahl, eds., Vol. 2, Marcel Dekker Inc., New York, 1985, pp. 237-252.

3. G. M. Cohen, A. C. Marchok, P. Nettesheim, V. E. Steele, F. Nelson, S. Huang, J. K. Selkirk, Cancer Res., 39: 1980-1984 (1979).

4. A. Bjorseth, G. Becher, PAH in Work Atmospheres: Occurrence and Determination, CRC Press Inc., Boca Raton, Florida, 1985, pp. 105-113.5. A. H. Conney, Cancer Res., 42: 4875-4917 (1982).

6. H. V. Gelboin, Physiol. Rev., 60: 1107-1166 (1980).

7. S. K. Yang, H. V. Gelboin, B. F. Trump, H. Autrup, C. C. Harris, Cancer Res., 37: 1210-1215 (1977).

8. J. Zaleski, A. R. Steward, H. C. Sikka, Carcinogenesis, 12: 167-174 (1991).

9. J. K. Selkirk, R. G. Groy, H. V. Gelboin, Science, 184: 169-171 (1974).

10. J. K. Selkirk, R. G. Groy, J. P. Whitlock Jr., H. V. Gelboin, Cancer Res., 35: 3651-3655 (1975).

11. J. K. Selkirk, R. G. Croy, F. J. Wiebel, H. V. Gelboin, Cancer Res., 36: 4476-4479 (1976).

12. E. A. Elnenaey, W. P. Schoor, Anal. Biochem., 111: 393-400 (1981).

13. R. G. Croy, J. K. Selkirk, R. G. Harvey, J. F. Engel, H. V. Gelboin, Biochem. Pharmacol., 25: 227-230 (1976).

14. R. Wang, J. W. O'Laughlin, Environ. Sci.Technol., 26: 2294-2297 (1992).

15. A. R. Steward, J. Zaleski, H. C. Sikka, Chem.-Biol. Interactions, 74: 119-138 (1990).

16. R. M. Mohseni, R. J. Hurtubise, J. Chromatogr., 514: 19-27 (1990).

17. J. Debowski, D. Sybilska, J. Jurczak, J. Chromatogr., 237: 303-306 (1982).

18. P. J. Sicard, M. H. Saniez, R. Freres, Cyclodextrin and Their Industrial Uses, D. Duchene, ed., Editions de Sante, Paris, 1987, PP. 77-103.

19. S. Li, W. C. Purdy, Chem. Rev., 92: 1457-1470 (1992).

20. J. Szejtli, Cyclodextrin Technology, Kluwer Academic Publishers, Netherlands, 1988, pp. 1-60.

21. R. Woodberry, S. Ransom, F. M. Chen, Anal. Chem., 60: 2621-2625 (1988).

22. V. C. Anigbogu, A. M. de la Pena, T. T. Ndou, I. M. Warner, J. Chromatogr., 594: 37-43 (1992).

23. A. M. de la Pena, T. T. Ndou, V. C. Anigbogu, I. M. Warner, Anal. Chem., 63: 1018-1023 (1991).

24. R. M. Mohseni, R. J. Hurtubise, J. Chromatogr., 499: 395-410 (1990).

25. D. Sybilska, J. Debowski, J. Jurczak, J. Zukowski, J. Chromatogr., 286: 163-170 (1984).

26. M. Rozbeh, R. J. Hurtubise, J. Liq. Chromatogr., in press.

27. R. J. Laub, J. H. Purnell, J. Chromatogr., 112: 71-79 (1975).

28. R. J. Laub, Physical Methods in Modern Chemical Analysis, T. Kuwana, ed., Academic Press, New York, 1983, Vol. 3, pp. 250-341.

29. A. J. Hsu, R. J. Laub, S. J. Madden, J. Liq. Chromatogr., 7: 599-614 (1984).

30. P. J. Schoenmakers, A. C. J. H. Drouen, H. A. H. Billiet, L. deGalan, Chromatographia, 15: 688-696 (1982).

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PROTEIN BAND DISPERSION IN AXIAL AND RADIAL FLOW CHROMATOGRAPHY

JOHN P. THARAKAN AND MARIO BELIZAIRE
Howard University
Department of Chemical Engineering
2300 6th Street, NW
Washington, D.C. 20059

ABSTRACT

Radial flow (RF) column configurations have been developed for larger scale production liquid chromatography, with the primary purpose of increasing throughput rates and decreasing trans-bed pressure drops in comparison to conventional axial flow (AF) columns. The RF columns have been quite successful in attaining these two objectives. In this work, we investigated the nature of protein band dispersion in both axial and radial flow chromatography column configurations, utilizing S-200 Sephacryl gel filtration media with bovine serum albumin as the applied protein. The effects of input feed flow rate as well as input feed albumin concentration on the nature of protein band dispersion were studied. Protein band dispersion was quantified by examining the shape of the eluting protein peak and measuring peak height-to-width (HTW) ratio's for the various flow rates and feed concentrations used in the study. Our results indicate that protein dispersion was larger in RF columns as indicated by smaller peak HTW ratio's than those obtained for AF columns, operated at the same flow rates and feed protein concentrations. We also found that moderately increasing the flow rate increased peak HTW ratio's and resulted in sharper peaks for both AF and RF columns.

INTRODUCTION

Radial flow (RF) columns were first developed to handle large gas flow rates through packed catalyst beds with minimal pressure losses across the bed

(1). Since their development, the fluid mechanics (2) and kinetics (3) of RF systems, as well as their extension to reverse osmosis (4) and cell culture (5) systems have been studied.

Columns for RF chromatography were soon developed (6) and their applications demonstrated that reduced trans-bed pressure drops as well as increased throughputs could be achieved. RF columns have been commercially available for some time (6,7), and they have been utilized for the purification of plasminogen (8) and other biologicals (9). In our laboratories we have documented decreased process times and trans-bed pressure drops and increased throughputs (10) and studied their application to affinity systems and shown that both RF and AF systems perform equivalently (11).

Schematics of the RF and AF columns are depicted in Figure 1a and 1b, respectively. As can be seen from the figure, the cross sectional area normal to the flow direction will either be increasing (as in the case of centrifugal [CF] flow) or decreasing (as in the case of centripetal [CP] flow). Thus, the linear velocity in CF flow in RF columns is decelerating while that in CP flow is accelerating. In an AF column, on the other hand, the cross-sectional area normal to the flow direction is constant, resulting in a flow with constant velocity. The implications of this is that mass transfer coefficients and radial dispersion cannot be assumed constant in the flow direction (12). Thus, protein band spreading will differ for the RF and AF columns.

In this study, we investigated the nature of protein band dispersion in both AF and RF columns. We utilized columns of the same total volume (50 mls) and similar bed height (AF: 2.9 cm; RF 3.0 cm) packed with Sephacryl S-

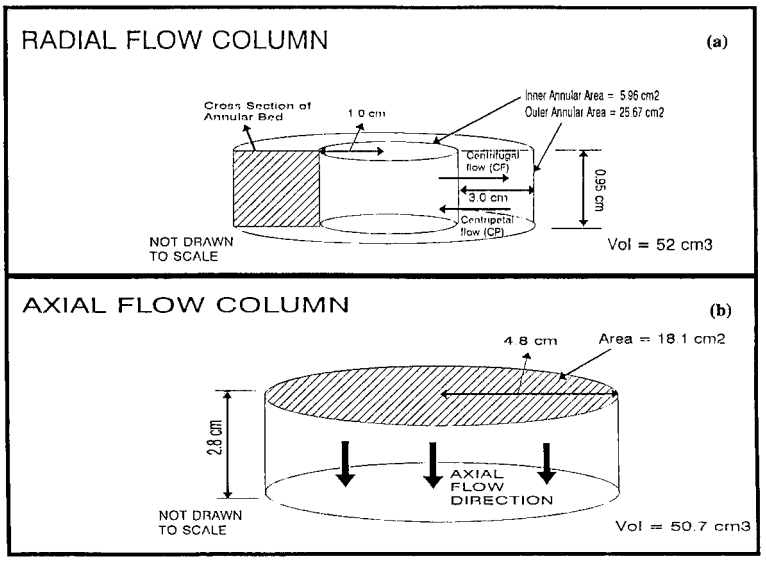

Figure 1: Schematic of radial flow (1a) and axial flow (1b) column flow configurations, indicating dimensions of experimental study columns.

200 gel filtration media. We utilize bovine serum albumin (BSA) to investigate the shape of the eluting peak from both columns packed with S-200 resin. Protein band dispersion was quantified by measuring peak height to width (htw) ratio's, and the effects of feed flow rate and inlet protein concentration on band spreading were also investigated.

MATERIALS AND METHODS

Columns

The axial flow column was purchased from Kontes Glass (Vineland, NJ). It had a diameter of 4.8 cm and was fitted with top and bottom flow adapters.

The resin was packed into the column by gravity settling and the height of the column was adjusted to yield a bed height of 2.8 cm, which resulted in a column of volume 50 cm^3. The area of the bed normal to flow was 18.1 cm^2.

The radial flow column was purchased from Sepragen (San Leandro, CA). The inner annulus had a radius of 1.0 cm and the outer annulus was 4.3 cm in radius, and the annular bed thickness (height) was 3.0 cm. The column was 0.95 cm high, resulting in a bed volume of 52 cm^3. The cross sectional area normal to flow at the inner annulus was 5.96 cm^2 and that at the outer annulus was 25.67 cm^2. Detailed diagrams of both columns are shown in Figure 1.

Resin

Sephacryl S-200 gel filtration resin was obtained from Sigma Chemical (St. Louis, MO).

Proteins and Chemicals

Bovine serum albumin (BSA) and blue dextran were obtained from Sigma. All chemical used were reagent grade and obtained from Sigma as well.

Experimental Protocol

The columns were packed with the resin according to the manufacturer's instructions. After packing, the resin beds were equilibrated with 5 column

volumes (CV) of 0.3M NaCl, 0.02M NaH_2PO_4, 0.02% NaN_3 at pH 7.0. A 1-ml solution of protein at a concentration of 1.0 mg BSA/ml was injected in-line to the column and the pump (Masterflex Digital Unified Drive, Cole Parmer, Chicago, IL) was switched on at a flow rate of 2 ml/min. The column effluent was monitored by a UV detector set at 280 nm (Gilson 112 UV/VIS, Gilson Medical Electronics, Middleton, WI), and the output from the detector was recorded on a strip chart recorder (BD40, Kipp and Zonen, Delft, Holland).

The protocol was repeated using protein concentrations of 2.5,5.0 and 7.5 mg/ml at the same flow rate of 2 ml/min. These experiments were then repeated again at a fixed protein feed concentration for flow rates of 5, 10 and 15 ml/min.

Data on the peak height of the eluting protein band was collected and the peak width at half height was also measured. Peak height-to-width ratio's were then calculated and the data is presented to show the effect of feed protein concentration and feed flow rate on peak htw ratio for both AF and RF column configurations.

RESULTS AND DISCUSSION

In an RF column, the linear velocity with which the mobile phase moves through the packed bed is not constant. Figure 2 shows the radial velocity profile for four different flow rates that were utilized in this study, for the centrifugal flow situation. These plots were obtained by dividing the flow rate by the area, which is a function of radial distance. As the figure demonstrates,

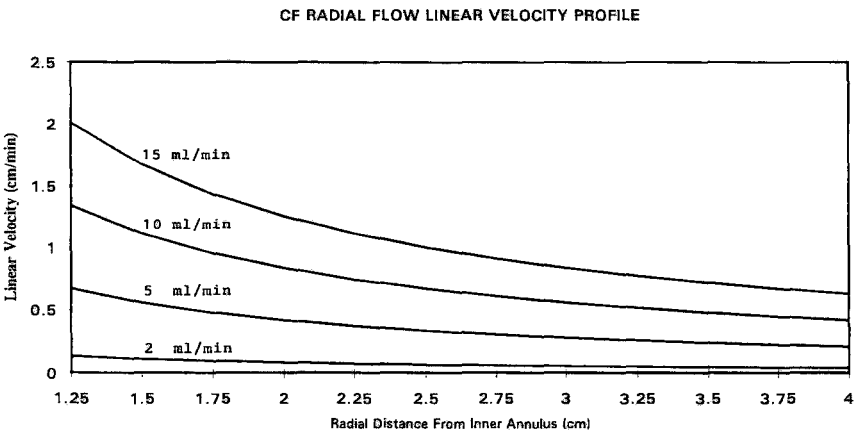

Figure 2: Radial velocity profile for centrifugal flow in an RF column.

larger flow rates result in larger differentials between the inlet velocity and the velocity at the outlet of the radial packed bed. In the case of the AF column, the cross sectional area normal to flow is constant and thus the linear velocity through the axial bed will be constant.

In the analysis of chromatography systems, the ratio of peak height to width provides information about the extent of protein band spreading that occurs as the protein flows through the chromatographic bed. When protein is introduced into a chromatography bed, it begins its passage as a tight plug that is subjected to radial and axial dispersion as it moves through the column. As it emerges from the column, a UV detector at the column outlet can monitor the spread of the protein band. Large peak HTW ratio's imply that the protein has remained in a relatively tight band and much dispersion has not occurred. As peak HTW ratio's decrease, the implication is that the protein band disperses

more in the direction of flow resulting in shorter and broader peaks, or peaks with a low peak HTW ratio.

In Figure 3a, the peak HTW ratio is plotted as a function of increasing inlet protein concentration for both AF and RF columns at two flow rates, 2 and 10 ml/min, respectively. Figure 3b shows similar data for the same columns for flow rates of 5 and 15 ml/min. As we can see from the figure, as the concentrations increase, the peak HTW ratio also increases for all four flow rates. However, as both Figures 3a and 3b demonstrate, the rate of increase for the AF columns is greater than that for the RF columns. Additionally, the peaks that were obtained from the AF column were sharper and less dispersed, in general.

In Figure 4a, the peak HTW ratio is plotted as a function of increasing flow rate for feed inlet protein concentrations of 2.5 and 7.5 mg.ml. Once again, the rate of increase of the peak HTW ratio is larger for the AF columns than for the RF columns. In all four cases plotted, the HTW ratio increases linearly for flow rates increasing from 1 to 9 ml/min. After that point, the rate of increase decreases. Figure 4b shows the HTW ratio for increasing flow rates for both the AF and RF system, in this case for protein concentrations of 1 and 5 mg/ml. Again, the data demonstrates that the increase in HTW ratio for AF is more rapid that for the RF columns.

Both the RF and AF columns were tested for integrity and flow uniformity by injecting a plug of Blue Dextran into the columns and observing the movement and dispersion of the plug. Our observations showed that the

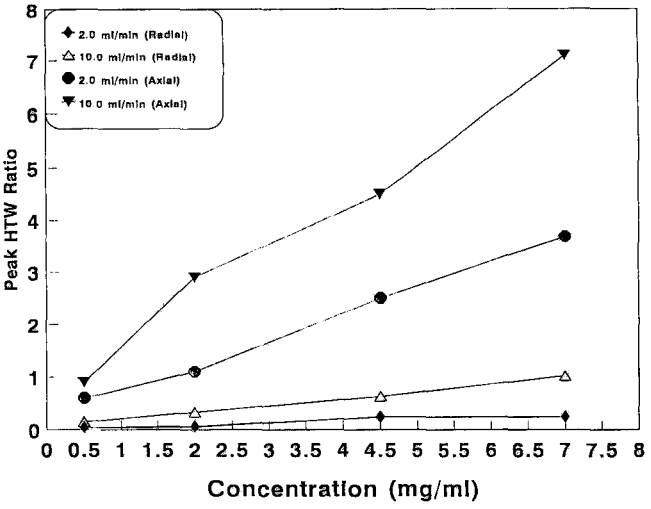

Figure 3a: Effect of concentration on peak HTW ratio for axial and radial flow.

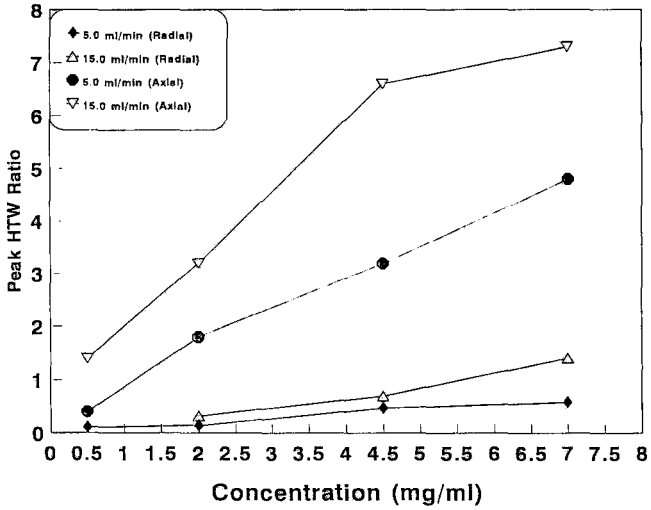

Figure 3b: Effect of concentration on peak HTW ratio for axial and radial flow.

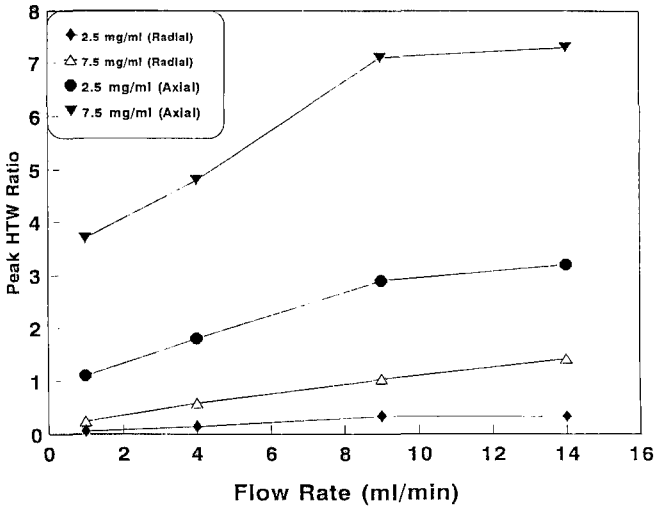

Figure 4a: Effect of flow rate on peak HTW ratio for axial and radial flow.

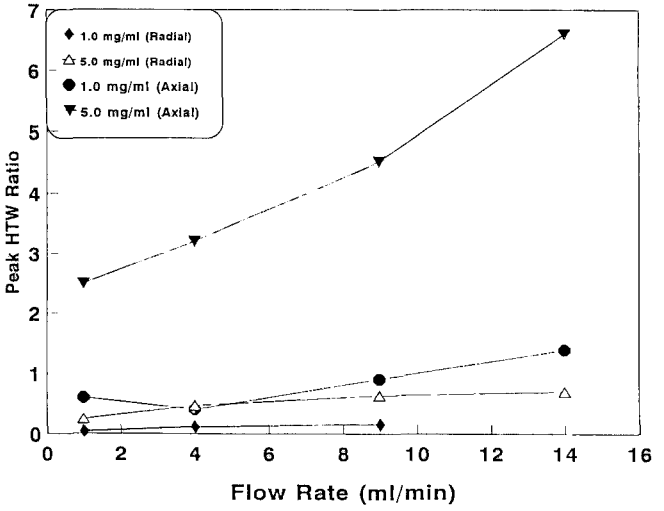

Figure 4b: Effect of flow rate on peak HTW ratio for axial and radial flow.

blue dextran moved uniformly through both the RF and AF columns. In this way, we ruled out the possibility of bed deformities or irregularities affecting the results.

Based on these data, it is clear that peaks will be flatter and broader for RF columns, confirming the theoretical determinations of Gu *et al* (12) that radial dispersion and mass transfer cannot be assumed constant for RF columns due to the accelerating and decelerating flows. Our data also shows that peaks become sharper in both AF and RF columns when the flow rates are increased. The results of these studies suggest that careful trial experiments need to be conducted to ascertain the effect of flow and protein loading on band dispersion before implementing an RF column at production scale.

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REFERENCES

1. Johansen, K., *Chemical Engineering World*, **5** (8), 8 (1970).

2. Genkin, V.S., V.V. Dil'man, and S.P. Sergeev, *Intl. Chem. Eng.*, **13**, 24 (1973).

3. Chang, H.C., and J.M. Callo, *ACS Symp. Ser.*, **168**, 305 (1981).

4. Gill, W.N., and B.N. Bansal, *AIChE J.*, **18**, 823 (1973).

5. Tharakan, J., and P.C. Chau, *Biotech. Bioeng.*, **28**, 435 (1986).

6. Saxena, V., K. Subramaniam, S. Saxena and M. Dunn, *Biopharm.*, **3**(3), 46 (1989).

7. Mandara, R.M., S. Roy, and K.C.Hou, *Bio/Technology*, **5**, 928 (1987).

8. Planques, Y., H. Pora, and F.D. Menozzi, *J.Chrom.*, **539**, 531 (1991).

9. McCormick, D., *Bio/Technology*, **6**, 158 (1988).

10. Tharakan, J.P., C. Orthner, B. Kolen, A.M. Ralston, D. Gee, D. Clark and W. Drohan, Paper presented at *International Chemical Congress of Pacific Basin Societies*, Honolulu, HW, December 1989.

11. Tharakan, J. and M. Belizaire, *Isolation and Purification*, In Press, 1994.

12. Gu, T., G.-J. Tsai, and G.T. Tsao, *Advances in Biochemical Engineering and Biotechnology*, **49**, 25 (1993).

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HPLC METHOD TRANSFER TO NARROW BORE COLUMNS: AN EVALUATION

TIMOTHY W. RYAN
Ganes Chemicals, Inc.
33 Industrial Park Road
Pennsville, New Jersey 08070

ABSTRACT

The use of organic solvents is common in high performance liquid chromatography (HPLC). Some of these solvents, such as acetonitrile, have a very high purchase price. In addition, because of the toxic nature of these solvents, waste elimination costs have soared. The use of narrow bore HPLC columns (2mm ID or less) represents an attractive solution to the problem of excessive solvent costs by reducing the amount of HPLC mobile phase consumed. An evaluation of analysis integrity following method transfer from a standard bore column (3.9 mm ID) to a narrow bore column (2 mm ID and 1 mm ID) is necessary to determine feasibility in a QC/analytical environment.

INTRODUCTION

High performance liquid chromatography (HPLC) is the preferred method of analysis for a number of important industries. Most HPLC mobile phases incorporate the use of an organic solvent. Generally, these solvents are toxic materials. They can also be quite expensive, both in terms of initial purchase cost

and in terms of the ever increasing cost of disposal. As such, solvents for HPLC can represent a significant portion of an analytical laboratory's annual budget.

A number of approaches have been developed to address both the costs of the solvents and the elimination of the waste. Commercially available (or laboratory fabricated) distillation apparatus allows the analytical lab to purchase less expensive technical or reagent grade solvents, which are then refined to an acceptable purity level. While this technique reduces the initial solvent purchase cost, it does not address the problem of disposal. It does, however, represent an increase in the routine lab housekeeping functions, something most analytical and quality control laboratories would rather avoid. Potentially, the same distillation technique could be applied to the waste mobile phase. But the possible difficulties are prohibitive. These include separating and purifying complex mixtures (even in the case of a single solvent with an aqueous phase, formation of an azeotrope would interfere with purity), and avoiding contamination from spent sample in the waste. This also represents an increase in housekeeping functions. Mobile phase recycling systems have also been commercially available for some time. These devices work on the principle that most of the mobile phase elutes from the HPLC column essentially uncontaminated, and that only those volumes that contain the injection void or the actual sample peaks are true waste. These systems can be very useful in an analysis where only a single peak in a product assay is encountered. Even in a QC lab, such an ideal situation is rare, almost all samples have some low level of impurities. As the number of peaks in the sample increases, the efficiency decreases. As the solvent is recycled, contamination eventually takes its toll. Increasing levels of chromaphoric "non-sample" material affects sensitivity and detection linearity. The presence of non-chromaphoric background material will affect the separation. Also there is some question as to whether or not the regulatory agencies would require additional testing of the

recycled mobile phase to demonstrate that it does not interfere with the integrity of the analysis. More importantly, the technique is useless for HPLC methods that requires any degree of gradient elution. Because of these inadequacies, this technique cannot be seriously regarded as a solution to the problem.

The easiest way to address both the costs of the solvents, and the cost and environmental impact of disposal, is to decrease the amount of solvent initially purchased and hence the amount of waste mobile phase generated. Liquid chromatography using narrow bore columns (2 mm internal diameter or less) represents an attractive solution to this problem. Column flow is proportional to the column internal diameter (ID). Thus the same linear velocities (and therefore retention times) are preserved when using a lower flow rate and a column with a reduced ID (if column ID is the only parameter changed). The following equation is commonly used to calculate flow rate changes based on a change in the column ID:

$$\frac{(\text{ID col 2})^2}{(\text{ID col 1})^2} \times \text{flow col 1} = \text{flow col 2}$$

The use of a 2 mm ID column in place of a traditional 3.9 mm ID column can reduce mobile phase consumption by a factor of 3-4 (assuming a typical flow rate of 1-2 ml/min). There are some difficulties with the technique, most of which are associated with incompatibilities between the narrow bore column and the chromatographic system.

The purpose of these experiments is to document the transfer of an HPLC method from a standard analytical column to a narrow bore technique. The object is to determine how much adjustment is required in order to execute an accurate method transfer. In order for the transfer to be practical, the only considerations addressed will be minor adjustments in flow rate and in injection volume. The only

hardware modification was the use of a semi-micro flow cell (volume 8 µl) in place of the standard flow cell (volume 14 µl) to maintain UV detector sensitivity with the narrow sample bandwidths that the smaller ID columns produced. The cell could also be used with the standard bore column

MATERIALS AND METHODS

The equipment used included a Model 1050 HPLC system (Hewlett Packard Co., Palo Alto CA). This system included an autosampler, pump and UV detector. The only other important change was slowing the syringe draw speed from 200 µl/min to 10 µl/min. This change was incorporated to accommodate the small injection volumes. Connecting tubing lengths were kept at an absolute minimum and the connecting tubing used had a 0.005" ID. Although even smaller ID's would be achieved using fused silica capillary tubing, the fragile nature of exposed capillary tubing on an LC precluded its use. Three different HPLC columns were evaluated. The first was the "standard" column, a 300 x 3.9 mm µBondapak C18 (Waters Division of Millipore, Milford MA). This column is routinely used for a number of our purity profile analyses and sample assays. The first narrow bore column evaluated was a 300 x 2 mm µBondapak C18 (Waters). The final narrow bore column evaluated was a 300 x 1 mm Hyperbond C18 (Keystone Scientific, Bellefonte PA). The Hyperbond column was used since the µBondapak is not available with a 1 mm bore (the Hyperbond packing is marketed as a comparable packing to the µBondapak). The sample was a mixture of 10 barbiturates made from in-house material (Ganes Chemicals, Inc., Pennsville NJ) and standards purchased from Aldrich (Milwaukee WI). The sample was prepared by dissolving 6-8 mg of each barbiturate in a single 25 ml volumetric flask and

diluting to volume with mobile phase. The mobile phase was 70/30 water/acetonitrile pH 3.0 (H_3PO_4). The mobile phase was filtered and degassed using sonication before use.

RESULTS & DISCUSSION

Ten injections were performed for each sample series. After calculation of the mean, the two injections with the highest deviation were eliminated. The sample series mean, standard deviation and % realtive standard deviation were then calculated using the remaining eight injections. The elimination of the two outliers is statistically acceptable given the number of injections. Figure 1 is an example analysis of the barbiturate sample analyzed using the 3.9 mm ID column. The flow rate was set at 1 ml/min, injection volume was 3 µl. All of the

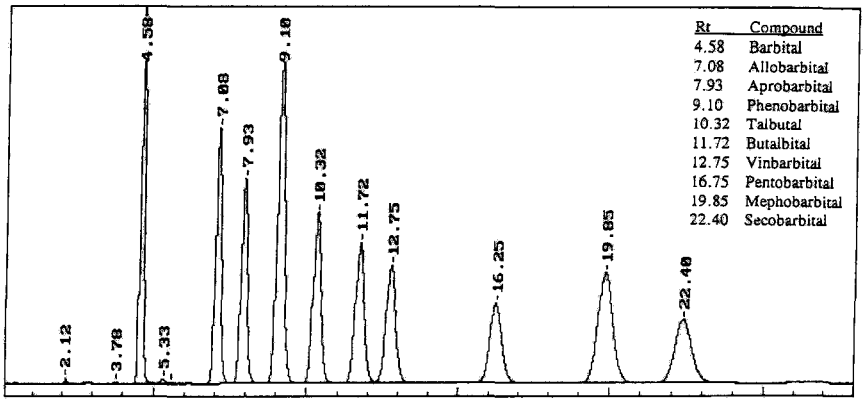

Figure 1. Analysis of barbiturate sample. Flow: 1 mL/min; Mobile Phase: 70/30 Water/Acetonitrile, pH 3.0 (H_3PO_4); Column: 300 x 3.9 mm µBondapak C18.

barbiturates are baseline resolved from one another. From Table 1, the reproducibility of the analysis is evident. This represents important information concerning the injection system. Injection volumes commonly used are 10 µl, so these analyses establish that at a 3 µl injection volume, the autosampler does provide reproducible injections. Because of the accuracy and reproducibility achieved using this column, no further experimentation was performed.

Figure 2 is an example of the barbiturate analyses using the 2 mm ID column. The flow rate was set at 263 µl/min, a comparable flow rate reduction based on the decrease in column cross sectional area. Table 2 lists the results of the initial analyses that used an injection volume of 0.8 µl. These results were not as good as those achieved using the 3.9 mm column. Although peak separation is comparable, area reproducibility is between 1 - 2 %. Most analytical methods specify RSD's of 2% or less, our methods typically provide RSD's of less than 1%.

TABLE 1
HPLC ANALYSIS OF BARBITURATES
3.9 MM ID COLUMN, 3 µl INJECTION

INJ#	Barb.	Allo.	Apro.	Pheno.	Tal.	Butal.	Vin.	Pento.	Mepho.	Seco.
1	354439	329744	295414	546253	322946	297960	270036	230622	386894	250018
2	355392	330048	295751	547646	322980	297605	270152	231460	387110	250280
3	353272	328179	**293816**	543556	**321211**	**295899**	269054	**228678**	386399	**248424**
4	357277	331686	296747	549010	324795	298964	271180	231990	387705	250947
5	356205	330881	296491	549307	324111	298636	270585	231131	387184	250124
6	**358591**	**332562**	**298061**	**551400**	324976	299811	**272299**	231769	389172	251889
7	353798	329046	294852	546220	322399	298158	269654	**229801**	385847	**249503**
8	354246	329822	294187	547557	324324	298900	270726	231316	386295	252395
9	357736	332030	297572	550862	**325553**	299492	271364	232464	388447	252035
10	**352436**	**327326**	294130	**546031**	321992	**296941**	**268841**	231123	386449	250180
mean	355296	330180	295643	547551	323565	298691	270344	231484	387067	250984
s	1643	1299	1239	2255	1130	757	776	576	729	980
% RSD	0.46	0.39	042	0.41	035	025	0.29	0.25	0.19	0.39

Bold entries were not used in calculations.

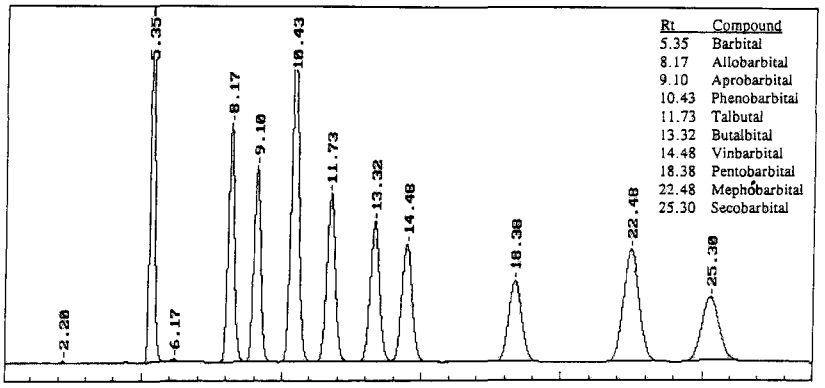

Figure 2. Barbiturate analysis. Flow: 263 µL/min; Mobile Phase: 70/30 Water/Acetonitrile, pH 3.0 (H_3PO_4); Column: 300 x 2 mm µBondapak C18.

TABLE 2
HPLC ANALYSIS OF BARBITURATES
2.0 MM ID COLUMN, 0.8 µl INJECTION

INJ#	Barb.	Allo.	Apro.	Pheno.	Tal.	Butal.	Vin.	Pento.	Mepho.	Seco.
1	**312776**	**290289**	**264096**	**478181**	**283012**	**262301**	**237853**	**201640**	**335389**	**217668**
2	323506	299375	272242	495702	293032	270145	244938	210161	346932	226372
3	318195	295330	266253	486908	287740	266648	242700	207522	342187	219542
4	325213	306145	274920	499073	295964	273365	248489	210809	349008	224665
5	325346	305271	271043	496477	291330	272896	249248	211785	348827	226549
6	324917	303052	271236	495983	291543	270825	246081	210408	346006	225606
7	327762	308805	275666	501941	297179	273329	249126	212101	346933	226096
8	**313069**	**290447**	**262277**	**478151**	**283071**	**260632**	**237232**	**200893**	**333866**	**215658**
9	320947	297574	268652	489582	290219	267857	243918	206032	341662	220065
10	328906	305145	276445	502219	298395	274501	250668	212485	350476	227587
mean	324349	302587	272058	495986	293175	271196	246896	210163	346504	224560
s	3427	4681	3534	5446	3692	2832	2885	2277	3165	3053
% RSD	1.07	1.55	1.30	1.01	1.26	1.04	1.17	1.08	0.91	1.34

Bold entries were not used in calculations.

From these results, it is apparent that the deviation is due to the small injection volume. This conclusion was reached by observing that in specific injections, all of the peaks have an area that is either higher or lower than the mean area. If the high RSD was due to chromatographic variations, a much more random distribution of peak areas would be expected (see Table 1). Since the smaller column has a lower sample capacity, and since the experimentation using the 3.9 mm ID column indicates that 3 µl is an accurately reproducible injection volume, the sample was diluted by a factor of 5 (2ml in a 10ml volumetric flask) and re-analyzed using a 3 µl injection volume. Results are listed in Table 3. Using the larger injection volume, the reproducibility of the injections is improved dramatically, and are now clearly comparable to those results achieved using the 3.9 mm ID column. This concluded the experimentation using the 2 mm ID column.

TABLE 3
HPLC ANALYSIS OF BARBITURATES
2.0 MM ID COLUMN, 3.0 µl INJECTION

INJ#	Barb.	Allo.	Apro.	Pheno.	Tal.	Butal.	Vin.	Pento.	Mepho.	Seco.
1	**270295**	**252049**	**228685**	**416014**	**246305**	**226476**	**204804**	**175611**	**295651**	**190130**
2	**269058**	**249865**	**224709**	**416720**	**245837**	**226386**	**204765**	**174795**	**296289**	**189795**
3	264284	245862	221237	409598	241707	222910	201261	171248	290096	184315
4	265923	247611	222732	412789	243220	224035	202811	174139	293082	187031
5	267414	247975	223143	413350	243675	225794	204522	174136	294271	187940
6	263759	245017	220857	409170	241544	223000	201344	171965	290063	185977
7	265397	246110	221874	410386	242093	223463	202956	173308	293004	185023
8	264960	246008	221422	410725	242413	223355	202398	173217	291959	185880
9	265293	246208	221518	410586	242182	223637	202073	173014	291986	185340
10	264899	245849	221453	409706	241895	223692	202821	173091	291995	186037
mean	265241	246330	221780	410789	242341	223736	202523	173015	292057	185943
s	1103	977	777	1511	744	909	1039	991	1448	1137
% RSD	0.42	0.40	0.35	0.37	0.30	0.41	0.51	0.57	0.50	0.61

Bold entries were not used in calculations.

