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OBSERVATION OF ABSENCE OF MECHANICAL DEGRADATION OF HIGH MOLECULAR WEIGHT POLYSTYRENES DURING ELUTION THROUGH A COLUMN IN SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Size exclusion chromatography of several polystyrene (PS) standards of a narrow molecular weight (MW) distribution was performed with an on-line light scattering detector (MALLS) using Shodex SEC column KF 806L packed with PS gel packings. Tetrahydrofuran was used as the mobile phase and the flow rate was 1.0 mL/min. Mechanical degradation of the sample PS was not observed even the PS sample having MW four million daltons. MW averages obtained after elution were comparable to those of vendor's values. Peak width of RI (refractometer) chromatograms increased with increasing sample concentrations and polymer components composed of the chromatograms had approximately the same MW except both

ends of the chromatograms. This observation is attributed to the overloading effect and the preferable sample concentration should be as low as possible. The breakthrough volume was independent on the sample concentration, although the peak retention volume increased with the sample concentration. The decrease in molecular size with increasing sample concentration was not observed and therefore, the increase in the peak retention volume with increasing sample concentration is considered to be attributed to the overloading effect, not to the decrease in the hydrodynamic volume.

INTRODUCTION

High molecular weight polymers are fairly sensitive to shear degradation when the polymer solutions flow through capillary. Barth and Carlin reviewed the polymer shear degradation in size exclusion chromatography (SEC).[1] They listed an injection valve, capillary connecting tubing, column frits, and packed columns as possible sources of polymer degradation in SEC and estimated the shear rates generated in these sources. The shear rates generated in columns as a function of particle diameter for a given flow rate in a given inside diameter (I.D.) column were calculated. They concluded from this calculation that a shear rate below about $1x10^4$ s^{-1}, which would be attained at a flow rate of 3.0 mL/min in a 8-mm I.D. column packed with high performance packings of 5 μm, was probably sufficiently low to avoid shear degradation of most polymers of molecular weight (MW) less than $1x10^6$ and that for ultrahigh MW (over 10^6) polymers, flow rate of 0.2 mL/min in a 8-mm I.D. column might be necessary.

A single pass through the conventional SEC system (polystyrene (PS) gel packings over 40 μm) seriously degraded $4.37x10^7$ MW PS by a twofold factor.[2] Polymer degradation is flow rate dependent and was first noticeable for poly(isobutylene) having MW of $1x10^6$ at flow rates greater than 0.15 mL/min.[3] At a flow rate of 2 mL/min, MW decreased by almost a factor of two. Shear degradation effects of PS of MW of $2.7x10^6$ to $7X10^6$ were studied using high performance columns (8 mm I.D.) packed with PS gel and silica gel at a flow rate of 2 mL/min.[4] No MW decrease was observed for the PS gel column, whereas, some degradation was observed for the silica gel column.

A small pore size and particle diameter of packings enhances the degradation.[5] Solvent compatibility with PS gels is also important. A recommendable SEC procedure was proposed;[5] use the lowest possible flow

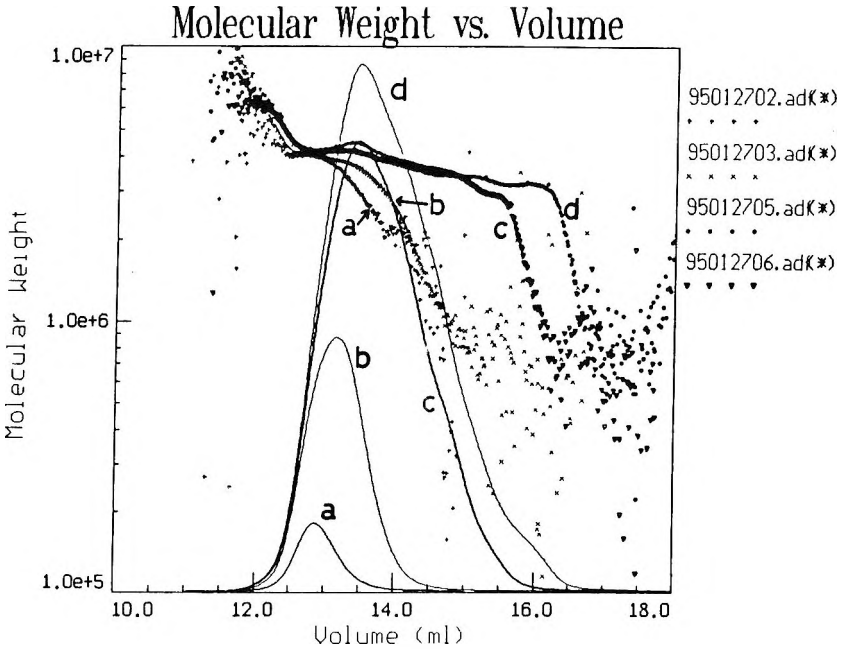

Figure 1. RI chromatograms for PS 4000K at different concentrations and the relationship between V_R and log MW (1). Concentration (%): a 0.0197; b 0.100, c 0.309; d 0.398.

rates and pressures; use the largest interstitial volumes; avoid low pore sizes; use swelling solvents; use concentrations well below the entanglement region and as low as can be detected. The degradation of PS standards of MW of 4.2×10^6 and 2.3×10^6 after elution through SEC columns was observed with calculated MW of $1.0 - 1.1 \times 10^6$ and the source of degradation was considered to be attributed to the 2 μm exit frit in a column.[6]

Tendencies in recent high performance SEC are to use column packings of lower particle diameter less than 5 μm and narrower I.D. columns of 6-mm I.D. instead of 8-mm I.D. Recent developments in polymer science require characterization of ultrahigh MW polymers by SEC. Under these circumstances, it is important to ascertain the shear degradation during elution through SEC columns of user's own.

The object of this work was to ascertain whether the degradation of PS standards having high MW was observed or not when the PS standards passed

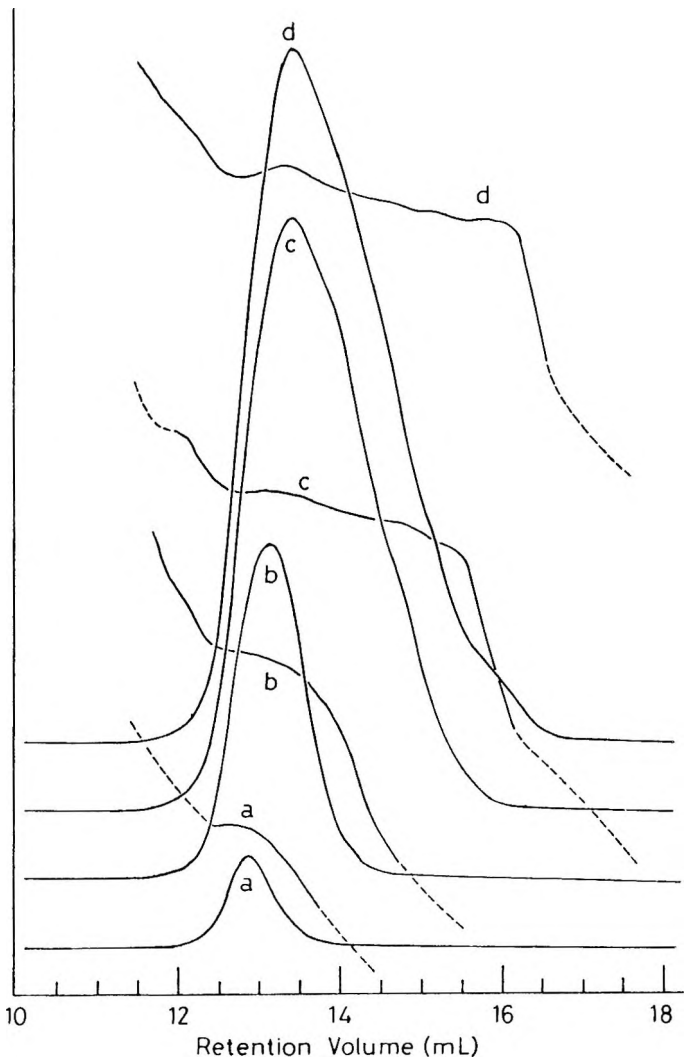

Figure 2. RI chromatograms for PS 4000K at different concentrations and the relationships between V_R and log MW (2). Concentration (%): same for Figure 1.

through commercially available SEC columns used in our laboratory and to study overloading and concentration effects by SEC/on-line light scattering photometry.

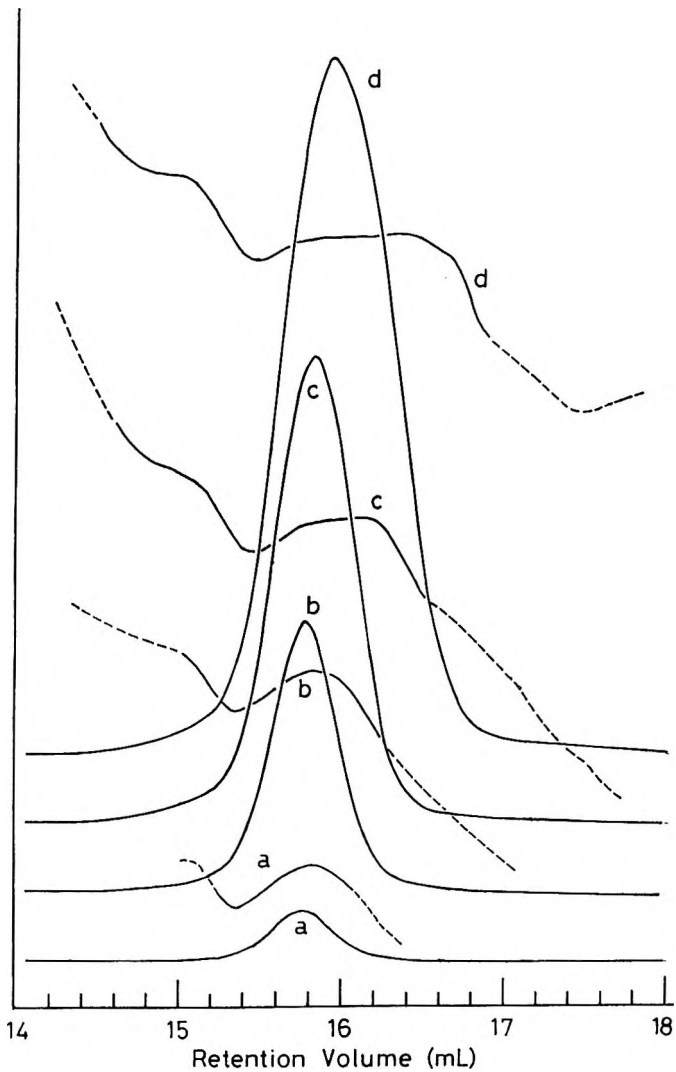

Figure 3. RI chromatograms for PS 200K at different concentrations and the relationships between V_R and log MW. Concentration (%): a 0.0203; b 0.101; c 0.203; d 0.406.

EXPERIMENTAL

Samples

Three PS standards of narrow MW distribution were used: PS 200K and PS 900K obtained from Pressure Chemical Co. (Pittsburgh, PA) and PS 4000K from Showa Denko Co. (Minato-ku, Tokyo, Japan). Vendor's values of MW averages determined by low-angle laser light scattering (LALLS) photometer were 2.16×10^5, 9.29×10^5, and 4.056×10^6, respectively. The PS standards were dissolved in tetrahydrofuran (THF) in the concentrations of 0.02, 0.1, 0.2 (or 0.3) and 0.4 (w/v)%. After being left for one day in a dark place, the sample solutions were filtered with a 0.5 μm membrane filter.

Size Exclusion Chromatography

SEC measurements were performed on a Shodex Size Exclusion Chromatograph Model System-II (Showa Denko) with a differential refractometer (RI) Model Shodex RI-71 and a multiple angle laser light scattering (MALLS) Model DAWN DSP-F (Wyatt Technology, Santa Barbara, CA). Columns were two Shodex KF806L (30 cm x 8 mm I.D.) which were linear columns packed with PS gel having the exclusion limit of PS MW over 1×10^7.

THF was used as the mobile phase. The flow rate was 1.0 mL/min and measurements were performed at room temperature (25-28°C). Injection volume of the sample solutions was 0.1 mL.

MALLS Parameters and Settings

Sixteen scattering angles ranging from 18.6° to 151.9° were used. ASTRA 3.0 software was used for data collection and further processing was done with the program EASI 6.0. Measurements were made at a wavelength of 632.8 nm. The specific refractive index increment for PS in THF (dn/dc, cm^3/g) was 0.185 measured with an interferometric refractometer Model Wyatt/Optilab 903. The second virial coefficients were 4.72×10^{-4} mL mol/g^2 for PS 200K, 3.33×10^{-4} for PS 900K , and 2.00×10^{-4} for PS 4000K, respectively. These values were taken from the literature.[7]

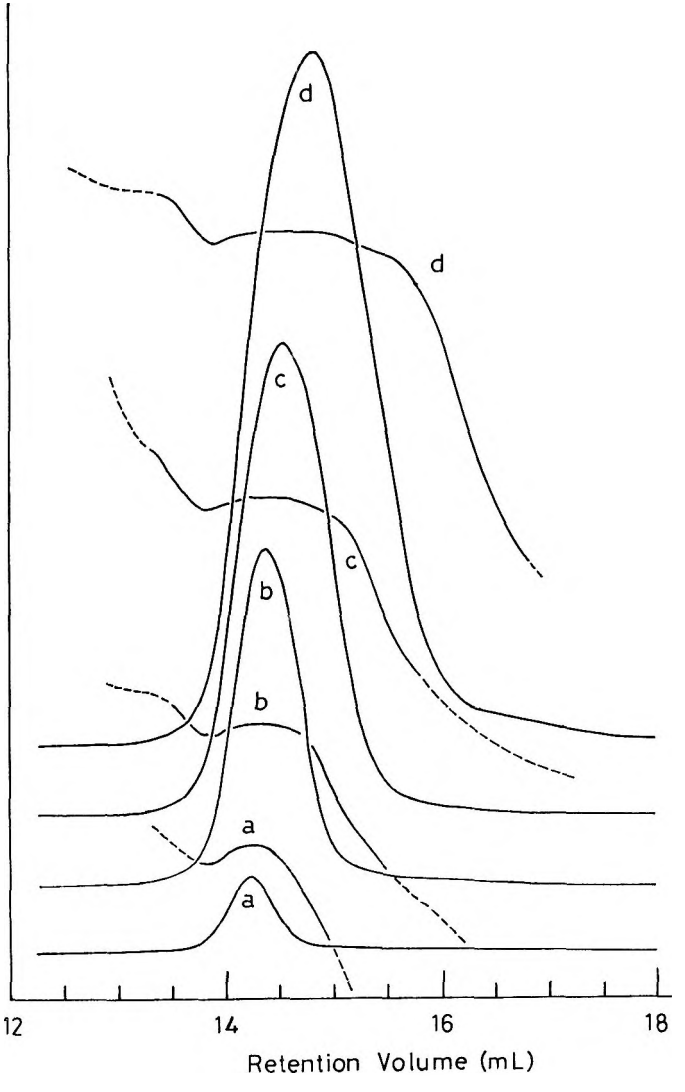

Figure 4. RI chromatograms for PS 900K at different concentrations and the relationships between V_R and log MW. Concentration (%): a 0.0201; b 0.107; c 0.201; d 0.403.

RESULTS

RI chromatograms and the relationships between retention volume (V_R) and log MW for PS 4000K at different concentrations were shown in Figure 1. Maximum MW near peak top was $4.09x10^6$ at 12.70 mL for Figure 1 (a), $4.09x10^6$ at 12.72 mL for (b), $4.22x10^6$ at 13.23 mL for (c), and $4.54x10^6$ at 13.3 mL for (d), respectively.

For the ease to see, the plots of the chromatograms and the relationship between log MW and V_R are shifted somewhat, and redisplayed in Figure 2. The abscissa is retention volume and the ordinate is the concentration of PS solutions for the RI chromatograms and log MW for the curves of the V_R - log MW relationship. Data points of log MW against V_R at the both ends of each peaks scattered as shown in Figure 1 and therefore, average points of these scattered data are drawn with a dotted line. Similarly, RI chromatograms and the relationships between retention volume (V_R) and log MW for PS 200K and PS 900K at different concentrations are shown in Figures 3 and 4, respectively, by shifting the plots of the chromatograms and the relationships.

Figures 5, 6 and 7 show RI chromatograms and the relationships between V_R and z-average root-mean square radius (RMS) for these three PS standards at different concentrations, respectively. Designation of the abscissa and the ordinate and the meaning of the dotted line are the same to those for Figures 1 to 3. The plots are also shifted somewhat for the ease to see.

Table 1 lists number- and weight-average MW, RMS in nm, and retention volume, MW, and RMS at peak top of the RI chromatograms for these three PS standards at different concentrations. As already stated, maximum MW for PS 4000K was not obtained at the peak top, but just before the peak top. However, for the case of PS 200K, maximum MW was obtained after the peak top: $2.42x10^5$ at 15.87 mL for Figure 3 (a); $2.37x10^5$ at 15.97 mL; $2.34x10^5$ at 16.13 mL; and $2.31x10^5$ at 16.38 mL. Maximum MW for PS 900K was nearly at the peak top in all concentrations tested.

DISCUSSION

Wang and Glasbrenner[6] reported the degradation of PS having MW of $4.25x10^6$ and $2.3x10^6$ after elution by SEC. MW observed after elution by on-line LALLS was 1.1 - $1.7x10^6$ and 0.95- $1.0x10^6$, respectively. The source of degradation was attributed to the 2 micron exit frit in the single Jordi mixed bed column.

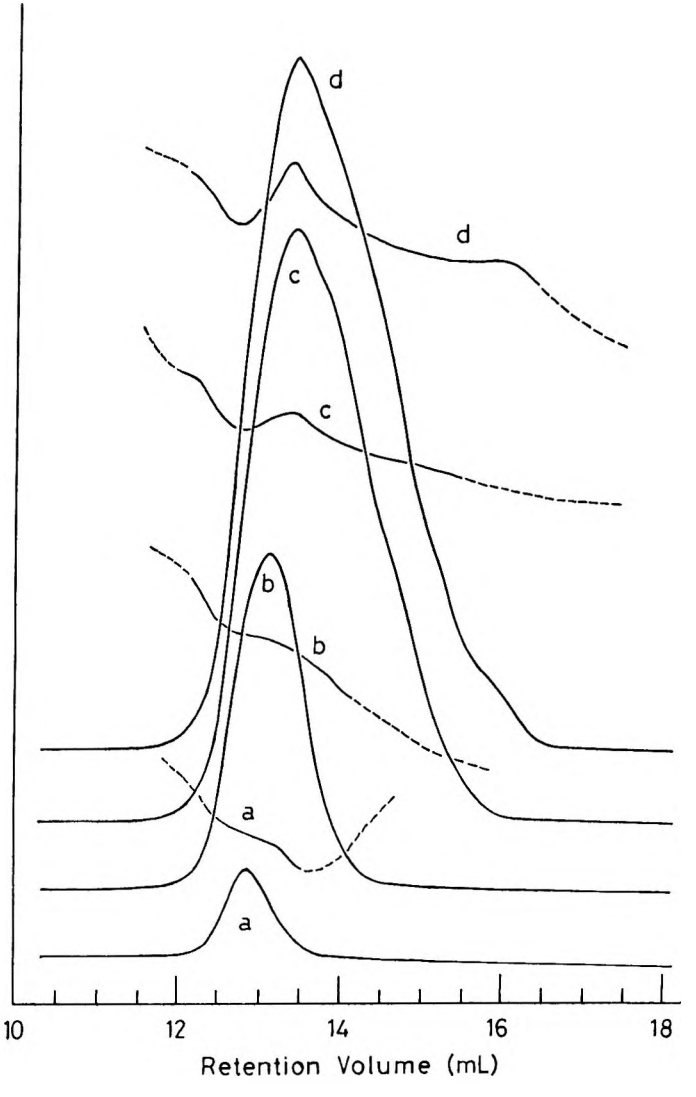

Figure 5. RI chromatograms for PS 4000K at different concentrations and the relationships between V_R and RMS. Concentration (%): a 0.0197; b 0.100; c 0.308: d 0.398.

Table 1

Molecular Weight, Root-Mean Square Radius and Retention Volume of PS Standards after Elution through SEC Columns

Standard	Conc. %	M_n x10^{-5}	M_w x10^{-5}	R_z nm	Vp mL	M_p xl 0^{-5}	R_p nm
200K	0.0203	2.17	2.20	16.7	15.75	2.28	16.2
	0.101	2.08	2.15	16.7	15.78	2.25	16.3
	0.203	2.11	2.19	17.4	15.85	2.26	17.0
	0.406	2.15	2.20	17.5	15.98	2.25	17.1
900K	0.0201	8.67	9.33	43.1	14.23	10.15	43.4
	0.107	9.16	9.57	44.4	14.38	10.15	44.5
	0.201	9.24	9.61	44.0	14.55	10.08	44.4
	0.403	7.97	9.50	44.7	14.83	10.30	46.0
4000K	0.0197	36.5	37.6	96.2	12.88	39.9	95.8
	0.100	35.7	37.4	96.8	13.15	39.0	97.0
	0.309	38.1	38.9	101.6	13.45	41.4	107.0
	0.398	39.1	39.9	107.1	13.45	44.7	123.0

M_n: number-average MW; M_w: weight-average MW; R_z: Z-average root-mean square radius; Vp retention volume at peak top of the RI chromatogram; M_p: MW at peak top of the RI chromatogram; R_p: RMS at peak top of the RI chromatogram.

However, as listed in Table 1, the degradation of PS after elution by SEC was not observed in our experiments. MW averages for PS 200K and PS 900k obtained after elution by SEC were comparable to those of vendor's values. In the case of PS 4000K, the former was somewhat lower than the latter, but not as much as observed by Wang and Glasbrenner.

The degradation of polymers accompanied by SEC measurements depends on packing materials in SEC columns as well as the exit frit in the column. Our experimental results were identical to those obtained by Ye and Shi.[4] As far as SEC measurements are performed using Shodex SEC column KF 806L packed with PS gel under the experimental conditions written in the **EXPERIMENTAL** section, the mechanical degradation of PS samples at least having MW less than $4x10^6$ is beyond the anxiety. Critical MW for other

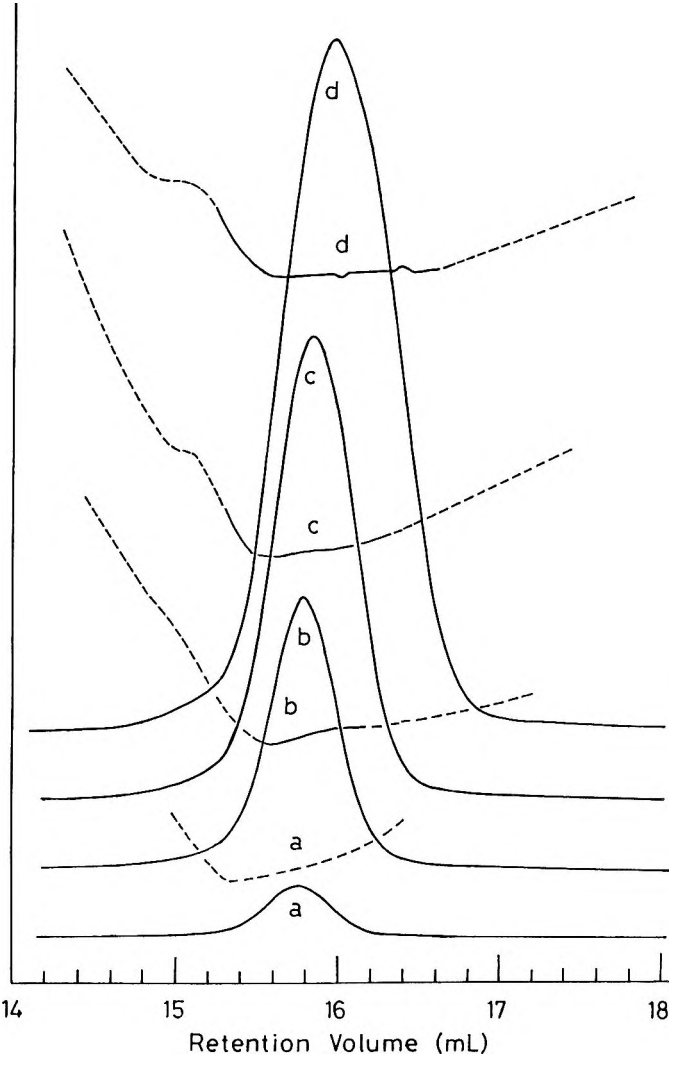

Figure 6. RI chromatograms for PS 200K at different concentrations and the relationships between V_R and RMS. Concentration (%): a 0.0203; b 0.101; c 0.203; d 0.406.

polymers must be determined experimentally.

When shear is applied, the most probable sites of stress concentration on the polymer chain are side chain linkages to main chain (branched points and

crosslinked points in network).[8] Therefore, branched polymers may degrade more easily than linear polymers.[9] The degradation of polyolefins having MW less than $1x10^6$ were reported[3,9,10] and the extent of the degradation was flow rate dependent. PS having MW $7x10^6$ was degraded using 8 μm-silica particles as a packing material[11] and therefore, silica particles are not adequate as high performance SEC packing materials.[1,4,12]

From RI chromatograms and the log MW - V_R relationships, it can be seen that polymer components composed of the RI chromatograms had approximately the same MW except both ends of the chromatograms. This phenomenon was independent on the polymer concentration in the sample solutions injected. The peak width of RI chromatograms became broader and also the range of the retention volume where polymer components having the same MW eluted was increased with increasing the sample concentration. The breakthrough volume was independent on the sample concentration. However, the peak retention volume V_P increased with increasing the sample concentration. The decrease in RMS or R_P with increasing the sample concentration was not observed as shown in Figures 5 - 7 and Table 1.

Theoretically, separation by SEC is based on hydrodynamic size and polymer molecules having the same molecular size (and MW for the same type of polymers) should have the same retention volume. Therefore, the increase in the range of the retention volume where polymer components having the same MW eluted can be attributed to the overloading effect in addition to the column broadening effects. The preferable sample concentration would be as low as possible.

The increase in the peak retention volume V_P with increasing the sample concentration has been considered to be attributed to the decrease in the hydrodynamic volume of the polymer in the mobile phase.[1] However, our experimental results could not approve the consideration and from the observation that the breakthrough volume was independent on the sample concentration, the increase in V_P with the increase in the sample concentration should be considered to be attributed to the overloading effect not to the decrease in the hydrodynamic volume. Delayed elution due to the overloading is also considered to be due to the macromolecular compression or viscous fingering effect.

Sample polymers used in this experiment were those having a narrow MW distribution and the elution of the polymers was not a monotonic decrease in MW as retention volume increased. Under the same experimental conditions, the PS NBS 706 which was broad in a MW distribution was

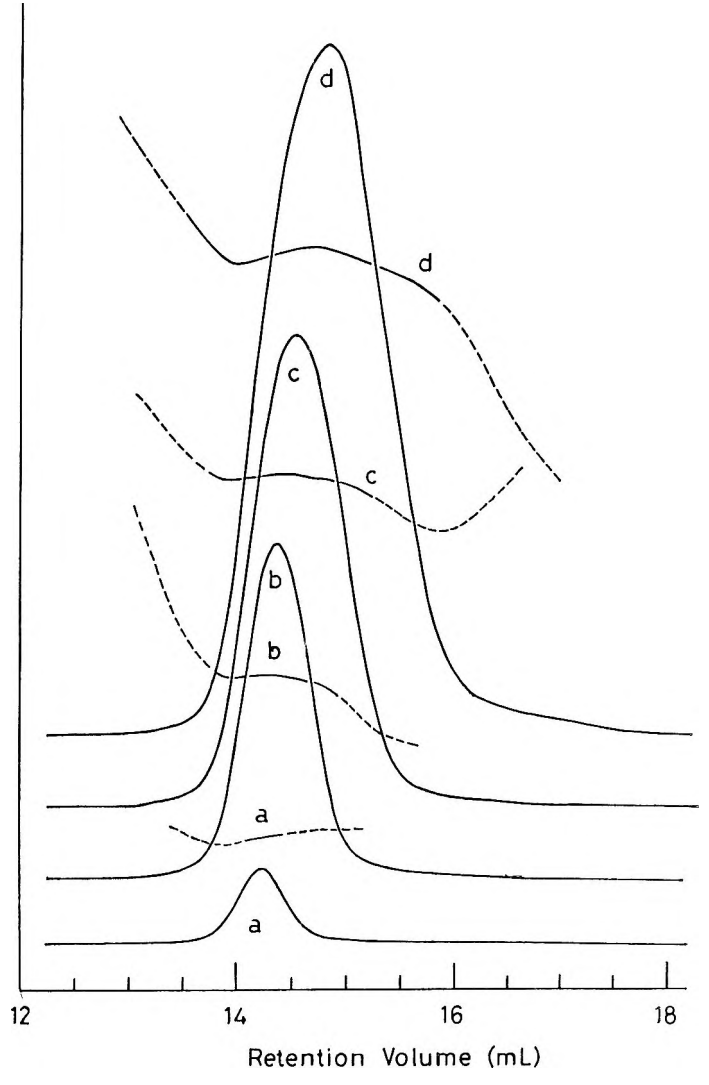

Figure 7. RI chromatograms for PS 900K at different concentrations and the relationships between V_R and RMS. Concentration (%): a 0.0201; b 0.107; c 0.201; d 0.403.

fractionated by the linear fractionation process, i.e., a monotonic decrease in MW as retention volume increased.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MICROASSAY FOR THE SIMULTANEOUS DETERMINATION OF FENTANYL AND ITS MAJOR METABOLITES IN BIOLOGICAL SAMPLES

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ABSTRACT

A simple high performance liquid chromatographic (HPLC) procedure for the simultaneous determination of fentanyl and its wo major metabolites in biological samples was used in blood samples from 6 preterm sick newborn infants. A 8 mm x 100 mm, 4 μm cyano column, 0.05 m phosphate buffer adjusted at pH 3.2 and acetonitrile (50:50 V/V) as mobile phase, were used at a flow rate 2.5 ml/min. Each run was completed within 10 minutes. The detection limits for the analysis were 0.10 ng, 0.15 ng and 0.10 ng for norfentanyl, fentanyl and despropiofentanyl, respectively, with 50 μl injection for all the compounds at a signal-to-noise ratio of 3. The respective retention times were 4.62±0.10, 6.20±0.76 and 6.71±0.73 minutes for norfentanyl,

fentanyl and despropiofentanyl. Microassay is simple, rapid and precise. This assay may be used for the therapeutic drug monitoring in newborn babies.

