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A VARIANT FOR ISOLATION OF SERUM GANGLIOSIDES

Peter P. Ilinov,[†] Denislava D. Deleva,[†] Svetoslav I. Dimov,[‡] Emilia T. Zaprianova[†]*

[†]Institute of Experimental Morphology and Anthropology
Bulgarian Academy of Sciences
block 25. Acad. G. Bonchev Street
1113 Sofia, Bulgaria
[‡]Institute of Molecular Biology
Bulgarian Academy of Sciences
block 21, Acad. G. Bonchev Street
1113 Sofia, Bulgaria

ABSTRACT

The serum gangliosides are of great diagnostic importance. A step-by-step procedure for their isolation from serum is described and it includes the following stages: a) dehydration of the sample; b) total lipids extraction; c) non-polar lipids removal by preparative TLC; d) elimination of the blood sugar by Sep-Pak technique; e) TLC of the ganglioside fractions. Azeotropic distillation of the mixture of serum water/n-propanol was used for sample dehydration. Triple extraction was carried out: with cyclohexane (I); chloroform : methanol = 1 : 1 (v/v) (II) and chloroform : methanol = 1 : 2 (v/v) (III). The extracts were combined and the non-polar lipids were removed. The eluate composition was checked out by HPTLC with a modified mobile phase: chloroform:methanol:0.1 M sodium lactate = 55 : 40 : 10 (v/v/v). Comparative data for the R_f values of the ganglioside fractions as well as recovery data are presented. This method is also applicable for milk samples.

INTRODUCTION

Gangliosides are glycosphingolipids that consist of sialic acid containing carbohydrate residue linked to ceramide, i.e. a N-fatty acyl derivative of a sphingoid linked carbon chain base.[1] Within vertebrates they occur in virtually all tissues and body fluids. In each species and each tissue their molecular patterns differ and undergo changes during development and under various physiological influences.[2] The majority of gangliosides are situated in the outer leaflet of the cell membrane. However, gangliosides also occur in non-cell associated form in blood plasma and other body fluids.[3] Although the concentrations of gangliosides reported in human serum vary, it is now established that elevated levels occur in some pathological conditions such as cancer, atherosclerosis and multiple sclerosis.[4,5]

In order to study the significance of gangliosides in the pathogenesis of different disorders it is very important to determine the concentration and composition of these lipids in the serum. Due to the low concentrations of gangliosides in serum (700 μg) ganglioside-bound sialic acid/100 mL sera,[5] it is difficult to obtain ganglioside extracts free from contaminants. Several methods for extracting, purifying and analysing gangliosides from serum or plasma have been reported.[6-16] All methods use a total lipid extraction as the first step. Gangliosides are then separated from other lipids by a partition procedure and further purified from contaminants. The separation of the lipids according to their polarity is usually performed by a phase distribution in a system of extraction solvents (mainly chloroform, methanol and water).[7-9] This step is followed by gel filtration and silicic acid column chromatography.[6,10] Most of the techniques used for ganglioside isolation are multistage and time consuming. A variant for isolation of serum gangliosides after suitable express dehydration and three-step extraction of the total lipids with solvents with increasing polarity followed by purification of the ganglioside fractions by selected TLC techniques is described in this article.

MATERIAL AND METHODS

One mL serum was pipetted in a distillation flask and mixed with 10 mL n-propanol. The flask was mounted to a rotary vacuum evaporator and the serum water was removed by azeotropic distillation at 35-40°C; 15-20 mm Hg for 10-12 min. The rest was suspended in 10 mL of cyclohexane which was vacuum evaporated at the same conditions. The dehydrated sample dissociated mainly in the form of fine granules.

Extractions

The granules were transferred in a centrifugation tube containing 1 g pure quartz sand and 0.3 g kieselguhr. The residue stuck to the flask wall was washed with 15 mL of cyclohexane (first extraction) and eventually ultrasonication could be used. The suspension formed was added to the centrifugation tube and the mixture was vigorously stirred for 10 min and centrifuged at 4000 min^{-1} for 10 min. The upper phase was transferred to a vial. The residue in the vacuum flask was washed repeatedly with 15 mL of a mixture of chloroform : methanol = 1 : 1 (v/v).

After sonication the solution was transferred to the tube and the extraction procedure was repeated (second extraction). The third extraction was performed as already described but with a mixture of chloroform : methanol = 1 : 2 (v/v). The combined extracts were evaporated at 35°C under nitrogen until 10-15 mL of solution were left in the vial. For analytical purposes the extracts could be processed separately.

Non-polar Lipids Removal

The concentrated extract was applied onto a preparative TLC plate (10x10 cm) and chromatographed with a mobile phase chloroform : methanol : 0.3% $CaCl_2$ as described in.[11] After peripheral marking of the ganglioside carrying zones (orcinol reagent) the sorbent was scrapped, placed in a column and successively eluted with a mixture (8 mL) of chloroform : methanol = 1 : 1 (v/v) and 12 mL of methanol. The eluate was concentrated at 35°C under nitrogen and its composition was checked out by HPTLC (aluminium plates, Merck, Germany). Modified mobile phase: chloroform : methanol : 0.1 M sodium lactate = 55 : 40 : 10 (v/v/v). After finishing the checking procedure the eluate was evaporated to dryness.

Sep-Pak Procedure

The Sep-Pak (Sep-Pak Cartridge, Millipore Corporation, Milford, Massachusetts, U.S.A.) extraction was performed according to.[12] The final eluate was concentrated and a sample was spotted onto a HPTLC glass plate (10x10 cm); mobile phase (see above). The spots were visualised by spraying with orcinol reagent (0.5% solution in 20% sulfuric acid) followed by local heating at 110°C. Bovine brain gangliosides could serve as a test mixture for identification.

Recovery Data

We have accomplished an additional experiment to determine the overall recovery. A standard mixture containing ganglioside fractions, obtained from bovine brain, was added to the native serum and the sample was processed as already described. The result was calculated according to the following equation:

$$\mu g \text{ sialic acid / mL serum} = \frac{A.F.Q}{a.b.g}$$

where:
A = absorbance at 580 nm
F = sialic acid content (μg) in the standard sample absorbance of the standard sample at 580 nm ratio
Q = whole ganglioside carrying area scrapped area ratio
a = percentage of the total extract applied onto the preparative TLC plate
b = percentage of the concentrated eluate after the Sep-Pak procedure used for sialic acid determination
g = serum sample (mL)

The technique for spectrophotometric determination of sialic acid is described in a previous work.[11]

RESULTS AND DISCUSSION

The equal amount of the ganglioside mixture added to the serum sample is:

$$A_1F = 0.4 \times 170 = 68.0 \ \mu g$$

where: A_1 - absorbance at 580 nm

A sample without addition of test mixture was worked separately:

$$\mu g \text{ sialic acid / mL Serum} = \frac{0.022 \times 170 \times 1.5}{0.8 \times 0.7 \times 2.0} = 10.0$$

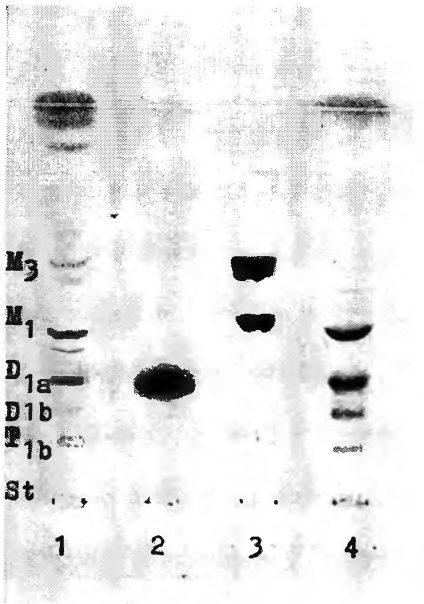

Figure 1. Thin-layer chromatogram of: (1) gangliosides after Sep-Pak procedure (test mixture added); (2) blood sugar (glucose); (3) GM1 and GM3 standards; (4) test mixture of bovine brain gangliosides; mobile phase: chloroform : methanol : sodium lactate = 55 : 40 : 10 (v/v/v); visualization by spraying with orcinol reagent.

A serum sample with test mixture was added for recovery:

$$\mu g \text{ sialic acid / mL serum} = \frac{0.164 \times 170 \times 1.5}{0.8 \times 0.7 \times 1.0} = 74.6$$

$$\frac{74.6 - 10}{68} \times 100 = 95\% \text{ recovery}$$

The control chromatogram of the first extract (cyclohexane) shows presence of cholesterol, neutral lipids and phospholipids, some blood sugar (glucose) and traces of gangliosides. Both second and third extracts contain traces of non-polar lipids, the main quantity of serum gangliosides and part of the blood sugar. The last one could be effectively removed by the Sep-Pak method. Control chromatograms illustrating the extraction procedure are given in Figure 1 (see text below).

Table 1

R_f (x 100) Values of Mobile Phases Containing 0.1M Sodium Lactate and 0.2% $CaCl_2$

	Sialic Acid	Glucose	Lactose	GT1b	GD1b	GM1	GM3
0.1M Na lactate	11	40	25	16	30	72	82
0.2% CaCl2	15	55	33	8	20	91	97

The choice of n-propanol for the azeotropic distillation proved to be successful. According to data cited in the literature the composition of the azeotropic mixture is water : n-propanol = 1 : 2.6. The sample dehydration was carried out at a ratio of 1 : 10 this was the optimal process to be completed within 8-10 min. The addition of cyclohexane at the dehydration stage was meant to remove the excess of n-propanol and to granulate the dry serum residue. Samples from rat serum, cow milk and human milk were dehydrated in a similar manner. Differences in the water, lipid and protein content did not affect the proposed method. The successive extraction of the dry serum residue with solvents with increasing polarity ensured a total drawing out of the sample gangliosides so that a four extraction step was not necessary. The sample solvents ratio in this three-stage procedure was 1 : 40 (v/v). Removing the total serum lipids (cholesterol, triacylglycerols and phospholipids) is necessary for simplifying and making the Sep-Pak procedure that follows more effective. A modified mobile phase consisting of chloroform : methanol : 0.1 M sodium lactate = 55 : 40 : 10 (v/v/v) was used in the analytical TLC and it was compared with a known one[5] consisting of chloroform : methanol : 0.2% $CaCl_2$ = 50 : 45 : 10 (v/v/v). The corresponding R_f (x100) values at 30°C ambient temperature are given in Table 1.

According to the chromatographic conditions (sorbent, mobile phase, ambient temperature), the glucose spot could overlap the GD1a and GD1b fractions and the same is valid for the lactose and GD1b and GT1b fractions respectively. In case this is avoided (as on the chromatogram in Figure 2) the information about the serum gangliosides is sufficient and the Sep-Pak procedure can be refrained.

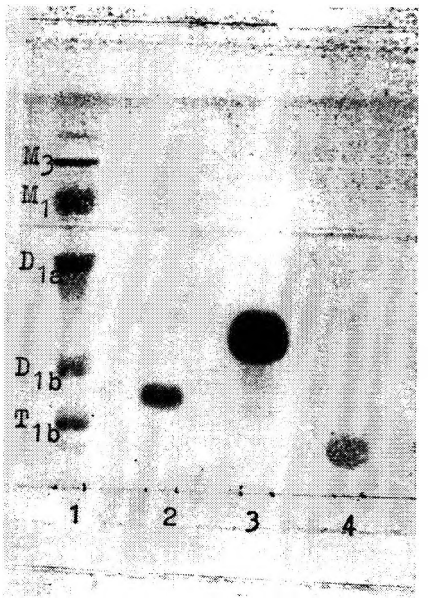

Figure 2. Thin-layer chromatogram of: (1) standard mixture; (2) lactose; (3) glucose; (4) sialic acid; mobile phase and visualization as in Fig. 1; for the R_f values see text.

The GM3 fraction forms about 50% of the total gangliosides and it is of great diagnostic importance.[5] According to the data for the R_f values, glucose does not overlap the GM3 spot and the Sep-Pak procedure could also be avoided for this reason. However, it is absolutely necessary when the serum gangliosides are quantified spectrophotometrically by the lipid-bound sialic acid.[11]

This variant for isolation of gangliosides from serum and also, from other biological fluids, is rapid and inexpensive and it is fairly suitable for routine diagnostic studies in the clinical practice.

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DETERMINATION OF VINCRISTINE AND VINBLASTINE IN *CATHARANTHUS roseus* PLANTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/ ELECTROSPRAY IONIZATION MASS SPECTROMETRY

I.-H. Chu,[‡] J. A. Bodnar,[†] R. N. Bowman,[§] E. L. White*[,†]

[†]R.J. Reynolds Tobacco Company
Bowman Gray Technical Center
Winston-Salem, NC 27102

[‡]Department of Chemistry
Wake Forest University
Winston-Salem, NC 27109

[§]Goldsmith Seeds, Inc.
PO Box 1349
Gilroy, CA 95021

ABSTRACT

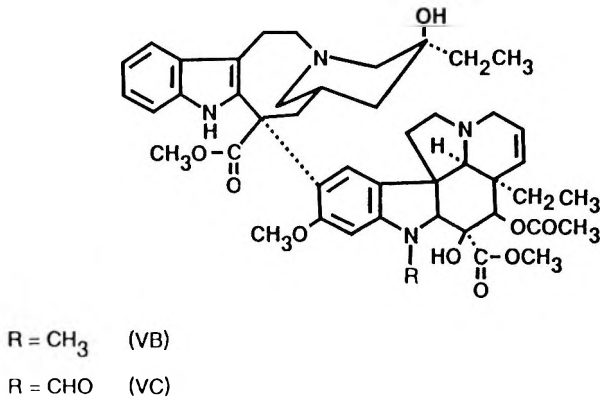
A reversed phase high performance liquid chromatographic/ mass spectrometric (LC-MS) method with an electrospray ion source was used in this study to determine the concentration of two dimeric alkaloids, vinblastine and vincristine, in near-isogenic lines of *Catharanthus* leaves. A linear gradient of 25 mM ammonium acetate in methanol (solution A) and the same concentration in water (solution B) served as the mobile phase. A C_{18} column was used to separate those two compounds from other components in the extracted plant samples. Mass spectra

generated by the electrospray ionization source were dominated by protonated molecules with little or no fragmentation being observed under the experimental conditions applied. Since very little fragmentation occurred, the method exhibited enhanced sensitivity over other LC-MS techniques. Product-ion mass spectra of vincristine and vinblastine were produced in a single quadrupole mass spectrometer through collision-induced dissociation (CID) between the capillary exit and first skimmer of the electrospray source. CID spectra can be used to unequivocally differentiate between vincristine, vinblastine and other alkaloids.

INTRODUCTION

Vincristine (VC) and vinblastine (VB) (Figure 1) are subjects of many biological and pharmaceutical studies, primarily because they exhibit anti-tumor activity. [1-3] Several high performance liquid chromatographic (HPLC) methods have been developed in the past few years[1,4-8] including some very promising HPLC/mass spectrometry (LC-MS) studies that utilized thermospray ionization.[9] Mass spectrometry as a detector offers several advantages over other detection devices for HPLC. These include; (1) molecular weight and/or fragmentation information about the compounds which allow structural elucidation and identification of unknown compounds, (2) the capability for further separation of ions with different mass-to-charge ratios for HPLC peaks that are not very well resolved, and (3) less sample preparation (e.g., destructive derivatization procedures) is required which reduces the possibility for artifacts.

In this study, a LC interfaced to an electrospray ionization (ESI) source was used to quantify by mass spectrometry the amount of VC and VB in near-isogenic lines of *Catharanthus* leaves. Electrospray ionization is recognized as one of the most versatile ionization techniques.[10,11] It is a soft ionization technique, therefore, most ions produced are protonated or otherwise cationized molecules for low molecular weight compounds (in the positive ion mode) or multiply charged ions for large molecules. Thus identification of analytes can be immediately confirmed, by comparison of elution time and the molecular weight. More informative collision-induced dissociation (CID) spectra can be generated, if needed, by electrospray ionization[12] in a single quadrupole mass spectrometer which provides characteristic product ion spectra for structure analysis. A comparison between HPLC-UV and LC-MS (using the full scan mode) was made in this report and the overall sensitivity of the two techniques is discussed.



R = CH_3 (VB)

R = CHO (VC)

Figure 1. Chemical structures of two dimeric indole alkaloids. (A) vinblastine and (B) vincristine.

EXPERIMENTAL

Reagents and Materials

Plant samples were obtained from Goldsmith Seeds, Inc. (Gilroy, CA). Vincristine sulfate, vinblastine sulfate and ammonium acetate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methanol (HPLC grade) and cyclohexane (spectroscopy grade) were purchased from Baxter Scientific Co. (McGaw Park, IL) and water (HPLC grade) was obtained from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). Glacial acetic acid was obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Glass fiber Acrodisc, 25 mm (Gelman Science, Ann Arbor, MI) and glass fiber syringe filters, 13 mm (Whatman Lab Division, Clifton, NJ) were used for filtration. All chemicals were used without further purification.

Standard Preparation

A 1.0 milligram sample of vincristine sulfate or vinblastine sulfate each was placed into a 25 mL volumetric flask, dissolved and diluted to volume with 0.01 M aqueous acetic acid. The standard solution was sonicated for 1 min.

Analytical standards were prepared by diluting the stock standard solution with 0.01 M acetic acid using an electronic digital pipette (Rainin, Woburn, MA) to give concentrations of 0.92, 2.29, 4.58, 9.17 and 27.51 ppm and 0.92, 2.31, 4.63, 9.27 and 27.80 ppm for VB and VC, respectively.

Sample Preparation

Ground *Catharanthus roseus* leaf samples (ca.0.6g) were accurately weighed into glass scintillation vials. Isopropyl alcohol (4 mL) was added and the vials shaken on a wrist action shaker for 15 min. The extracts were filtered through 25 mm glass fiber Acrodiscs into autosampler vials, taken to dryness under a stream of nitrogen, and reconstituted in 1 mL of 0.01 M acetic acid. The acid solutions were partitioned 3 times with 1 mL of cyclohexane. The cyclohexane solutions were discarded and the acid fraction was taken to dryness under a stream of nitrogen. The samples were finally reconstituted in 200 μL of 0.01 M acetic acid and sonicated for 1 min prior to a final filtration through 13 mm glass fiber syringe filters. The sample preparation should be accomplished as fast as possible, since a reduction in VB concentration was observed as a result of prolonged extraction process.

High Performance Liquid Chromatography

HPLC separations of extracted plant samples were performed on a Water's 600-MS system (Waters, Milford, MA) equipped with a Water's 484 tunable absorbance detector at 254 nm. The column used in this study was a 200 x 4.6 mm with 5 μm HP ODS hypersil (Hewlett-Packard, Palo Alto, CA).

During routine analysis, the flow rate was held at 1 mL/min and split with 0.02 in. i.d. PEEK tubing (Supelco, Bellefonte, PA) to the electrospray source and UV detector. Due to the small i.d. of the electrospray needle, the actual flow rates as determined by measuring the weight of mobile phase collected at the outlet of UV detector over a specified time period, were 400 μL/min and 600 μL/min to the electrospray source and UV detector, respectively.

A linear gradient mobile phase was used for the separation of extracted plant samples. Solution A was 25 mM ammonium acetate in HPLC grade MeOH and solution B contained the same concentration of ammonium acetate in HPLC grade water. Both ammonium acetate solutions were prepared daily and sonicated for 10 min prior to use. Although sodium phosphate is reportedly a better reagent[13] for alkaloid analysis, ammonium acetate was selected because of its volatility and compatibility with electrospray mass

spectrometry. A 20 μL aliquot of each sample was injected onto the HPLC column with a 25 μL syringe. Gradients over a 15 min period were used to separate VC and VB from other components in the plant sample. The HPLC mobile phase ratio was held at 60% A:40% B for 10 min, then the percentage of A was increased to 70% in 15 min and held for another 10 min. Most of the major components in the plant samples studied eluted from the column within 35 min.

Electrospray Ionization Mass Spectrometry

The LC-MS interface was a dual stage electrospray ion source purchased from Analytica of Branford, Inc., (Branford, CT) and recently upgraded to accommodate high flow rates. A HP5988A quadruple mass spectrometer (Palo Alto, CA) operating in the full scan mode with a scan range of 200-900 amu at 0.1 s/scan was used in all experiments. The electrospray needle was maintained at ground potential and the platinum-coated glass capillary at -4.6 kV. Nitrogen served both as drying gas (at 200°C) and sheath gas.

Ions were accelerated from the electrospray ion source into the mass spectrometer and focused through three electrical lenses and two skimmers. Applied voltages for those electrical elements were optimized while constantly infusing standard solution into the electrospray source The optimum entrance lens potential (EL) for this experiment was determined by monitoring the ion signal of direct flow injection of a mixture of VC and VB. The areas of VC and VB peaks were plotted against EL to determine the optimum capillary exit potential for this investigation. In some cases where structural information was desired, CID was carried out in the high pressure region between the capillary exit and the skimmer entrance to the mass analyzer by increasing the EL potential to a higher value (250 V in most cases).

Calibration Curves

Five-point calibration curves were constructed for VC and VB to determine the relationship between peak area and the concentration of samples. Standard solutions were prepared in 0.01 M acetic acid ranging from 0.9 to 27.5 ppm and 0.9 to 27.8 ppm for VC and VB, respectively. Calibration curves were generated by plotting the ion intensities versus the standard concentrations used. Each standard was analyzed, in triplicate, on two different days and the average of both sets of triplicate analyses was taken as the ion intensities of standards. The day-to-day relative standard deviations (%RSD) were measured by comparing the ion intensities of VB standard

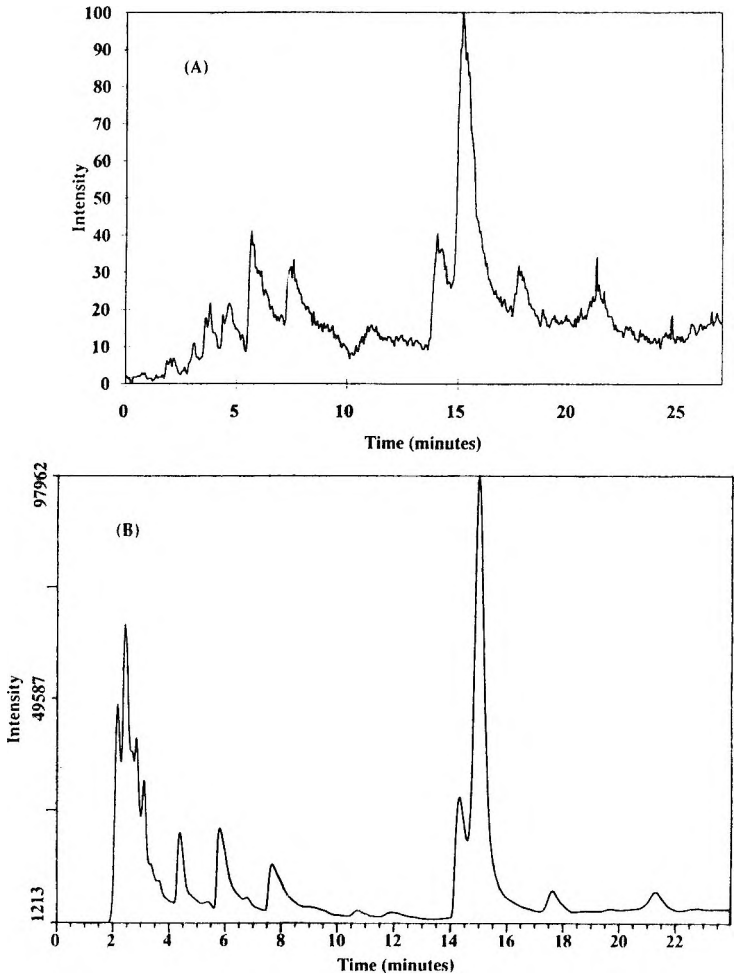


Figure 2. Chromatograms of extracted plant samples obtained by (A) LC-MS (shown as total ion chromatogram) and (B) HPLC-UV. Samples prepared in 0.1 M aqueous acetic acid. Elution times for vincristine and vinblastine are 14.8 and 21.3 min, respectively.

solutions obtained on three different days. The %RSD was approximately 10% for all the samples analyzed. The same set of ion intensity data was re-analyzed on a new calibration curve created after two weeks and similar %RSD were obtained by comparsion of the data with those measured earlier.

Table 1

Slopes and Linear Regression Data Obtained for VB and VC Calibration Curves by UV and LC-MS

	LC-MS		UV	
	Slope	**R^2**	**Slope**	**R^2**
VB	234.7	0.9839	24852	0.9998
VC	188.17	0.9995	37663	0.9987

Recoveries were determined by spiking plant samples with both vincristine and vinblastine, followed by extraction with the same procedure as the samples and comparison with authentic standards prepared in the acetic acid solution. The percent recovery ranged from 90-100% for both compounds.

RESULTS

The LC-MS total-ion-chromatograms (TIC) and the HPLC-UV absorbance chromatogram obtained for all the samples studied (e.g. Figure 2) were similar. The relatively high background in the TIC compared to that of the UV absorbance chromatogram, is the result of ammonium acetate cluster ions. VC, which has one more polar group (CHO) than VB (Figure 1) eluted earlier in the reversed phase HPLC system as expected. No indications of interference from either the sample matrix or mobile phases were observed, since the retention times (RT) and mass spectra of VC (RT = 14.8 min, $[MH]^+$ = 825.8) and VB (RT = 21.3 min, $[MH]^+$ = 811.5) in the spiked plant samples were essentially the same as that of the authentic standard compounds.

Linear regression parameters for the calibration curves of VC and VB measured by both UV and LC-MS are shown in Table 1. Six sample sets were analyzed by HPLC-UV and LC-MS to compare the two techniques. The results (Table 2) showed no appreciable differences between concentrations obtained by the two methods in any of the six samples investigated. Also, detection limits between these two detection methods were comparable. However, at lower sample concentration (especially at concentration <0.01 ppm), the detection limit of the UV suffered considerably from interferences of higher concentration components eluting in close proximity to VC and VB. Since little was known about the actual concentrations of these two dimeric alkaloids in the plant samples, we believed that mass spectrometry would be a more

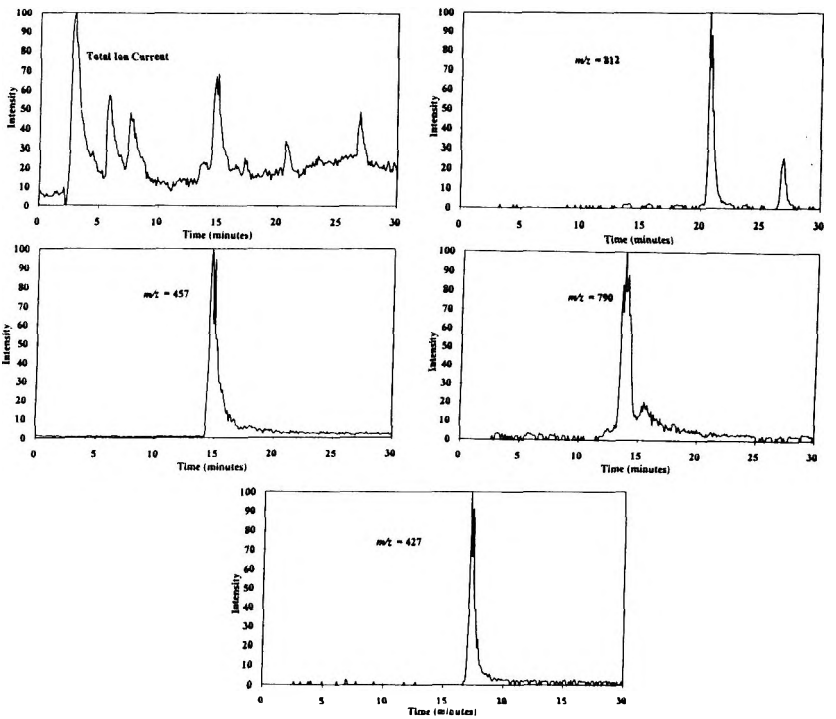


Figure 3. Selected ion chromatograms of protonated molecules for other compounds eluting from the column at close proximity to vinblastine and vincristine.

Table 2

Comparison of UV and LC-MS Concentrations (μg/g) for VB in Six Plant Samples (Mean Conc. ± S.D.)

Sample #	1	2	3	4	5	6
UV	5.9 ± 1.3	3.3 ± 0.2	9.4 ± 1.3	3.9 ± 0.3	4.4 ± 0.3	2.4 ± 0.1
LC- MS	7.0 ± 1.1	3.3 ± 0.3	8.1 ± 0.7	4.2 ± 0.1	4.8 ± 0.1	2.4 ± 0.1

reliable quantitative tool for our purpose. In addition, ions of different *m/z* can be distinguished by plotting selected ion chromatograms (Figure 3) or by selective ion monitoring (SIM), which will both greatly increase the detection limits of LC-MS. For this reason, LC-MS was selected for determination of VC and VB in all the plant samples investigated.

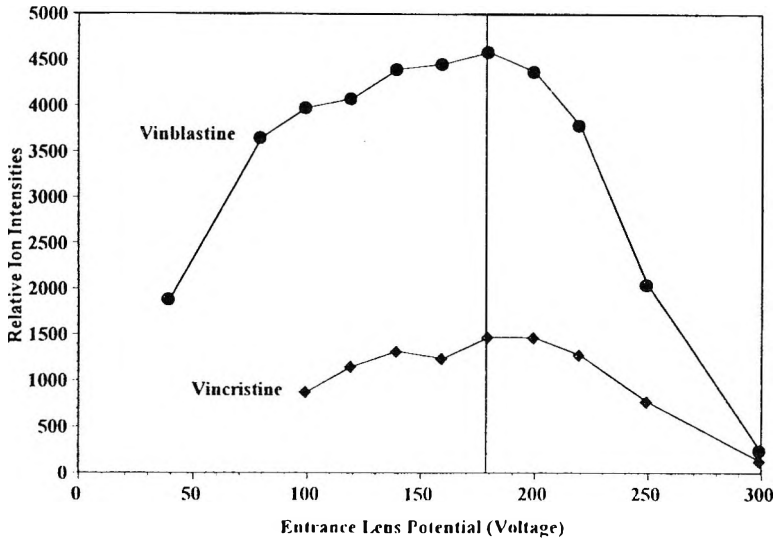


Figure 4. Plot of ion intensities vs. capillary exit potential for vinblastine and vincristine.

The optimum EL potentials for both VB and VC were determined to be approximately 180 V as shown in Figure 4. A higher EL value (250V) was used to provide more information-rich product-ion spectra for some samples which exhibited mass spectra similar to the alkaloids of interest.

DISCUSSION

The success in introducing a high gas flow coaxially around the emerging liquid stream has greatly increased the flow rate feasible for electrospray.[14] Flow rates as high as 1 mL/min are routinely employed in our laboratory so that conventional HPLC conditions can be applied to LC-MS without modification. Similarities between HPLC chromatograms obtained from LC-MS and UV (Figure 2) make direct comparison between these two techniques rather easy. Although the relative peak height is detection-principle-dependent, identification of unknown HPLC-UV peaks can be readily made by examination of the peak eluting at the same retention time in the total ion chromatogram of the LC-MS. This approach allows a more accurate selection of standard compounds than those based solely on retention time.

Similar detection limits were observed between the UV detector and the mass spectrometer for the molecules being studied. Since mass spectrometry has the potential to further distinguish eluted compounds by selecting mass chromatograms, peaks can be presented without background interference from solvent or other molecules (Figure 3). Therefore, more reliable quantitative results can be obtained.

Since very little heating is involved in the electrospray ionization process, most ions produced are protonated molecules (positive ion mode), which is one of the advantages of electrospray ionization over other ionization techniques. For clarification, a comparison between the mass spectra of a VC standard analyzed by both electron-impact ionization (EI) and ESI is presented in Figure 5. As can be seen, two series of small fragments which represent loss of *m/z* 13-14 were observed at *m/z* <149 in the EI spectrum. This could be the consecutive loss of CH or CH_2 groups from one of the monomers of the main VC skeleton. No molecular ion and no significant ions above *m/z* 200 were detected. On the other hand, in the LC-MS spectrum, the protonated molecular ion is the dominant species. Thermospray ionization, which is also a soft ionization technique, produced fairly extensive fragmentation for VB,[13] while essentially no fragmentation of this compound occurred in ESI-MS (Figure 6A). Also, some adduct-ion peaks were observed which resulted from either incomplete desolvation or the addition of alkali-metal ions in the electrospray ion source.