Figure 3 is an example of the barbiturate sample analyzed using the 1 mm ID column. The flow rate was reduced to 65 µl/min, and the initial sample volume was 0.3 µl. It is immediately apparent that the separation is not as good as that achieved using the other two columns. Unfortunately, the difference in separation could be due to the Hyperbond packing not performing as well as the µBondapak, or if the system void volumes are responsible. In either case, using a weaker mobile phase would probably restore baseline resolution, although this option was not explored. From Table 4, the effect of the 0.3 µl injection on area reproducibility is evident, RSD's ranged from 3 - 6%. This was not suprising since an injection volume of 0.8 µl was already observed to cause problems. In order to note changes in injection reproducibility, the sample was diluted by a factor of 25 (1ml in a 25ml volumetric flask) and re-analyzed using the 3 µl injection volume. These results are listed in Table 5. When using the larger injection volume, the area reproducibility improved significantly. Although the numbers are not quite as good as those achieved using the other two columns, the low RSD's indicate that the information from such an analysis should be quantitatively accurate.

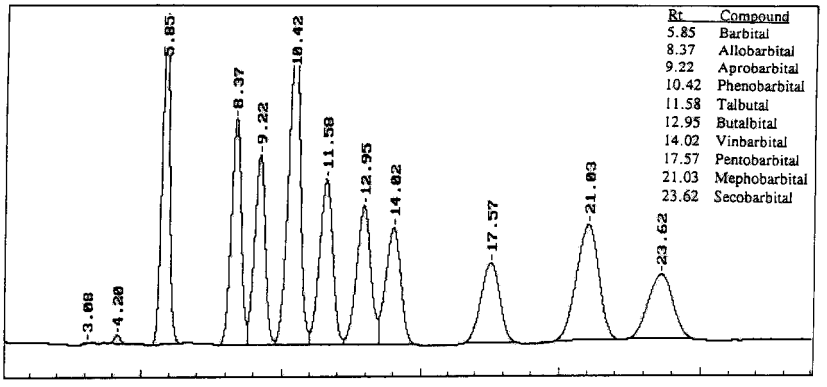

Figure 3. Barbiturate analysis. Flow Rate: 65µL/min; Mobile Phase: 70/30 Water/Acetonitrile, pH 3.0 (H_3PO_4); Column: 300 x 1 mm Hyperbond C18.

TABLE 4
HPLC ANALYSIS OF BARBITURATES
1.0 MM ID COLUMN, 0.3 µl INJECTION

INJ#	Barb.	Allo.	Apro.	Pheno.	Tal.	Butal.	Vin.	Pento.	Mepho.	Seco.
1	1180899	1168686	1001255	1782814	1067950	988876	899738	752711	1243252	848519
2	1188151	1209883	1022421	1815420	1085823	986471	891049	751654	1238571	838530
3	1088485	1056942	949435	1687601	1018341	927431	856532	714585	1170595	774813
4	1086278	1059138	961452	1680237	966416	882976	835252	713120	1186391	779738
5	1114745	1052680	961708	1725738	1081658	996259	895273	707251	1227165	858523
6	**872298**	**826099**	**752096**	**1338772**	**774842**	**709189**	**651416**	**579065**	**901454**	**596107**
7	1082325	1025078	927694	1658494	977675	874262	804929	716522	1133828	725930
8	1092671	1032814	948666	1682105	1008704	909205	806974	720034	1184685	764879
9	1168352	1123035	1022947	1824025	1090636	980115	874148	770788	1271588	812134
10	**865476**	**829858**	**739636**	**1315729**	**773823**	**688403**	**636016**	**568594**	**892777**	**580259**
mean	1125238	1090907	974447	1732054	1037150	943199	857987	730833	1207009	800383
s	45909	68290	36213	66108	50516	50597	38590	23796	45499	46559
% RSD	4.08	6.26	3.71	3.82	4.87	5.36	4.50	3.25	3.77	5.82

Bold entries were not used in calculations.

TABLE 5
HPLC ANALYSIS OF BARBITURATES
1.0 MM ID COLUMN, 3.0 µl INJECTION

INJ#	Barb.	Allo.	Apro.	Pheno.	Tal.	Butal.	Vin.	Pento.	Mepho.	Seco.
1	**214109**	194014	176690	328618	**186741**	177005	157636	**136472**	230514	**144910**
2	**207737**	**190827**	**174217**	**318102**	194756	181631	**161603**	131158	**231459**	143923
3	209962	191997	177449	323914	191052	175491	156977	134451	223297	142899
4	212537	196203	180028	334001	198095	182593	**160881**	132978	227464	140119
5	209456	194394	176827	325807	192397	178029	156970	132884	226795	143604
6	210955	193832	176202	326900	192778	178567	156970	134984	226623	**137042**
7	209141	195468	176661	324439	191505	**174372**	156893	132816	228121	142953
8	211669	192307	177304	324971	193372	179575	157672	132506	227996	142452
9	212909	**197011**	**181182**	**331647**	196925	181253	158131	**130784**	223450	141671
10	209650	194776	176222	322053	**186653**	**174552**	156369	134753	**230603**	139876
mean	210785	194124	177173	326338	193860	179268	157702	133316	226782	142178
s	1455	1442	1237	3669	2539	2452	563	1309	2418	1515
% RSD	0.69	0.74	0.70	1.12	1.31	1.37	0.36	0.98	1.07	1.07

Bold entries were not used in calculations.

CONCLUSION

Transfer of the HPLC method from the 3.9 mm ID column to the 2 mm ID column was very successful. The only consideration necessary was appropriate reduction in sample concentration in order to accommodate the injection volume without exceeding the column capacity, and the use of the semi-micro flow cell. This method is immediately applicable to any of the analytical methods performed using the 3.9 mm ID μBondapak columns, assuming that instrument performance is comparable. Based on availability of other C_{18} packings in a 2 mm ID column, other methods should transfer as successfully (this probably represents at least 90% of the HPLC analyses performed in most HPLC laboratories). To provide some idea of the potential savings, this analytical and associate QC lab spends approximately $1000 per month on acetonitrile which is used almost exclusively for LC. This figure could easily be reduced to less than $200 per month, yielding an annual savings of over $9000. Corresponding savings in methanol and other HPLC solvents would also be realized. This does not consider the savings realized from reduced waste disposal.

The transfer to the 1 mm ID column was not as successful. Although the separation could have been restored with some method development, the primary objective was method transfer without additional development. The evaluation of the performance must be tempered with the very rigorous separation that this sample requires. Most methods do not demand this degree of resolution for this many peaks. It is also possible that the sample bandwidths required a flow cell smaller than the 8 ul volume used, particularly to improve resolution between peaks 2-7. When comparing peak shape of pentobarbital, mephobarbital, and

secobarbital (which are clearly resolved), the broader bandwidth is obvious and is definately not due to the UV cell volume.

It is probable that the difficulties encountered with the low injection volume (< 1 μl) were beyond the instruments capabilities. More accurate injections with low volumes would be achieved fixed volume internal sample loop. The injection devices are not common on typical analytical HPLC's.

If a narrow bore column is substituted for a standard bore column, at least some aspects of the method validation package will need to be addressed. It is conceivable that in a rigorously regulated environment, such as pharmaceutical manufacturing, complete re-validation of the method may be necessary.

Narrow bore column analyses are extremely sensitive to extra-column volumes (commonly referred to as "dead" volumes), particularly those in the injection system. These volumes contribute to sample dispersion that, coupled with the low flow rates, can ruin peak shape and resolution. Narrow bore columns require that the liquid chromatograph can deliver low flow rates, have small internal volumes and handle small injection volumes. To facilitate widespread method transfer, it is important that the associated hardware be capable of performing the analysis in an unmodified state since the expertise to execute in-house modifications may be lacking in some laboratories. Some LC's, particularly older ones, do not provide the necessary minimum performance characteristics needed to accommodate narrow bore columns. Although future savings incurred by reduced solvent costs would more than pay for instrument modification, in those cases where modification is not possible it is difficult to consider outright instrument replacement based on projected budgetary surpluses.

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EFFECTS OF PUSHING AGENTS ON THE SEPARATION AND DETECTION OF DEBRANCHED AMYLOPECTIN BY HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

K. S. WONG AND J. JANE*
Department of Food Science and Human Nutrition
Iowa State University
Ames, Iowa 50011

ABSTRACT

The effects of acetate and nitrate as pushing agents on the high-performance anion-exchange chromatography with pulsed amperometric detector (HPAEC-PAD) for the separation and detection of debranched amylopectin were evaluated. Our experimental data indicated that nitrate could differentiate branched from linear isomers, whereas acetate could not. Therefore, the nitrate system had better separating power than the acetate system provided that the chromatograms under both gradient systems were completed within 100 min. Nitrate was more compatible with the detector than acetate; thus, a higher detector response resulted. Under our experimental conditions, with a signal-to-noise ratio of two, the number of DP (degree of polymerization) at various concentrations that was resolved by the nitrate system (62 at 0.5 mg/mL, 65 at 1.0 mg/mL, and 66 at 1.5 mg/mL) was larger than those resolved by the acetate system (56 at 0.5 mg/mL, 58 at 1.0 mg/mL, and 58 at 1.5 mg/mL). The chromatograms obtained from the nitrate gradient system were more reproducible than those from the acetate gradient system in terms of the consistencies of retention time and the peak area

*To whom correspondence should be addressed at 1333 Food Sciences Building, Iowa State University, Ames, IA 50011.

of the chromatographic peak. To various concentrations of the component with the same DP, the detector responses were more consistent with nitrate as a pushing agent compared with acetate. Neither pushing agent, however, resulted in a quantitative response for homologous debranched amylopectin.

INTRODUCTION

Starches from different botanical sources have diverse functional properties that make them useful for applications in food, pharmaceutical, paper, plastic, and other industries. The functionality of starch is allied to its chemical structure. Therefore, knowledge of its structure will help in the understanding of the relationship between the physicochemical properties and the functional properties of starch. The chain-length distribution is an important parameter for characterizing the molecular structure of amylopectin. Although size exclusion chromatography with refractive index detector (SEC-RI) is one of the most frequently used techniques, it cannot separate each debranched chain of a specific chain-length. Furthermore, the refractive index detector often lacks sensitivity and selectivity, and it cannot be used with a gradient. In the early 1980's, Hughes and Johnson [1,2] successfully applied a triple-pulsed amperometric detection method for the detection of carbohydrates. Rocklin and Pohl [3] had combined the high-performance anion-exchange chromatography with pulsed amperometric detection for the determination of carbohydrates. Since then, there has been a significant increase in the application of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in carbohydrate research [4-9].

The affinities of anions to the strongly basic anion-exchange resin are based on their charges and radia of the hydrated ions and are increased in the order of OH^- < acetate < nitrate < sulfate [10]. Therefore, sulfate is the strongest pushing agent and hydroxide is the weakest pushing agent in the anion-exchange chromatography. By using an isocratic system, Rocklin *et al.* [3] have evaluated acetate, carbonate, nitrate, and sulfate as pushing agents for the determination of carbohydrates by HPAEC-PAD. They preferred acetate

as a pushing agent because acetate and hydroxide have similar affinity for the anion-exchange resin, and all other pushing agents reduced the column loading capacity. Since then, acetate has become the most commonly used pushing agent in the HPAEC-PAD for the separation of carbohydrate. Recently, Lu *et al.* [11] have studied the effect of acetate, nitrate, and sulfate as pushing agents in the isocratic system on the determination of malto-oligosaccharides by HPAEC-PAD. They concluded that nitrate had a greater ability to reduce the capacity factor for better resolution and thus nitrate should be a better pushing agent than acetate. All the studies, so far, on pushing agents were performed under the conditions of an isocratic system. For the study reported here, we evaluated the function of acetate and nitrate as pushing agents in a gradient system for quantitative and qualitative analysis of debranched amylopectin. Our results should provide additional information on exploring the HPAEC-PAD in starch analysis.

EXPERIMENTAL

Materials

Sodium hydroxide solution (5 N), sodium acetate, sodium nitrate, and glacial acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium azide was obtained from Eastman Organic Chemicals (Rochester, NY). Glucose (G1), maltose (G2), and corn starch were obtained from Sigma Chemical Co. (St. Louis, MO). Maltotriose hydrate (G3, 95%), maltotetraose (G4), maltohexaose (G6, Tech., 90%), and maltoheptaose hydrate (G7, 90%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Maltopentaose (G5) and iso-amylase (EC 3.2.1.68, crystal, from Pseudomonas amyloderamosa, 59,000 units/mg protein) were obtained from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). All chemicals and enzymes were used without further purification. The corn amylopectin was fractionated from corn starch by the method of Schoch [12] and purified three times by

recrystallization. The isolated amylopectin was washed with methanol and oven dried at 50°C for 12 hr. The tapioca amylopectin and wheat amylopectin were provided by Dr. J.-F Chen. The acetate buffer was prepared by mixing 16 mL of 0.1 N acetic acid with 1 mL of 0.1 N sodium acetate solution. The standard solution containing G1 to G7 was composed of glucose (31 μg/mL), maltose (42 μg/mL), maltotriose hydrate (23 μg/mL), maltotetraose (42 μg/mL), maltopentaose (39 μg/mL), maltohexaose (33 μg/mL), and maltoheptaose hydrate (48 μg/mL). Water (18 MΩ cm) used in all of the preparations was obtained from the Milli-Q Reagent Water System (Millipore, Bedford, MA).

HPAEC-PAD

The HPAEC was performed on a Dionex (Sunnyvale, CA) DX30 gradient pump module equipped with a Dionex pulsed amperometric detector. Sample injection was *via* a Dionex microinjector valve with a 200 μL sample loop. Sample solution was filtered through a 0.45 μm Magna nylon membrane (Fisher Scientific, Fair Lawn, NJ) before injection. A CarboPac PA1 anion-exchange column (250 x 4 mm) and a CarboPac PA1 guard column (25 x 3 mm) were used for sample separation. Two HPLC gradient systems were applied for the separation of debranched amylopectin. The acetate gradient system employed two eluents. Eluent 1 was 150 mM sodium hydroxide and eluent 2 was a mixture of 150 mM sodium hydroxide and 500 mM sodium acetate. The gradient was programmed as follows: 0-0.1 min, 75% eluent 1 and 25% eluent 2; 0.1-15 min, linear gradient to 45% eluent 2; 15-45 min, linear gradient to 60% eluent 2; 45-80 min, linear gradient to 70% eluent 2; 80-100 min, linear gradient to 80% eluent 2. The nitrate gradient system employed eluent 1 and eluent 3, which was a mixture of 150 mM sodium hydroxide and 500 mM sodium nitrate. The following gradient was used: 0-5 min, 94% eluent 1 and 6% eluent 3; 5-10 min, linear gradient to 8% eluent 3; 10-30 min, linear gradient to 13% eluent 3; 30-80 min, linear gradient to 20% eluent 3; 80-100 min, linear gradient to 25% eluent 3. The flow rate for both

gradient systems was 1.0 mL/min throughout the program. The column was allowed to equilibrate for 10 min with the initial condition of the gradient system before each run. All eluents were filtered through a 0.45 μm Supor-450 membrane (Fisher Scientific, Fair Lawn, NJ) and degassed by sparging with helium gas before use. The debranched amylopectin was detected by a pulsed amperometry using a gold electrode with the following repeating sequences of potentials and durations: E1 = 0.05 V (480 ms); E2 = 0.6 V (120 ms); E3 = -0.6 V (60 ms). E1 was for sample oxidation, E2 was to clean the electrode surface, and E3 was to reduce gold oxide back to gold. The sampling period was set to 200 ms, and the response time was set to 1 s.

Enzymatic Debranching of Amylopectin

Amylopectin was debranched by using iso-amylase according to the procedures described by Jane *et al.* [13] with some modifications. Briefly, amylopectin (50 mg) was suspended in 9 mL of water and heated in a boiling water bath with constant stirring for 15 min. The suspension was cooled down to 25°C, then 1 mL of acetate buffer (0.1 N) was added and followed with a 5 μL of iso-amylase. The reaction mixture was incubated for 48 h in a shaker water bath (Versa Bath S, model 236, Fisher Scientific, Fair Lawn, NJ) at 40°C with 100 strokes/min. The mixture was then adjusted to pH 6 with 5 N NaOH solution, followed by the addition of sodium azide solution (10%, 20 μL), and heated in a boiling water bath for 15 min to inactivate the enzyme.

RESULTS AND DISCUSSION

Separation of Debranched Amylopectin

Debranched corn amylopectin with concentrations of 0.5 mg/mL, 1.0 mg/mL, and 1.5 mg/mL were subjected to HPAEC-PAD analysis by the acetate and the nitrate gradient systems. The resulting chromatographic

profiles of debranched corn amylopectin from the acetate and the nitrate gradient systems are shown in Figure 1. Both gradient systems were designed so that the separations for the debranched amylopectin were completed within 100 min. The standards G1 to G7 were used to identify the chromatographic peaks with the DP (degree of polymerization) from 1 to 7. The assignment for the chromatographic peaks with DP higher than 7 was based on the generally

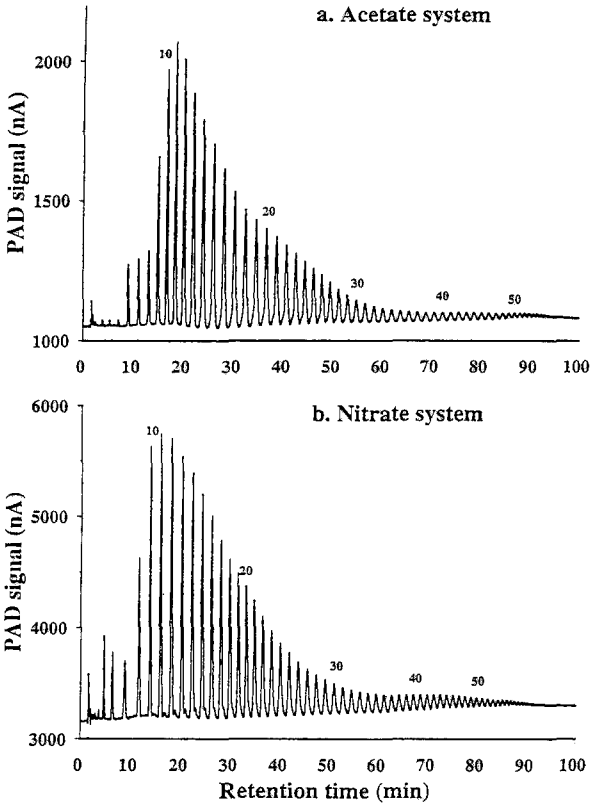

FIGURE 1. HPAEC-PAD chromatographic profiles of the enzymatic debranched corn amylopectin using (a) the acetate gradient system and (b) the nitrate gradient system. Peak numbers indicate the degree of polymerization.

accepted assumptions that (1) the retention time of the homologous series of saccharides increased as the DP increased, and (2) each successive peak represented a saccharide which was 1 DP longer than that of the previous peak. The detector response for the saccharides was much more sensitive under the nitrate system (Figure 1b) than the acetate system (Figure 1a). This observation suggested that the nitrate system was more compatible with the detection system (PAD). The number of DPs resolved by the acetate and nitrate systems is summarized in Table 1. With a signal-to-noise ratio of two or three, the number of DPs resolved by the nitrate system was always greater than that by the acetate system at all concentrations of the samples. The greater number of DPs detected in the nitrate system was attributed to the increase in the detection sensitivity of the system. Also, some minor peaks, which were suggested to be the isomers with maltosyl branches [14], were observed in the chromatograms using the nitrate system (Figure 1b) but not in those using the acetate system. For the acetate system, the isomers could be separated by increasing the resolution; however, the higher the resolution, the greater the disengagement of the two adjacent peaks, which meant a longer

TABLE 1. Degrees of Polymerization[a] Resolved by the Acetate and the Nitrate Gradient Systems

Sample concentrations mg/mL	Degree of polymerization			
	Acetate gradient system		Nitrate gradient system	
	S/N[b] = 3	S/N = 2	S/N = 3	S/N = 2
0.5	53	56	59	62
1.0	56	58	62	65
1.5	56	58	63	66

a. Average of triplicate analysis.
b. Signal-to-noise ratio.

separation period would be required. Thus, the nitrate system was more effective in differentiating the branched isomers, which were often found in the debranched amylopectin samples.

Reliability of the Systems

The reliability of the analytical system is an important issue especially when the characterization of amylopectin was based on the distribution of over a hundred saccharides, and standards for each saccharide are not always available. Therefore, the reliability of the acetate system and the nitrate system was investigated *via* the evaluation of the reproducibility and the accuracy of the acetate and the nitrate systems.

Reproducibility

The reproducibility of the chromatograms was judged by (1) the consistency of retention times (t_R) and (2) the consistency of detector responses determined by peak areas that were measured after repeated injections of the sample. Figure 2a is the plot of $\bar{t}_R$ *vs.* DP for both gradient systems. Each data point represented an average of retention time of the corresponding component resulted from the triplicate injections of the samples with concentration of 0.5 mg/ml, 1.0 mg/ml, and 1.5 mg/ml under the same chromatographic conditions. Figure 2b was the plot of the observed maximum shift (t_{Rmax} - t_{Rmin}) of the corresponding component *vs.* DP. The maximum shift of the chromatographic peaks ranged from 0.05 min to 3.72 min in the acetate system and from 0.02 min to 1.39 min in the nitrate system. The standard deviation plot of the $\bar{t}_R$ *vs.* DP for each system clearly (Figure 3) showed that the overlapping regions between peaks in the acetate system were significant. In contrast, the deviations of t_R were small in the nitrate system. For every sample analysis, the column was equilibrated with the initial chromatographic conditions for 10 min before each corresponding gradient

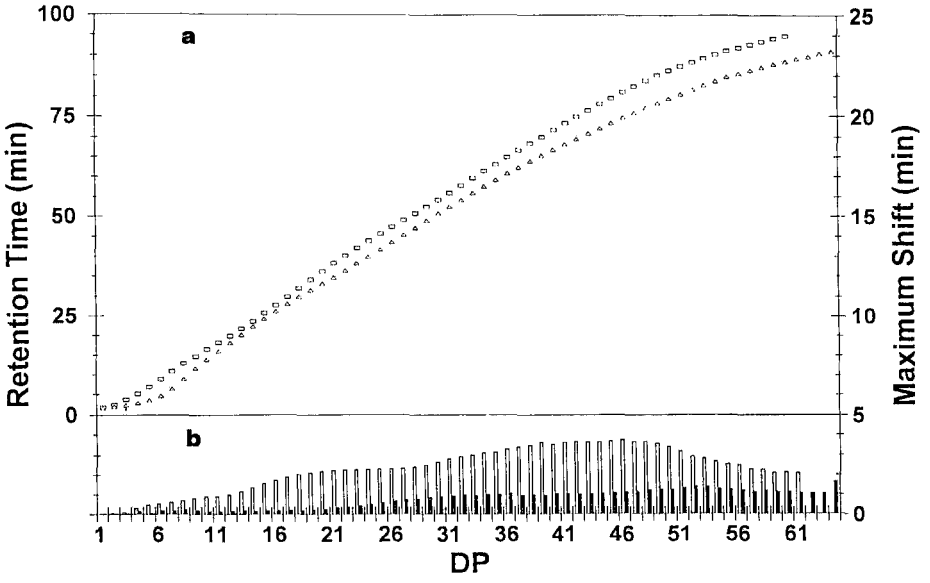

FIGURE 2. (a) Average retention time ($\bar{t}_R$) *vs.* DP of the corresponding component in the chromatograms of debranched corn amylopectin under the acetate gradient system (□) and the nitrate gradient system (△). (b) The observed maximum shift of each component in the acetate system (empty bar) and the nitrate system (shaded bar).

system was applied. The experimental results suggested that the acetate system might require a longer period of equilibration to condition the column. The initial and final concentrations of the acetate in the acetate gradient system were 125 mM and 400 mM, respectively, and those of the nitrate in the nitrate gradient system were 30 mM and 125 mM, respectively. The profound difference in the concentration of the pushing agent between the initial stage and the final stage (275 mM) of the acetate system also suggested that the column might need a longer time for equilibration.

The debranched tapioca and wheat amylopectin samples were also analyzed with the nitrate system. Their chromatographic profiles are shown in

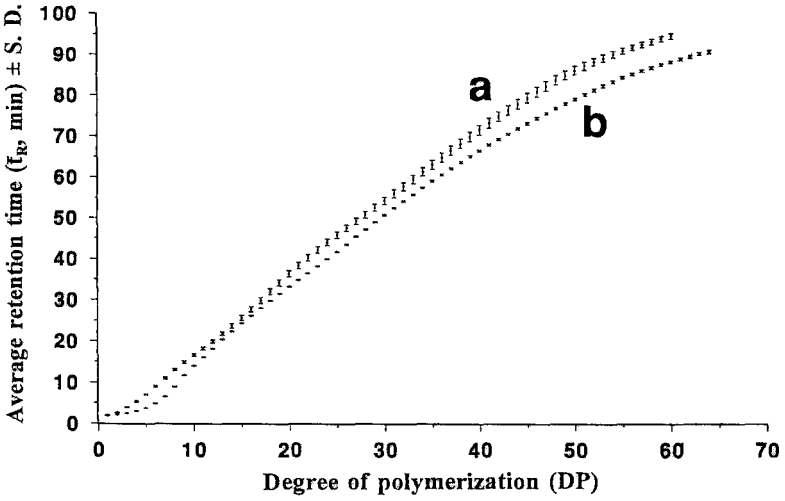

FIGURE 3. The plot of average retention time (from 9 analyses) and its standard deviation *vs.* DP of the corresponding component in the chromatograms of the debranched corn amylopectin with various concentrations under (a) the acetate system and (b) the nitrate system.

Figure 4. Figure 5 shows a plot of $\bar{t}_R$ with deviation *vs.* DP where $\bar{t}_R$ was an average of the retention time of the corresponding saccharide resulted from replicate injections of debranched corn, tapioca, and wheat amylopectin. As shown in Figure 5, the retention times of the saccharides with the same chain-length were very reproducible even though they were from different sources.

The reproducibility of the detector response was evaluated by the standard deviation of the peak areas, which resulted from the triplicate analyses of each sample. The peak area and its standard deviation for each component in the sample versus DP were plotted and are shown in Figure 6. Figure 6a is of the acetate system; Figure 6b is of the nitrate system. For the samples with concentration of 0.5 mg/mL (6ai), the standard deviations of the detector responses in the acetate system were comparable to those in the nitrate system (6bi), however, for the samples with a concentration of 1.0 mg/mL

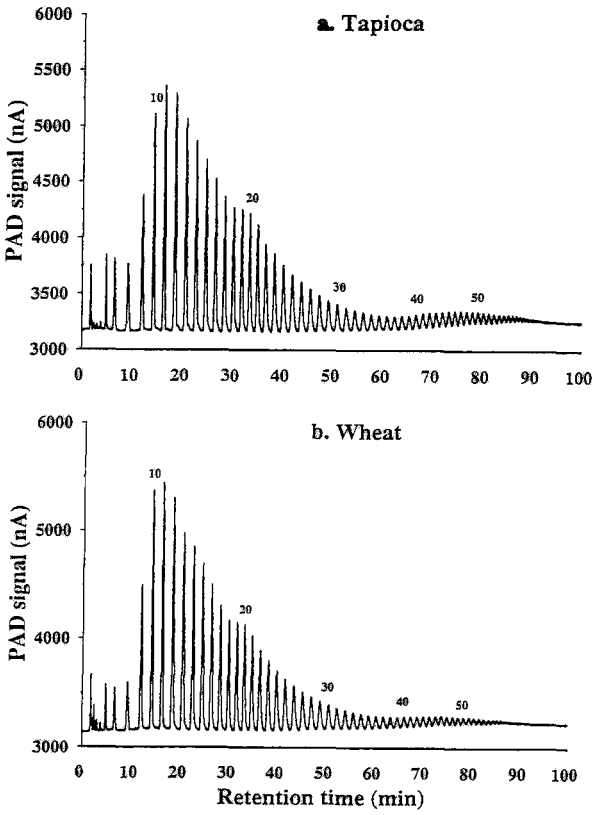

FIGURE 4. HPAEC-PAD chromatographic profiles of the enzymatic debranched (a) tapioca amylopectin and (b) wheat amylopectin under the nitrate system. Peak numbers indicate the degree of polymerization.

(6aii and 6bii) and 1.5 mg/mL (6aiii and 6biii), the deviations of the detector responses were relatively lower in the nitrate than in the acetate system.

Accuracy

To determine the accuracy of a measurement, knowledge of the true value from the sample is required. However, it is very difficult, if not

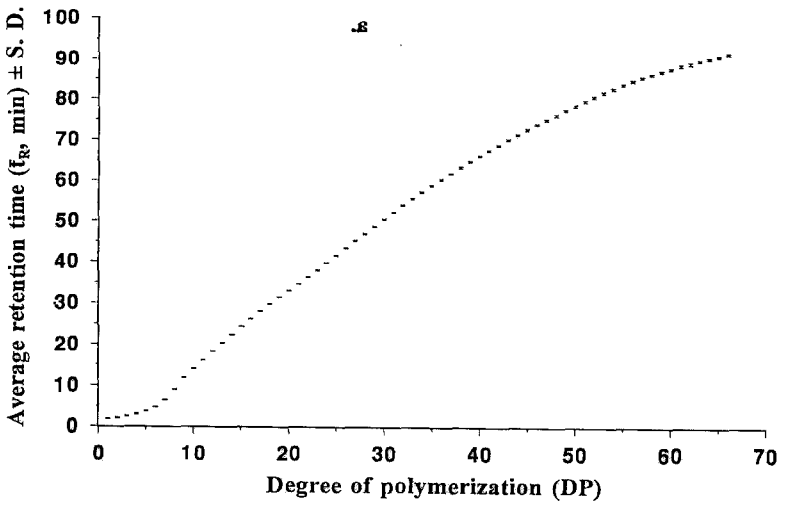

FIGURE 5. The plot of average retention time (from 6 analyses) and its standard deviation *vs.* DP of each corresponding component in the chromatograms of debranched amylopectin samples from corn starch, tapioca starch, and wheat starch.

impossible, to obtain standards for each of the saccharides in our samples. Therefore, the accuracy of the analysis of debranched amylopectin was determined by comparing the signal ratio of the corresponding component in the samples with different concentrations to the concentration ratios (i.e., true value) of the samples. The plots in Figure 7 are the signal ratios of the corresponding component of sample concentration ratios between 1.5 and 1 mg/mL, 1.0 and 0.5 mg/mL, and 1.5 and 0.5 mg/mL. With a concentration ratio of 1.5 (1.5 to 1 mg/mL), both systems exhibited a relatively consistent signal ratio with higher accuracy (7ai and 7bi). On the other hand, with a concentration ratio of 3 (1.5 to 0.5 mg/mL), both systems showed fluctuation in the response ratio with poor accuracy (7aiii and 7biii). These experimental results indicated that the appropriate sample size for the analysis would be 1 mg/mL to 1.5 ml/mL. Overall, the detector response was more consistent with nitrate as the pushing agent (Figure 7b) compared to that with acetate as

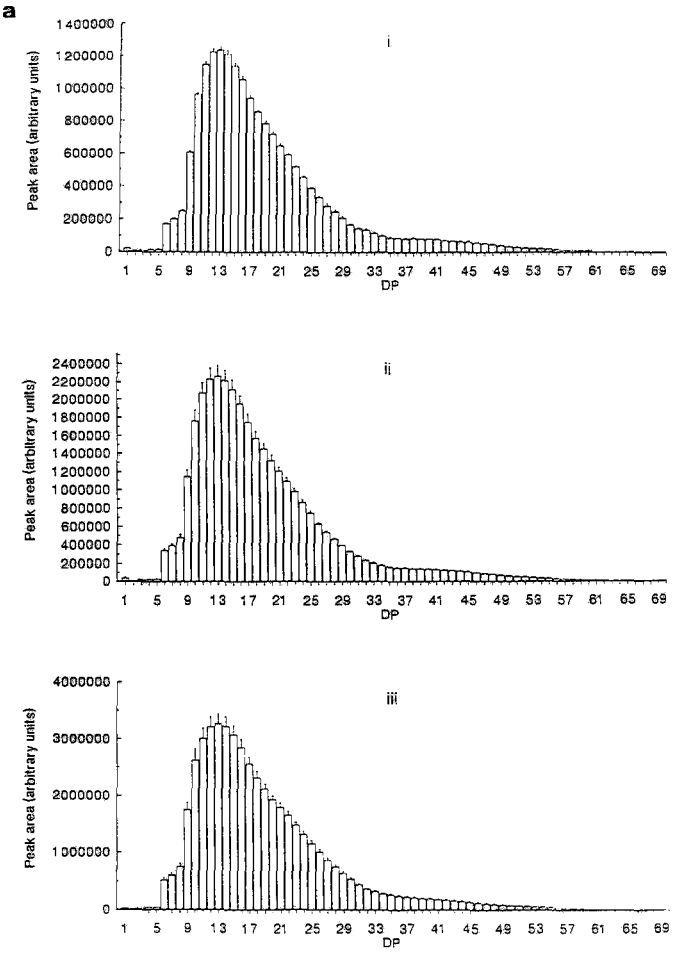

FIGURE 6. Peak areas and their standard deviations *vs.* DP in (a) the acetate system and (b) the nitrate system with sample concentrations of (i) 0.5 mg/mL, (ii) 1.0 mg/mL, and (iii) 1.5 mg/mL.

(continued)

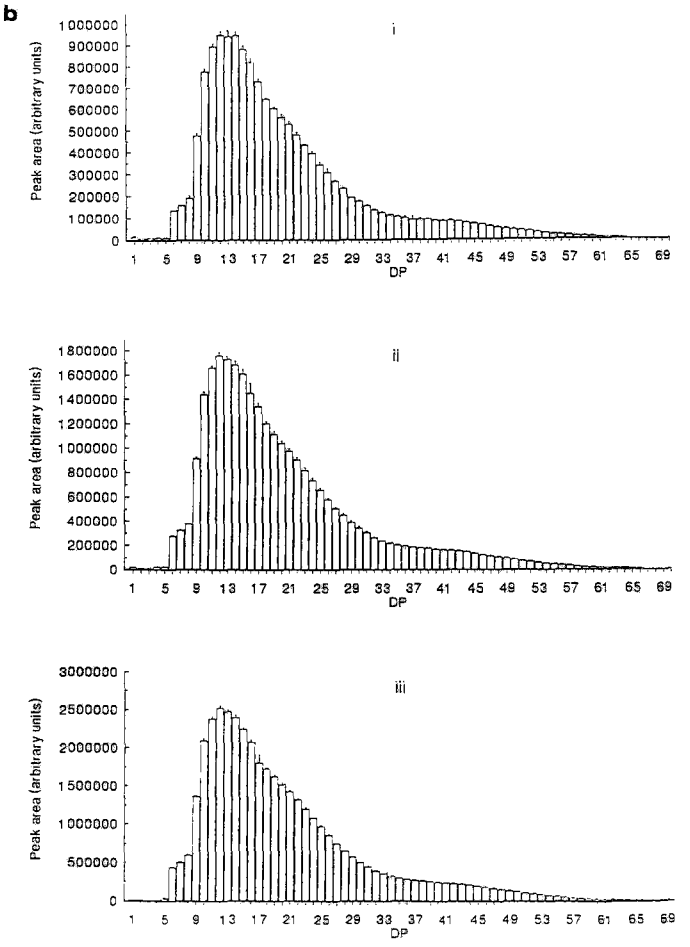

FIGURE 6 (continued).