INTRODUCTION

Fentanyl, N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl] propionamide, a potent and fast-acting narcotic analgesic, is widely used in neonatal anesthesia and intensive care. It is now the primary analgesic for both pediatric and adult cardiac surgery because of its wide margin of safety.[1,2,3,4,5] Due to its widespread use, studies requiring measurement of plasma fentanyl and its metabolites in biological fluids are increasing[6,7] and included such measurement as radioimmunoassay (RIA),[8-11] radioassays,[12,13] gas liquid chromatography (GLC),[14-18] thin layer chromatography (TLC),[19] infrared spectroscopy (IR),[20] nuclear magnetic resonance (NMR)[21] and high performance liquid chromatography (HPLC) with ultraviolet detection.[22,23]

In humans, fentanyl is metabolized primarily by N-dealkylation to norfentanyl (1-(2-phenethyl)-4-N-anilinopiperidine) (metabolite I) and by hydroxylation to despropiofentanyl (4-N-(N-propionyl-anilino)-piperidine) (metabolite II) respectively[25,26] as has been shown in rats.[24,25,26] Van Rooy et al.[16] were the first investigators to have worked on fentanyl and its two major metabolites, norfentanyl and despropiofentanyl, using GC-NPD in plasma of patients. Goromaru et al.[27] identified and quantitated fentanyl and its two major metabolites in human patients by GC-MS.

McClain and Hug[28] used paper chromatography to detect fentanyl and two metabolites in urine samples. Kintz et al.[17] reported simultaneous determination of fentanyl and its major metabolites using gas chromatography (GC) with nitrogen-selective detection.

Because of the high potency and low dosage of these compounds and small sample sizes available, extremely low (subnanogram) concentrations of drugs and their metabolites are present in biological specimens. There is a need to develop more and more sensitive, highly specific, analytical methods for drug monitoring, and its metabolites, in biological samples at therapeutic concentrations.

The aim of the present communication is to describe a suitable, highly specific and sensitive analytical method for determination of fentanyl and its

metabolites in body fluids for use in both clinical and research laboratories.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals used were of analytical grade. Sodium dihydrogen orthophosphate, sodium phosphate monobasic, orthophosphoric acid, sodium hydroxide, ammonium hydroxide, buffer solution pH 7.0, buffer pH 4.0, n-Hexane, acetonitrile (HPLC grade) were obtained from B.D.H. (Pooled, U.K.). Fentanyl was purchased from Sigma Chemical Company (U.S.A.). Norfentanyl (R U156) batch no. A0801 and despropiofentanyl (R 34853) batch no. V90799 were obtained from Janssen Pharmaceutical Company. Only HPLC-grade de-ionized water was used.

Equipment

HPLC instrumentation included Water 510 HPLC pump, 715 Ultrawisp autosampler, variable wavelength 994 UV/VIS detector and 820 inegrator plotter. A Waters 8 mm x 100 mm, 4 μm cyano column (Millipore Corporation, Milford, MA) was used. Additional equipment included a pH-M-82 standard pH meter, IEC Centra-8R centrifuge, and concentrator-Jouan RC - 1010.

Plasma and Urine Samples

Drug-free venous blood and urine were collected from six healthy adult human volunteers receiving no medications. In addition, arterial blood samples (0.2 ml) taken from umbilical artery catheters were obtained from six newborn infants receiving continuous intravenous infusion of fentanyl at 3.0 micrograms/kg/hour. Blood was collected into plastic tubes containing lithium heparin and centrifuged for 10 minutes at 3000 rpm. Plasma was separated and stored at -80°C until the time of analysis. Random urine samples were also collected in plastic bags and later stored at -80°C for the analysis of fentanyl and its metabolites.

Mobile Phase

The mobile phase consisted of a mixture of 50% acetonitrile and 50% phosphate buffer 0.05 M (pH 3.2) filtered through a 0.22 mm filter (Millipore) and degassed under suction.

Standard Solutions

Stock solutions of norfentanyl, fentanyl, and despropiofentanyl (50 mg/ml), respectively, were prepared by dissolving appropriate amounts of drug salts and metabolites. All stock solutions were prepared and stored at room temperature and protected from prolonged exposure to light. These were used as a stock solutions for the preparation of assay standards by serial dilution.

Chromatographic Conditions

The analysis of norfentanyl, fentanyl and despropiofentanyl was performed at room temperature (25°C) with the wavelength at 210 nm. The mobile phase was a mixture of acetonitrile and phosphate buffer (50:50 V/V) with a flow rate of 2.5 ml/min and a pressure of 1000 psi. The run time was less than 10 minutes.

Sample Preparations and Extraction Procedures

Extraction was performed in a silanized tube. Spiked plasma standards or sample plasma containing norfentanyl, fentanyl or despropiofentanyl (100 μl) in a silanized tube were added 50 μl, 5N NaOH, 100 μl acetonitrile and 600 μl of n-hexane. All samples were centrifuged at 2000 rpm for 5 minutes. The organic phase was transferred to a glass tube and the solvent was evaporated under nitrogen at 30°C for about 10 minutes. The residue was reconstituted in 100 μl of the acetonitrile and phosphate buffer (50:50 V/V) at pH 3.0 and 50 μl injected into a column.

Calculations

Standard curves and concentrations of norfentanyl, fentanyl and despropiofentanyl were calculated from peak heights. Calibration curves were constructed after the addition of known concentrations of norfentanyl, fentanyl

and despropiofentanyl to plasma and urine samples by linear regression analysis of peak height versus concentrations.

Recovery, Precision, Accuracy and Reproducibility

The precision and accuracy of the overall analytical procedure for the determination of norfentanyl, fentanyl and despropiofentanyl in plasma were assessed by processing spiked plasma samples (Table 1). The day-to-day precision and accuracy of the method were assessed by the repeated analyses of standard solutions of norfentanyl, fentanyl and despropiofentanyl over 5 days. Daily representative peak height for the three concentration levels tested was the mean value of five replicate injections. The inter- and intra-assay coefficient of variations of the method was < 10%.

Extraction recovery of the parent drug and its metabolites was estimated by comparison of peak height obtained from an extracted sample, containing a known amount of the compound. The peak height was obtained from direct

Table 1

Reproducibility, Precision and Accuracy of the Method

Drug	Amount Injected (ng/ml)	Drug Conc'n Measured (ng/mL)*	Confidence Interval 95%	Std Mean of Error**	Coeff of Variation (CV)%
Norfentanyl	10.0	8.66 ± 0.59	8.69 - 8.60	0.26	6.81
	25.0	25.44 ± 1.27	25.48 - 25.30	0.29	5.00
	50.0	49.40 ± 2.90	49.60 - 49.20	1.02	5.86
	100.0	100.40 ± 2.20	100.50 - 100.10	0.84	2.22
Fentanyl	10.0	9.70 ± 0.65	9.73 - 9.77	0.33	6.62
	25.0	25.90 ± 0.78	25.85 - 25.94	0.30	2.99
	50.0	48.40 ± 2.41	48.30 - 48.50	1.00	4.98
	100.0	100.30 ± 0.96	100.30 - 100.40	0.40	0.95
Despropio-	10.0	9.91 ± 0.86	9.97 - 0.85	0.29	8.68
fentanyl	25.0	25.00 ± 0.925	25.10 - 25.68	0.41	3.69
	50.0	48.50 ± 2.0	48.67 - 48.40	1.35	6.24
	100.0	99.80 ± 1.50	99.68 - 99.83	0.61	1.50

*Values are expressed as mean ± SD (n = 6 determinations). Volume injected was 50μL.

**Standard Mean of Error refers to the mean difference between the concentration injected and the concentration measured.

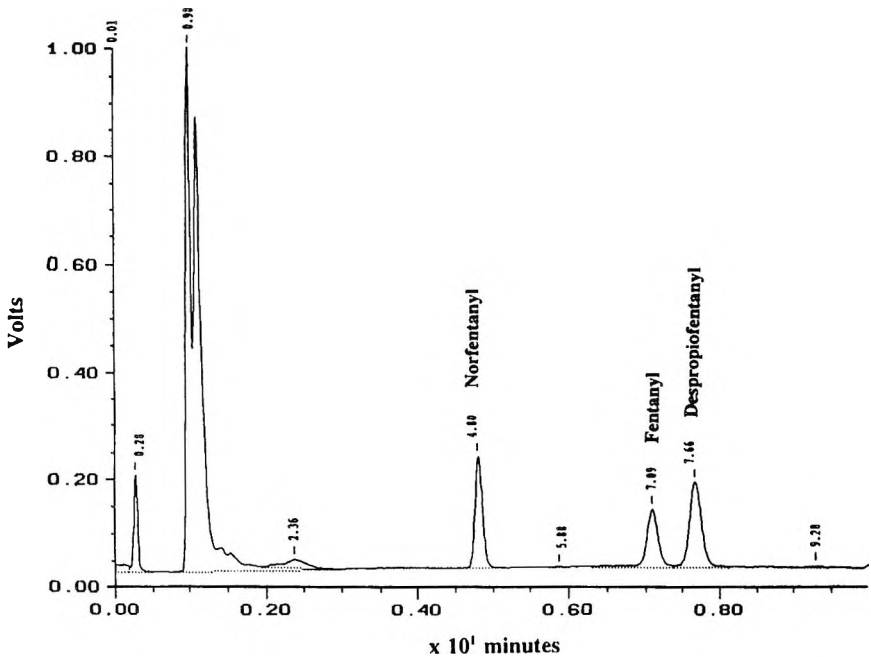

Figure 1. Chromatogram of Norfentanyl, Fentanyl and Despropiofentanyl from unextracted samples .

injection of a standard solution with different concentrations of the sample.

Statistical Analysis

Standard curves and concentrations of norfentanyl, fentanyl and despropiofentanyl were calculated from peak heights. Calibration curves, constructed after the addition of known concentrations of 1.0 to 200 ng/ml of norfentanyl, fentanyl and despropiofentanyl, were linear as analyzed by regression analysis of peak height versus concentrations. The correlation coefficients were 0.998, 0.994 and 0.997, for norfentanyl, fentanyl and despropiofentanyl, respectively.

RESULTS AND DISCUSSION

Using the chromatographic conditions for this method, the mixture of

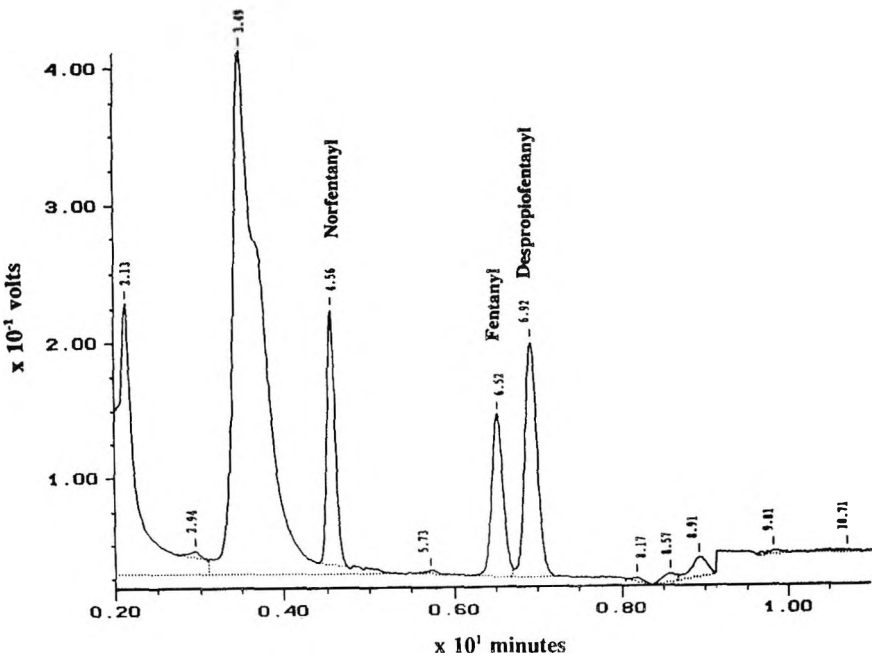

Figure 2. Chromatogram of blood sample spiked with Norfentanyl, Fentanyl and Despropiofentanyl from extracted samples

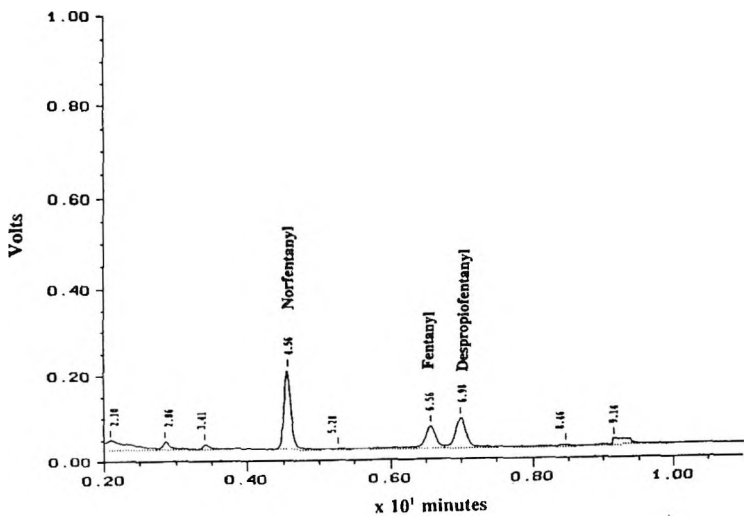

Figure 3. Chromatogram of urine sample spiked with Norfentanyl, Fentanyl and Despropiofentanyl from extracted samples

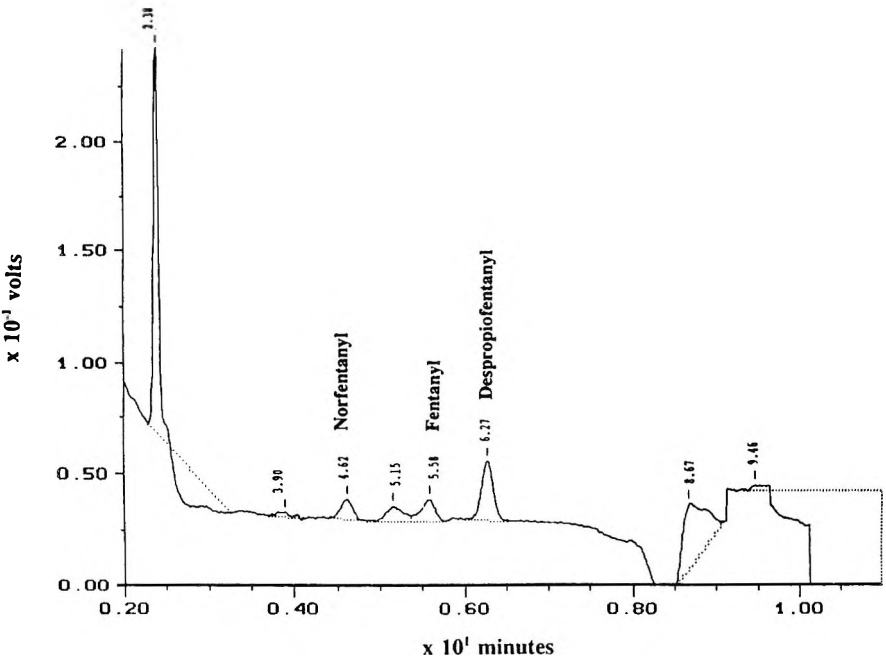

Figure 4. Chromatogram of Norfentanyl, Fentanyl and Despropiofentanyl from a newborn baby's blood sample, receiving a constant I.V. infusion of Fentanyl 3.0 microgram/kg/hour.

fentanyl and its major metabolites was separated in acetonitrile-phosphate buffer. The use of acetonitrile-phosphate buffer, pH 3.0, led to elution of all compounds. The application of acidic pH suppressed the ionization of acidic silanol groups, allowing the elution of drugs.

The chromatographic peaks of I, II, and III (norfentanyl, fentanyl and despropiofentanyl) were sharp and their retention times were 4.62±0.10, 6.20±0.76, and 6.71±0.73 minutes, respectively. The absolute peak heights of norfentanyl, fentanyl and despropiofentanyl were plotted against the concentration between 1.0 and 200 ng/ml of I (norfentanyl); 2.5 and 200 ng/ml of II (fentanyl); and 2.0 and 200 ng/ml of III (despropiofentanyl) respectively. The relationship was linear and passed through the origin. In the concentration range studied, the regression line was linear ($r=0.998$) with an intercept on the y-axis close to the origin. Results of the analysis of spiked serum for the determination of precision and accuracy of the method are given in Table 1.

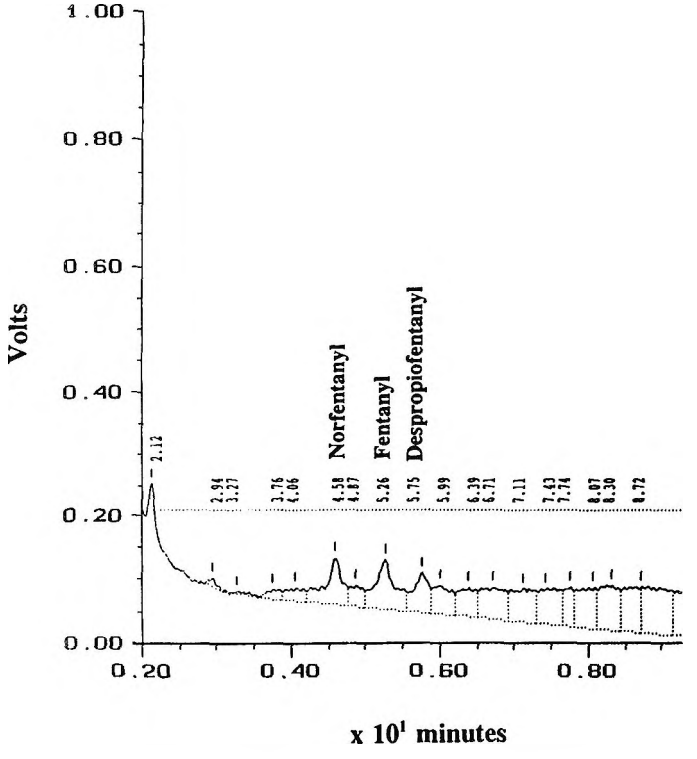

Figure 5. Chromatogram of Norfentanyl, Fentanyl and Despropiofentanyl from a newborn baby's urine sample, receiving a constant I.V. infusion of Fentanyl 3.0 microgram/kg/hour.

Using the described conditions, the analysis was completed within 10 minutes with complete separation of these three components. Figure 1 shows the chromatogram from the standard solutions. Figures 2 and 3 show the chromatograms from extracted blood and urine samples spiked with fentanyl and metabolites. Figures 4 and 5 show the chromatograms from the extracted blood and urine samples of a newborn baby. The resolution factor, between adjacent peaks, was calculated and found to be 4.28 between norfentanyl and fentanyl and 1.08 between fentanyl and despropiofentanyl. The extraction recoveries of all the compounds determined at all concentrations were 82.0±4.0, 83.3±3.84, and 79.5±3.20 % for norfentanyl, fentanyl and despropiofentanyl respectively for plasma and urine samples. There was no change in the peak height of the drug fentanyl and its two metabolites after

extraction by the described procedure. The inter- and intra-assay coefficent of variations of the method was < 10%. The precision of the method was evaluated in a blind study in the concentration range 10-100 ng/ml for fentanyl, norfentanyl and despropiofentanyl, respectively.

The specificity for this assay was tested by using those medications usually given to newborn infants that might be present in patient samples. Furosemide, morphine, calcium chloride, dobutamine, dopamine, midazolam, ampicillin, gentmicin, clafoxan, diazepam, phenytoin, pavulon, gentamicin, and vitamin K did not interfere with the measurement of norfentanyl, fentanyl and despropiofentanyl.

The method described is suitable for the simultaneous determination of norfentanyl, fentanyl and despropiofentanyl in a very small volume of plasma samples (50 ml). We have used the proposed technique for the therapeutic drug monitoring of fentanyl in small sick newborn babies receiving constant I.V. infusion of fetanyl 3.0 microgram/kg/h. Their plasma norfentanyl, fentanyl and despropiofentanyl concentrations at these doses were 2.8±1.2, 3.04±0.44, and 3.1±1.3 ng/ml, respectively. The proposed method is highly sensitive, precise and selective for fentanyl, norfentanyl and despropiofentanyl and is also rapid and simple to perform.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES OF SULPHONAMIDES AND DIHYDROFOLATE REDUCTASE INHIBITORS. I. SEPARATIONS IN METHANOL-MODIFIED SOLUTIONS

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ABSTRACT

Twenty-two sulphonamides and the three commonly used dihydrofolate reductase inhibitors have been subjected to an investigation of retention behaviour on a silica based reversed phase. Effects of variation in the percentage of methanol and the pH have been determined isocratically and the methanol gradients developed have been modified by variations in the concentration of the phosphate buffer. Significant variations in retention behaviour were observed such that the majority of combinations of drugs could be screened for. No set of conditions studied gave rise to a total separation of all drugs, but around twenty drugs were commonly resolved.

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INTRODUCTION

Assays for individual sulphonamides (SFA), often in combination with metabolite(s) or a dihydrofolate reductase inhibitor (DHFR) potentiator have been published extensively over the years. The early work in this area was largely concerned with theraputic studies and has previously been reviewed.[1] Since that time, the SFA have become the subject of residue analyses due to the prophylactic use of SFA in veterinary medicine which has led to traces of these drugs in milk, eggs, fish and meat. These studies have also been reviewed,[2,3] but many more recent publications have appeared.[4-22]

On the other hand, there have not been any investigations of instrumental methods for the broad screening for SFA and DHFR published since our capillary zone electrophoretic (CZE) work in 1993.[2] In this study it became apparent that conventional CZE (with the detector at the cathode) could not be successfully used to concurrently analyse for a wide range of SFA - or any other negatively charged compounds - if the span of pK_a values is too great. At low pH, some compounds are unionised and inseparable from each other and the neutral marker, whilst others overcome the electroosmotic flow (EOF) and migrate in the wrong direction. At high pH where all compounds may be ionised the EOF is far too great and the separation space is too small for multi-component mixtures.[23] Control of the EOF, perhaps including its reversal, or the use of micellar electrokinetic chromatography is required.

In the one recent publication concerning the separation of SFA,[22] micellar liquid chromatography was successfully employed, albiet only for twelve of the drugs. This paper reports the first part of a systematic examination of the HPLC separation and analysis of twenty-two SFA and the three commmonly occurring DHFR.

EXPERIMENTAL

Chemicals and Materials

The twenty-two SFA used in this study are sulphanilic acid (SNAC), sulphaguanidine (SG), sulphabenzamide (SB), sulphisoxazole (SISX), sulphacetamide (SAC), sulphamethizole (SMIZ), sulphachloropyridazine (SCP), sulphaquinoxaline (SQ), sulphamethoxazole (SMOX), sulphadimethoxine (SDIM), sulphadiazine (SDZ), sulphamethoxypyridazine (SMP), sulphameter (SM), sulphamerazine (SMRZ), sulphathiazole (ST),

sulphamethazine (SMAZ), sulphapyridine (SP), sulphanilamide (SAN), sulphamoxole (SAM), sulphisomidine (SISM), phthalyl sulphathiazole (PST) and succinyl sulphathiazole (SST). The three DHFR were diaveridine (DVD), pyrimethamine (PYR) and trimethoprim (TMP). The SFA and DHFR standards were all procured from Sigma (St. Louis, MO, U.S.A.). Their individual structures have previously been given.[2] Standard stock solutions for each drug were prepared by dissolving exactly 50 - 250 mg of the pure standard into 100 mL HPLC grade methanol. Combined standards were evaporated by N_2 purging, diluted with milli-q-water to give a final concentration of 25 ng/μL for each component and filtered with a 0.45 μm filter prior to injection. The resultant MeOH/H_2O ratio of the sample solution was 4:96.

Phosphate buffers were prepared using Na_2HPO_4 and KH_2PO_4 and adjusted to the desired pH with 20% KOH or 20% H_3PO_4. All chemicals were of AR grade and milli-q-water was used to prepare all solutions. Buffers were degassed by vacuum filtration.

Equipment

The HPLC system was a Varian (Walnut Creek, CA) Vista 5560 Series adapted for packed capillary operation . The pump had a flow rate range of 0.01 to 15 mL/min and was not modified. A tee piece was introduced downstream to split the flow. The bulk of the mobile phase was diverted through the parallel conventional column, leaving several μL/min flow to the packed capillary column. Both columns were housed in the oven of a cut-down Perkin-Elmer (Norwalk, CT) Model LC 65-T thermostatted detector. An air actuated automatic Valco (Houston, TX) 1/16" Model C14M injector with 1 μL sample loop was used at 80 psi nitrogen for all experiments.

The Varian UV-200 UV/Vis detector was modified by the replacement of the standard flow cell of 4.5 μL (4 mm path-length) with a 0.5 μL (0.5 mm path-length) flow cell. All absorbances were measured at 270 nm.

Dispersion in the transfer lines was minimised by mounting the injector at the foot of the oven door and using 10 cm of 0.13 mm (0.005") i.d. stainless steel tubing to the column head. Five cm of 0.005" i.d. tubing connected the column exit to the detector which was mounted directly above the column oven. Chromatographic data was collected, integrated and plotted using a Varian CDS 401/2 Vista Series system.

The Varian-packed, protein C_{18} columns were 30 cm stainless steel, 0.35 mm i.d. and 0.48 mm o.d. The stationary phase was the Separations Group Vydac IDI-TP 5 μm ± 1 μm (75%) silica with surface area 80 m^2/g, pore volume 0.63 cm^3/g and average diameter of 330 Å. The C_{18} bonded phase was TMS capped with a total carbon loading of 6-7%.

Experimental Methods

Flow rates through the packed capillary column were measured by collecting timed volumes of eluate into a 5 mL measuring cylinder via a piece of plastic tubing connected to the outlet of the restrictor column.

Peak identifications were established by adopting a time-saving peak tracking method based upon the minimum number of injections required to elucidate the maximum number of components.[24] In this case a minimum of five samples of varying compositions of the SFA and DHFR was required for full identification.

Spectroscopic grade KBr dissolved in Milli-q-water was injected to determine the hold-up time (t_0 = 2.83 minutes) and was used as a mobile phase to determine the delay time ($t_d = t_{SOLVENT\ FRONT} - t_0$ = 6.57 minutes). The mobile phase was delivered from the pump at the standard flow rate of 1 mL/min for all the packed capillary column analyses and the detector was set at 270 nm. Under the range of gradient conditions used, equilibration times of 20 minutes were found to be adequate.

Unless otherwise specified, the column oven was set to 30°C and the flow rate at the pump was 1.0 mL/min. This yielded a flow rate of approximately 6 μL/min through the packed capillary column.

RESULTS AND DISCUSSION

In an earlier study,[1] two reversed phases were used. For the primary stationary phase utilised, it was found that significant losses occurred for many of the twenty-five drugs. In the case of the SFA, losses varied from negligible with the early eluting, hydroplilic drugs through to 73% with the most hydrophobic (SQ). Each of the DHFR was totally lost. However, in the same study[1], an alternative stationary phase was also examined. This (10 μm diameter silica) stationary phase gave rise to relative peak areas consistent with the concentrations utilised and the measured molar absorbtivities for all drugs,

indicating that there were not any losses of either class of compound. For this investigation, the identically derivatised, 5 μm diameter silica from the same source was chosen as the stationary phase.

Methanol (MEOH) was chosen as the first organic modifier to test. It appeared to have been less extensively investigated previously and the weaker reversed-phase solvent (compared to acetonitrile) permitted easier experimental fine tuning of the net solvent strength. The initial pH chosen for the mobile phase was based upon previous studies[25,1] in which values of 2.5-3 were found to yield the best available separations (in conjunction with acetonitrile as the organic modifier and acetate or phosphate buffers, respectively). As phosphate buffers yielded the then best separations of sulphonamides obtained,[1] they were again employed in this study.

Isocratic Analyses

(a) The effect of % methanol

In this section, 0.1 M KH_2PO_4 was adjusted to pH 2.75 and trial chromatograms obtained at 10, 15, 20 and 25% MEOH. From the 25 compounds injected on each occasion, the respective results may be summarised in terms of the respective numbers of discernible peaks (or parts thereof) and the total analysis times: 21 peaks in 270 minutes, 19 peaks in 145 mins., 17 in 70, and, 13 in 40. As is usual with isocratic analyses, there is the trade-off between an acceptable number of separations and an acceptable time of analysis. With a view to later gradient procedures significantly modifying the long analysis times further, additional runs were performed in the intermediate range (15-20% MEOH) where large reductions in analysis time were offset by only modest losses in the numbers of peaks. Figure 1 shows the results of tracking all of the individual drugs at all of the percentages of MEOH examined.