Since relative ion intensities are inversely proportional to the degree of fragmentation; the detection limit of electrospray ionization for these dimeric alkaloids is likely to be lower for LC-MS than that of thermospray techniques. However, no direct comparison was made. The capability for generating predominantly molecular ions by LC-MS allowed us to separate molecules which have similar proton affinities but differ in molecular weights in a complex mixture. This was accomplished by simply examining their protonated molecules without further time-consuming HPLC separations.

As previously mentioned, CID for the compounds under investigation, can be generated between the capillary exit and skimmer entrance of the ESI source. The effect of EL on the molecular ion intensities was investigated and the results are shown in Figure 4. Both VB $[M+H]^+$ (*m/z* 811) and VC $[M+H]^+$ (*m/z* 825) ion intensities start low at EL = 40 V, reach a maximum at EL of ~180 V and then decreased dramatically at EL > 220V. The initial increase in the ion signals as EL increases is probably due to the fact that the ions are gaining sufficient energy for more efficient transfer to the mass analyzer by increasing the potential difference between capillary exit and the skimmer entrance. When this potential difference exceeds a certain value (in this case,

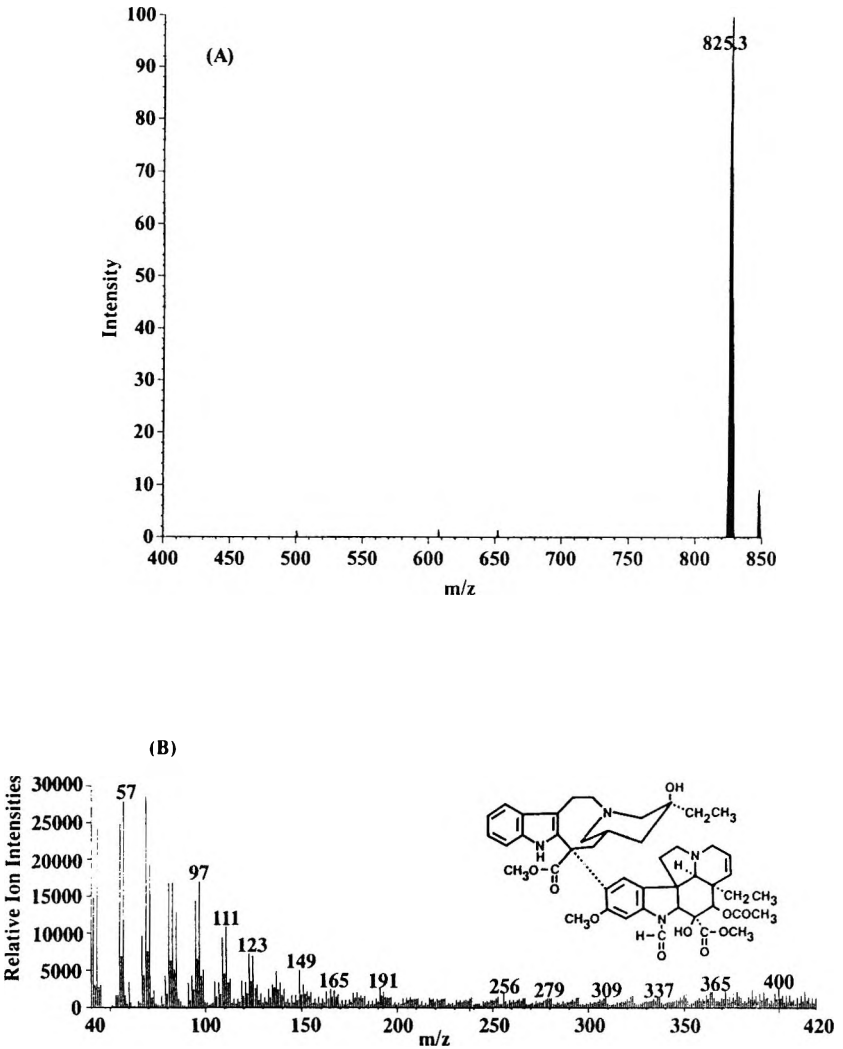


Figure 5. Mass spectra of vincristine by (A) electrospray and (B) electron-impact ionization mass spectrometry. Electrospray conditions: drying gas (N_2) at 200°C and capillary exit potential at 180 V.

EL >180V), high-voltage collision-induced dissociation (HV-CID) will occur (Figure 4) and the relative ion intensity of $[M+H]^+$ decreases, with an onset of fragment ions. Structural information can be obtained through HV-CID. For example, the mass spectrum of VB obtained at EL = 250V (Figure 6B) shows

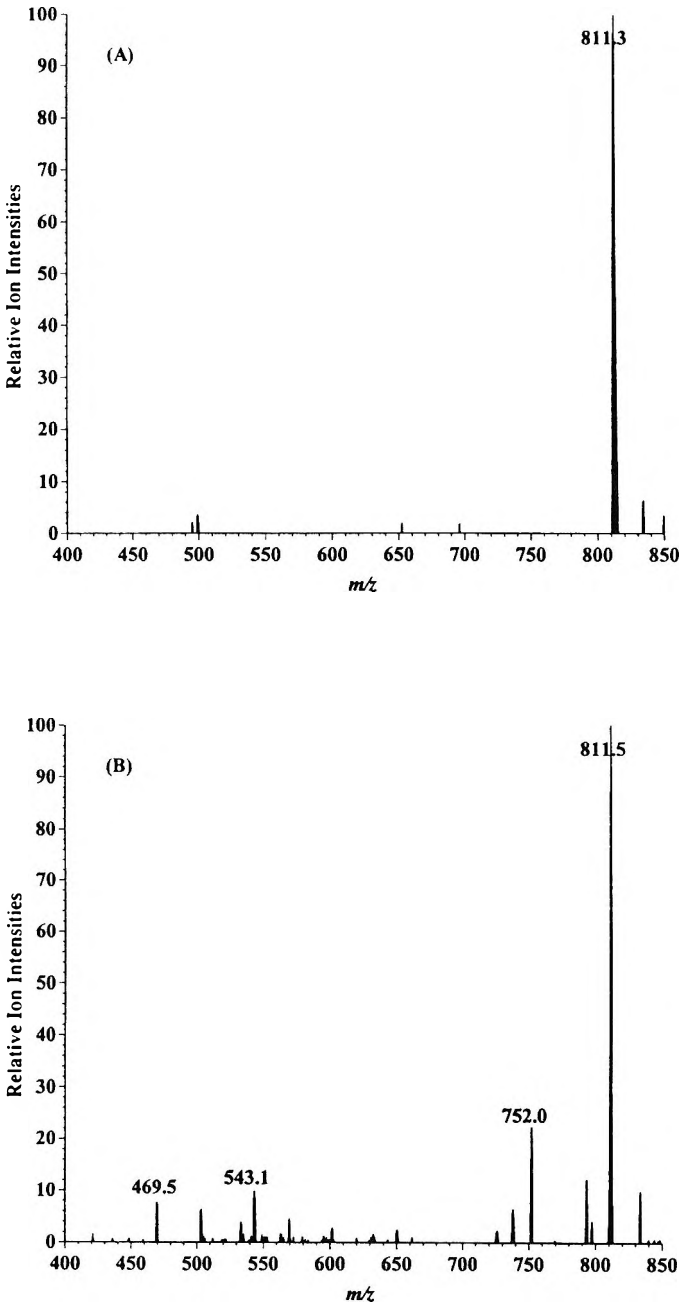


Figure 6. Electrospray mass spectrum of vinblastine at (A) EL = 180 V and (B) EL = 250 V.

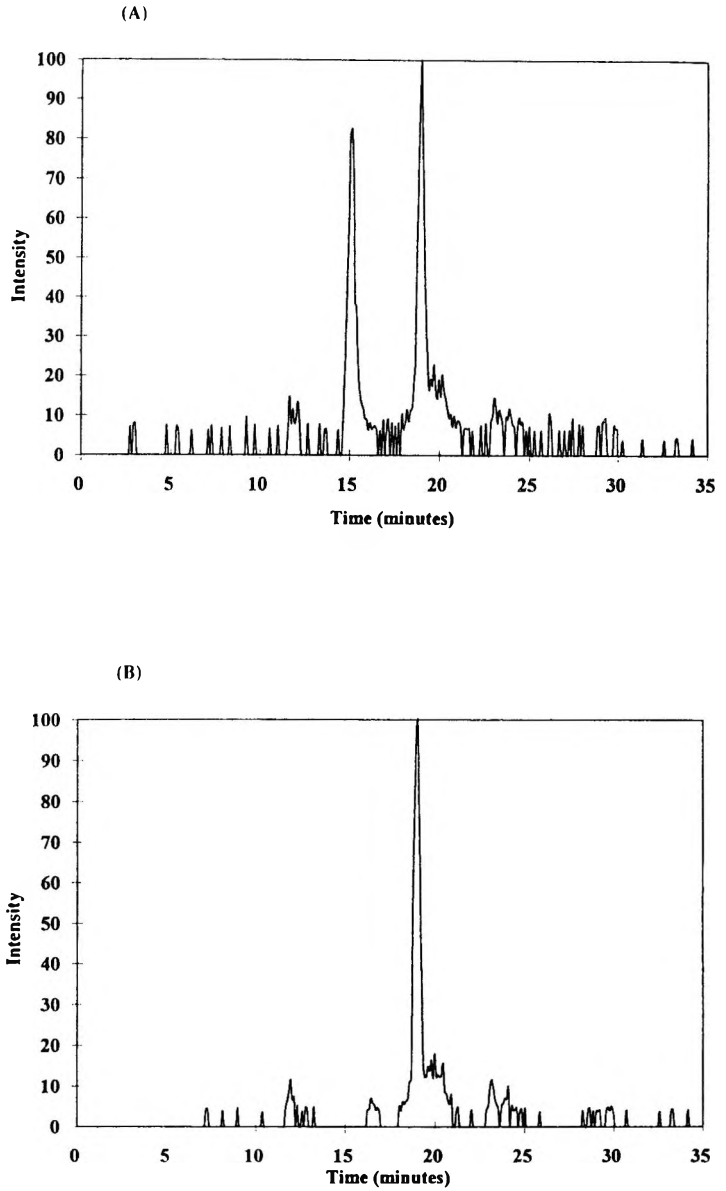


Figure 7. LC-MS ion chromatograms of m/z = 825 (A) plant extract sample spiked with vincristine and (B) unspiked plant extract sample.

fragment ions corresponding to the loss of H_2O (*m/z* 793) and $C_2H_3O_2$ (*m/z* 752) from the parent ion as were observed previously by desorption CI mass spectrometry.[16]

In some of the LC-MS chromatograms of extracts, a peak of *m/z* 825 eluted at a RT approx. 4.9 min. later than the standard VC peak (RT = 14.8 min.). In order to determine the nature of this peak, two approaches were taken. First, the samples were spiked with VC to determine if matrix effect might contribute to a change in RT for this compound. As shown in Figure 7A, a peak eluted at the same RT as the authentic VC standard in the spiked solution with $[M+H]^+$ of *m/z* 825. This indicated that the peak which eluted at RT = 19.7 (Figure 7B)is a different alkaloid than VC, possibly the product-ion of *m/z* 847, (depending on whether the ion at *m/z* 847 is a protonated molecular ion or a sodium adduct $[M+Na]^+$ ion. Next, we analyzed the spiked sample again with an EL of 250 V instead of 180 V to produce CID. The product-ion spectra of the two compounds were distinctly different. The VC standard, like VB, showed a loss of water (*m/z* 807) and $C_2H_3O_2$ (*m/z* 766) from the parent ion. It also had fragment ions at *m/z's* 798 and 780 which represent the $[M+H]^+$ - CHO and $[M+H]^+$ - CHO - H_2O. The other alkaloid (compound eluting later), had a major peak at *m/z* 790 in the product ion spectrum which was not detected in the VC standard. Since the goal of this study was to determine the concentration of VC and VB in near-isogenic lines of plant samples, identification of other possible alkaloids, which is the subject of continuing effort, will not be presented here.

SUMMARY

In this study, the potential of using an HPLC/ESI-MS technique for the analysis of weakly bonded dimeric alkaloids such as vincristine and vinblastine was investigated. These two structurally similar dimeric alkaloids were successfully separated on a C_{18} column from other possible alkaloids in the plant samples by gradient elution with 25 mM ammonium acetate in MeOH and water. Protonated molecules were always the dominant species produced in the electrospray ion source even though more informative spectra could be obtained by collision-induced-dissociation of the molecular ion.

The actual detection limit of this technique is dependent on the nature of the compound (e.g. ionization potential, proton affinity, etc.), but because of the "softness" of the electrospray ionization, the sensitivity of this technique is believed to be better than other LC-MS techniques.

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RETENTION PROFILE OF FENBENDAZOLE AND ITS SULPHOXIDE, SULPHONE, AND HYDROXYLATED METABOLITES IN ION-PAIR LIQUID CHROMATOGRAPHY

N. A. Botsoglou, D. J. Fletouris, I. E. Psomas, A. I. Mantis

School of Veterinary Medicine
Aristotle University
GR-54006 Thessaloniki, Greece

ABSTRACT

The retention behavior of fenbendazole and its sulphoxide, sulphone, and p-hydroxylated metabolites as a function of the nature and the concentration of the ion-pairing reagent in the mobile phase has been investigated. The influence of negatively and/or positively charged pairing ions on peak shape has been discussed, whereas the effect of column temperature on retention has been evaluated.

INTRODUCTION

Fenbendazole, a benzimidazole drug, has become an integral part of the animal producing industry for control of internal worm parasites.[1] Despite the large scale use of this anthelmintic, adequate methods for the analysis of fenbendazole and its metabolites as residues in edible tissues and milk do not currently exist. Reported analytical methods,[2-4] although valuable for studying the routes of metabolism and excretion of fenbendazole in cattle,[5] goat,[6] and

rabbit[7] they may be, cannot offer the sensitivity required to ensure that animal derived human foods do not contain residues that exceed legal levels. The maximum residue limit of total fenbendazole residues in edible tissues and milk has been recently set at 10 ng/g.[8,9]

In this regard we have been developing new methods for the determination of fenbendazole residues in milk.[10] As a result of these early approaches, we have developed various ion pair liquid chromatographic systems to better control the selectivity and elute fenbendazole and its sulphoxide metabolite in a single run without tailing.[11] We report here a further examination of this ion-pair methodology for the efficient liquid chromatographic separation of fenbendazole and its sulphoxide, sulphone, and p-hydroxylated metabolites at trace residue levels.

EXPERIMENTAL

Instrumentation

Liquid chromatography was carried out with a Gilson system consisting of a Model 805 manometric module, a Model 305 piston pump, a Model 119 UV-vis detector, and a Model TC 831 column heater (Gilson Medical Electronics, Villiers-le-Bel, France). Injections were made on a Hichrom, 250x4.6 mm, stainless-steel column packed with Nucleosil 120 C_{18}, 5-μm, through a Rheodyne, Model 7725, injection valve (Cotati, CA) equipped with 100-μL sample loop. Recordings were made with a Kipp & Zonen, Model BD 111, pen recorder (Delft, Holland).

A Model D7402 EasyPure UV compact ultrapure water system (Barnstead/Thermolyne Corp., Dubuque, IA) was also used for preparing highly purified water.

Chemicals

Lichropur grade octanesulphonate sodium salt and tetrabutylammonium hydrogen sulfate, analytical grade phosphoric acid, and LC grade acetonitrile were from Merck (Darmstadt, Germany). Standard fenbendazole sulphoxide, fenbendazole sulphone, p-hydroxyfenbendazole, and fenbendazole (Fig. 1) were a gift from Hoechst Hellas (Athens, Greece).

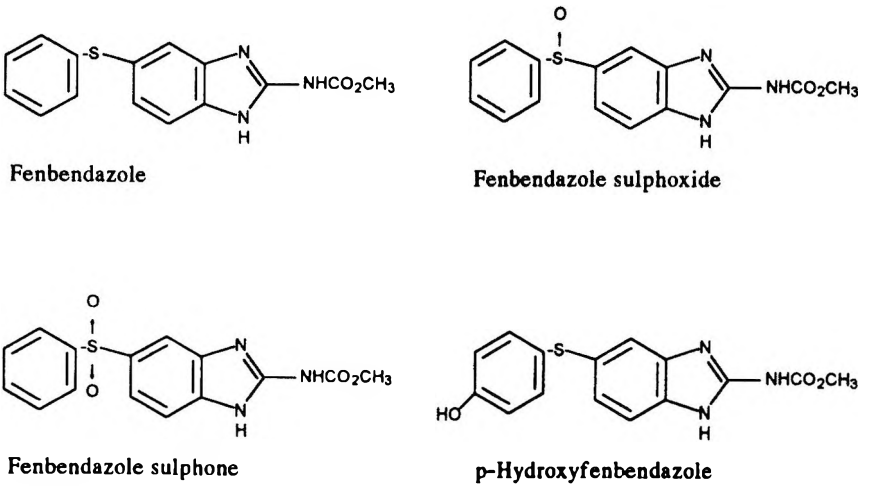

Figure 1. Chemical structures of investigated benzimidazoles.

Standard Solutions

Individual stock solutions of each reference standard were prepared in 10-mL volumetric flasks by weighing ca 5 mg of the compound and dissolving to volume with dimethylsulphoxide. A mixed standard intermediate solution containing all 4 analytes was prepared by combining appropriate aliquots (120-500 μL) from each of the stock solutions in a 10-mL volumetric flask and diluting to volume with acetonitrile. Mixed standard working solutions were prepared by diluting appropriate aliquots of the mixed intermediate solution in the mobile phase used each time.

Chromatographic Conditions

The mobile phases used in this study consisted of acetonitrile and 0.01 M phosphoric acid or phosphate buffer (pH 7). The acidic mobile phases differed from each other in both the type and the concentration of the contained ion-pair reagent. Some contained octanesulphonate sodium salt or tetrabutylammonium hydrogen sulfate, whereas others contained a mixture of these pairing ions at a molar ratio of 1/1 or 1/2. Following their preparation, each mobile phase was passed through 0.2 μm Nylon-66 filter (Anachem, Luton, UK) and degassed using helium. The mobile phase was delivered in the system at a rate of 1 mL/min.

The Nucleosil 120 C_{18} stationary phase was thoroughly equilibrated with mobile phase each time before use. Reproducible capacity factors (k′) could be obtained after passage through the column of at least 70 mL of mobile phase. When the mobile phase contained ion-pair reagents, passage of 150-mL volume was indispensable for column equilibration. On changing the mobile phase, successive column washings with at least 100 mL portions of water and acetonitrile were quite indispensable for removing the adsorbed pairing ions. Detection was made at 290 nm at a sensitivity setting of 0.05 a.u.f.s. Chart speed was set at 5 mm/min.

RESULTS AND DISCUSSION

Separation of the studied analytes by literature reversed phase liquid chromatographic methods[2-4] was generally characterized by poor chromatographic performance. The highly acidic mobile phase that has been described[4] in the analysis of fenbendazole residues in milk resulted in chromatograms with broad and tailing peaks. Attempts to resolve this problem by applying a flow rate program similar to that suggested[2,3] for the analysis of fenbendazole and its metabolites in plasma, urine, feces, and tissue homogenates, helped somewhat but failed to provide an acceptable procedure. In those ionization enhancement systems, where silanophilic interactions of the protonated analytes with the stationary phase occurs, peak distortion was more pronounced for the late eluted fenbendazole (Fig. 2A). To eliminate peak tailing and improve the chromatographic efficiency, a pH 7 mobile phase with similar elution strength was used instead.

However, excessive retention of all analytes was noted due to suppression of their ionization at this pH value. By increasing the elution strength of the mobile phase, retention could be drastically shortened but the tailing of the peaks could not be totally eliminated [Fig. 2B]. It might be of interest to note, that in these ion-suppression conditions the elution order of the p-hydroxy and sulphone metabolites reversed, the latter eluting now before the former.

Figure 2 also shows that, using a pH 7 mobile phase, peaks of both fenbendazole and p-hydroxyfenbendazole are higher than those expected on the basis of the recorded response with the pH 2.2 mobile phase. This was atributed to the significant absorbance difference between the protonated and unprotonated forms of these compounds. Upon acidification, the absorbance of both fenbendazole and p-hydroxyfenbendazole, unlike the other metabolites, undergoes a remarkable reduction (78-80 %).

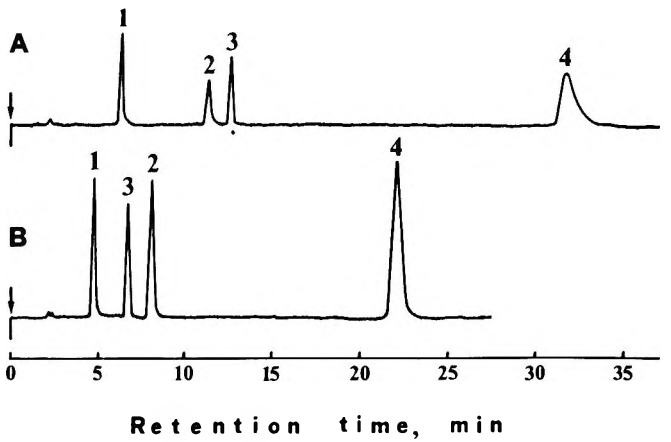

Figure 2. Chromatograms of a mixed standard working solution (2.5 μg/mL fenbendazole sulphoxide, 3.0 μg/mL p-hydroxyfenbendazole, 2.4 μg/mL fenbendazole sulphone, and 10.0 μg/mL fenbendazole) run with a mobile phase consisting of (A) acetonitrile-0.01 M phosphoric acid (30:70, v/v) or (A) acetonitrile-0.01 M phosphate buffer, pH 7, (40:60, v/v). Other LC conditions: column 250x4.6 mm, Nucleosil 120, C_{18}, 5μm; temperature, 30°C; flow rate, 1 mL/min; wavelength 290 nm; detection sensitivity, 0.05 a.u.f.s.; chart speed, 5 mm/min; injection volume, 10 μl. Peak identification: fenbendazole sulphoxide (1), p-hydroxyfenbendazole (2), fenbendazole sulphone (3), and fenbendazole (4).

To inactivate the residual free silanol action on the silica-based C_{18} packing material and eliminate peak tailing, modification of the LC partitioning process through addition to the mobile phase of various pairing agents was examined. In this investigation, an acidic mobile phase that consisted of acetonitrile and 0.01 M phosphoric acid (30/70, v/v) was selected as reference point. The effect of the pairing agents examined (type and concentration) on the retention and peak shapes of all analytes is best illustrated in Figure 3. In absence of ion-pair reagents, low height and badly tailed peaks appeared due, obviously, to strong silanophilic interactions of the protonated analytes with the stationary phase (Fig. 3A). Addition of negatively charged octanesulphonate ions in the mobile phase had a remarkable effect on both the shape and the elution order of the peaks (Fig. 3B). Despite the high increase of retention noted due to ion-pairing of the octanesulphonate anions with the positively charged benzimidazoles to more hydrophobic forms, peak heights were not significantly decreased, and peak distortion was reduced.

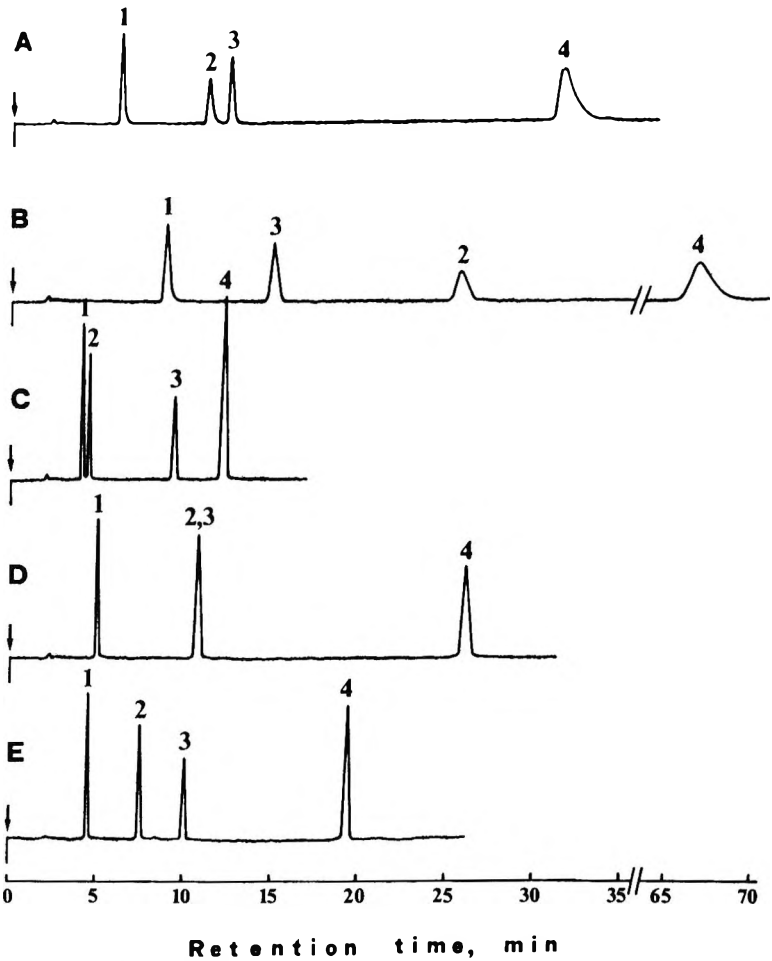

Figure 3. Chromatograms of a mixed standard working solution (concentrations as in Figure 2) run with an acetonitrile-0.01 M phosphoric acid (30:70, v/v) mobile phase containing or not ion-pair reagents; (A) without reagents; (B) with 5 mM octanesulphonate reagent; (C) with 5 mM tetrabutylammonium reagent; (D) with 5 mM octanesulphonate and 5 mM tetrabutylammonium reagents; (E) with 2.5 mM octanesulphonate and 5 mM tetrabutylammonium reagents.). Other LC conditions as in Figure 2.

Nevertheless, the tailing of the peaks could not be totally eliminated. Figure 3B also shows that octanesulphonate is an efficient means for regulating the elution order of the p-hydroxy and sulphone metabolites, as the former can elute far beyond the latter upon addition of the ion-pair reagent.

Figure 3C demonstrates that the most significant change in the chromatographic profile of the analytes is produced when positively charged tetrabutylammonium ions are added in the mobile phase. Retention was greatly decreased, peak tailing was totally eliminated, and peak heights were spectacularly increased. These effects may partly, at least, be attributed to efficient masking of the surface free silanol groups by the tetrabutylammonium reagent.[12,13] Electrostatic repulsion of the protonated analytes by the tetrabutylammonium cations adsorbed on to the octadecylsilica surface could also contribute to these effects.[14,15] At these conditions, however, p-hydroxyfenbendazole eluted quite close to the sulphoxide metabolite, as the elution time of the former had been largely shortened.

Considering that both octanesulphonate and tetrabutylammonium pairing ions could variably influence the chromatographic behavior of the fenbendazole metabolites, co-addition of these reagents in the mobile phase was investigated in an effort to achieve a desirable degree of separation between all analytes in a minimum time. It was found that addition of equimolar concentrations of these reagents could separate the sulphoxide and the p-hydroxy metabolites but the resolution of the latter from the sulphone metabolite was destroyed (Fig. 3D). In contrast, excellent resolution could be made possible when the molar concentration of the added octanesulphonate was half the concentration of the tetrabutylammonium reagent (Fig. 3E).

The retention behavior observed in this study, in which case the mobile phase in addition to tetrabutylammonium cations contains equal or lower concentration of octanesulphonate anions, is difficult to explain. The chromatographic mechanism in such systems has not yet been elucidated. The octanesulphonates might interact with both the anti-tailing tetrabutylammonium and solute ions; further, the two opposite charged surfactants might be co-adsorbed on to the column material.[16,17] Figure 3 indicates that the affinity of tetrabutylammonium cations to residual silanols should be more pronounced than to octanesulphonate anions, as the anti-tailing effect of the tetrabutylammonium reagent is not reduced by the presence of octanesulphonates. Figure 3 also suggests that negatively charged pairing ions capable to form ion pairs with solute cations are present, even if their concentration is equal to that of the positively charged pairing ions. Thus, it might be expected that the effect of variation in the concentration of the octanesulphonates would be as in reversed phase ion pair chromatography; further enhancement of retention occurs when the concentration of octanesulphonate anions increases. These observations that lend support to previous findings[11,18] may help in better understanding the retention mechanisms involved in ion-pair chromatography.

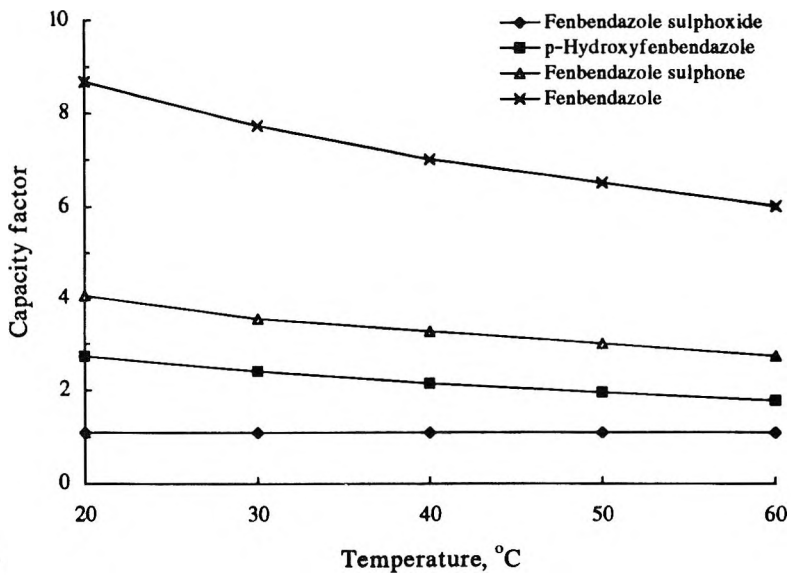

Figure 4. Effect of column temperature on the capacity factors of fenbendazole and its metabolites.

The effect of column temperature on retention time, when the mobile phase contains the optimized ratio of 2.5 mM octanesulphonate and 5 mM tetrabutylammonium reagents, was also investigated. Figure 4 shows that progressive reduction in retention for fenbendazole and its p-hydroxy and sulphone metabolites occurs when column temperature is increased up to 60°C, which is typical behavior for a reversed phase system. The retention, however, of the early eluted sulphoxide metabolite could not be affected by temperature change, a finding suggesting that control of temperature may aid in specific ion-pair separations.

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DETERMINATION OF SODIUM ACETATE IN ANTISENSE OLIGONUCLEOTIDES BY CAPILLARY ZONE ELECTROPHORESIS

Danhua Chen,* Pat Klopchin, Jodi Parsons, G. Susan Srivatsa

Isis Pharmaceuticals, Inc.
2292 Faraday Avenue
Carlsbad, California 92008

ABSTRACT

The determination of sodium acetate in antisense oligonucleotides purified by reverse phase chromatography in acetate buffer is important in both impurity profiling and drug concentration assay. A method has been developed for the quantitation of sodium acetate in a 20-mer phosphorothioate oligonucleotide drug substance employing capillary zone electrophoresis. Chloride was included as an internal standard to increase accuracy and precision of the measurement. Interference due to oligonucleotide matrix was eliminated by filtering the olignucleotide solution through a Centricon SR-3 column. The linear dynamic range for the method is 0.06-240 μg/mL. The electrophoretic separation requires only 6 min. The method has been demonstrated to be simple and reliable for the quantitation of acetate salts in oligonucleotide drug substances.