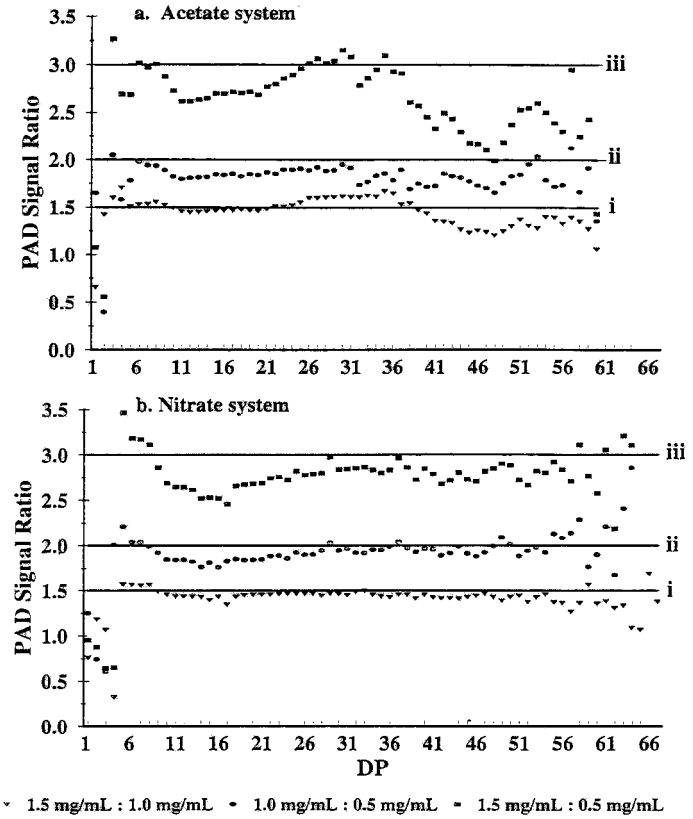

FIGURE 7. The signal ratios *vs.* DP of the corresponding components in the debranched corn amylopectin with sample concentrations of (i) 1.5 and 1 mg/mL, (ii) 1.0 and 0.5 mg/mL, and (iii) 1.5 and 0.5 mg/mL under (a) the acetate system and (b) the nitrate system. The solid lines represent the true values of the corresponding ratios.

the pushing agent (Figure 7a). This observation was supported by the mean signal ratios and the standard deviations (calculated from DP 5 to DP 56) for various concentration ratios of the sample (Table 2). In most occasions, the signal ratios obtained under the nitrate system were closer to the corresponding true values and the deviations were low.

TABLE 2. Statistic Data for the Signal Ratios from DP 5 to DP 56

Stats.[a]	Acetate System			Nitrate System		
True Value	1.5[b]	2[c]	3[d]	1.5[b]	2[c]	3[d]
Mean	1.46	1.83	2.67	1.45	1.93	2.80
Std. Dev.	0.12	0.08	0.29	0.05	0.09	0.17
Maximum	1.67	1.95	3.06	1.57	2.21	3.46
Minimum	1.20	1.65	2.10	1.34	1.76	2.46

a. Calculated from 52 data points.
b. Calculated from sample concentration ratio of 1.5 $mgmL^{-1}$/1.0 $mgmL^{-1}$
c. Calculated from sample concentration ratio of 1.0 $mgmL^{-1}$/0.5 $mgmL^{-1}$
d. Calculated from sample concentration ratio of 1.5 $mgmL^{-1}$/1.5 $mgmL^{-1}$

CONCLUSION

In comparison with the commonly used pushing agent, acetate, nitrate offered greater reproducibility and accuracy, and lower detection limit in the HPAEC-PAD; thus, it is a promising pushing agent for the quantitative and qualitative analysis of the debranched amylopectin. Our study further supported the view of Lu *et al.* [11] that nitrate was a better pushing agent than acetate for the separation of saccharides. However, neither pushing agent resulted in a quantitative response for homologous debranched amylopectin. In addition, the HPAEC-PAD can separate the saccharides but cannot provide the information of total carbohydrate for each saccharide. On the other hand, the size exclusion chromatography with refractive index detector (SEC-RI) can provide information of total carbohydrates; however, it cannot separate the saccharides as the HPAEC-PAD does. Therefore, an alternative detection technique for HPAEC is needed so that the information for both the fine chain-length distribution and the total carbohydrates for each saccharide in the debranched amylopectin sample can be obtained simultaneously.

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REFERENCES

1. Hughes, S., and Johnson, D. C. *Anal. Chim. Acta* **1981**, 132, 11.

2. Hughes, S., and Johnson, D. C. *J. Agric. Food Chem.* **1982**, 30, 712.

3. Rocklin, R. D., and Pohl, C. A. *J. Liq. Chromatogr.* **1983**, 6, 1577.

4. Koizumi, K., Kubota, Y., Tanimoto, T., and Okada, Y. *J. Chromatogr.* **1989**, 464, 365.

5. Martens, D. A., and Frankenberger, Jr., W. T. *Chromatographia* **1990**, 29, 7.

6. Koizumi, K., Fukuda, M., and Hizukuri, S. *J. Chromatogr.* **1991**, 585, 233.

7. Murugesan, G., Hizukuri, S., Fukuda, M., and Juliano, B. O. *Carbohydr. Res.* **1992**, 223, 235.

8. Suzuki, A., Kaneyama, M., Shibanuma, K., Takeda, Y., Abe, J., and Hizukuri, S. *Cereal Chem.* **1992**, 69, 309.

9. Murugesan, G., Shibanuma, K., and Hizukuri, S. *Carbohydr. Res.* **1993**, 242, 203.

10. Shpigun, O. A., and Zolotov, Y. A. *Ion Chromatography in Water Analysis* Ellis Horwood Ltd., England, **1988**, p. 41.

11. Lu, T.-J., Jane, J., and LaCourse, W. R. manuscript in preparation.

12. Schoch, T. J. *J. Am. Chem. Soc.* **1942**, 64, 2957.

13. Jane, J., and Chen, J. *Cereal Chem.* **1992**, 69, 60.

14. Ammeraal, R. N., Delgado, G. A., Tenbarge, F. L., and Friedman, R. B. *Carbohydr. Res.* **1991**, 215, 179.

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CHIRAL RECOGNITION OF ALKYL 2-ARYLOXYPROPIONATES BY HPLC

O. AZZOLINA* AND S. COLLINA
Dipartimento di Chimica Farmaceutica
Universita' degli Studi di Pavia
Via Taramelli 12-27100
Pavia, Italy

ABSTRACT

Chiral resolution of a series of antiphlogistic methyl and ethyl 2-aryloxypropionates on R-DNBPG and S-DNBL analytical columns was attempted. Most of the resolved enantiomers were eluted in a very short time on both CSPs and the α and K' values of the chromatographic separations performed by R-DNBPG phase were generally better than those using S-DNBL.
The elution order of the compounds was determined: the S isomer of all esters was eluted last from the R-DNBPG and viceversa from the S-DNBL column.
The role of the substituents on the chiral resolution was also elucidated. It was hypothesized that the chiral interactions between the solutes and the CSPs were more influenced by the electronic features than by their steric hindrance.
Finally, the chiral recognition mechanism which permitted resolution of the enantiomers was individuated.

INTRODUCTION

Our previous research (1,4) focused on the synthesis, chiral resolution of racemic mixtures and configurational assignment of enantiomers of a series of 2-aryloxypropionyl derivatives, which are biologically active as antinflammatory,

analgesic agents and as plant growth regulators (5-7). Some of these compounds can also be structurally related to chiral analogs of chlofibrate whose pharmacological activity has been already investigated (8). Furthermore, it was pointed out that the interaction of similar drugs with biological receptors is stereoselective (9-12).

To obtain the chiral resolution of the racemic compounds, chromatography on chiral stationary phases had been also performed. The enantiomers of some 2-aryloxypropionic N-(1-naphthyl)methyl amides had been resolved by HPLC on Pirkle type phases, *i.e.* CSPs of R - N - (3,5 - dinitrobenzoyl)- phenylglycine (R-DNBPG) and S -N-(3,5-dinitrobenzoyl)-leucine (S-DNBL), covalently bound to SiO_2 (3). The resolution of a more numerous series of 2-aryloxypropionic acids and their methyl and ethyl esters (partially corresponding to the amidic derivatives) had also been accomplished on a column of cellulose tris - (3,5-dimethylphenylcarbamate) Chiralcel OD (4).

Chromatographic separation of 2-aryloxypropionyl esters by Chiralcel OD has been shown to be better than separation of the amides using R-DNBPG and S-DNBL columns. As the Chiralcel OD phase is very expensive, we wanted to verify whether chiral resolution of the esters could also be accomplished on Pirkle's CSPs. If so, this could permit the enantiomeric resolution of the racemates on a preparative scale with the added advantage that the esters can be hydrolyzed under milder conditions than those needed for the amides to furnish the acids without racemization. Then we experimented the resolution of the above-mentioned methyl and ethyl esters on Pirkle's CSPs.

The analyzed compounds, apart from **26**, bear one or two substituents in the 2, 3 and 4 position of the aryloxylic portion directly linked to the chiral center. These substituents were selected to introduce atoms or groups of various dimensions and with electron-donating or electron-withdrawing properties, in order to verify whether their size, chemical features or position on the phenyl ring influence the chromatographic discrimination process. In this context, the unsubstituted compound **26** was also analyzed.

By critically evaluating the experimental results and studying molecular models of the solute-CSP interactions, we also investigated the relationship between the configuration and elution order of the enantiomers to determine a rationale for the chiral recognition mechanism.

MATERIALS AND METHODS

Materials

All racemates and optical antipodes of methyl and ethyl esters were prepared as previously reported (4).

Hexane and 2-propanol (HPLC grade) were purchased from Farmitalia Carlo Erba. All other chemicals and solvents were of analytical-reagent grade.

Methods

Melting points were determined with a Buchi apparatus and are uncorrected.

The elemental analyses were carried out with a Perkin-Elmer 240 C,H,N analyzer and were within $\pm$ 0.4 % of the theoretical values.

$[\alpha]_D$ measurements were made with a Perkin-Elmer 141 M photoelectric polarimeter at 22° C.

The chromatographic analyses were performed using a Gilson model 303 isocratic pump, equipped with Holochrome HM variable wavelength detector, and Reodyne model 7125 injection valve. Experimental data were analyzed with the Gilson 715 HPLC software.Analytical columns (250 x 4.6 mm, 5 µm) of covalently bound R-DNBPG and S-DNBL phases were used. The analysis of any compound was performed on both the racemate and the R enantiomer to determine the elution order of the antipodes.

Several mixtures of hexane or iso-octane, as the main apolar component, and lower amounts of ethanol or 2-propanol were tested as eluting systems; 0.25 % 2-propanol in hexane was found to be the most suitable mobile phase, at a flow rate of 1 ml/min. UV detection at 260 nm was used.

Tables 1 and 2 report the capacity factors K'_1 and K'_2 of the first and second eluted enantiomer, the selectivity factor α, the resolution factor R_s and the elution order of the solutes.

Methyl esters of methyl-, chloro- and nitro-substituted compounds had been already examined,on an R-DNBPG column by Dernoncour and Azerad (13) and their results are, in general, consistent with our findings.

TABLE 1 (part I)

Chromatographic Resolution of Methyl 2-Aryloxypropionates on R-DNBPG (upper values) and S-DNBL (lower values) CSPs.

Comp	Aryl	K'_1	K'_2	α	R_s	Last eluted
1 a	2-$CH_3C_6H_4$	0.88 0.94	1.14 1.03	1.29 1.10	1.03 0.84	S R
2 a	3-$CH_3C_6H_4$	1.41 1.36	1.58 1.47	1.12 1.08	1.06 0.78	S R
3 a	4-$CH_3C_6H_4$	1.55 1.50	1.71 1.60	1.10 1.07	1.19 0.60	S R
4 a	2-$C_2H_5C_6H_4$	0.80 0.80	0.90 0.89	1.12 1.12	0.95 0.95	S R
5 a	3-$C_2H_5C_6H_4$	1.19 1.26	1.31 1.37	1.10 1.09	0.94 0.88	S R
6 a	4-$C_2H_5C_6H_4$	1.32 1.25	1.47 1.34	1.11 1.07	1.12 0.68	S R
7 a	2,3-$(CH_3)_2C_6H_3$	1.11 1.09	1.30 1.24	1.17 1.13	1.56 1.06	S R
8 a	2,4-$(CH_3)_2C_6H_3$	1.06 0.97	1.25 1.10	1.18 1.13	1.23 0.99	S R
9 a	2,5-$(CH_3)_2C_6H_3$	1.00 0.95	1.20 1.06	1.20 1.12	1.69 0.88	S R
10 a	2,6-$(CH_3)_2C_6H_3$	0.97 1.02		1.00 1.00	— —	— —
11 a	3,4-$(CH_3)_2C_6H_3$	1.73 1.90	1.99 2.07	1.15 1.09	1.01 0.78	S R
12 a	2-ClC_6H_4	1.57 1.41	1.68 1.52	1.07 1.08	0.86 0.97	S R
13 a	3-ClC_6H_4	1.22 1.09	1.30 1.14	1.07 1.05	0.75 0.64	S R

TABLE 1 (part II)

Chromatographic Resolution of Methyl 2-Aryloxypropionates on R-DNBPG (upper values) and S-DNBL (lower values) CSPs.

Comp	Aryl	K'_1	K'_2	α	R_s	Last eluted
14 a	4-ClC_6H_4	1.31	1.39	1.06	0.69	S
		1.19		1.00	—	—
15 a	2-$NO_2C_6H_4$	8.19	8.34	1.02	0.52	S
		9.11	9.32	1.02	0.53	R
16 a	3-$NO_2C_6H_4$	4.67	4.81	1.03	0.70	S
		4.52		1.00	—	—
17 a	4-$NO_2C_6H_4$	7.43	7.58	1.02	0.50	S
		6.27		1.00	—	—
18 a	3-$(CH_3CO)C_6H_4$	12.23	12.95	1.06	0.90	S
		10.00	10.26	1.03	0.44	R
19 a	3-$(C_6H_5CO)C_6H_4$	13.24	13.79	1.04	0.81	S
		9.38	9.65	1.03	0.72	R
20 a	2-CH_3-3-(4-$ClC_6H_4CO)C_6H_3$	7.88	8.63	1.10	1.12	S
		5.73	6.27	1.09	1.16	R
21 a	4-$(C_6H_5)C_6H_4$	2.88	3.15	1.09	1.14	S
		2.29	2.40	1.05	0.80	R
22 a	4-(2,4-$F_2C_6H_3)C_6H_4$	2.54	2.71	1.08	1.03	S
		1.98	2.09	1.06	0.86	R
23 a	4-$(C_6H_5CH_2)C_6H_4$	2.35	2.50	1.06	0.79	S
		1.85	1.96	1.06	0.68	R
24 a	4-$(C_6H_5CO)C_6H_4$	17.69	18.86	1.07	1.05	S
		14.46	14.87	1.03	0.70	R
25 a	4-$(C_6H_5O)C_6H_4$	2.50	2.66	1.06	0.84	S
		2.02	2.15	1.06	0.87	R
26 a	C_6H_5	1.50	1.59	1.06	0.79	S
		1.55	1.62	1.05	0.35	R

TABLE 2 (part I)

Chromatographic Resolution of Ethyl 2-Aryloxypropionates on R-DNBPG (upper values) and S-DNBL (lower values) CSPs.

Comp	Aryl	K'_1	K'_2	α	R_s	Last eluted
1 b	2-$CH_3C_6H_4$	2.44 1.99	2.75 2.11	1.13 1.06	1.37 0.51	S R
2 b	3-$CH_3C_6H_4$	2.19 3.06	2.42 3.13	1.10 1.02	1.00 0.21	S R
3 b	4-$CH_3C_6H_4$	1.23 1.17	1.36 1.22	1.10 1.04	0.99 0.54	S R
4 b	2-$C_2H_5C_6H_4$	0.47 1.90	0.55 2.09	1.16 1.10	1.00 0.97	S R
5 b	3-$C_2H_5C_6H_4$	0.76 2.16	0.85 2.27	1.11 1.05	1.12 0.66	S R
6 b	4-$C_2H_5C_6H_4$	2.73 3.67	2.92 3.81	1.07 1.04	0.89 0.53	S R
7 b	2,3-$(CH_3)_2C_6H_3$	1.09 1.07	1.29 1.17	1.18 1.09	1.74 0.81	S R
8 b	2,4-$(CH_3)_2C_6H_3$	0.98 1.13	1.15 1.24	1.17 1.10	1.20 0.79	S R
9 b	2,5-$(CH_3)_2C_6H_3$	2.01 2.58	2.33 2.68	1.16 1.04	1.79 0.38	S R
10 b	2,6-$(CH_3)_2C_6H_3$	0.65 1.14	0.68	1.05 1.00	0.59 —	S —
11 b	3,4-$(CH_3)_2C_6H_3$	1.43 0.79	1.65 0.84	1.15 1.06	1.71 0.56	S R
12 b	2-ClC_6H_4	1.57 1.60	1.68 1.70	1.07 1.07	0.86 0.77	S R
13 b	3-ClC_6H_4	1.11 1.07		1.00 1.00	— —	— —

TABLE 2 (part II)

Chromatographic Resolution of Ethyl 2-Aryloxypropionates on R-DNBPG (upper values) and S-DNBL (lower values) CSPs.

Comp	Aryl	K'1	K'2	α	Rs	Last eluted
14 b	4-ClC_6H_4	1.04		1.00	—	—
		2.65		1.00	—	—
15 b	2-$NO_2C_6H_4$	8.93		1.00	—	—
		8.54		1.00	—	—
16 b	3-$NO_2C_6H_4$	3.89		1.00	—	—
		2.98		1.00	—	—
17 b	4-$NO_2C_6H_4$	5.43		1.00	—	—
		5.10		1.00	—	—
18 b	3-(CH_3CO)C_6H_4	26.16	27.62	1.06	0.90	S
		8.43		1.00	—	—
19 b	3-(C_6H_5CO)C_6H_4	10.84	11.30	1.04	0.91	S
		8.30	8.46	1.02	0.57	R
20 b	2-CH_3-3-(4-ClC_6H_4CO)C_6H_3	6.15	6.72	1.09	1.71	S
		4.53	4.84	1.07	1.35	R
21 b	4-(C_6H_5)C_6H_4	3.15	3.46	1.10	1.04	S
		2.96		1.00	—	—
22 b	4-(2,4-$F_2C_6H_3$)C_6H_4	1.80	1.93	1.07	1.01	S
		1.53		1.00	—	—
23b	4-($C_6H_5CH_2$)C_6H_4	2.68	2.88	1.07	0.86	S
		2.53	2.60	1.03	0.34	R
24 b	4-(C_6H_5CO)C_6H_4	18.32	18.93	1.03	0.81	S
		12.58	12.77	1.02	0.38	R
25 b	4-(C_6H_5O)C_6H_4	2.60	2.80	1.08	1.03	S
		2.36	2.42	1.03	0.48	R
26 b	C_6H_5	1.23	1.31	1.07	0.83	S
		1.35		1.00	—	—

RESULTS AND DISCUSSION

Chromatographic separation of the racemic compounds and R enriched samples (arising from the synthesis of the optically active compounds) was performed to determine the correct elution order of the enantiomers.

As expected , the chromatographic chiral resolution of ethyl and methyl esters on both R-DNBPG and S-DNBL phases was very similar. Nearly all the esters appeared to be more suitable derivatives than the corresponding amides (3) for the chiral interaction with CSPs. This is because in general they show comparable α and R_s values, but shorter elution times. K' values of both ethyl and methyl esters are much lower than those of the amides, even if the eluting system contains much less 2-propanol (0.25 % *vs* 5 or 10 %), normally used for avoiding excessively strong interactions of solutes with stationary phases by means of hydrogen bonding. Apart from the sites involved in the mechanism of chiral discrimination by hydrogen bonding, on the CSP there are other sites which can give this type of interaction; the amount of 2-propanol must thus be enough to saturate these sites.

Methyl and ethyl esters showed better values of α and R_S parameters on R-DNBPG than on S-DNBL phase, in contrast to the amides.

On comparison of the elution order of the esters and amides it appeared that, while the S enantiomer of almost all amides was eluted last on both the CSPs, the S enantiomer of all methyl and ethyl esters was more retained than the R enantiomer on R-DNBPG and vice versa on S-DNBL phase.

We had already observed that the enantiomers of the amides, bearing a second carbonyl group on the aryloxylic portion (acetyl or benzoyl derivatives), were eluted with longer retention times and were less resolved than the others. Higher K' values can be also observed for the corresponding esters **18a**-**20a**, **24a** and **18b**-**20b**, **24b**. These results could signify that these compounds strongly interact with the stationary phases by means of the acetyl or benzoyl moieties that can give rise to hydrogen bonds or dipole-dipole bonding which do not involve the chirality of the interaction between the molecules and the CSPs.

The influence that the substituents of the aryloxylic portion have on the extent of the chiral resolution was pointed out. We observed that the presence of electron-donating methyl and ethyl groups produced an increase in α and R_s values. This is in agreement with the assumption that the strength of the π–π interaction of the solute with the π acidic moiety of CSP (dinitrobenzoyl group) is increased because of the π basic property of the methyl or ethyl substituted aryl group.

In contrast, compounds bearing electron-donating groups with greater steric hindrance (*e. g.* phenyl, benzyl, phenoxyl), as well as the unsubstituted derivative and derivatives with electron-withdrawing substituents were less resolved chromatographically.

The poor or complete lack of resolution of the compounds with electron-withdrawing substituents indicate that the π–π interaction between their aryloxylic portion and the dinitrobenzoyl group of CSP is unlikely because the π electrons of the substituted phenyl ring are less available. The higher K' values of the nitro derivatives show the presence of other interactions, which are not determinant for chiral separation.

It was also observed that of the compounds with electron-donating groups, the esters bearing a second aromatic ring (phenyl, benzyl, phenoxyl), showed higher K' values with respect to methyl, dimethyl or ethyl derivatives. This could signify a stronger interaction with the stationary phase because of the higher stability of the solute-CSP complex when a second aromatic ring was present. In fact, another π–π interaction, which does not involve the chirality of the molecule and delays the elution of the solutes through the column, can take place.

Chiral Discrimination Mechanism

Apart from the analysis of the experimental results, we also investigated interactions of the esters with the chiral phases using molecular models, which enabled us to recognize more probable interaction sites of both the CSPs and solutes.

We assumed (Figg. 1 and 2) that in the conformation by which the CSPs moiety interacts with the solutes, the dinitrobenzoyl carbonyl oxygen is near the methyne hydrogen, presumably for reasons of carbinyl hydrogen bonding (as already proposed by Pirkle *et al.* (14)). Further hydrogen bonding is also possible between the carbonyl next to the chiral center and the aminoacidic NH group.

Figures 1 and 2 show that the possible interactions for the more retained enantiomer (S for R-DNBPG and R for S-DNBL) are:

a) dipole-dipole stacking between the carbonyl of 3,5-dinitrobenzoyl group of the stationary phase and the carbonyl of the propionyl group of the solutes;

b) π–π interactions of the dinitrobenzoyl group of CSPs and the aryloxyl portion of the solutes;

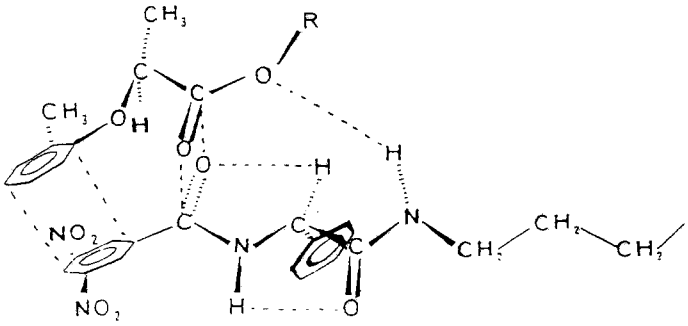

FIGURE 1. Interactions of enantiomer S-1 with R-DNBPG phase

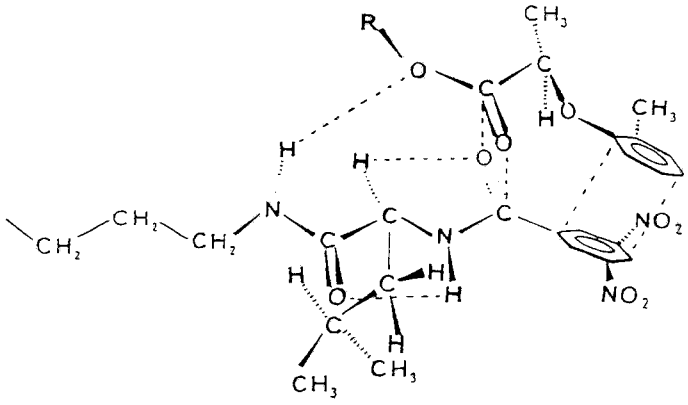

FIGURE 2. Interactions of enantiomer R-1 with S-DNBL phase

c) hydrogen bonding between the carbamic NH group of CSP and the oxygen of the ester group of the solutes.

All these interactions are possible depending on the configuration of the solutes. They can thus give rise to chiral discrimination because the methyl group, directly attached to the asymmetric carbon atom, prevents optimum alignment of the solute and CSP for less retained enantiomers whose diastereomeric complexes with CSPs have lower stability.

This discrimination mechanism agrees with experimental data.

For the unresolved esters, other interactions (also those already mentioned for the nitro-derivatives), can obviously prevent the chiral interaction.

CONCLUSIONS

We experimented chiral resolution of methyl and ethyl esters of twenty-six 2-aryloxypropionic acids and, for numerous compounds, this was accomplished in a very short time. Compounds bearing a carbonyl group at the aryloxylic moiety showed highest K' values.

The degree of chiral resolution did not appear to depend on the steric hindrance of the substituents, but rather on their electronic features. Furthermore, the electron-donating properties of methyl and ethyl groups improved the resolution, as shown by the comparison of the α and R_s values of the methyl and ethyl substituted compounds, those of the unsubstituted **26** and of the compounds with electron-withdrawing substituents.

Critical analysis of the experimental results and the study of the solute-CSP interactions, by means of molecular models, allowed us to establish the chiral recognition mechanisms which lead to the enantiomeric separation of the examined compounds.

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REFERENCES

1. Azzolina O., Vercesi D., Ghislandi V., *Il Farmaco Ed. Sc.*, **40**, 145; 1985.

2. Azzolina O., Vercesi D., Ghislandi V., *Il Farmaco Ed. Sc.*, **43**, 469; 1988.

3. Azzolina O., Ghislandi V., *Il Farmaco*, **48**, 713 ; 1993.

4. Azzolina O., Collina S., Ghislandi V., *Il Farmaco*, **48**, 1401 ; 1993.

5. Brit. Pat. 916,242 (Jan. 23, 1963, Appl.Jan. 7 and Sept. 16, 1959) : Boots Pure Drug Co., Ltd. (Designated inventors : Lewis H R., Housley J.R., Richards H.C., Nicholson J.S., Baker M.W., Adams S.S.).

6. Marzo A., Quadro G., Bruno G., Treffner E., Reali F. L., Borrelli G., Stradi R., *Arzneim.-Forsch.*, **33**, 198; 1983.

7. Worthing C.R., **The Pesticide Manual**, BCPC Publications, London, 6th ed., 329; 1979.

8. Witiak D.T., Ho C.-L.T., Hackney R.E., Connor W.E., *J. Med. Chem.*, **11**, 1086; 1968.

9. Bettoni G., Bryant S.H., Conte Camerino D., Feller D.R., Grasso G., Kamanna V.S., Loiodice F., Mambrini M., Newmann H.A.I., Romstedt K.J., Tortorella V., Witiak D.T., *Actual Chim. Ther.*, **15**, 125; 1988.

10. Feller D.R., Kamanna V.S., Newmann H.A.I., Romstedt K.J., Witiak D.T., Bettoni G., Bryant S.H., Conte Camerino D., Loiodice F., Tortorella V. , *J. Med. Chem.*, **30**, 1265; 1987.

11. Bettoni G., Loiodice F., Tortorella V., Conte Camerino D., Mambrini M., Ferrannini E., Bryant S.H., *J. Med. Chem.*, **30**, 1267; 1987.

12. Lien E.J., Rodrigues de Miranda J.F., Ariens *J., Mol. Pharmacol.*, **12**, 598; 1976.

13.Dernoncour R., Azerad R., *J. Chromatography* ,**410**, 355; 1987.

14. Pirkle W. H., House D. W., Finn J. M., *J. Chromatography*, **192**, 143; 1980.

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A RAPID DETECTION AND IDENTIFICATION OF HEMORPHINS RELEASED FROM BOVINE HEMOGLOBIN ENZYMATIC HYDROLYSIS BY USE OF HPLC COUPLED WITH PHOTODIODE ARRAY DETECTOR

Q. ZHAO[1], F. SANNIER[1], G. RICART[2], AND J. M. PIOT[1]*

[1]*Laboratoire de Génie Protéique*
Pôle des Sciences et Technologies
Université de La Rochelle
Av. Marillac, 17042 - La Rochelle Cédex 1, France
[2]*Laboratoire de Spectroscopie de Masse*
Lille I, 59655 - Villeneuve d'Ascq Cédex, France

ABSTRACT

Identification of hemorphins issued from a complex hemoglobin enzymatic hydrolysate was carried out by UV-spectra comparison. Two hemorphins,VV-hemorphin-7 and LVV-hemorphin-7, were detected in a single step by the use of HPLC coupled with photodiode array detector. This technique greatly simplified the the multistage identification and purification strategy. This method could also be efficiently applied to the identification of peptides containing aromatic amino acids.

INTRODUCTION

High performance liquid chromatographic technique greatly enhanced the separation and quantification abilities in the bioactive peptides studies area [1 - 3]. Therefore, the measure of retention or mobility derived from this technique typically yield insufficient information in order to verify or quantify the presence of expected peptides

Three dimensional ancillary spectroscopic technique have made great contribution to the effectiveness of chromatographic system in a variety of application areas [4]. In recent years, one of them, HPLC combined with photodiode array detector technology, extended many of advances of ancillary spectroscopy in liquid chromatography [5-10]. The parallel detection nature of the

reverse optics diode array detection system provides the potential for absolute wavelength accuracy. This means that the UV spectra of even compounds with no prominent peaks in the UV spectra could be compared, and true differences resulting from the sample under study have been obtained [11 -12]. This technique has been successfully applied in peak purity verification and determination of aromatic amino acid content of protein and peptides [4].

We have previously reported isolation of two opioid peptides, LVV-hemorphin-7 and VV-hemorphin-7 from a very complex bovine hemoglobin peptic hydrolysate [13]. These isolated peptides or those obtained either from the bovine or human beta- chain of hemoglobin [14] contained important amounts of aromatic amino acids. This conferred peculiar spectra differing from that of other peptides. These properties allowed us to set up, using UV spectra comparison, a chromatographic method for identification of hemorphins from this peptic mixture. The aim of our study was to simplify the purification procedure of such expected bioactive peptides.

EXPERIMENTAL

MATERIALS AND CHEMICALS

All common chemicals and solvents were of analytical grade from commercial sources. Rabbit lung angiotensin converting enzyme (ACE) was purchased from Sigma Chemicals. Hemorphin-7 was synthesized by C.Guillon, Laboratoire de Technologie Enzymatique, University of Compiègne Compiègne, France

Hydrolysate, fraction F VII, LVV-hemorphin-7 and VV-hemorphin-7 preparation

Bovine hemoglobin hydrolysate was obtained at pilot- plant scale by peptic proteolysis in an ultrafiltration reactor as previously described [15]. Active fraction FVII was prepared by gel permeation HPLC using TSK G2000 SWG column (19mm i.d. X 600 mm) and analyzed by reversed phase (RP) HPLC in order to obtain LVV-hemorphin-7 and VV-hemorphin-7 as described previously [13].

LVV-hemorphin-5 and VV-hemorphin-5 preparation

1 mg of either VV-hemorphin-7 or LVV-hemorphin-7 were dissolved in 1 ml 0.05 M Tris - HCl buffer pH 7.4 and incubated at 37 °C with angiotensin converting enzyme (ACE) (7.5 mU) during 7 h. according to LANTZ [16]. The reaction mixture was resolved on a Nova-Pak C-18 column (3.9 mm i. d. X 150 mm). LVV-hemorphin-5 and VV-hemorphin-5 were identified by mass spectrometry.

HPLC system

The liquid chromatographic system consisted of Waters 600 automated gradient controller-pump module, Waters Wisp 717 automatic sampling device and Waters 996 photodiode array detector. Spectral and chromatographic data were

stored on NEC image 466 hard disc using NEC image 466 computer. Millennium software was used to plot, acquire and treat chromatographic data.

METHODS

Mobile phase for Delta Pak C-18 column (19 mm i.d.X 300 mm)

The mobile phase comprised 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 12 ml/min. Samples were dissolved in buffer A, filtered through 0.20 µm filters and injected. The gradient applied was 0-40% B in 80 min.

Mobile phase for Nova-Pak C-18 column (3.9 mm i. d. X 150 mm)

The mobile phase comprised: 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. A linear gradient (15-30% B in 15 minute) was applied.

Procedure

Hemorphin-7, VV-hemorphin-7 and LVV-hemorphin-7 were chromatographed at room temperature on the Delta Pak C-18 and Nova Pak C-18 respectively with conditions described above. Total hydrolysate and fraction FVII were also injected on Delta Pak C-18 column under the same conditions. On-line instantaneous UV absorbance spectral scan were performed between 190 nm and 350 nm with a rate of one spectrum/second. Then the results of chromatographic analyses were completed by using Millennium software. Spectrum matching results (comparison spectra of the peaks in the chromatographic profile with library spectra of the hemorphins.) were reported by Waters Millenium system as:

Match angle - A measure of the difference in spectral shapes between an unknown spectrum and a library spectrum. Match angle can range from 0 to 90 degrees. Lower values indicate that spectra are similar. Larger values indicate greater degrees of spectral difference.

Match threshold - Sensibility of the measurement. It can range from 0 to 180 degrees. Larger values indicates a lower sensibility of the measurement. In general, if the match angle is greater than the match threshold, it indicates that two spectra are different. If the match angle is less than the match threshold, it does not indicate that the two spectra are different.

Amino acid analysis

Amino acids were analyzed using a Waters Picotag Work Station. Peptide hydrolysis was achieved with constant-boiling HCl containing 1% phenol, for 24 h. at 100 °C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids on a Waters RP-Picotag column(150 mm x 3.9 mm i.d.) were performed according to Bidlingmeyer et al. [17]. The detection wavelength was 254 nm and the flow rate 1 ml/min.

Mass spectrometry analysis

Mass spectra, generated from Fast Atom Bombardment (FAB) mass spectrometry of the peptides, were recorded on a four sector "Concept II" tandem mass spectrometer (Kratos, Manchester, UK). Ions were produced in a standard FAB source by bombarding the sample with xenon atoms having a kinetic energy of 8 KeV and the instrument was operated at an accelerating voltage of 8 KeV. The peptide was dissolved in water (1 μg/μl) and 1μl of the solution was loaded on the stainless steel tip with thioglycerol as matrix. The mass range was scanned at 10 s/decade with a mass resolution of 3000. Caesium iodide was the standard for mass calibration.

RESULTS AND DISCUSSION

COMPARISON OF HEMORPHINS SPECTRA

Hemorphin-7 (50 μg), VV-hemorphin-7 (50 μg), LVV-hemorphin-7 (15 μg), or a mixture of VV-hemorphin-7 (50 μg) or LVV-hemorphin-7 (50 μg) incubated with ACE (15) were successively loaded on the Nova Pak C18 column.

The UV spectra of protein and peptides are greatly dependent on their amino acids content. Unlike aromatic amino acids (phenylalanine, tryrosine and tryptophan) the others have very similar spectra. Only the aromatic amino acids exhibit maximal absorbances between 250 and 300 nm, nevertheless, their spectra are broad and they overlap.

Absorbance UV spectra of hemorphins are shown in figure 1. They were very similar especially between 250 and 300 nm. This was due to the same amount of Try and Trp in these molecules. Only the mathematical analysis and comparison of spectra allowed to differentiate them. The differences between spectra are displayed in table 1. When one spectrum was matched with itself, match angle was zero. The smaller match angle indicated that two molecules were almost similar. For example, as far as LVV-hemorphin-7 was concerned, it matched more or less with the other hemorphins. Thus, match angle values could be classified following an increasing order: LVV-hemorphin-7 < VV-hemorphin-7 < hemorphin-7 < LVV-hemorphin-5 < VV-hemorphin-5. It confirmed that, regarding hemorphins primary structure, the degree of similarity were inversely related with match angle values.