However, it must be kept in mind that the log k' plots are easily over interpreted. For example, in Figure 1, at 15% MEOH with log k' 0.2-0.3, SDZ (open square) and SISM (closed square) appear to be just separated. They are not. Finite peak widths ensure that individual compounds with distinguishable retention times in separate chromatograms may not even appear as 'shoulders' in the mixture. Indeed peak widths for the sulphonamides are a serious impediment to separation. Using a method adapted from the literature,[26] the estimated[27] theoretical plates for the column used in this study was 8550. On the other hand, the realisable plates in the crowded region of the chromatogram (16% MEOH) varies from only 1700 for SAC at log k' = 0.1 to 3000 for SMP at

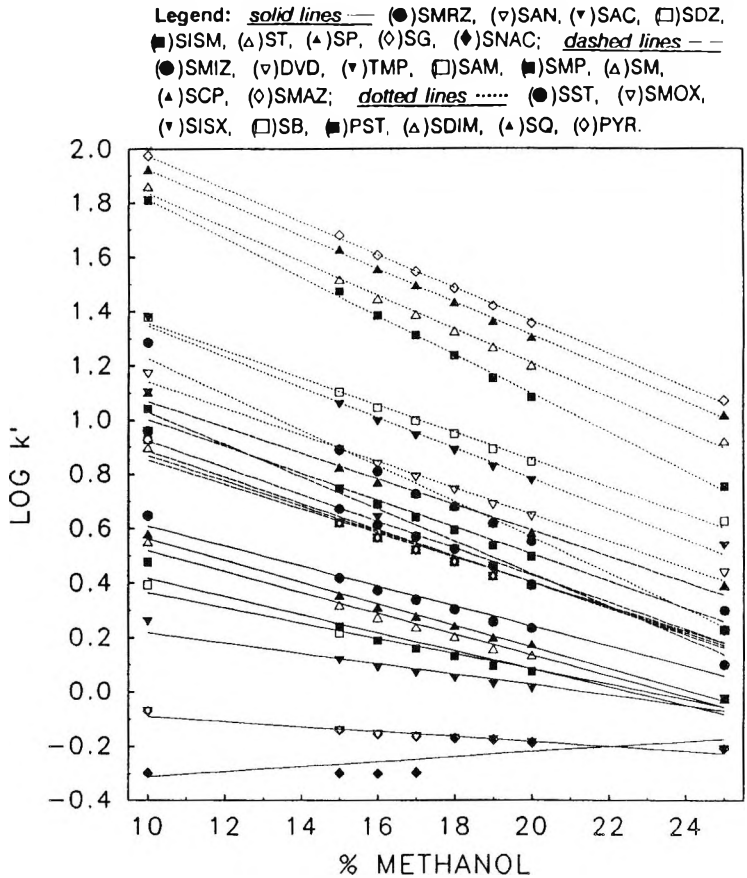

Figure 1. Plot of log k' vs % methanol for the 22 SFA and 3 DHFR compounds using a 0.1M phosphate buffer at pH 2.75.

log k' = 0.7. In Figure 1, at 10% MEOH (with log k' 0.4-0.5) SDZ and SISM appear to be well separated; and in the main, they are. But near the baseline they are not. This is due to the non ideal peak shapes typical of these compounds with highly polar functional groups. Further exaccerbation of the misinterpretation of log k' plots can also arise due to the imperfect lines of best fit drawn by the software.

Nonetheless, the value of the plots is clear in providing an overview. Given the convergence of the log k' plots, one crossover at about 15% MEOH and several others above 17%, 16% MEOH appeared to be the optimum

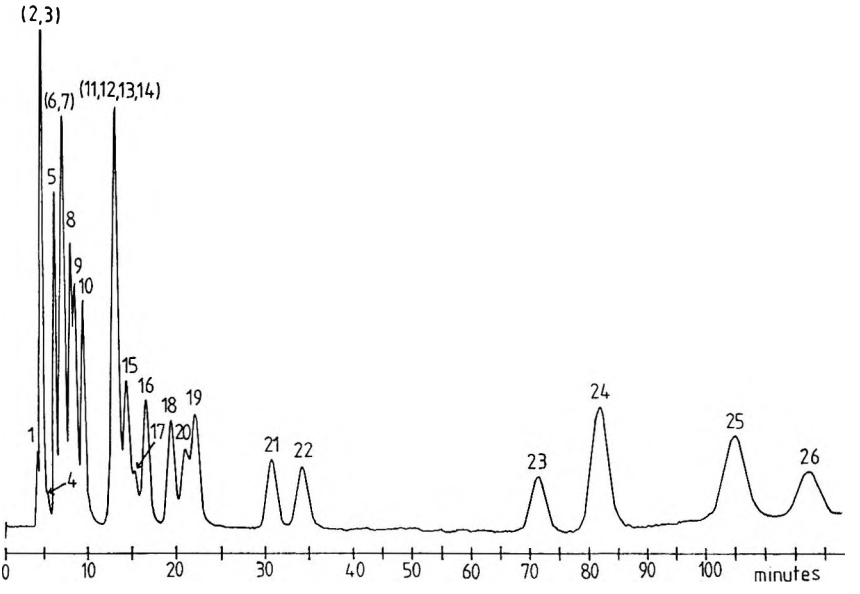

Figure 2. Isocratic 16% methanol/84% 0.1M phosphate buffer (pH 2.75) chromatogram. The compounds are: **(1)**SNAC, **(2)**SG, **(3)**SAN, **(4)**SAM hydrolysis product, **(5)**SAC, **(6)**SDZ, **(7)**SISM, **(8)**ST, **(9)**SP, **(10)**SMRZ, **(11)**SM, **(12)**SAM, **(13)**SMAZ, **(14)**DVD, **(15)**SMIZ, **(16)**SMP, **(17)**TMP, **(18)**SCP, **(19)**SMOX, **(20)**SST, **(21)**SISX, **(22)**SB, **(23)**PST, **(24)**SDIM, **(25)**SQ, **(26)**PYR.

composition for further studies (especially remembering that the 10% chromatogram took 270 minutes). Figure 2 shows the chromatogram at 16% MEOH.

(b) The effect of pH

As stated above, the initially chosen pH of the mobile phase (2.5-3) was based upon previous studies. However, this is not what would logically be expected on the basis of the known pK_a data. (For structures and pK_a values see reference 2.) Firstly, several of the $pK_{a,1}$ values are in the vicinity of 2.5 so that differential diffusion/migration of the acid-base conjugates would be expected to give rise to band-broadening and loss of resolution. This was found particularly to be the case for SP;[1] $pK_{a,1}$ = 2.6. Secondly, $pK_{a,1}$ values range from 1.5-2.6 and generally[23] refer to the deprotonation of the anilinium group, and, $pK_{a,2}$ values span 4.6-10.4 with the majority between 5.4 and 7.4. With the exception of SA, these $pK_{a,2}$ values refer to the ionisation of the

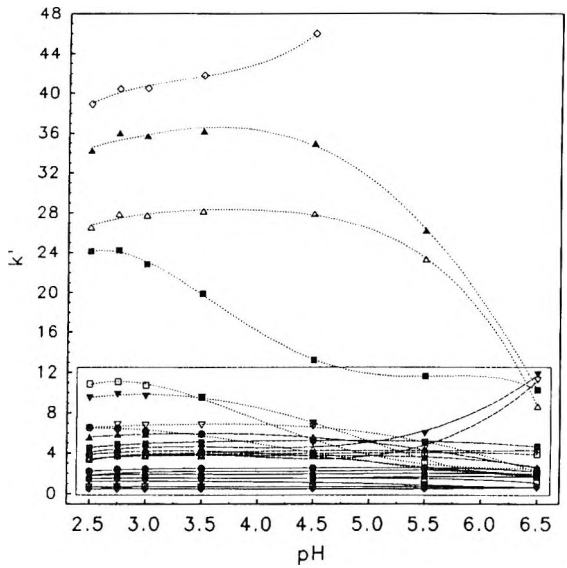

Figure 3(a). Plot of k' vs pH for the 22 SFA and 3 DHFR using isocratic methanol/0.1M phosphate buffer (16:84) conditions. Legend as in Figure 1.

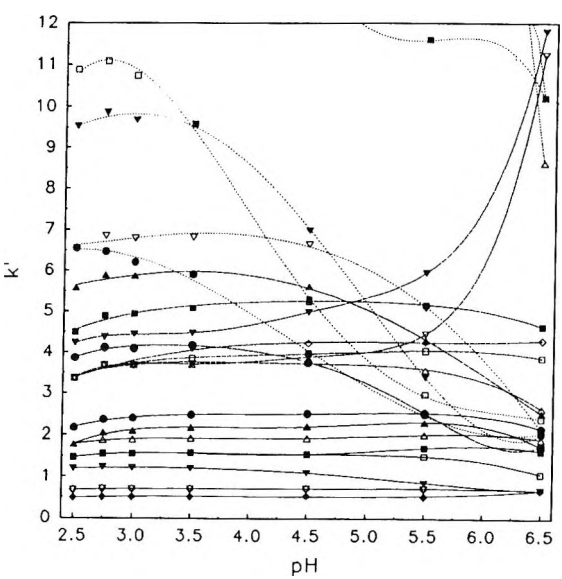

Figure 3(b). Plot of k' vs pH. Enlargement of boxed area in figure 3(a) for the antibiotics in the k' region between 0.0 and 12.0. Legend as in Figure 1.

sulphonamide nitrogen. Thus, for these moderately polar drugs, maximum retention and separation would be expected in the vicinity of pH 4. Hence, there is good reason to examine the pH dependence of the separation.

All of the runs in this section were carried out in 16% MEOH and 84% aqueous 0.1 M phosphate buffer blended from two reservoirs at pH 2.5 and 6.5. The required proportions were calculated and then verified at the purge outlet. Figure 3(a) shows the plots of k' versus pH for all of the drugs and Figure 3(b) is an enlargement of the crowded, boxed area at the bottom of Figure 3(a). We have chosen to display the data in this way (rather than as the more evenly spaced log k' plot) in order to clearly show the shapes of the variations. (The data is also slightly less crowded.) In all cases where pK_a data is known, the shapes of the retention plots are in accord with expectation.

Figure 3(b) contains some very encouraging information. Two of the extremely difficult groups of peaks at low pH are separated at higher pH. At k' 1.4 SDZ and SISM separate above pH 5 and the coincident quartet of compounds at pH 2.5 and k' 3.3 (DVD, SAM, SM and SMAZ) move apart as pH increases. Contrary to these gains are the excessive number of crossovers among the retention plots, the number of which increases dramatically with increasing pH. Above pH 4.5, the situation is chaotic. It is unfortunate that several of the moderately retained SFA at pH 2.5 (SB, k'=11; SISX, k'=10; SST, k'=6.5; SMIZ, k'=3.9) have low $pK_{a,2}$ values (4.6, 5.1, 4.16($pK_{a,1}$ but ionising) and 5.4, respectively) so that large losses in retention with increasing pH cause many of the crossovers. DVD and TMP (k' 3.3 and 4.2 at pH 2.5) add to the problem by deprotonating and increasing retention against the trend. Scanning the pH range in Figure 3(b) does reveal some pH windows that appear promising. PH 5.20 is one of these. However, closer inspection of the data is not encouraging.

Firstly, the pH window is very narrow. There are multiple crossovers within $\pm$0.2 pH units. Secondly, again it is important to keep peak widths in mind to avoid over interpretation of the data. Thirdly, some irreproducibility of the experimental data and some inexactness in the graphical lines of best fit must be taken into account. Finally, when it is realised that the difficult to separate pairs at k' 1.5 and 0.7 are still fused, pH 5.2 is not at all attractive. The most logical pH (4) is similarly difficult, although fewer crossovers of retention plots are in that vicinity. The original pH region appeared as good as any. Further runs and close inspection of the chromatograms indicated a marginal preference for pH 2.8.

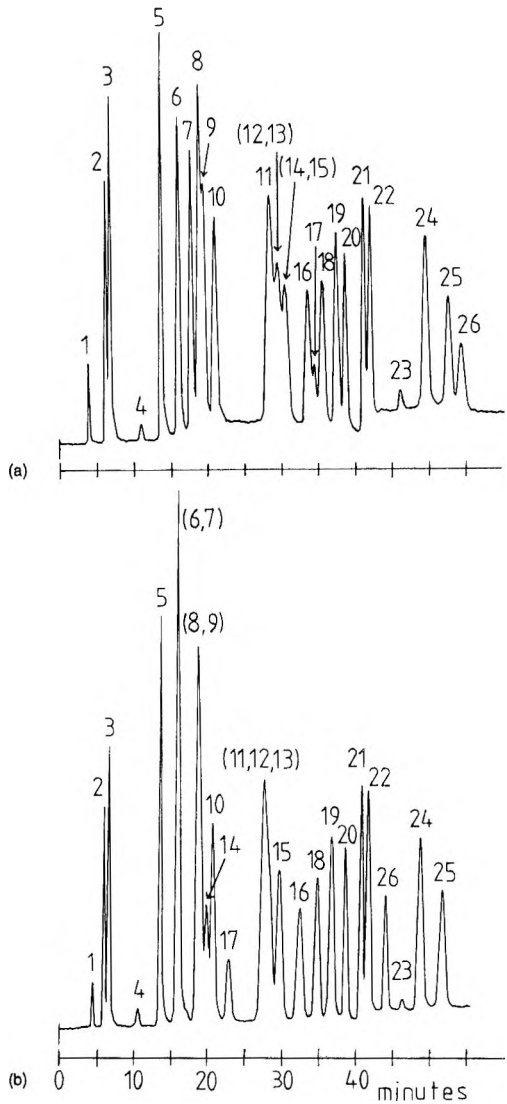

Figure 4. Chromatograms showing the effect of phosphate buffer concentration on the SFA and DHFR separation using gradient #9. The exact pH was 2.72. The numbering of the compounds is as in Figure 2.

(a) 0.1M phosphate buffer. (b) 0.001M phosphate buffer.

Gradient Analyses

(a) 0.1 M phosphate

During the early isocratic separations it became apparent that some of the early eluting peaks were only likely to be separated at greater k' values so that 10% MEOH and less would be required for the initial conditions. By consultation of the log k' versus % MEOH plot, and with the pre-determined delay and column holdup times, a series of solvent gradients were tested and modified. All runs were done at pH 2.8.

Compared to the best compromise isocratic separation (16% MEOH), all of the gradients were highly successfull in the following aspects. (a) Total separation time was reduced from 120 minutes to 50-60 minutes for the solvent programs, plus 15-20 minutes re-equilibration. (b) Although only seen as a leading spike on the combined second (SG) and third (SAN) peaks at 16%, the first peak (SNAC) was always far better than baseline resolved. (c) SG and SAN were always 50-90% separated. (d) Figure 4(a) (gradient details below) also shows the first main group of peaks at t_r 14-21 minutes to be well separated (SAC, SDZ, SISM and SMRZ) with one exception. ST is the peak at 19 minutes and SP is the following shoulder. Under some conditions and on some occasions, the SP peak could be observed as a clear spike on the side of the ST peak. (e) The last eight to nine peaks were always largely resolved.

To obtain the chromatogram in Figure 4(a), a multi-stage gradient in MEOH was employed. Equilibration took place at 0% MEOH and was jumped to 10% at the start of the run. This was followed by linear stages to 12% MEOH at 15 minutes, to 16% at 20 minutes, to 18% at 25 minutes and finally to 30% at 30 minutes. In figure 4(a), from 28-35 minutes, the difficult central section of the chromatogram contained seven compounds in the five peaks: SM, SAM+SMAZ, DVD+SMIZ, SMP and TMP. TMP was generally not resolved from SMP, but did sometimes appear as a shoulder. The first five compounds in this group were clearly the most difficult to separate in the context of the 25 compounds and often appeared as only two peaks.

(b) Effect of phosphate concentration

Figure 5 shows the variation in retention time with (log) phosphate concentration (mM). The 1.0M phosphate run terminated at 28 minutes when the maximum pressure load of 410 atm was exceeded. The 1.0 M solution was too high in salt concentration to be employed with increasing MEOH

concentrations. The eight compounds that did elute are included. Note that SP is almost baseline resolved from ST. This particular pair had previously been very difficult to separate.

The results in Figure 5 are striking in the dichotomy between the behaviour of the SFA and the DHFR. The former show little in the way of salt effects. In view of the moderate polarity of these compounds and the partial charges carried by the majority, this is not suprising. On the other hand, the effect on the DHFR is relatively dramatic. With $pK_{a,2}$ values for the first protonation of the electrically neutral base from 6.6-7 and $pK_{a,1}$ values for the second protonation around 1.3,[28] the DHFR have greater than one charge and their behaviour must be considered as that of ions in a swamping electrolyte. A plot of t_r versus the square root of the buffer concentration yields a convex curve, the inverse of which would represent the variation of the activity coefficients of the analytes in the increasing concentration of the buffer. This half-parabolic shape is consistent with the expected behaviour, if displaced a little further towards low salt concentrations than expected.

From the point of view of the separation, the size of the salt effect on the retention of the DHFR is very favourable. In reducing the phosphate concentration from 0.1 M to 0.001 M, (a) the last eluting peak (PYR) is eluted earlier but with baseline resolution maintained, thus reducing the total run time by about 5 minutes. Also, (b) the difficult to resolve TMP is removed to a vacant part of the chromatogram and is baseline resolved. (c) DVD, one of the compounds previously inseparable from the most difficult group of 5 located in the middle of the chromatogram, is moved forward to an earlier group (but causes a deterioration in that region). Figure 4(b) shows the chromatogram in 0.001 M phosphate.

Comparison of Figures 4 (a) and (b) is revealing. At higher salt concentrations the front of the chromatogram is almost resolved, whilst at lower salt concentrations the end of the chromatogram is resolved. Two isoionic elution stages joined by a gradient(s) in salt concentrations across the 20-30 minute period suggests itself. However, the timing and the gradient of the salt programming across this critical central period could be difficult. (Again the significance of peak widths should not be forgotten.)

(c) The final gradient (0.001 M phosphate)

With the improvements observed in the second half of the separation in 0.001 M phosphate, some further investigations of the front end of the gradient

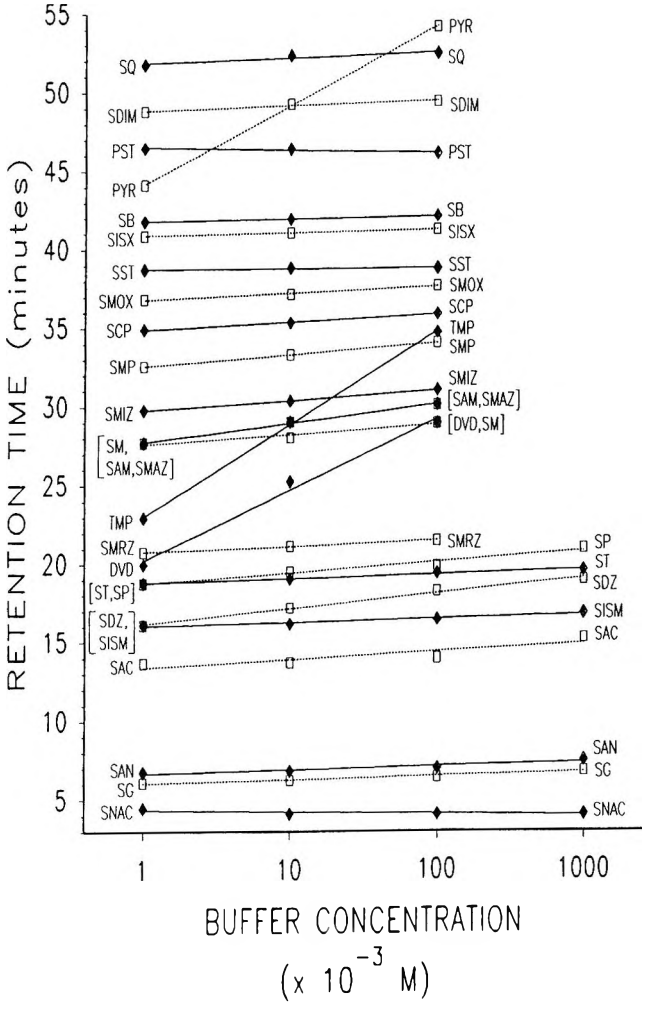

Figure 5. The influence of the variation of the phosphate buffer concentration on the retention time for the 25 antibiotics. Legend as in Figure 1.

were carried out. The best result was obtained by a variation of the gradient described in part (a) of this section. The 10-12% step in MEOH from 0 minutes was extended out to 30 minutes, then to 18% MEOH at 35 minutes and to 30% at 40 minutes. Figure 6 shows the result. Only two coelutions remain, compounds 8 (ST) and 9 (SP) [of which the later appears as a clear shoulder],

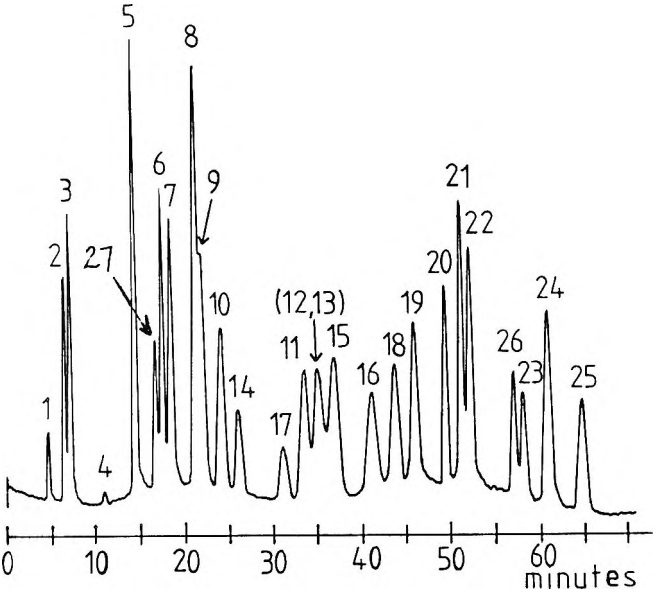

Figure 6. The chromatogram obtained using the final gradient described in the text (0.001M phosphate buffer). The numbering of the compounds is as in Figure 2. **27** is the second SAM hydrolysis product.

and, compounds 12 (SAM) and 13 (SMAZ). A further advantage lies in the revelation of the second SAM hydrolysis product (27) which had hitherto been unresolved.

CONCLUSIONS

The systematic and detailed examination of the retention behaviour of 22 sulphonamides and 3 dihydrofolate reductase inhibitors indicates that most combinations of the drugs could be successfully screened under an appropriately chosen set of conditions. For example, one of the groups of drugs most difficult-to-separate under the majority of conditions investigated are sulphameter(SM), sulphamoxole(SAM), sulphamethazine(SMAZ), sulphamethizole(SMIZ) and diaveridine(DVD). At pH 6.5 these are baseline resolved. Separation of all 25 drugs simultaneously has not been achieved, but may be possible with simultaneous methanol and salt (or pH) gradients.

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HPLC SEPARATION OF NADOLOL AND ENANTIOMERS ON CHIRALCEL OD COLUMN

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ABSTRACT

Nadolol (SQ 11725) is a β-adrenergic blocker which has two chiral centers, this allows for the presence of two racemates known as racemate A (SQ 12181) and racemate B (12182) and a total of four enantiomers. A simple isocratic HPLC method is developed for the separation of nadolol racemate A (SQ 12181) to its corresponding enantiomers RSR-nadolol (SQ 12148) and SRS-nadolol (SQ 12150) and racemate B (SQ 12182) to its corresponding enantiomers RRS-nadolol (SQ 12149) and SSR-nadolol (SQ 12151). Hexane with different percentage of ethanol containing 0.4% diethylamine is used as a mobile phase.

The effluents are monitored by ultraviolet detector set at 254 nm. The method showed good efficiency in identification, separation and optical purity determination of nadolol racemates and individual enantiomers in bulk materials and pharmaceutical formulations.

INTRODUCTION

The increased emphasis on research into enantiospecific drug action has been accompanied by increased activity in the field of chromatographic chiral separations. The β-adrenergic blocking agents have received particular attention as they are a pharmacologically important class of drug (cardiovascular drugs). They are used in treatment of angina pectoris, cardiac arrthymias and other heart conditions.

Nadolol 2, 3 cis-1, 2, 3, 4-tetrahydro-5 [2-hydroxy-3-(tert-butylamino) propoxy-2, 3 napthalenediol], is a long acting β-blocker used for the treatment of hypertension. Although nadolol possesses three chiral centres, the ring hydroxy groups at position 2 and 3 of tetrahydronaphthalene ring are in the cis configuration. In turn, there are only four enantiomers available since there are only two stereogenic centers. Nadolol (SQ 11725) is marketed and administered as a mixture of two racemic mixtures, racemate A (SQ 12181) and racemate B (SQ 12182). Racemate A (SQ 12181) consists of two enantiomers RSR (SQ 12148) and SRS (SQ 12150), while racemate B (SQ 12182) is composed of two enantiomers RRS (SQ 12149) and SSR (SQ 12151). The chemical structure of nadolol, its racemate and four available enantiomers are shown in Figure 1. Nadolol is stable to long exposure of normal levels of heat and light.[1] Several methods for the assay of nadolol in bulk material and pharmaceutical formulation based on colorimetry,[2] titration,[3] HPLC[1] and NMR[4] have been reported. Mutsutera et al.[5] described a method for the separation of nadolol diastereomers by reverse phase HPLC.

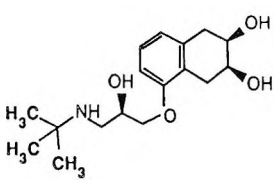
Recently, the enantiomers of several β-blockers are successfully separated using cellulose tris (3, 5-dimethylphenyl carbamate) known as Chiralcel OD as the chiral stationary phase (CSP).[6,7,8,9,10]

The aim of this study is to present a direct and simple separation of the four nadolol enantiomers on Chiralcel OD CSP under isocratic conditon for the purpose of identification and quality control of nadolol in bulk material.

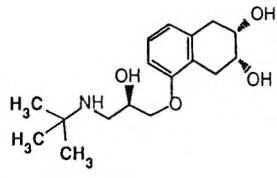
EXPERIMENTAL

Chemicals

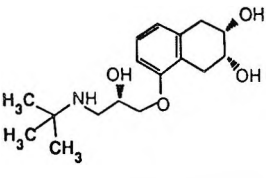
Nadolol (SQ 11725), the two racemates, racemate A (SQ 12181), racemate B (SQ 12182) and the four enantiomers (SQ 12148, 12150, 12149, 12151) are kindly supplied by Bristol-Myers, Squibb Pharmaceutical Research



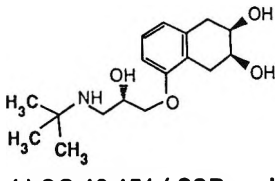
(+) SQ 12 148 (RSR-nadolol)



(+) SQ 12 149 (RRS-nadolol)



(+) SQ 12 150 (SRS-nadolol)



(-) SQ 12 151 (SSR-nadolol)

Figure 1. Chemical structure of nadolol enantiomers.

Institute (Princeton, NJ, USA). HPLC grade hexane was obtained from Fisher Scientific (Fairlawn, New Jersey, USA). Ethanol and diethylamine were purchased from BDH Chemicals (Poole, England).

Apparatus

The HPLC analysis is performed at room temperature with a Bio-Rad 1350 Solvent Delivery Pump, a Rheodyne Model 7125 Injector, a Waters Lambda Max 481 Variable Wavelength Detector set at 245 nm and a Hewlett-Packard 3394A Integrator. The cellulose tris (3,5-dimethylphenyl carbamate) Chiralcel OD column (25 cm x 4.6 i.d. coated on silica gel of 10 μm particle size) was obtained from Daicel Chemical Industries, Tokyo, Japan.

Chromatographic characteristics:

The separation factor (α), which represents a measure of relative peak separation is expressed as follows:

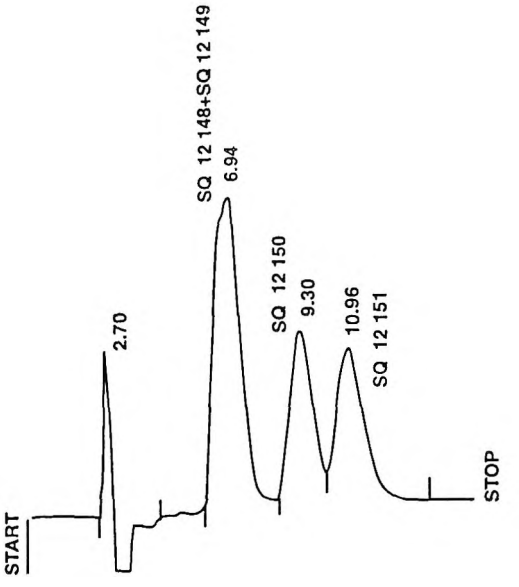


Figure 2. Chromatogram of nadolol SQ 11725. Column: Chiralcel OD (250 x 4.6 mm I.D.); mobile phase: hexane:ethanol:diethylamine (85:15:0.4); flow rate: 1 ml/min; chart speed: 0.5 m/min; temperature: 23^{0}C; detector UV 254 mn; sensitivity; 0.01 aufs; sample quantity 10 nmol.

$\alpha = k_2'/k_1'$ where k_1' and k_2' are capacity factor for the first and second eluting enantiomers, respectively. The capacity factor (k') is calculated as follows:

$k_1' = (t_{R1} - t_{R0})$ and $k_2' = (t_{R2} - t_{R0})$ where t_{R0}, t_{R1} and t_{R2} refer to the retention time in seconds for the solvent peak, the first and second eluting enantiomers, respectively.

The stereochemical resolution factor (Rs) is calculated by following formula:

$Rs = 2 (t_{R2} - t_{R1}) / (w_1 + w_2)$ where w_1 and w_2 are the peak width for the first and second eluting enantiomer peaks, respectively.

Determination of elution order

Peak identification for nadolol and its four enantiomers were established using Shodex OR-1 optical detector (JM Sciences, NY, USA) under the same chromatographic conditions described in the figures. It is of interest to mention that the peaks eluted with lower capacity factors were the dextroratatory (+) enantiomers followed by levorotatory (-) enantiomers (see Results and Discussions).