INTRODUCTION

Antisense oligonucleotides have been developed in recent years as potential drugs to treat human diseases ranging from HIV to cancer.[1] The specific binding of oligonucleotides to their corresponding RNA targets via Watson-Crick hybridization can inhibit RNA splicing[2] and mRNA translation,[3] and cause the degradation of RNA by RNase H.[4] Phosphorothioate oligonucleotides are one class of first-generation antisense analogs of natural oligonucleotides, modified to enhance nuclease resistance while maintaining hybridization to the target mRNA.[5] Phosphorothioate oligomers are generally synthesized on a solid phase DNA synthesizer and purified by preparative reverse phase chromatography. Sodium acetate is introduced by the elution buffer and removed by subsequent ultrafiltration and/or precipitation. The determination of residual acetate in the purified oligonucleotide drug substance is important for drug purity analysis.[6,7]

There is a lack of simple methodology for determination of acetate at low concentrations.[8,9] Various chromatographic techniques have been applied for the quantitative determination of acetate. However, they are less advantageous due to requirements for (i) extensive sample preparation or (ii) appropriate derivatization in order to introduce a detectable chromaphore or to reduce the analytical polarity.[10-13] A hyphenated GC-MS technique has been proposed for the determination of acetate in dried solid samples, in which the acetate was converted to acetic acid by heating with oxalic acid, followed by chromatographic purification, combustion and mass spectrometric detection of carbon dioxide. The recoveries are only 81 $\pm$ 5 % in the linear dynamic range of 3-50 μmoles of acetate.[14] Nonaqueous titration has been suggested by the United States Pharmacopeia for the determination of acetate salts, but the method is only suitable for distinctly anhydrous salts in the absence of other carboxylate anions,[15] not applicable to oligonucleotides. Membrane electrode sensors for the determination of acetate suffer from a narrow dynamic range, poor sensitivity and serious interferences.[16,17] A potentiometric gas sensor was proposed for the determination of acetate in medical solutions, metal acetates and vinegar. The device is composed of a gas-permeable teflon membrane and an internal pH sensing electrode cell. Acidifying the acetate solution to pH < 2 allows the free acetic acid vapor to diffuse through the membrane and changes the pH of the electrode.[8] ^{1}H NMR spectroscopy has been used for the determination of acetate in intravenous infusions and haemodialysis solutions, based on the singlet spectrum of the methyl protons of acetate in D_2O at δ 1.92 with N-methylurea as internal standard.[9] Unfortunately, the NMR method can only be used for solid samples at high acetate concentrations, and is not feasible for quantitating acetate in oligonucleotides due to interference by the oligonucleotide matrix. An enzymatic spectrophotometric method for direct

kinetic assay of acetate in serum or plasma is based on three sequential enzymatic reactions involving acetic kinase, pyruvate kinase and lactate dehydrogenase. The acetate concentration was measured by the decrease in absorbance at 340 nm due to NADH consumption.[18] The requirement of using three enzymes for the determination made this method less attractive.

Capillary electrophoresis is one of the fastest growing separation techniques today.[19,20] Analytes include simple organic and inorganic molecules, peptides, proteins, oligonucleotides, and DNA fragments.[21] One advantage of this technique is the high analytical efficiency, which is orders of magnitude greater than those obtained by HPLC and is achieved in a similar or shorter period of time.[21] Capillary Zone Electrophoresis (CZE), the simplest and most widely used CE mode, provides separation based on differences in free-solution mobility. In the present work, CZE has been developed as a simple and reliable method for the determination of residual acetate salts in phosphorothioate oligonucleotides.

MATERIALS AND METHODS

Deionized (Milli-Q) water was used for the entire experiment unless otherwise stated. The CIA-Pak™ Anion-BT solution was purchased from Waters Corporation (Milford, MA). Potassium hydrogen phthalate and sodium acetate trihydrate (HPLC grade) were purchased from Sigma (St. Louis, MO). Sodium chloride was purchased from J.T. Baker (Philipsburg, NJ). Centricon-SR3 (3,000 MW) tubes and 0.2 micron filters were purchased from Amicon, Inc. (Beverly, MA) and Nalge Company (Rochester, NY), respectively. The oligonucleotide sample is a 20-mer phosphorothioate of the sequence 5' GTT CTC GCT GGT GAG TTT CA 3'. It was synthesized using a Milligen 8800 DNA synthesizer.

(1) Preparation of Electrolyte and Reference Standard Solutions

Phthalate electrolyte solution was prepared weekly by weighing 0.204 g potassium hydrogen phthalate into a beaker and adding 5 mL of Waters CIA-Pak™ OFM Anion-BT solution and about 150 mL Milli-Q water. The solution was mixed thoroughly and the pH was adjusted to 5.6, using 1 N NaOH. The solution was transferred into a 200 mL volumetric flask, filled to the mark with Milli-Q water and filtered through a 0.2 micron filter. Acetate stock solution (2.00 mg/mL) was prepared by weighing 4.611 g sodium acetate ($CH_3COONa \cdot 3H_2O$, MW 136.08, HPLC grade) into a clean 1000 mL

volumetric flask, dissolved and filled to the mark with Milli-Q water. The stock solution was stored at 2-8°C when not in use and kept for a month. Chloride stock solution (4.00 mg/mL) was prepared by weighing 6.594 g sodium chloride into a clean 1000 mL volumetric flask, dissolved and filled to the mark with Milli-Q water. The solution was stored at 2-8°C when not in use and kept for 6 months. Reference standard solutions of acetate in the range of 0.06-240 μg/mL, containing 40 μg/mL chloride as internal standard, were prepared by transferring appropriate volumes of the acetate and chloride stock solution to a volumetric flask and diluting to the mark with Milli-Q water.

(2) Preparation of Sample Solutions

Solutions of oligonucleotide drug substance were prepared by accurately weighing 5 ± 0.1 mg of oligonucleotide sample. Concurrently, an additional sample was weighed for the determination of water content by capillary gas chromatography. About 3 mL of Milli-Q water were added into a 5 mL volumetric flask containing the oligonucleotide. The sample was dissolved and mixed well by vortexing. 50 μL of the 4.00 mg/mL chloride stock solution was added and the sample solution was diluted to the mark with deionized Milli-Q water and mixed well by shaking.

The sample solution was filtered through Centricon-SR3 3,000 MW tubes to remove the oligonucleotide matrix. Prior to fitration of oligonucleotide solution, the Centrion SR-3 tube was washed twice by centrifugation of 2 mL deionized Milli-Q water at 5,000 rpm for 60 min, and the residual water was removed by inverting the tube and centrifuging for 5 min at the same speed. About 2 mL of oligonucleotide solution was added to the clean Centricon tube and centrifuged at 5,000 rpm for 60 min at 4°C. The collected solution in the bottom of the tube was analyzed by capillary zone electrophoresis to determine acetate.

(3) Capillary Electrophoresis Apparatus

A polyimide coated fused silica capillary tube of 100 μm i.d. was cut to 60 cm length. A detection window was made by burning an approximate 5 mm section at 15 cm from one end using an electrical heating device to remove the coating. The column was rinsed for 10 min with 1 N NaOH and 10 min with Milli-Q water, filled with a 0.2% (v/v) solution of γ-methacryloxypropyltrimethoxysilane in 50% (v/v) ethanol/water, and allowed to stand for 1 hour.

The column was then flushed with air to remove residual reagent and rinsed with Milli-Q water for 10 min. The capillary column was placed in a capillary cartridge. Both ends were cut to form a 47 cm column (40 cm from inlet to the detection window).

A Beckman P/ACE 5000 instrument (Fullerton, CA) was used for the CZE experiments. As the capillary cartridge was inserted, the column was rinsed with Milli-Q water for 2 min before use. Under high pressure, the capillary column was filled with electrolyte solution for 30 seconds before each sample injection and was rinsed with Milli-Q water for 30 seconds after each run. The sample solution was injected hydrostatically for 10 seconds and separated at 15 kV for 6 min at 30°C. The background absorbance was measured at 254 nm.

RESULTS AND DISCUSSION

Phthalate is an intermediate mobility electrolyte which absorbs UV light at 254 nm. CIA-Pak™ Anion-BT is an electroosmotic flow modifier used to reverse the normally cathodic direction of the electroosmotic flow found in fused-silica capillaries.[22] Phthalate (5 mM) containing 2.5% (v/v) CIA-Pak™ OFM Anion-BT was applied as cathode and anode electrolytes and filled into the capillary tube. As the sample solution was injected hydrostatically and the electric field was applied, all anions migrated toward the anode. Since the capillary tube contained the same concentration of phthalate, a steady absorbance of phthalate was obtained. Acetate in the sample plug migrates faster than phthalate. Since the anions repel each other, phthalate ions were replaced by the acetate ions during the migration process. Acetate exhibits no absorption of UV light, therefore the relative decrease in phthalate concentration is attributed to a decrease in absorbance as acetate ions reach the detector.

Using negative detector polarity, a peak is obtained whereby the peak area is proportional to the acetate concentration in the sample. Since the oligonucleotide samples do not contain chloride, chloride has been selected as an internal standard and added to the sample solution. The chloride ions migrate faster than acetate ions (Figure 1). The area of acetate has been normalized against the area of chloride, the internal standard and the normalized area of acetate has been used for quantitation. The relative migration time of acetate to that of chloride (the normalized migration time) is used as an identity for acetate.

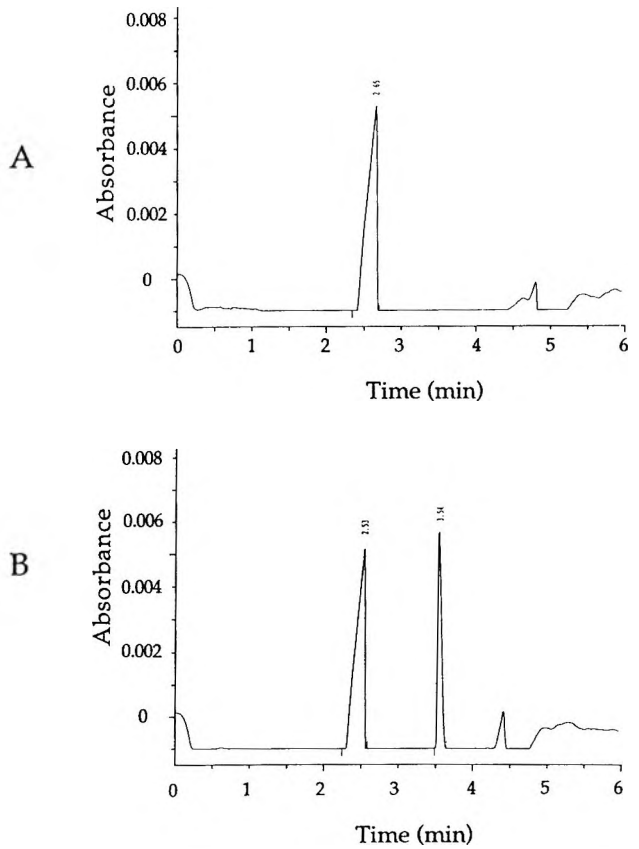

Figure 1. Electropherograms of a reference standard solution containing 40 μg/mL chloride as internal standard and (A) 0 μg/mL acetate, (B) 20 μg/mL acetate.

Oligonucleotides are negatively charged and migrate under the electric field. They have an absorption at 254 nm, and hence interfere with the determination of acetate in two ways: positive response due to replacement of phthalate ions and negative response due to absorption at high concentrations. Figure 2 depicts the electropherogram of a 1 mg/mL oligonucleotide sample solution where the peaks of acetate and added chloride in the sample solution were fully covered. The strong interference of the oligonucleotide with acetate was removed by prior centrifugation of the sample solution through a Centricon-SR3 (3,000 MW) filter. As the molecular weight of the oligonucleotide is significantly larger than 3,000 Da, the oligonucleotide cannot pass through the filter and is separated from acetate ions.

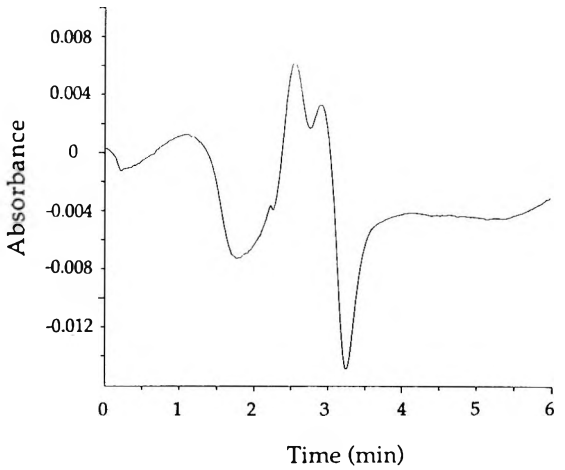

Figure 2. Electropherogram showing the interference of ISIS 3521 oligonucleotide matrix.

Before use, the Centricon SR-3 column was cleaned by washing twice with deionized Milli-Q water. After centrifugation, the collected solution in the bottom of the tube was used for the analysis by capillary zone electrophoresis. Figure 3 demonstrates the effectiveness of the Centricon SR-3 filter for the isolation of acetate and chloride ions from the oligonucleotide matrix without any contamination of the sample solution. No signal was obtained as Milli-Q water was eluted from the column (Figure 3-A). An electropherogram of the sample solution obtained following passage through the Centricon SR-3 filter (Figure 3-B) indicates that the interference by oligonucleotide is fully eliminated and chloride and acetate peaks obtained are similar to those of an acetate standard solution as depicted in Figure 1-B.

The purpose of adding chloride as an internal standard is to identify the acetate by relative migration time and to improve precision. Table 1 shows the migration time of acetate and chloride. The migration time of acetate varied during the measurements (3.217-3.487) while the relative migration time, which is defined as the ratio of the migration time of acetate to that of chloride, was approximately 1.38. The relative standard deviations (n=6) were 2.95 % for migration time of acetate and 0.66 % for the normalized migration time, respectively, indicating that migration time relative to chloride can be used to effectively identify the position of the acetate peak in the electropherogram.

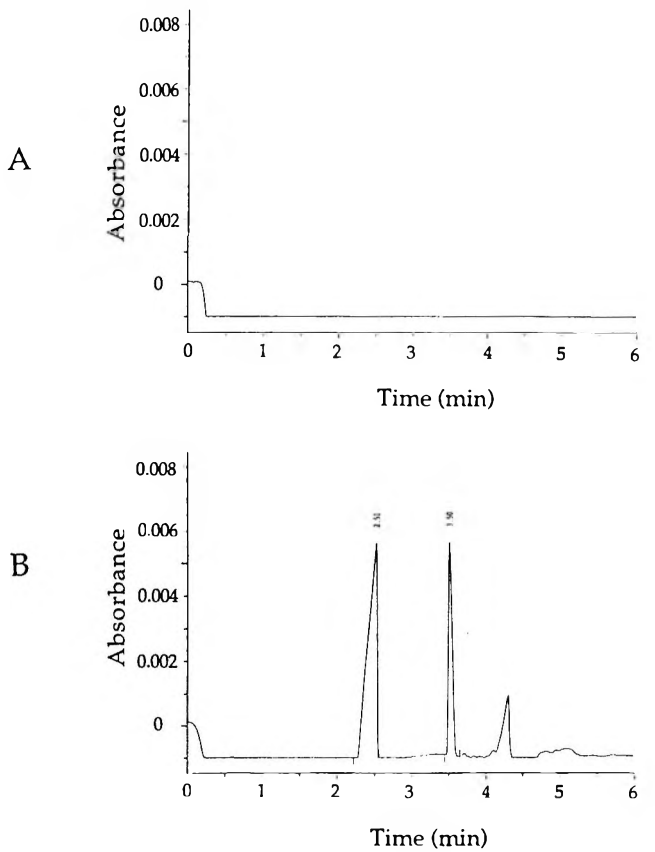

Figure 3. Electropherograms of (A) a deionized water blank and (B) an ISIS 3521 (Lot #0027) sample solution following passage through the Centricon SR-3 column.

The relative percent difference of the normalized migration time between the sample and the reference standard was less than 2%. Table 2 lists the results obtained for five repetitive injections of the same sample. The relative standard deviations were 4.39% for the acetate peak area and 1.88% for the normalized peak area. The precision of the method was improved considerably by using normalized peak area for quantitation.

Acetate samples were injected in hydrostatic mode. The injection time was tested in the range of 0-20 seconds and a linear relationship between the peak area and the sample injection time was found between 0-15 seconds. A sample injection time of 10 seconds was chosen as a compromise between

Table 1

The Migration Time of Acetate and Chloride in the Sample Solution

Lot Number	Migration Time (Min) Chloride	Acetate	Normalized Migration Time
	2.403	3.317	1.380
0027	2.453	3.427	1.397
	2.513	3.487	1.388
	2.333	3.217	1.379
0028	2.353	3.297	1.401
	2.463	3.407	1.383
RSD (%):	2.86	2.95	0.66

Table 2

Reproducibility for Repetitive Injections (n = 5)

	Acetate	Chloride	Normalized (Acetate/Chloride)
	0.2855	1.123	0.2542
Peak	0.2598	1.030	0.2522
Area	0.2808	1.104	0.2542
	0.2883	1.186	0.2432
	0.2901	1.142	0.2539
RSD (%):	4.39	5.12	1.88

sensitivity and resolution. Voltage was evaluated in the range of 0-20 kV. The higher the voltage, the shorter the separation time, but high noise was observed as the voltage was increased to 20 kV. Therefore, a voltage of 15 kV was selected for the separation. Under these conditions, the retention time for acetate is about 3.5 min. A calibration curve is obtained by plotting normalized area of acetate versus the concentration of acetate standard solutions in μg/mL. Linearity was demonstrated in the range of 0.06 to 240 μg/mL acetate with a y-intercept of -0.252, slope of 0.0624 and a correlation coefficient of 0.994. The recovery experiment was studied by the quantitation of Centricon-SR3 column

filtrates having known concentrations of acetate and by the standard addition method. The recovery is above 96%. Detection limit, estimated from the lowest concentration standard (0.06 μg/mL), is approximately 0.0012% w/w acetate, equivalent to 0.0017% w/w sodium acetate. Three typical oligonucleotide samples analyzed showed sodium acetate concentration of 1.43%, 2.60% and 0.058%, respectively.

CONCLUSION

Capillary Zone Electrophoresis has been demonstrated to be a simple, sensitive and reliable method to detect and quantitate the acetate salt present in oligonucleotide drug substances. Chloride was included as an internal standard for the identification and quantitation of acetate. As a result, both accuracy and precision have been increased as shown in Table 1 and 2. Although a single 20-base phosphorothioate oligonucleotide is shown as an example, the method can be applied to the determination of acetate in other oligonucleotides having molecular weights above 3,000 Da. Smaller molecular weight cut-off membranes are required to filter oligonucleotides with molecular weights less than 3,000 Da.

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LIQUID CHROMATOGRAPHIC SEPARATION OF CALIXARENES

I. Rodriguez,[†] S. F. Y. Li,[†,*]
B. F. Graham,[‡] R. D. Trengove[‡]

[†]Department of Chemistry
National University of Singapore
10 Kent Ridge Crescent
Singapore 119260, Republic of Singapore

[‡]Research Center for Advanced Mineral and Materials Processing
Department of Chemistry
University of Western Australia
Nedlands, W.A.6907, Australia

ABSTRACT

In this paper, the retention behavior of p-t-butyl calixarenes: p-t-butylcalix[4]arene, p-t-butylcalix[5]arene, p-t-butylcalix[6]-arene, p-t-butylcalix[7]arene, p-t-butylcalix[8]arene, p-t-butyl-calix[9]arene, p-t-butylcalix[10]arene and the bis-homo-oxacalix[4]arene was examined by reverse phase liquid chroma-tography, on a silica based octadecyl bonded phase with non-aqueous tertiary eluent in a linear gradient elution. The effect of various mobile phase compositions on the capacity ratio, peak asymmetry and resolution were studied. It was found that log k' did not vary linearly with the percentage of methanol, and that the symmetry of peaks was improved when shorter elution times and/or methanol percentage were decreased.

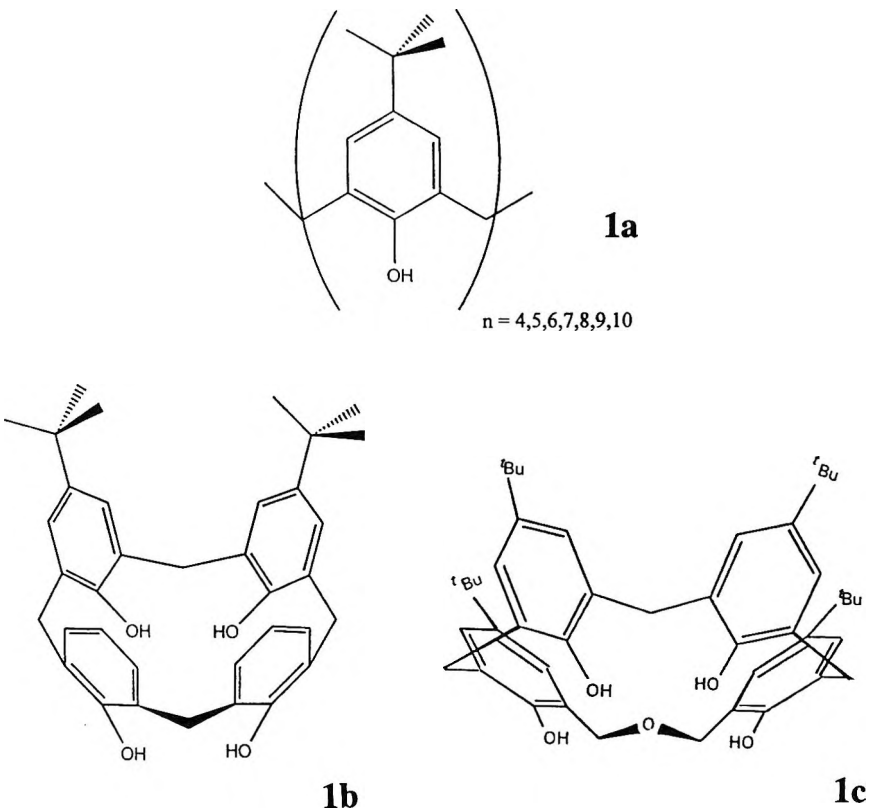

Figure 1. Structures of calixarenes: (1a), basic monomeric structure, (1b), *p*-t-butylcalix[4]arene (front t-butyl groups are omitted for clarity), (1c) bis-homo-oxacalix[4]arene.

INTRODUCTION

Calixarenes are macrocyclic oligomers which are obtained from the condensation of p-t-butylphenol and formaldehyde.[1] The name calixarene[2] is due to the cone conformation adopted by the smallest oligomers, p-t-butylcalix[4]arene. *p*-t-butylcalix[5]arene and bis-homo-oxacalix[4]arene. Their structures can be seen in Figure 1 In order to maximize their intramolecular hydrogen bond, when the numbers of phenol units increase the structures become flatter.

Calixarenes can undergo conformational conversion in solution, as evidenced by NMR studies,[3] by free rotation about the σ bonds of the Ar-CH_2-Ar groups. The energy barrier for the ring conversion is decreased by more polar solvents and particularly by solvents which can break hydrogen bonds.[3]

The growing interest in calixarenes derives from their ability to reversibly include smaller molecules and ions,[4] and to act as hosts of neutral organic molecules[5] as well as their selective complexation capability for metal cations such as cesium,[6] and organic cations such as amines.[7,8] These properties are more pronounced in the case of special derivatives than in the parent calixarenes.[9] The high interest in these compounds is also due to the large accessibility of these compounds from inexpensive and readily available starting materials. Accordingly, the synthetic chemistry of calixarenes is widely investigated to explore new aspects in their chemistry.

In order to analyze the reaction mixture and isolate specific compounds, the first chromatographic methods were developed more than 10 years ago, including thin layer chromatography (TLC),[10] flash column chromatography on silica gel,[11] and high performance liquid chromatography (HPLC).[12] In their work on HPLC, Ludwig and Bailie[12] separated reaction mixtures containing up to the heptamer linear oligomer and the octamer cyclic oligomer using a RP-18 reverse phase column and gradient elution with methanol, ethyl acetate and acetic acid. Under these conditions the separation took 35 min, baseline resolution between the trimer and tetramer linear oligomers was not achieved, and calix[8]arene had a very asymmetric peak shape.

In this work we report an alternative HPLC method for the analysis of calixarenes up to the 10^{th} cyclic oligomer. Furthermore, attempts are made to improve the symmetry of peaks and to shorten analysis time.

MATERIALS

Reagents and Materials

P-t-butylcalix[n]arenes standards (Calix[n], n = 4,6,8) and bis-homo-oxacalix[4]arene were synthesized in one of our laboratories (University of Western Australia) by literature methods, whereas calix[5]arene, calix[7]arene, calix[9]arene and calix[10]arene were supplied by Prof. C. D. Gutsche and Dr. D. Stewart. All solvents were of HPLC grade.

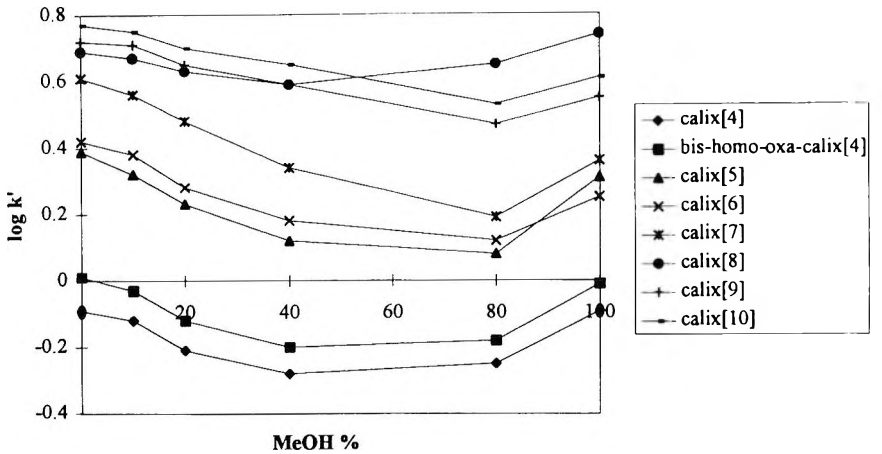

Figure 2. Variation of log k' with the percentage of methanol added in the mobile phase.

Toluene, acetonitrile (ACN), methanol (MeOH) and ethylacetate (EtAc) were purchased from J.T. Baker (Phillipsburg, USA). Chloroform was purchased from Lab Scan (Dublin, Ireland). Dichloromethane was purchased from Fisher Chemical (New Jersey, USA).

HPLC Instrumentation and Conditions

The column used was a Spherisorb ODS (3 μm; 4.6x150 mm) from Phase Separations (Deeside, UK). The flow rate was maintained at 1mL/min. HPLC experiments were conducted at ambient temperature in a Waters 600 E solvent delivery system. Absorbance was measured at 288 nm with a 0.001 absorbance range in a Waters 486 UV detector. Usually injection was of 20μl, performed by a Waters 700 Satellite WISP injector. Data was processed by a Waters Maxima 820 chromatography workstation equipped with version 3.3 software (Waters, Massachusets, USA).

METHODS

To achieve greatest solubilities, calix[4] was dissolved in toluene, calix[6] was dissolved in dichloromethane, and calix[8], calix[9], calix[10] were dissolved in chloroform. Bis-homo-oxacalix[4], calix[5], calix[7] were

dissolved in ethylacetate. Before injection the standard solution was diluted in the mobile phase to the appropriate concentration. Separation was performed using linear gradient elution of 15 min and a tertiary mobile phase of acetonitrile: methanol: ethylacetate. 0.1% of trifluoroacetic acid (TFA) was added to deactivate the free silanols of the partially end-capped column.

RESULTS AND DISCUSSION

The effect of various mobile phase compositions on the retention behavior was studied. The variation of the values of log k' with the percentages of methanol can be seen in Figure 2. It is evident that the retention curves of the calix[5]arene cross over that of calix[6] and the retention curves of calix[8] cross over those of calix[9] and calix[10], i.e. selectivity reversals occur. On the other hand, in contrast to the regular reverse phase behavior, as the percentage of ACN is reduced by increasing volumes of MeOH, the retention factors decrease until between 50% to 80% of MeOH is reached, then retention factors increase again. The retention of the calixarenes in principle is expected to be governed by their hydrophobic properties, π-electron interactions, and attractive interactions of the t-butyl substituents of the solute, and the C_{18} chain of the stationary phase. Methyl groups at the termini of the long aliphatic chains are expected to have very little interaction in terms of inclusion into the hydrophilic cone cavity.[8,13] When we used increased percentages of methanol, additional polar effects were expected to come into play that decrease k' values. We can expect that after the addition of methanol, polar silanols become solvated and therefore silanophilic interactions are lowered. This leads to a decrease in retention times until saturation is reached, then retention times start to increase.

Therefore, we may say that in addition to solvophobic interactions, silanophilic interactions between the solute and accessible silanols are important mechanisms for the separation. There is no evidence of a specific inclusion process that might influence the order of retention of the calixarenes.

Generally, surface silanol interactions can be significant particularly when the eluent has a high organic solvent concentration. We found that the use of a surface modifier is absolutely necessary, and that trifluoroacetic acid is appropriate. Without the addition of TFA, the peaks of calixarenes tailed and finally split indicating a strong interaction with the stationary phase. We have tested the effect of triethylamine (TEA) as a silanol masking agent. We obtained only one peak which was not retained by the column indicating that calixarenes did not interact with the reverse phase stationary phase.

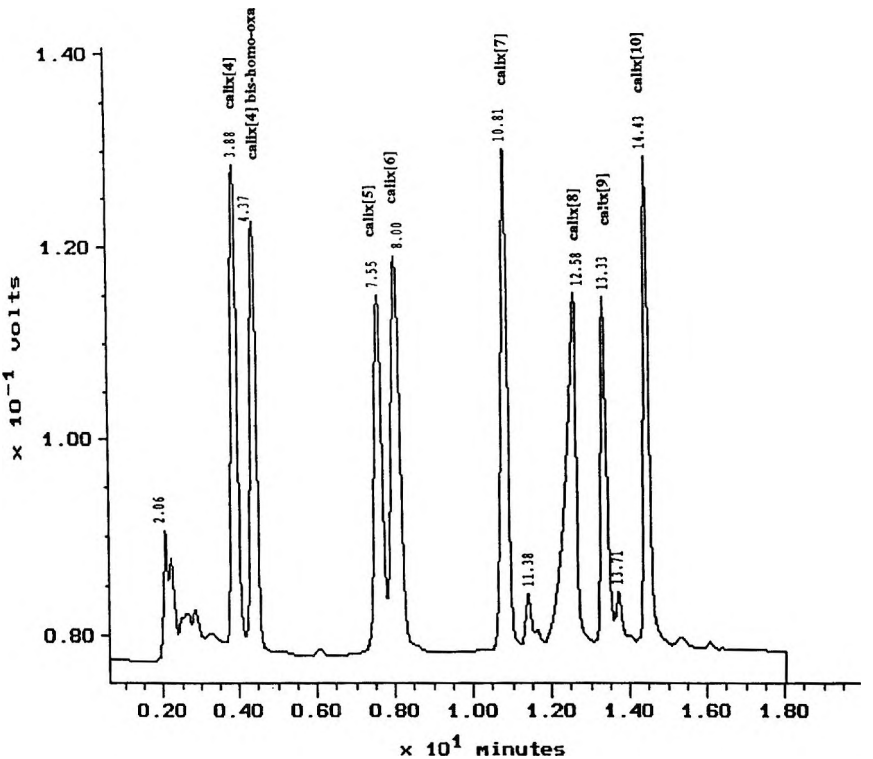

Figure 3. Liquid chromatogram of calixarenes. Conditions: Column, Sperisorb ODS (3μm; 4.6 x 150 mm); flow rate, 1 mL/min; linear gradient elution time, 15min; initial solvent composition 100% ACN, 0% EtAc; final solvent composition 40% ACN, 60% EtAc.

Interaction of TEA with calixarenes has been reported before[8] and it was concluded that interaction between p-t-butylcalix[4] and TEA occurred predominantly through hydrogen bonding or ion pairing formation. However, due to the increased acidity of the hexamer and octamer their interaction resulted in the formation of ions and ion-pairs.

The symmetry of calix[9], calix[10] and especially calix[8] peaks is the most affected when methanol is introduced in the mobile phase. The chromatograms obtained for a linear gradient of 15 min and different percentages of MeOH in the mobile phase are shown in Figures 3 to 7. Calix[8]arene peak does not show a very good symmetry in Figure 3 where the mobile phase did not contain MeOH.