It could be noticed that hemorphin-7 and VV-hemorphin-5, in spite of the same chain length, showed different match angles when compared with LVV-hemorphin-7. According to the match angle values, hemorphin-7 was much similar to LVV-hemorphin-7 than VV-hemorphin-5. So, when peptides similarity is required, if aromatic amino acids seems to prevail, the chain length should also be considered (Table 2)

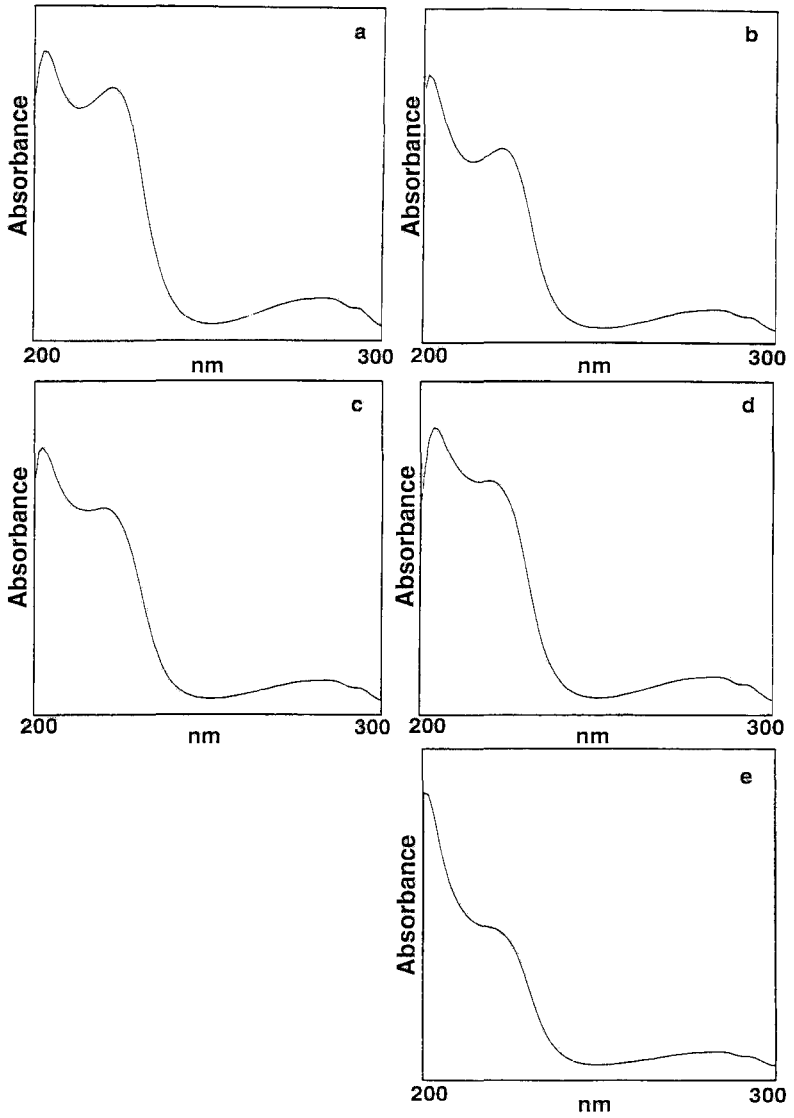

Figure 1: UV-spectra of the hemorphins obtained by photodiode array detector during their chromatographic analyses on a Nova Pak C18 column with the conditions described in experimental section. a) VV-hemorphin-5; b) LVV-hemorphin-5; c) Hemorphin-7; d) VV-hemorphin-7; e) LVV-hemorphin-7.

TABLE 1
Match Angle (MA) and Match Threshold (MT) Calculated by Millenium System from the Comparison of UV Spectra of Hemorphins during Chromatographic Analyses on Nova Pak Column.

Library spectra	VV-hemorphin-5		LVV-hemorphin-5		Hemorphin-7		VV-hemorphin-7		LVV-hemorphin-7	
Sample spectra	MA	MT	MA	MT	MA	MT	MA	MT	MA	MT
VV-hemorphin-5	0.00	0.051	0.784	0.073	2.695	0.045	3.366	0.212	3.175	0.181
LVV-hemorphin-5	0.784	0.073	0.00	0.084	1.583	0.066	2.748	0.219	2.694	0.187
Hemorphin-7	2.693	0.045	1.581	0.065	0.00	0.034	1.466	0.021	1.717	0.179
VV-hemorphin-7	3.366	0.212	2.748	0.219	1.466	0.211	0.00	0.288	0.559	0.274
LVV-hemorphin-7	3.175	0.182	2.693	0.188	1.717	0.179	0.559	0.274	0,00	0,244

TABLE 2
Primary Structure of Hemorphins

Hemorphins	Structure									
VV-hemorphin-5		Val	Val	Tyr	Pro	Trp	Thr	Gln		
LVV-hemorphin-5	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln		
Hemorphin-7				Tyr	Pro	Trp	Thr	Gln	Arg	Phe
VV-hemorphin-7		Val	Val	Tyr	Pro	Trp	Thr	Gln	Arg	Phe
LVV-hemorphin-7	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln	Arg	Phe

VERIFICATION OF THE METHOD RELIABILITY

The method was tested by searching VV-hemorphin-7 and LVV-hemorphin-7 in fraction FVII of the hemoglobine hydrolysate by use of UV spectral comparison

Hemorphin-7 (50 µg), VV-hemorphin-7 (50 µg) and LVV-hemorphin-7 (15µg) were injected on a Delta Pak column under the conditions described in experimental section. Chromatographic profiles and UV spectra of hemorphins were recorded (Fig. 2). With photodiode array detector, two chromatographic parameter were obtained in the same time: retention time and real time spectra. Then, fraction FVII was injected on the same column under the same conditions. Figure 3 exhibits its chromatographic profile. The hemorphins UV spectra were matched with every peak in this fraction. Table 3 exhibited the results. When we matched VV-hemorphin-7 UV-spectrum as library spectrum towards all the peaks of the profile, peak 3 was identified as VV-hemorphin-7. In the same manner, peak 4 was identified as LVV-hemorphin-7. This corroborated the results obtained with the same column from previous studies involving time consuming purification steps [14].

Verification was also carried out by use of hemorphins UV spectra obtained from Nova Pak column. Under these conditions it was impossible to detect VV-hemorphin-7 and LVV-hemorphin-7 only by use of their retention time. Therefore, matching of their spectra could bring more informations. The results are presented

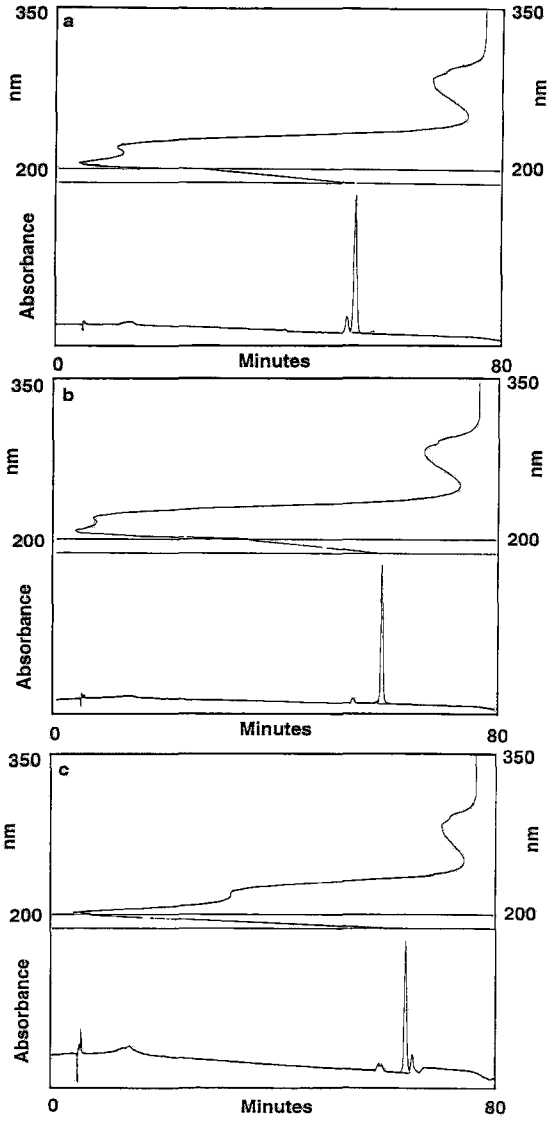

Figure 2: UV-spectra and correspondent chromatographic profiles of the hemorphins obtained by a photodiode array detector and a HPLC Delta Pak C18 column with the conditions described in experimental section. a) Hemorphin-7; b) VV-hemorphin-7; c) LVV-hemorphin-7.

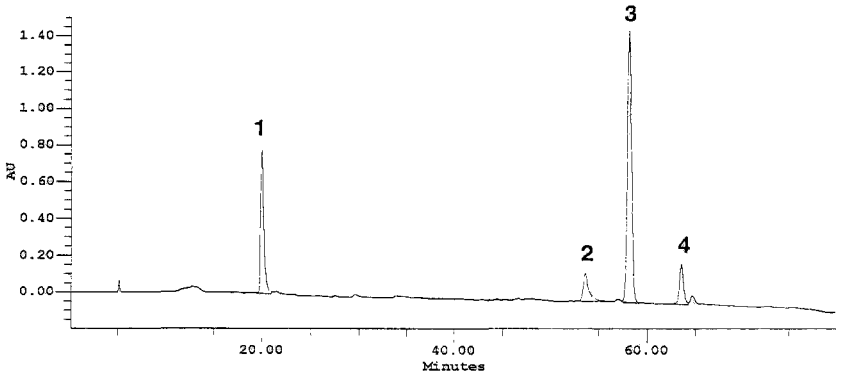

Figure 3: Chromatographic profile of fraction FVII, issued from the TSK G2000 SWG column, on a Delta Pak C18 column with the conditions described in experimental section. Peak 3 and 4 were identified as VV-hemorphin-7 and LVV-hemorphin by UV-spectra comparison.

TABLE 3
Spectral Matching Results of the Hemorphins with the Peaks of Fraction VII (fig.3) Separated by Delta Pak C18 Column. Peak 1 and 2 Were Rejected. Peak 3 and 4 Were Identified as VV-Hemorphin 7 and LVV-Hemorphin 7 Respectively Match Angle (MA), Match Threshold (MT)

Library spectra	Hemorphin-7		VV-hemorphin-7		LVV-hemorphin-7	
Sample spectra	MA	MT	MA	MT	MA	MT
Peak 3	1.754	1.003	**0.551**	**1,004**	1.330	1.006
Peak 4	2.829	1.013	1.950	1.014	**0.148**	**1.103**

in Table 4. Peak 3 and peak 4 were identified as VV-hemorphin-7 and LVV-hemorphin-7 by their match angles. So, it became an evidence that matching spectra were not limited to strict conditions: whatever the column, the method was still efficient.

IDENTIFICATION OF VV-HEMORPHIN-7 AND LVV-HEMORPHIN-7 FROM A COMPLEX PEPTIC BOVINE HEMOGLOBIN HYDROLYSATE

The previous experiments represented analyses performed on a simple fraction. Our purpose was to know whether it was possible to extract similar information from a more complex hydrolysate. So, peptic bovine hemoglobin hydrolysate (10 mg) was loaded on a Delta Pak C18 column. The profile is presented in figure 4. About 60 peaks could be resolved. Each peak was matched with UV spectra of hemorphins obtained from Delta Pak column. The results are shown in Table 5. Among 60 peaks, only 4 were selected by this calculation (peaks 27, 41, 51, 52), the other peaks exhibited match angles greater than than 90

TABLE 4
Spectral Matching Results of the Hemorphins with the Peaks of Fraction VII (fig.3) Separated by Nova Pak column. Peak 1 and 2 Were Rejected. Peak 3 and 4 Were Identified as VV-Hemorphin 7 and LVV-Hemorphin 7 Respectively. Match Angle (MA), Match Threshold (MT)

Library spectra	VV-hemorphin-5	LVV-hemorphin-5	Hemorphin-7	VV-hemorphin-7	LVV-hemorphin-7
Sample spectra	MA	MA	MA	MA	MA
Peak 3	2.643	2.051	1.063	0.51	1.677
Peak 4	4.394	3.332	2.794	3.332	0.562

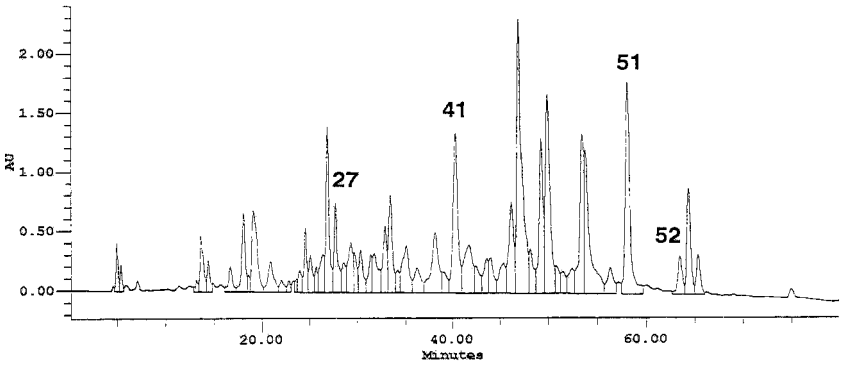

Figure 4: Chromatographic profile of on a Delta Pak C18 column with the conditions described in experimental section. Peaks 27, 41, 51 and 52 were selected by matching spectra with hemorphins. Peak 51 and 52 were identified as VV-hemorphin-7 and LVV-hemorphin-7.

degrees. It signified that their UV spectra were incomparable with those of hemorphins, probably because their primary structure were quite different from the hemorphins. When LVV-hemorphin-7 was used as library spectrum, the peak 52 was identified as LVV-hemorphin-7. In the same way, peak 51 was identified as VV-hemorphin-7. When hemorphin-7 was used as library spectrum, the minimum match angle was peak 51, identified as VV-hemorphin-7. It indicated that, as already described, VV-hemorphin-7 was found the most similar molecule to hemorphin-7 (Table 1). Consequently, hemorphin-7 was not present in total hydrolysate.

We used also the UV-spectra of the hemorphins obtained from Nova Pak column as library spectra. Similar results were obtained. So, the procedure of purification of LVV-hemorphin-7 and VV-hemorphin-7 was greatly simplified by only one step. The four peaks were collected and analyzed.

TABLE 5
Spectral Matching Results of the Hemorphins with All the Peaks of the Bovine Hemoglobin Hydrolysate (fig.4).Separated by Delta Pak C18 Column. Peak 27, 41, 51, 52 Were Selected. Peak 51 and 52 Were Identified as VV-Hemorphin 7 and LVV-Hemorphin 7 Respectively. Match Angle (MA), Match Threshold (MT)

Library spectra	Hemorphin-7		VV-hemorphin-7		LVV-hemorphin-7	
Sample spectra	MA	MT	MA	MT	MA	MT
Peak 27	9.196	0.005	9.811	1,006	9.612	1.006
Peak 41	4.956	0.003	6.093	1.003	5.236	1.103
Peak 51	0.824	0.003	0.398	1.004	1.707	1.008
Peak 52	2.856	0.012	2.999	1.012	0.332	1.024

IDENTIFICATION OF VV-HEMORPHIN-7 AND LVV-HEMORPHIN-7 BY BIOCHEMICAL ANALYSIS

By analysis of amino acid composition, FAB mass spectrometry and in regard of alpha and beta chains of bovine hemoglobin, the sequences of the four peaks selected among 60, were found out as following:

P27: Asn - Val - Lys - Ala - Ala - Trp - Gly - Lys - Val
(9 -17 of alpha chain of bovine hemoglobin)
P41: Ser - Ala - Ala - Asp - Lys - Gly - Asn - Val - Lys - Ala - Ala - Trp
(3 - 14 of alpha chain of bovine hemoglobin)
P51: Val - Val - Tyr - Pro - Trp - Thr - Gln - Arg - Phe
(32 - 40 of beta chain of bovine hemoglobin, **VV-Hemorphin 7**)
P52: Leu - Val - Val - Tyr - Pro - Trp - Thr - Gln - Arg - Phe
(31 - 40 of beta chain of bovine hemoglobin, **LVV-Hemorphin 7**)

As a result, peaks 51 and 52 were identified as VV-hemorphin-7 and LVV-hemorphin-7. These results validated the results obtained by matching spectra. We may suppose that Peaks 27 and 47 were retained by the spectral detection owing to their tryptophan content. Meanwhile, their match angle values were too much high to put them in the same category as hemorphins. Trp seemed to play a great role in spectrum matching because other peptides containing other aromatic amino acids, which are numerous in hemoglobin hydrolysate [18-19], were not assimilated to hemorphins by match spectra.

In this work, a rapid method for identification of hemorphins issued from a complex bovine hemoglobin hydrolysate was established. Its accuracy was verified by biochemical analysis. As mentioned above, aromatic amino acids played a very important role in matching spectra. This property could also be applied to the detection of rich aromatic amino acid peptides.

REFERENCES

1. Mant C.T., and Hodges R.S., J. Liq. Chromatogr., 12, 139-172 (1989).
2. Kopaciewicz W., and Regnier F.E., Anal. Biochem., 126, 8-16 (1982).
3. Pfannkoch E., Lu K.C., Regnier F.E., and Barth H.G., J. Chromatogr. Sci., 18, 430-441 (1980).
4. Zavitsanos P., and Goetz H., in High Performance Liquid Chromatography of peptides and proteins. Mant C.T. and Hodges R.S. pp 553-561 (1991).
5. Grego B., Nice E., and Simpson R., J. Chromatogr., 352, 359-368 (1986).
6. Nyberg F., Pernow, C., Moberg U., and Eriksson R., J. Chromatogr., 359, 541-551 (1986).
7. Fell A., Clark B., and Scott H., J. Chromatogr., 297, 203-214 (1984).
8. Goetz H., Hewlett packard HPLC Application Note, 12-5954-8920, April, (1987).
9. Hearn M.T.W., Aguilar M.I., Nguyen T., and Fridman M., J. Chromatogr., 435, 271-284 (1988).
10. Krull I.S., Stuting H.H., and Krzysko S.C., J. Chromatogr., 442, 29-52 (1988).
11. Hopkins W.H., Nordman R.G., and Willis B.G., Proc. Soc. Photo-optical Instrumentation Engineers, 191, 48 (1979).
12. Willis B.G., Fustier D.A., and Bonelli E.J., Am. Lab., June (1981).
13. Piot, J.M., Zhao,Q.Y., Guillochon, D., and Thomas, D., Biochem. Biophys. Res. Commun., 189, 101 - 110 (1992).
14. Glämsta, E.L., Marklund, A.., Hellman, U., Wernstedt, C., Terenius,L., and Nyberg, F., Reg. Peptides 34, 169-179 (1991).
15. Piot, J.M., Guillochon, D., Leconte, D., and Thomas, D., J. Chem. Tech. Biotechnol. 42, 147 - 156 (1988).
16. Lantz, J., Glämsta, E.L., Talback, L., and Nyberg, F., FEBS Letters 287. 39-41 (1992)
17. Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L., Napier, J.R., and Hancock, W.S., J. Chromatogr., 336, 93- 104 (1984).
18. Piot, J.M., Guillochon, D., Zhao,Q.Y., Leroy Y., Ricart G., Fournet B., and Thomas, D., Chromatographia 27, 128-142 (1989).
19. Piot, J.M., Guillochon, D., Zhao,Q.Y., Ricart G., Fournet B., and Thomas, D., J. Chromatogr. 481, 221-231 (1989).

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TLC ASSAY OF THYMOQUINONE IN BLACK SEED OIL *(NIGELLA SATIVA LINN)* AND IDENTIFICATION OF DITHYMOQUINONE AND THYMOL

LAILA I. ABOU BASHA, MOHAMED S. RASHED, AND HASSAN Y. ABOUL-ENEIN*
Bioanalytical and Drug Development Laboratory
Biological and Medical Research Dept. (MBC-03)
King Faisal Specialist Hospital and Research Centre
P.O. Box 3354, Riyadh 11211
Kingdom of Saudi Arabia

ABSTRACT

A simple quantitative TLC method for the determination of thymoquinone in commercially available black seed oil obtained from *Nigella Sativa L.* (Ranunculaceae) using a scanning densitometer is described. Also the identification of thymol, dithymoquinone in this sample was established. The R_f values for thymoquinone, thymol and dithymoquinone are 0.77, 0.37 and 0.52 respectively. The identification of the thymoquinone spot, obtained from the methanol extract of oil is confirmed by GC/MS which is essentially identical to thymoquinone standard. The solvent system consisted of benzene: isopropyl ether (1:1). All the spots were visualised and quantitated at 254 nm. The method proposed is simple, reproducible with a lower limit of detection of 100 nmoles/ml and can be used in routine analysis of thymoquinone in black seed oil for quality control purposes.

*Author to whom correspondence should be addressed

INTRODUCTION

Nigella Sativa L. (Ranunculaceae), it was first identified and described by Linnaneus, 1753.

Nigella Sativa L. grows in Mediterranean countries and is cultivated in others. The black seed oil has a long history of folklore medicine in Arabian and other countries for the treatment of various diseases (1,2). Its main constituents are fixed oil, volatile oil and alkaloids.

Tapozada et al (3) and Rathee et al (4) demonstrated that the volatile oil content of black seed oil is equivalent to 1.4-1.9% based on the weight of total oil extracted.

The percentage of thymoquinone isolated from *Nigella sativa L.* volatile oil was only 24% w/w of the volatile oil weight (5). Thymoquinone was isolated in a yield of 18.4 w/w from the volatile oil (6). Other constituents detected in the volatile oil were thymol and thymoquinone dimer (dithymoquinone) (7). The chemical structure of thymoquinone, thymol and dithymoquinone are shown in Figure 1. Aboutabl et al, (8) reported on the presence of monoterpenes, phenols and some esters in black seed oil.

It has been shown that the volatile oil revealed some pharmacological activities such as bronchodilators (9,10,11), increases bile flow and concentration of bile salts (12), decreases blood pressure in dogs (13) and rats (14). Accordingly, it was considered worthwhile to establish an analytical method for the determination of thymoquinone to which the pharmacological activities of black seed oil are attributed.

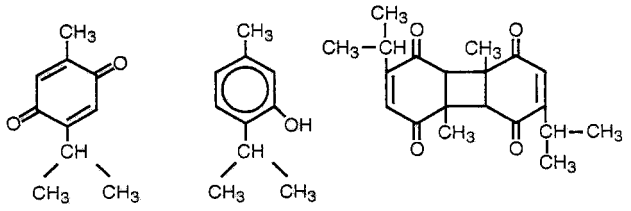

Figure 1. The chemical structure of thymoquinone, thymol and dithymoquinone.

MATERIALS AND METHODS

Chemicals

Methanol, benzene, isopropyl ether were HPLC grade (Springfield, New Jersey, USA). TLC, Alumina GF plates (20 cm X 20 cm, 250 microns, Analtech, Inc. Newark, USA) were used. Authentic thymoquinone obtained from Aldrich Chemical Co., Milwaukee with 99% purity was used without further purification. The three black seed oil samples obtained from the local market. Thymol was obtained from BDH Pool, UK. Dithymoquinone was prepared according to the method described by Smith & Tess (15).

Uniscan video densitometer (Analtec. Newark. DE, USA) was used in the analysis of thymoquinone and it consist of viewbox fitted with the appropriate light sources, video camera and an IBM compatible Central Processing Unit (CPU) with appropriate computer boards and densitometer software.

The solvent system used for TLC runs was benzene: isopropylether 1:1. A saturation time of 30 min is allowed before each run (Chamber dimensions are:

30 cm, 10 cm, 25 cm length, width and height respectively). All sample spots were quantitated using the Uniscan integration software at wavelength 254 nm.

Extraction procedure for oil:

One ml of methanol was added to 1 ml of oil (commercial black seed oil) in a glass centrifuge tube with cover. Vortex mix for 2 mins, the methanol top layer was transferred to a small glass vial and 5 μl was spotted on TLC plate.

The methanol extract of black seed oil and the spots corresponding to thymoquinone scrabed from the plates extracted with methanol, for further identification by GC/MS.

Gas chromatography mass spectrometry (GC/MS):

Electron impact (EI)-GC/MS analysis of the samples were carried out using a Hewlett-Packard (Palo Alto, CA, USA) 5988A GC/MS system equipped with a Hewlett-Packard 5890 GC and a 7673A autosampler. An Ultra-1 crosslinked methylsilicone capillary column (Hewlett-Packard, 25mx0.2mm i.d. x 0.33 μm film thickness) was used for analysis. The column oven was programmed as follows: 1 min at 80^{o}C, followed by an increase of 6^{o}C/min up to 290^{o}C followed by 5 min at 290^{o}C and injections were made in the splitless mode. The mass spectrometer interface was maintained at 280^{o}C and the mass spectrometer was scanned after a 4 min delay from m/z to 550.

Variability and Percentage Recovery:

The black seed oil sample 3 (1 ml) spiked with 500 nmol internal thymoquinone standard. The spiked sample extracted and 5 μl was spotted on TLC plate. The spiked sample assayed 6 times during two weeks period to evaluate the precision of the assay.

RESULTS AND DISCUSSION

The TLC solvent described above gave optimum separation of thymoquinone, its dimer dithymoquinone and thymol. Identification of thymoquinone, dithymoquinone and thymol was achieved by using their corresponding R_f values, as shown in Table 1.

GC/MS

Both the oil extract and the TLC purified sample showed a peak at 11.8 min. This peak had the same retention time as that obtained by injecting a solution of standard thymoquinone. The spectra obtained from the extract and the purified sample were essentially identical with the thymoquinone standard and also matched with the NIST library spectrum. The spectrum showed a strong molecular ion at m/z 164 and a strong M-CH_3 at m/z 149 as well as a strong ion corresponding to M-CO at m/z 136 and also showed M-C_3H_7 at m/z 121 and also a strong ion at m/z 93, possibly corresponding to M-C_3H_7-CO. A typical spectrum of the scrabed thymoquinone spot is shown in Figure 2.

Table 1. R_f values of Thymoquinone, Dithymoquinone and Thymol

	$R_f$values*
Thymoquinone	0.77
Dithymoquinone	0.52
Thymol	0.37

* Average of 6 determinations.

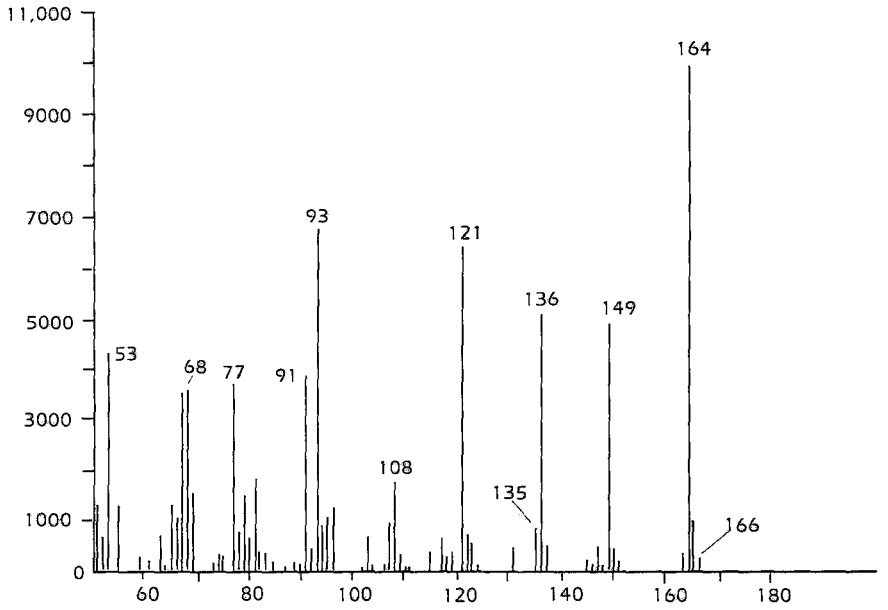

Figure 2. A mass spectrum of scraped thymoquinone TLC spot.

Linearity

Linear regression curves were constructed over the range of 1 - 30 nmoles with the correlation coefficient 0.979, (n=6) (Figure 3). Each determination (n=6) for the thymoquinone contents in black seed oil consisting of calibration curve and extracts of interest, was on one TLC plate. The lower limit detection of the method was 100 nmoles/ml as shown in Figure 4.

Percentage Recovery and Variability

The percentage recovery of spiked oil sample 3 was 90, with a coefficient of variation 5.6. Table 2 summarizes the results for the quantitative assay of thymoquinone in black seed oil.

Thorough literature review revealed that thymoquinone, was constantly the main active constituent of volatile oil contents of black seed oil (5,6,8).

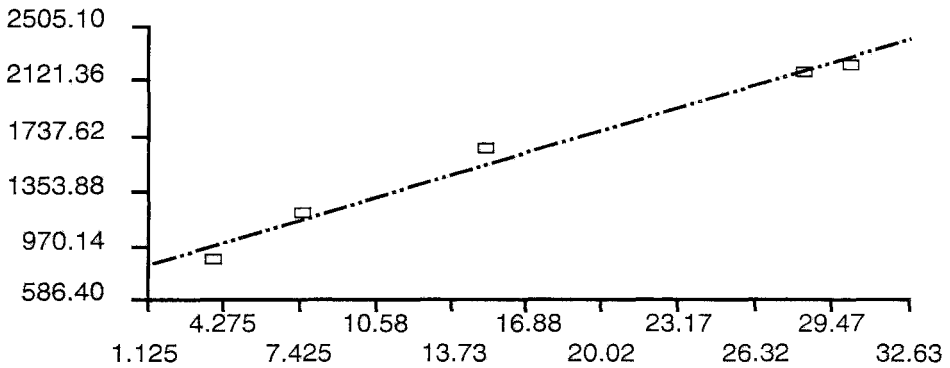

Figure 3. Linear regression curve for the quantitative determination of thymoquinone.

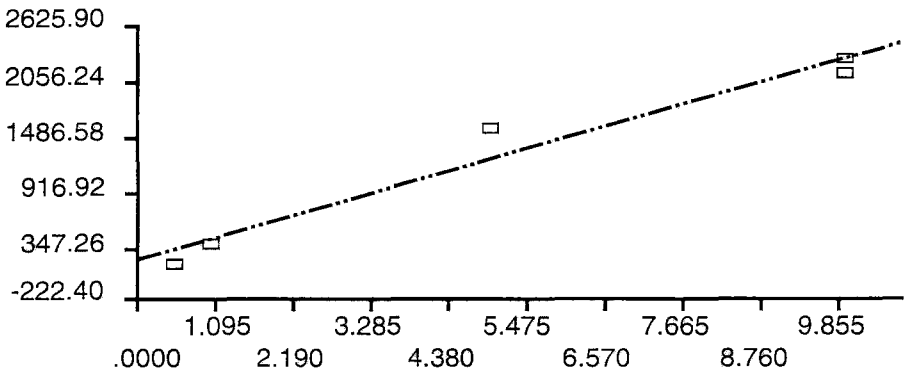

Figure 4. Linear regression curve for the quantitative determination of the lower limit of thymoquinone.

Table 2: Thymoquinone content in black seed oils (3 commercial samples) and sample 3 spiked with thymoquinone internal standard.

Black seed oil sample	Mean* ± S.D (nmoles/5μl)	C.V.	Thymoquinone nmoles/ml
1	14.42 ± 1.07	7.4	2.9×10^3
2	4.46 ± 0.6	6.0	0.9×10^3
3	undetected	-	< 100
3 spiked	2.25 ± 0.13	5.7	0.45×10^3

*Average of 6 determinations.

Marozzi et al. (16) claimed that the pharmacological activities of the volatile oil is due to its thymoquinone contents. The previous statement suggest that the quality of black seed oil was related to its thymoquinone contents. This content may vary according to the method of processing.

CONCLUSION

The quantitative TLC determination for thymoquinone described in this study provides a simple, rapid, reproducible method of analysis which can be used to establish the criteria required for the quality control of this constituent.

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REFERENCES

1. Chopra, R.N., Nayar, S.L., Chopra, I.C. Glossary of Indian Medicinal Plants; CSIR, New Delhi, 175 (1956).

2. Nadkarni, A.K. Indian Materia Medica; Popular Prakashan: Bombay, 1, 824 (1976).

3. Topozada, H.H., Mazloum, H.A. and El-Dakhany, M. The antibacterial properties of Nigella Sativa seeds. Active principle with some application. J. Egypt. Med. Ass. 48, Suppl. 187-202 (1965).

4. Rathee, P. S., Mishra, S. H. and Kaushal, R. Antimicrobial activity of essential oil, fixed oil and unsaponifiable matter of Nigella Saliva L. J. Pharm, Sci. 44, 1, 8-10 (1982).

5. El-Dakhakny, M. Studies on the chemical constitution of Egyptian Nigella Sativa L. seed II. The essential oil. Planta Med. 11, 4, 465-470 (1963).

6. Canonica, L., Jommi, G., Scolastico, C., and Bonati, A. The pharmacologically active principle in *Nigella sativa*. Gazz, Chim. Ital, 93 (11), 1404-1407 (1963).

7. El-Alfy, T.S., El-Fatatry, H.M. Toama, M.A. Isolation and structure assignment of an antimicrobial principle from the volatile oil of *Nigella sativa L.* seeds. Pharmazie 30 (2), 109-111 (1975).

8. Aboutabl, E.A., El-Azzouny, A.A., Hammerschmidt, F.J. Aroma volatiles of *Nigella sativa L.* seeds. Prog. Essent, Oil-Res., Proc. Int. Symp. Essent. Oils, 16th (ed. Brunke. E.J.), 49-55 (1986).

9. Badr-El-Din, M.K. Anti-asthmatic activity of "Nigellone". Gazette of the Egyptian Paed. Assoc. 8, 864-866 (1960).

10. Mahfouz, M., Abdl-Maguid, R. and El-Dakhakny, M. The effects of "Nigellone therapy" on the histominopexic power of blood sera of asthmatic patients. Arzneimm. Forsch (Drug Res.) 15, 1230-1232 (1965).

11. El-Tahir, K.E.H., Ashour, M.M.S., Al-Harbi, M.M. The respiratory effects of the volatile oil of the black seed (*Nigella Sativa*) in guinea-pigs; elucidation of mechanism of action. Gen. Pharmac. 24, 5, 1115-1122 (1993).

12. El-Dakhakny, M. Studies on the Egyptian *Nigella Sativa* L. IV. Some pharmacological properties of the seed's active principle in comparison to its dihydrocompound and its polymer. Arzneim. Forsch. (Drug Res.), 15, 1227-1229 (1965).

13. Mahfouz, M., El-Dakhakhny, M., Gemel, A., and Moussa, H. Choleretic action of *Nigella sativa* seed oil. Egyptian Pharm. Bull., 44 (4), 225-229 (1962).

14. El-Tahir, K.E.M., Ashour, M.M.S., Al-Harbi, M.M. The cardiovascular actions of the volatile oil of the black seed (*Nigella Sativa*) in rats; elucidation of the mechanism of action. Gen. Pharmac. 24, 5, 1123-1131 (1993)

15. Smith, L. J., and Tess, W. H. Dithymoquinone. J. Am. Chem. Soc. 66, 1323 (1944).

16. Marozzi, F.J., Kocialski, A.B., Malone, M.H. Studies on the antihistaminic effects of thymoquinone and querceting. Arzneim. Forsch. (Drug Res.) 10, 1574-1577 (1970).

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HPLC DETERMINATION OF A METOCLOPRAMIDE AND ONDANSETRON MIXTURE IN 0.9% SODIUM CHLORIDE INJECTION

T. G. VENKATESHWARAN, D. T. KING, AND JAMES T. STEWART
Department of Medicinal Chemistry
College of Pharmacy
The University of Georgia
Athens, Georgia 30602-2352

ABSTRACT

A high performance liquid chromatography procedure has been developed for the assay of a metoclopramide and ondansetron mixture in 0.9% sodium chloride injection. The separation and quantitation are achieved on a base deactivated octylsilane column at ambient temperature using a mobile phase of 77:23 v/v 0.01 M phosphate buffer, pH 4 - acetonitrile at a flow rate of 1.0 mL/min with detection of analytes at 273 nm. The separation is achieved within 15-20 min with sensitivity in the ng/mL range for each analyte. The method showed linearity for metoclopramide and ondansetron in the 12.5 - 50 and 5-20 μg/mL ranges, respectively. Accuracy and precision were in the 1 - 2% and 0.3 - 1.3% ranges, respectively, for both drugs. The limits of detection for metoclopramide and ondansetron were 49 and 20 ng/mL, respectively, based on a signal to noise ratio of 3 and a 20 μL injection.

INTRODUCTION

A mixture of metoclopramide and ondansetron can be used as a perioperative injection in operating rooms in U.S. hospitals. Interest in

our laboratories in the stability and compatibility of the drug mixture over time in 0.9% sodium chloride injection required the development of an HPLC method. A search of the literature indicated that an HPLC method was not available to assay for the mixture concurrently in a single injection.