RESULTS AND DISCUSSION

Nadolol is a peculiar β-blocker in that it has three chiral centers, the 2-3 hydroxy groups are fixed in the cis configuration thus it possesses two chiral centers which allow a total of four enantiomers. Lee et al.[11] reported the direct separation of these four enantiomers using supercritical liquid chromatography under subcritical conditions with carbon dioxide as an eluent and were unable to resolve more than three enantiomers when using Chiralcel type or Pirkle type stationary phases. However, they obtained full resolution of all four enantiomers using an α_1 - acid glycoprotein.[11] Following a chiral derivatization of nadolol with 1-napthylisocyanate, a method has been developed for the separation of all four nadolol enantiomers on (R)-N-(3-5-dinitrobenzoyl)-L-leucine CSP.[12]

The method described by Lacroix et al.[13] can be used to determine total nadolol and related compounds and racemate composition of drug raw material. Numerous HPLC assays had been reported for determination of nadolol in biological fluid.[14,15,16,17] The Chiralcel OD-CSPs has been used for the resolution of several racemic β-adrenergic blockers that belong to the aryloxyaminopropane-2-ol class of compounds including propranolol[18] and others[6,20] into their corresponding enantiomers in normal phase mode. Nadolol is partially resolved into three peaks on Pirkle type[19] phases known as Chirex 3018 and 3022.[19]

In the method described Chiralcel OD column in the normal phase mode was used for separation of nadolol SQ 11725 which shows three separate peaks (Figure 2). These peaks were identified by the injection of individual enantiomers onto the HPLC system under the same chromatographic conditions. The two enantiomers SQ 12148 and SQ 12149 were overlapping in the first peak. We tried to optimize the separation of these overlapping peaks under isocratic condition but it was unsuccessful. However, the method can separate the enantiomers of the racemate A (SQ 12181), and the

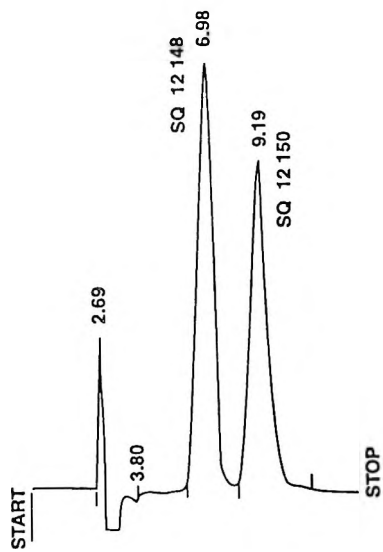


Figure 3. Chromatogram of nadolol SQ 12181. Column: Chiralcel OD (250 x 4.6 mm I.D.); mobile phase: hexane:ethanol:diethylamine (80:20:0.4);other chromatographic conditions are the same as in Figure 2.

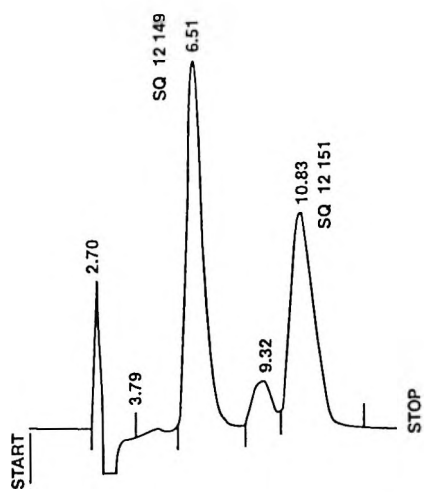


Figure 4. Chromatogram of nadolol SQ 12182. Column: Chiralcel OD (250 x 4.6 mm I.D.); mobile phase: hexane:ethanol:diethylamine (80:20:0.4); other chromatographic conditions are the same as in Figure 2.

enantiomers of racemate B (SQ 12182) (the chromatographic parameters were shown in Table 1) and verified by subsequent injection of the four individual enantiomers. It was found that SQ 12181 consists of 1:1 ratio of enantiomers SQ 12148 and SQ 12150 (Figure 3) while SQ 12182 consists of 1:1 ratio of enantiomers SQ 12149 and SQ 12151 (Figure 4). The mobile phase used contains 0.4 diethylamine to improve the peak sharpness, symmetry and to some extent the stereochemical resolution factor (Rs).[20]

Table 1

Chromatographic Parameters, Capacity (k′), Separation (α) and Resolution (Rs) Factors for the Nadolol Racemates.*

Nadolol Racemate	k'_1	k'_2	α	Rs
SQ12181 Racemate A	1.6 SQ 12148	2.42 SQ12150	1.5	2.6
SQ12182 Racemate B	1.4 SQ 12149	3.0 SQ 12151	2.14	5.4

* Mobile phase composition: Hexane:ethanol:diethylamine (80:20:0.4% v/v); see Figure 2 for other chromatographic conditions.

Shodex OR-1 optical detector was used to identify the optical rotation of nadolol enantiomers. It was found that the first two peaks are (+) dextrorotatory while the last peak is (-) levorotarory. The optical rotation was verified by injection of individual enantiomer under the same chromatographic condition (Table 2), enantiomer RSR nadolol (SQ 12148) and RRS nadolol (SQ 12149) which overlapped under the chromatographic condition used and represented the first peak both of these enantiomers are (+) dextrorotatory. The second (+) dextrorotatory peak belongs to SRS nadolol (SQ 12150) while the third peak is (-) levorotatory identified as SSR nadolol (SQ 12151). It is of interest to mention that there is no relationship between the sign of rotation and absolute configuration of the substance. Thus the method desribed can be used for the separation, identification and optical purity determination of nadolol racemates and enantiomers in the bulk material and pharmaceutical dosage forms.

Table 2

The Chromatographic Parameters; Capacity (k′) Separation (α) Factors and Optical Rotation of Nadolol enantiomers.

Nadolol Enantiomers	k′	α	Optical Rotation
SQ 12148	4.24	1.13	(+)
SQ 12149	3.73	0.87	(+)
SQ 12150	6.5	1.55	(+)
SQ 12151	8.25	1.25	(-)

Mobile phase composition: Hexane:ethanol:diethylamine (85:15: 0.4% v/v), see Figure 2 for other chromatographic conditions.

CONCLUSION

The two racemates and four enantiomers of nadolol were separated by a simple, isocratic and fast (required 15 minutes) method using Chiralcel OD column. The method can be used for the identification and optical purity determination of nadolol in bulk material and in pharmaceutical dosage form. It can also be used to separate the individual enantiomers of nadolol from their corresponding racemates on a preparative scale using a semi-preparative or preparative Chiralcel OD column.

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SOLID PHASE EXTRACTION/HIGH PERFORMANCE LIQUIDCHROMATOGRAPHY METHOD FOR THE DETERMINATION OF METHYL ANTHRANILATE RESIDUES IN BLUEBERRIES

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ABSTRACT

Methyl anthranilate fortified blueberries were extracted with methanol, cleaned up by solid phase extraction and analyzed by reverse phase high performance liquid chromatography. Methyl anthranilate was quantified by UV absorbance and fluorescence detection. Recoveries of 70 ± 13% and 14% were obtained for blueberry samples fortified with methyl anthranilate at 5 and 0.15 ppm respectively. The method limits of detection for ultraviolet and fluorescence detection were 0.04 and 0.009 μg methyl anthranilate/g, respectively.

INTRODUCTION

Methyl Anthranilate (MA) is a methyl ester used as an additive in the food and cosmetic industry. It is used commonly in chewing gum and

beverages as grape flavoring and odor. It is also used frequently as a fragrance in perfumes.

Methyl anthranilate is also an effective feeding deterrent to many species of birds[1-3] and rodents.[4] As MA is on the Food and Drug Administration list of compounds that are generally recognized as safe (GRAS), it has excellent potential as a non-toxic bird repellent. MA is being tested as a bird repellent for a variety of bird management uses.[5] One such application involves using MA to deter bird feeding on blueberries. Bird depredation to ripening blueberries is a major problem for growers in the United States and Canada with annual losses estimated at $8.8 million in 1989 for the United States.[6]

To determine the effectiveness of MA as a bird deterrent on blueberries, microencapsulated MA formulation was mixed with water and applied with air blast sprayers. The field test was conducted at 5 different sites in Washington and Oregon by the Denver Wildlife Research Center. To support efficacy studies, an analytical method utilizing High Performance Liquid Chromatography (HPLC) coupled with ultraviolet detection (uv) was developed to determine the minimum methyl anthranilate residue levels associated with bird repellency. This analytical method had a limit of detection of 0.4 μg MA/g[7] which was sufficient to quantify methyl anthranilate in the spray formulation and the methyl anthranilate residue levels associated with bird repellency. However, a more sensitive analytical method was needed to quantify the lower methyl anthranilate residue levels on blueberries harvested for consumption. For this harvest residue study, a more sensitive method was developed which utilizes solid phase extraction (SPE) and fluorescence detection to achieve the required lower limits of detection for long term residue studies.

MATERIALS

Methanol and acetonitrile (Fisher Scientific, Denver, CO) were liquid chromatography grade. Deionized water was purified using a Milli-Q water purification system (Millipore, Bedford, MA). The solvents were degassed by the Hewlett-Packard 1050 series on line degasser. Concentrated sulfuric acid (Fisher Scientific, Denver, CO) was used to make the 5% sulfuric acid solutions in methanol.

Methyl anthranilate was obtained from Aldrich (Milwaukee, WI) and PMC Specialties (Cincinnati, OH). Concentrated stock solutions of MA were prepared from the commercial products, without further purification, by

dissolving 100 mg in 25 mL of methanol. Working solutions were prepared weekly by dilution with 1% sulfuric acid in 86:13, methanol to water. All standard solutions were stored in the dark at 5°C.

Isolute NH_2 SPE cartridges containing 500 mg aminopropyl sorbent and 10 mL reservoir volume were obtained from Jones Chromatography (Lakewood, CO). SPE cartridges were used with a VacMaster sample processing station (Jones Chromatography).

METHODS

Formulation Application And Sample Collection

Prior to application, control samples were collected from the control and treated plots. A microencapsulated methyl anthranilate solution was applied at a concentration of 2.5 to 3.0% MA with air blast sprayers. Residue samples were carefully collected by randomly selecting blueberry bushes and then clipping a stem with a cluster of blueberries. Each blueberry was clipped and allowed to drop into the sample container until approximately 100 grams of sample were collected. The container was immediately sealed and placed in a cooler with ice, five replicates were taken each sampling day. Within one to two hours the samples were transferred to a -25°C freezer and eventually shipped with dry ice to the Denver Wildlife Research Center for analysis.

Sample Preparation

For quality control and method development samples, blueberries harvested from untreated control plots were spiked with solutions of MA in methanol at levels ranging from 0.15 to 125 ppm. Prior to analysis, the frozen blueberry samples were partially thawed at room temperature for 15 to 20 minutes. The lids were removed, followed by removal of the lid liners which were rinsed into the sample jars with 0.75 mL of 5% sulfuric acid in methanol. Another 0.25 mL portion of the sulfuric acid solution was used to rinse the inside rim of the sample jar. The blueberry samples were then homogenized to a puree with a hand blender. Each homogenized sample was then extracted by weighing 1.50 to 1.60 grams of the puree into a 15.0 mL graduated 15-mL glass screw cap centrifuge tube. Methanol was added to the tube to give a total volume of 15.0 mL. The samples were vortexed for 10 seconds and then sonicated three times for 15 minutes each. Between each sonication period the

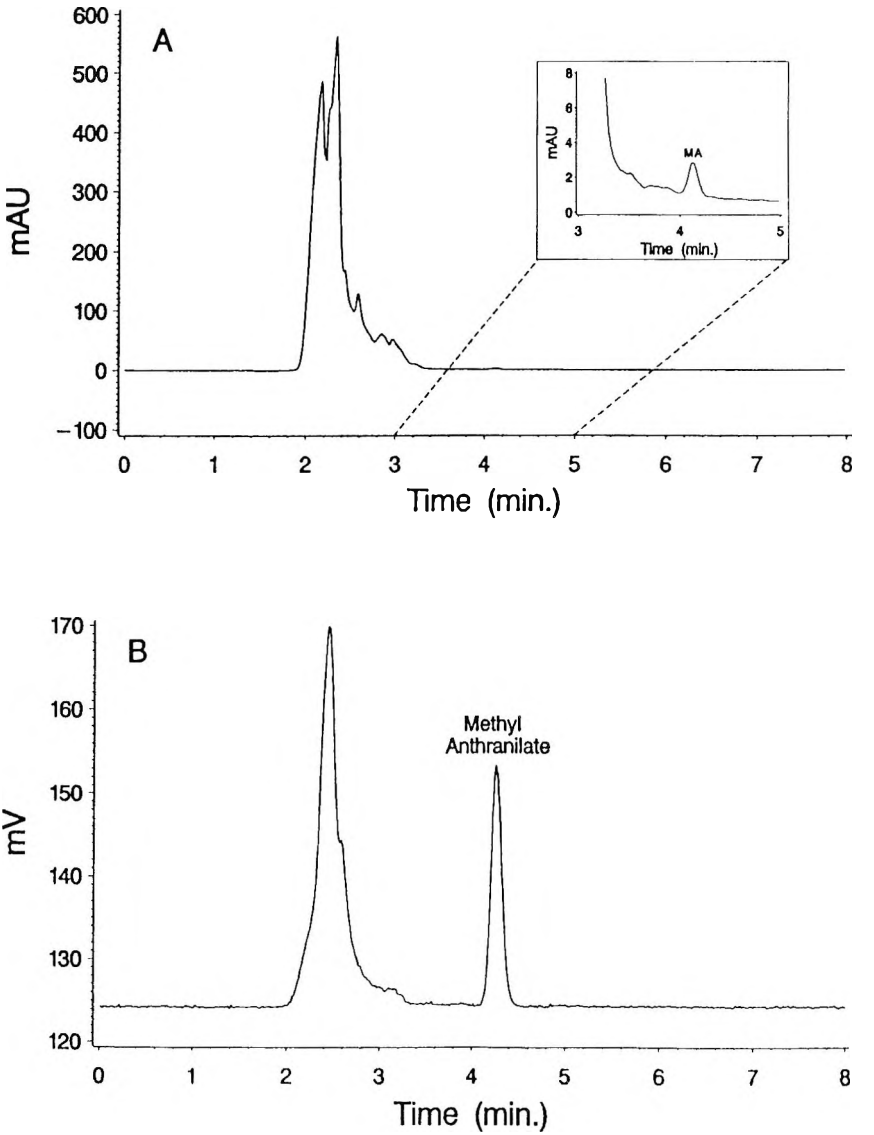

Figure 1. Chromatogram of a 1.0 mg/g MA fortified control blueberry extract with detection:

(A) UV and (B) Fluorescence

samples were shaken by hand for 5 seconds. The samples were then centrifuged for five minutes at approximately 2500 rpm and 5 mL of the supernatant transferred to an NH_2 column which had been preconditioned with 6 mL each of water and methanol. Matrix interferences were retained on the SPE column and the MA containing eluant was collected in a 10 mL centrifuge tube. The volume of the eluant was reduced to approximately 0.5 mL by evaporation at 25°C under a gentle stream of nitrogen. The contents of the tube were then brought to 1.0 mL total volume with HPLC mobile phase which was then transferred to an autosampler vial and analyzed by HPLC.

High Performance Liquid Chromatography

The HPLC system consisted of a Hewlett-Packard 1050 liquid chromatograph (Palo Alto, CA) operated at ambient temperature. A Hewlett-Packard 1050 variable wavelength detector was used at 220 nm to detect MA. A SpectroVision Inc.(Cambridge, MA), dual monochromator fluorescence detector placed serially in-line with the UV detector was used with an excitation wavelength of 338 nm and an emission wavelength of 424 nm. The sample extracts (15 μL) were chromatographed with a acetonitrile:water (70:30) mobile phase at 1.00 mL/min. The MA was separated on a 25-cm x 0.46-cm i.d. stainless steel analytical column packed with 5-μm Alltech Econosil C_{18} (Deerfield, IL). To prolong column life, a 1.5-cm x 0.46 cm i.d. Keystone Octyl-H (Bellefonte, PA) guard column was used. The MA peak was identified and quantified on the fluorescence chromatogram by comparison of retention time and chromatographic response with a MA standard. When possible, qualitative confirmation was also accomplished by comparison of the uv chromatographic response to the uv spectrum and retention time of a standard. A Hewlett-Packard 386 Vectra computer work station with an Epson printer was used to collect, process, store, and print the chromatographic data.

RESULTS AND DISCUSSION

HPLC chromatograms of extracts from blueberries spiked with MA at 1.0 and 0.15 μg/g are presented in Figures 1 and 2, respectively. These extracts were neither cleaned up by SPE nor concentrated. The retention time of MA is 4.18 minutes. Coextracted matrix constituents eluted primarily between 2 and 3.5 minutes. Both figures clearly illustrate the improved sensitivity of fluorescence detection as compared with uv detection. Figures 1A and 2A show that uv detection is sufficient to detect MA in blueberries at a level of 1.0

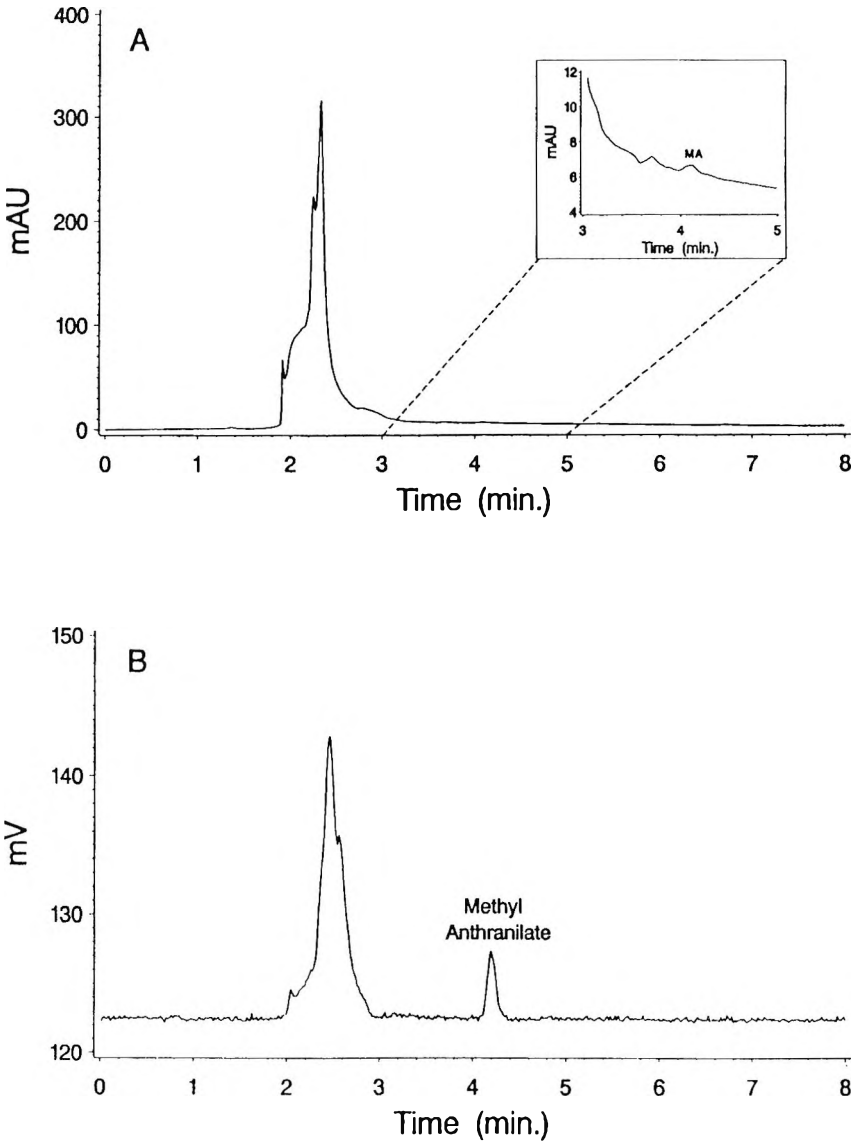

Figure 2. Chromatogram of a 0.15 mg/g MA fortified control blueberry extract with detection:

(A) UV and (B) Fluorescence

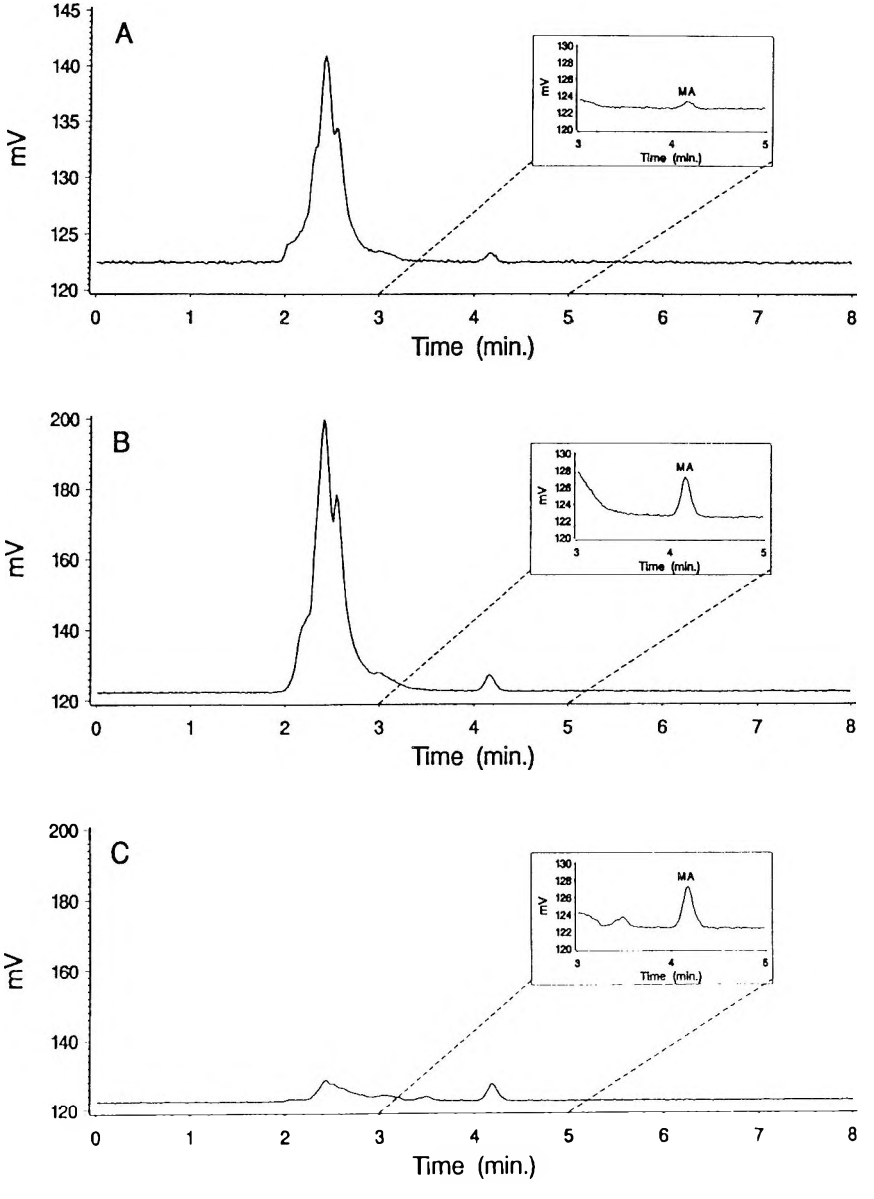

Figure 3. Fluorescence chromatogram of a 0.04 mg/g MA fortified blueberry extract with (A) no clean up or concentration, (B) concentration step and (C) clean up and concentration.

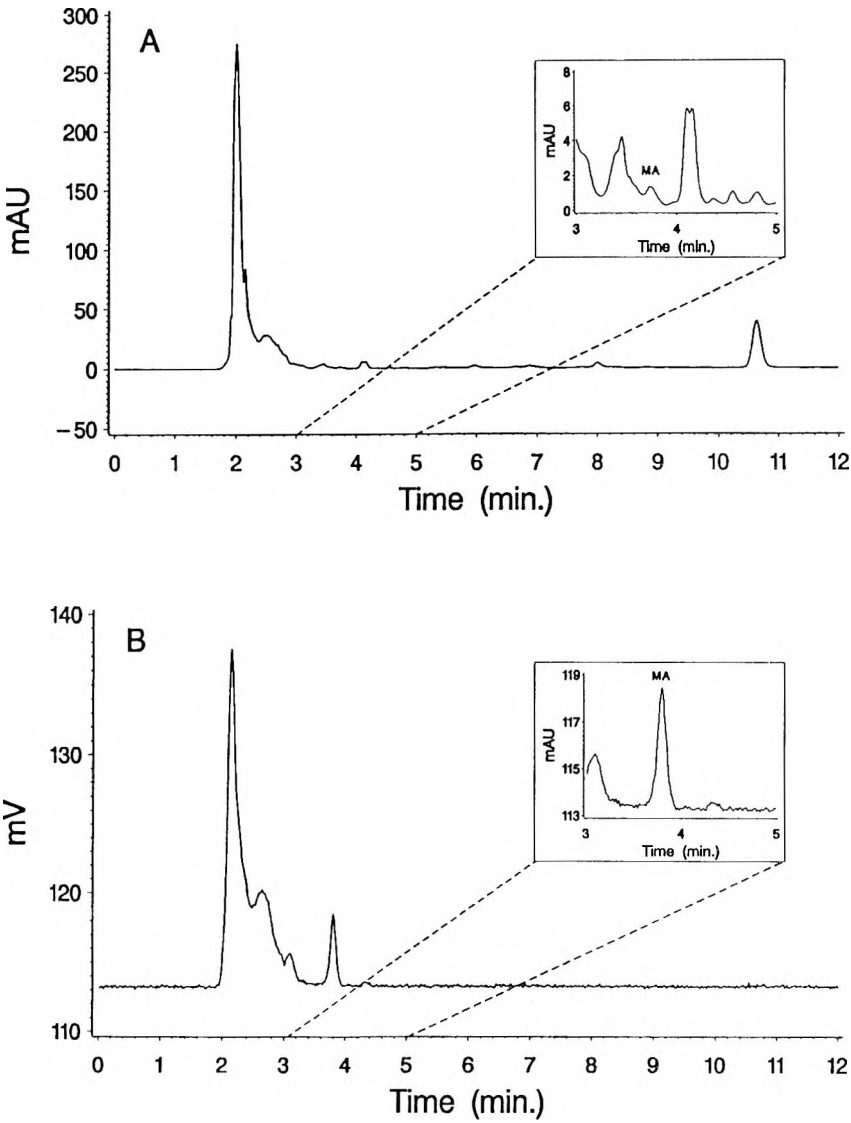

Figure 4. UV (A) and fluorescence (B) chromatograms of extracts from blueberries harvested from control plots.

μg/g but that the uv response for MA in blueberries at 0.15 μg/g is too low to quantify. Comparison of these uv chromatograms with the fluorescence

chromatograms in Figures 1B and 2B illustrate that fluorescence detection provides about a 5 fold increase in sensitivity. Furthermore, the relative response of interfering compounds with respect to the MA is significantly less with fluorescence detection.

The three fluorescence chromatograms presented in Figure 3 illustrate the advantages of the concentration and cleanup sample preparation steps. All three chromatograms are extracts from blueberries spiked with MA at 0.04 μg/g. Chromatogram A resulted from the HPLC analysis of an extract without concentration or cleanup. The addition of the concentration step to the sample preparation resulted in chromatogram B. Comparison of chromatograms A and B illustrate that the concentration step improved the response of MA by a factor of approximately 5. Chromatogram C illustrates the advantages of both the concentration and sample cleanup. The SPE cleanup removed the majority of matrix coextractants without removing MA. With the clean up and concentration step, the method limit of detection for MA in blueberries was determined to be 0.009 μg/g.

The uv and fluorescence chromatograms presented in Figure 4 resulted from the analysis of extracts of blueberries harvested from control plots. Control plots were situated at least 30 meters from the nearest treated plot. Most chromatograms of blueberry extracts from control plots had no detectable MA residues. However, the chromatograms presented in Figure 4B show MA residues of 0.031 μg/g. Minor variations in retention times were due to the use of different HPLC columns. The advantages of selectivity and sensitivity afforded by fluorescence vs uv detection are especially evident in trace level analyses such as these. The low levels of MA detected on these control blueberries by fluorescence detection are probably the result of drift during or shortly after application.

MA residue levels on blueberries were determined for blueberries harvested from 5 different sites. The mean residue level at 6 days after application were approximately 2 μg/g. The mean half-life for MA on blueberries was 3 days. As the method limit of detection for this method was 0.009 μg/g, this method should be able to detect MA residues on blueberries harvested up to 4 weeks post application.

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Mention of commercial products does not constitute endorsement by the

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APPLICATION OF COLUMN SWITCHING IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CHLORTHALIDONE ENANTIOMERS IN UNTREATED URINE

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ABSTRACT

A liquid chromatographic method incorporating column-switching for the separation and determination of chlorthalidone enantiomers in urine is described. Untreated urine samples (50 μL) were directly introduced into a 20 mm x 2.1 mm I. D. precolumn packed with a Hypersil ODS-C_{18}, 30 μm, stationary phase. Polar urinary compounds were removed by flushing the precolumn with 4 mL of water (pumped at a flow rate of 1 mL/min), and the racemic analyte was then transferred to a LiChroCART ChiraDex, 5 μm, 250 mm x 4 mm I. D. column, where the enantiomers were separated by means of a methanol/0.05 M acetate buffer adjusted to pH 4 mobile-phase (40:60, v/v), and quantified at 230 nm. The system shows good linearity and reproducibility in the 0.25 - 5.0 μg/mL, thus covering therapeutic levels of chlorthalidone in urine. The usefulness of the described procedure has been tested by analyzing urine samples obtained after drug administration.

INTRODUCTION

In drug analysis, stereochemical of separation is currently of great interest, because the separation and determination optical isomers is essential to understand the mechanism of drug action. Chiral separations can be also of interest in the development of new drugs.