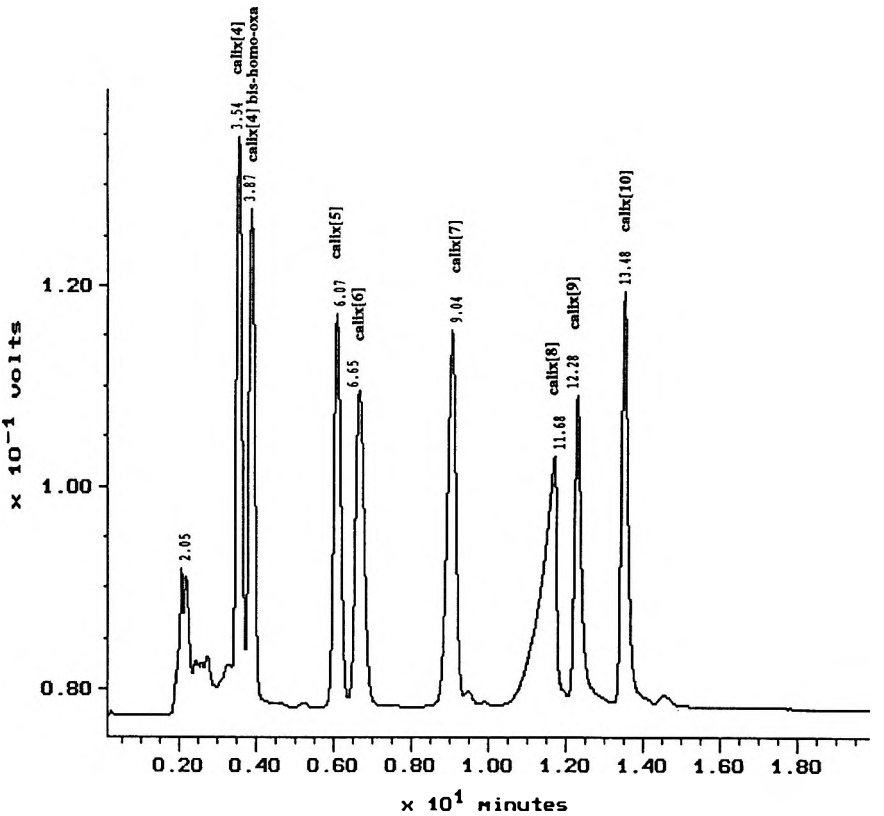

Figure 4. Liquid chromatogram of calixarenes. Conditions: Column, Sperisorb ODS (3μm; 4.6 x 150 mm); flow rate, 1 mL/min; linear gradient elution time, 15min; initial solvent composition 80% ACN, 20% MeOH, 0% EtAc; final solvent composition 40% ACN, 60% EtAc.

The symmetry was strongly affected when increased amounts of methanol were added in the mobile phase as can be seen from Figures 4 to 7. This fact may be due to a special interaction with the stationary phase.[11] Peak symmetry was also influenced by the gradient elution time. Asymmetry values for three different gradient elution times, 15 min, 30 min and 60 min and for two mobile phases; without the addition of methanol and with 10% of methanol, can be seen in Table 1 (only the four calixarenes which have variable asymmetry values are listed). From the asymmetry values we can see that calix[7] and calix[8] exhibit fronting whereas calix[9] and calix[10] exhibit tailing.

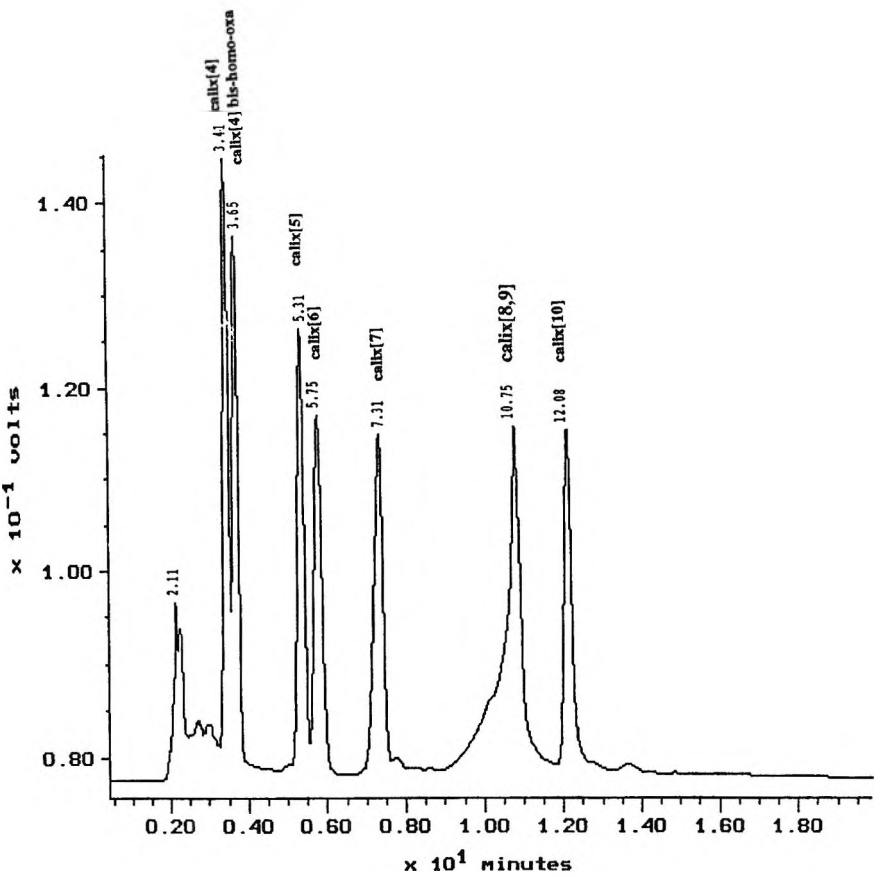

Figure 5. Liquid chromatogram of calixarenes. Conditions: Column, Sperisorb ODS (3μm; 4.6 x 150 mm); flow rate, 1 mL/min; linear gradient elution time, 15min; initial solvent composition, 60% ACN, 40% MeOH, 0% EtAc; final solvent composition 40% ACN, 60% EtAc.

Calix[8] shows very low asymmetry values as the gradient elution time is increased to 30 min and 60 min. The symmetry of calix[7], calix[9] and calix[10] decreases as well, with the increase in the gradient elution time, without improvement in resolution.

Separation of the calixarenes can be achieved without the use of methanol although 10% of methanol gives better baseline separation between calix[5] and calix[6] as can be seen by comparing Figure 3 and Figure 8, and from the log

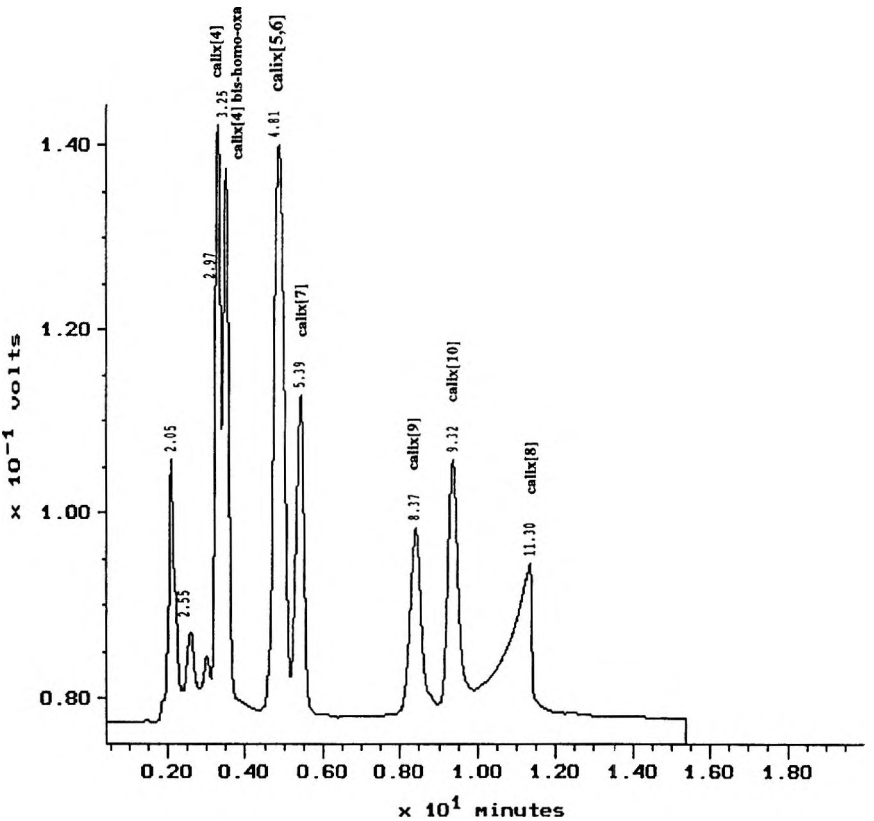

Figure 6. Liquid chromatogram of calixarenes. Conditions: Column, Sperisorb ODS (3μm; 4.6 x 150 mm); flow rate, 1 mL/min; linear gradient elution time, 15min; initial solvent composition 20% ACN, 80% MeOH, 0% EtAc; final solvent composition 40% ACN, 60% EtAc.

Table 1

Asymmetry Values for Different Gradient Elution Times and for Two Mobile Phase Compositions

	15 min		30 min		60 min	
	0% MeOH	10% MeOH	0% MeOH	10%MeOH	0% MeOH	10%MeOH
Calix[7]	1	1	0.79	0.96	0.76	0.76
Calix[8]	0.50	0.45	0.27	0.18	0.20	0.13
Calix[9]	1.16	1.19	1.57	1.79	1.77	2.50
Calix[10]	1.10	1.30	1.33	1.66	1.46	2.45

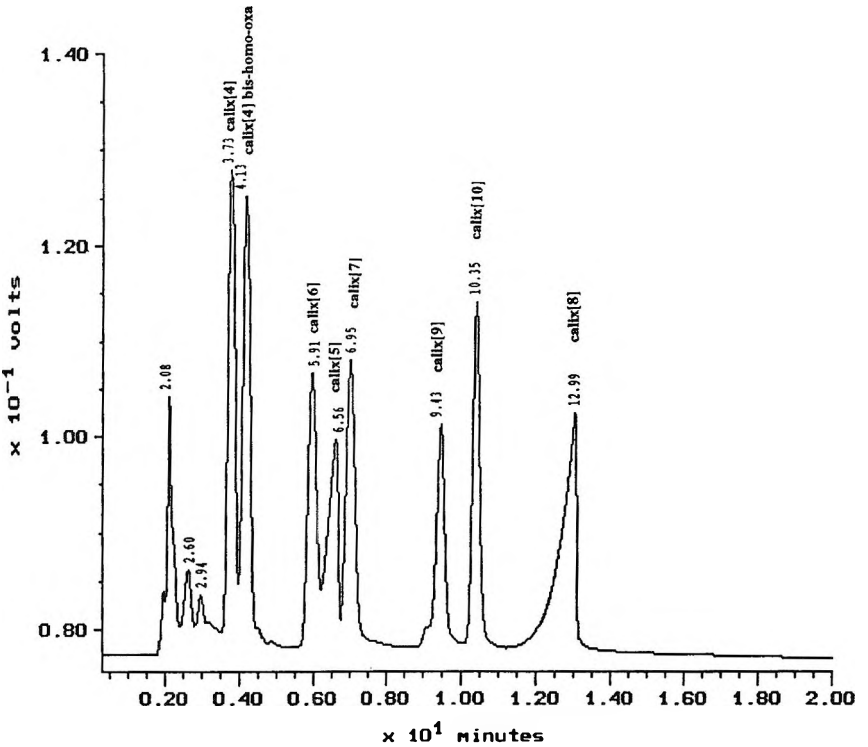

Figure 7. Liquid chromatogram of calixarenes. Conditions: Column, Sperisorb ODS (3μm; 4.6 x 150 mm); flow rate, 1 mL/min; linear gradient elution time, 15min; initial solvent composition 0% ACN,100% MeOH, 0% EtAc, final solvent composition 40% ACN, 60% EtAc.

Table 2

% RSD for the Peak Area and the Retention Time

Calixarene	[4]	oxa	[5]	[6]	[7]	[8]	[9]	[10]
RT	0.19	0.26	0.50	0.36	0.29	0.32	0.28	0.29
PA	2.16	2.37	2.61	2.51	3.44	1.89	4.63	2.52

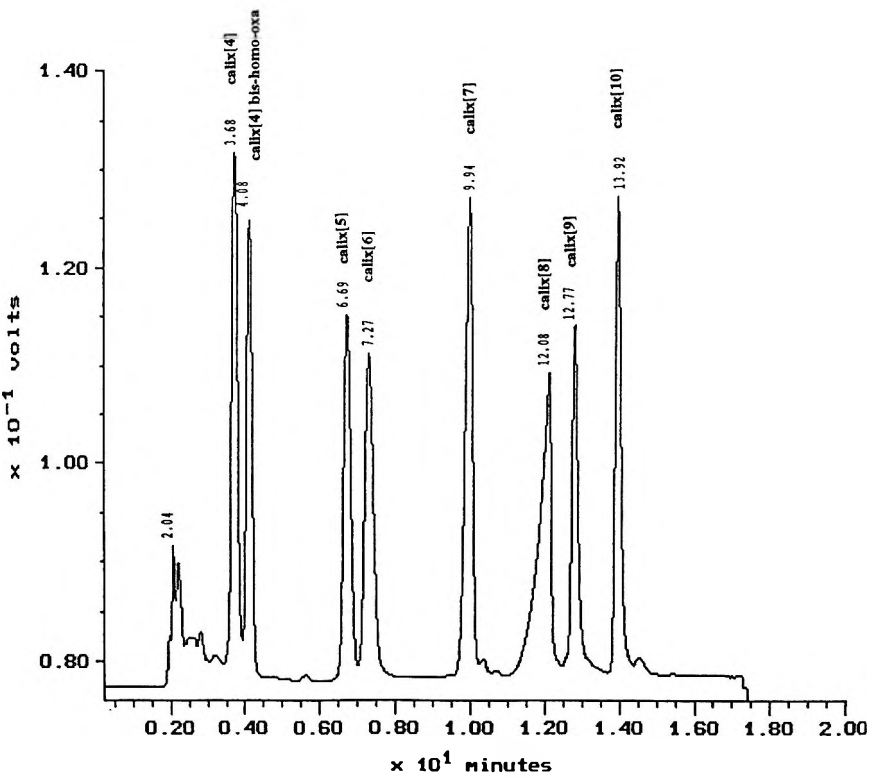

Figure 8. Liquid chromatogram separation of a mixture of calixarenes. Conditions: Column, Sperisorb ODS (3μm; 4.6 x 150 nm); flow rate 1mL/min; linear gradient elution time, 15 min; initial solvent composition 90% ACN, 10% MeOH, 0% EtAc; final solvent composition 40% ACN, 60% EtAC; UV detection at 288 nm; injection 20μL; temperature: abient. Concentration of each peak is ca. 5ppm.

k′ values in Figure 2. Therefore, reductions of the gradient elution time and the percentage of methanol in the mobile phase were attempted. Optimum separation was obtained by using linear gradient elution time of 15 min and a tertiary mobile phase of ACN: MeOH: EtAc. The gradient was set as follows: Initial solvent composition was 90% ACN, 10% MeOH, 0% EtAc. Final solvent composition was 40% ACN, 0% MeoH, 60% EtAc. A chromatogram obtained using the optimum conditions is shown in Figure 8. The reproducibility of the separations was determined by repetitive analysis (n=5) of the calixarene standard sol'n. The values of the relative standard deviation, % RSD for peak area (PA), and retention time (RT) are in Table 2. The % RSD for peak area and retention time were less than 5% and 0.5%, respectively.

In summary, the decreases of the percentage of methanol in the mobile phase and the gradient elution time resulted in improvement in the separation of the calixarenes. Optimized separation was achieved by employing gradient elution time of 15 min with an initial solvent composition of 90% ACN, 10% MeOH, 0% EtAc and final solvent composition of 40% ACN, 0% MeoH, 60% EtAc. These conditions provided good resolution and satisfactory peak symmetry, and the analysis time was reduced to 14 min.

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STUDIES OF CRUDE ETHANOL EXTRACT OF ROOTS OF SALVIA PRZEWALSKII MAXIM BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

Z. D. Hu,* Li Jia, Z. P. Zhang, Y. P. Shi, K. T.Wang

Department of Chemistry
Lanzhou University
Lanzhou 730000, China

ABSTRACT

A crude ethanol extract of roots of *Salvia przewalskii* Maxim was studied by micellar electrokinetic chromatography. Optimum conditions for the separation were established. Three peaks belonging to cryptotanshinone, dihydrotanshinone I and tanshinone IIA were identified by known standards. The three substances are valuable pharmaceutical ingredients of the plant.

INTRODUCTION

The air dried roots of *Salvia przewalskii* Maxim is a Chinese traditional herb. Many chemists have studied the physiologically active constituents of this herb and have isolated more than 30 substances and have determined their structures.[1] The tanshinone species are major components in *Salvia przewalskii*[2] and have acquired considerable importance in the treatment of heart disease and inflammation.[1,3-5]

Tanshinone IIA plays a major part in the treatment of heart disease.[6] Cryptotanshinone and dihydro-tanshinone I exhibited stronger inhibition agains *staphylococcus aureus* and its drug resistant species.[7] Tanshinone IIA and cryptotanshinone were found to have an inhibitive effect against $H_{37}RV$.[7]

Thin layer chromatography (TLC)[3-6] and high performance liquid chromatography (HPLC)[5] have been used for the separation and determination of tanshinone species. TLC and HPLC are not only limited in separation power, but also time consuming, since a number of prior steps are often required. For HPLC, the chromatographic column is easily contaminated and hard to clean.

Micellar electrokinetic capillary chromatography (MECC) was developed by Terabe et al.[8] in 1984 and Cohen et al.[9] in 1987. It is the most common mode of operation of capillary electrophoresis (CE). In MECC, resolution occurs because of a combination of kinetic and thermodynamic phenomena. So it is a highly efficient and fast technique, the application of which in the biological and pharmaceutical fields has developed rapidly in recent years.[10-12] For MECC, very complicated samples can be analysed directly without much pretreatment, because any contaminants in the CE tube can be rinsed away with a suitable solvent after each analysis.

The application of MECC to the analysis of roots of *Salvia przewalskii* Maxim has not hitherto been reported. In this work, roots of *Salvia przewalskii* Maxim were analysed directly after ethanolic extraction without further purification, and three peaks belonging to cryptotanshinone, dihydrotanshinone I and tanshinone IIA were identified by known standards. The effects of the addition of N,N-dimethyl-formamide (DMF) and tetra ethylenemglycol (TEG) in the electrolyte on separation selectivity are also discussed.

EXPERIMENTAL

Instrumentation

The CE system employed was a Quanta 4000 (Waters Chromatography Division of Millipore, Milford, MA, USA) with a positive power supply. Waters AccuSep fused silica capillaries (60 cm x 75 μm I.D.) were used throughout. Direct UV detection was achieved with the use of a Hg lamp and a 254 nm optical filter. A window for on column detection was created 7.6 cm from the end of the capillary.

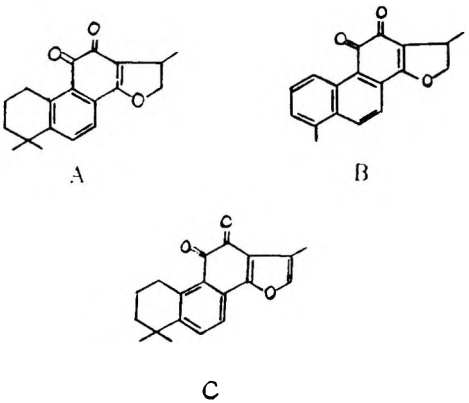
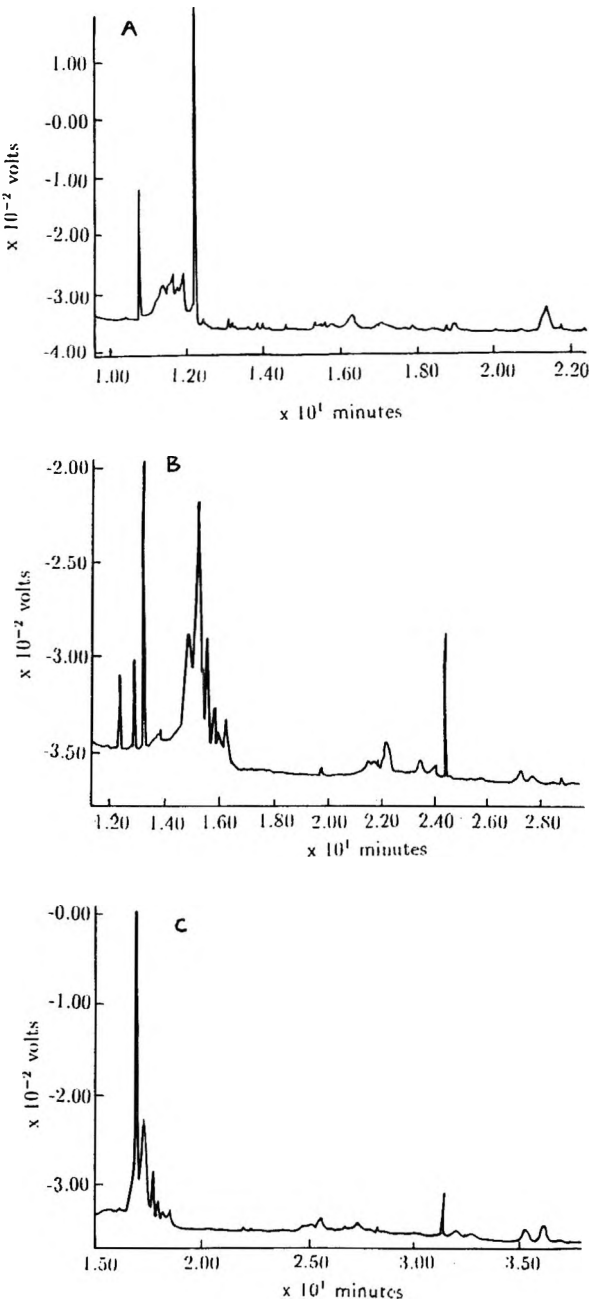


Figure 1. Chemical structures of cryptotanshinone (A), dihydrotanshinone I (B) and tanshinone IIA (C).

Hydrostatic sampling mode was selected for injection. Data acquisition was carried out with a Maxima 820 chromatography workstation (Waters) with a system interface module connecting the CE system to the station. Data acquisition rate was 20 points s^{-1}. Collection of electropherographic data was initiated by a signal cable connection between the Quanta 4000 and the system interface module (SIM).

Materials and Reagents

The sample of the roots of *Salvia przewalskii* Maxim was collected from Gansu, China. The air dried roots of *Salvia przewalskii* Maxim (1 kg) were extracted four times with ethanol (95%) at room temperature. After evaporation, the crude material (50 g) was chromatographed on silica gel column eluted with a gradient of benzene -- ethyl ether (10:1-1:1-1:5-ethyl ether) and three fractions were obtained. Then, from the three fractions, the pure compounds, cryptotanshinone, dihydrotanshinone I and tanshinone IIA, were obtained by recrystallization in methanol.[13] The crude ethanol extract of *Salvia przewalskii* Maxim and the three compounds isolated from the extract were dissolved in N,N-dimethylformamide to give appropriate concentrations for their separation by MECC. The chemical structures of cryptotanshinone, dihydrotanshinone I and tanshinone IIA are illustrated in Figure 1.


A
1.00
-0.00
-1.00
-2.00
-3.00
-4.00
x 10⁻² volts
1.00 1.20 1.40 1.60 1.80 2.00 2.20
x 10¹ minutes
B
-2.00
-2.50
-3.00
-3.50
x 10⁻² volts
1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80
x 10¹ minutes
C
-0.00
-1.00
-2.00
-3.00
x 10⁻² volts
1.50 2.00 2.50 3.00 3.50
x 10¹ minutes

Sodium tetraborate, sodium dodecyl sulfonate (SDS) (chemical grade), N,N-dimethylformamide (DMF) and tetra ethylene glycol (TEG) were purchased in China.

The run buffer solutions were prepared as to contain 25.0 mmol L^{-1} of sodium tetraborate, 10 mmol L^{-1} of SDS, 10% (v/v) of DMF and 0.2% (v/v) of TEG, whose pH was adjusted to 9.5 by addition of appropriate volume of concentrated KOH solution. Unless otherwise specified, all chemicals were of analytical reagent grade. All solutions were prepared using filtered, degassed and deionized distilled water.

Procedure

At the beginning of each day, the capillary was rinsed with sufficient deionized water for about 15 minutes. It was also flushed with 0.5 mol L^{-1} KOH solution after being used for three or four days.

Experiments were performed to optimize the CE system. Unless otherwise specified, the standard conditions used were: 10 KV applied voltage, 15 s hydrostatic loading time and a working electrolyte consisting of 25.0 mmol L^{-1} of sodium tetraborate, 10 mmol L^{-1} of SDS, 10% (v/v) of DMF and 0.2% (v/v) of TEG. Before each run, the capillary was purged for 3 minutes under vacuum. All operations were at room temperature.

RESULTS AND DISCUSSION

Effect of pH

The pH of the buffer solution containing 25.0 mmol L^{-1} of sodium tetraborate, 10 mmol L^{-1} of SDS and 10% (v/v) of DMF was varied by addition of concentrated HCl or concentrated KOH solution.

Figure 2. (left) Effect of DMF concentration on MECC of the crude ethanol extract of the roots of *Salvia* (*przewalskii* Maxim. Buffers: 25.0 mmol L^{-1} sodium tetraborate (pH 9.5) containing 10 mmol L^{-1} SDS and a) no DMF (b) 10 % (v/v) DMF and (c) 15% (v/v) DMF; capillary: 60 cm x 75 μm; voltage: 10 KV; gravity injection: 10cm for 15 s; detection: absorption at 254 nm.

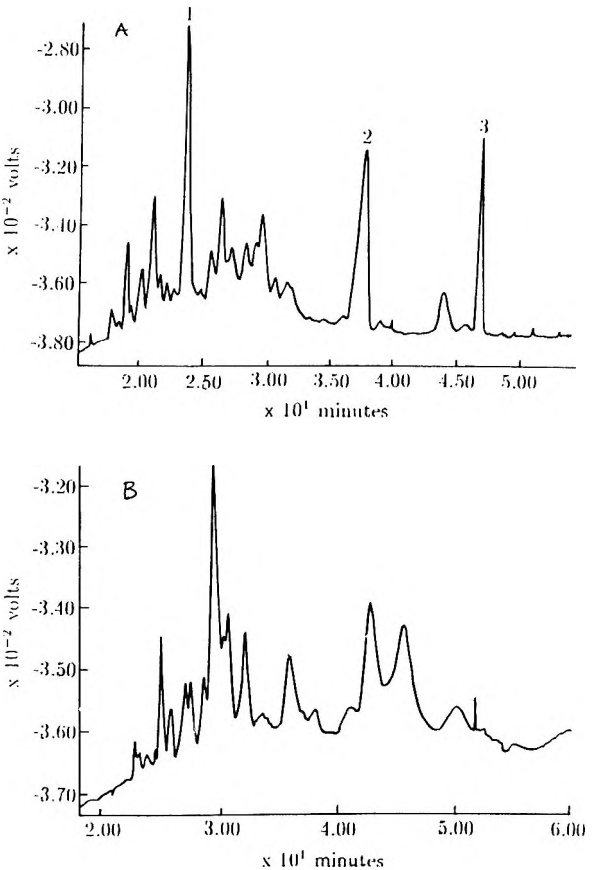
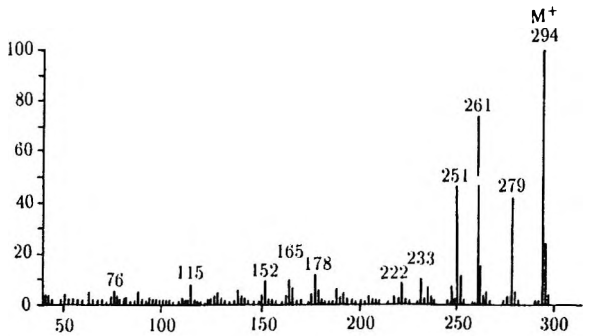


Figure 3. Effect of TEG concentration on MECC of the crude ethanol extract of roots of *Salvia przewasskii* Maxim.
Buffers: 25.0 mmol L^{-1} sodium tetraborate (pH 9.5) containing 10 mmol L^{-1} SDS, 10% (v/v) DMF and (a) 0.2% (v/v) TEG and (b) 0.5% (v/v) TEG; capillary: 60 cm x 75 μm; voltage: 10 KV; gravity injection: 10 cm for 15 s; detection: absorption at 254 nm. Peaks: 1 = cryptotanshinone, 2 = tanshinone IIA, 3 = dihydrotanshinone I, other peaks are not identified.

Figure 4. (right) MS spectra of the three substances. A: tanshinone IIA. B: dihydrotanshinone. C: cryptotanshinone, M^+: peak of molecular ion.

xl Bgd=1 2AB-HS EI+ Bpm=0 I=10v Hm=0 TIC=438532992 Ront: s212/360 Sys: SYSTEMDEF PT=0 Cal: 0331PFK



A

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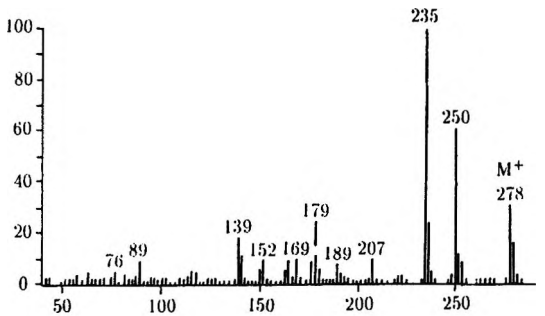

B

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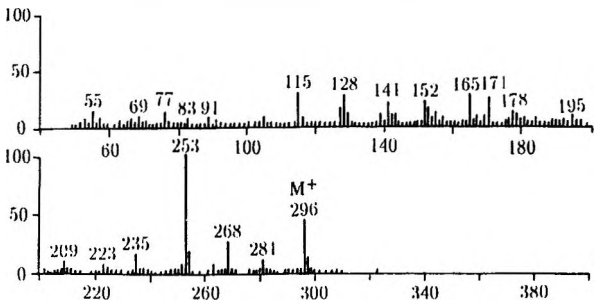

C

The effect of pH on the separation of the crude ethanol extract of the roots of *Salvia przewalskii* Maxim was investigated. It was observed that, at lower pH, poor separation was obtained, whereas when the pH was increased from 9.2 to 9.8 many peaks were separated. As the pH of the buffer was further increased, results of the separation did not improve. For subsequent work, the pH of 9.5 was employed.

Effect of DMF

MECC of the ethanol extract of the roots of *Salvia przewalskii* Maxim uses SDS as micelles. In MECC the separation mechanism involves partitioning between the aqueous phase and the micellar phase. Therefore, any manipulation to the buffer system will have some effect on the distribution coeffecient and thus on the separation selectivity.

Organic modifiers are often used in MECC to decrease the affinities of hydrophobic solutes for the micellar phase. In addition, organic solvents reduce electroosmotic flow and subsequently expand the migration window.[14-17] As a result, resolution of highly hydrophobic compounds in MECC is enhanced. Addition of DMF to the SDS buffer improved the separation, as shown in Figure 2.

The best separation was observed when 10% (v/v) of DMF was added to the buffer, and this concentration was used in subsequent experiments.

Effect of TEG

In order to obtain a better separation, some additives to the buffer containing 25.0 mmol L^{-1} of sodium tetraborate, 10 mmol L^{-1} of SDS and 10% (v/v) of DMF were tried. β-Cyclodextrin (β-CD) was added to the buffer at different concentrations (0-20 mmol L^{-1}), but no improvement of the separation wasobserved. Addition of tetra ethylene glycol (TEG) to the buffer improved the separation considerably, as shown in Figure 3.