Metoclopramide has been previously analyzed by ultraviolet and fluorescence spectroscopy and gas-liquid and high performance liquid chromatography. The UV assay measured the drug at 305 nm in a chloroform extract of commercial tablets (1). The fluorescence method measured the drug in a pH 2 solution using excitation and emission wavelengths of 310 and 360 nm, respectively (2). The GC assay provided separation of the drug on a 6 ft 3% OV-1 stationary phase with flame ionization detection (3). One HPLC method reported the separation of metoclopramide on an octadecylsilane column with a methanol-water-ammonia mobile phase and detection at 308 nm (4). The most recent HPLC method used an acetate buffer-acetonitrile mobile phase to assay for the drug in commercial tablets with the UV detector set at 273 nm (5).

Ondansetron has been assayed by high performance thin-layer chromatography (HPTLC), HPLC methods and radioimmunoassay methods. The HPTLC method was developed especially for plasma samples, but the sample throughput was low and the equipment is not generally available in most laboratories (6). The HPLC assays used either

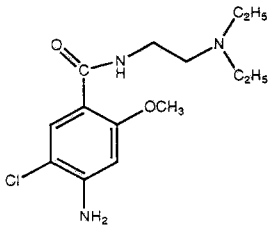
a silica column with an aqueous-organic mobile phase or a cyanopropyl column operated in the reverse-phase mode (7,8). Detection of the analyte was either by UV at 305 nm or radiochemical detection. The radioimmunoassay was combined with sample cleanup using a cyanopropyl solid phase extraction cartridge to provide a subnanogram per mL determination of ondansetron (9).

In this paper, an isocratic HPLC assay is presented that will simultaneously analyze for metoclopramide and ondansetron in 0.9% sodium chloride using a single injection. The compounds are separated on a base deactivated octylsilane column using a buffered aqueous-acetonitrile eluent. The separation is achieved within 15-20 min at ambient temperature with sensitivity in the ng/mL range.

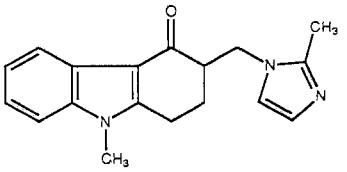
EXPERIMENTAL

Reagents and Chemicals

The structure formulae of the compounds studied are shown in Figure 1. Metoclopramide hydrochloride (Lot 75F-0603) was purchased from Sigma Chemical Co. (St. Louis, MO). Ondansetron hydrochloride (Lots AWS17 or AWS332A) was a gift from Glaxo, Inc. (Research Triangle Park, NC 27709). Acetonitrile (J.T. Baker, Phillipsburg, NJ 08865) was HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell, GA 30076). Monobasic potassium phosphate and potassium hydroxide were Baker analyzed reagents.



METOCLOPRAMIDE



ONDANSETRON

Figure 1 - Chemical structures of compounds studied.

Instrumentation

The chromatographic separation was performed on an HPLC system consisting of a Waters Model 501 pump (Milford, MA 01757), an Alcott Model 728 auto-sampler (Norcross, GA 30093) equipped with a 20 μL loop, a Beckman Model 163 variable wavelength UV-VIS detector (Fullerton, CA 92634) and a Shimadzu Model CR-3A integrator (Columbia, MD 21046). Separation was accomplished on a 25 cm base deactivated octylsilane (4.6 mm i.d., 5 μm particle size, Zorbax Rx-C8 Mac-Mod Analytical, Chadds Ford, PA 19317). The mobile phase consisted of 77:23 v/v 0.01M aqueous monobasic potassium phosphate,

pH 4.0 (adjusted with 1 N potassium hydroxide)-acetonitrile. The mobile phase was filtered through a 0.45 μm Nylon-66 filter (MSI, Westborough, MA 01581) and degassed by sonication prior to use. The flow rate was 1 mL/min and the detector was set at 273 nm.

Preparation of Standard Solutions

A combined standard solution containing metoclopramide and ondansetron was prepared by accurately weighing 2.8 mg of metoclopramide hydrochloride and 1.1 mg of ondansetron hydrochloride, transferring to a 50-mL volumetric flask, manually shaking for 10 min and 0.9% sodium chloride injection added to volume. This combined standard solution along with 1:1 and 1:4 dilutions made from the combined standard solution gave solutions containing 50.0, 25.0 and 12.5 μg/mL of metoclopramide and 20.0, 10.0, and 5.0 μg/mL of ondansetron expressed as the free base concentrations. Three point calibration curves were constructed for each analyte. Additional dilutions (7.5:10 and 3.8:10) of the combined standard solution were prepared in 0.9% sodium chloride injection to serve as spiked samples for each analyte to determine accuracy and precision of the method. Quantitation was based on linear regression analysis of analyte peak height versus analyte concentration in μg/mL.

RESULTS AND DISCUSSION

There were no reports in the scientific literature describing a separation of metoclopramide and ondansetron in a single mixture. Initial

studies to develop a single isocratic HPLC method for the two analytes involved the use of underivatized silica, phenyl, octyl, and octadecyl columns with various mobile phases containing methanol-aqueous phosphate buffers and/or acetonitrile-aqueous phosphate buffers at 1 mL/min. The best resolution of the analytes was obtained on a base deactivated octylsilane column using a 77:23 v/v phosphate buffer pH 4-acetonitrile mobile phase with a total run time of 15-20 min. The column also allowed the separation of methylparaben (preservative found in most commercial injections) from the analytes (Rt of 17.8 min). A typical chromatogram showing the separation of the two analytes is shown in Figure 2.

The absorption maximum for metoclopramide in the phosphate bufer-acetonitrile mobile phase was 273 nm. Even though this was not the maximum absorption wavelength for ondansetron, 273 nm was selected as the detection wavelength for the assay since it provided both good accuracy and precision data for the two component mix.

The HPLC method showed concentration versus absorbance linearity for metoclopramide and ondansetron in the 12.5-50 and 5-20 μg/mL ranges, respectively, at 273 nm. Table 1 gives other analytical figures of merit for each analyte. A photodiode array detector (Model 990, Waters Associates, Milford, MA 01757) was used to verify that none of the degradation products of the analytes would interfere with the quantitation of each drug at 273 nm. These experiments were performed on solutions of both drugs in 0.9% sodium chloride injection

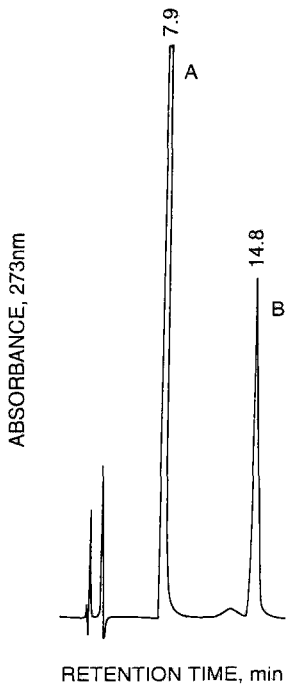


Figure 2 Typical HPLC chromatogram of metoclopramide (A) and ondansetron (B) on a base deactivated octylsilane with an aqueous phosphate buffer pH 4.0 - acetonitrile mobile phase. See Experimental Section for assay conditions.

after they had been degraded for 6 hr at 80°C in both 1.0N hydrochloric acid and 1.0N sodium hydroxide.

Percent error and precision of the method were evaluated using spiked samples containing each analyte. The results shown in Table 2 indicate that the procedure gives acceptable accuracy and precision for both analytes.

Table 1

Analytical Figures of Merit for Metoclopramide and Ondansetron

Analyte	r^{2a}	System Suitability[b]	LOD[c] ng/mL	k'	Theoretical Plates[d]	Tailing Factor[e]	Rs
Metoclopramide	0.9994	0.75	49.0	1.78	4456	1.5	
							9.4
Ondansetron	0.9992	0.43	20.0	3.93	4577	2.0	

[a] Range examined from 12.5-50 μg/mL metoclopramide (n = 9) and 5.0 - 20 μg/mL ondansetron (n = 9). Mobile phase consisted of 77:23 v/v 0.01M phosphate buffer, pH 4-acetonitrile at 1.0 mL/min with detection at 273 nm.
[b] Mean RSD% of 6 replicate injections at 25.0 μg/mL metoclopramide and 10.0 μg/mL ondansetron at 273 nm.
[c] Limit of detection, S/N = 3.
[d] Calculated as $N = 16\ (tr/w)^2$
[e] Calculated at 10% peak height

Table 2

Accuracy and Precision Using Spiked Drug Samples

	Concn Added (μg/mL)	Concn Found[a] (μg/mL)	Percent Error	RSD (%)
Metoclopramide	37.65	38.10 ± 0.12	1.20	0.31
	18.83	19.08 ± 0.25	1.33	1.31
Ondansetron	14.87	15.07 ± 0.04	1.34	0.27
	7.44	7.56 ± 0.05	1.61	0.66

[a] Based on n = 3.

Intra-day variabilities of the assay for metoclopramide and ondansetron expressed as % RSD were 0.75 and 0.43% (n = 6), respectively. Inter-day variabilities of the assay for these drugs were 2.2 and 2.4% (n = 18 over 3 days), respectively.

In summary, a base deactivated octylsilane column with an aqueous 0.01 M pH 4 buffer-acetonitrile mobile phase has been shown to be amenable for the separation and quantitation of a metoclopramide-ondansetron mixture in 0.9% sodium chloride injection. This study suggests that the HPLC method can be used to investigate the chemical stability of a mixture of the drugs in sodium chloride injection.

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REFERENCES

1. Metoclopramide monograph, British Pharmacopeia 1993, Vol II London, Her Majesty's Stationary Office, 1993, pp 1010-1011.

2. Baeyens, W. and de Moerloose, P., Fluorescence Properties of Metoclopramide and Its Determination in Pharmaceutical Dosage Forms, Analyst, 103, 359 (1978).

3. Daldrup, T., Susanto, F. and Michalke, P., Combination of TLC, GLC (OVI and OVI7) and HPLC (RP18) for a Rapid Detection of Drugs and Related Compounds, Fresenius Z. Anal. Chem, 308, 413 (1981).

4. Block, W., Pingoud, A., Khan, M. and Kjeldrap, P., The Pharmacokinetics, Bioequivalence and Bioavailability of Different Formulations of Metoclopramide in Man, Arzneim.Forsch/Drug Res., 31, 1041 (1981).

5. Foda, N.H., Quantitative Analysis of Metoclopramide in Tablet Formulations By HPLC, Anal.Lettr., 27, 549 (1994).

6. Colthup, P.V., in Recent Advances in Thin-Layer Chromatography, Dallas, F.A., Read, H. Ruane, R.J. and Wilson, I.D., Eds., Plenum, New York (1988) pp179-185.

7. Personal Communication, Fox, J., Glaxo, Inc., Research Triangle Park, NC 27709 (1991).

8. Colthup, P.V., Felgate, C.C., Palmer, J.L. and Scully, N.L. Determination of Ondansetron in Plasma and its Pharmacokinetics in the Young and Elderly, J. Pharm. Sci., 80, 868 (1991).

9. Wring, S.A., Rooney, R.M. Goddard, C.P., Waterhouse, I., and Jenner, W.N., A Sensitive Radioimmunoassay Combined with Solid Phase Extraction, For the Subnanogram per mL Determination of Ondansetron in Human Plasma. J. Pharm. Biomed. Anal., 12, 361 (1994).

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DEVELOPMENT OF A HPLC METHOD FOR THE DETERMINATION OF 17β-ESTRADIOL-3-PHOSPHATE IN PHARMACEUTICAL PREPARATIONS

R. BRENT MILLER*, GERALD DELKER, CHUAN CHEN, AND CHARLES H. SHERWOOD
IOLAB Corporation (a Johnson & Johnson Company)
Materials Characterization Department
500 Iolab Drive
Claremont, California 91711

ABSTRACT

A validated reversed-phase high-performance liquid chromatographic (HPLC) procedure for the analysis of 17ß-estradiol-3-phosphate is reported. In the development of this assay, several factors were evaluated including buffer ionic strength, mobile phase pH, ion-pairing concentration, organic composition, and column type. The described method is rapid and coupled with standard HPLC procedures leads to a selective, accurate, and reproducible assay. The peak area versus 17ß-estradiol-3-phosphate concentration is linear over the range of 0.1 - 100 μg/mL, with a detection limit of 0.02 μg/mL.

INTRODUCTION

An analytical method for the determination of the conjugated estrogen, 17ß-estradiol-3-phosphate (E_p), in the presence of 17ß-estradiol (E_2), estrone (E_1), and estriol (E_3) is reported. E_p can hydrolyze to E_2 and,

since E_2 is readily oxidized to E_1, which in turn can be hydrated to E_3 [1], the proposed method must be selective for these analogues.

The analyses of estrogen conjugates typically requires enzyme hydrolysis or solvolysis, which increases the time and cost of each analysis. Furthermore, following chemical cleavage, it is not possible to simultaneously measure both the conjugated and unconjugated species.

Several methods exist for the determination of estrogens in pharmaceutical preparations [2-12]. However, to our knowledge, no method exists for the determination of E_p in the presence of E_1, E_2, and E_3 and, moreover, that satisfies the USP XXII guidelines under Assay Category I [13]. Data elements required for Assay Category I include precision, accuracy, selectivity, range, linearity, and ruggedness. The method described herein for 17ß-estradiol-3-phosphate satisfies all of these requirements.

The development of this reversed-phase HPLC method required investigating several factors including buffer ionic strength, mobile phase pH, ion-pairing concentration, organic composition, and column type.

EXPERIMENTAL

Chemicals and Reagents

17ß-Estradiol-3-phosphate, disodium salt, was purchased from Research Plus (Bayonne, NJ, USA). Estrone, estriol, and 17ß-estradiol were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, 1 **N** hydrochloric acid, and ACS reagent grade potassium phosphate, monobasic, monohydrate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Tetrabutylammonium chloride hydrate (TBAC) was purchased from Aldrich (Milwaukee, WI, USA). The water was deionized

and distilled. All reagents were used without further purification.

Apparatus

The chromatographic system consisted of a Waters Model 600E system controller and pump, a WISP 712 autosampler, and a 486 variable-wavelength UV detector set at 220 nm (Waters Associates, Milford, MA, USA). Columns which were investigated included a Zorbax Rx C18 (4.6 x 250 mm, 5 μm, MAC-MOD Analytical, Inc., Chadds Ford, PA, USA), a Keystone CPS Hypersil-1 cyano (4.6 x 150 mm, 3 μm, Keystone Scientific, Inc., Bellefonte, PA, USA), a Keystone ODS Hypersil (4.6 x 250 mm, 5 μm) and an Alltech Adsorbosphere HS C18 (4.6 x 150 mm, 3μm, Alltech Associates, Inc., Deerfield, IL, USA). All columns were maintained at ambient temperature.

Mobile Phase

The mobile phase consisted of buffer-acetonitrile-methanol (35:15:50, V/V/V) adjusted to pH 3.0 (apparent) with 1**N** HCl. The buffer was comprised of 10 m**M** potassium phosphate, monobasic, monohydrate and 50 m**M** TBAC. The mobile phase was filtered through a 0.45 μm filter and degassed for 10 minutes and maintained under a helium atmosphere. The flow rate was 1.0 mL/minute with a typical operating pressure of **ca.** 93 bar.

Data Acquisition

The peak area of E_p was measured using a PE Nelson 900 series interface and down-loaded to a PE Nelson Turbochrom II workstation (Perkin-Elmer Corporation, Cupertino, CA, USA). The chromatographic data was automatically processed for peak area followed by a weighted (1/C) linear regression analysis.

Preparation of 17ß-Estradiol-3-Phosphate Solutions

An E_p stock solution was prepared at 100 µg/mL in water. Appropriate dilutions of the E_p stock solution were made with water to prepare standards ranging from 0.1 - 100 µg/mL.

System Suitability Requirements

The system suitability results are calculated according to Chromatography <621> of the USP XXII from typical chromatograms [14]. The instrument precision as determined by six successive injections of an E_p standard solution should provide a relative standard deviation (RSD) not greater than 1.0%. The tailing factor should not exceed 1.5 at 5% peak height. Finally, the resolution between the analyte peaks should be greater than 1.5.

RESULTS AND DISCUSSION

Chromatography

A reference solution containing E_p, E_1, E_2, and E_3 each at a concentration of 100 µg/mL in methanol/water (1:1, v/v) was used to verify that the method met all the suitability limits.

To obtain the best overall chromatographic conditions, the mobile phase was optimized by examining the effect of capacity factor (k') on mobile phase pH (Figure 2), KH_2PO_4 ionic strength (Figure 3), and TBAC concentration (Figure 4). By adjusting the organic composition from acetonitrile to methanol (Table 1), the dead time between E_1 and E_2 was reduced by approximately 4 minutes, while having little effect on E_p and E_3. However, increased band broadening was observed as the amount of methanol increased. Therefore, a compromise between the ratio of acetonitrile and methanol ensued. Finally, four different columns were evaluated in optimizing the method. The Keystone CPS Hypersil-1 cyano column was too

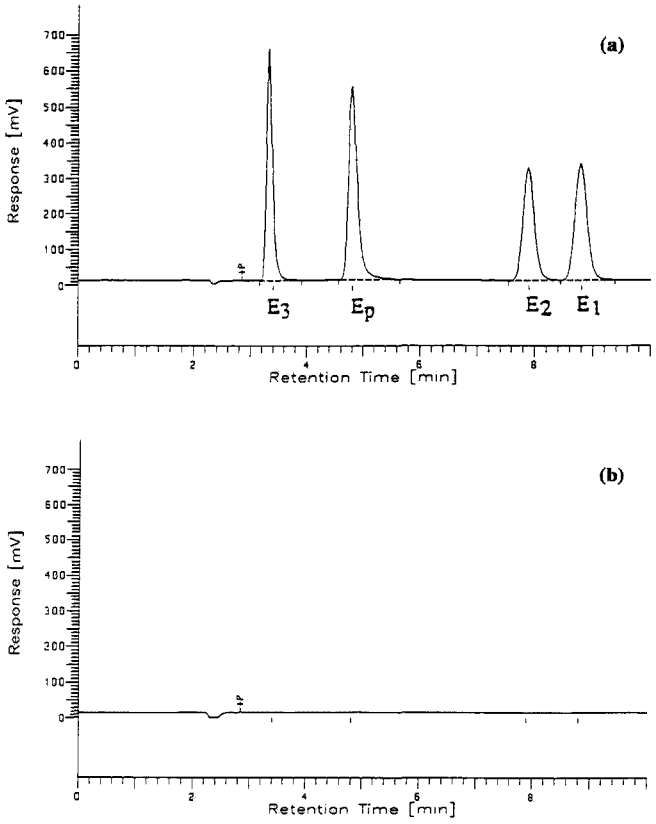

FIGURE 1. Typical chromatograms; (a) reference solution and (b) blank.

polar and did not provide sufficient retention of the analytes. The Keystone ODS Hypersil column provided adequate resolving power, but, severe peak tailing resulted with E_p. Both the Alltech Adsorbosphere HS C18 and Zorbax Rx C18 columns provided sufficient system suitability, however, the latter provided a shorter chromatographic run time and, consequently, was selected as the column of choice.

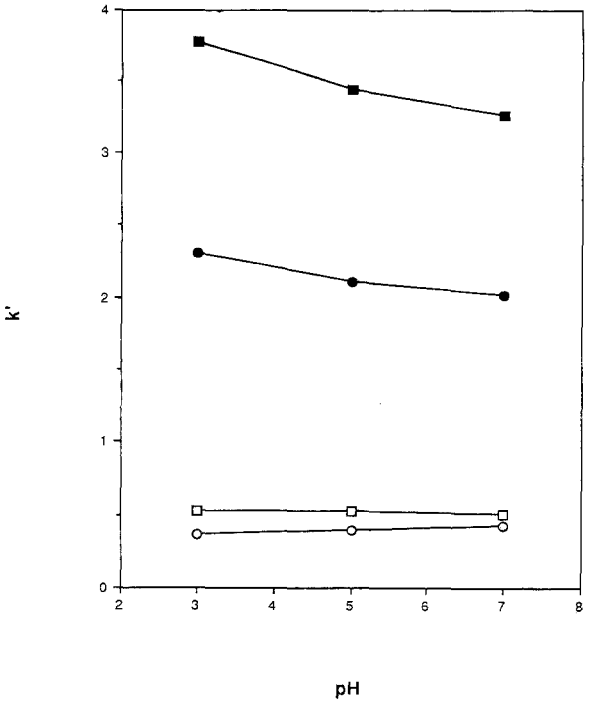

FIGURE 2. Effect of mobile phase pH on k': (■) E_1, (●) E_2, (□) E_p and (○) E_3.

Typical chromatograms of a reference and blank solution are illustrated in Figure 1 using a Zorbax Rx C18 column and a 50 μL injection. The retention times of estriol, 17β-estradiol-3-phosphate, 17β-estradiol, and estrone were 3.3, 4.8, 7.9, and 8.8 minutes, respectively. The overall chromatographic run time was 10 minutes.

System Suitability

The column efficiency for E_p was 3856 theoretical plates. The tailing factor of E_p was 1.2. The resolution between E_3 and E_p, E_p and E_2, and E_2 and E_1 was 4.4,

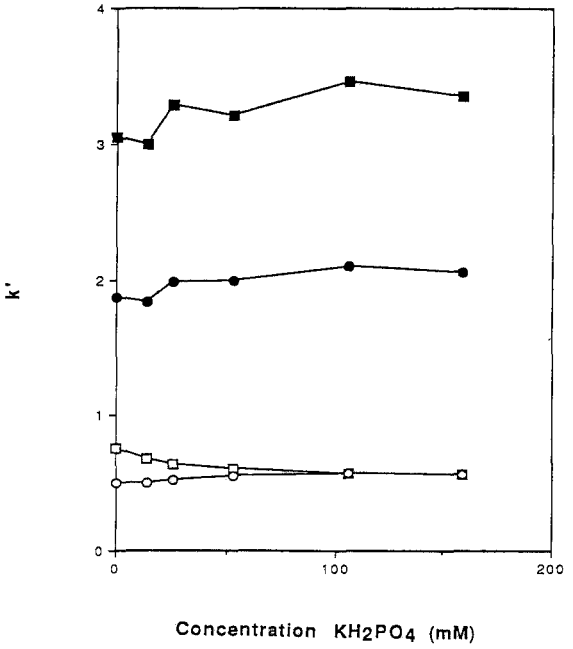

FIGURE 3. Effect of KH_2PO_4 ionic strength on k': (■) E_1, (●) E_2, (□) E_p and (○) E_3.

7.8, and 2.0, respectively. The instrument precision, determined by 6 replicate injections of the E_p standard solution, exhibited a RSD of 0.3%.

Precision and Accuracy

The precision (RSD) and accuracy (relative error, RE) was determined by analyzing 17ß-estradiol-3-phosphate standards ranging from 0.1 - 100 μg/mL, in replicates of six (Table 2).

Linearity

A linear response in peak area for E_p over the range of 0.1 - 100 μg/mL was observed. The correlation coeffi-

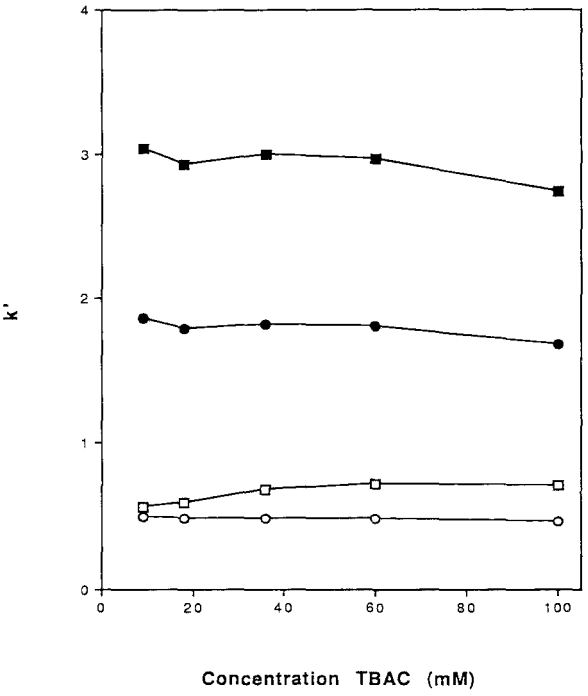

FIGURE 4. Effect of TBAC concentration on k': (■) E_1, (●) E_2, (□) E_p and (○) E_3.

TABLE 1

Effect of Mobile Phase Composition on Retention Time

Composition (%)			Retention Time (min)			
Buffer	ACN	MeOH	E_3	E_p	E_2	E_1
60	40	--	3.1	4.4	8.8	14.3
35	--	65	3.6	5.5	9.7	10.2
55	40	5	3.0	4.0	7.6	11.7
35	10	55	3.3	4.8	7.9	8.9
35	15	50	3.2	4.6	7.3	8.2

TABLE 2

Accuracy and Precision of 17ß-Estradiol-3-Phosphate

Nominal Conc. (μg/mL)	n	Mean Found Conc. (μg/mL)	%RSD	%RE
0.10	6	0.12	8.0	20.0
0.25	6	0.24	2.9	-4.0
0.50	6	0.48	1.8	-4.0
1.00	6	1.01	1.4	1.0
5.00	6	4.98	0.9	-0.4
10.00	6	10.20	1.0	2.0
50.00	6	49.83	0.5	-0.3
100.00	6	100.07	0.3	0.1

cients were 0.998 or better (n=6). The limit of detection for E_p, defined as 3 times the signal-to-noise ratio, was 0.02 μg/mL.

Conclusion

The described assay for the analysis of 17ß-estradiol-3-phosphate in the presence of estriol, 17ß-estradiol, and estrone is selective, sensitive, and robust. With exception of the limit of quantitation, the precision of the method is below 3.0%, while the accuracy is within 4.0%. The method is rapid and requires no sample pretreatment, resulting in **ca.** 100 samples being analyzed daily. More than 1000 injections can be made on a single analytical column with minimal loss in chromatographic integrity.

Furthermore, it is anticipated that this method could be used for the analysis of 17ß-estradiol-3-phosphate in pharmaceutical preparations designed for hormone replacement therapy.

REFERENCES

1. F. Murad and R.C. Haynes, Jr., in The Pharmacological Basis of Therapeutics, 7th edition, (A.G. Gilman, L.S. Goodman, T.W. Rall, and F. Murad, eds.), pp. 1412, Macmillian, New York (1985).

2. Sj. van der Wal and J.F.K. Huber, J. Chromatogr., 149, 431-453 (1978).

3. Sj. van der Wal and J.F.K. Huber, J. Chromatogr., 135, 305-321 (1977).

4. B. Flann and B. Lodge, J. Chromatogr., 402, 273-282 (1987).

5. P.J. Kelly and P.A. Sewell, Chromatographia, 25, 957-960 (1988).

6. K.Y. Chong, T.H. Khoo, F.S. Koo, C.P. Ong, S.F.Y. Li, H.K. Lee, B. Venkatesh, and C.H. Tan, J. Liq. Chromatogr., 14, 2445-2455 (1991).

7. J. Wei, J. Wei, and X. Zhou, Biomed. Chromatogr., 4, 34-38 (1990).

8. L. Jiang, Z. Wang, and S.A. Matlin, J. Liq. Chromatogr., 13, 3473-3479 (1990).

9. J. Wei, J. Wei, and X. Zhou, J. Chromatogr., 552, 103-111 (1991).

10. A.A. Fasanmade and A.F. Fell, Anal. Lett., 25, 363-378 (1992).

11. P.A. Lane, D.O. Mayberry, and R.W. Young, J. Pharm. Sci., 76, 44-47 (1987).

12. P.A. Asmus and J.B. Landis, J. Chromatogr., 316, 461-472 (1984).

13. The United States Pharmacopeia XXII, pp. 1558-1567, U.S. Pharmacopeial Convention, Rockville, MD (1990).

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CHROMATOGRAPHIC ANALYSIS OF RESIDUAL ACETATE IN BULK DRUGS

MARJORIE M. SEE, R. THOMPSON, N. GRINBERG*, HOLLY J. PERPALL, G. BICKER, AND PAT TWAY
Merck Sharp & Dohme Research Laboratories
R80Y-115, P.O. Box 2000
Rahway, New Jersey 07065-0914

ABSTRACT

An ion chromatographic method for the determination of the residual acetate in bulk drug was developed. The drug was MK0476, an LTD_4 antagonist. The compound also has a carboxyl functionality, which would interfere with the detection of the acetate ion. A solid phase extraction through a Sep-Pak cartridge was pursued for the removal of MK0476 from the matrix. Since the analyte does not have a chromophore, a mobile phase containing trimesic acid facilitated its detection by indirect photometric detection. The separation was performed on a polymeric strong anion exchange column. The influence of pH, concentration of trimesic acid, and temperature were studied. Chloride ion was found to be a contaminant that was interfering in the analysis. To improve the separation between chloride and acetate ions, methanol was added to the mobile phase, leading to complete separation between the two species. Recovery of the acetate ion was determined as 92.3%. The method was applied to real samples with good results. It was shown to be sensitive for the determination of less than 0.001 mg/ml of acetate with a linear range of 0.00036 to 0.074 mg/ml.

* Author to whom the correspondence should be addressed

I. INTRODUCTION

The use of ion chromatography for the analysis of inorganic and organic ions has shown an exponential increase since the mid 1970's due to the implementation of suppressor systems [1]. Prior to this time, the concept of ion exchange chromatography was well known [2-6], but its application was hindered by the lack of a good detector. Many ions were unsuitable for UV detection due to lack of chromophores, and the use of conductivity detection was rendered impossible due to the high background conductivity of the eluents used. Suppressor systems removed most of the background conductivity by exchanging ions such as sodium and nitrate by protons and hydroxide ions respectively. With the introduction of suppressor systems ion chromatography using conductivity detection grew rapidly and at present conductivity is still the most popular choice of detection used.

Several alternate detection systems have been developed since the introduction of suppressor systems. They include direct conductivity detection using low conductivity eluents [7], UV detection following post or pre-column derivatization [8], amperometric detection [9,10], and indirect photometric detection [11-13].

Indirect photometric detection (IPD) or "vacancy detection" offers an alternative that is as sensitive as conductivity detection [14,15], does not require derivatization, and utilizes standard HPLC pumps and detectors. Aromatic carboxylic acids are typically used as additives to

the mobile phase because of their high absorptivity in the UV-Vis range. At a chosen wavelength where the eluent absorbs strongly and the analyte does not, the presence of the analyte in the detector cell will result in a decrease in absorbance. This decrease will be manifested as a negative peak. Many inorganic and organic ions have been analyzed using IPD [15-18].

In a previous paper, our laboratory showed the effectiveness of using trimesic acid as an eluent for the separation of some organophosphonates [19]. The work described in this paper is an extension of the previous study dealing with the quantitation of trace acetate ion in the presence of the bulk drug MK0476. The compound, MK0476, is a molecule with a carboxyl functionality, Fig. 1.

This drug is a potent receptor antagonist of leukotriene D_4 (LTD_4) which is being developed as a therapeutic agent for bronchial diseases such as asthma [20-22]. In the penultimate step of its synthesis,

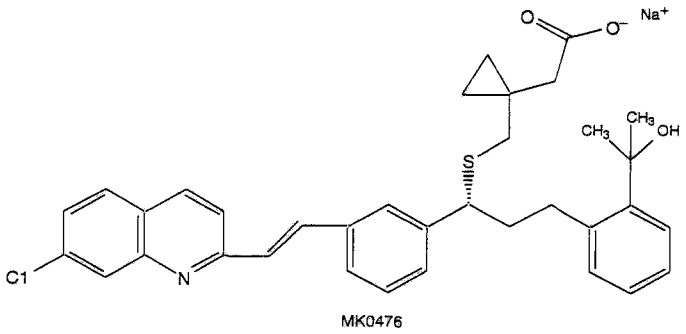

Fig. 1. Structure of MK0476.

MK0476 is obtained as a dicyclohexyl amine (DCHA) salt. Addition of acetic acid to the reaction mixture produces an exchange of the DCHA leading to the free acid dissolved in the organic layer. An ethanolic solution of sodium hydroxide is added to convert the MK0476 free acid to the sodium salt which then precipitates out. The salt is subsequently washed with acetonitrile to remove residual acetic acid and is then analyzed for trace acetate by ion chromatography.

II. EXPERIMENTAL

Sample preparation.

Ten batches of samples were prepared by weighing 100 mg of MK0476 sodium salt that were then introduced into 10 ml volumetric flasks. Five ml of deionized water were added and the volumetric flask was introduced into an ultrasonic bath and sonicated until the compound was completely dissolved. The flask was then diluted to volume with water. Batch MO35 was used for the recovery experiment. An aliquot was removed from this sample for sample treatment (as described in the next section). Then, 2 ml of 0.006 mg/ml acetate standard was spiked into 2 ml of the sample solution. The solution obtained was also subjected to the same sample treatment as the unspiked solution.

Sample treatment.

To remove MK0476 from the samples, each sample was passed

through a Sep Pak C18 cartridge (Waters, Marlborough, MA) that was previously conditioned in the following manner. First, the cartridge was washed with 10 ml of methanol followed by 20 ml of water deionized water. Then the sample solutions and the solutions spiked with acetate were passed through a cartridge. The first 2 ml were then discarded. Twenty-five microliters of sample were injected into the HPLC system.

HPLC conditions.

An HP1050 HPLC system equipped with an autosampler and a variable wavelength detector was used for the analysis of the residual acetate in the MK0476 sodium salt.

The stationary phase, manufactured by Hamilton under the trade name PRP-X-100, consisted of a polymeric strong anion exchange packed in a 25 cm x 0.4 cm I.D. column. The column was maintained at constant temperature of 30 °C.

The optimal mobile phase consisted of a mixture of an aqueous solution of 0.5 mM trimesic acid. The pH was adjusted to 5.0 with LiOH, and methanol at 90:10 (v/v) ratio.

The analyte was detected by UV at 254 nm. Due to the negative peaks obtained with the indirect photometric detection method the polarity of the detector was reversed such that the peaks were inverted and facile integration of the peaks was achieved.

III. RESULTS AND DISCUSSION

To analyze residual acetate in MK0476 final product a strong anion exchange column was selected. MK0476 has a carboxylic functionality that can interact with the stationary phase. Under our chromatographic conditions MK0476 elutes as a large peak close to the void volume. Consequently, a solid phase extraction was necessary to remove the compound from the system prior to the chromatographic analysis.

Acetate is an analyte that has very little absorbance in the UV-Vis region, and the detection of trace amounts of this analyte thus represents a challenge. Indirect photometric detection was chosen to analyze the acetate ion. The method is based on the use of a mobile phase that has a high absorbance at a particular wavelength; at the same time the analyte presents no or minimal absorbance at that same wavelength. Upon equilibration of the stationary phase with the mobile phase ions, the detector senses a strong steady absorbance. When the analyte ion is injected and eventually eluted from the stationary phase, its transparency results in a localized region of lower absorbance and is reflected in the detector as a negative analyte peak.

Trimesic acid is a benzene tricarboxylic acid. It absorbs strongly at 254 nm while acetate ion does not, and is therefore suitable for indirect photometric detection. Injection of a solution of acetate into the chromatographic system produces a negative peak. The polarity of the detector can be inverted to obtain a positive peak.

Several parameters, such as the concentration of trimesic acid in the mobile phase, the pH of the mobile phase, the concentration of methanol in the mobile phase and the temperature were varied to optimize the method.

Influence of the pH of the mobile phase on the peak parameters. Trimesic acid is a tricarboxylic acid and consequently it has three pK values of 3.1, 3.9 and 4.7. It is therefore expected that the net charge of the acid, and consequently its displacing power, will vary with the changes in the pH of the mobile phase. Consequently, the retention of acetate on the anion exchange stationary phase will be strongly influenced. Thus, the pH of the mobile phase was varied between 4.0 and 6.25 as follows: 4.0, 4.25, 4.6, 5.0 and 6.25. As expected, the k' of the analyte is strongly affected by the pH changes. Between 4.0 and 4.25, it is almost constant, and decreases sharply at the rest of the pH intervals studied (Fig. 2).