Owing to their potential for separation, High Performance Liquid Chromatography (HPLC) is presently the most widely used technique for enantiomeric analysis. In this respect, different approaches have been reported: (i) indirect methods based on formation of the diastereomeric derivatives and subsequent separation in a conventional (achiral) stationary phase, and direct methods based on (ii) separation in a conventional stationary phase by means of a chiral mobile-phase, or (iii) separation in a chiral stationary phase.

Although HPLC is an increasingly popular technique for direct separation of enantiomers (specially through the development of new chemically bonded chiral stationary phases), some complications are encountered when analyzing biological samples. For example, most applications described are based on normal phase chromatography. This is a major difficulty in the analysis of biological samples because the analyte must be transferred from an aqueous to an organic phase before injection. Otherwise, chiral selectivity rapidly decreases.[1] Important progress has been made since the introduction of cylclodextrin bonded phases, which can be used in a conventional reverse phase mode.

On the other hand, a very selective clean-up is necessary because chiral separations increase the number of peaks to be resolved (every endogenous compounds can lead to a pair of peaks).[1,2] Moreover, low efficiencies associated with chiral columns limit the sensitivity. This means that some kind of enrichment and/or derivatization of the analytes are often required.[3]

Switching chromatography has become especially important in the context of chiral separation because highly selective separations can be achieved by coupling chiral columns to conventional reverse phase systems. Several successful applications of this technique in drug analysis have been reported. However, most of the described assays have not been applied to real samples,[4,5] or involve off-line clean-up or preconcentration steps before injection into the chromatographic systems.[2,6] In addition, relatively sophisticated set-ups are sometimes required to obtain adequate resolution and sensitivity.[7-9] In such cases, several (chiral and a chiral) columns, pumping systems and/or detectors must be combined within the same network to effect enantiomeric resolution and peak compression; peak compression is necessary to overcome band broadening due to chiral separation and

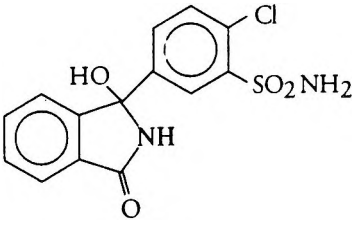

Figure 1. Chemical structure of chlorthalidone.

analyte transfer from one column to another.

In this work we have evaluated the usefulness of column-switching for the on-line sample conditioning, analyte enrichment and enantiomeric separation using a cylclodextrin bonded phase column. The diuretic chlorthalidone (Figure 1), has been used as a model of substance because we previously evaluated the potential of switching techniques for sample pretreatment in the analysis of some diuretics (including chlorthalidone) in urine,[10] and also because the recent increase in the consumption of chlorthalidone in combination with other antihypertensive agents have resulted in very low doses of drug; therefore, very sensitive and highly specific methods to measure chlorthalidone levels in biological samples are required. On the basis of these studies, a procedure for the determination of chlorthalidone enantiomers in urine has been developed.

MATERIALS

Apparatus

The chromatographic system used consisted of two quaternary pumps (Hewlett-Packard, 1050 Series, Palo Alto, CA, USA), an automatic sample injector (Hewlett-Packard, 1050 Series) with a sample loop injector of 100 μL, and a high pressure six port valve (Rheodyne model 7000). A diode array detector (Hewlett Packard, 1040 series) linked to a data system (Hewlett Packard HPLC Chem Station) was used for data acquisition and storage. The detector was set to collect a spectrum every 640 ms over the range 200-400 nm, and the chromatographic signal was monitored at 230 nm. All the assays were carried out at ambient temperature.

Reagents

All the reagents were of analytical grade. Methanol was of HPLC grade

(Scharlau, Barcelona, Spain). Water was distilled, deionized and filtered in nylon membranes, 0.45 μm (Teknokroma, Barcelona, Spain). Racemic chlorthalidone was obtained from ICI-Pharma (Pontevedra, Spain). Propylamine hydrochloride (Fluka, Buchs, Switzerland), sodium acetate (Panreac, Barcelona, Spain) and acetic acid (Probus, Badalona, Spain) were also used.

METHODS

Standard Solutions

Standard solutions of chlorthalidone were prepared by dissolving 50 mg of the racemic compound in 25 mL of methanol; these solutions were stored in the dark at 2^0C. Under such conditions, the standard solutions are stable at least for a month. Working solutions were prepared daily by dilution of the stock solutions with the appropriate volumes of purified water.

Columns and Mobile-Phases

The pre-column (20 mm x 2.1 mm I.D.) was dry-packed with a Hypersil ODS-C_{18}, 30 μm, stationary-phase (Merck, Darmstadt, Germany). Purified water was used as washing solvent to eliminate the biological matrix from the precolumn. The analytical column was a LiChroCART ChiraDex, 250 mm x 4 mm I.D., 5 μm, column (Merck). A methanol/0.05 M acetate buffer (pH = 4) mixture was used for the enantiomeric separation. The acetate buffer was prepared by dissolving 2 g of sodium acetate in 500 mL of purified water, after the addition of 0.5 mL of propylamine hydrochloride. The pH was adjusted to 4 by adding concentrated acetic acid.

The mobile-phases were prepared daily, filtered with a nylon membrane, 0.45 μm, (Teknokroma) and degassed with helium before use.

Column Switching Operation

The system used for the on-line sample pretreatment and enantiomeric separation is shown in Figure 2. At the beginning of each assay 50 μL of sample were injected from the sample injector to the precolumn. Polar components of the matrix were directly washed-out with water by means of Pump 1 (at a flow rate of 1 mL/min). At the same time, the analytical column was being reequilibrated with an

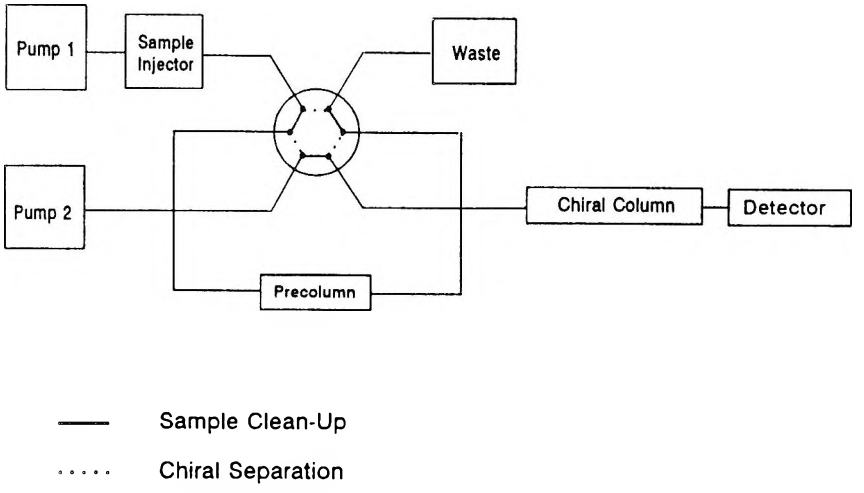

Figure 2. Schematic representation of the switching system used for the determination of chlorthalidone enantiomers in urine.

acetonitrile/acetate buffer (pH = 4) eluent (40:60, v/v) delivered by Pump 2 at a flow rate of 1 mL/min. At t = 4 min, the switching valve was rotated, so the trapped analyte was transferred from the precolumn to the chiral column, where chlorthalidone enantiomers were separated. At t = 9 min the switching valve was turned back to the original position to regenerate and reequilibrate both the precolumn and the analytical column.

Recovery Studies

Blank urine samples were spiked with chlorthalidone standard solutions reproducing different concentrations for each enantiomer in the 0.25 - 5.0 μg/mL range. The percentage of drug recovered for a particular injection was calculated by comparing the peak areas obtained for each isomer in the spiked samples, with the values obtained for a direct injection of 50 μL of an aqueous solution containing the same concentration of analyte. Each concentration was assayed in triplicate.

Preparation of Standards for Calibration

Standards for calibration were prepared by spiking urine samples with the appropriate volumes of the chlorthalidone standard solutions reproducing different

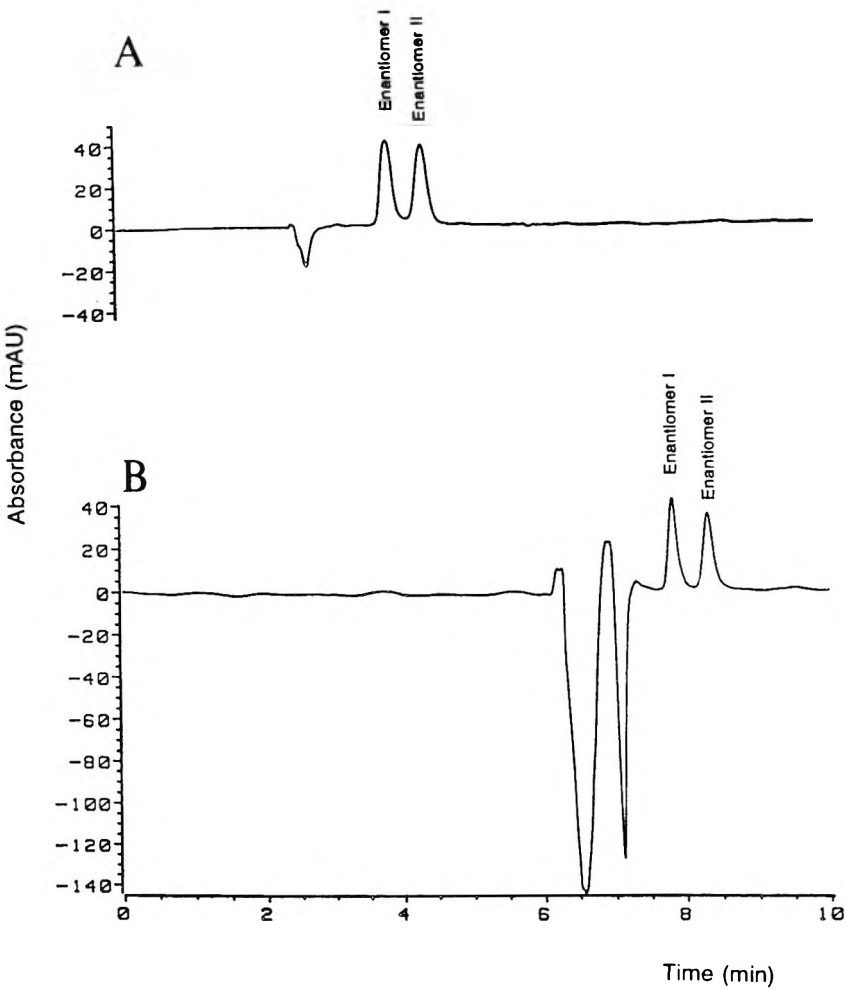

Figure 3. Chromatograms obtained for an aqueous solution of racemic chlorthalidone by (A) direct injection into the chiral column and (B) injection in the switching system (duration of the flushing stage, 4 min). Concentration of each enantiomer, 2.5 μg/mL. For experimental details, see text.

concentrations for each enantiomer in the 0.25 - 5.0 μg/mL range. These samples were processed as described above. Peak areas at 230 nm were plotted versus the analyte concentration, and the resulting calibration curves were used to calculate the concentration of each enantiomer in the unknown samples. Each concentration was assayed in triplicate.

Table 1

Analytical Data for the On-Line Determination of Chlorthalidone Enantomers in Urine

Enantiomer	Recovery[a] (n = 3) (%)	Linearity				Intra-day Precision[a] (n = 6) (%)	Inter-day Precision[a] (n = 15) (%)	Limit of Detection (ng/mL)
		$y = a + bk$	S_{xy}	n				
I	92 ± 2	a = 1 ± 4 b = 88.9 ± 1.1	8.83	18	4		7	20
II	92 ± 3	a = 2 ± 4 b = 89.0 ± 1.5	10.7	18	4		8	20

[a] Determined at half of highest concentration in tested range.xxxx

Human Studies

Urinary excretion studies were performed with a human healthy volunteer after a single dose administration of racemic chlorthalidone (25 mg). Urine samples were collected at appropriate time intervals post-dose, and analyzed as described above.

RESULTS AND DISCUSSION

Chiral Separation

Conditions for the resolution of chlorthalidone enantiomers were optimized by direct injection of aqueous standard solutions into the chiral column. Initially, different water/methanol and water/acetonitrile eluents were tested as mobile phase for separation. However, in all instances, every enantiomer leaded to a pair of peaks, which were partially overlapped. The relative intensities of the peaks observed for each enantiomer were found to be highly dependent on the pH of the aqueous component of the mobile phase, as well as on the eluent strength. This effect was not observed when chlorthalidone was chromatographed with conventional (achiral)

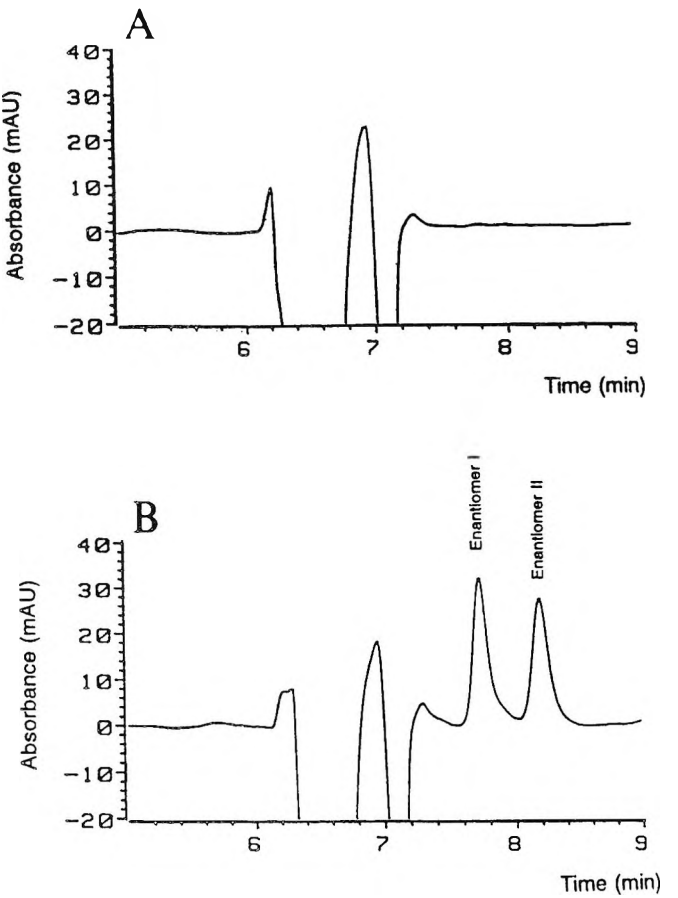

Figure 4. Chromatograms obtained for (A) blank urine and (B) urine spiked with racemic chlorthalidone. Concentration of each enantiomer, 2.5 μg/mL. For experimental details, see text.

reverse phase columns,[10,11] and it is most probably due to the partial ionization of the drug.[1] Since the pH is a critical variable, we tested acetate and phosphate buffers adjusted to different pH in the 3 - 7 interval. Best results were obtained with a mixture methanol/acetate buffer adjusted to pH 4, (60:40, v:v), at a flow rate of 1 mL/min. Under these conditions, each enantiomer was eluted as a single peak, and both enantiomers were base-line resolved (Figure 3A).

Table 2

Accuracy for Chlorthalidone in Urine (n = 3)

Added Concentration (μg/mL)	Subject Number	Isomer I		Isomer II	
		Determined Concentration (μg/mL)	E_r (%)	Determined Concentration (μg/mL)	E_r (%)
0.375	1	0.36 ± 0.01	- 4.0	0.38 ± 0.01	+ 1.3
	2	0.38 ± 0.02	+ 1.3	0.38 ± 0.01	+ 1.3
	3	0.39 ± 0.01	+ 3.0	0.375 ± 0.001	0.0
1.25	1	1.28 ± 0.09	+ 2.4	1.28 ± 0.03	+ 2.4
	2	1.35 ± 0.09	+ 8.0	1.37 ± 0.07	+ 9.6
	3	1.36 ± 0.06	+ 8.8	1.29 ± 0.06	+ 3.2
2.5	1	2.49 ± 0.01	- 0.4	2.54 ± 0.06	+ 1.6
	2	2.56 ± 0.09	+ 2.4	2.60 ± 0.06	+ 4.0
	3	2.48 ± 0.02	- 0.8	2.48 ± 0.04	- 0.8
3.75	1	3.79 ± 0.06	+ 1.1	3.80 ± 0.09	+ 1.3
	2	3.80 ± 0.05	+ 1.3	3.7 ± 0.1	- 1.3
	3	3.78 ± 0.04	+ 0.8	3.70 ± 0.04	- 1.3

Column-Switching System

The described configuration (Figure 2) allows the quantification of the total amount of each enantiomer present in the samples. On the basis of previous experiences, we selected a Hypersil stationary phase for packing the precolumn and water was used as mobile-phase for washing-out the urinary endogenous components.[10] The breakthrough volume of chlorthalidone in this precolumn was found to be higher than 15 mL of water, which means that large volumes of water can be used to remove matrix components from the precolumn.

As can be seen by comparing figures 3A and 3B, with the set-up used we did not observe additional peak dispersion compared with direct injection of the analytes

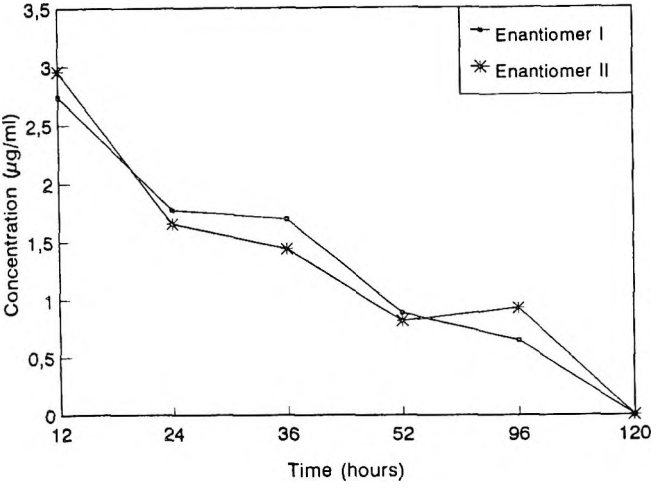

Figure 5. Urinary excretion-time profile of chlorthalidone enantiomers. Dose administered, 25 mg. For experimental details, see text.

into the chiral column. Negative peaks observed can be explained by the absorption properties of the acetate buffer used for elution.[12] Indeed, these peaks were much more intense when using the switching approach because the volume of water introduced in the flow scheme of Pump 2 is larger. However, these system peaks do not interfere with the quantitation of chlorthalidone enantiomers.

Analysis of Urine Samples

A vast majority of the urinary endogenous compounds are eliminated from the precolumn with the first 1.5 mL fraction of water used in the clean-up step. However, in order to prevent the injection of medium polarity matrix components into the chiral column, the flushing stage was extended to 4 min, so the precolumn was flushed with 4 mL of water. As a result, excellent selectivity is achieved. This is illustrated in Figure 4, which shows typical chromatograms obtained for blank urine and urine spiked with racemic chlorthalidone.

Some relevant analytical parameters obtained with the described procedure are summarized in Table 1. Linearity and reproducibility were satisfactory over the studied interval, analyte recoveries being also reasonable. No significant differences between enantiomers were observed.

Accuracy of the method was evaluated by determining the concentration of chlorthalidone enantiomers in spiked urine samples. The results of these studies are shown in Table 2. As can be seen from this table, the method provides concentrations close to the real ones in all cases tested, with relative errors ranging from -4 % (for the enantiomer I at a concentration 0.375 μg/mL) to +9.6 % (for enantiomer II at a concentration 1.25 μg/mL). The limit of detection (calculated as the concentration required to generate a signal-to-noise ratio of 3) was 20 ng/mL for both enantiomers (Table 1). This sensitivity is about 5 times lower than that obtained for a racemic mixture of chlorthalidone when an achiral C_{18} column (of the same length) and a similar switching device were used.[10]

Utility

Since chlorthalidone is clinically used as a racemate, the utility of the described approach was tested by measuring the concentration chlorthalidone enantiomers levels in urine after a single dose administration of the racemic drug. The results obtained are shown in Figure 5. Both enantiomers can be detected at least 96 hours after drug administration. Therefore, the sensitivity of the described approach can be considered satisfactory for most applications concerning the determination of chlorthalidone enantiomers in urine, taking into account the pharmacological properties of this diuretic.[12]

The total analysis time, including precolumn reequilibration, takes about 10 min, and the system can be repeatedly used with satisfactory stability for several injections. However, occasional precolumn replacement (every 100 injections) is recommended to ensure suitable performance of the chiral column.

CONCLUSIONS

The described approach illustrates the potential of column-switching for the on-line determination of chlorthalidone enantiomers in urine. The described system does not introduce peak dispersion, which means that additional columns to effect peak compression are not required. Therefore, determination of the total amount of both isomers can be achieved using a very simple set-up. In addition, since sample clean up and enrichment are on-line performed, no manipulations of the samples are involved, so, the system is well suited for fully automation. Compared with conventional columns, the employment of a chiral phase diminishes the sensitivity by a factor of about 5. However, chlorthalidone enantiomers can be determined with suitable accuracy and precision, the sensitivity being also satisfactory for most applications.

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PROTEIN SEPARATION BY IMPROVED CROSS-AXIS COIL PLANET CENTRIFUGE WITH ECCENTRIC COIL ASSEMBLIES

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ABSTRACT

The new prototype of the cross-axis coil planet centrifuge (X-axis CPC) fabricated in our laboratory provides various improvements over the original unit such as ambient temperature control, good visibility of the rotary frame and substantial reduction of the torque by a round transparent case, direct motor shaft coupling to the rotary frame to stabilize the system, and ease of belt tension adjustment using idler pulleys. The

capability of the system was demonstrated in the separation of stable proteins with a polymer phase system using a pair of eccentric coil assembly separation columns. Cytochrome C, myoglobin and trypsinogen were well resolved and eluted in 5.5 h at a partition efficiency of 200 theoretical plates. The method provides a gentle environment for proteins without causing their deactivation or loss.

INTRODUCTION

Countercurrent chromatography (CCC) is a continuous liquid-liquid partition method which does not require a solid support. The stationary phase is retained in the column by the aid of gravity or, in more modern versions, centrifugal force. All of the complications arising from the use of solid supports (deactivation, sample loss, etc.) are thereby avoided. Among various CCC systems developed in the past, high-speed CCC has proven most useful since it provides the advantages of high peak resolution and short separation times while using a durable and stable instrument.[1] However, when applied to aqueous-aqueous polymer phase systems that are characterized by low interfacial tension and high viscosity, the usual form of the apparatus does not properly retain the stationary phase.

The cross-axis coil planet centrifuge (X-axis CPC) was introduced to solve the above problem by providing a unique mode of planetary motion in which the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge. This motion satisfactorily retains the stationary phase when used with polymer phase systems required for protein separations.[2]

Previous studies on the separation of stable proteins by the X-axis CPC[3] have demonstrated that the ratio between the revolution radius (X) and the lateral deviation (L) of the coiled column is an important parameter determining both retention of stationary phase and peak resolution. Our recent studies further indicated that an improved version of the X-axis CPC which provides two positions for the column was especially useful in the separation of proteins.[4]

The present paper describes the performance of our new prototype X-axis CPC which provides various improvements over the original unit. The capability of the apparatus was demonstrated in separation of stable proteins with a polymer phase system using a pair of eccentric coil assemblies mounted in an off-center position.

Figure 1. Photograph of the apparatus.

EXPERIMENTAL

Apparatus

The X-axis CPC employed in the present studies was constructed at the machine shop of Nihon University, Chiba, Japan. The basic feature of the apparatus was previously described in detail[4] and a brief description is given here. The apparatus produces a synchronous planetary motion of column holder which rotates about its horizontal axis and simultaneously revolves around the vertical axis of the apparatus at the same angular velocity. The column holder was mounted at an off-center position (X = 10 cm and L = 15 cm) which provides efficient mixing of the two solvent phases and stable retention of the stationary phase in the column.

Figs. 1-3 show the design of the present apparatus. The following improvements were made over the original unit:

Visibility of the rotary frame, the torque and windage were improved by a round transparent centrifuge case (Figs. 1 and 2); a cooling fan is installed on

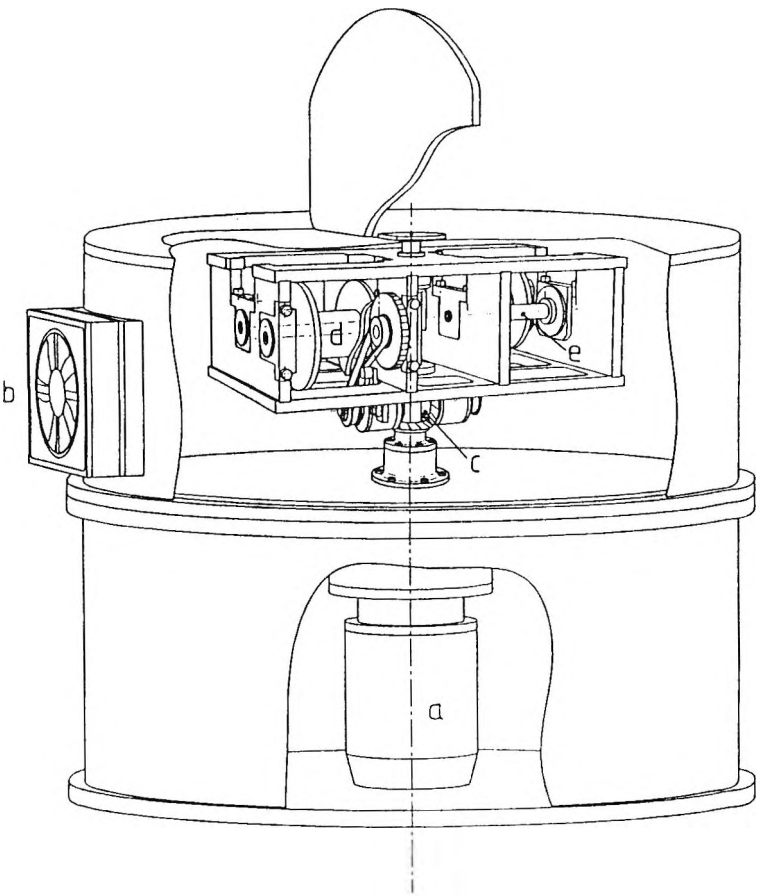

Figure 2. Schematic drawing of the side view of the apparatus. a: motor; b: cooling fan; c: spiral miter gear; d: column holder (off-center position); e: tube holder shaft (central position).

the centrifuge wall to maintain the steady ambient temperature (Fig. 2); the rotary frame is directly driven by the motor mounted at the bottom to improve the stability of the machine (Figs. 1 and 2); and tension of the toothed belts on the rotary frame is precisely adjusted by idler pulleys in both column positions (Fig. 3). These features substantially improved the performance of the apparatus.

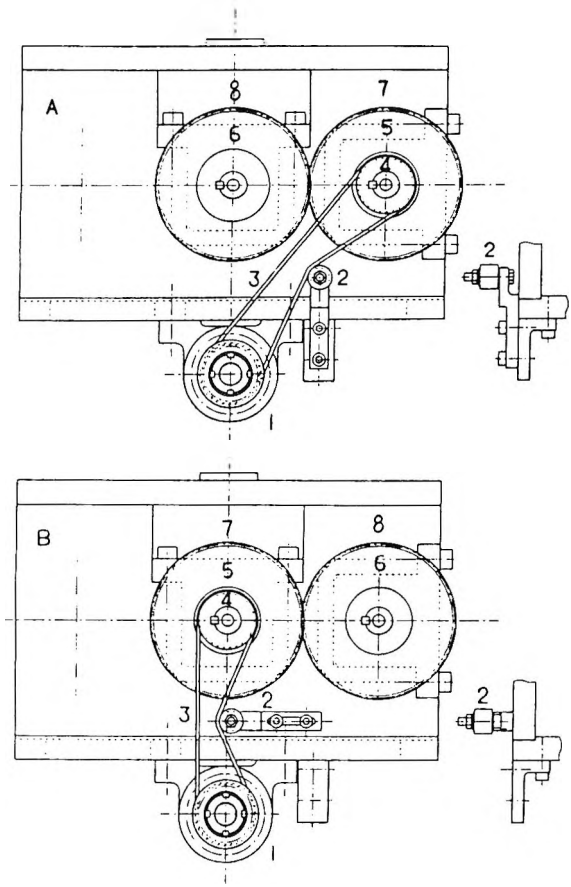

Figure 3. Driving mechanism of column holder and tube holder shaft and adjustment of the tension of the toothed belt by an idler pulley for off-center (A) and central (B) column positions. A set of miter gears at the bottom of the centrifuge axis (not shown) drives the coaxially mounted toothed pulley[1] on the rotary frame. This motion is conveyed through a toothed belt[3] to the identical pulley[4] on the holder. The gear engagement[5,6] between the holder[7] and the tube holder shaft[8] counterrotates the latter to unwind the flow tubes. The tension of the toothed belt is conveniently adjusted by an idler pulley[2] in both positions.