The best separation was obtained when 0.2% (v/v) of TEG was added to the buffer. Using this buffer (25.0mmol L^{-1} sodium tetraborate + 10 mmol L^{-1} SDS + 10% (v/v) DMF + 0.2% (v/v) TEG), many peaks were separated and the three tanshinone compounds were also separated.

Identification of the Components of Interest in Roots of *Salvia przewalskii* Maxim

The three peaks belonging to cryptotanshinone, dihydrotanshinone I, and tanshinone IIA were identified by comparing their migration times with those of standards. Pure standards were also added to the samples so that the peak heights of related compounds were increased in order to improve their detectability. The ratio of absorbances at different wavelengths between the sample and the standard in UV detection mode was compared. Figure 4 showed the MS spectra of the three substances. By the above methods, the three components of interest in roots of *Salvia przewalskii* Maxim were identified.

CONCLUSION

The successful separation of the main tanshinone substances in the crude ethanol extract of the roots of *Salvia przesalskii* Maxim shows that MECC is a useful method for the determination of pharmaceutical compounds in natural product isolation procedures. The addition of modifiers to the SDS buffers, such as organic solvents and TEG greatly improved the separations.

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SIMULTANEOUS SEPARATION OF INORGANIC CATIONS AND ANIONS BY ION CHROMATOGRAPHY USING STRONG ANIONIC/ZWITTERIONIC MIXED MICELLES AS THE STATIONARY PHASE

Wenzhi Hu,[†,*] Kiyoshi Hasebe,[‡] Darren Michael Reynolds,[§] Hiroki Haraguchi[¶]

[†]Division of Chemistry
Graduate School of Science
Hokkaido University
Sapporo 060, Japan
[‡]Division of Material Science
Graduate School of Environmental Earth Science
Hokkaido University
Sapporo 060, Japan
[§]National Institute for Resources and Environment
Hydrospheric Environment Protection Dept.
16-3 Onogawa, Tsukuba-shi
Ibaraki 305, Japan
[¶]Division of Applied Chemistry
Graduate School of Engineering
Nagoya University
Nagoya 464-01, Japan

ABSTRACT

Mixed micelles obtained by mixing SDS (sodium dodecylsulfate) with Zwittergent-3-14 (N-tetradecyl-N, N-dimethyl- 3-ammonio 1-propanesulfonate) are used as a

dynamic stationary phase for the simultaneous ion chromatography of inorganic cations and anions. Analyte cations are separated by the negative charges in the SDS based upon ion-exchange interactions while analyte anions are separated by both the positive and negative charges in Zwittergent-3-14 based upon simultaneous electrostatic attraction /repulsion interactions. The ratio of SDS/Zwittergent-3-14, which constitutes the stationary phase is an important factor in the determination of the retention times and elution orders of the analyte ions. The investigation of the mechanisms involved and the practical applications are discussed in this paper.

INTRODUCTION

A previous study[1] has demonstrated that a single, column, injection, eluent and detector could form the basis for the simultaneous separation of inorganic cations and anions when using a weak-positively/strong-negatively charged zwitterionic surfactant (W/S ZWITS) as the stationary phase. The mechanism involved in the simultaneous separation of the cations and anions when using a W/S ZWITS stationary phase differs to those observed for other similar 'simultaneous' IC methods.[2-10] When a W/S ZWITS stationary phase is used for IC, the analyte cations separate as a result of ion-exchange interaction while the analyte anions separate due to simultaneous electrostatic attraction and repulsion interactions.

Previously, other cations-anions separation methods[2-10] involved the use of ion exchange. When a cation-separation column and an anion separation column are used in series[2-4] then complete separation of cations and anions is achieved independently, i.e. in two stages. However, if both the cation and the anion exchangers are interspersed throughout a column[5-7] and/or if the stationary phase has both chemically bonded anionic and cationic exchangers,[7] then the separation of both cations and anions is achieved simultaneously (simultaneous IC). When chelating agents are used for the mobile phase,[8-10] the analyte cations are converted into anionic complexes and are separated along with other inorganic and/or organic anions in a single stage of anion-exchange.

The W/S ZWITS stationary phase reported in our previous study[1] was obtained by the protonation of taurine-conjugated bile surfactants using an acidic aqueous solution as the mobile phase. In this method, the mobile phase pH was extremely important for obtaining the W/S ZWITS stationary phase.

Furthermore, a simpler method which does not require protonation and which also separates inorganic cations and anions simultaneously (based on the combined effects of cation-exchange and simultaneous electrostatic attraction/repulsion interactions) is also proposed.[11] This is achieved by using a mixed micelle solution containing sodium taurodeoxycholate (NaTDC) and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) as the stationary phase. NaTDC acts as a cation-exchanger and separates the analyte cations while the CHAPS acts as a simultaneous electrostatic attraction/repulsion provider and separates the analyte anions.

Although a number of inorganic cations and anions were successfully separated when using the two aforementioned stationary phases,[1,11] nitrate, a very important and common inorganic ion, could not be separated nor detected using either the W/S ZWITS stationary phase or NaTDC/CHAPS mixed stationary phase. When a W/S ZWITS stationary phase is used nitrate is separated from the other analyte ions, however, its retention time is very close to that of water. When UV-Visible detection is used, its UV absorbance is obscured by the negative UV absorbance of water.[1]

When the NaTDC/CHAPS stationary phase is used, nitrate ions were co-eluted with the mono-valent cations.[11] Unfortunately, the conditions which determine the separation abilities during this particular type of IC analysis were not investigated in previous studies.[1,11]

To further improve the separation abilities of simultaneous IC a new type of stationary phase is used. This is obtained by immobilizing a mixed micelle of SDS (sodium dodecylsulfate, a strong negatively charged surfactant) with Zwittergent-3-14 (N-tetradecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate, a strong/strong zwitterionic charged surfactant).

Present studies have demonstrated that by careful selection of the strongly charged surfactants which are used as the stationary phase, the separation abilities of the simultaneous IC technique are vastly improved. Present experimental results also show that the elution order of the analyte ions can be manipulated by adjustment of the SDS/Zwittergent-3-14 ratio in the stationary phase. This provides a new and convenient approach for avoiding technical problems such as elutionoverlapping, which are often encountered in the IC of target ionic species in a concentrated salt matrix. The investigation of the separation mechanism and the practical applications of this new proposed method are outlined in this paper.

EXPERIMENTAL

Apparatus

The HPLC system used in this study was a Shimadzu (Kyoto, Japan) LC-6A system. It was equipped with an LC-7A pump, a SIL-6A auto injector, a SCL-6A system controller and an UV-visible detection (SPD-M6A). ODS packed columns (L-Column, 46 X 250 mm, Chemical Inspection & Testing Institute, Tokyo, Japan) were used as the support columns for the preparation of SDS/Zwittergent-3-14 mixed micellar stationary phase. A commercial cation separation column, Shim-pack IC-C3 (4.6 X 100 mm, Shimadzu), and anion separation column, Shim-pack IC-A3 (4.6 X 150 mm, Shimadzu) were also used for conventional IC measurements.

Materials

Sodium dodecylsulfate (SDS) and N-tetradecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent-3-14) were used to prepare a stationary phase for the simultaneous IC of inorganic cations and anions. These were obtained from Wako (Osaka, Japan) and Calbiochem (La Jolls, CA, USA), respectively. Inorganic salts, which were used as the standard analytes were also obtained from Wako, all reagents were used as received.

Stationary Phase Preparation

Aqueous solutions containing mixtures of SDS and Zwittergent-3-14 were passed through the ODS-packed columns for 60 minutes using a flow rate of 1.0 mL/min. These columns were then conditioned with an aqueous solution containing 2.0 mM cerium (III) chloride for at least 60 minutes using the same flow rate.

RESULTS AND DISCUSSION

The concentration of the mobile phase ions, flow rate of the mobile phase, and the ratio of SDS/Zwittergent-3-14 which is immobilized on the ODS surfaces, will determine the resolution abilities. To detect both analyte cations and anions using a single UV-visible detector, UV-absorbing anions were chosen as the typical analyte anions and were detected directly while analyte cations were detected 'indirectly'. Indirect UV absorption is based upon the

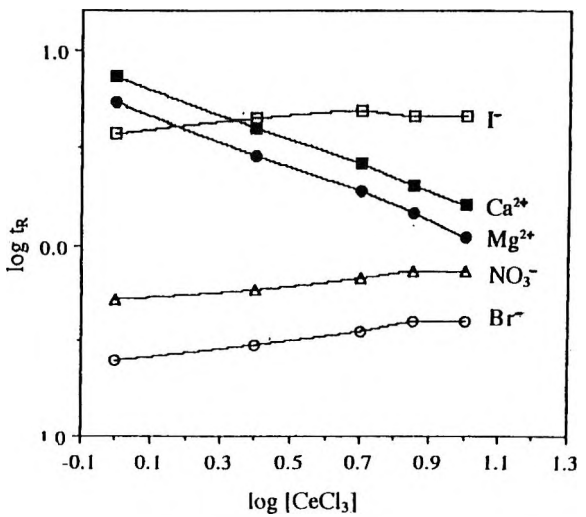

Figure 1. The relationship between the logarithms of the adjusted retention times of analyte ions (log t_R) and the logarithms of $CeCl_3$ concentration (log[$CeCl_3$]) in the mobile phase. Column: ODS-packed column (46 X 250 mm) coated with SDS/Zwittergent-3-14 (20/40, mM/mM). Mobile phase: $CeCl_3$. Flow rate: 1.0 mL/min. Sample injection volume: 20 μL. Detection UV-visible at 253 nm.

measurement of the decreased UV-absorbency of the mobile phase. When non-UV absorbing analytes are eluted from the stationary phase, the UV-absorbency of the mobile is diluted by the eluting analyte, resulting in a negative signal. Cerium (III) are UV-absorbing ions commonly used as the mobile phase ions when detecting inorganic cations using the indirect UV-absorption method.

Five aqueous solutions containing, (i) 1. 0, (ii) 2. 5, (iii) 5. 0, (iv) 7.0 and (v) 10, mM of cerium (III) chloride were prepared and respectively used for the mobile phase to investigate the effects of mobile phase concentration on the retention behavior of the analyte ions. An aqueous solution containing 1.0 mM each of $MgBr_2$, $Ca(NO_3)_2$, and CaI_2 was chosen to represent a 'typical' sample. Adjusted retention times were calculated as the difference between the observed retention time of the analyses and the void time (t_o) of the column. The relationships between the logarithms of the adjusted retention times of Mg^{2+}, Ca^{2+}, Br^-, NO_3^-, and I^- and the logarithms of cerium (III) chloride concentration are shown in Figure 1. The values of the logarithms for the adjusted retention times of the analyte cations linearly decreased with increasing concentration of $CeCl_3$ in the mobile phase. For this, the separation of the analyte cations is achieved as a result of ion exchange interactions.[12]

On the contrary, the value of the logarithms for the adjusted retention times of the analyte anions are initially increased with increasing concentration of $CeCl_3$ in the mobile phase. However at around 7.0 mM concentration of $CeCl_3$ the logarithmic values stabilized and remained almost constant. These experimental results suggest that the separation for the analyte anions is not based on ion-exchange interactions.

It was initially mentioned in our previous paper that when a strong positively/negatively charged zwitterionic stationary phase is used for the IC of inorganic anions, the retention time of the analyte ions is increased with increasing concentration of mobile phase ions.[13] The IC of ionic chemicals while using a strong positively/negatively-charged zwitterionic stationary phase is a new approach proposed by the present authors.[14-16] When analyte ions are passed through a stationary phase where the positive and negative charges are fixed in close proximity, i.e., the zwitterionic stationary phase, both of the analyte cations and anions will receive electrostatic attraction and repulsion from the stationary phase, simultaneously. This results in an extremely weak electrostatic affinity between the analyte ions and the stationary phase. The effective distribution of the analyte ions between the stationary phase and the mobile phase can be achieved without the need for ion-exchange. The IC of ions based on simultaneous electrostatic attraction and repulsion interactions is termed electrostatic IC.[14]

For ion-exchange IC, the retention time of the analyte ions is decreased with increasing concentration of mobile phase ions. The potential for ion-exchange is increased by increasing the concentration of replacing ions present in the mobile phase. For electrostatic IC, the retention time of the analyte ions is increased with increasing concentration of the mobile phase ions due to the following; when a strong positively/negatively charged zwitterionic stationary phase is conditioned with a mobile phase containing electrolytes (ions), a moving (mobile) electrical double layer consisting of the mobile phase ions is created with the stationary phase. The levels of the mobile phase ions in the moving electrical double layer are proportional to the concentration of mobile phase ions. This becomes critical when the concentrations of the mobile phase ions are at sufficiently high levels. The separation of analyte ions while using a strong positively/negatively charged zwitterionic stationary phase which has been conditioned with a mobile phase containing the electrolytes, requires an effective distribution of the analyte ions between the mobile phase and the moving electrical double layer. The distribution rate of the analyte ions decreases with increasing levels of mobile phase ions that are initially involved in the moving electrical double layer. This results in longer retention times for the analyte ions with increasing concentration of the mobile phase ions.

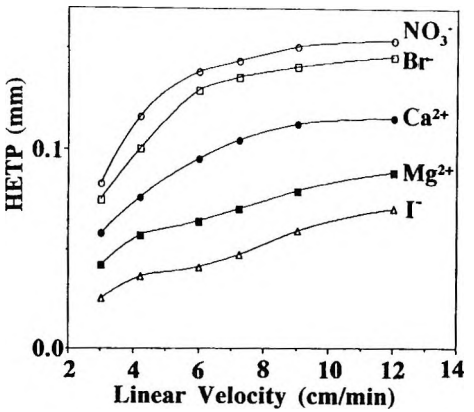

Figure 2. Relationship between HETP and the linear velocity for a strong anionic/zwitterionic mixed micellar stationary phase. Mobile phase: 5.0 mM $CeCl_3$. Conditions are the same as described in Figure 1.

To investigate the effects of flow rate on the theoretical plate heights (HETP) of the column, the flow rate of the mobile phase was incremented from 0.5 mL/min to 2.0 mL/min (3.01 cm/min to 12.04 cm/min). An aqueous solution containing 5.0 mM $CeCl_3$ was used for the mobile phase. As the experimental results in Figure 2 show, the HETP of this column depends on the flow rate of the mobile phase in a similar way as conventional packed columns.[17] The optimum flow rate which allowed for high resolution and a relatively short analytical time was found to be 1.0 mL/min (6.02 cm/min).

Three aqueous solutions, containing (i) 40 mM Zwittergent-3-14 and 10 mM SDS, (ii) 40 mM Zwittergent-3-14 and 20 mM SDS, and (iii) 40 mM Zwittergent-3-14 and 40 mM SDS, were prepared as SDS/Zwittergent-3-14 mixed micellar stationary phases. An aqueous solution containing Mg^{2+}, Ca^{2+}, Ba^{2+}, Br^-, NO_3^-, I^- and SCN^- was prepared and used to represent a 'typical' sample. This sample was then analyzed using the three respective stationary phases. When the concentrations of Zwittergent-3-14 and SDS in the mixed micellar stationary phase preparatory solutions are 40 and 10 mM respectively, Br-, NO_3^-, I^-, SCN^-, Mg^{2+}, Ca^{2+} and Ba^{2+} can be base-line separated and the divalent cations elute between NO_3^- and I^- (Figure 3A). However, when the concentrations of SDS were increased to 20 mM (Figure 3B) and 40 mM (Figure 3C), the retention times of the analyte cations are increased while the retention times of the analyte anions are decreased. The increased and decreased retention times of respective analyte cations and anions results in elution overlapping and a change in elution order.

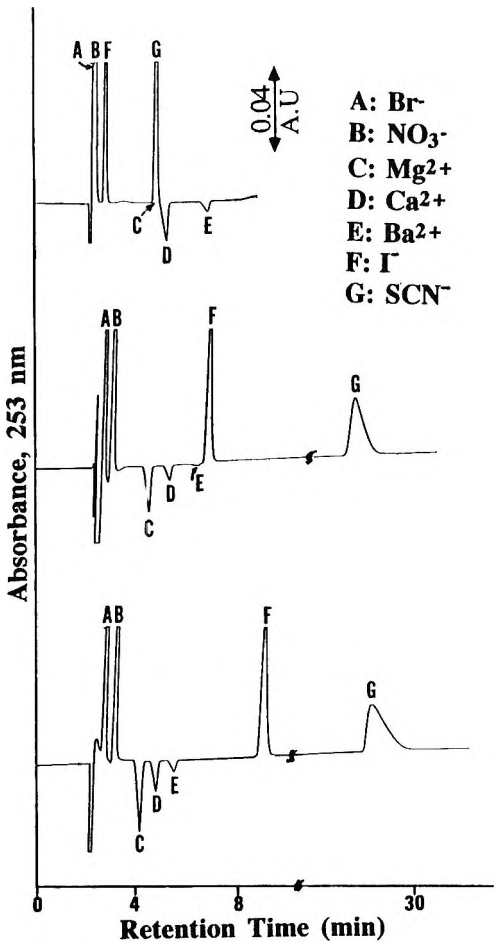

Figure 3. Chromatograms of an aqueous solution containing Br^-, NO_3^-, I^-, SCN^-, Mg^{2+}, Ca^{2+} and Ba^{2+} obtained using the SDS/Zwittergent-3-14 mixed micellar stationary phases. Ratio of SDS/Zwittergent-3-14 concentration is 10/40 (A), 20/40 (B) and 40/40 (C), mM/mM. In Figure 3B, the negative peak due to Ba^{2+} is partly obscured by the positive peak due to I^-. In Figure 3C, Br^- is not baseline separated from NO_3^-; the negative peak due to Mg^{2+} is completely obscured by the positive peak due to SCN^-. Flow rate of the mobile phase: 1.0 mL/min. Other conditions are the same as described in Figure 1.

For a certain ODS-packed column, the total amount (maximum) of SDS and Zwittergent-3-14 found in the stationary phase is a constant. By increasing the concentration of SDS in the preparatory solution containing 40 mM Zwittergent-3-14 the amount of SDS immobilized on the stationary phase is increased. This results in a decrease in the amount of Zwittergent-3-14 immobilized in the stationary phase. The retention times for the analyte cations and anions are proportional to the levels of SDS and the level of Zwittergent-3-14, respectively, in the stationary phase. By adjustment of the SDS/Zwittergent-3-14 ratio in the solutions (stationary phase) the retention time and/or the elusion order of the analyte ions can be manipulated.

While using the Zwittergent-3-14/SDS (40/10, mM/mM) mixed micellar coated stationary phase and 5.0 mM $CeCl_3$ mobile phase, the mono-valent cations elute with a zero retention time (void time). Subsequently, this provides a simple and convenient method for the determination of divalent cations and UV-visible absorbing inorganic anions in a concentrated salt matrix. To illustrate this, sea water diluted ten-fold with pure water, was directly injected into the IC system and magnesium, calcium and iodide ions were simultaneously determined. The cations and the magnesium ions are easily detected, however, the iodide ions were not detected, due to the low concentration of iodide found in sea water (sub-μM levels). Figure 4 shows the chromatogram of a real sea water sample spiked with iodide.

An aqueous solution containing 10μM each of $CaBr_2$, $Ca(NO_3)_2$, CaI_2 and $Ca(SCN)_2$, 40 μM $MgCl_2$ and 40 μM $BaCl_2$ was analyzed ten times to test the reproducibility and the detectability of this method. The standard deviation (in %) was found to be 0.73, 0.45, 0.52, 0.55, 0.52, 0.42 and 0.43 for the retention times and 0.85, 0.55, 0.55, 0.53, 0.62, 0.52 and 0.61 for the concentration (peak areas) of Br^-, NO_3^-, Mg^{2+}, Ca^{2+}, Ba^{2+}, I^- and SCN^-, respectively. The detection limit for Br^-, NO_3^-, Mg^{2+}, Ca^{2+}, Ba^{2+}, I^- and SCN^-, using a signal to noise ratio of 3, was found to be 0.60, 0.65, 6.1, 6.3, 7.2, 5.5, and 6.3 μM, respectively.

Tap water was directly analyzed using this IC system. As the chromatogram in Figure 5 shows, the UV-absorbing anion, nitrate, and divalent cations, magnesium and calcium, were simultaneously detected. The concentrations of NO_3^-, Mg^{2+} and Ca^{2+} that were found in tap water were 0.026, 0.61 and 0.57 mM, respectively. This tap water was also analyzed using the conventional anion-separation and cation-separation IC, respectively (the measurement was achieved under the conditions as recommended by the manufacturer). The concentration of NO_3^- was found to be 0.029 mM, using a conventional anion-separation IC.

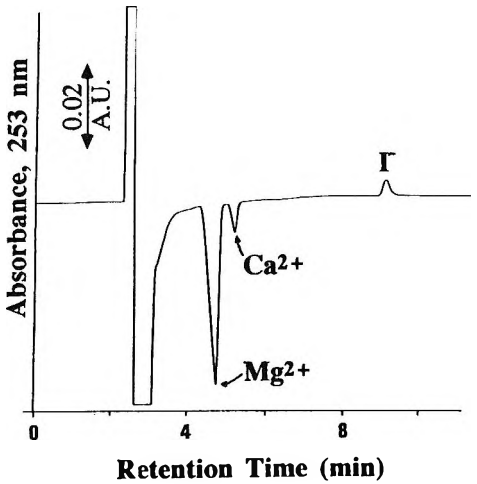

Figure 4. Chromatogram of 10-fold diluted real sea water spiked with 0.1 mM of I⁻. Data obtained using a SDS/Zwittergent-3-14 mixed micellar stationary phase, conditions are the same as described in Figure 3A.

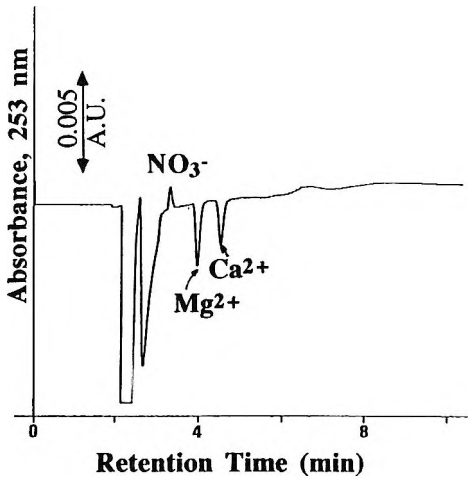

Figure 5. Simultaneous determination of UV-Visible inorganic anions and divalent cations in tap water. Separation conditions are the same as described in Figure 3A.

The concentrations of Mg^{2+} and Ca^{2+} were found to be 0.59 mM and 0.58 mM, respectively, using a conventional cation separation IC. Hence, good agreements on the analytical results obtained using the present method and the conventional IC were obtained. Although, using conventional IC techniques other inorganic ions, such as Na^+, K^+, NH_4^+, Cl^-, and SO_4^{2-} can also be measured along side Mg^{2+}, Ca^{2+} and NO_3^-, two analytical stages are required for cations and anions respectively. On the contrary, Mg^{2+}, Ca^{2+} and NO_3^- were measured simultaneously using the proposed IC system, and analysis was achieved using a single sample injection and a single IC system. The separation-ability of the proposed IC system could further be improved by using a more effective anionic/zwitterionic mixed micellar stationary phase. It is also possible to further improve the separation/detection abilities by using a more appropriate detection method

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OPTIMIZED AND VALIDATED HPLC METHODS FOR COMPENDIAL QUALITY ASSESSMENT I. METHYLXANTHINE DERIVATIVES

Zs. Budvári-Bárány, Gy. Szász, K. Gyimesi-Forrás

Semmelweis University of Medicine
Institute of Pharmaceutical Chemistry
Budapest, Hungary

ABSTRACT

The HPLC method suggested here is suitable for the detection and quantitative determination of 0.1-1 % caffeine, theobromine and theophylline impurities in each of these methylxanthine derivatives. The system optimization includes organic modifier content, ionic strength, eluent flow rate and concludes to the use of a simple C_{18}/methanol-water 1:4 (I = 0.1) chromatographic system.

The limit of impurity detection was found to be 15 ng (theobromine, theophylline) and 30 ng (caffeine). The range of quantitation extends from 31.25 to 250 ng. The accuracy (percentage of recovery) and precision (repeatability) of the method are tabulated. The method, considering validation requirements of USP XXIII, demonstrates the suitability of HPLC for selective compendial purity tests.

INTRODUCTION

The three methylxanthine (MX) alkaloids — caffeine (1,3,7-trimethylxanthine),theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine) — have reserved their therapeutic importance through several decades and they are monographed in all of the pharmacopeias, even for the present. At the purity test for "related substances" (i.e., detection of the other two MX's as impurity in the third MX derivative) the European Pharmacopeia applies thin layer chromatography; USP XXIII does not prescribe a test for related impurities. At the same time, for the RP-HPLC assay of theophylline, as internal standard, USP prescribes theobromine. In caffeine, USP XXIII applies a non-selective chemical reaction for the detection of "other alkaloids" impurity.

In our opinion, the application of HPLC for the detection (quantification) of "related impurities" (i.e., related MX impurities in the MX samples) can provide a good solution.

Although a number of works dealing with the HPLC separation of the three MX's can be found in the literature, no reference to drug purity control was found.

In a previous paper[1] dealing with purity control of MX's, the relevant literature till 1987 was compiled and tabulated.

Reviewing the papers of the period between 1988-1995, there may be found HPLC separation of the MX's in biological samples[2-12] and in foods.[13,14] The publication of Vree et al.[15] deals with the relationship of structure and chromatographic behavior of purine derivatives, and only one paper[16] was found in which MX's were separated in pharmaceutical preparations.

The present work aims to complete the cited paper[1] by chromatographic system optimization and method validation, thereby enabling the presented procedure for compendial application, including also the quantitative determinations of the detected impurities.

Even our first experiments definitely showed that the very simple RP-HPLC system C_{18}/methanol-water, after suitable optimization, provides a good solution of the task. A similar system was used in our previous work[1] and, by some other authors, for the separation of caffeine and theobromine in foods,[17] as well as for the three MX's in biological samples.[18]

The present paper demonstrates that the optimized system allows the resolution of the three (isomeric and homologous) MX's with a method which is also suitable for sensitive purity control. At the system optimization and method validation, the general principles of USP XXIII[19] and some other authentic sources[20,21] were considered.

EXPERIMENTAL

Chromatography

The HPLC apparatus comprised a Waters pump Model 510 (Millipore, USA) combined with a Rheodyne injector unit Model 7125 (Cotati, California, USA) equipped with a 10 μL loop. A Hewlett-Packard variable wavelength absorbance detector Model 1030B (220-800 nm) was used.

The equipment modules, subsequent to the pump, were thermostatted (Column Chiller, Model 7955 - Jones Chromatography, Ltd., Wales) at 25°C±0.1°. The chromatograms were recorded and the data handling was effected by a Hewlett Packard integrator Model 3396, Ser. II.

The C_{18} sorbent, Hypersil 5 ODS (Shandon), particle size 5 μm, was packed into a stainless steel column (250 x 4.0 mm I.D.; BST, Budapest, Hungary).

As mobile phase, sonically degassed and filtered methanol-water mixtures with 20,40 and 60 v/v% of methanol were applied. All ready-made eluents contained sodium chloride in an amount to adjust the ionic strength to 0.075, 0.1, 0.125. The column void time was signalled by the injection of water.

Each retention data point was calculated as an average of at least three parallel runs. The eluent flow rate was adjusted to 0.6, 0.8, 1.0 mL/min. The effluent was monitored at the optimum wavelength, i.e., at 270 nm.

The columns were brought to the initial state by washing with 50 mL of methanol-water 1:4 mixture, 50 mL of 0.001 M HCl and then 100 mL of water, followed by purging with 50 mL of methanol. After each experiment, the loop was washed with 5-10 times repeated injection of 0.1 M HCl, then water and, finally, methanol.

Table 1

Influence of Methanol Content on the Retention* of MX's

Compound	Methanol Content of Eluent, % 20	40	60
Theobromine	5.49 (1.9)	3.88 (0.21)	2.94 (≤0.1)
Theophylline	8.05 (2.6)	4.58 (0.4)	3.24 (≤0.1)
Caffeine	12.63	5.45	3.43

*t_R in minutes.
R_s values in parentheses; I (NaCl): 0.1; Eleuent flow rate: 0.8 mL/min.;
Injected: 10 μL of methanolic solution (equivalent to 125 ng MX);
Preparation: see Experimental.

Materials

Caffeine, theobromine and theophylline samples were obtained from the manufacturers and met the requirements of the US, European, and Hungarian Pharmacopeias. The "related MX content " of these samples was controlled by HPLC and was found to be ≤0.01%. Methanol, RS HPLC grade, (Carlo Erba); water was double-distilled. Sodium chloride, 99.99% was obtained from Aldrich Chemical Co..

Standard Solutions

The 0.1 g of MX bulk substances (see Table 1) were dissolved in 200 mL of a 1:3 mixture of 0.1 M HCl and methanol. This solution (c = 0.05%) was used, after dilution with the HCl-methanol mixture, to prepare the standard solutions.

Test Solutions

The 1 g of the MX sample, spiked with 0.1 % of the two related MX's (see Table 4) was sonicated for 15 minutes with 10 mL of methanol and then

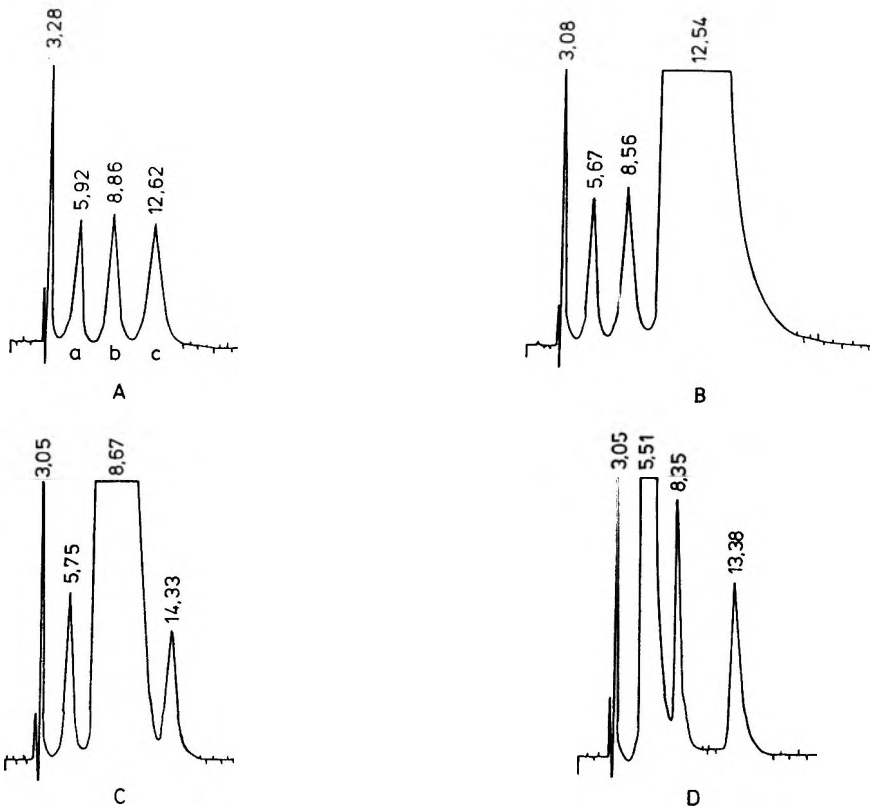

Figure 1. Separation of MX's and detection of related MX impurity in caffeine, theobromine and theophylline samples. **A**: 125 ng of theobromine (a) theophylline (b) and caffeine (c). **B**: 0.1% theobromine and 0.1% theophylline in caffeine. **C**: 0.1% caffeine, 0.1% theobromine in theophylline. **D**: 0.1% caffeine, 0.1% theophylline in theobromine. Injected: 10 μL of solution (125 ng of MX impurity). Preparation: see Experimental section.

filtered. To a 5 mL portion of the filtrate, was added 5 mL of 0.1M HCl and 30 mL of methanol (equivalent to 125 ng impurity/10 μL). One gram of theophylline or caffeine sample, spiked with 1% of the other two MX's, was sonicated for 25 minutes with 40 mL of methanol.