Since the present study deals with the analysis of trace amounts of acetate ions, the method should be optimized such that the half peak width ($W_{1/2}$) should be at a minimum. This optimization will insure lower detection limits. The influence of the pH on the $W_{1/2}$ is presented in Fig. 3. From this data the minimum $W_{1/2}$ was obtained at pH 5. Therefore, in the following experiments the pH was maintained at this value.

Influence of the concentration of trimesic acid. The concentration of the trimesic acid in the mobile phase was varied between 0.25 mM

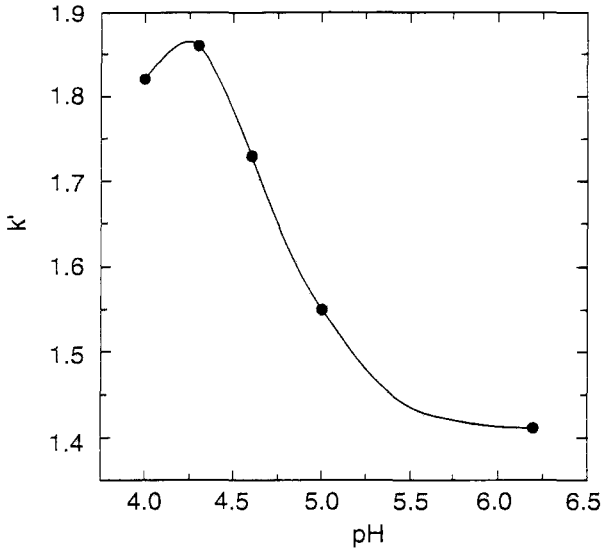

Fig. 2. Influence of pH on the k'.

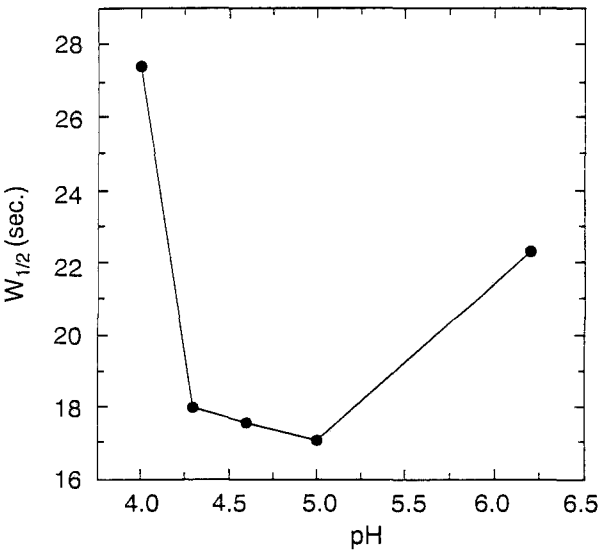

Fig. 3. Influence of pH on the $W_{1/2}$.

and 1.5 mM as follows: 0.25, 0.5, 1.0 and 1.5 mM. The influence of the concentration of trimesic acid on the k' of the acetic acid is presented in Fig. 4. Upon increasing the trimesic acid concentration from 0.25 mM to 1.5 mM the k' values decreases steadily.

The influence of the concentration on $W_{1/2}$ is presented in Fig. 5. The increased concentration of the additive into the mobile phase produces a decrease in the mass transfer of the acetate ion. This will lead to an increase in $W_{1/2}$ up to a concentration of 1 mM, decreasing after this concentration.

The increase of the concentration of trimesic acid into the mobile phase also has a negative effect on the detection of the acetate ion. The results are presented in Fig. 6. From 0.25 mM to 0.5 mM an increase in the area counts for the acetate is observed. A further increase of concentration of the additive into the mobile phase produces a decrease in area counts. Based on these results the concentration of trimesic acid in the mobile phase was maintained constant at 0.5 mM for further optimization studies.

Influence of the temperature. The influence of temperature on the k' is presented in Fig. 7. Upon increasing the temperature, an increase in k' is observed. Such behavior is due to a shift in the partition equilibrium of trimesic acid toward the mobile phase, leaving more sites in the stationary phase to interact with the acetate ion. Due to an increased interaction of the acetate ion with the stationary phase an

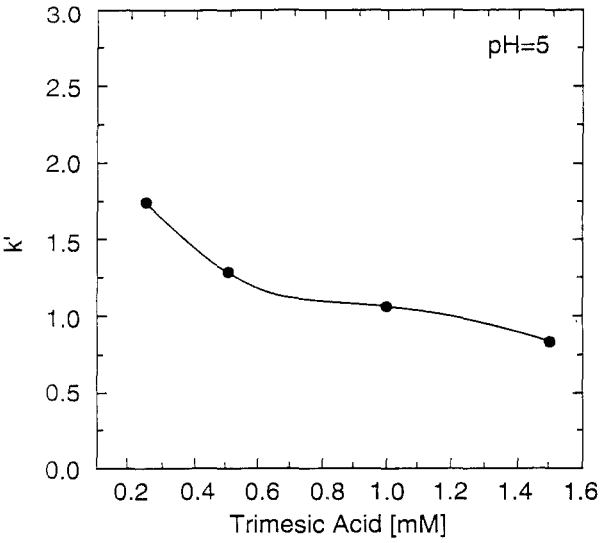

Fig. 4. Influence of the concentration of trimesic acid on the k' values.

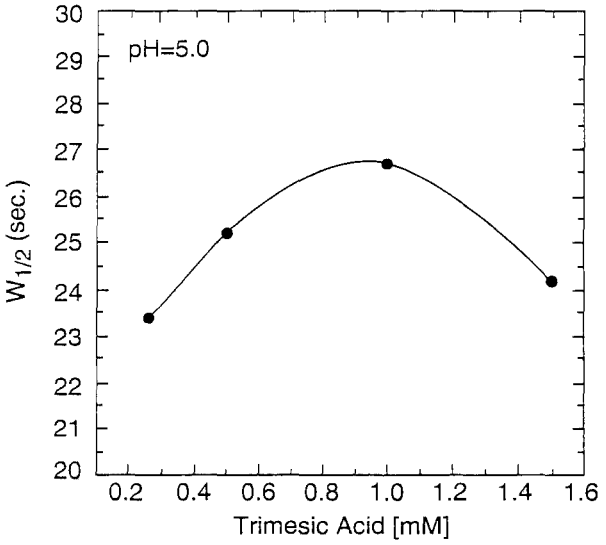

Fig. 5. Influence of the concentration of trimesic acid on the $W_{1/2}$.

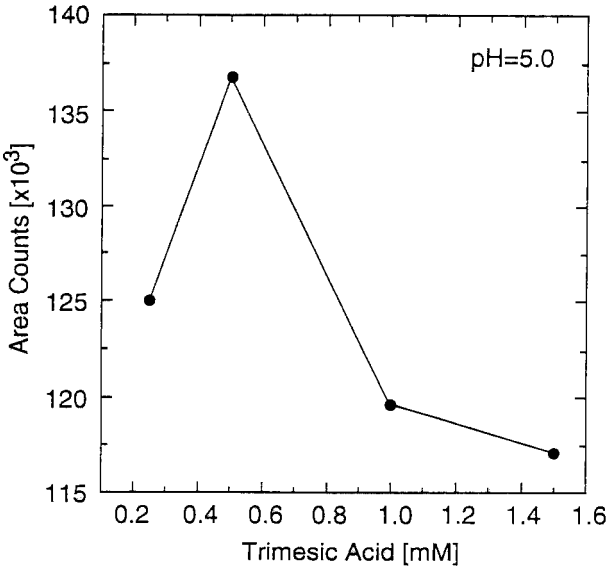

Fig. 6. Influence of the concentration of trimesic on the area counts.

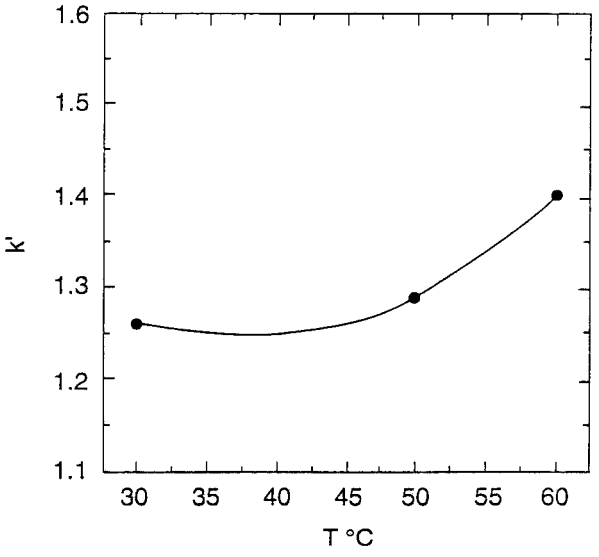

Fig. 7. Influence of the temperature on the k'.

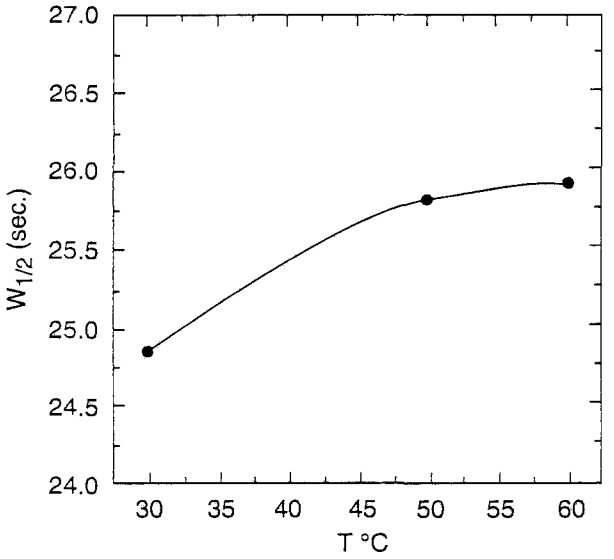

Fig. 8. Influence of the temperature on the $W_{1/2}$.

increase of $W_{1/2}$ was also observed (Fig. 8). Therefore, 30 °C was chosen as the temperature for the subsequent experiments.

Influence of the concentration of methanol in the mobile phase. Chloride was observed as an impurity in all the standard solutions, as well as in the samples. Its presence is due to inherent laboratory contamination. Special care was placed on improving the resolution between the acetate ion and chloride. With no organic modifier the two peaks were partially separated. It is known that decreasing the polarity of the mobile phase (by adding an organic modifier to the mobile phase) will lead to an increase of the ionic character of the stationary phase [18]. The interaction of the analyte with the

stationary phase will then be enhanced. Acetonitrile could not be used because it contains trace levels of acetic acid. Consequently, methanol was used as an organic modifier. An increase in the concentration of methanol in the mobile phase produced an increased resolution between the two ions (Fig. 9). Upon increasing the methanol concentration, a slight decrease in the retention time of acetate and an increase in the retention time of chloride was observed. The observed decline in the retention of acetate ion can be attributed to a slight elevation in its solubility with increasing methanol. The increase retention of chloride is due to an enhanced polarity of stationary phase relative to the mobile phase. Similar results were obtained by Walker et al. [18].

Determination of the linear dynamic range of the detector. To determine the linear dynamic range of the detector, a series of solutions of sodium acetate in a range of concentrations varying from 0.00036 mg/ml to 0.07416 mg/ml was prepared and injected into the HPLC system. The results are presented in Fig. 10. A r^2 of 0.9996 was obtained, which is sufficient for the range of concentrations studied. The method was capable of detecting less than 0.00036 mg/ml.

Determination of the recovery through the Sep-Pak cartridges. An important step in the quantitation of the real samples of MK0476 consists in determining whether the acetate ion is retained on the Sep-Pak cartridge during the sample preparation process. Standard

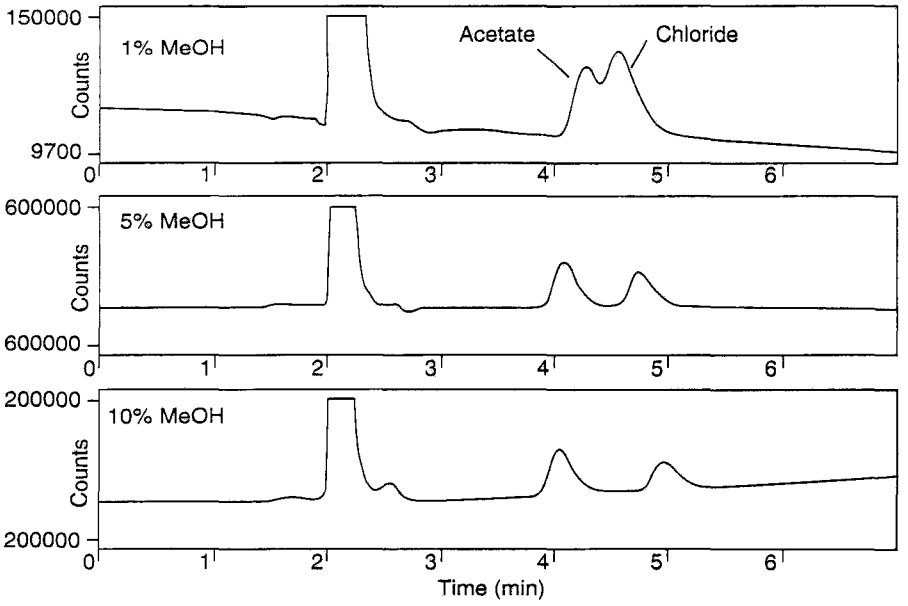

Fig 9. Influence of methanol concentration on the separation of chloride and acetate.

solutions of acetate in increasing concentrations were prepared. Half of each solution was subjected to HPLC analysis without further treatment. The remaining solutions were passed through a Sep-Pak cartridge before injection into the HPLC system. A plot was constructed to determine the recovery. Area counts obtained from the HPLC analysis without filtration were plotted on the X axis, and area counts from the analysis with filtration through the Sep-Pak cartridge were plotted on the Y axis. A linear plot was obtained (Fig. 11) with a slope of 0.93977 and a $r^2 = 0.9992$, suggesting that no or few acetate ions were retained on the cartridge.

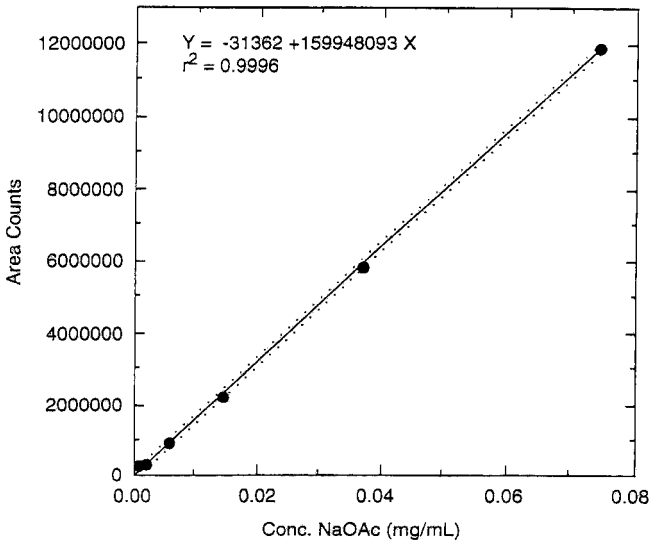

Fig. 10. Linear regression for the sodium acetate standards at 95% confidence limit.

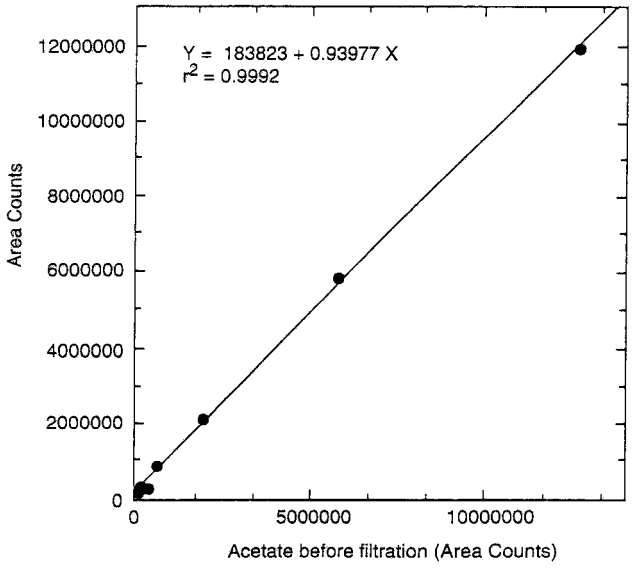

Fig. 11. Recovery of acetate through Sep-Pak cartridges.

Table 1

Results of the Analysis of Residual Acetate in MK0476*

MK0476 Batch #	%NaOAc	%RSD
MO22	0.079	0.62
MO23	0.034	2.68
MO24	0.054	1.24
MO25	0.066	0.58
MO26	0.050	0.97
MO27	0.019	1.74
MO29	0.020	7.66
MO33	0.074	0. 29
MO34	0.089	0.21
MO35	0.031	1.15

*Each sample was analyzed in triplicate

Results of final product batches. Several samples representing final product batches were analyzed for the residual acetate. The results are presented in Table 1. To determine the influence of the sample matrix on the sample recovery during the sample treatment procedure, batch

MO35 was spiked with 0.006 mg/ml solution acetate standard. The sample, the spiked sample and the 0.006 mg/ml acetate solution were subjected to the HPLC analysis according to the experimental section. A recovery of 92.3% was obtained.

III. CONCLUSIONS

A new method for the analysis of residual acetate was developed. The sample preparation provided for the removal of the final product in a way that did not interfere with the analysis of the residual acetate. The method has been demonstrated to be sensitive for the determination of less than 0.001 mg/ml of acetate with a linear range of 0.00036 to 0.074 mg/ml.

REFERENCES

1. H. Small, T.S. Stevens, and W.C. Bauman, *Anal. Chem.* ,47, 1801- 1809, 1975.

2. F.H. Spedding, A. F. Voight, E.M. Gladrow, and N.R. Sleight, J. Am. Chem. Soc. , 69, 2777-2781, 1947.

3. J.A. Marinsky, L. E. Glendenin , and C.D. Coryell, J. Am. Chem. Soc. , 69, 2781-2786, 1947.

4. E.R. Thompkins, and S.W. Mayer, J. Am. Chem. Soc. , 69, 2859-2866, 1947.

5. S. W. Moore and W. H. Stein, J. Biol. Chem., 192, 663-681, 1951.

6. P.B. Hamilton, Anal. Chem. 32, 1779-1781, 1960.

7. D. J. Gjerde, J.S. Fritz, and G. Schmukler, J. Chromatogr., 186, 509-519, 1979.

8. J. Weiss, Handbook of Ion Chromatography, Dionex Corporation, Sunnyvale, California, 148-150, 1986.

9. R.D. Rocklin and E.L. Johnson, Anal. Chem., 55, 4-7, 1983.

10. P. Edwards and K. Haak, Amer. Lab, April 1983.

11. M. Denkert, L. Hackzell, G. Schill, and E. Sjogren, J. Chromatogr., 218, 31-43, 1981.

12. H. Small and T. E. Miller, Anal. Chem., 54, 462-469, 1982.

13. R. A. Cochrane and D. E. Hillman, J. Chromatogr., 392-394, 1982.

14. S. Wilson and E. Yeung, Anal. Chim. Acta, 157, 53- , 1984.

15. N. Chauret, and J. Hubert, J. Chromatogr., 329-338, 1989.

16. T. A. Walker, T.U. Huo, and N. Akbari, J. Liq. Chromatogr., 14(7), 1351-1366, 1991.

17. S. Motomizu, M. Oshima, and T. Hironaka, Analyst, 116, 695-700, 1991.

18. T. A. Walker, T.U. Huo, and N. Akbari, J. Liq. Chromatogr., 12(7), 1213-1230, 1989.

19. R. Thompson, N. Grinberg, H. Perpall, G. Bicker, and P. Tway, J. Liq. Chromatogr., 17(11), 2522-2531, 1994.

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DETERMINATION OF TRACE METHOTREXATE AND 7-OH-METHOTREXATE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

GUANGWEI LU AND H. WON JUN*

Department of Pharmaceutics
College of Pharmacy
The University of Georgia
Athens, Georgia 30602

ABSTRACT

A sensitive and reliable HPLC method consisting of a solid-phase extraction, post-column photoreaction of the analytes in a polyethylene tubing by UV lamp, and fluorimetric detection for the simultaneous determination of methotrexate (MTX) and 7-OH-methotrexate (7-OH-MTX) in plasma was developed. The linear relationships between the peak area and MTX (0.1 ng/ml-1000 ng/ml, r=0.9997) or 7-OH-MTX (6.25 ng/ml-400 ng/ml, r=0.9995) concentrations were obtained. The average coefficients of variation for the intraday and interday replications were less than 11% and the absolute recoveries were 96.8%-101.2% for MTX and 76.4%-86.7% for 7-OH-MTX for the calibration ranges used. The limit of detection for MTX in plasma was 0.05 ng/ml. This most sensitive HPLC method reported so far was used for the trace assay of plasma MTX concentrations in dogs following a topical application of MTX in gel.

* Corresponding author

INTRODUCTION

Methotrexate(MTX), an antifolate used for the treatment of several types of cancer, has frequently been prescribed in the therapy of rheumatoid arthritis. Although the complete mechanism of MTX for the treatment of rheumatoid arthritis is yet to be known, the actions of antiinflammation and immunosuppression of this drug have been found [1, 2]. The recommended dose of MTX for the patients with rheumatoid arthritis is 10 - 20 mg per week by oral, intravenous or intramuscular administration. In order to maintain an effective therapeutic concentration of MTX in plasma for a longer duration and reduce the side effects due to a dose dumping, a transdermal formulation of MTX is being developed in our laboratory. Since the drug concentrations in plasma following transdermal application were expected to be much lower than that following oral administration due to the barrier effect of the stratum corneum, a sensitive analytical method which allows the quantitation of the drug concentration in plasma down to 10^{-11} g/ml was needed to evaluate the bioavailability and pharmacokinetic profiles of the transdermal formulation in the body. Various methods have described the determination of MTX in biological fluids which include an enzyme multiplied immunoassay [3], fluorescence polarization immunoassay [4], radioimmunoassay [5,6], capillary zone electrophoresis

[7] and HPLC [8-20]. In terms of high degree of specificity and sensitivity, HPLC provides a powerful technique for monitoring the drug concentration in biological samples. HPLC methods with UV [8-10], fluorescence [11-13] and electrochemical detection [14-16] have been reported for this compound, and various procedures for sample treatment such as protein precipitation with TCA or PCA [17], liquid extraction [18] and solid extraction [18-20] were used. Salamoun et al. [12] first reported the use of a PTFE capillary tubing for the post-column photodecomposition of MTX. Nuernberg et al.[19] described a HPLC method using a solid-phase extraction procedure and UV detection for MTX and its metabolites in biological samples. Another recently developed HPLC method using solid-phase extraction and a similar post-column photoreaction procedure with fluorescence measurement offered the detection limit down to 0.095 ng/ml [20]. Based on the work of these previous authors [12,19,20], we were able to improve the method with respect to the sensitivity and photoreaction procedures. In this paper, a very sensitive and reliable HPLC method for the quantitation of MTX and 7-OH-MTX consisting of a solid-phase extraction and fluorimetric detection following post-column photochemical degradation of the parent compounds by UV irradiation in a polyethylene tubing is presented.

EXPERIMENTAL

Chemicals

MTX (obtained from American Cyanamid Company, Pearl River, NY) and 7-OH-MTX (kindly provided by Dr. David Johns of the National Cancer Institute, Rockville, MD) were used as received. HPLC-grade acetonitrile and methanol and all the other analytical-grade chemicals used were purchased from commercial sources. The water was treated with a Millipore water purification system. Bond-Elut cartridges (100 mg) were obtained from Varian Analytical Supplies Company (Harbor City, CA).

Solid-phase extraction

Each Bond-Elut cartridge was conditioned by washing with 1 ml of methanol first and with 1.5 ml of phosphate buffer (0.05 M, pH 2.7) before the application of sample. One ml of plasma (spiked or dosed) samples was mixed with an equal volume of a phosphate buffer (0.05 M, pH 6.5), and applied to the cartridge. The flow rate was maintained approximately at 2 ml/min by a mild vacuum. The cartridge was washed sequentially with 2 ml of phosphate buffer (0.05 M, pH 2.7), 1 ml of sodium hydroxide (0.1 M) and 1 ml of the phosphate buffer. The adsorbed compounds were eluted with 1.5 ml of methanol. The eluate was evaporated to dryness under nitrogen at an ambient temperature. The residue was dissolved with 0.2 ml of the mobile phase and 0.1 ml of the aliquot was injected onto the column.

Apparatus

An Altex Model 110A pump, equipped with a Rheodyne 7125 injector and a reversed-phase column (Novapak C_{18}, 5μm, 150 × 3.9 mm I.D., Phenomenex, Rancho Palos Verdes, CA), a guard column (C_{18}, spherisorb, 30 μm, 30 x 3.2 mm I.D.), a Spectroline pencil UV lamp (254 nm) inserted in a polyethylene tubing coil (PE-20, I.D. 0.38 mm, O.D. 1.09 mm, length 3 m) which was connected between the column and the monitor for the photodecomposition of MTX and 7-OH-MTX, a Shimadzu RF-530 fluorimetric monitor and a Shimadzu C-R 3A integrator (Shimadzu Co. Tokyo, Japan) were used.

Chromatographic conditions

The mobile phase consisted of a 0.014 M of phosphate buffer (pH 6.5) containing 4% of N,N-dimethylformamide, 3.3% of acetonitrile and 0.5% of 3% hydrogen peroxide. The operation was conducted at ambient temperature and the flow rate was 1 ml/min. The fluorescence excitation and emission wavelengths were set at 350 nm and 465 nm, respectively.

Quantitation

The calibration curves for MTX and 7-OH-MTX in plasma were constructed daily by spiking blank plasma samples with known amounts of the compounds. The MTX and 7-OH-MTX concentrations in the plasma samples were obtained by comparison of the peak area of the sample chromatogram with the calibration plots.

RESULTS

Chromatogram

The typical chromatographic profiles of MTX and 7-OH-MTX in human and dog plasma were shown in Fig 1. Using the solid-extraction method, clean baselines without any interfering peaks were obtained. The retention times of MTX and 7-OH-MTX were 4.0 and 5.5 min, respectively. A chromatogram of a blank human plasma sample spiked with 0.1 ng/ml of MTX was shown in Fig 1 (C).

Linearity and limit of detection

As shown in Fig 2 and Fig 3, the responses to MTX and 7-OH-MTX concentrations in the human plasma were highly linear over the range of 0.1 - 1000 ng/ml ($r=0.9997$, $n=7$) and 6.25 - 400 ng/ml ($r=0.9995$, $n=6$) for MTX and 7-OH-MTX, respectively. The similar linearity was also obtained in the dog plama. The limit of detection which is the smallest concentration that can be distinguished from the blank (the ratio of signal/baseline noise = 3) was 0.05 ng/ml of plasma for MTX and 2 ng/ml for 7-OH-MTX when 1 ml of plasma sample was used. The CV for the quantitaton of 0.1 ng/ml of MTX in plasma was 17.3% ($n=3$).

Recovery and precision

The absolute recoveries from plasma were found to be 96.8% - 101.2% for MTX over the range of 1 ng/ml to 100 ng/ml and 76.4% - 86.7% for 7-OH-MTX over the range

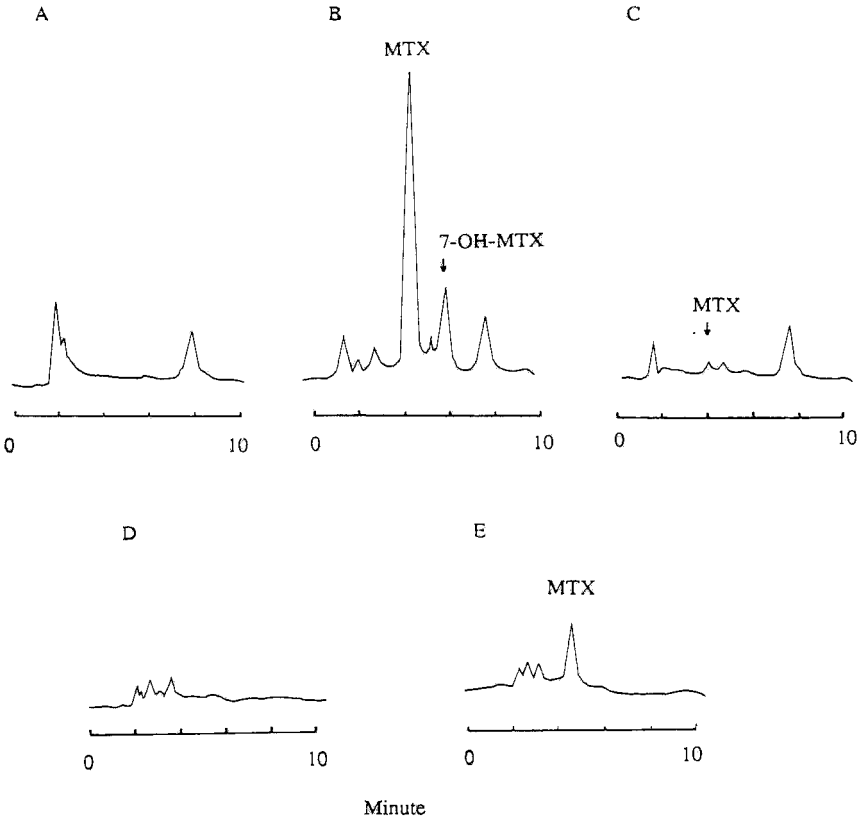

Figure 1. Chromatograms of MTX and 7-OH-MTX in human and dog plasma. A: blank human plasma; B: human plasma containing 10 ng/ml of MTX and 20 ng/ml of 7-OH-MTX; C: human plasma containing 0.1 ng/ml of MTX; D: blank dog plasma; E: dog plasma containing 1 ng/ml of MTX.

of 10 ng/ml to 100 ng/ml, as shown in Table 1.

The variation of intraday and interday assays for the determination of MTX and 7-OH-MTX were shown in Table 2. The CV's for the assays were 3.9% - 8.9% for MTX in the range of 1 ng/ml - 200 ng/ml and 5.6% - 10.8% for 7-OH-MTX in the range of 20 ng/ml - 200 ng/ml. The average

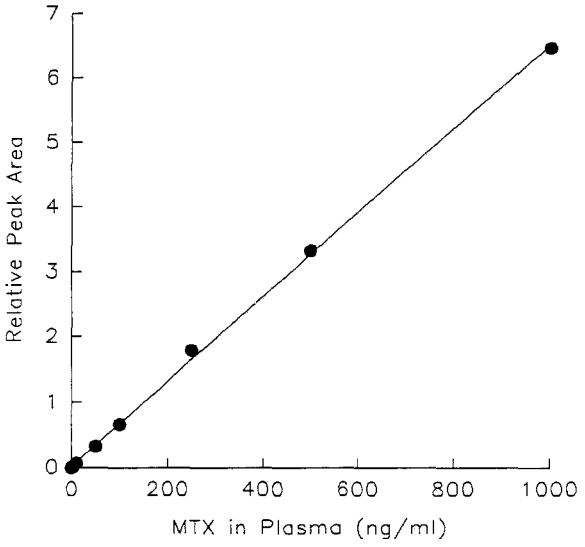

Figure 2. Standard curve for MTX in human plasma. Each point is the mean of duplicate samples. (r=0.9997)

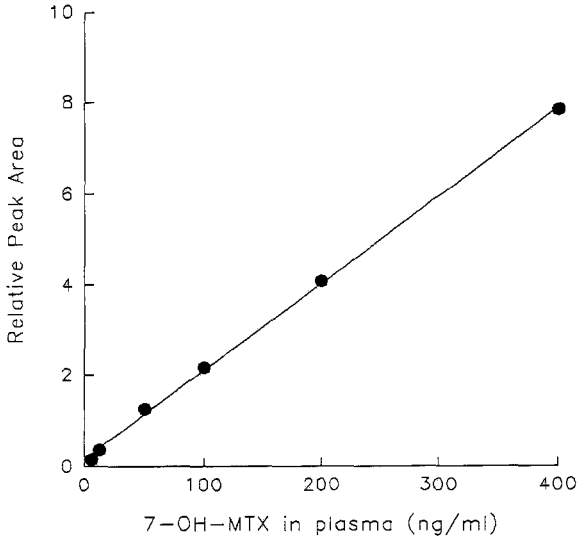

Figure 3. Standard curve for 7-OH-MTX in human plasma. Each point is the mean of duplicate samples. (r=0.9995)

TABLE 1. Recoveries of MTX and 7-OH-MTX (mean ± SD)

MTX			7-OH-MTX		
Conc. (ng/ml)	Recovery (%)	n	Conc. (ng/ml)	Recovery (%)	n
1	97.2 ± 8.1	6			
10	101.2 ± 9.1	6	20	76.4 ± 4.3	6
100	96.8 ± 3.9	6	200	86.7 ± 9.4	6

TABLE 2. Precision of the Assay for MTX and 7-OH-MTX

		conc. (ng/ml)	n	CV (%)
Intraday	MTX	1	6	8.3
		10	6	8.9
		100	6	3.9
	7-OH-MTX	20	6	5.6
		200	6	10.8
Interday	MTX	10	9	7.1
	7-OH-MTX	25	5	5.5

CVs for the determination of interday assays were 7.1% for MTX and 5.5% for 7-OH-MTX.

Effects of coil material on photoreaction

Three coils which were made with teflon (I.D. 0.88 mm, O.D. 1.75 mm, length 1 m), quartz (I.D. 1 mm, O.D. 2.5 mm, length 0.9 m) and polyethylene(I.D. 0.86 mm, O.D. 1.27 mm, length 1 m) were used to compare the efficiency of the photochemical reaction of MTX. The chromatogram with the coils made by different materials were shown in Fig 4. The coil made by polyethylene tubing was shown to

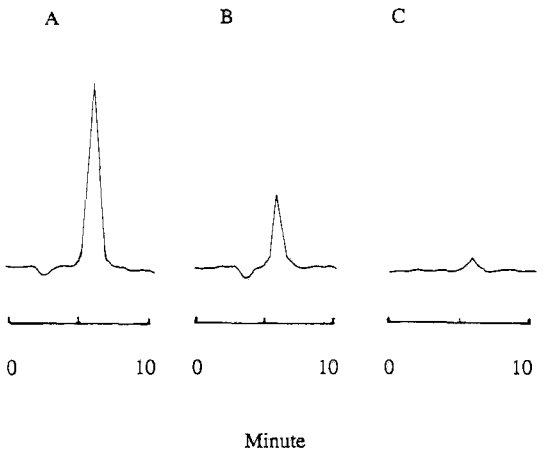

Figure 4. Chromatograms of MTX (50 ng/ml) with different coils used for the photochemical reaction. A: polyethylene tubing; B: quartz tubing; C: teflon tubing.

be the most suitable photoreaction device among the three coils tested. It was also found that the sharper chromatographic peaks for MTX and 7-OH-MTX were observed when a coil made by a thinner polyethylene tubing (PE-20 tubing, I.D. 0.38 mm, O.D. 1.09 mm) was used.

The effect of the length of PE-20 tubing on the photoreaction of these compounds was investigated. The relative peak areas (n=2) after injection of 50 μl of MTX (20 ng/ml) onto the column were 6981, 17239 and 13945 for the coils of the different length of 2 m, 3 m and 4 m, respectively. Thus the PE-20 coil with the length of 3 m was chosen for the assay of the compounds throughout this study.

Selection of the fluorescence excitation and emission maxima

The excitation wavelengths from 330 nm to 370 nm were tried for the comparison of the fluorescence intensity of the MTX products. The highest response was observed at 350 nm which was the same as the excitation wavelength used by Beck et al.[20].

The variation of the peak area of the photodegraded MTX with different emission wavelength used was shown in Fig 5. The maximum peak area was observed at 465 nm which was different from 436 nm used by Beck et al.[20].