Preparation of the Column

The columns used in the present studies were a pair of eccentric coil assemblies described earlier.[4] Each assembly was prepared by winding a single

Table 1

Partition Coefficients of Proteins in Aqueous Two-Phase Solvent Systems

pH:	**9.2**	**7.2**	**9.4**	**7.3**	**7.0**	**6.6**
PEG 1000 (g/100g)	12.5	12.5	16.0	16.0	16.0	16.0
K_2HPO_4 (g/100g)	12.5	9.4	12.5	9.5	8.3	6.3
KH_2PO_4 (g/100g)	-----	3.4	-----	3.1	4.2	6.3
Protein						
BSA (68,000)	1.95	0.58	10.8	0.82	0.48	0.24
Ovalbumin (45,000)	1.26	0.96	3.16	1.36	1.21	0.91
Cytochrome C (12,000)	0.02	0.08	0.04	0.01	1.36	2.10
Hemoglobin (67,000)	25.0	1.16	33.7	2.10	0.97	0.36
Myoglobin (17,000)	0.59	0.30	0.71	0.13	0.09	0.09
γ-globulins	112	14.9	4.13	17.0	52.0	26.0
Trypsinogen (24,000)	1.79	1.02	5.41	1.46	1.32	1.24
Trypsin Inhibitor (20,100)	9.45	10.5	20.3	19.8	17.5	16.3
α-Chymotrypsinogen A (25,635)	6.00	5.01	29.1	11.8	9.50	6.48
apo-Transferrin	0.17	0.04	0.12	0.06	0.05	0.03
Carbonic Anhydrase (29,000)	4.46	2.53	9.88	5.00	3.67	1.83
Lactalbumin (14,200)	4.06	1.59	5.94	3.50	2.12	1.33

Partition coefficients were calculated from the absorbance of the upper phase divided by that of lower phase.

piece of 1 mm ID PTFE (polytetrafluoroethylene) tubing (Flon Kogyo Co., Tokyo, Japan) onto 7.6 cm long, 5 mm OD nylon pipes forming 20 units of serially connected left handed coils, which were then arranged around the holder with their axes parallel to the holder axis. A pair of identical coil assemblies was connected in series to obtain a total capacity of 28 mL.

Reagents

Polyethylene glycol (PEG) 1000 (M.W. 1000), cytochrome C (horse heart), myoglobin (horse skeletal muscle), ovalbumin (chicken egg), hemoglobin (human), trypsinogen (bovine pancreas), apo-transferrin (bovine), carbonic anhydrase (bovine erythrocytes), trypsin inhibitor (soybean), lactalbumin, α-chymotrypsinogen A (bovine pancreas), γ-globulins (human) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Another ovalbumin (chicken egg), monobasic and dibasic potassium phosphates and bovine serum albumin (BSA) were purchased from Wako Pure Chemicals, Osaka, Japan. All other reagents were of reagent grade.

Preparation of Two-Phase Solvent Systems and Sample Solutions

The compositions of PEG 1000 - potassium phosphate solvent systems are listed in Table 1. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated after two clear phases formed. Samples were prepared by dissolving each protein mixture in 0.4 mL of each phase of the two-phase solvent systems used for separation.

Measurement of the Partition Coefficients of Protein Samples

The partition coefficient of each protein was measured spectrophotometrically using a simple test tube procedure. Two milliliters of each phase of the equilibrated two-phase solvent system was delivered into a test tube and about 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the two clear layers formed, a 1 mL aliquot of each phase was diluted with 2 mL of distilled water and the absorbance was measured at 280 nm using a spectrophotometer (Model UV-160, Shimadzu Corporation, Kyoto, Japan). The partition coefficient (K) was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

CCC Separations of Proteins

For each separation, the coil was completely filled with the PEG-rich upper stationary phase and the sample solution (ca. 1 mL) was charged into the column through the sample port. Then, the phosphate-rich lower mobile phase was pumped into the column at 0.2 mL/min, while the column was rotated at 800 rpm in a counterclockwise direction. The effluent from the outlet of the column was collected in test tubes (0.4 mL/tube) using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each fraction was diluted with 2.5 mL of distilled water and the absorbance was measured at 280 nm.

Evaluation of Partition Efficiency

The partition efficiencies of protein separations were computed from the chromatogram and expressed in terms of theoretical plate number (N) and peak resolution (Rs). Both values are based on an assumption that each peak represents the distribution of a single component.

RESULTS AND DISCUSSION

Table 1 shows partition coefficients of various proteins in the PEG 1000 - potassium phosphate systems over a broad range of pH values. This data is useful for predicting the retention time of various protein samples.

Fig. 4A illustrates the separation of cytochrome C (2.5 mg), myoglobin (8 mg) and ovalbumin from Sigma Chemical Company (30 mg) by the X-axis CPC. In previous studies[4] it was found that ovalbumin from this source gave an extremely broad peak compared with those of the other two proteins. In the present experiment, the partition efficiency computed from the myoglobin peak is 101 TP (theoretical plates) and the resolution between cytochrome C and myoglobin is 1.2. The myoglobin and ovalbumin peaks, however, are once again only partially resolved due to the broad ovalbumin peak. The breadth of this peak is apparently due to heterogeneity of the ovalbumin as described in detail in the accompanying paper.[5] Fig. 4B illustrates the separation of cytochrome C, myoglobin and a sample of ovalbumin obtained from the Wako

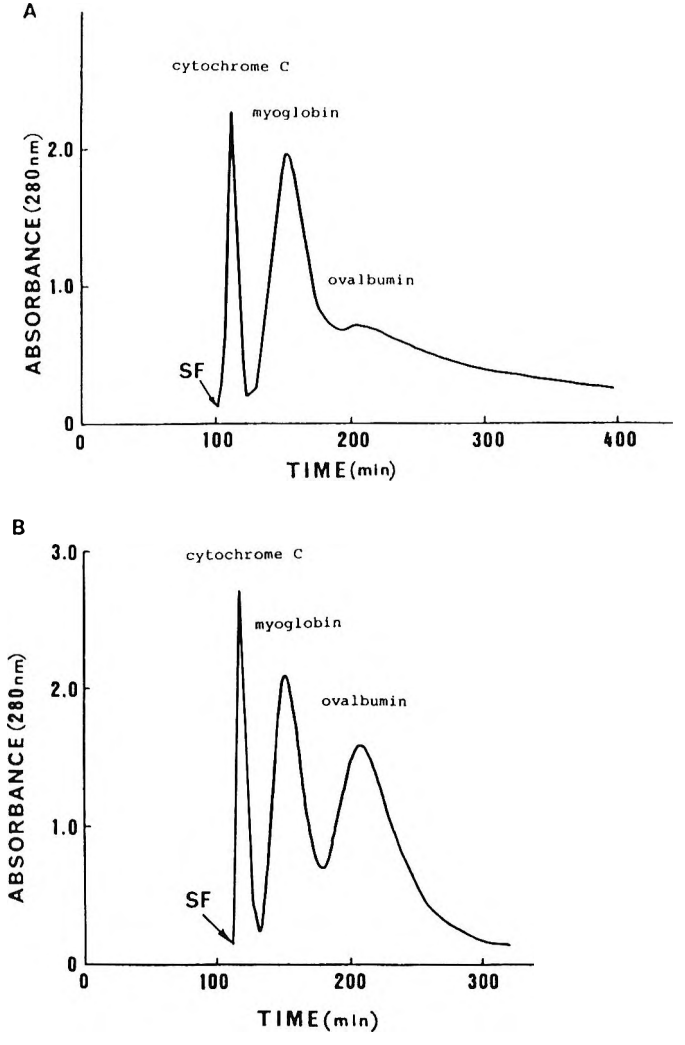

Figure 4. CCC separation of cytochrome C, myoglobin and two different commercial ovalbumin products by X-axis CPC.

Experimental conditions: Apparatus: X-axis CPC equipped with a pair of eccentric coil assemblies, 1 mm ID and 28 mL capacity; Sample: (A) cytochrome C (2.5 mg), myoglobin (8 mg), and ovalbumin (Sigma) (30 mg); (B) cytochrome C (2.5 mg), myoglobin (8 mg) and ovalbumin (Wako) (30 mg); Solvent system: 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate; Mobile phase: lower phase; Flow rate: 0.2 mL/min; Revolution: 800 rpm. SF = solvent front

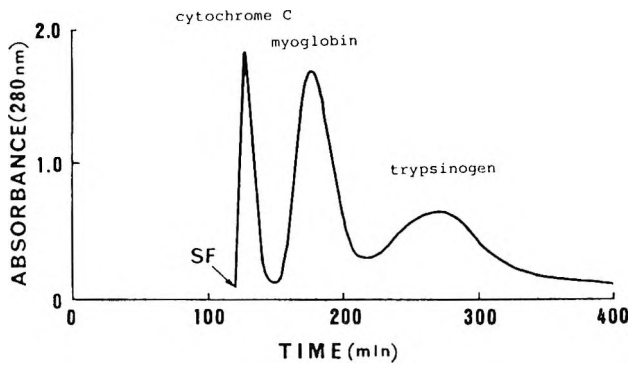

Figure 5. CCC separation of proteins by X-axis CPC.
Experimental conditions: Sample: cytochrome C (2.5 mg), myoglobin (8 mg) and trypsinogen (10 mg). Other experimental conditions are same as those described in Fig. 4 caption. SF = solvent front

Chemical Company under the same experimental conditions. The partition efficiency computed from the myoglobin peak is 183 TP, the resolution between the cytochrome C and myoglobin peaks is 1.1 and that between the myoglobin and ovalbumin peaks, 0.9. In this separation the resolution between myoglobin and ovalbumin peaks is much improved because the ovalbumin (Wako) consists exclusively of monomer in contrast with the ovalbumin from Sigma which contains both dimer and monomer forms.

Fig. 5 shows a separation of cytochrome C, myoglobin and trypsinogen where all components are well resolved. The partition efficiency computed from the myoglobin peak is 200 TP. The peak resolution between the cytochrome C and myoglobin peaks is 1.3 and that between the myoglobin and trypsinogen peaks, 1.1.

The overall results of the present studies indicate that the new prototype of the X-axis CPC yields an efficient CCC separation of 2.5 - 30 mg of proteins in several hours. The method provides a gentle environment for biological samples without loss or deactivation caused by the solid support matrix.

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A LARGE SCALE PROCESS FOR PACLITAXEL AND OTHER TAXANES FROM THE NEEDLES OF *TAXUS* X *MEDIA HICKSII* AND *TAXUS FLORIDANA* USING REVERSE PHASE COLUMN CHROMATOGRAPHY

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ABSTRACT

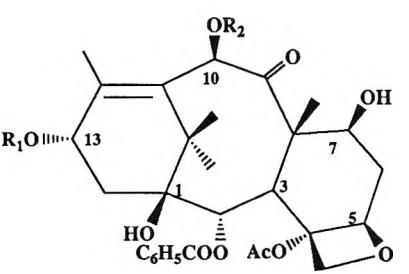
Although the renewable, needle bio-mass of *Taxus* x *media* Hicksii was proposed as the future source for paclitaxel in 1990, no details for the actual isolation of paclitaxel and other taxanes have been published, other than testing the extracts by HPLC analysis. Compared to the bark, the needle source poses additional problems, eg. chlorophylls, waxes and co-eluting taxanes, and thus, there is a need for an efficient method.

We have developed a new large-scale process based on a single reverse phase column, which takes the $CHCl_3$ extract of *T* x *media* Hicksii directly, and is eluted with acetonitrile (30-60%) in water. Paclitaxel and five other taxanes crystallize out from the fractions, and they are filtered and recrystallized. The taxanes that co-elute with paclitaxel are removed by ozonolysis and a short silica column to give paclitaxel in a yield of 0.012-0.015 % from the dry needles.

The needle mass of *T. floridana* was also processed by the same method on a pilot-plant scale. From the fresh needles, paclitaxel (0.01%) and 10-deacetyl baccatin III (0.06%) and two other taxanes are obtained. A discussion on the two plants as sources for paclitaxel is presented.

INTRODUCTION

On the basis of its demonstrated activity, paclitaxel **1** is currently viewed with much interest as an effective antitumor drug[1-3]. Paclitaxel is being produced at present by isolation from the bark of *Taxus brevifolia* (Pacific yew), although for future isolation, the bark will be replaced by a renewable source such as the needles of various *Taxus* spp., one such source being the cultivar, *Taxus* x *media* Hicksii (ornamental yew)[4,5]. In spite of such importance, there appear to be little or no published data, with details on the actual isolation of paclitaxel (and other taxanes) from these needles, other than testing of various plant samples and their parts by analytical HPLC.[6,7] In the extracts of this cultivar, paclitaxel co-elutes with several unrelated taxanes[8,9]. Minute amounts of four of these taxanes were isolated as two equilibrium (inseparable) mixtures and their structures elucidated by spectroscopic means[8].



1: $R_1 = C_6H_5CH(NHCOC_6H_5)CH(OH)CO$
$R_2 = CH_3CO$

7: $R_1 = R_2 = H$

The current large-scale process for paclitaxel from the bark of *T. brevifolia* is said to involve two or more chromatographic steps using silica and/ or Florisil columns. Isolation from the needles must pose additional challenges due to the

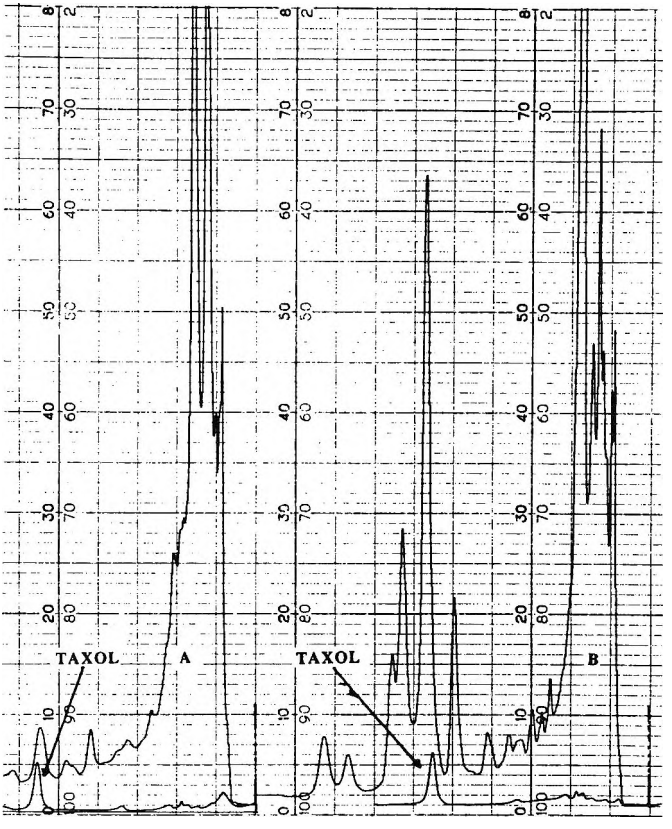


Figure 1. An analytical trace of the needle extract of A) *T.* Floridana, and B) *T. x media* Hicksii.

increased content of waxes, chlorophylls, carotenoids etc., as well as the co-eluting taxanes[8,9]. An analytical HPLC trace of the needle extract of *T.* x *media* Hicksii (Fig. 1A), shows the relatively large amounts of the co-eluting taxanes that accompany paclitaxel, and which must be separated from it.

We reported a new process for paclitaxel and related taxanes from the bark of *T. brevifolia* by the use of a single reverse phase column[10,11], both on a laboratory and pilot-plant scale, which was simpler and gave higher yields. The present paper deals with a pilot-plant scale chromatographic process for the needles of *Taxus* x

media Hicksii and of *Taxus floridana* (Florida yew) with details for the isolation of paclitaxel and several other taxanes, on a scale of 50-200 lb of plant material.

MATERIALS AND METHODS

Plant Material

The needles of *Taxus* x *media* Hicksii (200 lbs of the dried material) were kindly supplied by Hauser Inc., Boulder CO, during May/June 1993.

The needles of *Taxus floridana* were collected (50 lbs, fresh) from the campus of the University of Florida during April/May, 1993 and used fresh.

Step 1: Extraction and Concentration

The extraction was carried out using methanol, in stainless steel tanks of capacity 100 or 200 gal, equipped with lids that could be clamped tightly and with an outlet (1" dia) at the bottom for draining, fabricated by Pillsbury Alloy Fabrication, Inc. Jacksonville, FL. 200-250 Lbs of the needles could be extracted in the 200 gal tank and 70-90 lbs in the 100-gal tank. For several hours during the day, the extract was recycled by being pumped back into the tank as a rotating spray. Three to four such extractions, each running for a day, were carried out. The progress of the extraction was monitored by uv-absorbance at 275 nm.

The methanolic extract was concentrated under reduced pressure ($<30^0$ C) using a semi-continuously operated still with a receiving capacity of 220 gal. The extract was fed into a jacketed still-tank (100 gal) heated by steam, introduced into the jacket. The vapors were led into a vertical tube-type condenser (4 ft long and 1 foot in diameter with 1" pipes for the cooling water), where they were condensed by chilled water at 10^0 C and the condensate taken to a series of four receiving tanks, each of 55 gal capacity.

The system was kept under reduced pressure by using a vacuum pump, and the rate of distillation maintained by the steam introduced into the jacket as needed. Distillation was carried out until the volume of the concentrate reached 20-25 gal from the extract obtained from a 200 lb batch of the plant material.

Step 2: Solvent-Partition

Partition of the concentrated methanolic extract with chloroform ($CHCl_3$) was performed in 50 or 100 gal tanks, equipped with an air-driven stirrer. The concentrate from a 200 lb batch (25 gal) was stirred with water (20 gal) and $CHCl_3$ (or dichloromethane) (20 gal) for about 30 minutes and, after 2-14 hours to allow for any emulsion to clear, the solvent layer was drained off from the bottom into stainless steel containers. Two additional extractions were carried out by using 15 and 10 gallons respectively, of $CHCl_3$.

Concentration of the combined $CHCl_3$ extract under reduced pressure was carried out in an all-glass, steam heated, circulating evaporator (Ace Glass Co., Vineland, N.J.). The resulting concentrate (app. 2 gal) was further stripped of its solvent in a rotary evaporator to a thick syrup which was then poured into glass trays and converted to a brittle, dark green glassy solid, using a vacuum oven maintained at 35-40^0 C. This glassy solid represented a yield of 50-55 g per Kg of the dried needles of *Taxus* x *media* Hicksii and about 25 g per Kg of the fresh needles of *T. floridana*.

Optional Pretreatment of the Extract

In preliminary trials, attempts were made to "preclean" the $CHCl_3$ extract (remove chlorophylls, waxes etc.) through selective solvent partition methods, but they were not very satisfactory. The best method found to be was, passing a solution of the $CHCl_3$ extract in a mixture of methanol, acetone and water (7:1:2) through a column of C_{18} bonded reverse phase silica (15-35 micron particles, 3 g per gram of the sample).

The column was washed with the same solvent mixture until the uv absorbance became negligible ("Fraction I") and then with a mixture of methanol, ligroin and ethyl acetate (2:1:1) until the column and the wash became colorless ("Fraction II"). "Fraction I" (taxane-containing, 40% of the total) was concentrated to a syrup and applied to the column as described below. "Fraction II" (60% of the total) contained no paclitaxel but the lipophilic constituents: chlorophylls, carotenoids, waxes, steroids etc.

Subsequent trials showed that such pretreatment was not necessary and that the chloroform extract solid could be applied directly on to the column as described below.

Step 3: Chromatography

For the chromatography, stainless steel columns of two sizes were used: a 4"x 4' and a 6" x 6', both fabricated by Fluitron Inc. Ivyland, PA. The columns were rated for operation up to 200 psi. Each of the columns was equipped with a lid that could be sealed tightly and containing an inlet connection (0.5"). A three-way valve was attached to this inlet, to allow for feeding the column, for venting and for measuring the pressure. A circular stainless steel mesh was inserted securely at the lower end of the column to support a wad of glass wool. The lower, tapered end of the column was connected to a nipple (1/4 inch), to which was attached a rubber tube for collecting the column effluent into fraction bottles.

The columns were packed with C_{18} bonded silica (Spherisorb, 15-35 micron diameter, Phase Separations Inc., Norwalk CT) as a slurry in methanol. Approximately 3-4 Kg and 12-13 Kg of silica gel were required for the 4" and 6" columns respectively. After a thorough wash with methanol, the columns were equilibrated with 25% acetonitrile in water.

For running the 6" diameter column, the dark green glassy solid from the chloroform extract of the needles (2.3-2.7 kg) was dissolved in acetonitrile (AN, 5 L) and, while the mixture was being stirred with the equilibrated silica (1-2 L), diluted with water to make 20 L. After 15 minutes of stirring, the mixture was allowed to stand for 15-30 minutes and the clear supernatant siphoned off into another container. The slurry was applied to the column, followed by part of the supernatant, after which, the column was sealed. The remaining supernatant was pumped into the column using a diaphragm metering pump (Pulsa 680, Pulsafeeder Inc. Rochester, NY), maintaining a pressure of 30-80 psi. After the sample has been pumped, the column was eluted with a step gradient of 35, 40, 45, 50 and 60 % acetonitrile (AN) in water. The change of solvent was dictated by the results of TLC and HPLC analysis of the fractions, but generally, 40-50 L of each solvent was used. After this, the column was washed with methanol, followed by a mixture of ethyl acetate and ligroin (1:1) until the effluent was nearly colorless. Following this, the column was again washed with methanol and equilibrated with 25% acetonitrile in water, ready for reuse.

Fractions of approximately 2 L were collected and these were monitored by uv absorbance at 275 nm, TLC and analytical HPLC. The column fractions were allowed to stand at room temperature for 2-10 days, by which time, many of them showed a substantial degree of crystallization. Soon after, the crystals were filtered in groups, analyzed for purity and composition (TLC and analytical HPLC) and recrystallized from the appropriate solvent.

A similar procedure was used with the 4" diameter column, on which was applied approximately 500-700 g of the $CHCl_3$ extract solids dispersed in 5-6 l of the solvent mixture as was described above.

The chromatography was also run using aqueous methanol as the solvent, starting with 30 % methanol in water and continuing up to 65 %. The results obtained were comparable to those seen with the AN/ water system, except that the rate and extent of crystallization of the various components was less.

ANALYTICAL AND OTHER METHODS

Analytical HPLC was performed using two different units. For routine use, a combination of a Waters 501 pump, with a U6K injector, a 486 tunable absorbance detector and a Goerz Servogor 120 recorder was used. For determinations of purity and quantitative information on composition etc., a setup containing a Waters 600 E pump with gradient control system, a 996 photodiode array detector, a 717 autosampler, coupled with an NEC-386 computer and printer was used. Waters Millennium 1.1 program was used with the instrument. Standard columns (4.6 mm x 25 cm, Whatman, Partisil) packed with C_8 bonded silica, 5 micron diameter were used with either of the solvents: 50% AN/ water, or a 5:4:1 mixture of AN, water and methanol. The flow rate for both was 0.5 mL per min. For routine use, the detector was set at 254 nm, and for purity determinations using the photodiode array detector, the data were collected both at 254 and 220 nm.

Thin-layer chromatography was carried out using silica gel HF-60, 254+366 (EM Science/Fisher) and solvent systems consisting of acetone/$CHCl_3$ or MeOH/$CHCl_3$. Visualization was by a uv-lamp and by charring with 1 N H_2SO_4. Column chromatography was performed using silica gel (Fisher, 100-200 and 235-425 mesh) and Florisil (Fisher F-101, 100 mesh) were used, with a solvent sequence consisting of ligroin/$CHCl_3$, $CHCl_3$, 2-5% acetone and finally, 2-10% MeOH in $CHCl_3$.

Ozonolysis was carried out using the ozonizer made by Ozone Research and Equipment Company, Phoenix, AZ.

Melting points were determined on Fisher-Johns apparatus and are uncorrected. The following instruments were used to record the spectra described here: uv, Perkin-Elmer, Lambda 3B; ir, Perkin-Elmer, PE-1420; and nmr, Varian VXR-300, Varian Gemini-300 and General Electric QE-300 spectrometers. Mass spectra (FAB) were obtained on a Finnegan Mat 95Q spectrometer using a cesium gun operated at 15 Kev of energy.

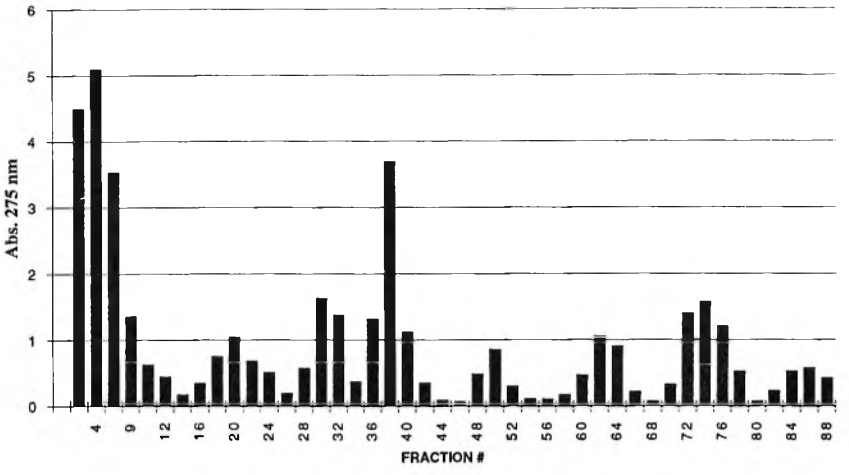


Figure 2. Column on *T. x media* Hicksii.

RESULTS

Elution Sequence for *Taxus* x *media* Hicksii:

The yield data given below are for the 4" columns, with 3 kg of the C_{18} silica and 600 g of the $CHCl_3$ extract obtained from 12 kg of the dry needles. Data for the 6" diameter column are presented below under the heading, paclitaxel.

After the sample was loaded, elution with 30-35% AN/ water gave large amounts of water-soluble, polar constituents, which accounted for the bulk of the uv absorbance at 275 nm. Elution with 35-40% solvent mixture gave a number of minor components, followed by a major component, identified as brevifoliol[12,13]. Washing with 45-50% AN/ water started the elution of taxanes I and II, followed by paclitaxel, all of which crystallized together from the fractions, as they stood for 2-8 days. From the eluates with 55-60% AN/ water, there was another region of crystallization, from which taxane III was isolated. As the column was being washed with MeOH, the fractions again deposited crystals, consisting of taxane IV. Further elution with methanol produced large amounts of crystals consisting of sitosterols. The progress of the column, followed by absorbance at 275 nm is shown in Fig. 2.

CHARACTERIZATION OF THE TAXANE CONSTITUENTS of *T.* x *MEDIA* HICKSII

Brevifoliol 2:

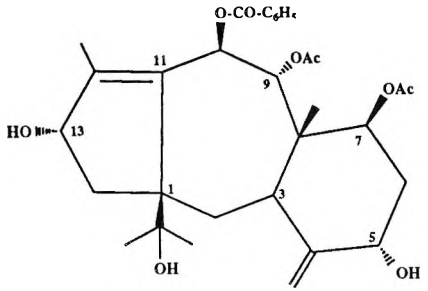
Fractions from the 40% AN/ water were partially concentrated, the solid which separated was filtered, and decolorized by dissolving in CH_2Cl_2 and passage through a column of Florisil (3 g per gram of the sample). The product was crystallized from acetone/ ligroin (2:1), yield, 2.5 g (0.02%). Brevifoliol **2** (also called brevitaxane[13]) is a colorless crystalline solid, m.p. 220-222^0 C (lit.[12], 200-205^0 C); specific rotation -27^0. Anal. calc for $C_{31}H_{40}O_9$: C, 66.89; H, 7.24. Fd. C, 67.13; H, 7.35.

Taxanes I and II 3 and 4:

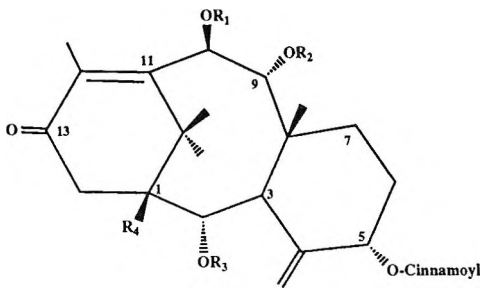
The crude crystals (25 g) consisting of **1**, **3** and **4** were processed by two methods. In one, a solution in $CHCl_3$/ ligroin (3:1, 250 mL) was chromatographed on silica (150 g), with the eluent changed to $CHCl_3$, 2% acetone, 5% acetone, 2% MeOH and 5% MeOH, all in $CHCl_3$. The mixture of **3** and **4** appeared first (2-5% acetone/ $CHCl_3$), followed by **1** (2% MeOH/ $CHCl_3$). Concentration of the appropriate fractions gave the mixture of **3** and **4** (12 g, 0.1%). (For the second method, see under paclitaxel)

A portion of this mixture of **3** and **4** (1 g) was applied to a C_{18} reverse phase column (25 g) in 40% AN/ water, and elution with 45 and 50% AN/ water. After one week, the fractions with the crystals were filtered in groups. Although **3** and **4** were separated, such that each contained the other to the extent of 10% or less, further recrystallizations gave worse mixtures, thus suggesting that isomerization was taking place in solution. Data on a crystalline 90:10 mixture of **3** and **4**: m.p. 136-138^0 C, specific rotation ($CHCl_3$): 214 (lit.[14], m.p. 163-165^0 C, rotation, 185, on a powder form of the sample); Anal. Calc. for $C_{31}H_{38}O_8$, H_2O: C, 66.89; H, 7.24. Fd. C, 66.51; H, 7.19.

The ^{1}[H] and the ^{13}[C] nmr spectra of the crystalline **3** and **4** gave evidence for mixtures of two compounds. From the spectral data, these two were identified as a mixture of 5-O-cinnamoyl-9-acetyltaxicin I **3** and 5-O-cinnamoyl-10-acetyltaxicin I **4,** as described by Chmurney et al.[9] from *Taxus* x *media* Hicksii, and by Appendino et al.,[14] from the needles of *Taxus baccata.* The latter authors obtained the two as amorphous powders by using HPLC and preparative TLC.