The solution, which became warm, was cooled, then filtered. To 1 mL of the filtrate, was added 4 mL of 0.1M HCl and 15 mL of methanol (equivalent to 125 ng MX impurity/10 μL).

Table 2

Detection Limit for MX's

Compound	Detection Limit (ng)	Peak Area S.D. (%)
Theobromine	8.0	2.8
Theophylline	8.0	2.8
Caffeine	30.0	5.5

Chromatographic conditions: Eluent, water/methanol 40:10; I, 0.1 (NaCl); Flow rate, 0.8 mL/min.; Detection, 270 nm.

One gram of theobromine, spiked with 1% of the other two MX's, was sonicated for 25 minutes with 20 mL of methanol. The cooled mixture was filtered. To 1 mL of the filtrate, was added 4 mL of 0.1 M HCl and 35 mL of methanol (equivalent to 125 ng MX impurity/10μL).

RESULTS

System Optimization

The main working parameters for the optimization of the C_{18}/methanol-water system were selected by applying the screening sequence which is shown in Table 1. The table shows, also, the effectiveness of the system by comparing the influence of eluent methanol content on the chromatographic behavior of MX's. In conclusion, as optimized parameters, 1:4 methanol - water content in eluent, 0.1 ionic strength (NaCl) 0.8 mL/min. eluent flow rate and 270 nm as wavelength of detection were accepted. Figure 1 illustrates that the high resolution achieved allows the sharp separation of MX's, even in the case when they are present in a ratio 1000:1:1.

Method Validation

Limit of detection was determined by using the signal-to-noise ratio method. For this, the peak area of a solution with known concentration of the

Table 3

Quantification Range and Linearity of Conc'n./Peak Area Relationship for MX's

	S.D. % (Peak Area)		
Injected	**Theobromine**	**Theophylline**	**Caffeine**
31.25	1.9	2.5	---
62.50	3.6	3.9	2.6
93.75	1.9	1.7	3.4
125.0	2.1	3.1	2.5
187.0	2.3	1.4	2.3
250.0	2.1	2.1	1.1
r:	0.9996	0.9986	0.9956

MX, and the noise (background response of a blank experiment) were determined. The average of 6-6 runs was taken for the evaluation. As the limit of detection, a signal-to-noise ratio of 3:1 was accepted (see Table 2).

Limit of quantitation: (the lowest amount of MX impurity can be determined with acceptable precision and accuracy) was determined using the value of the background response.[19] The latter was obtained by analyzing eight blank experiments and calculating the standard deviation of background response. This value, multiplied by ten, gives the limit of quantitation. In this work, considering the requirements of repeatability, as limit of quantitation, 31.25 ng was chosen.

Linearity and range: the upper level of the range of quantitation was chosen as the highest amount of MX appearing in a peak, with acceptable peak shape and symmetry (Table 3). The linearity was characterized as the regression coefficient of test results of peak area *vs.* analyte concentration relationship (Table 3). The amount of impurity can be quantitated between 31.25 and 250 ng.

Precision (repeatability): was determined by dissolving 0.01 g of MX in a mixture of 150 mL methanol and 50 mL 0.1 M HCl. This solution (MX content = 0.005%) was used for the preparation of the calibration curve in the range 31.25-250 ng (Table 3). The precision was determined by assaying the MX content of five aliquots at each concentration within the above range.

Table 4

Accuracy of MX-Related Impurity Determination

Bulk Substance	Impurity Content, %					
	Caffeine		Theobromine		Theophylline	
	Calc.	Found	Calc.	Found	Calc.	Found
Caffeine	---	---	0.10	0.092	0.10	0.091
			1.0	0.90	1.0	0.91
Theobromine	0.10	0.088	---	---	0.10	0.09
	1.0	0.85			1.0	0.93
Theophylline	0.10	0.092	0.10	0.089	---	---
	1.0	0.91	1.0	0.089		

Accuracy was examined by the determination "related MX" content of the samples spiked homogenously with 0.1 and 1 % of MX impurities. In conclusion, the MX's can be determined in the selected range within S.D.±2-4%. As Table 4 shows, the recovery ranges between 8-10 % in case of 0.1-1% of impurity content.

DISCUSSION

The selected chromatographic system may be applied for compendial purity testing in two ways:

(1) performing a limit test,when the area of the related MX peak is limited to a maximum of 3 times larger then that of the noise, i.e., detection limit, (see Table 2),

(2) quantification of MX impurity content; for the evaluation a calibration curve is to be used (Table 3).

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CHIRAL HPLC SEPARATIONS OF 1-AZABICYCLO[2.2.1]HEPTAN-3-ONE AND 1-ALKOXYCARBONYLALKYL-PYRROLIDINE-3-CARBOXYLIC ACID ALKYL ESTER ENANTIOMERS ON POLYSACCHARIDE-BASED STATIONARY PHASES

Sechoing Lin,* Cara E. Engelsma, Nancy J. Maddox,
Brian K. Huckabee, Denis M. Sobieray

Chemical Development Department
Parke-Davis Pharmaceutical Research Division
Warner-Lambert Company
188 Howard Avenue, Holland, Michigan 49424

ABSTRACT

Chiralcel OD-H, OJ, Chiralpak AD and AS columns were screened for the enantiomeric separation of 1-azabicyclo-[2.2.1]heptan-3-one (**5**) and 1-alkoxycarbonylalkyl-pyrrolidine-3-carboxylic acid alkyl ester intermediates (**1**, **2**, **3** & **4**, see Figure 1 for structures) during the large-scale synthesis of PD 151832. PD 151832 is a highly potent m1 subtype selective muscarinic agonist expected to be useful for patients with Alzheimer's disease. Cellulose-based columns such as Chiralcel OD-H and OJ are in general less efficient than amylose-based columns such as Chiralpak AD and AS for separation of these types of compounds. The optimal column for separation of compounds **3** and **5** is a Chiralpak AD column using hexane/2-propanol/diethylamine as mobile phase, while a Chiralpak AS column works the best for compounds **1**, **2** and **4** using the same solvent mixture.

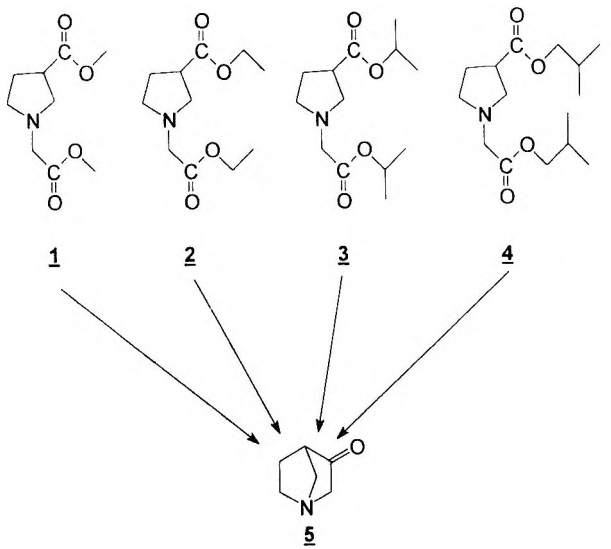

Figure 1. Scheme for Synthesis of 1-Azabicyclo[2.2.1]Heptan-3-One (**5**) from 1-Alkoxycarbonylalkyl-Pyrrolidine-3-Carboxylic Acid Alkyl Esters (**1**, **2**, **3** and **4**).

INTRODUCTION

Enantiomerically pure (R)-1-azabicyclo[2.2.1]heptan-3-one is a synthetic intermediate for making potentially useful biologically active agents.[1] In particular, (R)-1-azabicyclo[2.2.1]heptan-3-one is an important intermediate towards the synthesis of PD 151832, a potential cognition activator under active development for the treatment of neurodegenerative disorders.[2] One synthetic approach to this intermediate involved the cyclization of 1-alkoxycarbonylalkyl-pyrrolidine-3-carboxylic acid alkyl esters (**1**, **2**, **3** & **4**) to give 1-azabicyclo[2.2.1]heptan-3-one (**5**). It was our desire to resolve these early intermediates. Chiral analytical methods are vital in exploring resolution conditions for these intermediates and to ensure the enantiomeric purity of compound **5**.

Chiralcel OD-H, OJ, Chiralpak AD and AS columns were evaluated for the enantiomeric separation of compounds 1, 2, 3, 4 and 5. The effect of the structure of mobile phase alcohol modifier on enantiomeric resolution was also studied.

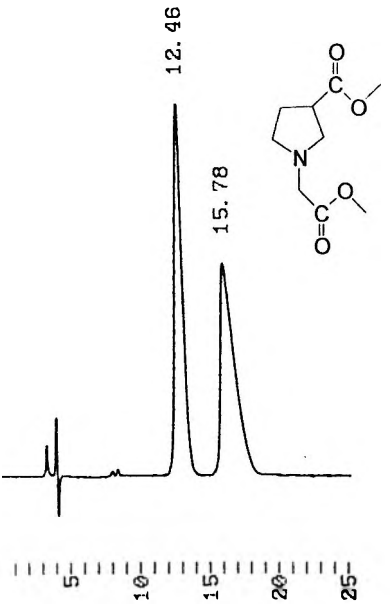

Figure 2. Separation of a Racemic Mixture of Compound 1; Column: Chiralpak AS, Mobile Phase: Hexane/IPA/DEA (950/50/1), Flow Rate: 1.0 mL/min, Detection: UV @ 230 nm, Sample Amount Injected: 90 μg.

EXPERIMENTAL

Equipment

The liquid chromatographic system consisted of a Hitachi L-6200 intelligent pump, a Micromeritics 728 autosampler, a Valco injector with a 20 μL loop, a Hitachi L-4000H variable wavelength UV detector, a Waters 410 Differential Refractometer equipped with a column oven, and a Hitachi D-2500 Chromato-integrator.

The analytical columns were Chiralcel OD-H, OJ, and Chiralpak AS and AD. All of the columns were 250 x 4.6 mm I.D., and 10 microns in particle size except OD-H which was 5 microns. They were purchased from Chiral Technologies, Inc, Exton, PA.

Chemicals

Hexane and 2-propanol (HPLC grades) were obtained from EM Science, Gibbstown, NJ. Ethanol (absolute) was purchased from Aaper Alcohol and Chemical Company, Shelbyville, KY. Diethylamine (redistilled, 99.5%) was obtained from Aldrich Chemical Company, Milwaukee, WI. Racemic 1-azabicyclo[2.2.1]heptan-3-one, (R)-1-azabicyclo [2.2.1]heptan-3-one, (S)-1-azabicyclo[2.2.1]heptan-3-one, and racemic 1-alkoxycarbonylalkyl-pyrrolidine-3-carboxylic acid alkyl esters were synthesized in the Chemical Development Department, Parke-Davis Pharmaceutical Research Division, Holland, MI.

HPLC Conditions

The mobile phase was either hexane/2-propanol (IPA)/diethylamine (DEA) or hexane/ethanol (EtOH)/diethylamine (DEA) in an appropriate volume ratio. The flow rate was either 1.0 or 0.6 mL/min. The detection was UV @ 230 nm for compounds **1**, **2**, **3** and **4**, and 220 nm for compound 5 when the modifier was 2-propanol (IPA). Refractive index detection (RI) was used for compound **5** when the modifier was ethanol (EtOH). The column temperature was maintained at 30°C. The sample was dissolved in mobile phase. The amount of sample injected was 10 to 100 μg unless otherwise stated.

The capacity factor of the first eluted peak, k_1', the separation factor, α, and the resolution factor, R_s were calculated as follows: $k_1' = (t_1 - t_0)/t_0$; $\alpha = (t_2 - t_0)/(t_1 - t_0)$; $R_s = 2(t_2 - t_1)/(w_1 + w_2)$; where t_0 is the time at void volume, t_1 is the retention time of the first eluted peak, t_2 is the retention time of the second eluted peak, w_1 and w_2 are the widths at baseline for the first and second eluted peaks, respectively, and they were obtained by extrapolating the relatively straight sides of the peaks to the baseline.

RESULTS AND DISCUSSION

The ability of polysaccharide derivative based stationary phases to achieve separation of enantiomers appears to depend on the conformation of the polysaccharide chain and the structure of the substituents.[3] Okamoto et al.[4] and more recently Wainer et al.[5] have provided further insights into the chiral recognition mechanism. However, the actual chiral recognition mechanism remains far from clear.

Table 1

Enantiomeric Separation of Compounds 1, 2, 3, and 4, and Effect of 2-propanol Concentration in Mobile Phase With a Flow Rate of 1.0 mL/min

	Compound 1			Compound 2			Compound 3			Compound 4		
Column	k_1'	α	R_S	k_1'	α	R_S	k_1'	α	R_S	k_1'	α	R_S
Hexane/IPA/DEA: (950/50/1)												
OD-H	1.51	1.07	0.85	----	----	<0.5	No Separation			No Separation		
OJ	----	----	<0.5	No Separation			No Separation			No Separation		
AD	----	----	<0.5	0.93	1.10	1.19	0.55	1.11	1.09	0.62	1.13	1.07
AS	2.80	1.34	4.01	1.28	1.22	2.23	No Separation			0.56	1.25	1.65
Hexane/IPA/DEA (980/20/1)												
OD-H	2.70	1.08	1.20	1.89	1.05	0.67	No Separation			No Separation		
OJ	No Separation			No Separation			----	----	<0.5	No Separation		
AD	----	----	<0.5	1.67	1.09	1.39	1.00	1.11	1.26	1.13	1.11	1.38
AS	4.95	1.38	4.72	2.35	1.24	2.95	No Separation			1.03	1.28	2.59

Table 2

Effect of the Flow Rate on the Enantiomeric Separation of Compounds 1, 2, 3 and 4 Using a Mobile Phase of Hexane/IPA/DEA (950/50/1)

	Compound 1			Compound 2			Compound 3			Compound 4		
Column	k_1'	α	R_S	k_1'	α	R_S	k_1'	α	R_S	k_1'	α	R_S
1.0 mL/min												
OD-H	1.51	1.07	0.85	----	----	<0.5	No Separation			No Separation		
OJ	----	----	<0.5	No Separation			No Separation			No Separation		
AD	----	----	<0.5	0.93	1.10	1.19	0.55	1.11	1.09	0.62	1.13	1.07
AS	2.80	1.34	4.01	1.28	1.22	2.23	No Separation			0.56	1.25	1.65
0.6 mL/min												
OD-H	1.56	1.07	1.20	----	----	<0.5	No Separation			No Separation		
OJ	----	----	<0.5	No Separation			No Separation			No Separation		
AD	1.35	1.05	0.65	0.96	1.10	1.42	0.58	1.11	1.07	0.65	1.12	1.32
AS	2.86	1.35	4.42	1.32	1.22	2.53	No Separation			0.59	1.24	1.97

Table 3

Effectof Mobile Phase Alcohol Modifier on the Enantiomeric Separation of Compounds 1, 2, 3 and 4 with a Flow Rate of 1.0 mL/min

	Compound 1			Compound 2			Compound 3			Compound 4		
Column	k_1'	α	R_S	k_1'	α	R_S	k_1'	α	R_S	k_1'	α	R_S
Hexane/IPA/DEA (980/20/1)												
OD-H	2.70	1.08	1.20	1.89	1.05	0.67	No Separation			No Separation		
OJ	No Separation			No Separation			----	----	<0.5	No Separation		
AD	----	----	<0.5	1.67	1.09	1.39	1.00	1.11	1.26	1.13	1.11	1.38
AS	4.95	1.38	4.72	2.35	1.24	2.95	No Separation			1.03	1.28	2.59
Hexane/EtOH/DEA (980/20/1)												
OD-H	----	----	<0.5	No Separation			No Separation			No Separation		
OJ	No Separation			No Separation			No Separation			----	----	<0.5
AD	4.46	1.04	0.90	----	----	<0.5	1.17	1.07	1.04	1.39	1.11	1.31
AS	1.93	1.23	3.09	0.99	1.13	1.33	No Separation			0.46	1.12	0.77

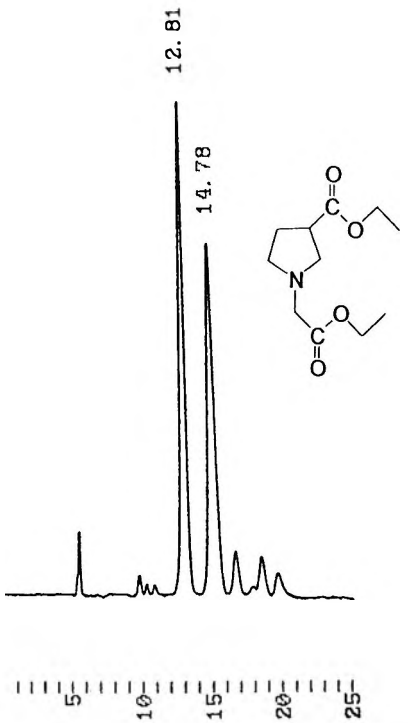

Figure 3. Separation of a Racemic Mixture of Compound **2**; Column: Chiralpak AS, Mobile Phase: Hexane/IPA/DEA (950/50/1), Flow Rate: 0.6 mL/min, Detection: UV @ 230 nm, Sample Amount Injected: 25 μg.

The enantiomeric separation for compounds **1**, **2**, **3** and **4** was carried out using hexane /IPA/DEA. A small amount of diethylamine added in the mobile phase reduced the peak tailing. As shown in Table 1 with hexane /IPA/ DEA (950/50/1), the best results for compounds **1**, **2** and **4** were obtained using a Chiralpak AS column. On the other hand, the same column would not resolve compound **3**.

The drastic difference observed in chiral recognition for compound **3** is probably caused by the added bulk of the methyl substituent on the carbon directly linked to the oxygen on the carboxylic acid ester. This appears to prevent the necessary interactions for chiral recognition to occur. Nevertheless, compound **3** can be resolved using a Chiralpak AD column.

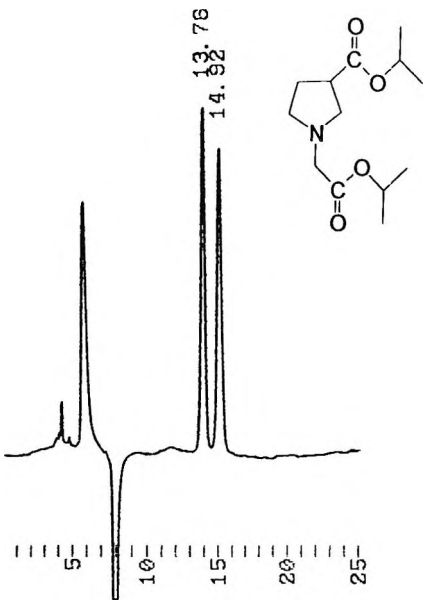

Figure 4. Separation of a Racemic Mixture of Compound 3; Column: Chiralpak AD, Mobile Phase: Hexane/IPA/DEA (990/10/1), Flow Rate: 0.6 mL/min, Detection: UV @ 230 nm, Sample Amount Injected: 30 μg.

Table 4

Enantiomeric Separation of Compound 5 and Effect of Mobile Phase Alcohol Modifier on a Chiralpak AD Column with a Flow Rate of 1.0 mL/min

Column	k_1'	α	R_S
Hexane/IPA/DEA			
700/300/1	1.47	2.15	11.91
800/200/1	2.17	2.13	12.46
900/100/1	4.28	2.09	13.25
Hexane/EtOH/DEA			
700/300/1	3.45	1.48	5.46
800/200/1	4.67	1.47	6.60

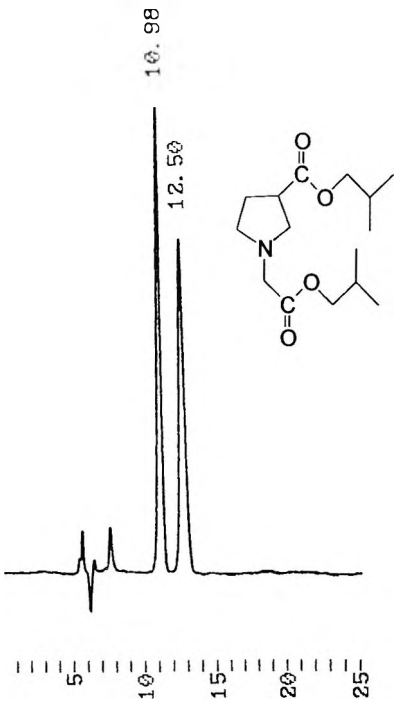

Figure 5. Separation of a Racemic Mixture of Compound **4**; Column: Chiralpak AS, Mobile Phase: Hexane/IPA/DEA (980/20/1), Flow Rate: 0.6 mL/min, Detection: UV @ 230 nm, Sample Amount Injected: 30 μg.

With hexane/IPA/DEA (980/20/1), a decrease in IPA concentration in mobile phase resulted in a corresponding increase in retention and the resolution was generally improved as seen in Table 1. The effect of flow rate was also studied (Table 2).The resolution is increased at 0.6 mL/min for those compounds which give resolution at 1.0 mL/min. The only exception is compound **3** on a Chiralpak AD column where the resolution remains unchanged.

The structure of the mobile phase alcohol modifier is observed to change enantiomeric resolution depending on compound and column type.[6-16] When 2-propanol was replaced with ethanol, resolutions of all four compounds on all four different columns were significantly decreased except for compound **1** where resolution was slightly increased (Table 3). In addition, retention for all four compounds on a Chiralpak AD column increased with the more polar ethanol while it decreased on Chiralcel OD-H, OJ and Chiralpak AD columns.

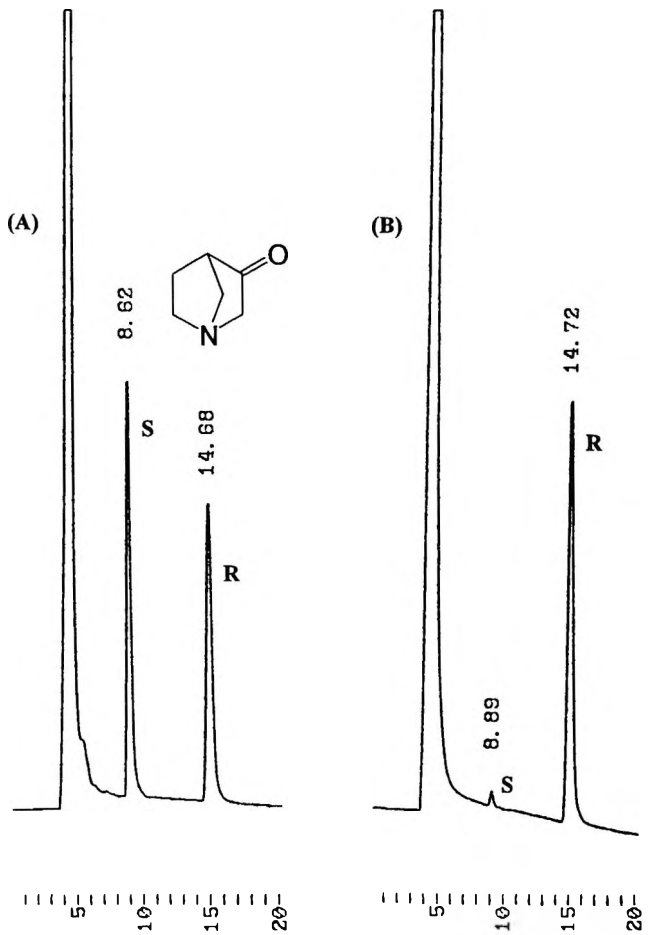

Figure 6. Separations of (A) a Racemic Mixture of Compound **5** and (B) an Enriched Sample of Compound **5**/Chiral Acid Salt; Column: Chiralpak AD, Mobile Phase: Hexane/IPA/DEA (700/300/1), Flow Rate: 1.0 mL/min, Detection: UV @ 220 nm, Sample Amount Injected: 90 μg (Sample was dissolved in IPA).

Separation of the enantiomers of target compound **5** was unsuccessful on Chiralcel OD-H and OJ columns. A Chiralpak AS column gave only partial resolution. Excellent resolution was achieved using a Chiralpak AD column. A decrease in IPA concentration in mobile phase significantly increases retention of compound **5**, and to a lesser extent, resolution. The use of ethanol in mobile phase increases retention of compound **5** while drastically decreasing the resolution (Table 4).

As a result of this study, we were able to select conditions to perform efficient separations of all five compounds of interest. Representative chromatograms for all five compounds are shown in Figures 2-6, respectively.

CONCLUSIONS

Four different polysaccharide derivative-based stationary phases were rapidly screened for enantiomeric separation of five different intermediates in the synthesis of PD 151832. Amylose-based Chiralpak AD and AS columns appear to be superior to the cellulose-based Chiralcel OD-H and OJ columns for separation. A Chiralpak AS column works the best for compounds **1**, **2** and **4**, and a Chiralpak AD column works best for compounds **3** and **5**. 2-Propanol is superior to ethanol as a mobile phase modifier.

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COLUMN-SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR MINOCYCLINE OF NUDE MICE SERUM

S. Bompadre, L. Ferrante, L. Leone,* M. Montesi, L. Possati

Institute of Biomedical Sciences
Faculty of Medicine
University of Ancona
60100 Ancona, Italy

ABSTRACT

A rapid, direct, accurate and sensitive liquid chromatographic assay with on-line extraction for minocycline of nude mice serum is described. Serum was directly injected onto the extraction column where the drug was separated from the serum concomitants using a solid phase extraction procedure based on cation exchange. Using an on-line column switching system, minocycline was quantitatively transferred and separated on the analytical column. Ultraviolet absorption at 352 nm was used for detection. The assay was linear from 0.2 to 10 μg/mL with a correlation coefficient of 0.999. Detection limit was 50 ng/mL. Recovery was 97%.

The method has been developed for the determination of minocycline levels of nude mice serum collected from a study designed to evaluate the potential for tumor anti-angiogenic effects of this drug.

INTRODUCTION

Minocycline is a semisynthetic tetracycline antimicrobial. It is rapidly absorbed from the gastrointestinal tract, is widely distributed in body tissues and fluids, and has a prolonged half-life.[1] Tetracycline derivatives, long used clinically for their antibiotic effects, have recently been shown to have potential benefit in the treatment of cancer.[2,3] Although the mechanism of this antitumor effect has not yet been definitively elucidated, tetracyclines have been shown to inhibit tumor angiogenesis,[4] presumably due to inhibition of collagenase.[5,6]

Our purpose was to develop a simple and sensitive assay for quantitation of minocycline in serum of nude mice as part of a study programmed to investigate the correlations between serum levels and the effects of the drug on human tumors transplanted into nude mice. Previously, the determination of minocycline in biologic specimens has been difficult because of the poor specificity of microbiological and fluorometric assays. High pressure liquid chromatography (HPLC) has become the preferred method of assay, but previous techniques involved complicated and time consuming procedures.[7,8]

We propose a column-switching HPLC method involving direct injection of serum samples into the chromatographic system to simplify the assay and decrease the potential of introducing bias into the results. A similar chromatographic system has been used to determine other drugs in biological fluids[9,10] which gave excellent results.

EXPERIMENTAL

Reagents and Chemicals

Minocycline hydrochloride was purchased from Sigma Chemical Co. (St.Louis, MO, USA). Acetonitrile was HPLC grade and purchased from E. Merck (Darmstadt, Germany).

Both sodium dihydrogenphosphate mono-hydrate and di-sodium hydrogen orthophosphate dihydrate were supplied by Carlo Erba Farmitalia (Milan, Italy). All chemicals were reagent grade.

Analytical grade, filtered water was obtained fresh daily from an Elgastat UHQ PS apparatus (ELGA, High Wycombe, Bucks, UK). Pooled drug-free serum of nuce mice was used to prepare standards.

Apparatus

The liquid chromatograph consisted of a Beckman 126 Programmable Solvent Module (Beckman, Fullerton, CA, USA), a Varian model Vista 5500 pump (Walnut Creek, CA, USA), and a Beckman 166 Programmable Detector Module. The injector was a Rheodyne model 7725i manual injection valve, fitted with a 200 μL sample loop. The chromatograms were integrated with a System Gold Laboratory Data System (Beckman, Fullerton, CA, USA). The coupled-column system was operated by a pneumatic, six-port, automated switching valve (Valco, Schencon, Switzerland) controlled by the HPLC system.

Chromatographic Conditions

The extraction column (50 mm x 4 mm I.D.) was dry-packed with a 40 μm WCX stationary phase obtained from an ion-exchange SPE cartridge (LC-WCX) from Supelco (Bellefonte, PA, USA). The analytical column was an Ultrasphere C_{18} (25 cm x 4.6 mm, particle size 5 μm) from Beckman (Fullerton, CA, USA). Mobile phase 1 consisted of a mixture of 2.5% methanol in 0.01 M phosphate buffer (pH 7.0); mobile phase 2 consisted of 14% acetonitrile in 0.1 M phosphate buffer (pH 3.0). Sample aliquots of 200 μL (mouse serum + mobile phase 1, 1:3, v/v) were injected directly onto the pre-column. Mobile phase 1 was used as washing solvent to eliminate the biological matrix from the extraction column. The flow-rate for both columns was set at 1 mL/min. The effluent from the analytical column was monitored by UV at a wavelength of 352 nm. All analyses were performed at ambient temperature.

Column Switching Procedure

Figure 1 shows the scheme for the column switching procedure. Serum samples were diluted 1:3, v/v, with phosphate buffer 0.01 M (pH 7.0) to avoid clogging of column frits. Sample aliquots of 200 μl were injected directly into the chromatograph. Sample was brought onto the pre-column by mobile phase 1 (2.5% methanol in phosphate buffer 0.01 M) delivered by pump 1, at a flow-rate of 1 ml/min, and directed to waste, while the pump 2 delivered the mobile phase 2 (14% acetonitrile in phosphate buffer 0.1 M) to the analytical column. The components of the serum matrix were removed whilst minocycline was trapped in the column (Figure 1A). After two minutes the valve was switched and mobile phase 2 from pump 2 eluted the analyte of interest to the analytical column (Figure 1B). The low pH of the mobile phase 2 promotes the elution of

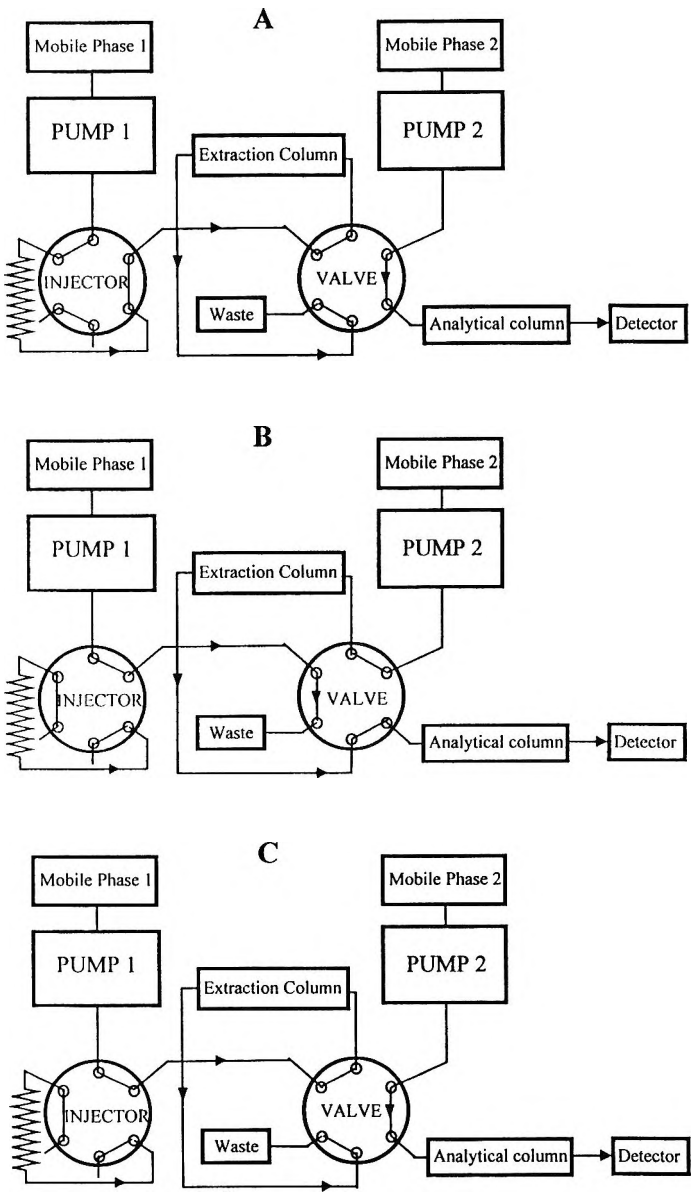

Figure 1. Scheme of column switching.

the analyte from the cation-exchange sorbent of the extraction column. The connection time of the pre-column to the secondary column was 2 minutes. At this stage, the valve was switched to the initial position and pump 1 delivered the mobile phase 1 to the extraction column to prepare it for the next sample, while pump 2 maintained the flow of mobile phase 2 through the analytical column (Figure 1C) where minocycline was separated and detected by UV. The retention time of minocycline was 6.5 minutes and cycle time of one analysis was 10 minutes.