Application

Four beagle dogs (10-14 kg) were used for the bioavalability study of a transdermal gel formulation of MTX. The hair on the two right legs were carefully shaved and the 8% of MTX gel (2.5 mg/kg) was applied on the joints (100 cm^2). The blood samples were obtained at 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 hr after the application and analyzed by the present method. The lowest concentration which was quantitated by this method was 1 ng/ml of MTX and one of the chromatograms was shown in Fig 1 (E). The steady state concentration of 47 ng/ml was reached at 4 hr after the dosing and maintained for about 30 hrs. The plasma profile was shown in Fig 6. The 7-OH-MTX concentration was undetectable in these plasma samples.

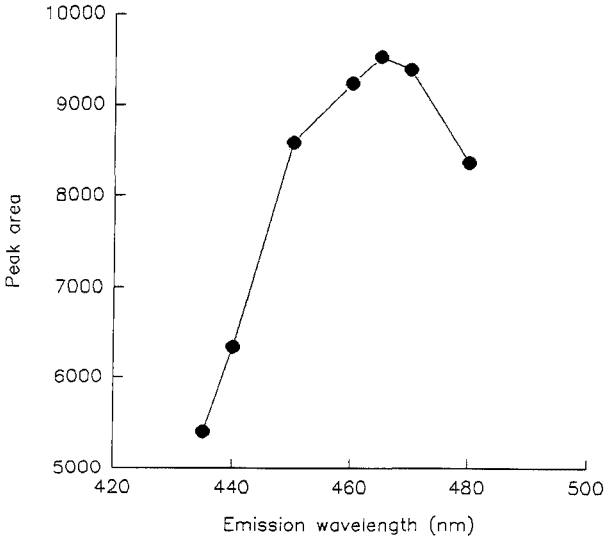

Figure 5. The effect of emission wavelength on the relative peak area of MTX (10 ng/ml).

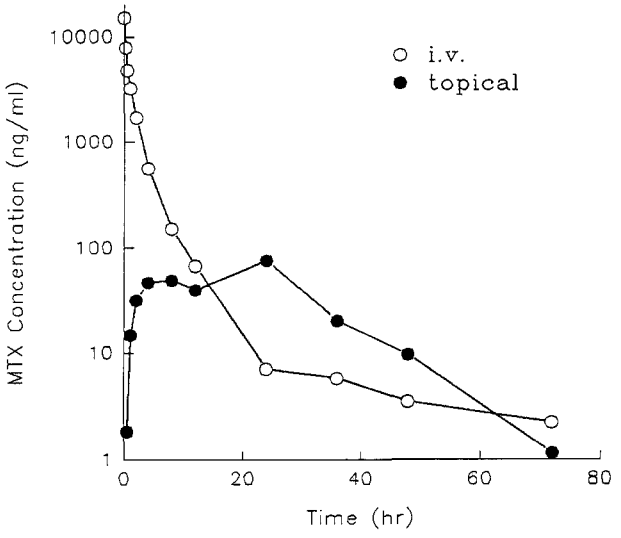

Figure 6. Plasma level versus time profiles of MTX in a dog after an i.v. injection and a topical application with 3 g of gel containing 25 MTX.

DISCUSSION

Various procedures for the sample preparation were tested. Plasma deproteinization with acetonitrile, trichloroacetic acid or perchloric acid provided a simple and fast preparation of samples, but resulted in a low recovery of the compounds and interfering peaks in the chromatogram, especially at the lower concentration range used. The solid-phase extraction method was more effective not only in terms of removing the interfering peaks but also with respect to the specificity and sensitivity of the assay. The results observed in this study were generally in agreement with those reported in the literature [17,19,20]. As compared to the solid-phase extraction reported by Nuernberg et al.[19], the current method used a smaller Bond-Elut cartridge (100 mg) which required smaller volumes of the liquids for extraction, and therefore a shorter sample preparation time. In addition to the reduced expenses for the cartridges and solvents used, excellent linear relationships between the analyte concentrations and peak areas over a wide range of concentration were obtained.

The major photodegradation product of MTX which shows an intense fluorescence was reported to be 2,4-diaminopteridine-6-carboxaldehyde [12]. The post-column photodecomposition of MTX and 7-OH-MTX to the fluorescent products was achieved by the UV irradiation of the samples in the polyethylene tubing coiled around the UV

lamp which was connected between the analytical column and the detector. The effect of the tubing materials on the photoreaction of the compounds was examined and the efficiency of photodegradation among the polyethylene, teflon and quartz tubing was shown in Fig 4. The smallest peak was observed when the teflon tubing was used which was probably due to a limited penetration of the UV light through the walls of teflon tubing. When the quartz tubing was used for the photoreaction, the fluorescence response of the MTX product was only a one-third of that of the polyethylene tubing. The high fluorescence intensity observed for the MTX degradation with the polyethylene tubing indicated that not only the UV light sufficintly penetrated through the walls of polyethylene tubing, but also effectively induced the photochemical reaction of the analytes in the coil. The efficiency of the polyethylene coil for the photoreaction appeared to remain unaffected during 250 hr of use. The peak heights for 50 μl sample of MTX (50 ng/ml) were 2.32 ± 0.2 cm for the new coil and 2.45 ± 0.18 cm for the old coil which was used for about 250 hr. Although either the polyethylene or quartz tubing could be used for the post-column photoreaction of MTX and 7-OH-MTX, the former was chosen because of its availability, low cost and easy handling.

The maximum fluorescence intensity of MTX was obtained at the excitation wavelength of 350 nm and the

emission wavelength of 465 nm [20]. At this emission wavelength, it was found that nearly 40% increase in the fluorescence response was observed as compared to the emission wavelength of 435 nm previously used. A chromatogram of a plasma sample containing 0.1 ng/ml of MTX was shown in Fig 1 (c). A small but sharp and symmetrical peak for the MTX degradation product was observed at this low concentration. Using this method we have determined the bioavailability of the MTX transdermal gel applied in dogs, which was approximately 12% of the dose applied for the first 72 hrs. Fig 6 shows the plasma level versus time profiles of MTX for the i.v. and transdermal gel formulation.

In conclusion, a very sensitive, reliable and economical HPLC method for the determination of MTX in plasma using a solid-phase extraction, post-column photoreaction of the analytes in a polyethylene tubing by UV irradiation, and fluorimetric detection was presented. Both MTX and its main metabolite, 7-OH-MTX, were quantitated simultaneously with high accuracy and precision. The method was used for the analyses of MTX in the dosed plasma samples of dogs.

REFERENCES

1. J.E. Stevens and D.A. Willoughby, J. Phathol., 97(1969) 367-373.

2. D. Mathers and A.S. Russell, Methotrexate, In: J.S. Dixon and D.E. Furst (eds.), Second-line agents in the

treatment of rheumatic diseases, Marcel Dekker, Inc., New york, p301.

3. C.De Porcer-Morton, J. Vhuang, M. Specker and R. Bastiani, Performance Evaluation of EMIT Methotrexate Assay, Syva,Palo Alto, CA. November 1978.

4. M.A. Pesce and S.H. Bodourian, Ther. Drug Monit., 8(1986) 115-121.

5. V. Raso and R. Schreiber, Cancer Res., 35(1975) 1407-1410.

6. C. Bohuon, F. Duprey and C. Bondene, Clin. Chim. Acta, 57(1974) 263-267.

7. M.C. Roach, P. Gozel and R.N. Zare, J. Chromatogr., 426 (1988) 129-140.

8. A.E. Yazigi and R.M. Cazemiro, J. Liq. Chromatogr., 7(1984) 1579-1591.

9. H.J. Ahr, D. Becker and H.E. Keller, Anal. Chem., 318 (1984) 251-252.
10. N. So, D.P. Chandra, I.S. Alexander, and V.J. Webster, J. Chromatogr. 337 (1985) 81-90.

11. J.A. Nelson, B.A. Harris, W.J. Decker and D. Farquhar, Cancer Res., 37 (1977) 3970-3973.

12. J. Salamoun and J. Frantisek, J. Chromatogr., 378 (1986) 173-181.

13. J. Salamoun, M. Smrz, F. Kiss and A. Salamounova, J. Chromatogr. 419 (1987) 213-223.

14. B.K. Birmingham and D.S. Greene, J. Pharm. Sci., 72 (1983) 1306-1309.

15. F. Palmisano, T.R.I. Cataldi and P.G. Zambonin, J. Chromatogr., 344 (1985) 249-258.

16. A.C. Garcia, A.J.M. Ordieres, M.A.G. Fernandez and P.T. Blanco, Anl. Proc. 22 (1985) 349-350.

17. O.V. Tellingen, H.R.V.D. Woude, J.H. Beijnen, C.J.T.V. Beers and W.J. Nooyen, J. Chromatogr., 488 (1989) 379-388.

18. M. Stout, Y. Ravindranath and R. Kauffman, J. Chromatogr., 342 (1985) 424-430.

19. B. Nuernberg, M. Kohlbrenner, R. Faulkner and D.E. Furst, J. Chromatogr., 487 (1989) 476-482.

20. O. Beck, P. Seideman, M. Wennberg and C. Peterson, Ther. Drug Monit., 13 (1991) 528-532.

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A RAPID HPLC METHOD FOR THE DETERMINATION OF FREE FUCOSE IN URINE. A MARKER OF MALIGNANCY IN CHILDREN

S. C. FLEMING, M. GILL, AND M. F. LAKER
Department of Clinical Biochemistry
University of Newcastle upon Tyne
Framlington Place
Newcastle upon Tyne NE2 4HH, United Kingdom

ABSTRACT

The concentration of free fucose in urine has been shown to be elevated in adults with malignancy. Current analytical methods include enzymatic analysis and high pressure liquid chromatography (HPLC) with fluorescence detection. We describe a simple HPLC method using anion exchange chromatography with pulsed electrochemical detection for the determination of free fucose in urine of children with malignancy. Separation of sugars is achieved within 7 minutes. The method is linear to 200 μmol/l fucose with a detection limit of 0.5 μmol/l in urine. Mean recovery was 96.6 % ± 11.8%. Precision was good with coefficient of variation (CV) of 4.3% (25 μmol/l), 3.7% (50 μmol/l) and 4.9% (100 μmol/l). The median fucose : creatinine ratio in healthy children was 13.6 μmol / mmol (5.5 - 128.5). Children with malignancy had a median fucose : creatinine ratio of 8.1 μmol / mmol (2.6 - 66.4) which was significantly lower than the control subjects (p=0.038). The method enables rapid determination of free fucose in urine with minimal sample preparation and without the need for derivatization. Urine free fucose was lower in children with malignancy than in their age-matched controls and requires further investigation.

INTRODUCTION

L-fucose is a monosaccharide found at the non-reducing end of oligosaccharides on glycoproteins and is important in their function. It has been reported that plasma and urinary free fucose concentrations are elevated in patients with diabetes mellitus, cystic fibrosis and a variety of malignancies [1-6]. Increased excretion of fucose has been demonstrated in a wide range of cancers including liver, pancreatic, breast and oesophagus [5,6].

Analytical methods used for the assay of fucose include enzymatic [4,5] and high pressure liquid chromatography [6]. The HPLC method of Suzuki et. al. [6] employs fluorescence detection with the derivatization of fucose with 2-aminopyridine. This requires time-consuming sample preparation coupled with an elution time of 100 minutes for each sample.

The use of anion exchange chromatography together with pulsed electrochemical detection [7] allows the sensitive estimation of sugars within biological matrices without the need for extensive sample preparation or derivatization [8].

It has been postulated that elevated urinary fucose excretion may provide a suitable marker for some forms of cancer. We have applied this technique to investigate the concentration of urinary free fucose in children with malignancy, a group not previously studied.

MATERIALS

Fucose,3-O-methyl glucose, mannitol, dulcitol, arabinose and α-methyl glucose were obtained from Sigma Chemical Co. (Poole, UK.) Sodium hydroxide (500g/l), Amberlite IR 120 H^+ and IRA 400 Cl^- were obtained from BDH. (Poole, UK.) Deionized water (18Mohm cm^{-1}) was prepared by an in-house deionizer (Millipore, MA, USA).

Equipment

High pressure liquid chromatography equipment was composed of a quaternary gradient pump, Carbopac PA1 40mm x 250 mm anion exchange column with associated guard column, and pulsed electrochemical detector all supplied by Dionex UK Ltd. (Camberley, UK). Signal recording was provided by a Dionex 4100 integrator.

Subjects

Samples of urine from 35 healthy subjects (21 female, 14 male) age range 1-17 years and 22 cancer patients (7 female, 15 male) age range 4-21 years, were obtained. All samples were taken fasting and stored at -20^{o}C prior to analysis. Ethical permission was granted for the study by the local hospital ethical committee.

METHODS

Sample preparation

Samples of urine were diluted 1 in 10 with deionized water to a total volume of 1ml. One millilitre of the internal standard, 3-O-methyl glucose (100 μmol/l) was added. The mixture was desalted with mixed ion-exchange resin (IR 120 H^{+} and IRA 400 Cl^{-} in a mass ratio 1:1.5). After de-salting the mixture was centrifuged and 25 μl of supernatant was injected onto the chromatographic system.

HPLC analysis

Samples were eluted with 50mM NaOH at 1 ml /min at 20^{o}C, with an analysis time of 7 minutes. Following this the column was washed with 1M NaOH for 5 min, to ensure stability of retention times, and then was re-equilibrated

for a further 8 min in 50mM NaOH. The total time between injection of samples was 20 minutes. (Figure 1.)

Detection was by integrated amperometry using a gold working electrode with a Ag/Ag Cl reference electrode. The following potentials were utilised: detection potential +0.1V (0-0.5s) : oxidation potential +0.75V (0.5-0.65s) : reduction potential -0.75V (0.65-0.75s) with an integration period of 0.05-0.5s. Quantification was by peak height analysis with internal standardisation.

Creatinine estimation

Creatinine concentration in the undiluted urine samples was measured by alkaline picrate method as adapted for an automated discrete analyser. (Hitachi 717).

RESULTS

Complete resolution from other closely related monosaccharides and sugar alcohols was achieved using 50mM NaOH as eluent in an isocratic mode. (Figure 1)

A range of standards in deionized water was analysed from 7.8 μmol/l to 1mmol/l, with internal standardisation. The method was found to be linear to 200 μmol/l fucose. The detection limit of the assay in urine was 0.5 μmol/l.

Analytical Recovery

To assess recovery three urine samples from control subjects were assayed ten times to determine the endogenous fucose concentration. Each neat urine was diluted 1:5 with deionized water and a known concentration of fucose added at three levels (100, 50 and 25 μmol/l) to each urine. The 'spiked' samples were analysed as previously described and the measured concentration

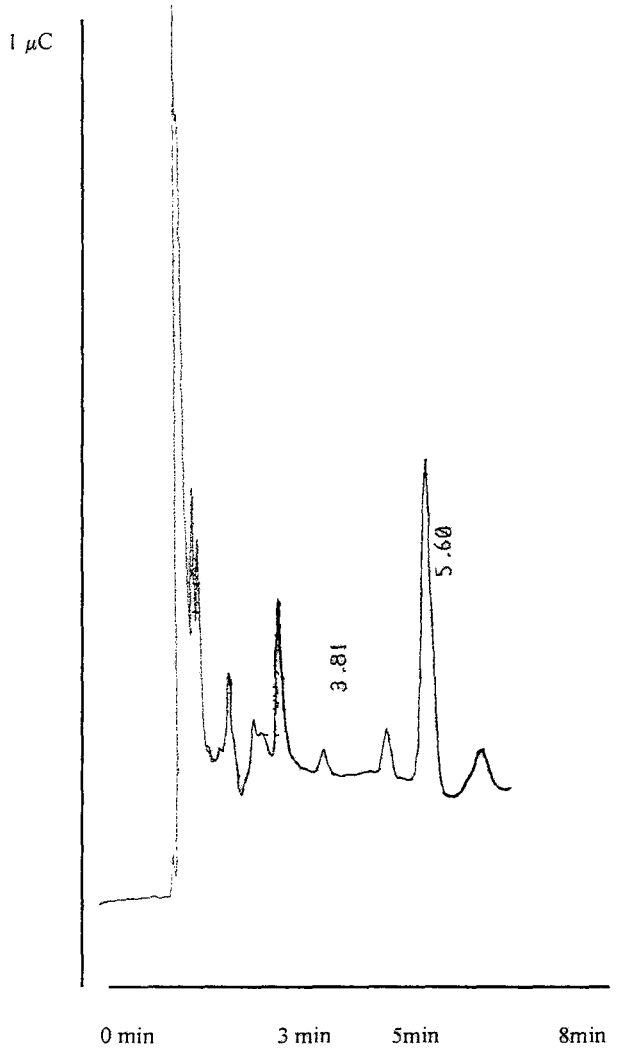

Figure 1. Chromatogram from a child with malignancy. Fucose =3.81 mins. 3-O methylglucose (internal standard) = 5.60 mins. Full scale deflection = 1μC

Table 1.
Analytical Recovery of Fucose in Urine.

Sample	Amount Added	Total (μmol/l)	% Recovery
Basal Concentration			
1. 4.2 μmol/	25μmol/l	24.8	85%
	50μmol/l	39.0	72%
	100μmol/l	99.0	95%
2. 16.5 μmol/l	25μmol/l	41.5	100%
	50μmol/l	69.8	105%
	100μmol/l	109.5	94%
3. 4.8 μmol/l	25 μmol/l	31.0	104%
	50 μmol/l	58.6	107%
	100μmol/l	113.2	108%

Overall mean recovery : 96.6% ± 11.8% (sd)

was compared to the theoretical value and the percentage recovery calculated. The results are summarised in table 1.

Precision

Imprecision of the assay was assessed by repeatedly analysing samples of known fucose concentration at three separate levels and the results are summarised in Table 2. The overall coefficient of variation did not exceed 5% at any concentration and was considered acceptable for this type of assay.

Measurement of Fucose in Urine

Fucose was measured in urine of 35 healthy children by the methods described previously. Fucose concentrations were corrected for creatinine

Table 2.
Estimate of Imprecision of fucose measurement in urine

Mean Fucose	Standard Deviation	CV %
29.9μmol/l	1.3	4.3%
62.9 μmol/l	2.4	3.7%
85.1 μμoλ/l	4.2	4.9%

concentration within each sample to yield a fucose : creatinine ratio for each child. Fucose : creatinine ratios ranged from 5.5 μmol/mmol to 128.5 μmol / mmol with a median ratio of 13.6 μmol/mmol. Forty nine samples analysed from 21 children with malignancy (7 female, 14 male) showed a median fucose : creatinine ratio of 8.1 μmol/mmol. (range 2.8 to 66.4 μmol/mmol) This was significantly lower than in the control group (p= 0.038 Mann Whitney U Test). Creatinine concentrations in urine did not differ significantly between the two groups, and thus the drop in the ratio could be attributed to the fall in the fucose concentration in the subjects with malignancy.

DISCUSSION

In a previous study by Suzuki et al [6] urinary free fucose concentrations were found to be elevated in adults with various types of malignancy. Both enzymatic and HPLC methods for the determination of free fucose in urine have been described [4-6]. The method described in this paper utilises the potential of anion exchange chromatography coupled with pulsed amperometric detection. Separation from other compounds in urine is achieved within a short analysis time, and thus many samples can be analysed within one working day. Sample preparation is simple and requires no extensive purification procedures.

The use of pulsed amperometric detection allows for sensitive and specific detection of fucose without the need to resort to fluorescene derivatization. Urinary free fucose concentrations in children were found to be lower in subjects with malignancy when compared to healthy controls. This is in contrast to the findings in adults [6]. Little is known about the course of metabolic events during malignancy, particularly in relation to glycoproteins, however there is little to suggest that metabolism should differ so markedly between adults and children.

The method described here allows rapid, sensitive and specific measurement of free fucose in urine and will allow the further investigation of both children and adults with malignancy.

REFERENCES

1. A.M. Marshall, R.J. Georges, B.F. Clarke, L.G. Whitby, L.J.P. Duncan. Metabolism 19:114-119 (1970).

2. C.B. Howard, P.C. Kelleher. Clin Chim Acta 31: 75-80 (1971)

3. H Kollberg, A. Lundeblad, G Ekbohm. Acta Paedriatr. Scand 62 : 279-82 (1973)

4. M Endo, H. Matsui, S. Sato, M Majima, N. Hiyama Clin Chim Acta 103 : 269-275 (1980)

5. T. Sakai, K. Yamamoto, H. Yokata, K. Hakozaki-Usui, F. Kino, I. Kato. Clin Chem. 35 : 474-476 (1990)

6. J. Suzuki, A. Kondo, I. Kato, S. Hase, T. Ikenada. Clin Chem 38 : 752-755 (1992)

7. R.D. Rocklin, C.A. Pohl. J. Liq. Chromatogr 6 : 1577-1590 (1983)

8. S.C. Fleming, M.S. Kapembwa, M.F. Laker, G.E. Levin, G.E. Griffin. Clin Chem 36 : 797-799 (1990)

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PURIFICATION OF 25-HYDROXY-CHOLECALCIFEROL FROM IRRADIATION OF CHOLESTA-5,7-DIOL BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

QIZHEN DU[1], TIAN-YOU ZHANG[2], AND YOICHIRO ITO[3]

[1]Tea Research Institute
Chinese Academy of Agricultural Sciences
Hangzhou, Zhejiang 310008
The People's Republic of China
[2]Beijing Institute of New Technology Application
Beijing, 100035
The People's Republic of China
[3]Laboratory of Biophysical Chemistry
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, Maryland 20892

ABSTRACT

High-speed countercurrent chromatography (HSCCC) was used for the separation of 25-hydroxycholecalciferol (25-HCC) from a post-irradiation mixture of cholesta-5,7-diene-3ß,25-diol. With a two-phase solvent system composed of hexane/ethyl acetate/methanol/water (5:1:5:1, v/v/v/v), 500 mg of reaction mixture containing 245 mg of 25-HCC was separated with 207 mg of the product obtained at a purity of 97.7%. The separation was completed within 2 hours.

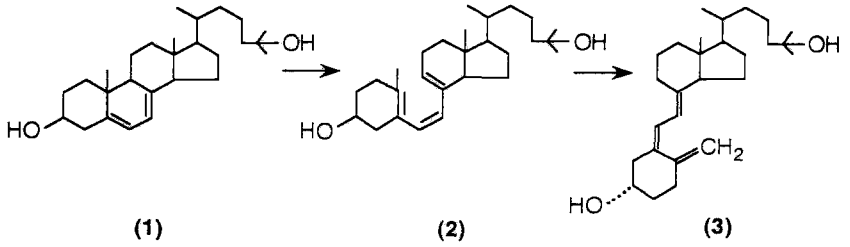
INTRODUCTION

25-Hydroxycholecalciferol (25-HCC) is a major circulating metabolite of vitamin D_3. This metabolite was shown to be more potent than vitamin D_3 itself for curing rickets and is able to stimulate calcium transport faster than a similar amount of vitamin D_3 (1). The conventional method of synthesizing 25-HCC utilizes radiation of cholesta-5,7-diene-3ß,25-diol (Fig. 1), followed by purification of the products by silicic acid column chromatography (2-4). However, we found this technique results in considerable loss of the product on the silicic acid column. In order to alleviate this problem, we used high-speed countercurrent chromatography (HSCCC) because it eliminates a sample loss from this source (5).

EXPERIMENTAL

Apparatus

HSCCC experiments were performed using a coil planet centrifuge equipped with a multilayer coil separation column that was designed and fabricated at the Beijing Institute of New Technology Application, Beijing, P. R. China. The multilayer coil was prepared by winding a 1.6mm ID PTFE (polytetrafluoroethylene) tube coaxially



(1) (2) (3)

Figure 1. Irradiation reaction of cholesta-5,7-diene-3ß,25-diol. Compound 1: cholesta-5,7-diene-3ß,25-diol; Compound 2: 25-hydroxycholecalciferol (25-HCC); Compound 3: 25-hydroxyprecholecalciferol.

onto the column holder hub. The total column capacity measured 230 ml. The HSCCC centrifuge was rotated at 800 rpm with an 8cm revolution radius. The system was equipped with an FMI pump (Zhejiang Instrument Factory, Hangzhou, P.R. China), a variable wave-length UV detector (UV-752, Shanghai Analytical Instrument Factory, Shanghai, P.R. China), a recorder and a sample injection valve.

Reagents

All organic solvents were of analytical grade and purchased from Shanghai Chemical Factory, Shanghai, P.R. China. Cholesta-5,7-diene-3ß,25-diol and standard 25-hydroxycholecalciferol (25-HCC) were gifts of Hangzhou Mingsheng Medicine Factory, Hangzhou, P.R. China.

Irradiation of Cholesta-5,7-diene-3ß,25-diol

In each treatment, 1000 mg of cholesta-5,7-diene-3ß,25-diol dissolved in 1L of diethyl ether was irradiated in a double-walled, water-cooled jacketed quartz immersion well. A high-pressure quartz mercury vapor lamp, Model 1000 (Yixin Optics Instrument Factory, Yixin, P.R. China), was lit 1 minute before placing the solution in the immersion well. During 1.5 h of irradiation the ether was constantly flushed and stirred with nitrogen. The reaction solution was then evaporated to dryness in a rotary evaporator at room temperature.

HSCCC Procedure

The HSCCC experiment was performed with a two-phase solvent system composed of hexane/ethyl acetate/methanol/water (5:1:5:1, v/v/v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use. In each separation, the multilayer coil column was first entirely filled with the upper stationary phase. Then the lower mobile phase was pumped into the inlet of the column at a flow rate of 2.0 ml/min, while the apparatus was rotated at 800 rpm. After the mobile phase front emerged and the two phases had established

hydrodynamic equilibrium in the column, the sample solution, containing 500 mg of the reaction mixture in 10 ml of the mobile phase, was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 280 nm and the fractions collected (Fig. 3).

HPLC Analysis

HPLC analysis of the reaction mixture and CCC fractions were performed with Waters HPLC equipment (Waters Associates/Millipore Chromatography Co., Milford, MA, USA) consisting of a Model 510 pump, a Model 717 auto-injector, a Model 996 PDA detector and a Millennium 2010 data processor. HPLC separations were performed on a μ-Bondapak C_{18} column, 0.46 x 25 cm ID. (Waters Associates). The mobile phase, composed of methanol-water (90:10, v/v), was isocratically eluted at 1 ml/min and the effluent was monitored at 280 nm.

RESULTS AND DISCUSSION

HPLC analysis of the post-radiation reaction mixture (Fig. 2) shows five major components three of which are: 25-HCC (R.T.: 7.08 min)(49.0% of the total), cholesta-

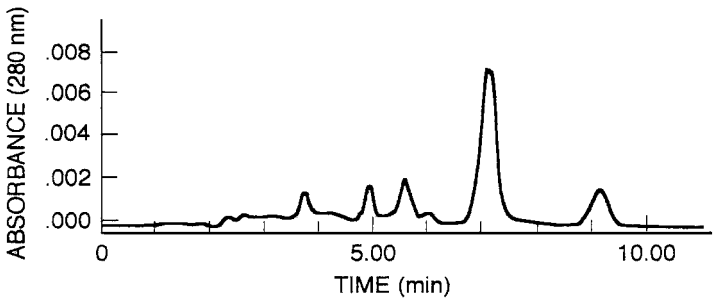


Figure 2. HPLC analysis of the reaction mixture. Retention time: cholesta-5,7-diene-3ß,25-diol (Compound 1): 5.57 min; 25-HCC (Compound 2): 7.08 min; and 25-hydroxyprecholecalciferol (Compound 3): 9.12 min.

5,7-diene-3ß,25-diol (R.T.: 5.57 min) and 25-hydroxyprecholecalciferol (R.T.: 9.12 min). The HSCCC separation of the reaction mixture is shown in Fig. 3. A 500mg amount of the sample was separated into peaks A, B and C in a relatively short elution time of 110 min. The fractions corresponding to peak B contained 25-HCC as confirmed by the HPLC analysis with the standard sample. Peak B fractions were combined, dehydrated by $MgSO_4$, and evaporated to dryness in rotatory evaporator. The 25-HCC present in the residue was extracted with dichloromethane which was in turn evaporated in vacuum. This yielded a net amount of 204 mg of 25-HCC of 97.7% purity as determined by HPLC analysis.

Separation of the reaction mixture by silicic acid column chromatography performed according to the method described in refs. 2-4 resulted in 148mg of the product

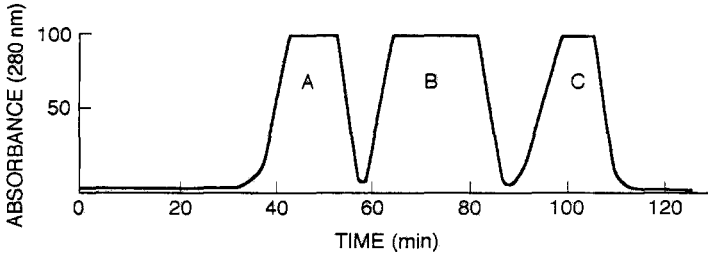


Figure 3. HSCCC separation of the reaction mixture. Peak B corresponds to 25-HCC (Compound 2) and peak C to 25-hydroxyprecholecalciferol (Compound 3).

and the purity of the product was 90-94 % as determined by HPLC. This necessitated recrystallization of the products with additional sample loss to raise the purity to the 97%, requirement of the Federal Drugs Administration of P.R. China. In the present HSCCC method, the loss of the product is no more than 17.5% of the total (HPLC) while purity of the product was over 97%.

We believe that pharmacy-scale production can be realized by the use of multiple sets of HSCCC instruments, since an adult daily dose of 25-HCC is only 20 micrograms (6).

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REFERENCES

1. Andrews, D.R., Barton, D.H.R., Hesse, R.H. and Pechet, M.M., Synthesis of 25 hydroxycholecalciferol and 1α, 25-dihydroxyvitamin D_3 from vitamin D_2, J. Org. Chem., 51, 4819, 1986.

2. Blunt, J.W. and Deluca, H.F., The synthesis of 25-hydroxycholecalciferol, a biologically active metabolite of vitamin D_3, Biochem., 8, 671, 1969.

3. Suda, T., Deluga, H.F. and Hallick, R.B., Synthesis of [26,27-^{3}H]-25-hydroxycholecalciferol, Anal. Biochem., 43, 139, 1971.

4. Campbell, J.A., Squires, D.M. and Babcock, J.C., Synthesis of 25-hydroxycholecalciferol, the biologically effective metabolite of vitamin D_3, Steroids, 13, 567, 1969.

5. Ito, Y., High-speed countercurrent chromatography, CRC Crit. Rev. Anal. Chem. 17, 65, 1986.

6. Thing, M.J., Peter, B. and Lise, B., Toxicity of vitamin D analogs in rats fed diets with standard or low calcium contents, Pharmacol, Toxicol., 72, 124, 1993.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF HISTAMINE, ITS PRECURSOR, AND METABOLITES: APPLICATION TO BIOLOGICAL SAMPLES

K. HERMANN, G. FRANK, AND J. RING
Department of Dermatology
University Clinic Eppendorf
University of Hamburg
Hamburg, Germany

ABSTRACT

Histamine was separated from its precursor L-histidine and its metabolites 1-methylhistamine and methylimidazole acetic acid on a TSK SP-5 PW cation exchange column and gradient elution. A baseline separation of histamine, 1-methylhistamine and methylimidazole acetic acid was also achieved under isocratic elution conditions on a reversed phase C_8 column with a mobile phase of 15 % methanol and 85 % of an aqueous solution of 0.05 M NaH_2PO_4 pH 3.1 which contained 0.5 mM EDTA-Na_4 and 0.005 M octan-1-sulfonic acid sodium salt as an ion paring reagent. The most sensitive UV detection of histamine and 1-methylhistamine with the highest detector signal was obtained at a wavelength of 210 nm. In the absence of the ion pairing reagent and the organic modifier methanol, histamine and 1-methylhistamine were not retained on a reversed phase C_{18}, C_8 or C_4 column. Octadecasilyl-silica cartridges were used to purify histamine from other constituents present in human urine and a commercially available heparin formulation. The analytical recovery of ^3H-labeled histamine after the purification on octadecasilyl-silica cartridges was 95.16 ± 0.92 % (mean ± SEM, n=22). The concentration of the

*Correspondence to (current address): Klaus Hermann Ph.D., Sanofi Diagnostics Pasteur, 1000 Lake Hazeltine Drive, Chaska, MN 555318, USA. Tel: (612) 368-7616; Fax: (612) 368-1397.

histamine-like material in urine samples from healthy volunteers was 46.28 ± 15.42 µg/24h (mean ± SEM; n=7). In the heparin formulation the histamine concentrations were 81.93 ng/ml before and 279.24 ng/ml after the purification on octadecasilyl-silca cartridges. The histamine-immunoreactive material in urine samples could be characterized on a TSK SP-5 PW cation exchange column as 19.69 ± 7.86 % histamine and 46.74 ± 12.89 % 1-methylhistamine (mean ± SEM, n=7). Rechromatography of the 1-methylhistamine peak from the ion exchange column on the reversed phase C_8 column disclosed a substance of unknown nature with a different retention time than 1-methylhistamine. Histamine purified from heparin eluted from the ion exchange column as a single peak with the same retention time as the histamine standard.

INTRODUCTION

The biogenic amine histamine (H) is predominantly found in mast cells and basophil leukocytes. Besides its numerous biological effects on blood vessels, the heart, the bronchial tract and the stomach, H is an important mediator of immediate type hypersensitivity and a marker for allergic reactions (1-4). The determination of histamine in blood, serum, urine or cell cultures as a diagnostic parameter is therefore not only of scientific interest but also of significance for the routine allergy practice. Numerous analytical procedures such as radioenzymatic (5), photometric (6) or gas chromatographic-mass spectrometric assays (7) have been developed for the qualitative and quantitative measurement of H in plasma, urine and cerebrospinal fluid. These approaches are either tedious or require expensive equipment and specially trained personal. Likewise, radioimmunoassays (RIA) have been introduced to determine H in urine, whole blood and cell supernatants after in-vitro H-release (8-10). Although the radioimmunological determination possess high sensitivity and specificity, crossreactions with H-metabolites, the H-precursor or other components may occur. Therefore, other procedures such as HPLC are needed for the accurate evaluation of the radioimmunological data.

In this study we report the use of HPLC to separate H from its precursor L-histidine and its metabolites 1-methylhistamine (MH) and methylimidazole acetic acid (MIAA) using ion exchange and reversed phase paired ion chromatography. The influence of C_{18}, C_8 and C_4 reversed phase columns and penta-1- and octan-1-sulfonic acid as ion pairing reagents upon the resolution of H from MH and the effect of different wavelengths on the detector signal was investigated. Octadecasilyl-silica gel (ODS) cartridges were used to purify H in urine and heparin. HPLC in combination with a specific radioimmunoassay (RIA) for H was applied to measure and characterize H in partially purified human urine samples and a commercially available heparin formulation.

EXPERIMENTAL

High performance liquid chromatography (HPLC)

An ERC HPLC equipment model ABEO 88 (ERC, Alteglofsheim, FRG) was used. All separations were performed at room temperature. The mobile phases were passed through a RC 55 membrane filter (Schleicher and Schüll, Dassel, FRG) prior to use.

Ion exchange chromatography

Ion exchange chromatography was carried out on a cation exchange TSK SP-5 PW column (75 x 7.5 mm, TasoHass, Stuttgart, FRG) with gradient elution. The mobile phases were solution A: HPLC water and solution B: 0.5 M NaH_2PO_4 solution in HPLC water, adjusted to pH 5.0 with concentrated phosphoric acid. Initially, 10 % of solution B corresponding to 0.05 M was delivered for 9 min followed by an exponential increase in solution B to 60 % corresponding to 0.325 M for 16 min. At 25 min the column was flushed isocratically with 60 % of solution B. The column was equilibrated under the initial conditions for 20 min. The separations were carried out at a flow rate of 0.6 ml/min. The column was calibrated with H, MH, L-histidine and MIAA using UV detection at 210 nm and a sensitivity of 0.1 absorbance units full scale (AUFS).

Paired ion chromatography

Paired ion chromatography was conducted on a reversed phase C_{18}, C_8 or C_4 column (Nucleosil C_{18}, C_8, C_4, 300 Å , 7 µm, 250 x 4 mm, Macherey and Nagel, Düren, FRG). The column was developed isocratically at a flow rate of 1.0 ml/min. The mobile phase was 15 % methanol and 85 % of an aqueous solution of 0.05 M NaH_2PO_4 pH 3.1 which contained 0.5 mM EDTA-Na_4 and 0.005 M pentan-1-sulfonic acid or octan-1-sulfonic acid sodium salt. The column was calibrated with MIAA, L-histidin, H and MH using UV detection at 205, 210, 215, 225 and 254 nm and a sensitivity of 0.1 AUFS.