2: BREVIFOLIOL

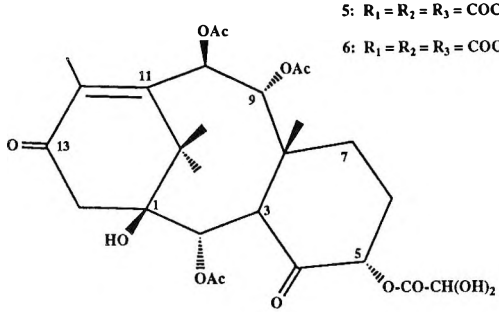


3: $R_1 = COCH_3$, $R_2 = R_3 = H$, $R_4 = OH$

4: $R_1 = R_3 = H$, $R_2 = COCH_3$, $R_4 = OH$

5: $R_1 = R_2 = R_3 = COCH_3$, $R_4 = OH$

6: $R_1 = R_2 = R_3 = COCH_3$, $R_4 = H$

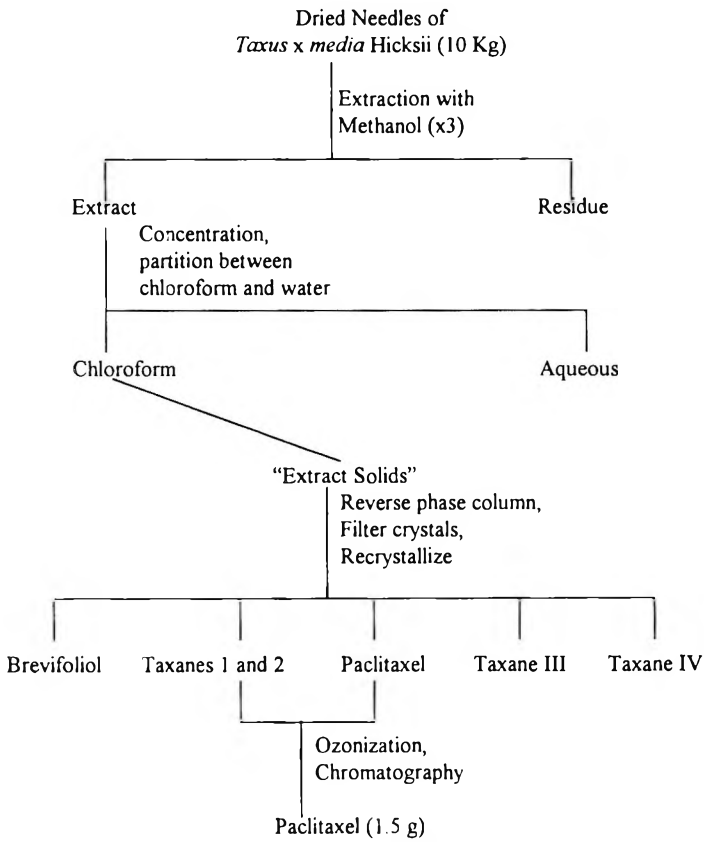


13

The mixture of **3** and **4** on acetylation (Ac_2O / pyridine, r.t. 6 h) gave the acetate, m.p. 238-241^0 C, the nmr spectrum of which showed it to be a single entity, unlike the starting mixture. It was identical with taxane III (**5** see below).

Taxane III 5:

Crude crystals of taxane III were filtered and recrystallized from acetone/hexane to give colorless needles, yield, 2.5 g (0.02%), m.p. 238-241^0 C; specific rotation ($CHCl_3$): 214 (lit[15]. 218); MS (FAB): 645 (M^- + Na), 623 (M^+ + H), 475 [(MH^+)-148 (cinnamoyl)], 415 (475-AcOH), 355 (415-AcOH), 295 (355 - AcOH). The spectral data showed that it is the 5-O-cinnamoyl 2a,9a,10b-triacetyl taxicin I (Appendino et al.,[14] and Baxter et al.[15]. Anal. Calc. for $C_{35}H_{42}O_{10}$, H_2O: C, 65.61; H, 6.92. Fd. C, 66.00; H, 6.72.



Scheme 1

Taxane IV 6:

This compound was recrystallized from acetone/ligroin, yield, 2.6 g, (0.02) %, m.p. 265-267^0 C; specific rotation ($CHCl_3$): 133 (lit.[15], 137); MS (FAB): 607 (MH^+), 459 (607 - 148 (cinnamate), 399 (459 - HOAc), 339 (399 - HOAc), 279 (339 - HOAc); Anal. Calc for $C_{35}H_{42}O_9$: C, 69.02, H, 7.03. Fd. C, 69.29, H, 6.98.

The nmr spectral data that it is 5(O)-cinnamoyl taxicin II-2a,9a,10b-triacetate (Appendino et al.[14] and Baxter et al.[15]).

Paclitaxel

After the SiO_2 chromatography described under **3** and **4**, the paclitaxel still contained these two (<5%) and for their removal, the sample (2.5 g) was ozonized in $CHCl_3$/ MeOH (9:1, 30 mL) at -70° C for 10-15 min. After completion of the reaction (HPLC), the mixture was treated with $(CH_3)_2S$ and let stand for 2-3 h. After concentration, the mixture was chromatographed on silica gel (65 g) in $CHCl_3$ Elution with 2-5% acetone in $CHCl_3$ gave pure **1**, which was crystallized from acetone/ligroin, yield, 1.5 g, (0.012 %), m.p. 219-221° C. Its spectral and physical data were the same as those of an authentic sample.

In subsequent trials, a second method of processing was used, involving a 6"-diameter column run on 2.5 Kg of the extract from 50 Kg of dried needles. The crude mixture of **1**, **3** and **4** (95 g) was processed by direct ozonization (without the intermediate silica column) in 30 g portions (9:1 $CHCl_3$/MeOH, 400 mL) and the product chromatographed on silica as before. By this method, the yield of pure **1** was 7.5 g (0.015 %),

A summary of the process used in connection with *T.* x *media* Hicksii is given in Scheme **1**.

Ozonolysis of 5:

The ozonolysis converted **3**,**4** and **5** to other products and to characterize one of these, ozonolysis was carried out on pure **5** (1 g), as described above. The product was crystallized from acetone/ligroin to give colorless needles, yield, 0.8 g., m.p.168-170° C, specific rotation (pyridine), 130; HR/MS: 569.2239, Calc. for $C_{27}H_{36}O_{13}$, 569.2234; [H] nmr ($CDCl_3$:

Taxus Floridana

The 45 lb (20 kg) batch of fresh needles gave 500 g of the $CHCl_3$ extract which was applied on to a C_{18} silica column (4" diameter). The solvent sequence used for elution was the same as that given under *Taxus* x *media* Hicksii.

After the initial polar (phenolic) components, the first taxane to elute, 10-deacetyl baccatin III (**7**) appeared with the 35% AN/ water and crystallized almost immediately from the fractions forming glistening plates. The next area which showed significant crystallization was the 40-45 % AN/ water eluate, and it contained a new 11(15→1)-abeotaxane, which was designated as taxiflorine (**8**).

With the 50 % AN/ water was eluted paclitaxel **1**, accompanied by baccatin VI (**9**).

Characterization and yield data on each of the preceding compounds are given below.

10-Deacetyl baccatin III 7

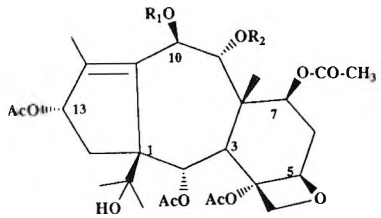
The crude crystals (17 g) after recrystallization gave 12 g (0.06 %) of pure 7, m.p. 232-234^0 C. The spectral and HPLC data were identical with those of an authentic sample[10].

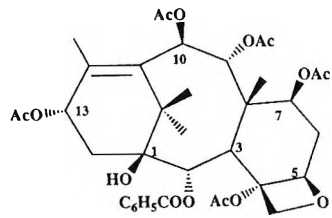
Taxiflorine 8

The crude crystals (2.5 g) were recrystallized from acetone/ligroin to yield colorless prisms, yield, 1.2 g (0.006 %), m.p. 254-255^0 C, specific rotation, [α]D23, -26.1. Anal. Calc. for $C_{35}H_{44}O_{13}$: C, 62.48; H, 6.59. Fd. C, 62.12; H, 6.63.

The ^{1}[H] nmr spectrum of pure crystalline (**8**) in $CDCl_3$ showed broad peaks with poor resolution, and double sets of peaks, The spectrum was sharper in DMSO, but it still had the extra peaks, ^{13}[C] spectrum also showed the extra peaks, which suggested the existence of either an equilibrium mixture[16], or a conformational inversion[17]. The spectrum gave evidence for one benzoate, four acetates, and an oxetane ring. The spectrum of the mono acetate (**10**) showed that it is a single compound. Although isomeric with baccatin VI (**9**), it was different from it. The striking difference between the ^{1}[H] spectra of the two was with the H-13 signal: in **10** at δ 5.60, and in **9**, at δ 6.3. A comparison with other related taxanes showed that in those with a 6-membered A-ring, the H-13 signal appears at 6.2-6.5[18], whereas in those with a 5-membered A-ring, as in the 11(15→1)-abeotaxanes, it appears at 5.4-5.7[19].

Positions 9 and 10 in **8** carry the free OH, and the benzoate. Of the signals at δ 6.30 and 5.90 in **8**, acetylation shifts one of these down-field from δ 5.9 to d 6.2, while the other stays essentially unchanged (δ 6.37). Since the allylic H-10 must be more down-field than H-9, the signal at δ 6.30 in **8** (and 6.37 in **10**) can be assigned to H-10 and the one at δ 5.9 in **8** (and 6.2 in **10**) to H-9, thus showing that the benzoate in **8** is at C-10 and the OH at C-9. This leaves positions 2 α,4 α,7 β and 13 α for the four acetate functions, which were confirmed by the COSY and HETCOR spectra.





8 TAXIFLORINE: $R_1 = C_6H_5CO$, $R_2 = H$

9 BACCATIN VI

10 $R_1 = C_6H_5CO$, $R_2 = CH_3CO$

11 $R_1 = R_2 = C_6H_5CO$

12 $R_1 = R_2 = CH_3CO$

Benzoylation of **8** gave the monobenzoate **11**, which was also a single entity as shown by the ^{1}[H] nmr spectrum, This structure **8** with the hydroxyl at 9, has the potential for intramolecular trans-esterification between the 10-benzoate as well as the 7-acetate[9,19]. The fact that both acetylation and benzoylation readily gave single products discounts the trans-esterification possibility as being responsible for the anomalous nmr spectrum of **8**. To verify if the appearance is due to an equilibrium between two rotamers, the spectrum was taken in DMSO-d_6 at temperatures ranging from -20° to 100°. At lower temperatures, the spectra were sharper and showed two sets of peaks. At higher temperatures the peaks coalesced into a single set, as well as became broad to the extent that some were barely seen. This behavior suggested that the conformational equilibrium between rotamers is responsible and that the presence of the 9-OH facilitates this process.

Baccatin VI 9

The fractions from the 50% AN/ water elution contained two components: **9** and **1**. The crude crystals from the first portion (3.5 g) containing mostly **9** were taken up in $CHCl_3$ (50 mL) and decolorized by passage through a column of Florisil (20 g). The solid was crystallized from acetone/ligroin to yield **9**, 1.6 g. With the additional amount from the purification of paclitaxel (see below), the yield was 1.95 g (0.01 %), m.p. 250-252° C, specific rotation, $[\alpha]^{D,23}$, -11°, MS (FAB), 714; Anal. Calc. for $C_{37}H_{46}O_{14}$ (714): C, 62.18; H, 6.49. Fd. C, 61.83; H, 6.49. The nmr spectral data agreed with those Senilh et al. [20]

Table 1

Protein NMR Spectra of Taxiflorine Esters

H AT	10	11	12
2	6,19, d, J=7,8	6.26, d, J=7.8	6.07, d, J=7.8
3	2.99, d, J=7.8	3.06, d, J=7.8	2.92, d, J=7.8
5	4.98, d, J=7.5	5.01, d, J=7.5	4.98, d, J=7.5
6	2.68, m	2.7, m	2.52, m
	1.84, m	1.84, m	1.84, m
7	5.52, m	5.64, m	5.49, t, J=7.8
9	6.32, d, J=10.8	6.48, d, J=10.8	6.04, d, J=10.8
10	6.44, d, J=10.8	6.72, d, J=10.8	6.27, d, J=10.8
13	5.62, t, J=7.8	5.64, m	5.61, t, J=7.8
14	2.30, dd, J=7.4, 14.2	2.34, dd, J=7.5, 14.7	2.30, m
	1.72, dd, 7.4, 14.2	1.78, m	1.72, m
16	1.16, s	1.24, s	1.15, s
17	1.19, s	1.21, s	1.13, s
18	1.72, s	1.72, s	1.83, s
19	1.64, s	1.95, s	1.66, s
20	4.5, 4.42, d, J=7.9	4.52, 4.44, d, J=7.2	4.47, 4.38, d, J=7.5
Ph(2', 6')	7.93, d	u	--
Ph(3', 5')	7.45, t	7.24, m	--
Ph(4')	7.62, t	7.37, m	--
Ph'(2",6")	--	7.63, d, J=7.2	--
Ph'(3",5")	--	7.24, m	--
Ph'(4")	--	7.37, m	--
OAc	2.02, s	2.14, s	2.11, s
OAc	2.14, s, (2x)	2.05, s	2.1, s
OAc	1.86, s	--	2.08, s
OAc	1.8, s	--	2.03, s
OAc	--	--	2.01, s
OAc	--	--	1.95, s

Paclitaxel 1

The crude crystals (4.5 g) from the second part of the peak, which was mostly **1** but had some **9**, were taken up in $CHCl_3$ (60 mL) and applied to a short column of Florisil (40 g). Elution with $CHCl_3$ gave **9** and subsequent elution with 2-5 %

acetone/ $CHCl_3$ gave **1** which was recovered and crystallized from acetone/ ligroin, yield, 1.98 g (0.01 %), m.p. 220-222^0 C. The spectral and HPLC data showed that it was identical with paclitaxel.

DISCUSSION

Taxus x *media* Hicksii

Isolation of paclitaxel and other taxanes has been carried out on a pilot-plant scale from the needles of *Taxus x media* Hicksii. Briefly, methanol extraction, concentration and partition of the concentrate between water and $CHCl_3$, followed by concentration of the $CHCl_3$ layer gave approximately 5% of the $CHCl_3$ extractives, from the dried needles. This extract is applied directly on to a reverse phase (C_{18}) silica column. For example, on a 6"x 6' column, 2.5 kg of the extract (from 50 kg of the dry needles) can be loaded using the 25% AN/ water medium. The column was then developed with a step gradient: 30-60% AN/ water (in increments of 5%), during which, most of the taxanes of interest were eluted. The column was then washed free of the chlorophyll, waxes etc., re-equilibrated and reused.

It is generally understood that, compared to the bark, large-scale processing of the needles of *T.* x *media* Hicksii would have more challenges because of the presence of the lipophilic chlorophylls, carotenoids and waxes, as well as unrelated taxanes that co-elute with paclitaxel[8,9] (Fig. 1A). For this reason, methods such as fractional extraction with nonpolar solvents[21], or extraction of the plant material with aqueous alcohol[22] have been proposed. If one must employ such a pretreatment step, the procedure given here: passage through a reverse phase column in a methanol/ acetone/ water mixture (see methods) appears to be the most efficient and convenient.

However, our experience has shown both with the bark extract (*T. brevifolia)*[10,11] and with the needle extract (3 species of *Taxus*), that the chlorophylls or waxes present in the needles pose no problem with the column performance. One explanation may be that the C_{18} bonded silica gel seems to behave as a long-chain hydrocarbon and thus, exhibits great affinity for these non-polar components, which appear to be taken up by the silica and almost "dissolved" in the gel matrix. Thus, the waxes are not present as an insoluble powder at the top of the column to block the column flow. Because of the apparently high distribution coefficients for the chlorophylls and waxes as compared to the taxanes, in favor of the silica and against the 30-60% AN/ water mixtures (or even a 7:1:2 methanol/acetone/water mixture as

seen above), they remain on the column until all of the taxanes are eluted. After the elution of the taxanes is over, the column can be completely stripped of these lipophilic components, then equilibrated with the AN/ water and made ready for reuse.

It is important to distinguish the process described here, which uses a reverse phase column, from being characterized as an "HPLC" process. Although reverse phase column chromatography is used extensively in analytical HPLC-methodology, its use in preparative work is relatively less frequent, and when it is used, the methods are carried out almost as an extension of the analytical technique. For example, the compounds to be separated have already been purified by other means, the columns are selected to reflect the sample / adsorbent ratio of 1:1000 or (much) more, the sample is applied in a minimum volume as a clear solution, and the columns are usually run at high pressures (500-1500 psi).

In contrast, applying a crude extract of the plant, which is essentially insoluble in the mobile phase, on the column; using a sample / adsorbent ratio of 1:5, and a large sample volume, all show that the method given here is clearly not an extension of the HPLC protocol. In spite of these unfavorable changes, the column appears to perform normally, i.e., giving acceptable resolution (Fig. 2, the compounds being eluted in the order of their retention times), a good flow rate at medium pressures (2-4 l per hour at 40-120 psi) and for many of the products, the purity increases from 1% or less to 50% or more, after this single reverse-phase column.

As an illustration of this last point, a very important advantage of the reverse phase column as described here is that when the fractions are allowed to stand for about a week, a number of the taxanes crystallize out directly, leaving many impurities in solution. Based on the HPLC analysis of the fractions before and after the crystallization, it appeared that the degree of crystallization was better than 75%. Thus, the crystals, although still relatively crude, are significantly purer than what one might obtain by concentration of the whole fraction to dryness, or extraction of the fraction with a solvent (eg. dichloromethane) and concentration. In many cases (eg. compounds **2**, **5**, **6** and **7** from *T.* x *media* Hicksii, and **8, 9** and **1** from *T. floridana*), further purification of the crude crystals involves only a recrystallization, or a simple "filtration" type column and no additional chromatography. Thus, this advantage of crystallization is lacking in the normal phase silica column chromatography, with commonly used solvents such as dichloromethane or acetone.

Next to the chlorophyll/ wax problem, the problem of co-eluting taxanes is of importance as far as the yields of paclitaxel from the needles of *Taxus* x *media* Hicksii are concerned. These taxanes, primarily **3** and **4**, occur to the extent of 4-6

times as much as paclitaxel in these needles (Fig. 1A). Because of their higher uv-extinction values (due to the cinnamate moiety), even trace amounts of these appear as significant impurities in the usual uv-based HPLC analyses. Thus, to reach the 99+% level of purity for paclitaxel, at least two, if not three, successive columns are needed. Hence, as an alternative, ozonization of the crude mixture of **1**, **3** and **4** was used here for the final purification, in which **3** and **4** are converted to more polar products, which are more readily separable from **1**. The ozone also causes extensive bleaching of the sample, thus assisting further in the purification.

To learn of the identity of the product after ozonization, the reaction was carried out with pure **5** (acetate of **3** and **4**). The analytical and spectral data of the crystalline product indicated the presence of the C=O function at 4 and a hydrated glyoxalyl ester group at C_5.

Thus, the process described here for the needles of *Taxus* x *media* is simpler because, the extract does not require pretreatment, essentially a single column gives crystalline products and, because of fewer steps, the yields are higher than those reported earlier[4]. The reverse phase column can be run on a large scale economically, because of the high capacity loading of the column, re-usability of the adsorbent and the solvent(s).

T. floridana

In the earlier studies conducted to examine various species and cultivars of *Taxus* as sources for paclitaxel[4,5,23], the species *T. floridana* has received scant, if any attention. As a source of paclitaxel, this plant must be counted as one of the important ones because, in several tests by actual isolation, both on laboratory and pilot-plant scale, the yield of paclitaxel has been consistently 0.01% on a fresh basis, and hence, potentially 0.02% or more on a dry basis. To make the plant even more appealing, 10-deacetyl baccatin III can also be isolated, again consistently in yields of 0.05-0.06% from the fresh leaves.

Additionally, this plant yields two other compounds: baccatin VI (**9**) and taxiflorine (**8**), both of which contain the oxetane ring and hence can serve as precursors for active compounds; **9** to produce 9-dihydropaclitaxel[24] type analogues, and **8**, to produce analogues with the 11(15→1)-abeotaxane skeleton. This is not the case with *Taxus* x *media* Hicksii, where the unrelated (4/20 methylene) taxanes which have no declared use at present, exceed paclitaxel in yield by 4-6 fold. Finally, isolation of paclitaxel and the other useful taxanes is considerably easier from *T. floridana* than that of paclitaxel alone from *Taxus* x

media Hicksii, because there are no co-eluting taxanes in *T. floridana* (Fig. 1A and B) and paclitaxel and the other three components are readily obtained pure after a single reverse phase column.

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EVALUATION OF A NEW PEPSIN ENZYME CHIRAL STATIONARY PHASE FOR THE OPTIMIZED SEPARATION OF SEPROXETINE (S-NORFLUOXETINE) FROM R-NORFLUOXETINE

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ABSTRACT

A thorough analysis of a new commercially available pepsin chiral stationary phase (CSP) has been completed using seproxetine (S-norfluoxetine) hydrochloride bulk drug substance and R-norfluoxetine hydrochloride as the test analytes. Chromatographic properties of this new Ultron ES-Pepsin column were investigated by varying key mobile phase parameters (pH, flow rate, buffer strength and organic concentration), column temperature and sample loading. After observing and plotting changes in retention, resolution and

theoretical plates based on corresponding variation in these parameters, it is possible to choose conditions for the separation that are optimum and robust. The subsequent method validation demonstrated acceptable precision, linearity, recovery, selectivity, limit of detection and ruggedness for the determination of R-norfluoxetine in seproxetine hydrochloride bulk drug substance.

INTRODUCTION

Pharmaceutical compounds are routinely evaluated for bulk drug substance purity, including quantitation of the unwanted enantiomer for chiral compounds. Typically, two analytical test methods are used. An achiral method is developed to determine impurities, including process related compounds and degradation products.

A second method, preferably a direct separation using a CSP, is used to determine the unwanted enantiomer. The technology of CSPs is an area of continual improvement and development, especially in light of increased regulatory requirements in the marketing of pharmaceutical products with stereogenic centers. In 1992, the FDA issued a formal guideline requiring compounds containing stereogenic centers be recognized and the activity of specific enantiomers be identified.[1] In response, the research community has introduced a wide selection of CSPs for high performance liquid chromatography (HPLC), including Pirkle type, cellulose-based, inclusion complexes, ligand exchange, macrocyclic antibiotics and protein bonded phases.[2-13] Protein-based columns have gained much attention due to their broad scope of chiral recognition and compatibility with aqueous mobile phases. Several protein CSPs are available as albumins, glycoproteins and enzymes, including bovine serum albumin (BSA), human serum albumin (HSA), α-acid glycoprotein (AGP), ovomucoid (OVM), avidin, fungal cellulase and α-chymotrypsin.[14-20]

A new CSP utilizing pepsin, a digestive enzyme, was investigated in this report. Pepsin is an acid protinase enzyme found in the gastric fluids of mammals.[21] The enzyme is bound to a 5 μm aminopropyl silica with a pore size of 120 Å using N,N-disuccinimidyl carbonate. Pepsin has an isoelectric point of less than 1, and is therefore targeted for the enantiomeric analysis of basic compounds. In this evaluation, pepsin as a CSP is analyzed for the separation of seproxetine from its unwanted enantiomer, R-norfluoxetine. The

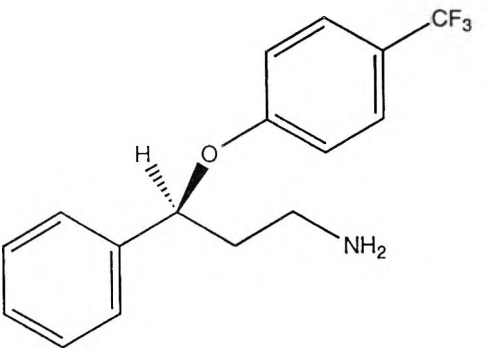

Figure 1. Structure of seproxetine.

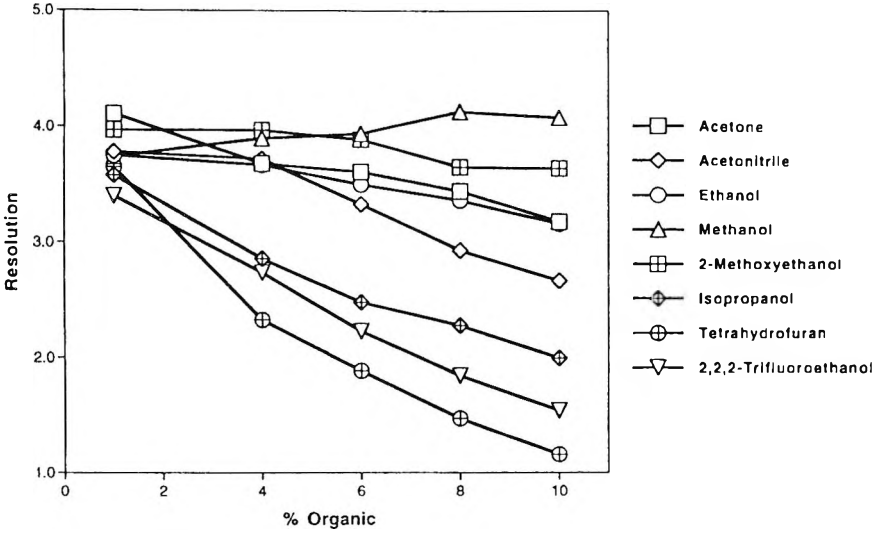

Figure 2. Th effect of organic modifier on the resolution of seproxetine and R-norfluoxetine. Aqueous mobile phase component: 20 mM KH_2PO_4 buffer.

structure of seproxetine is shown in Figure 1. Chromatographic conditions for the separation of seproxetine from R-norfluoxetine on the ES-Pepsin column were optimized, and final method conditions were validated.

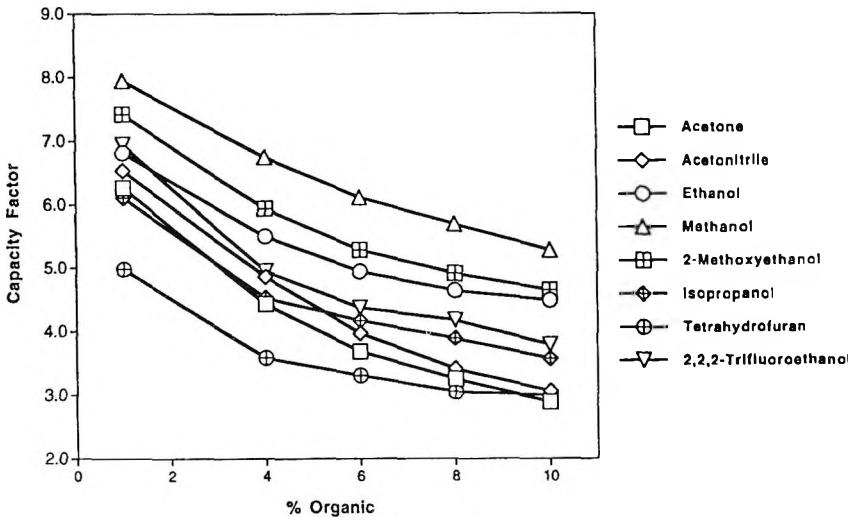

Figure 3. The effect of organic modifier on the capacity factor (K') for R-norfluoxetine. Aqueous mobile phase component: 20 mM KH_2PO_4 buffer.

EXPERIMENTAL

The Ultron ES-Pepsin column (15 cm x 4.6 mm) was obtained from Mac-Mod Analytical (Chadds Ford, PA) and manufactured by Shinwa Chemical Industries (Tokyo, Japan) for Rockland Technologies, Inc. (Newport, DE). A Hewlett-Packard (Wilmington, DE) 1050 series autoinjector and pump were used with an Applied Biosystems 1000S diode array detector (Foster City, CA). The column temperature was maintained with a Model 7950 Column Chiller from Jones Chromatography (Lakewood, CO). Chempure™ Brand organic modifiers distributed through Curtin Matheson Scientific (Houston, TX) were purchased. Ethanol (200 proof) was purchased from Quantum Chemical Corporation (Tuscola, IL) and the 2-methoxyethanol and 2,2,2-trifluoroethanol were obtained from Sigma-Aldrich Company (Milwaukee, WI). The potassium phosphate monobasic salt (KH_2PO_4) was obtained from Mallinckrodt® (Paris, KY) and the 85% o-phosphoric acid (H_3PO_4), 1.0 N hydrochloric acid and 0.1 N sodium hydroxide were acquired from Fisher Scientific (Fair Lawn, NJ). The water was deionized and filtered through a Millipore Milli-Q™ water purification system (New Bedford, MA). Seproxetine hydrochloride, R-norfluoxetine hydrochloride and fluoxetine hydrochloride were synthesized and degradation products were isolated at Eli Lilly and Company (Indianapolis,

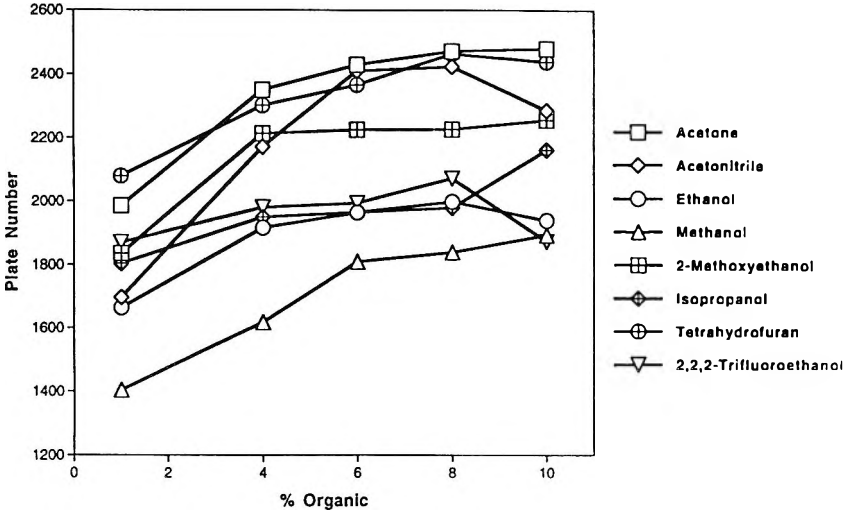

Figure 4. The effect of organic modifier on the plate numbers for R-norfluoxetine. Aqueous mobile phase component: 20 mM KH_2PO_4 buffer.

IN).[22-24] Unless otherwise noted, the sample for injection consisted of a mixture of 0.03 mg/mL of seproxetine plus 0.02 mg/mL of R-norfluoxetine prepared in 20 mM KH_2PO_4 buffer, the flow rate was 1.0 mL/min. and the column temperature was ambient. An injection volume of 10 μL and UV detection set at 225 nm were used throughout this study.