Recovery

Standard solutions were prepared by dissolving minocycline in distilled water and stored in the dark at +4 °C. The drug was found to be stable for up 1 week. Working solutions were prepared daily by dilution of the stock-solutions. Control serum samples were spiked with minocycline standard solutions to reproduce different concentrations in the 0.2 - 10.0 μg/mL range. The percentage of drug recovered for a particular injection was calculated by comparing the peak area obtained from the spiked sample with the value obtained from direct injection onto the analytical column of aqueous solution containing the same concentration of drug.

Quantification

Calibration standards were prepared by spiking control serum at concentrations of minocycline ranging from 0.2 to 10.0 μg/mL. Each spiked serum standard was injected eight times. These samples were processed as described above. Peak areas were plotted *versus* minocycline concentrations and the resulting calibration curve was used to calculate the drug concentrations of the unknown samples.

RESULTS

Optimization

Various experiments were conducted to establish the optimal conditions. In order to optimize the extraction process, C_8 and C_{18} stationary phases were tested for retention and elution. Serum was deproteinized with trichloroacetic acid. Both C_8 and C_{18} gave a satisfactory retention of the drug, but elution of the analyte was difficult. The compound of interest exhibited a broad tailing

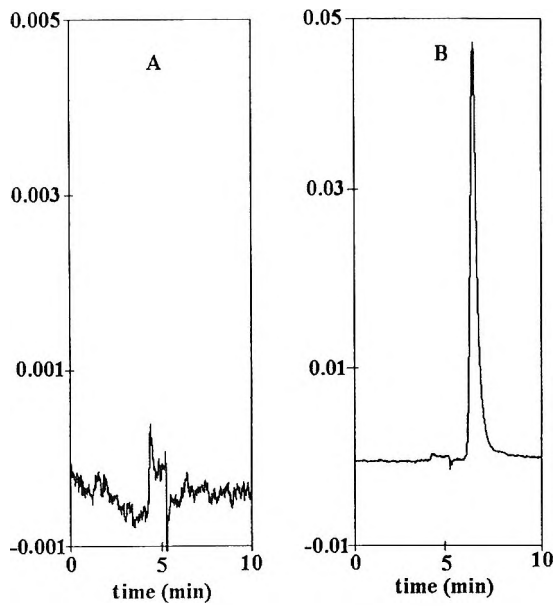

Figure 2. Chromatograms of (A) drug-free serum of nude mice and (B) serum spiked with 5 μg/mL minocycline.

peak, and many interfering substances were present. To enhance the chromatographic profile, a weak cationic exchanger was tested. The packing chosen, a WCX (carboxylic acid, Na^+ counterion), was obtained from a solid phase extraction cartridge from Supelco. Because of its pKa value of 4.8, the functional group is negatively charged at neutral pH and it can bind amino groups as those of the minocycline molecules. In fact, the dimethylamino group at C-4 has a pKa value of 9.3[11] and, at neutral pH, it exhibits a positive charge. The column was washed for two minutes with 2% methanol in 0.01 M phosphate buffer (pH 7.0) that washed off most unwanted material and did not remove the drug.

After retaining the isolate, the pre-column can be washed with up to 5 mL of mobile phase without displacing the isolate from the packing. The analyte can then be eluted from the extraction column with an acidic buffer. Phosphate buffer, 0.1 M, pH 3.0, containing 14% acetonitrile, eluted minocycline by suppressing ionization of the tertiary amine at C-4 of the molecule. In this way, we obtained peaks better resolved from the extraction column using a concentration of organic solvent still optimal for the subsequent separation in the analytical column. In preliminary experiments, it was found that the

Table 1

Linearity, Precision, and Accuracy for Minocycline*

Nominal (μg/ml)	Actual value (mean ± SD, n=8) (μg/ml)			Precision %	Accuracy %
0.2	0.209	±	0.005	2.39	+4.50
0.5	0.472	±	0.017	3.60	-5.60
1.0	1.047	±	0.045	4.30	+4.70
2.0	1.930	±	0.098	5.08	-3.50
5.0	4.890	±	0.120	2.45	-2.20
10.0	10.230	±	0.230	2.25	+2.30

*$Y = (3.67 \pm 0.09)X + (-0.22 \pm 0.15)$

concentration of acetonitrile at 14% gave the optimum separation with a reasonable chromatographic time. The connection time of the pre-column to the analytical column was optimized by stepwise reduction until the peak area of minocycline started to decrease. To avoid clogging column frits, serum was diluted with phosphate buffer.

The equilibration of both the pre-column and the analytical column after an analysis can be achieved in a few minutes so that total analysis time was 10 minutes.

Representative chromatograms from blank and spiked serum of nude mice with minocycline are shown in Figure 2. In the chromatograms, no peak was present that might interfere with the determination of minocycline, thus demonstrating the clean-up efficiency.

Recovery

The column-switching procedure gave nearly complete recovery of minocycline from serum. Absolute recovery of the drug was calculated by comparing the peak areas after direct injections of minocycline with those of equal amounts of analyte "extracted" from serum. Values obtained were 97% (n=8) for the concentrations that were examined.

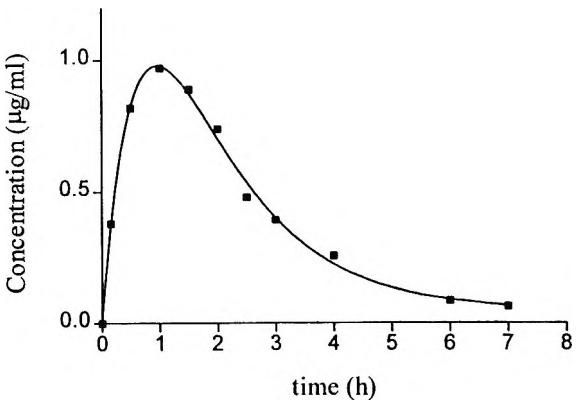

Figure 3. Serum concentration-time curve of minocycline from a nude mouse after 10 mg/Kg oral administration.

Linearity, Precision, Accuracy, and Sensitivity

The calibration curves obtained were linear over the working interval 0.2 - 10.0 µg/mL of minocycline. The regression line obeyed the equation $Y = (3.67 \pm 0.09)X + (-0.22 \pm 0.15)$; the correlation coefficient being $r^2 = 0.999$. The accuracy was +4.50% for the lower limit of quantitation and +2.30% for 10 µg/mL. Values for precision (coefficient of variation) at these concentrations were 2.39 and 2.25%, respectively. The results obtained are summarized in Table 1. The detection limit with 50 µL of serum was 50 ng/mL. This limit corresponds to a serum concentration resulting in a peak height equal to 3 times the signal-to-noise ratio.

Application

The procedure described above was employed to determine the serum concentrations of minocycline after oral administration to 5 nude mice. The pharmacokinetic parameters (means ± SD) calculated in this study, terminal half-life ($t_{1/2b}$), area under the serum concentration-time curve (AUC), absorption rate constant (Ka), serum clearance (Cl_s), volume of distribution of the central compartment (V_1) and mean residence time in the central compartment (MRT_1) are given in Table 2. Figure 3 shows a typical serum profile of minocycline obtained after oral administration of a 10 mg/Kg dose to a nude mouse.

Table 2

Pharmacokinetic Parameters of Minocycline after Oral Administration of 10 mg/Kg*

$t_{1/2b}$ (h)	AUC (mg h/L)	K_a (h)	V_1 (L/Kg)	Cl_s (ml/min Kg)	MRT_1 (h)
4.90 ±1.22	3.20 ±0.09	1.15 ±0.01	3.20 ±0.06	52.06 ±1.49	1.03 ±0.02

*Means ± S.D.

DISCUSSION

Only a few HPLC techniques have been published which analyze minocycline in biological fluids. They involve complicated and time-consuming procedures which also increase the potential of introducing a bias in the results. Previous methods have necessitated ethyl acetate extraction and evaporation,[7] ion-exchange systems, or complex mobile phases and gradient elution.[8] Birmingham's method[12] requires time-consuming sample preparation and the use of an internal standard, which prolongs analysis time. Moreover the sensitivity of the method is lower than that obtained with the proposed technique.

Classical preparation techniques involve numerous steps, which can cause loss of the compounds of interest. In recent years, an increasing number of HPLC methods incorporating on-line sample clean-up by solid phase extraction, using column switching, have been developed. With column-switching techniques, the manual sample preparation steps are drastically reduced, or even eliminated. As a result, the accuracy and precision that can be obtained in the determination of drugs in biological fluids is generally improved Additionally, the time required for processing the samples with this latter technique is greatly shortened, and the selectivity that can be achieved is comparable with, and sometimes better than, that obtained with traditional sample pre-treatment. There are additional advantages of column-switching over classical procedures for sample clean-up. Good precision and accuracy can be achieved without the need for an internal standard. Moreover, sample clean-up by column switching protects light-sensitive analytes from light during the analysis. The proposed method has all these advantages.

CONCLUSIONS

In conclusion, the proposed method is extremely simple and rapid since it requires no extraction or cleanup steps and no internal standard. The diluted sample is directly injected onto the chromatographic column. The assay offers appreciable accuracy and precision. The selectivity of the method, in regard to endogenous compounds, is very satisfactory; no interference was found. High sensitivity can be achieved in a relatively short analytical time. Moreover the method is relatively inexpensive.

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DEVELOPMENT OF AN HPLC-UV METHOD FOR DETERMINATION OF TAURINE IN INFANT FORMULAE AND BREAST MILK

I. M. P. L. V. O. Ferreira,[†] M. V. Nunes,[†] E. Mendes,[†]
F. Remião,[‡] M. A. Ferreira[†]

[†]CEQUP
[‡]Laboratório de Bromatologia
Laboratório de Toxicologia
Faculdade de Farmácia, Universidade do Porto
Rua Aníbal Cunha 164
4050 Porto, Portugal

ABSTRACT

A rapid and accurate high performance liquid chromatographic (HPLC) procedure was proposed for routine and selective determination of taurine in infant formulas and breast milk. The sample preparation was simple, involving protein removal and filtration operations. Afterwards, taurine was derivatised with o-phthalaldehyde/2-mercaptoethanol prior to injection onto a reversed phase column C_{18} ($S_{10}ODS_2$). Isocratic elution was carried out using 0.05 M phosphate buffer pH 5.3/ methanol (60:40) mixture. The effluent was monitored by a UV detector at 350 nm. A linear relationship was found between peak area and a concentration range of 1-70 μg/mL. The detection limit was 0.3 μg/mL. Effective separation and quantification was achieved in under six minutes. No interference of other amino acids was observed.

Extensive validation of the proposed method was carried out both by the standard additions method and by comparison with a fluorimetric method of known accuracy. The precision was better than 1.4%.

INTRODUCTION

Taurine, 2-aminoethanesulphonic acid, is a sulphur-containing β-amino acid that is not incorporated into proteins. However, it takes part in biochemical reactions of major importance, such as conjugation with bile acids to form bile salts essential for fat absorption, cell membrane stabilization, antioxidation, detoxification, osmoregulation, neuromodulation, and brain and retinal development.[1]

Considerable evidence has accumulated that neonates and infants, who have a very limited ability to synthesize taurine, are especially prone to develop taurine deficiency and that they depend on an external taurine supply.[2]

Taurine concentrations in milk are very variable, depending on species. While breast milk is an excellent source of taurine in the developing infant, cow's milk has a very low taurine content. Nowadays the supplementation of infant formulae with taurine in concentrations similar to those found in human milk is therefore recommended.[3,4]

Many methods for measuring taurine in several matrices viz. biological samples, such as tissue homogenates, urine, and serum have been published, including gas chromatography[5] and HPLC analysis of diferent amino acid derivatives.[6-12] One of the most sensitive and commonly used derivatising agent techniques is the use of o-phthaldehyde / mercaptoethanol, which, originates a fluorescent adduct that enables the fluorimetric determination of taurine.[6,11]

Other HPLC fluorimetric determinations in similar matrices include derivatising reaction with thiamine[8] and fluorescamine.[9]

It is also possible to use UV absorbance detection at 350 nm with different derivatisation techniques, including o-phthaldehyde / mercaptoethanol[7] and dinitrofluorobenzene.[10,12] Interestingly, research work developed by Chen at al.[12] is distinguished from the rest since one of the matrices used was human milk.

Some of these methods require time-consuming pre-treatment of the samples, in order to eliminate interfering substances or complicated derivatising treatment of the sample before assay.

This paper describes a rapid and accurate method for the determination of taurine in infant formulae and breast milk. Although, the sample matrix is of complex nature, the pre-treatment applied is simple and the method is suitable for rapid routine assays of a large number of samples and uses a UV detector which is much more common in control laboratories than the fluorimetric detector.

MATERIALS

Apparatus

The chromatographic analysis was carried out in a Gilson, high performance liquid Chromatograph (Gilson Medical Electronics, Villiers le Bel, France) equipped with a type 305 pump, a type 302 pump and a type 7125 Rheodyne Injector with a 20 μl loop. A Gilson 118, variable long wave ultra violet detector was also used.

The chromatographic separation was achieved with a Spherisorb S_{10} ODS_2 Chromatographic column, 10 μm. The integrator used was a Gilson 712 HPLC System Controller Software.

A Gilson 121 Spectrofluorometer was used for detection of the fluorescent adduct.

Reagents and Standards

Taurine and o-phthaldehyde were obtained from Sigma Chemical Co. L-Amino acids kit was also from Sigma (Kit No. LAA-21). Sulphosalicylic acid, boric acid, sodium hydroxide and mercaptoethanol, all p.a., were obtained from Merck. Methanol (LiCrosolv) was Merck "gradient grade".

Water used for chromatography possessed a resistance greater than 15 MΩ, and was filtered through a membrane of 0.45 μm porosity which was subsequently degassed.

Sample Preparation

After homogenisation, 5.0 g of infant formulae were dissolved in 40 mL of warm (40°C) water. 5.0 mL of the infant formulae solution or breast milk were added to 5.0 mL of sulphosalicylic acid solution 0.2 M, mixed thoroughly, and allowed to stand for 10 min. All samples were filtered through W42 paper and thereafter, through 0.2 μm filter paper.

The use of sulphosalicylic acid as a protein precipitating agent has been shown to result in a higher % recovery of taurine.[11]

Derivatisation Procedure

Taurine was derivatised with o-phthalaldehyde (OPA, 40 mg, 0.8 mL absolute ethanol) and 2-mercaptoethanol (40μl) in 0.5 M borate buffer (10 mL, 3.1 g boric acid in 90 mL water, adjusted to pH 10.4 with 5M NaOH, and made up to 100 mL)[6] prior to injection onto a reversed phase column C_{18} ($S_{10}ODS_2$).

The derivatizing solution was prepared daily and filtered through a 0.20 μm filter before use.

A 100 μL volume of standard or sample solution (after preparation) was placed in an eppendorf and 100 μl of derivatizing reagent were added. The derivatization reaction was allowed to proceed for exactly 1.5 min, at which time an aliquot was injected into the HPLC.

Chromatography

The HPLC elution required a mixture of two solvents. Solvent A, 0.05 M phosphate buffer pH 5.3, and solvent B, methanol. The two solvents were filtered and degassed before use. Isocratic elution was carried out at a flow rate of 1.5 mL/min, using 60% solvent A combined with 40 % solvent B. The injection volume was 20 μl and the chromatographic analysis was conducted at ambient temperature.

Taurine concentration was determined at an absorbance of 350 nm, which is the maximum wavelength on the excitation spectrum of OPA-derivatized amino acids. Taurine peak was identified by coelution with a standard and by comparison of the retention time.

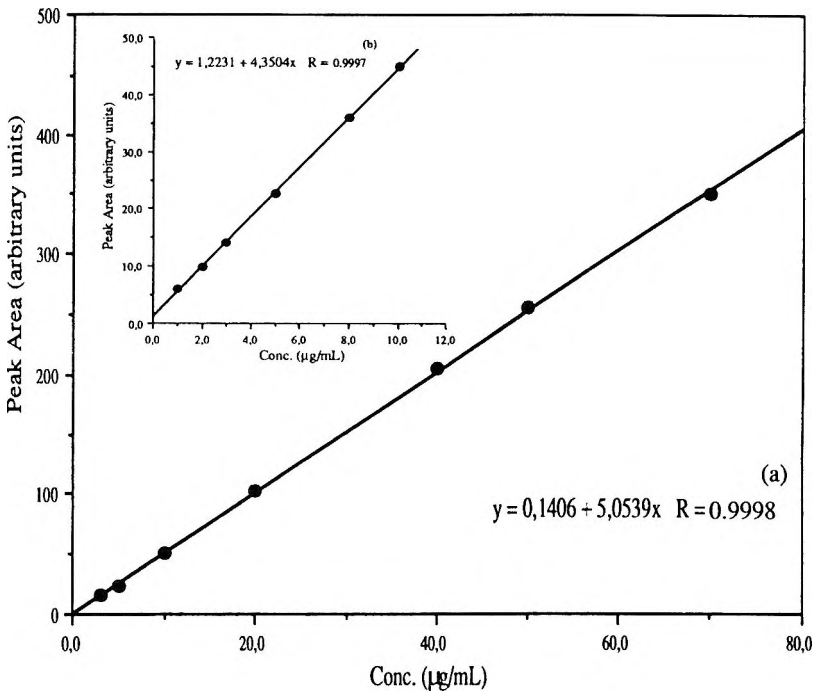

Figure 1. Standard curves for taurine. Taurine was derivatised and chromatographed by the method described in the text. Each point represents the average of triplicate determinations. (a) Sensitivity of 0.06 AUFS, detection limite 1 µg/mL. (b) Sensitivity of 0.03 AUFS, detection limite 0.3 µg/mL.

RESULTS

Analytical Curve and Detection Limit

Under the assay conditions described, a linear relationship between the concentration of taurine and the UV absorbance at 350 nm was obtained. This linearity was mantained over the concentration range 3-70 µg/mL with a sensitivity of 0.06 AUFS and 1-10 µg/mL with a sensitivity of 0.03 AUFS. The detection limit value was calculated as the concentration corresponding to three times the standard deviation of the background noise and was 1 µg/mL and 0.3 µg/mL, respectively. The calibration curves for taurine are ilustrated in Fig. 1.

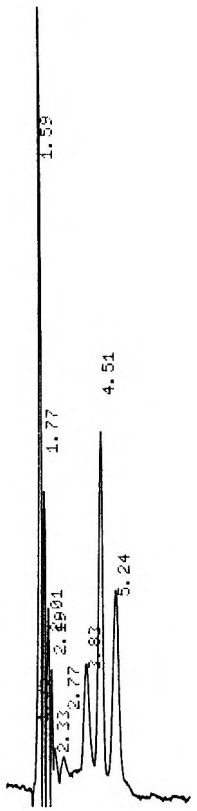

Figure 2. Typical chromatogram obtained for taurine extracted from infant formulae. Retention time of taurine 4.51.

Triplicate determinations were made on each of the calibration standards, the peak area values (arbitrary units) that were plotted are average values. The relative percent average deviations of triplicates were less than 3 % in all cases.

Validity of the Method

The repeatability of the derivatization procedure was examined. A standard taurine solution was derivatized and chromatographed six times. When reaction and chromatographic conditions were carefully optimized, the standard deviation was 0.02 (n=6 and concentration of taurine 20 μg/mL).

Table 1

Recovery of Taurine from Spiked Infant Formulae

	µg/mL Added	µg/mL Found[a]	Standard Deviation	CV%	Recovery %
	2.00	2.03	±0.05	2.46	101.5
Sample 1	4.00	3.92	±0.08	2.04	98.0
	6.00	5.82	±0.09	1.54	97.0
	2.00	1.99	±0.03	1.51	99.5
Sample 9	4.00	4.10	±0.07	1.71	102.5
	6.00	5.73	±0.06	1.05	95.5

[a]Mean value found for 3 assays for each studied concentrations.

The interference of other amino acids was examined next. For this purpose, different amino acids were injected after derivatization with OPA. They included L-Alanine, L-Valine, L-Leucine, L-Isoleucine, L-Proline, L-Phenylalanine, L- Tryptophan, L- Methionine, Glycine, L-Serine, L-Threonine, L-Tyrosine, L-Asparagine, L-Glutamine, L-Aspartic acid, L-Glutamic acid, L-Lysine, L-Arginine, L-Histidine. Different retention times were obtained for each amino acid.

Fig. 2 shows a typical chromatograph of taurine extracted from infant formulae. Peaks corresponding to several amino acids were detected in the chromatograms obtained for infant formulae determinations, but as shown, these peaks were well separated from those of taurine under the conditions used. A similar behaviour was observed with regards to breast milk.

The precision of the analytical method was evaluated by measuring the peak chromatographic area 10 times on the same sample. The relative standard deviation (RSD) ranged between 0.7 and 1.4 % (concentration of taurine in infant formula 42.6 and 28.6 mg of taurine/100 g of infant formula, respectively).

Recovery studies were carried out on two different samples of infant formulae, sample 1 with high concentration of taurine, and sample 9 with very low concentration of taurine. The samples were analysed in triplicate before and after the addition of known amounts of taurine and they were spiked with

Table 2

Comparison Between Taurine Determined in Infant Formulae and Breast Milk by HPLC-UV and a Comparison Method of Known Accuracy[a]

Sample No.	Taurine Determined (mg/100 g of Infant Formula) HPLC-UV(y)	Comparison method (x)
1	39.4 ± 0.7[b]	40.6 ± 0.5
2	34.4 ± 1.3[b]	32.7 ± 0.2
3	28.6 ± 0.3[b]	27.7 ± 0.7
4	39.0 ± 0.9[b]	40.3 ± 0.5
5	38.0 ± 0.2[b]	38.8 ± 0.6
6	44.7 ± 0.7[b]	43.1 ± 0.3
7	32.7 ± 0.9[b]	32.9 ± 0.4
8	30.2 ± 1.1[b]	31.0 ± 0.9
9	1.01 ± 0.08[c]	0.80 ± 0.04
10	3.99 ± 0.21[c]	4.32 ± 0.11
	Taurine Determined (mg/100 mL of Breast Milk)	
11	4.13 ± 0.06[b]	4.23 ± 0.10
12	4.25 ± 0.08[b]	4.32 ± 0.05
13	2.28 ± 0.11[b]	2.43 ± 0.08
14	2.18 ± 0.09[b]	2.08 ± 0.12

[a]Values are expressed as mean ± standard deviation of three determinations.
[b]Sensitivity of 0.06 AUFS.
[c]Sensitivity of 0.03 AUFS.
Samples 1 to 10 are from infant formulae, samples 11 to 14 are from breast milk.

three different concentrations of taurine. The results are listed in Table 1. The addition of taurine to the infant formulae was made before the protein removal. Thus, this procedure proved the effectiveness of the extraction step and the accuracy of the proposed method. Recovery values were between 95.5 and 102.5 %, which confirm no interference effects due to matrix composition.

In order to validate the accuracy of the analytical method and because there was no certified reference material available, not only the standard addition method was made, but also a comparison with a method of known accuracy.[11] The results of these determinations of taurine in infant formulae and breast milk are presented in Table 2. In both cases quantification was based on the external standard method.

No significant differences between the results obtained with the present method (y) and the comparison method (x) were obtained when determined by ANOVA methodology, followed by Fisher's PLSD test. (Differences were considered significant for $p < 0.01$). Regression lines between the two methods were $y = 0.996\ x + 0.108$, ($r = 0.997$) and $y = 1.01\ x - 0.042$, ($r = 0.993$) for infant formulae and breast milk, respectively.

CONCLUSION

An accurate and precise method to quantify taurine in infant formulae and breast milk is presented.

Despite the complexity of the matrix, the sample pre-treatment is simple and this approach only requires a basic HPLC system without extra reagent pumps, mixing manifolds, reaction coils, etc. which is an obvious advantage of precolumn derivatisation relatively to post column derivatisation.[7] However, it requires rigorous control of the OPA-reagent and reaction time in order to obtain the high degree of reproducibitily shown here.

The chromatographic run time of 5 minutes is comparable with the lowest reported time for fluorimetric determinations[11] and less than the run time reported for UV determinations.[7]

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AN IMPROVED SAMPLE PREPARATION METHOD FOR THE QUANTITATIVE HPLC DETERMINATION OF 5-METHYL-DEOXYCYTIDINE IN ANIMAL TISSUE DNA

Craig A. Cooney,*,† Carolyn K. Wise, Lionel A. Poirier

Division of Nutritional Toxicology
3900 NCTR Road
National Center for Toxicological Research
Jefferson, Arkansas 72079

ABSTRACT

Techniques are presented for the purification of DNA from mammalian tissues, its enzymatic hydrolysis to deoxyribonucleosides and the separation and quantification of these by high pressure liquid chromatography (HPLC). The method is used to quantify 5-methyldeoxycytidine (5MdC) and deoxycytidine (dC) in DNA. From this data the molar %5MdC, i.e. 100 x 5MdC/(dC + 5MdC), is calculated for DNA. The precision of the method matches or exceeds that of other published HPLC methods for quantifying the %5MdC. The DNA obtained is extremely clean, and the enzymatic hydrolysis provides deoxyribonucleosides without contamination so there are no extraneous peaks in the region of interest, and peaks that are obtained have sufficient area to eliminate errors from variation in integration. The %5MdC is a quantitative and absolute measure of the genome wide DNA methylation. This method is suitable to quantify small changes in mammalian enzymatic DNA methylation due to age, diet, drugs or carcinogens.

INTRODUCTION

Measurement of 5-methyldeoxycytidine (5MdC) content in DNA by enzymatic digestion and HPLC is an excellent technique with a number of advantages. The data generated are both absolute and quantitative, and the measurement of both 5MdC and deoxycytidine (dC) is made from the same chromatogram and thus requires no internal standard. It is a convenient choice because the method uses standard laboratory equipment and nontoxic, nonradioactive reagents and enzymes.

A number of effective techniques have been described for quantifying 5MdC by HPLC.[1-5] In particular, the techniques of Kuo *et al.*[1] and Gehrke *et al.*[3] are often used. Here we describe a combination of techniques for careful purification, quantification and hydrolysis of animal tissue DNA which produces extremely uniform peaks, and eliminates extraneous peaks, in chromatograms. The method described avoids a number of variables often encountered with DNA preparation, digestion and chromatography and gives precise quantification of 5MdC in animal tissues.

Enzymatic DNA methylation as 5MdC has essential roles in the organization and control of the mammalian genome from genetic imprinting and embryonic development[6-10] through adulthood and aging. Changes in adult mammalian 5MdC occur with manipulation of methyl metabolism (e.g. S-adenosylmethionine and S-adenosylhomocysteine)[11-12] carcinogenesis[11-15] and aging.[16-20] The DNA of vertebrate tissues contains between 2% and 10% 5MdC.[1,21] The sensitivity of the method presented here allows for the determination of the %5MdC from just a few micrograms of mammalian DNA. The precision of the method is suitable to follow changes in genome wide %5MdC occurring with carcinogenesis, aging and manipulation of methyl metabolism.

MATERIALS AND METHODS

Reagents and Enzymes

Reagent or molecular biology grade chemicals were used throughout. Deoxyribonucleosides used for external standards in HPLC were from Sigma Chemical, St. Louis, MO (5MdC # M-3155; dC # M-3897). NaOH, used for base treatment of DNA, and acetic acid, used to titrate NaOH before DNA digests, were commercially standardized solutions of 1.0 M NaOH and 1.0 M acetic acid (Mallinckrodt Chemical, Paris, KY). HPLC grade methanol was

from J.T. Baker, Phillipsburg, NJ (# 9093-03). Enzymes were from Sigma Chemical, St. Louis, MO (proteinase K # P-0390) and from Boehringer Mannheim Biochemicals, Indianapolis, IN (RNase, DNase free, # 1119915; P1 nuclease # 236225; alkaline phosphatase # 713023).

Isolation of Nuclei from Frozen Tissue

Frozen tissue (e.g. 300 mg of liver at -70°C) was crushed and ground to a powder with liquid nitrogen and a pestle in a ceramic mortar. Subsequent nuclear isolation steps were performed on ice with solutions at ~4°C. Nuclei were isolated by an adaption of conventional means.[22] Briefly, the powdered tissue was transferred to a Potter homogenizer to which was added 20 volumes of "nuclear isolation buffer" (250 mM sucrose, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4, 0.1% Triton X-100) at 4°C. Tissue was homogenized with several strokes of a glass "B" pestle. The homogenate was filtered twice. The first filtration was through cheese cloth (held in broad plastic mesh) into a 50 mL centrifuge tube and the next through a plastic fine mesh strainer (Falcon, Becton-Dickinson, Franklin Lakes, NJ #2350) into a 50 mL centrifuge tube. Nuclei were pelleted from the filtered homogenate by centrifugation at 1500 x *g* for 15 minutes. The supernatant was carefully poured off and the nuclear pellet was resuspended in nuclear isolation buffer and spun again. This last step was repeated once.

Double-Stranded DNA Preparation

DNA was prepared from isolated nuclei by an adaption of conventional means.[23] Briefly, the nuclear pellet (e.g. 100 μL) was resuspended in four volumes (e.g. 400 μL) of "RNase buffer" (100 mM NaCl, 30 mM Tris-HCl, 10 mM EDTA pH 8.4) at room temperature. An equal volume (e.g. 500 μL) of "proteinase mix" (100 mM NaCl, 50 mM EDTA pH 8.0, 1% SDS, 300 μg/mL proteinase K) was then added. This was mixed and incubated at 37°C overnight (16 hours). The next morning, the sample was treated with 2 μL of RNase (DNase free, 500 μg/mL), mixed and incubated at 37°C for 3 hours. The sample was then extracted twice with phenol:chloroform:isoamyl alcohol (24:24:1) and then once with chloroform:isoamyl alcohol (24:1). Subsequently, 0.10X volume of 3.0 M potassium acetate (pH 5.4 with acetic acid) was added (to a final concentration of 0.3M potassium acetate), and DNA was precipitated with ethanol. DNA was collected as a fluff and rinsed twice with 70% ethanol. DNA was dried (SpeedVac, Savant, Farmingdale, N.Y.) and redissolved in 10 mM Tris-HCl, 0.1 mM EDTA pH 7.4 (TE). After three days at 4°C, the DNA

was mixed to homogeneity with mild vortexing and/or pipetting. A small aliquot of each DNA sample was quantified by fluorescence using Hoechst 33258 dye on a DyNAquant 200 (Hoefer Pharmacia Biotech, Inc., San Francisco, CA). Alternatively the DNA concentration could be estimated at this step by UV spectrophotometry (A260-A320) using TE as a blank (double stranded DNA is 50 μg/mL @ 1 O.D. @ 260nm).

DNA Purification

DNA was treated with base, dialyzed and concentrated. Approximately 200 μg or less of DNA, was used in the following procedure: The DNA sample was treated with NaOH according to the procedure of Singer *et al.*[24] with modifications. Briefly, water and 1 M NaOH (standardized solution, Mallinckrodt) were added to a DNA aliquot such that DNA was in 1 mL of 0.1 M NaOH. This was lightly vortexed and incubated at 37°C overnight.