The standard substances were dissolved in 0.01 N HCl to yield a concentration of 1 mg/ml. For all separations the injection volume of each standard was 1-2 µl corresponding to 1-2 µg.

Radioimmunoassay (RIA)

RIA for H was carried out using a test kit delivered by IBL (Immuno Biological Laboratories, Hamburg, FRG). Briefly, 0.1 ml samples or standards were incubated for 30 min at room temperature with an acylation reagent to form acyl-H. Then 0.05 ml ^{125}I-labeled H and 0.05 ml anti-H-antiserum were added and the mixture was incubated over night in the refrigerator at 6-8°C. The next day 1.0 ml of second antibody solution was added. After incubation at room temperature for 15 min the precipitate was spun for 15 min at 2 000 x g and 20°C. The supernatant was aspirated and the pellet was counted in a gamma counter. The crossreactivity of the antibody with acyl-H was 100 % and with MH

0.01 %. L-histidine, imidazole acetic acid, MIAA, serotonin and 5-hydroxyindole acetic acid did not crossreact with the antibody.

Collection of urine samples

Urine was collected in individual fractions over 24 h from 7 healthy volunteers, 4 females and 3 males, mean age 30 ± 6 years. Each time the individual had to urinate the urine was collected in a 1 L plastic flask. The time and the volume was recorded. From the total urine volume 40 ml were poured from the 1 L flask into a 50 ml plastic tube which contained 0.32 ml of 25 % HCl. The samples were thoroughly mixed and stored at -80°C.

Purification of histamine from urine and heparin

H was purified from urine using octadecasilyl-silica cartridges (ODS, Sep Pak C_{18}, Millipore Waters, Eschborn, FRG). The cartridges were first rinsed with 3 ml methanol followed by 3 ml 0.01 N HCl.

Urine samples were thawed at 4°C and centrifuged for 10 min at 1 000 x g and 4°C. The pellets were discarded and the clear supernatants of the urine samples were further processed. The distinct urine samples collected over 24 h from the 7 volunteers were pooled to obtain a 24 h urine pool of the 7 volunteers. From the 7 pooled urine samples, 2 ml of the clear supernatants were applied to the primed cartridges and the flow through of the cartridges were collected (fraction # 1). The cartridges were washed 3 times with 1 ml 0.01 N HCl and the eluates of the 3 washing steps were collected in single fractions (fraction # 2 to 4). The samples (fractions # 1 to 4) were dried in a vacuum centrifuge. The dried residues, fractions 1 and 2 were dissolved in 1 ml HPLC water each. Fractions 1 and 2 were pooled and spun at 20°C, 10 000 x g and 2 min.

H was also purified from a heparin formulation using the ODS cartridges. The heparin sample (1.0 ml) was applied to the primed cartridge and 1 ml of the flow through was collected. The cartridge was then washed once with 1 ml 0.01 N HCl and the eluting fraction was collected. The two eluting fractions were combined and dried in a vacuum centrifuge. The dried residues were dissolved in 1.0 ml HPLC water and spun at 20°C, 10 000 x g for 2 min. The heparin formulation contained 25 000 I.E. heparin/5ml.

H was measured radioimmunologically in the reconstituted urine or heparin samples.

Recovery of ^{3}H-Histamine in urine samples

The analytical recovery of the ODS cartridges for H in urine samples was determined by the addition of ^{3}H-labeled H (^{3}H-H) to the urine samples before purification. Fractions 1 to 4 were collected and aliquots of 0.1 ml of the radioactive ^{3}H-H were mixed with 5.0 ml scintillation fluid and counted for 1 min in a liquid scintillation counter.

HPLC characterization of H-like material

For the characterization of H-like material, 1 ml urine or 1 ml of heparin which were purified on ODS cartridges were injected and separated on the cation exchange TSK SP-5 PW column. Fractions of 0.6 ml were collected. Aliquots of 0.1 ml of each fraction were withdrawn for the radioimmunological measurement of H. Rechromatography was performed on a reversed phase C_8 column using paired ion chromatography as mentioned above.

Chemicals

All chemicals used were of analytical grade. L-Histidine, H and MIAA were purchased from Sigma Chemie, Deisenhofen, FRG. MH was delivered by Calbiochem, Frankfurt, FRG. All other chemicals including HPLC solvents were delivered by Merck AG, Darmstadt, FRG.

RESULTS

HPLC separation of histamine on an ion exchange column

On the cation exchange TSK SP-5 PW column and gradient elution, a baseline separation was achieved for H, its metabolites MH and MIAA and its precursor L-histidine (Fig. 1A). The retention time for H was 32.54 ± 0.04 min, for MH 30.57 ± 0.02 min, for L-histidine 8.11 ± 0.12 min and for MIAA 4.05 ± 0.01 min (mean ± SEM; n=3).

HPLC separation of histamine on different reversed phase columns

Using an aqueous mobile phase of 0.05 M NaH_2PO_4 which contained 0.5 mM EDTA-Na_4 (in the absence of an organic modifier and the ion pairing reagent pentan-1-sulfonic acid or octan-1-sulfonic acid), H and MH were not retained on a reversed phase C_{18}, C_8 or C_4 column and eluted in the void volume of the columns. The addition of pentan-1-sulfonic acid had no influence of the retention times for H and MH on C_{18} column. H and MH still eluted in the void volume of the column with retention times of 1.78 and 1.96 min, respectively. Although, the retention times of H and MH increased on a C_8 or C_4 column, H could not be separated from MH (Tab. 1). Replacement of the pentan-1-sulfonic acid by the octan-1-sulfonic acid significantly increased the retention time of H (62.79 min) and MH (82.48 min) on the C_8 column (Tab. 1). Substitution of the C_8 column with a C_4 column induced a notable reduction of the retention times of H and MH from 62.70 to 18.16 min and 82.48 to 21.76 min (Tab. 1).

The retention times for H and MH could be significantly reduced from 62.70 to 11.14 and 82.48 to 12.60 min on a reversed phase C_8 column using a mobile phase which was composed of a mixture of 15 % methanol as an organic modifier and 85 % of an aqueous

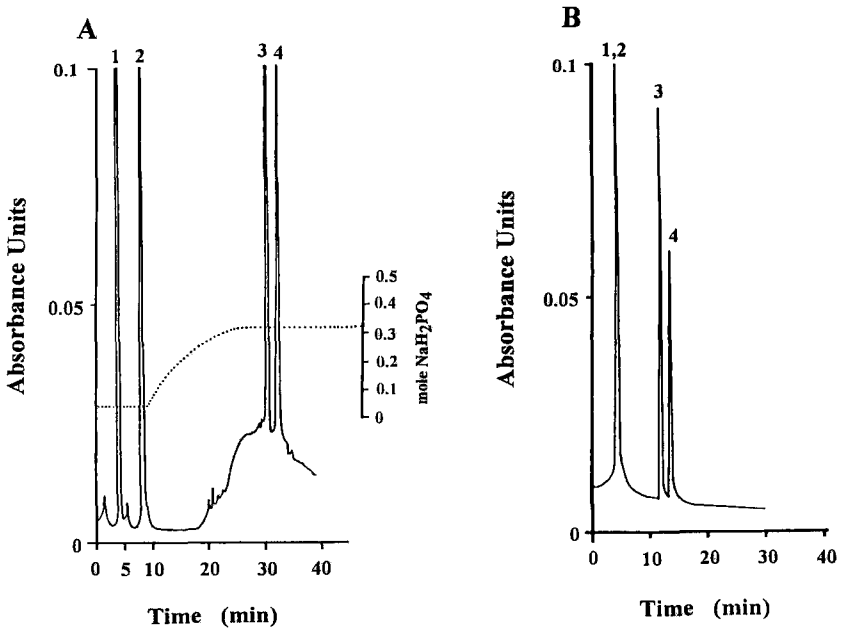

Figure 1

HPLC separation of H, L-histidine, MH and MIAA on an ion exchange TSK SP-5 PW column (A) and a reversed phase C_8 column (B) and UV detection at a wavelength at 210 nm. A: 1=MIAA, 2=L-histidine, 3=MH, 4=H. B: 1,2=MIAA and L-histidine, 3=H and 4=MH. For more detailed information see text.

solution of 0.05 M NaH_2PO_4 which contained 0.5 mM EDTA-Na_4 and 0.005 M octan-1-sulfonic acid (Tab. 1). With the use of a reversed phase C_8 column in combination with this mobile phase H could be separated from its precursor L-histidine and its metabolites MH and MIAA. However, MIAA and L-histidine could not be separated from each other (fig. 1B). The retention time for H was 11.14 ± 0.08 min, for MH 12.60 ± 0.09 min, for L-histidine 4.10 ± 0.02 min and for MIAA 4.34 ± 0.03 min (mean ± SEM; n=3). For this HPLC setup the detection limit for H was 20 ng at a detector sensitivity of 0.01 AUFS.

The influence of different wavelengths on the detector signal for H and MH for this HPLC setup were investigated. The highest detector signals for H and MH were obtained at a wavelength setting of 210 or 215 nm. The detector signals were significantly lower at 205 or 225 nm. At a wavelength of 254 nm no detector signal was obtained for H or MH (Fig. 2).

Table 1

Influence of pentan-1-sulfonic acid and octan-1-sulfonic acid on the retention times of H and MH using C_{18}, C_8 or C_4 columns in the absence of the organic modifier methanol. For more detailed information see text.

	Retention Times (min)			
Ion Pairing reagent	Histamine	1-Methylhistamine	Column	Experiments
Pentan-1-sulfonic acid	1.78	1.96	C18	n=1
	3.50 +/- 0.01	3.95 +/- 0.04	C8	n=3
	2.83 +/- 0.01	2.94 +/- 0.01	C4	n=3
Octan-1-sulfonic acid	62.7	82.48	C8	n=1
	18.16 +/- 0.45	21.76 +/- 0.19	C4	n=3

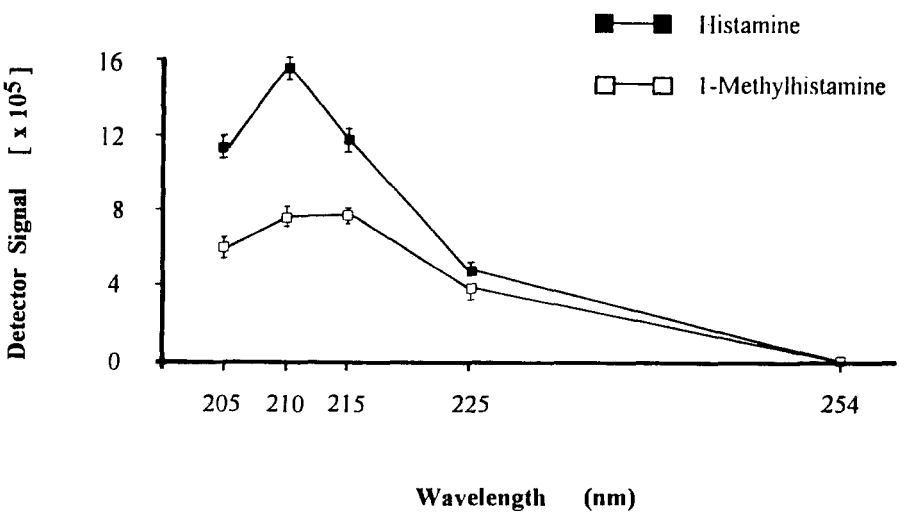

Figure 2
Relationship between the wavelength and the detector signals on the reversed phase C8 column. The highest detector signal for H and MH was noted at a wavelength of 210 nm.

Recovery of ^{3}H-H

The overall analytical recovery of ^{3}H-H in urine samples on ODS cartridges was 95.16 ± 0.92 % (n=22). In the fraction which was collected at the same time the sample was applied to the cartridge 63.10 ± 1.23 % of the radiolabeled H was recovered (fraction # 1). In the 1st wash 27.32 ± 1.26, the 2nd wash 3.79 ± 0.49 and the 3rd wash 0.99 ± 0.13 % could be recovered (Fig. 3 top panel). HPLC separation of ^{3}H-H in a pool of the application and the 1st wash fractions showed a single peak of intact ^{3}H-H indicating that H has not been degraded in the acidified urine (Fig. 3 bottom panel).

Histamine in human urine samples

H-immunoreactivity was present in human urine samples purified on ODS cartridges. The concentration of H-like material excreted in 24 h was 29.57 ± 10.16 µg (mean ± SEM; n=7).

HPLC characterization of H-like material on the cation exchange column showed the presence of H, a compound with the same retention time as MH and a variety of substances which crossreacted with the anti-H-antibody (Fig. 4).

With regard to the capability of the HPLC system separating H from MH, the urine

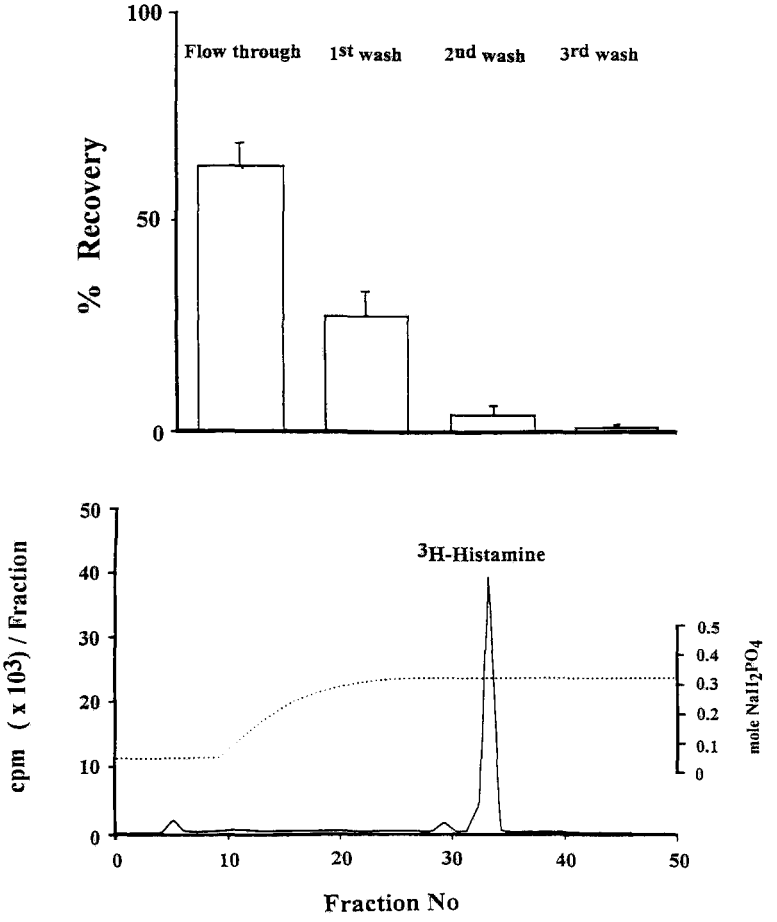

Figure 3

Top panel: Analytical recovery of ^{3}H-H in human urine samples on ODS cartridges. The overall recovery of ^{3}H-H on ODS cartridges was 95.16 ± 0.92 (n=22). Bottom panel: HPLC characterization of ^{3}H-H which was added to human urine samples prior to ODS purification on a TSK SP-5 PW cation exchange column. The chromatogram showed a single peak of intact H indicating that H was not degraded in the acidified urine samples. For more detailed information see text.

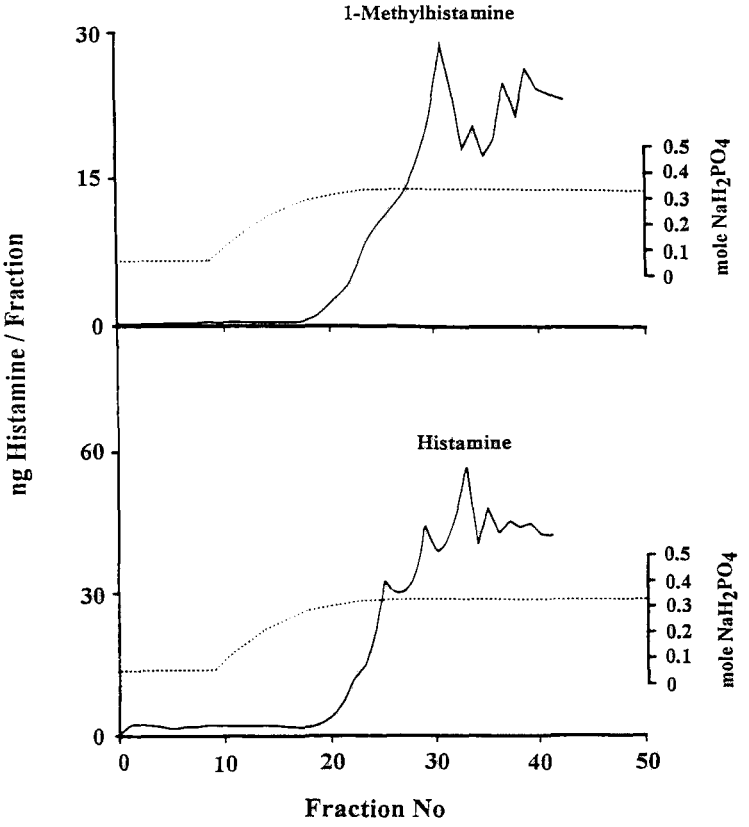

Figure 4

HPLC characterization of H-immunoreactive material in human urine samples on a TSK SP-5 PW cation exchange column. Two substances with the same retention times as H and MH were found in the HPLC fractions. For further information see text.

samples could be divided into 2 distinct types: one with mainly H (Fig. 4 bottom panel) and the other with MH (Fig. 4 top panel) as the prominently excreted product. Only H and no MH was identified in one subject (#4) while MH and no H was detected in another subject (#5). In one subject (subject #2) H was identified with minute amounts of MH. The H metabolite MH was mostly found in 4 of the 7 subjects (#1, 2, 6 and 7). The ratio of H and MH to the total amount of H-like material present in 7 urine samples from healthy volunteers was 19.69 ± 7.86 % for H and 46.74 ± 12.89 % for MH (Tab. 2).

Table 2

Concentration of H-immunorective material in partially purified human urine before and after HPLC separation on a TSK SP-5 PW cation exchange column. Numbers in parenthesis gives the percentage of H and MH in the HPLC fractions in relation to the H-immunorective material before HPLC separation. For further information see text.

Subject	before HPLC	after HPLC	
	H-like	MH	H
# 1	83.69	69.25 (82.75)	1.76 (2.10)
# 2	45.55	35.71 (78.39)	4.30 (9.43)
# 3	10.33	0.26 (2.56)	3.31 (32.05)
# 4	24.18	0	13.93 (57.59)
# 5	9.98	4.57 (45.83)	0
# 6	20.75	9.86 (47.55)	5.88 (28.33)
# 7	12.49	8.76 (70.13)	1.04 (8.35)
	29.57 ± 10.16	18.35 ± 9.65 (46.74 ± 12.89)	4.32 ± 1.77 (19.69 ± 7.86)

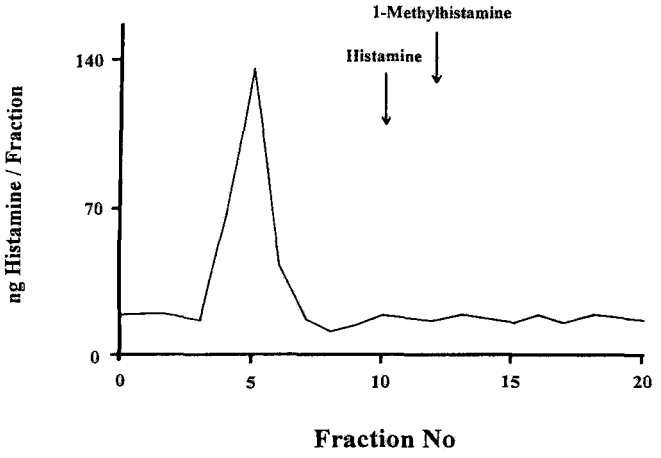

Figure 5

Rechromatograpy of the MH peak from the separation on the TSK SP-5 PW cation exchange column on a reversed phase C_8 column. The compound eluted with a retention time of 5 min which was different from the retention time of MH. Arrows indicate the retention times of H and MH. For further information see text.

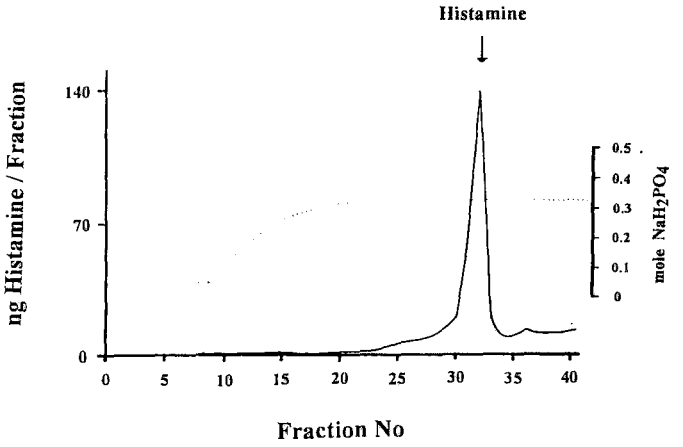

Figure 6
HPLC characterization of H extracted from a commercial heparin preparation using ODS cartridges on a TSK SP-5 PW cation exchange column. A compound with the same retention time as H could be identified in the HPLC fractions. For further information see text.

The substance which eluted from the cation exchange TSK SP-5 PW column with the same retention time as MH was submitted to a rechromatography on the reversed phase C_8 column with octan-1-sulfonic acid as anion pairing reagent. This compound showed a retention time different from MH (Fig. 5).

Histamine in heparin

In a commercially available heparin formulation 81.93 ng/ml H-immunoreactive material was detected. After ODS purification the concentration of the H-immunoreactive material was 279.24 ng/ml. HPLC characterization of the H-immunoreactive material on an ion exchange TSP SP-5 PW column demonstrated the presence of a substance with the same retention time as H (Fig. 6).

DISCUSSION

An ion exchange and reversed phase HPLC procedure for the separation of H from MH was developed and a combination of HPLC and RIA was utilized to characterize H and

MH in human urine samples and a heparin formulation. With an ion exchange TSK SP-5 PW column and gradient elution using a phosphate buffer pH 5.0 as a mobile phase H was separated from its precursor L-histidine and its metabolites MH and MIAA. In addition, H was separated from MH and MIAA on reversed phase C_8 column with isocratic elution in the presence of methanol as an organic modifier and octan-1-sulfonic acid sodium salt as an ion pairing reagent.

The effects of different stationary phases and the composition of the mobile phases upon the retention times of H and MH were studied. The results indicated a close interaction between the polarity of the mobile phases and the polarity of the column matrix which strongly effected the elution behavior of H and MH. The less polar the stationary phase was the less polar the ion pairing reagent had to be in order to retain H and MH. H and MH are very polar substances. They did not interact with the apolar matrix of a reversed phase C_{18}, C_8 or C_4 column in the absence of methanol and an ion paring reagent and eluted without retention in the void volume of the columns. In addition, H and MH were not retained on reversed phase C_{18} column even when the ion pairing reagent pentan-1-sulfonic acid was added to the mobile phase. Switching from a reversed phase C_{18} column to a C_8 or a C_4 column by decreasing the apolar column matrix induced an increase in the retention times for H and MH. However, no separation between H and MH was obtained. The use of octan-1-sulfonic acid which differs from the pentan-1-sulfonic acid only by 3 additional C-atoms had a marked influence on the retention of H and MH on the reversed phase C_8 column. H and MH were retained on the column for 63 and 82 min, respectively. Replacing the C_8 column with a C_4 column by increasing the polarity of the column matrix significantly reduced the retention times of H and MH to 18 and 22 min. The best chromatographic conditions with respect to an optimal retention time for H and MH and the ability for separating H from MH were obtained on a reversed phase C_8 column and a mobile phase which was composed of 15 % methanol in the presence of octan-1-sulfonic acid as an ion pairing reagent.

The detection of H and MH was highly influenced by the wavelength setting of the UV detector. A wavelength below 210 nm or above 225 nm resulted in a decrease in the detector signal. The most sensitive detection with the highest detector signal on the UV detector for H and MH was obtained at a wavelength of 210 nm.

The HPLC procedures were applied to characterize H in human urine samples and a heparin formulation. HPLC separation of native urine or heparin was hampered by the fact that these samples are composed of a variety of different substances which interfered with the radioimmunological identification of H in the HPLC fractions. Therefore, an extraction step to isolate and purify H from other constituents present in these samples was necessary. For this purpose, H was purified on ODS cartridges which have been used

previously for the purification of angiotensin peptides from brain tissue (11). Whereas angiotensin peptides were retained by the ODS cartridges, H was not. H passed through the cartridges leaving the yellow colored material in urine in the cartridges. With this purification step 63 % of ^{3}H-H was recovered in the flow through and another 27 % in the 1st washing of the cartridges. The overall recovery of ^{3}H-H which was added to the urine samples was 95 %. HPLC characterization of the recovered ^{3}H-H showed a single peak in the chromatogram indicating that H was not degraded in the acidified urine samples or during the sample handling. Moreover, the addition of HCl to the urine samples was essential to prevent degradation of H. In contrast, native urine samples with pH values between 5 to 6 displayed considerable degradation of H (data not shown).

H was identified radioimmunologically in the purified urine samples. The H-immunoreactive material could be characterized on the ion exchange column with subsequent radioimmunological detection as H and a substance with the same retention time as MH. However, the identity of MH could not be confirmed upon rechromatography of the MH peak on the reversed phase C_8 column. The nature of this substance is still unknown. Since the ion exchange column allowed a separation of H from MH the ratio of H to MH in human urine was calculated. H constituted about 20 % and MH about 47 % of the total H-immunoreactive material found in the urine samples without HPLC separation. The rest obviously accounted for material of unknown nature which crossreacted with the anti-H antiserum. This confirms findings of our previous study in urine of healthy volunteers where we also identified 50 % of the H-immunoreactive material as MH (12). It has been shown that H and MH were separated on a reversed phase C_{18} column with the addition of sodium dodecyl sulfate to the mobile phase using electrochemical detection (13). The authors could demonstrate a baseline separation and a highly sensitive detection for H and MH. Although, electrochemical detection is very sensitive it is also very susceptible to various interferences. We have tried to use electrochemical detection in our experiments but obtained variable and discouraging results and were unable to reproduce the results by Houdi et al (13).

H-immunoreactivity could be identified radioimmunologically in a commercially available heparin formulation. ODS cartridges were also used to purify H from other constituents. The partially purified H-immunoreactivity was clearly identified as H on the ion exchange TSK SP-5 PW column. Previously, Sjodin and Svensson reported the contamination of heparin with H. Administration of heparin in cats showed vasodepressor activity which could be blocked by a H_1-antagonist indicating the involvement of H (14). According to their study they estimated that 5 000 I.U. of heparin can be contaminated with as much as 100 ng H. Likewise, Adt et al showed that injection of H induced cardiopulmonary reactions and an increase in plasma H (15).

In summary: ion exchange and reversed phase HPLC in combination with a specific H RIA is fast, reproducible, specific and sensitive method for the determination of H in biological samples. This approach was successfully applied for the qualitative and quantitative determination of H in human urine and a commercially accessible heparin formulation.

Acknowledgments
The authors wish to thank Mrs. J. Grosch and P. Decker-Hermann for their excellent technical assistance. H-RIA-Kits were generously provided by Dr. B. Manz, IBL, Hamburg, FRG. The editorial help of Mrs. P. Decker-Hermann is gratefully acknowledged.

REFERENCES

1. J. Ring, F. Sedlmeier, D. von der Helm, T. Mayr, U. Walz, H. Ibel, H. Riepel, B. Przybilla, H-J. Reimann, W. Dorsch, Histamine and allergic disease, in New Trends in Allergy II, J. Ring, G. Burg, eds., Springer Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo, 1986, pp. 44-77.

2. G. Katz, Science 91: 221 (1940)

3. L.M. Liechtenstein, A.G. Osler, J. Exp. Med. 120: 507-530 (1964)

4. W. Lorenz, A. Doenicke, Mount Sinai J. Med. 45: 357-386 (1978)

5. S.H. Snyder, R. Baldessarini, J. Axelrod, J. Pharmacol. Exp. Ther. 153: 544 (1966)

6. P.A. Shore, A. Melvin, V.H. Cohn, J. Pharmacol. Exp. Ther. 127: 182-191 (1959)

7. J.K. Khandelwal, L.B. Hough, A.M. Morrioshow and J.P. Green, Agents and actions 12: 583-590 (1987)

8. V. Stephan, A. Zimmermann, J. Kühr and R. Urbanek, Allergy Clin Immunol 86:862-868 (1990)

9. B. Rotz, A. Savaser, I. Werthmann, S. Lau and U. Wahn, Allergy 46: 529-533 (1991)

10. K. Schübeler, W.-M. Becker, W.W. Schaubschläger and M. Schlaak, Allergologie 12: S 322-S 325 (1989)

11. D. Ganten, K. Hermann, C. Bayer, Th. Unger and R.E. Lang, Science 26: 869-871 (1983)

12. K. Hermann, B. Hertenberger, J. Ring, Int Arch Allergy Immunol 101: 13-19 (1993)

13. A.H. Houdi, P.A. Crooks, G.R. Van Loon and C.A.W. Schubert, J Pharm Sci 76: 389-401 (1987)

14. L. Sjodin, U. Svensson, Acta Pharmaceutica Nordica 2: 89-100 (1990)

15. Adt M, et al, Anaesthesist 36: 529-530 (1987)

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(1), 205-206 (1995)

THE BOOK CORNER

THIN-LAYER CHROMATOGRAPHY, TECHNIQUES AND APPLICATIONS, Third Edition, Revised and Expanded, by B. Fried and J. Sherman, Marcel Dekker, Inc., New York, NY, 451 pages, 1994. Price: $165.00.

This new edition of the book has been written to update and expand coverage of the topics in the second edition. It provides extensive coverage of qualitative and quantitative thin-layer chromatography (TLC) and high-performance TLC, and compares modern TLC with other separation methods, especially gas and column liquid chromatography.

The authors state that they have maintained the overall organization of the second edition, namely, a series of initial chapters on theory and practice, and a second section of chapters concerned with applications to important compound types. As in the previous two editions, practical, rather than theoretical, aspects of TLC have been stressed. Coverage of certain modern principles, techniques, and instrumentation (such as optimization of separations and densitometry, overpressurized and multiple development, and preparative layer chromatography) has been expanded.

This edition updates and extends references to the literature. The TLC literature is voluminous and references in this book are selective. Citations are mainly to the 1980-1993 literature.

The second part of this book is concerned with the applications of TLC to various classes of compounds. The third edition also provides extensive coverage of sample preparation.

Overall, the book is a good introduction to TLC. The discussion of multi-modal TLC and use of cyano and amino phases as multi-modal supports is inadequate, however. The section on biphasic layers is very weak and almost non-existent. Recent reviews were not included in this updated edition.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(1), 207-208 (1995)

MEETING REPORT

THE INTERNATIONAL CONGRESS ON NATURAL PRODUCTS RESEARCH

Halifax, Nova Scotia, Canada
July 31 - August 4, 1994

The 1994 International Congress on Natural Products Research, which is held once every five years, which is a joint meeting of the American Society of Pharmacognosy (35th Annual Meeting), Association Francaise pour l'Enseignement et la Recherche en Pharmacognosie, Gesellschaft fur Arzneipflanzenforschung, and Phytochemical Society of Europe, was held at the World Trade and Convention Centre in Halifax, Nova Scotia, Canada. The theme of the Congress was "Natural Toxins and Their Impact on Society."

The Congress was attended by over 600 conferees. The excellent program was divided into the following categories: thirteen symposium lectures by leaders in natural products research, each talk was 45 minutes; four award speakers; 45 oral communications (20 minutes each); four lectures by outstanding young investigators, which were awarded scholarships to attend; and 267 posters.

The posters were divided into four sessions, which allowed ample time for review and discussion. The day started with a continental breakfast from 7:00-8:00 AM, followed by symposium lectures, a refreshment break for 30 minutes, and by another symposium. After the lunch break from 12:00-1:30 PM, oral presentations were given until 3:00 PM, followed by another refreshment break for 30 minutes. At 3:30 p.m. another symposium (or poster session) and from 4:15-6:00 p.m. a poster session (or a series of oral presentations).

On the first day, an additional poster session was held from 8:00-11:00 p.m. This resulted in the conferees becoming saturated by the end of the Congress, dreaming

of natural toxins, drugs, poisons and pharmaceuticals. The program was excellent and, for the novice, this was quite an introduction to an exciting and complex field which is, with all honesty, a very important area of research. It is by no means an easy area, it is complex and the search for a toxin or a drug is tedious, difficult and time consuming. It is comparable to searching for a needle in a hay stack, if not harder.

It was clear to me, as a separation scientist, that separation science is an integral and important part of natural products research. TLC, HPLC, GC and column chromatography are important tools which are used extensively. However, capillary electrophoresis and micellar electrokinetic chromatography are extremely important and useful tools in this complex area of natural products research, due to their high resolving power. This was clear from one of the posters, which showed that CE is superior to HPLC in resolving a complex crude extract.

The scientific and organizing committees deserve all the praise that one can shower on them. Dr. F. Chandler, the host and Chairman of the Congress, deserves much credit for a very successful and enjoyable Congress, for the quality of the presentations and for the smooth running of the scientific and social programs. Dr. Chandler and his colleagues were very generous and caring to the conferees and offered them their "money's worth," both scientifically and socially. They did not try to save the registration fees for their own purposes or to invite their own friends to fancy dinners, as we often see at other meetings around the world. They offered the conferees breakfast and refreshments all day including juice, coffee, tea, and cold drinks. They even gave the conferees a free lunch when the poster sessions ran from 11:30-1:30 PM. The social events were of the highest quality and I am sure that most of the registration fee was spent on the conferees.

Scientifically, the Congress was enlightening and socially it was rewarding. Dr. Chandler and his committee should be congratulated on a job well done.

Haleem J. Issaq, Ph.D.
Associate Editor

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(1), 209 (1995)

MEETING ANNOUNCEMENT

Prep'95
1995 International Symposium, Exhibit
& Workshop on Preparative Chromatography

June 11 - 14, 1995

Washington, DC

The Prep'95 Symposium will address all aspects of preparative chromatography from empirical laboratory applications to large scale process optimization. Some sessions will be devoted to practical applications by authors from industry. The program will incorporate lectures; poster sessions; discussions; seminars; workshops; an instrumentation exhibit.

Specific topics which will be covered include

* Industrial Applications
* Recombinant Technology
* Peptide & Protein Purification
* Chiral Separations
* Instrumentation
* Economic Considerations
* Simulated Moving Bed
* Overload Elution
* Bulk Adsorption Processes
* Case Studies
* Isolation of Impurities
* Development of Preparative Methods
* Drugs & Biopolymers
* Column Technologies
* Experimental Optimization
* Regulatory Aspects of GMP
* Displacement Chromatography
* Ion-Exchange Chromatography
* Low Pressure Applications
* High Pressure Applications

Concerns which will be discussed:

* What's new in preparative Chromatography?
* Is displacement going to remain a technique of the future?
* Should I use a large preparative column?
* Is a moving bed column the answer to my separation problem?
* What about the bed compression approach?
* What are the trends in stationary phase developments?
* Will chiral phases become economical for preparative separations?

Further information about Prep'95 may be obtained from Ms. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(1), 211-216 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

FEBRUARY 13 - 15: PrepTech '95, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. Brian Howard, ISC Technical Conferences, Inc., 30 Controls Drive, Shelton, CT 06484-0559, USA.

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MAY 21: Techniques for Polymer Analysis and Characterization, a short course, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

MAY 22 - 24: 8th International Symposium on Polymer Analysis and Characterization, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 23: Miniaturization in Liquid Chromatography versus Capillary Electrophoresis, Pharmaceutical Institute, University of Ghent, Ghent, Belgium. Contact: Dr. W. R. G. Baeyens, Univ of Ghent, Pharmaceutical Inst, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

MAY 31 - JUNE 2: 27th Central Regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco.. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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