DNA in 0.1 M NaOH was transferred to a Centricon 30 spin dialysis unit (Amicon, Beverly, MA), and spin dialyzed at least seven times. Each spin dialysis was done with 1 mL of 20 mM NaOH (made from 1 M NaOH standardized solution). For each spin dialysis, the DNA was reduced to the Centricon 30 default stopping volume of about 50 μL. Standard Amicon instructions were followed for the Centricon 30 in a fixed angle rotor and two additional steps were added. The first additional step, done between spin dialyses, was rotating of the microconcentrator 180° on its long axis. This allowed for efficient dialysis of the DNA by the next 1 mL of 20 mM NaOH. After at least seven successive washes, the DNA, in 20 mM NaOH, was spun off the membrane into the recovery cap and DNA was then transferred to a microcentrifuge tube. The second additional step is washing of the membrane. Specifically, about 50 μL of 20 mM NaOH was added to the membrane, allowed to soak at room temperature for 10 minutes and spun off the membrane into the recovery cap. This recovered material was then added to the DNA in the microcentrifuge tube. This washing and recovery step was repeated once. We have also used Centricon 100 and Microcon 30 and 100 devices (Amicon) for spin dialysis with good results (not shown).

Recovered DNA was mixed and stored at 4°C. A small aliquot was diluted in 20 mM NaOH and quantified by UV spectrophotometry (A260-A320) using 20 mM NaOH as a blank (single stranded DNA is 33 μg/mL @ 1 O.D. @ 260nm). From this the concentration of the stock DNA was determined.

An exact standard volume and concentration of DNA was chosen for the series of digests (e.g. 100 μg/100 μL). DNA solutions of exactly the same concentration were prepared from the stock solutions using 20 mM NaOH.

DNA Digestion

Instructions which follow are for DNA samples (e.g. 100 μg) each in 100 μL of 20 mM NaOH. Each DNA solution was neutralized with exactly 12 μL of 200 mM acetic acid (1.20 moles of acetic acid/mole of NaOH). Acetic acid at 200 mM was made from standardized 1.0 M acetic acid (Mallinckrodt). The DNA solution was mixed, centrifuged down, heat denatured at 95°C for 5 minutes and cooled quickly on ice.

P1 nuclease solution was prepared from solid (300 units phosphodiesterase activity per mg) by dissolving at 5U/μL (1 mg in 60 μL) in 30 mM sodium acetate (pH 5.3 with acetic acid). This can be used for at least 6 months when stored frozen (-20°C).

A hydrolyzation mixture was prepared on ice just before use. The preparation of the hydrolyzation mixture for ten neutralized and heat-denatured DNA samples (100 μg in 112 μL each) is as follows: 650 μL H_2O, 130 μL sodium acetate (300 mM pH5.3 with acetic acid) and 20 μL $ZnSO_4$ (10 mM) were mixed and then 40 μL P1 nuclease solution and 40 μL alkaline phosphatase solution (1U/μL) was added and thoroughly mixed. The alkaline phosphatase stock solution was stored at +4°C.

To each DNA sample, 88 μL of the hydrolyzation mixture was added, the solution was mixed, and spun down. The final volume of each digest was 200 μL, and the final buffer and enzyme concentrations in each digest were: 30 mM sodium acetate (pH 5.3), 0.1 mM $ZnSO_4$, P1 nuclease 100 units/mL (or 0.2 unit/μg DNA, whichever is greater) and alkaline phosphatase 20 units/mL (or 0.04 unit/μg DNA, whichever is greater).

The DNA digest was incubated at 37°C for 5 hours, vortexed, two volumes of room temperature EtOH (95 to 100%) were added, and the samples mixed and spun at room temperature at 17,000 *xg* for 15 min. A small pellet or streak of protein was formed in each sample. The supernatant containing the deoxyribonucleosides (dNs) was collected, dried in a Speed Vac and redissolved in H_2O such that the dN (formerly DNA) concentration was 1.0 μg/μL.

For example, if the digestion was started with 100 μg of DNA, the dN was redissolved in 100 μL of H_2O. This digestion procedure was adapted from Palmgren *et al*[5] and Gehrke *et al.*[3] Mixtures of dNs (digested DNA or standards) were stored frozen (-20°C).

HPLC

Samples and external standards of the same volume and concentration (with respect to dC + 5MdC) were injected in each run of a series. That is, the same amount (±10%) of dN (e.g. from 10 μg of DNA) was injected for each sample. The external standards had combined peak areas of dC and 5MdC closely matching those of the samples. The samples were injected in a small volume (usually 10 μL).

HPLC was performed as described by Wise and Hass[25] as adapted from Gehrke *et al.*[3] Briefly, the samples and standards were injected into a Hewlett-Packard 1090 HPLC, equipped with a Beckman Ultrasphere ODS, 4.5 mm x 25 cm (5 μm particle size) column held at 30°C in a temperature controlled column compartment. A pre-column filter (ChromTech, Apple Valley, MN #C-751) was also used. HPLC buffer A was 0.05 M potassium dihydrogen phosphate containing 2.5% methanol while buffer B contained 0.05 M potassium dihydrogen phosphate with 9% methanol. HPLC mobile phase was delivered to the column at 0.9 mL/min. Prior to each injection, the column was equilibrated with 100% buffer A for 5 minutes. After the injection the HPLC was programmed to pump buffer A for 22 minutes, after which time it switched to buffer B over a 5 minute linear gradient. After 10 minutes of 100% buffer B, the mobile phase was changed back to 100% buffer A in preparation for the next injection. Before the run, a single batch of buffer A was made that was sufficiently large for the entire series of samples and standards. A photodiode array detector was used and data were collected at 280 nm. Under these conditions, the retention time of the 5MdC was 15-17 minutes and dC was 7.5-8.5 minutes. The purpose of buffer B was to elute the remaining dNs and to clear the column in preparation for the next analysis. The HPLC was equipped with an autosampler which allowed automatic injection of about 25 samples in a 24-hour period.

Peak Identification

In the development of these methods we verified the identity of peaks derived from DNA digests by using both internal and external 5MdC and dC

standards. Peaks were identified by their elution positions and by their 280nm/254nm absorbance ratios relative to standards as described elsewhere.[3,26]

Molar Standards for Quantifying %5MdC

The standard mixtures used here were external, i.e. they were separated and quantified in separate chromatograms from the DNA derived samples. Most of the standard mixtures and all of the samples contained both 5MdC and dC. The relative proportions of 5MdC and dC (and later the %5MdC) were determined without an internal standard.

Standard mixtures of 5MdC and dC were made as concentrated stock solutions in water. Aliquots of these were diluted in HPLC buffer or in the solution resulting from processing a mock digestion mixture (to which no DNA had been added). Using the methods described in this paper we found no quantitative difference between standards injected in HPLC buffer or injected in a processed mock digestion mixture (not shown). Standard mixtures were stored frozen (-20°C).

Standard mixtures of 5MdC and dC at relative molar concentrations of 0.0, 1.0, 2.0, 4.0, 8.0, 16.0 and 100.0% 5MdC were injected with each sample series. Standards in the range being measured were injected at the beginning and at the end of each series. Our deoxycytidine standard contained a trace (i.e. 0.5%) of 5MdC and the percent 5MdC of dC standard (no added 5MdC) was subtracted from the percent 5MdC of the other molar standard mixtures.

Quantification of Molar %5MdC

The percent 5MdC [100 x 5MdC/(5MdC + dC)] in DNA was first calculated based on the peak areas (absorbance) at 280 nm. At least two injections of each sample were done and mean of these was taken as the percent 5MdC for that sample. The molar absorbtivities of 5MdC and dC were very similar under these conditions and little or no correction was needed to obtain percent 5MdC by moles from percent 5MdC by peak areas. When necessary the percent 5MdC by peak areas at 280 nm of molar standard mixtures of 5MdC and dC were used to convert peak area percentages for DNA to molar percentages. A calibration curve can be used for this purpose when absorbtivities of 5MdC and dC differ significantly.[26] The accuracy of quantification was monitored by including DNAs of known %5MdC, such as calf thymus or salmon sperm DNA,[1,4,26] in each series of digests and standards.

Statistical Analysis

Groups were compared for significant differences by the two tailed t-test. This and other statistical analysis was done using Quattro Pro software. Groups were considered statistically different when $p < 0.05$.

RESULTS AND DISCUSSION

The methods described here are reliable and precise in comparing animal groups for the genome wide %5MdC in their DNA. These methods avoid the introduction of variables that can reduce the precision of measurement.

DNA Preparation and Purification

We used a multistep conventional method for the preparation and purification of DNA because we have found this most reliable. In particular, the purification steps are important to remove RNA that remains after RNase treatment. Residual RNA in DNA samples is a persistent nuisance in chromatographic determination of %5MdC.[3] Chromatograms of DNAs digested prior to purification (i.e. prior to NaOH treatment and dialysis) have numerous peaks in addition to the five deoxyribonucleosides expected for most eukaryotic DNA (not shown). After base treatment, dialysis and concentration, the only significant peaks are the expected dC, 5MdC, thymidine, deoxyguanosine and deoxyadenosine. In particular, there are no other significant peaks in the region of the 5MdC peak (Figure 1).

Other methods, sometimes elaborate, have been used for DNA purification before %5MdC determination by HPLC. These include cesium chloride gradient centrifugation,[4,27] gel filtration,[5] hydroxyapatite chromatography or gel electrophoresis.[3,28] The base treatment and spin dialysis purification method we use here is highly reproducible, convenient and requires no specialized equipment.

DNA Quantification and Digestion

After purification, we carefully quantify DNA by ultraviolet spectrophotometry. This provides a measure of the DNA concentration (RNA has been removed) by a very reliable method, absorbance at 260nm, that works on single stranded DNA (base treatment denatures DNA) and is done after manipulations that could result in DNA loss are finished.

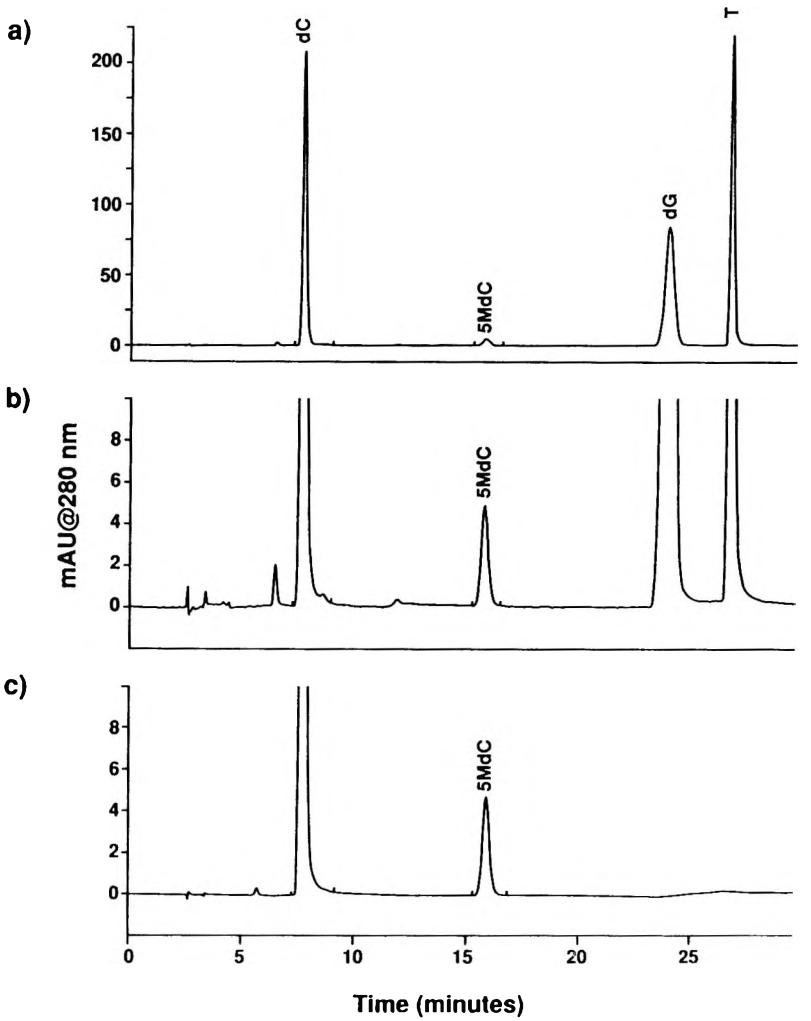

Figure 1. Chromatograms of deoxyribonucleosides from mouse liver DNA as described in the text (a, b) and of 4% 5MdC standard mixture (c). dG= deoxyguanosine, T= thymidine. Deoxyadenosine elutes after 30 minutes and is not shown. a) Full scale (from mouse liver DNA); b) Enlargement 15X to show detail around the 5MdC peak (from mouse liver DNA); c) 4% 5MdC standard mixture (shown at same scale as in b).

We used an adaption of Palmgren et al.[5] and Gehrke et al.[3] methods, in which digestion is achieved in a short, two enzyme, single buffer, single step procedure. This digestion was conducted with a digestion mix such that the same amounts and concentrations of the same enzymes and buffers were added to DNA samples, each of which are also of the same concentration and DNA amount. The removal of enzymes by ethanol precipitation at room temperature helped assure clean chromatograms as described by Palmgren et al.[5]

Both alkaline phosphatase and P1 nuclease from some commercial sources contain significant amounts of adenosine deaminase activity[3,5,29] (and our unpublished results). This activity converts deoxyadenosine from DNA to deoxyinosine. Deoxyinosine would elute just after 5MdC in our chromatograms and could interfere with quantifying 5MdC. Using the P1 nuclease and alkaline phosphatase enzymes and conditions as described above we saw no deoxyinosine peak in our chromatograms (i.e. <0.05% of dC peak area). Other combinations of commercial enzymes and digestion conditions have been described which largely avoid adenosine deaminase activity when digesting nucleic acids.[1,4,5,29] Kuo *et al.*[1] and Gehrke *et al.*[3] heated alkaline phosphatase before DNA digestion to destroy most adenosine deaminase. We did not need to heat or otherwise treat the P1 nuclease or alkaline phosphatase before DNA digestion.

HPLC

We injected the same amount (± 10%) of dN for each sample and used standards whose dC and 5MdC combined peak areas closely matched that of the samples. Thus peak sizes of dC were about the same in each chromatogram for all samples and for most standards. Peak sizes of 5MdC were also similar for samples with the same %5MdC. By digesting and injecting very similar amounts of each sample, no correction for possible variations in linearity of the overall method was necessary.

We used a single batch of HPLC buffer A that was sufficiently large for the entire series of samples and standards. This avoided small variations in pH, buffer concentration etc. that could occur between batches. Molar absorbtivities of 5MdC and dC are pH dependent,[3] and thus small pH changes could affect quantification.

Typical chromatograms from mouse liver DNA and of 4.0% 5MdC standard are shown in Figure 1. Chromatograms using this method are extremely consistent in such features as baseline and peak shape.

Because we apply these methods to vertebrate DNA, where the %5MdC varies between 2% and 10%, we can easily detect and quantify the %5MdC in 10 μg of DNA. Within these parameters the 5MdC peak at 280nm has sufficient area for precise integration. For quantification of %5MdC in DNA with less than 1% 5MdC a larger amount of DNA would need to be used (e.g. 40 μg or more) or the more sensitive ^{32}P-postlabeling method could be used.

More sensitive methods for quantification of genome wide DNA methylation include the ^{32}P-postlabeling method[30] and the methyl acceptor assay.[31,32] Both of these methods use radioisotope detection and can be used with less than 1 μg of DNA. The %5MdC can be determined when as low as 0.01% with less than 1 μg of DNA using the ^{32}P-postlabeling method.[30] This method is quantitiative, absolute and extremely sensitive but uses large amounts of ^{32}P and is laborious. It is the method of choice to determine %5MdC when only small quantities of DNA are available or when the %5MdC is very low, as in insect DNA.[30] The methyl acceptor assay gives a relative measure of genome wide DNA methylation but does not give data to determine the %5MdC. This method uses about 1 μg of DNA per determination and is probably the least laborious of the three methods. It is the method of choice when only small quantities of DNA are available and when only a relative measure of DNA methylation is required.

Percent 5MdC Quantification

Using the method described in this paper, the quantification of the %5MdC in DNA is excellent. For example, groups (four each, assayed as individuals) of B6C3F1 male mice fed NIH-31 diet had liver DNA levels of 4.13 ± 0.02 and 4.00 ± 0.03 %5MdC (± standard errors) at 8 weeks and 19 weeks of age respectively. While the difference in %5MdC at these two different ages is small (0.13), the animal groups are consistent enough, and the above described methods precise enough, that the difference is significant ($p < 0.01$). A difference in liver global DNA methylation is expected with age in mice.[17-20]

The precision of our method compares well with other methods. For example, we used deoxyribonucleosides from 10 μg of DNA for each injection in collecting the above data on 8 and 19 week mice. The mean precision for these %5MdC measurements expressed as percent relative standard deviation is 0.79%. This is similar to the percent relative standard deviation of 1.1% reported by Gehrke *et al.* 1984,[3] for the 5MdC peak when injecting deoxyribonucleosides from 7.5 μg of calf thymus DNA.

We do not measure our accuracy based on mouse liver DNA because huge variations in literature values do not provide a clear basis for comparison. Mouse liver DNA %5MdC as reported in the literature varies between 3% and 8%.[17-19, 21] As a check of accuracy we use calf thymus DNA. The %5MdC in calf thymus DNA has been extensively literature reviewed and carefully quantified by Kuo *et al.*[1] at 6.3%. We found a value of 6.44 ± 0.04% (± standard error) which is within the range of Kuo *et al.*[1] and of the literature. The accuracy of our method compares well with other extensively standardized HPLC methods.

CONCLUSION

The DNA purification and digestion methods we describe here easily and predictably avoid the introduction of variables that can reduce the precision of %5MdC measurement by HPLC. The precision of our method matches or exceeds that of other published HPLC methods for quantifying the %5MdC.[1-5] We have used these methods with numerous groups of animals with similar precision. This method has the precision needed to quantify small changes in global DNA methylation that occur in animals during aging as well as those occurring with modified diets or exposure to certain pharmaceuticals or carcinogens.

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†Email address: ccooney @ nctr.fda.gov

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A FACILE SEPARATION OF NONACTIN AND ITS HOMOLOGUES

Tony Herlt

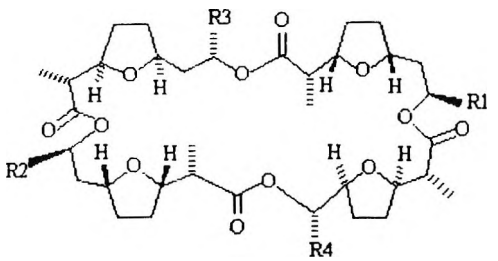
Research School of Chemistry
Australian National University
Canberra
ACT 0200, Australia

ABSTRACT

An analytical reverse phase HPLC separation of Nonactin and its homologues: Monactin, Dinactin, Trinactin and Tetranactin using Evaporative Light Scattering Detection is described.

INTRODUCTION

As part of the ongoing screening of natural products for biological activity, we needed to establish the purity of Nonactin and its homologues which had been isolated by conventional chromatography of an extract from a *Streptomyces* species. The structure of Nonactin and its homologues (collectively known as Actins) are shown in Figure 1. As one ascends the homologous series, four of the methyl groups in Nonactin are sequentially replaced by ethyl groups. The compounds are potent ionophores for alkali metals and might be expected to be chromotographically challenging. A search of the literature revealed only one preparative HPLC method.[1] Attempts to adapt this as analytical method failed, as did attempts to derive an HPLC method based on TLC conditions.[2]



NONACTIN:	R1 = R2 = R3 = R4 = **Me**
MONACTIN:	R1 = **Et**, R2 = R3 = R4 = **Me**
DINACTIN:	R1 = R2 = **Et**, R3 = R4 = **Me**
TRINACTIN:	R1 = R2 = R3 = **Me**, R4 = **Me**
TETRANACTIN:	R1 = R2 = R3 = R4 = **Et**

Figure 1. The structure of Nonactin and its homologues.

Described below is an HPLC method which separates Nonactin, Monactin, Dinactin, Trinactin and Tetranactin to baseline with good peak shape. There is also no need for mobile phase additives, as long as the HPLC system is kept as free of metal ions as possible.

EXPERIMENTAL

THF (stabilised with 0.04% butylated hydroxytoluene) and disodium EDTA were obtained from Ajax Chemicals, 9 Short Street, Auburn, NSW 2144, Australia. All other solvents were obtained from the same source and were distilled and 0.45μ filtered before use. Water was obtained from a Milli-Q purification system. Nonactin and tropolone were obtained from Sigma-Aldrich Pty Ltd, Unit 2, 14 Anella Avenue, Castle Hill, NSW 2154, Australia. Tetranactin as a 6:3:1 mixture of Tetranactin, Trinactin and Dinactin, was obtained from Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co Ltd, 135 Komakodo 1-chome, Gotemoa-shi, Shizuoka 412, Japan. Monactin, Dinactin and Trinactin were obtained by column chromatography[2] of an ethyl

acetate extract of a culture produced from *Streptomyces* A515, grown by the RSC mycology unit. Tetranactin was also purified by the same method. The identities of the purified Actins were established by FAB-MS and ^{1}H NMR spectroscopy.

The HPLC system consisted of a Waters 510 pump, Rheodyne 7125 injector with 20μL sample loop and a Varex Mark III Evaporative Light Scattering (ELS) detector kindly loaned by Alltech Associates Pty Ltd, Baulkham Hills Business Centre, NSW 2153, Australia. The drift tube temperature was set to 96°C and the nitrogen flow to 2.9 SLPM. The other detectors used were a Waters 481 variable wavelength uv detector, and a Waters 410 RI detector. The instruments were interfaced with Waters Maxima software.

The HPLC columns were Merck Lichrosorb Si 60 7μ 250mm x 4mm obtained from Merck Pty Ltd, 207 Colchester road, Kilsyth, Victoria 3137, Australia; a Waters μ-Porasil 10μ 300mm x 3.9mm and a Waters Resolve 5μ 150mm x 3.9mm obtained from Waters Australia Pty Ltd, PO Box 84 Rydalmere, NSW 2116, Australia; a YMC ODS-AQ 3μ 150mm x 4.6mm obtained from YMC Inc, 3233 Burnt Mill Drive, Wilmington, NC 28403, USA; and an Alltech Alltima C18 5μ, 250mm x 4.6mm obtained from the Alltech address given above. Mobile phase compositions are described later in the text. The Actins were dissolved in the mobile phase and 5μL containing approximately 40μg injected.

TLC was run on Merck silica gel 60 glass backed plates, gel thickness 0.25mm and size 10cm x 5cm, obtained from the above Merck address, with 2:1 ethylacetate:chloroform. After development the plates were dipped in an ethanol solution of 6% vanillin and 1% concentrated sulfuric acid, and then heated to 150°C for 5 minutes. The Actins appear as dark blue zones on a pale grey background.

RESULTS AND DISCUSSION

The HPLC method in the literature[1] used a Lichrosorb Si 60 5μ column, a mobile phase of 80:20 hexane:isopropanol (containing 4% water) and detection at 215nm. Attempts to reproduce this separation with an analytical, 7μ version of this column and a mobile phase flow of 0.8mL/min were unsuccessful, with the Actins eluting very close to the void volume and with very poor resolution. Increasing the hexane content did increase retention but at the expense of excessive peak broadening.

The TLC method described in the literature used silica gel 60 and 2:1 ethyl acetate:chloroform. Attempts to develop an HPLC method based on this used a Waters μ-Porasil column, 1:2 ethyl acetate:chloroform at 0.8mL/min and RI detection. The Actins again all eluted very close to the void volume with very poor resolution. Increasing the chloroform content led to excessive peak broadening. A similar result was obtained with the Waters Resolve column. It should be noted that on silica TLC the Actins elute as diffuse streaks rather than as well defined spots, so the poor elution behaviour in normal phase HPLC, in hindsight, is not unexpected.

The next strategy to adopt seemed to be reverse phase HPLC, although solubility tests were not encouraging, as the Actins were insoluble in both acetonitrile and methanol. They were, however, readily soluble in THF, and the first reverse phase method used a YMC ODS-AQ column, 60:40 THF(unstabilised):water at 0.8mL/min and detection at 215nm. The five Actins did separate within 20 minutes but peak shape for the last two, (Trinactin and Tetranactin) was poor. At this low wavelength the absorbance of the mobile phase was very high and, and the photomultiplier close to cutting out. Adding 1mM disodium EDTA to the mobile phase improved peak shape dramatically.

It appears that the disodium EDTA works by providing sodium ions for the Actins to complex with, as well as by chelating with trace metals, because a similar improvement in peak shape was obtained if the mobile phase was made 1mM in sodium acetate. However, the difficulties with working at this low wavelength with THF, and low sensitivity due to the low uv absorbance of the Actins, instigated a further search for a better method.

The Actins are high molecular weight, thermally stable, involatile compounds and as such, should be potentially detectable by ELS. However ELS cannot be used with involatile mobile phase additives which rules out adding sodium salts to improve peak shape. The Alltech Alltima C18 column has very low metal ion content in its silica matrix, and indeed with 60:40 THF(stabilised):water at 1mL/min and ELS detection, separated all five Actins to baseline with excellent peak shape (Figure 2). The elution order is that expected, with the retention time increasing with the number of ethyl groups in the Actin.

When trying to separate potential metal chelators it is important to not only have a metal ion-free column but also to eradicate as many metal ions as possible from the entire HPLC system. On one occasion, switching to a different pump produced a deterioration in peak shape and resolution. This

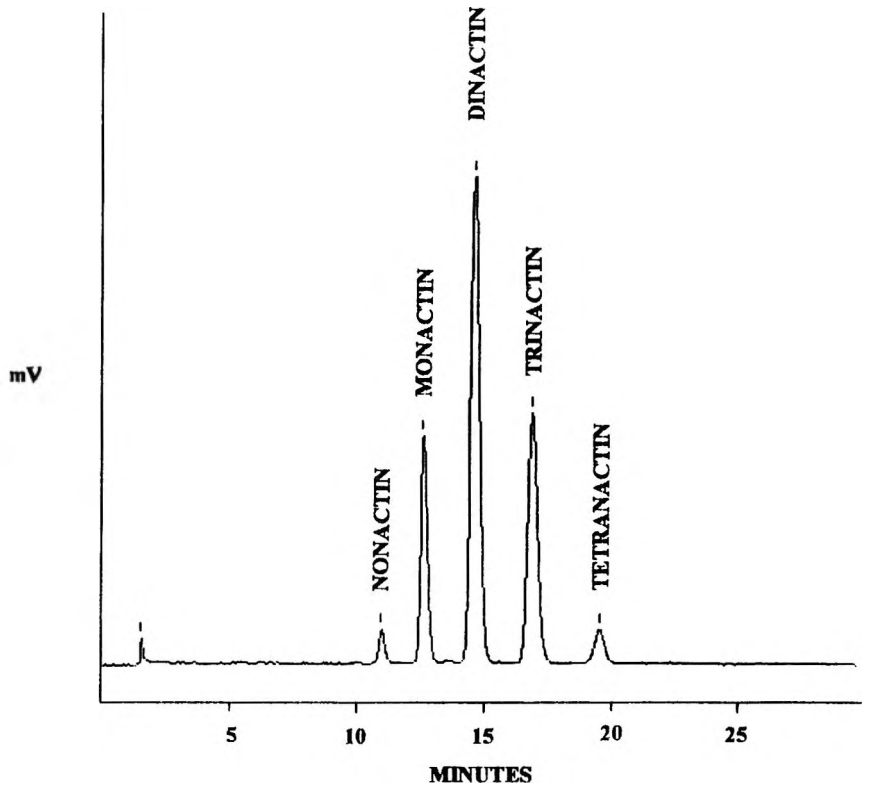


Figure 2. Separation of all five Actins from an ethyl acetate extract of *Streptomyces* A515. Retention times in minutes: Nonactin 11.1, Monactin 12.9, Dinactin 14.9, Trinactin 17.1, Tetranactin 19.8. Column: Alltech Alltima C18 5µ 250mm x 4.6mm. Mobile phase: 60:40 THF:Water at 1mL/min. Detection: ELS, Drift tube temperature 96°C, Nitrogen flow 2.9 SLPM.

pump had previously given problems with metal ions leaching into the mobile phase. Making the solution 1mM in tropolone restored separation quality. Tropolone is a metal scavenger but unlike disodium EDTA is volatile and can be used in ELS detection.

CONCLUSION

A reverse phase HPLC separation of Nonactin and its homologues at the microgram level has been achieved. The method could be adapted to semi-

preparative HPLC using either ELS detection with a post-column splitter, or the non-destructive, RI detection. It is hoped this separation will prove useful to all who work with these chromatographically challenging compounds.

ACKNOWLEDGMENTS

I would like to thank the following people for their assistance: Christine Andrews of Alltech Associates Pty Ltd, NSW, Australia; Dr Akinori Kawamura of Fuji Gotemba Research Laboratories, Chugai Pharmaceuticals, Shizuoka, Japan, and Jenny Rothschild of the RSC mycology unit.

REFERENCES

1. M. Beran, J. Jizba, M. Blumauerova, J. Nemecek, J. Novak, J. Zima, N. V. Kandybin, G. V. Samoukina, Journal of Chromatography, **442**, 431 (1988).

2. J. Beck, H. Gerlach, V. Prelog, W. Voser, Helvetica Chimica Acta, **45**, 620 (1966).

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J. LIQ. CHROM. & REL. TECHNOL., 20(8), 1301 (1997)

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J. LIQ. CHROM. & REL. TECHNOL., 20(8), 1303–1309 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 12 - 14: 18th Annual Introductory HPLC Short Course, sponsored by the Chromatography Forum of the Delaware Valley, West Chester University, West Chester, PA. Contact: Bill Champion, DuPont Merck Pharm. Co., PRF Bldg., Chambers Works, Deepwater, NJ 08023, USA. Tel: (609) 540-4826; Email: champiwl@al.lldmpc.umc.dupont.com.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 28: IInd Miniaturisation in Liquid Chromatography versus Capillary Electrophoresis Conference, University of Ghent, Ghent, Belgium. Contact: Prof. Dr. W. Baeyens, Faculty of Pharmaceutical Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 1 - 4: 1997 International Symposium, Exhibit & Workshops on Preparative Chromatography: Ion Exchange, Adsorption/Desorption Processes and Related Separation Techniques, Washington, DC. Contact: J. Cunningham, Barr Enterprises, 10120 Kelly Road, Box 279, Walkersville, MD 21793, USA. (301) 898-3772; FAX: (301) 898-5596.

JUNE 2 - 4: Advanced HPLC Short Course, sponsored by the Chromatography Forum of the Delaware Valley, Widener University, Chester, PA. Contact: Jim Alexander, Rohm & Haas Labs, 727 Norristown Rd., Spring House, PA 19477, USA. Tel: (215) 619-5226; Email: rsrjna@rohmhaas.com.

JUNE 16 - 19: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach. L'Electrophorese Capillaire, Methode de Routine pour le Contrôle Qualité des Medicaments: Approche Pratique, Monpellier, France. (Training course given in two languages) Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Fac. de Pharmacie, F-34060 Montpellier Cedex 2, France. Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ.montp1.fr.

JUNE 20: Enantiomeric Separation in Capillary Electrophoresis, a short course given by Dr. K. Altria, Glaxo-Wellcome, Ware, UK, in Montpellier, France. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Fac. de Pharmacie, F-34060 Montpellier Cedex 2, France. Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ.montp1.fr.

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce, Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

AUGUST 2 - 9: 39th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency Denver, Denver, Colorado. Contact: Patricia Sulik, Rocky Mountain Instrumental Labs, 456 S. Link Lane, Ft. Colleins, CO, USA. (303) 530-1169; Email: gebrown@usgs.gov.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (703) 231-8222.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 6 - 10: Validation d'une Procedure d'Analyse, Qulification de l'Appareillage; Application a la Chromatographie Liquide, Montpellier, France. (Training course given in French) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

OCTOBER 26 - 29: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Doubletree Hotel, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish

Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

1998

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Res. Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Seattle, Washington. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596.
AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street. NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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Secondary headings, if any, should be placed flush with the left margin, and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The **first line of each paragraph** within the body of the text should be indented a half inch.

6. **Acknowledgments**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

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Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

Book:

1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used. **Hand-drawn characters are not acceptable.**

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2. All text should be **typed single-spaced**.

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