METHOD OPTIMIZATION

Effect of Organic Modifier

The Ultron ES-Pepsin column was evaluated for the separation of seproxetine and its unwanted enantiomer, R-norfluoxetine, using the eight organic solvents: acetone, acetonitrile, ethanol, methanol, 2-methoxyethanol, isopropanol, tetrahydrofuran and 2,2,2-trifluoroethanol. The organic composition was increased from 1 to 10% with a buffer comprised 20 mM KH_2PO_4 with no pH adjustment. The resolution of the enantiomers was greater than 1.5 (baseline) for all the organic modifiers tested as shown in Figure 2.

Generally, resolution decreased with increasing organic composition in the mobile phase. In the case of methanol, a resolution of approximately 4.0 was maintained as the methanol composition was increased. The effect of organic mobile phase composition on the capacity factor (K') for the first eluting peak, R-norfluoxetine, is shown in Figure 3. The use of methanol resulted in the greatest overall K' values whereas tetrahydrofuran yielded the lowest. The effect of changing organic mobile phase composition on the number of theoretical plates is shown in Figure 4. Acetone resulted in the highest theoretical plate numbers and methanol the lowest. 2-Methoxyethanol was an excellent organic modifier when considering the small resolution change from 1 to 10% organic modifier, the number of theoretical plates and overall peak shape. However, this solvent is not commonly used for chromatographic work due to safety concerns, expense and questionable long-term effects on the column longevity. The spectral properties of acetone and 2,2,2-trifluoroethanol make them inappropriate choices when considering the need for trace analysis of the unwanted enantiomer (R-norfluoxetine) with an UV maximum at 225 nm. Although methanol resulted in the lowest theoretical plate numbers of all the organic modifiers tested, it exhibited comparable resolution and K' values to 2-methoxyethanol and the advantage of its consistent resolving capability is important, especially when developing a rugged and universal method. A mobile phase comprised 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer was used for further optimization of other parameters. This composition produced substantial resolution, along with reasonable theoretical plates and a desirable K' value.

Effect of pH

Hydrochloric acid and sodium hydroxide were used to attain the various pH adjustments of the 20 mM KH_2PO_4 buffer solutions. The resolution of the enantiomers was very sensitive to changes in pH. The resolution increased with increasing pH as did K' as illustrated in Figure 5. In fact, pH appears to be the most selective parameter to enhance the chiral separation for this column. The large resolution values between seproxetine and R-norfluoxetine over a wide pH range allowed for some flexibility in choosing the pH for this method but the tradeoff is an increasing K' value with increasing resolution. Generally accepted guidelines for determining adequate retention is to establish a K' range from 2 to 20; thus from the data in Figure 5, the pH range from 3.9 to 5.3 would be acceptable. Other considerations, such as, sharp peaks for adequate detection, large resolutions for robustness and shorter run time for multiple sample analyses, are also important. To keep the analysis time under 30 minutes and resolution above two, any pH from above 3.8 to below 4.9

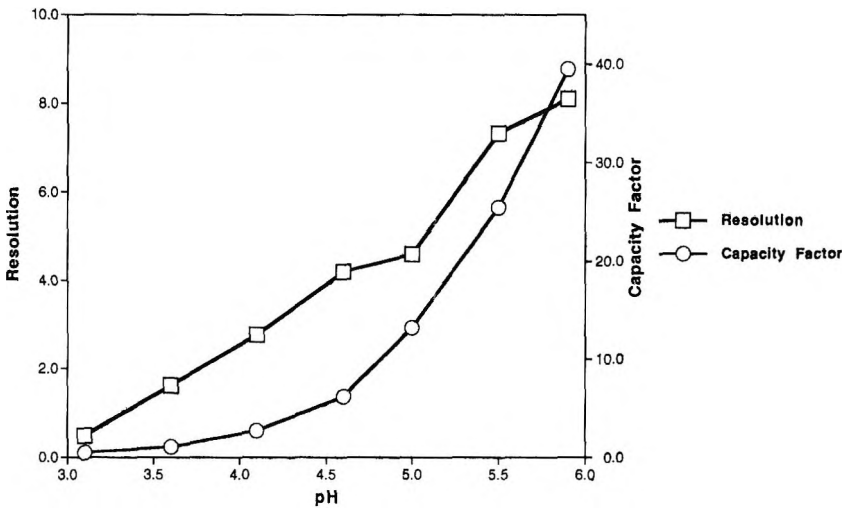

Figure 5. The effect of pH on the resolution of seproxetine and R-norfluoxetine and on the capacity factor (K') for R-norfluoxetine. Mobile phase: 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer (pH adjusted with either 1.0 N HCl or 1.0 N NaOH).

would be sufficient. Using 20 mM KH_2PO_4 buffer resulted in a pH of approximately 4.6 and therefore eliminated a procedural step for further pH adjustment; thus a simpler and more rugged method could be obtained. Further evaluation of other parameters used 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer without pH adjustment.

Effect of Buffer Concentration

Potassium phosphate monobasic was the only buffer tested in concentrations ranging from 1 to 40 mM for this evaluation of the ES-Pepsin column. Although varying the buffer concentration did not have the striking effect that pH variations produced, there were trends in the resulting chromatography. The concentration resulting in the maximum resolution was from 5 to 10 mM. The best peak shape, however, was obtained using 10 to 20 mM KH_2PO_4 concentrations. The total resolution change over the buffer concentration range tested was approximately 0.7 with resolution decreasing below 5 mM or above 10 mM (Figure 6). The effect of buffer concentration on column plate number peaked at an approximate concentration range from 10 to 20 mM. The number of theoretical plates quickly decreased when the buffer

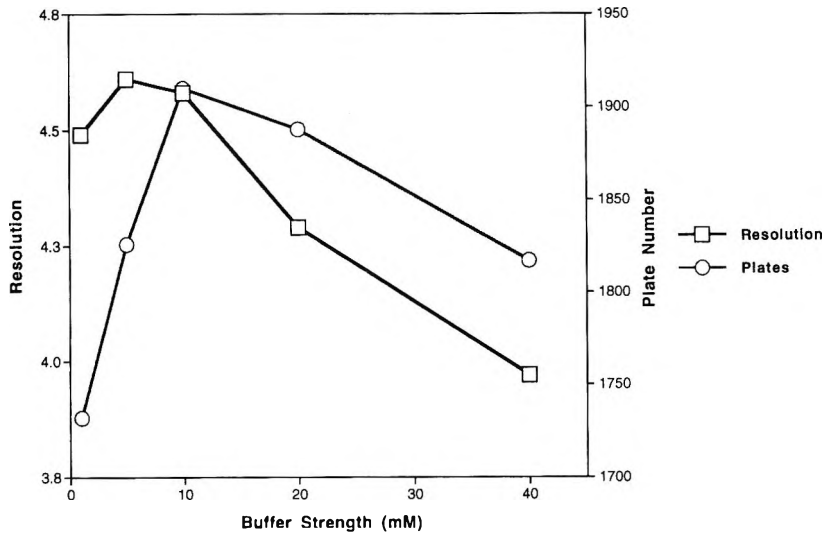

Figure 6. The effect of KH_2PO_4 buffer strength on the resolution of seproxetine and R-norfluoxetine and on the plate numbers for R-norfluoxetine. Organic mobile phase component: 6% methanol.

strength drops below 10 mM (Figure 6). Likewise, peak shape deteriorated and tailing increased at buffer strengths below 5 mM. Capacity factor values decreased with increasing buffer concentration, and therefore buffer strength can be used for retention control. Reasonable K' values were seen throughout the range tested. The large resolution value for the separation of seproxetine from R-norfluoxetine minimized the importance of varying buffer concentration. Such high resolutions may not always be obtained, therefore this parameter could be used to optimize separations at or near baseline resolution. A 20 mM KH_2PO_4 buffer concentration for this analysis was used due to a reasonable K', good peak shape and a more rugged working range.

Temperature

Holding all other parameters constant, a mixture of seproxetine and R-norfluoxetine was injected onto the ES-Pepsin column conditioned at temperatures ranging from 10°C to 35°C in increments of 5°C. The resolution and theoretical plates increased with increasing temperature as illustrated in Figure 7. The resolution only changed about 0.4 units over the range tested.

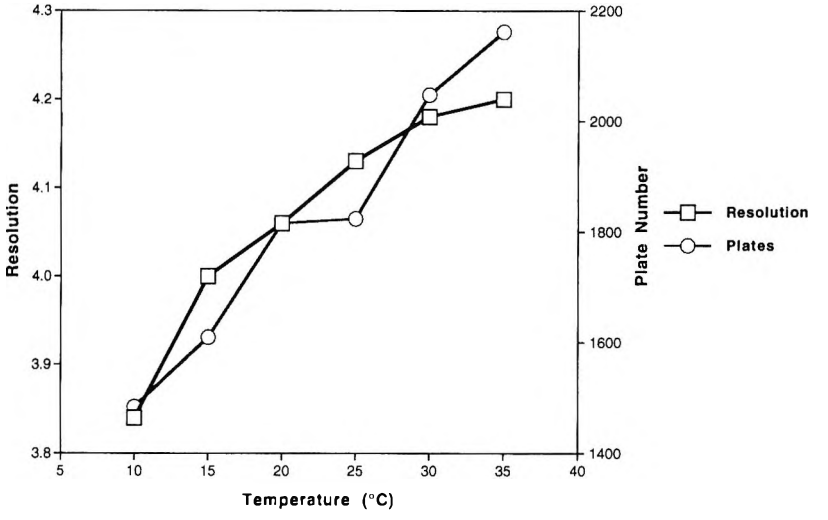

Figure 7. The effect of temperature on the resolution of seproxetine and R-norfluoxetine and on the plate numbers for R-norfluoxetine. Mobile phase: 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer.

Column temperature was shown to have a greater impact on theoretical plate values than either mobile phase composition or buffer strength. Both peak tailing and K' values decreased linearly with increasing column temperature. In general, higher temperature provided better chromatography, however, the results did not account for the potential of decreased column life at prolonged elevated temperatures. Although there was an advantage in using an elevated column temperature, this was outweighed by the need to obtain a simple and rugged analytical method. If resolution or other factors were not adequate, altering the column temperature was another parameter that could be used for affecting method optimization. For the purposes of optimizing a method for the enantiomeric analysis of seproxetine and R-norfluoxetine, ambient conditions were selected.

Flow Rate

A sample mixture of seproxetine and R-norfluoxetine was injected at different flow rates ranging from 0.2 mL/min. to 1.0 mL/min. All other parameters described earlier were held constant during this analysis. As expected, the resolution and theoretical plate numbers decreased with

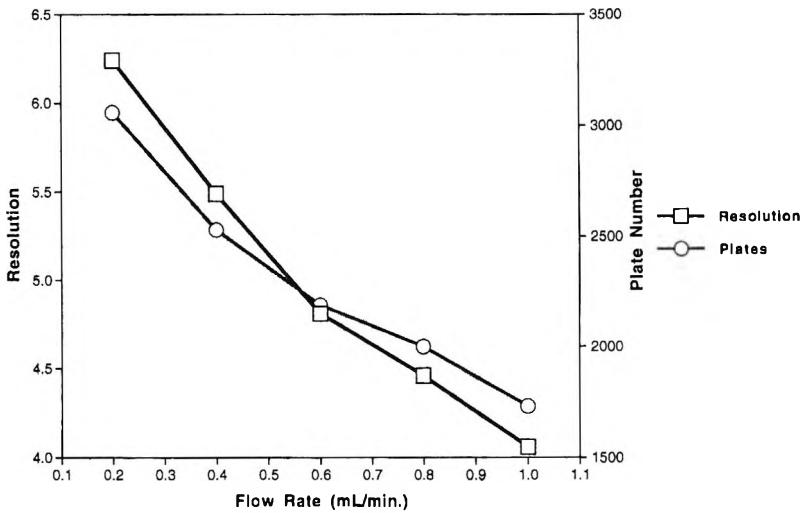

Figure 8. The effect of flow rate on the resolution of seproxetine and R-norfluoxetine and on the plate numbers for R-norfluoxetine. Mobile phase: 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer.

increasing flow rate as illustrated in Figure 8. Although a lower flow rate would yield better resolution, the tradeoff was sample analysis time; at a flow rate of 0.2 mL/min. the seproxetine peak eluted at 95 minutes. In the case of these enantiomers, the excellent resolution obtained under various conditions allowed for the higher flow rate of 1.0 mL/min. in light of a quicker analysis time.

Sample Loading

Consistent with other protein-based columns, the ES-Pepsin column has a relatively low sample loading capacity. The recommended analyte injection is 1 µg on column. Sample loading was tested by injecting five samples of the enantiomers from 1 µg to 6 µg onto the column. As the analyte concentration was increased over this range, resolution decreased by 70%, tailing approximately doubled and the number of theoretical plates decreased by 30%. Based on experimental data for seproxetine, a maximum sample loading of 1.5 µg on the ES-Pepsin column was the limit before resolution, tailing and plate number began deteriorating.

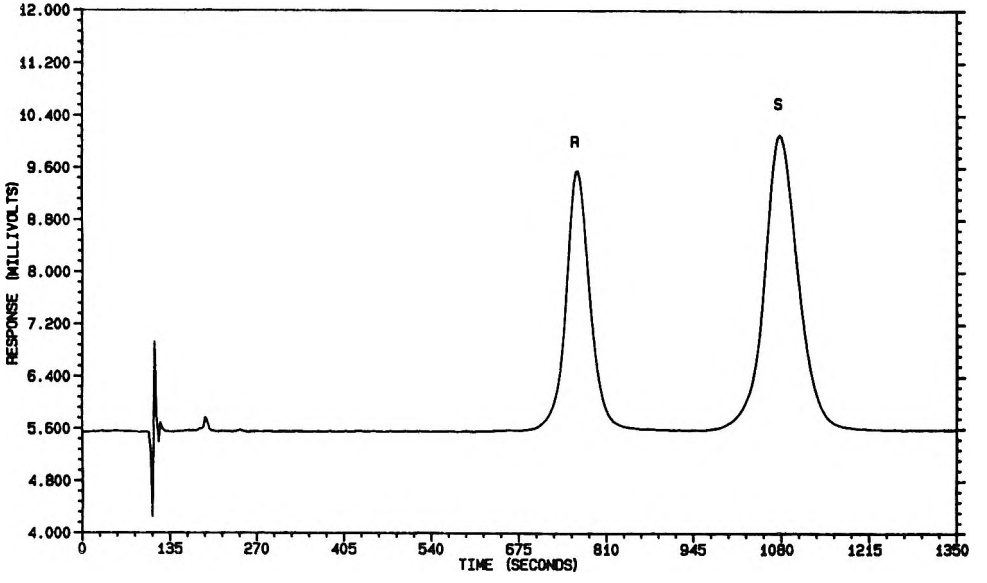

Figure 9. A sample chromatogram of the separation of seproxetine and R-norfluoxetine. Mobile phase: 6:94 (v/v) methanol /20 mM KH_2PO_4 buffer; detection: UV absorbance, 225 nm; flow rate: 1.0 mL/min.; injection volume: 10 µL; sample: 0.05 mg/mL of a 3:2 mixture of seproxetine:R-norfluoxetine; temperature: ambient. Peak identification: (R) = R-norfluoxetine, (S) = seproxetine.

METHOD VALIDATION

HPLC Conditions

The mobile phase was comprised 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer. A flow rate of 1.0 mL/min., UV detection at 225 nm and sample injections of 10 µL were used with the Ultron ES-Pepsin column. A sample chromatogram of a 0.05 mg/mL solution (3:2; seproxetine:R-norfluoxetine) is shown in Figure 9.

Linearity

It is usual practice to perform linearity determinations over a wide range of sample concentrations to fully assess the linear dynamic range of the

detection system. The linearity of the method was determined by injecting 20 samples prepared from seproxetine hydrochloride and R-norfluoxetine hydrochloride which were serial dilutions from stock solutions of these compounds. The samples encompassed a range of 0.01 - 406 μg/mL for each of seproxetine and R-norfluoxetine. The linear working range for the method was determined to be 0.1 - 150 μg/mL. This range included 16 samples and resulted in a correlation coefficient of 0.9999 for both seproxetine and R-norfluoxetine.

Precision

The precision of the method was evaluated in two ways. First, ten replicate injections of the same sample were injected to determine the reproducibility of the method apart from analyst error. Second, ten separate sample preparations were injected singly to determine the overall precision of the method. Approximately 11.5 mg from a bulk drug substance lot of seproxetine hydrochloride was transferred into respective 100 mL volumetric flasks and diluted to volume with 20 mM KH_2PO_4 buffer resulting in the target nominal concentration of 0.1 mg/mL of seproxetine. The samples were quantitated using a peak versus total peak area approach. The samples averaged 0.9% R-norfluoxetine in the validation lot with a 2.3% relative standard deviation (RSD) for the ten replicate injections and a 2.7% RSD for the ten separate sample preparations.

Selectivity

As part of the USP guidelines for validation, a method must be proven to be selective for the analyte of interest. For this method selectivity was assessed by separating seproxetine from R-norfluoxetine and three degradation products: p-trifluoromethylphenol, 3-phenyl-3-hydroxypropylamine and 3-amino-1-phenyl-1-propene. The degradation pathways have previously been reported.[20] The relative retention times for 3-phenyl-3-hydroxypropylamine, p-trifluoromethylphenol, 3-amino-1-phenyl-1-propene and R-norfluoxetine as compared to seproxetine were 0.15, 0.21, 0.22 and 0.71, respectively.

Surprisingly, the enantiomers from a racemic mixture of fluoxetine hydrochloride could not be separated using any of the conditions tested in this report. The main metabolite of fluoxetine is norfluoxetine which is identical in

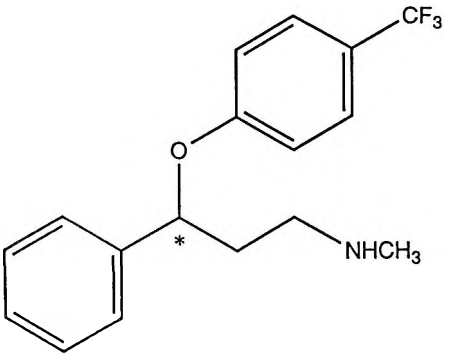

Figure 10. Structure of fluoxetine.

structure to fluoxetine except it is demethylated. The structure of fluoxetine is shown in Figure 10. Fluoxetine had a relative retention of 0.85 as compared to seproxetine.

Recovery

The recovery was determined by a standard addition technique whereby three separate preparations of two seproxetine hydrochloride bulk drug substance lots containing 0.1% and 0.9% of R-norfluoxetine initially were spiked with an additional 1% of R-norfluoxetine from a stock solution of this enantiomer. The average percent recovery for these two sample lots were 91.5% and 101.1%, respectively.

Limit of Detection/Quantitation

The Limit of Detection (LOD) can be defined as the lowest concentration of sample that can be clearly detected above baseline noise. Typically this value is three times the level of baseline noise. The LOD for this method was determined to be 0.05 μg/mL. The limit of quantitation (LOQ) might be estimated as three times the LOD or can be determined from the linearity validation experiments. For this method the LOQ was determined experimentally to be 0.1 μg/mL. When using a nominal sample concentration of 0.1 mg/mL of seproxetine, quantitation of R-norfluoxetine can be achieved at a level of 0.1%.

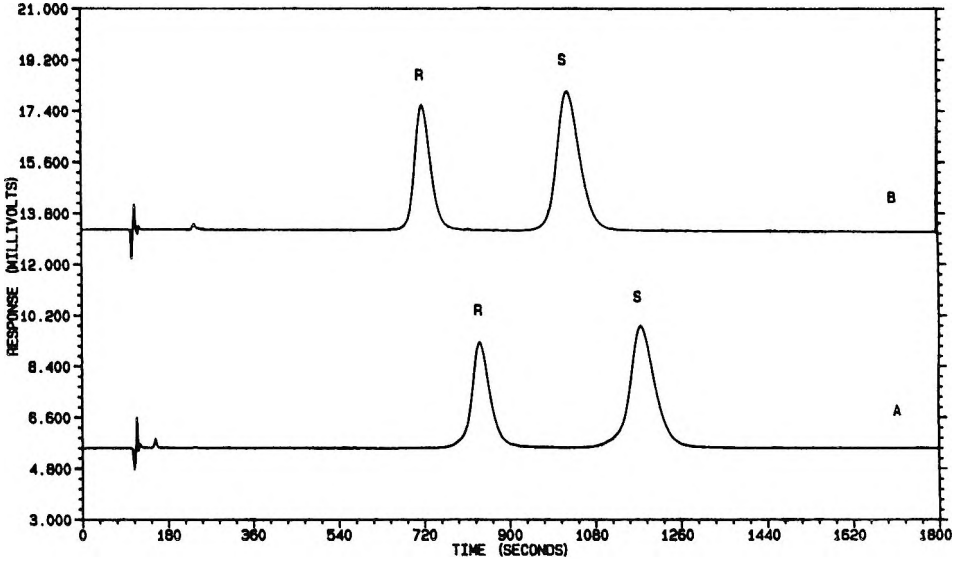

Figure 11. Sample chromatograms for the separation of seproxetine and R-norfluoxetine using two different batch numbers of the Ultron ES-Pepsin column with identical instrument and chromatographic conditions: (A) Ultron ES-Pepsin column with > 250 injections, lot # 2061023 and (B) Ultron ES-Pepsin column with < 10 injections, lot # 2061030. Mobile phase: 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer; detection: UV absorbance, 225 nm; flow rate: 1.0 mL/min.; injection volume: 10 µL; sample: 0.05 mg/mL of a 3:2 mixture of seproxetine:R-norfluoxetine; temperature: ambient. Peak identification: (R) = R-norfluoxetine, (S) = seproxetine.

Ruggedness

The effects of the key mobile phase parameters (pH, flow rate, buffer strength and organic concentration), column temperature and sample loading have been discussed in the method optimization section (Figures 2-8). Although the excellent separation of seproxetine and R-norfluoxetine was easily achieved, these parameters were also important for maintaining consistency of the separation over long-term use of the method.

In addition to studying these effects, another ES-Pepsin column, from a different batch number, was compared to the column used for the optimization and validation. Figure 11 shows a comparison of the chromatograms generated by the two columns using identical conditions. The favorable comparison of

the two chromatograms in Figure 11 is a good indication of the ruggedness of the method for long-term use.

CONCLUSION

The Ultron ES-Pepsin column has been demonstrated to be an excellent CSP for the separation of seproxetine and R-norfluoxetine. The column was shown to be compatible with several different organic modifiers and a relatively wide range of other key mobile phase parameters (pH, buffer strength and organic concentration).

Although this report focused on the separation of seproxetine and R-norfluoxetine, we have separated other basic chiral drug substances using this column, thus the ES-Pepsin column is likely to be applicable to the separation of many other basic chiral compounds.

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A PRACTICAL APPROACH TO IMPROVE THE RESOLUTION OF DANSYL-AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

We describe an HPLC procedure for the separation of dansyl (Dns)-derivatives of all common amino acids present in polypeptide hydrolyzates. A linear gradient of solvent B [methanol: water, 70:30, (v/v)] in solvent A [30 mM sodium phosphate buffer, pH 7.4, containing 5 mL methanol and 6.5 mL tetrahydrofuran (THF)], from 0 to 100% B in 30 min, at 1.0 mL/min and 25 °C, was used to elute the Dns-amino acids from a 4 μm NovaPak C_{18} column with good resolution. The Dns-amino acids were detected fluorometrically at 338 nm wavelength excitation and 455 nm emission.

The concentration of sodium phosphate buffer was critical for the resolution of the dansyl-derivatives of: Arg from Ser and Thr, NH_3 from Val and Met and of cysteic acid from Asp and Glu. THF mainly improved the resolution of the dansyl-derivatives of: Arg from Ser and Thr, NH_3 from Val, and Leu from Ile and Trp.

The effects of sodium phosphate buffer and THF on the column capacity factor provided a convenient and reproducible manner to adjust elution conditions for Dns-amino acid separation when using new columns.

Using this procedure, we showed that Asx is the N-terminal amino acid residue of human pancreatic secretory trypsin inhibitor, in agreement with sequence data.

INTRODUCTION

A general aspect of HPLC methodology is the necessity of adjusting a given elution protocol to each new column to be used, to obtain a good resolution.[1] This is particularly valid for the HPLC separation of 1-dimethylamino-naphthalene-5-sulfonyl- (dansyl, Dns) amino acids. The separation of Dns-amino acids requires the use of application-selected columns[2] and presents difficulties to resolve some Dns-amino acids, such as: Glu/cysteic acid, Thr/Gly, Ile/Leu, Trp/Ile/Leu, Ile/Phe.[3,4]

None of the methods for the separation of Dns-amino acids[2,4-7] that we have tried yielded good resolution with 10 μm μBondapak C_{18} and 4 μm NovaPak C_{18} (Waters), and with 5 μm Spherisorb C_{18} (Applied Science Division) columns. In addition to be able to resolve Dns-amino acids with good efficiency and peak simmetry, it is also convenient that such analytical procedure can be adjustable for use with new columns, by predictable changes in solvent composition.

To address this issue, we have developed an HPLC procedure for the separation of the dansyl-derivatives of all the common amino acids present in polypeptide hydrolyzates. The procedure is based on a linear gradient of increasing methanol and decreasing tetrahydrofuran (THF) and sodium phosphate concentrations.

The effects of THF and sodium phosphate concentrations on column capacity factors (k') of the Dns-amino acids described here have been used to develop a new procedure for the separation of Dns-amino acids that fullfills the above requirements. The applicability of this procedure was evaluated using it to identify the N-terminal amino acid residue of homogeneous human pancreatic secretory trypsin inhibitor (hPSTI).

MATERIALS AND METHODS

Equipment

The liquid chromatograph used here (Waters Associates, Milford, MA) consisted of: two Model 6000A pumps, a Model 660 programmer, a Model U6K Universal injector and a Model 420-AC fluorescence detector fitted with a F4T5 BL lamp, and 338 and 455 nm wavelength excitation (band-pass) and emission (long-pass) filters, respectively. Chromatograms were recorded using a RB201 recorder (ECB, São Paulo, SP). pH values were determined using a Micronal (São Paulo, SP) model B-222 digital pH-meter.

Standards and Reagents

Dansyl-chloride was from Sigma, St. Louis, MO. LC-grade solvents and all other reagents were from Merck (Darmstadt, DFR). Distilled water was purified using a Milli-Q apparatus (Millipore, Bedford, MA). Homogeneous hPSTI was a gift from Dr. L. J. Greene, Laboratório de Química de Proteinas, Faculdade de Medicina de Ribeirão Prêto, Universidade de São Paulo.

A stock solution of L-amino acids was prepared either by diluting Amino Acid Standard H (containing, per mL, 1.25 μmoles L-cystine and 2.50 μmoles of: ammonia, L-Ala, L-Arg, L-Asp, L-Glu, L-Gly, L-His, L-Ile, L-Leu, L-Lys, L-Met, L-Phe, L-Pro, L-Ser, L-Thr, L-Tyr, L-Val; Pierce, Rockford, IL) or dissolving weighed amounts of each L-amino acid (Pierce) in 0.1 N hydrochloric acid to provide a final concentration of 50 nmol/mL. The stock solutions were stored at -20 °C. A given amount of the amino acid mixture was taken by volume into a pyrolyzed[8] glass vial and evaporated to dryness. The dry sample was dansylated as described below. Standard Dns-amino acids were from Pierce. Nor-leucine (Ahx, 2-aminohexanoic acid) was used as an internal standard.

Mobile Phase Preparation

Solvent A was prepared by mixing: 30 mL 100 mM sodium phosphate buffer, pH 7.4, 5 mL methanol and 6.5 mL tetrahydrofuran in a volumetric flask and adding water to 100 mL, unless otherwise stated. Solvent B was prepared by mixing methanol and water, 70 : 30 (v/v). Mobile phases were degassed by sonication under reduced pressure before use.

Chromatographic Conditions

Dansyl-amino acids and reaction by-products were separated by gradient elution chromatography (0 to 100% B in 30 min, curve 6), at a flow rate of 1.0 mL/min and at 25 °C, using a stainless steel 4 μm NovaPak C_{18} column (3.9 mm x 150 mm). A 5 cm Bondapak precolumn (37-50 μm C18/Corasil packing; Waters) was placed between the injector and column. The effluent was monitored fluorometrically. The retention time of Dns-Asp, eluted with 100 % methanol, was taken as t_0.

At the end of a run, initial conditions were restored by running a reversed gradient in 10 min, at 1.0 mL/min. Then, about 10 min were required for column equilibration. This procedure was found necessary for maintaining optimal column performance.

The overnight shut down procedure consisted in washing the column with about 35 mL water, followed by 20 mL solvent B, at 1 mL/min. Washing the buffer pump, tubing and the column with water was found essential because solvent A contained phosphate buffer.

Dansylation Procedure

Dansylation was carried out by a modification[2] of the procedure described by Tapuhi et al.[9] Samples (up to 20 nmol total NH_2) were transfered to pyrolyzed (4.4 mm I.D. x 6 mm) glass tubes, dried under reduced pressure and dissolved in 40 μL 40 mM lithium carbonate buffer, pH 9.5. Then, 20 μL of 1.5 mg/mL (5.56 mM) Dns-Cl solution in acetonitrile were added to the sample, shaken for 2 min, and the mixture was incubated for 40 min at 37 °C in the dark. The reaction was stopped by adding 2 μL 8.9 M ethylamine hydrochloride, and further incubating for 10 min at 37 °C. The derivatization mixture was evaporated to dryness under reduced pressure, at room temperature. The dry sample was either stored in the dark at -20 °C for up to one week or diluted in 100 μL solvent A immediately before injection.

N-terminal Determination of Human Pancreatic Secretory Trypsin Inhibitor

hPSTI (200 pmol) and nor-leucine (Ahx, 200 pmol) were transfered to a hydrolysis tube, dried under reduced pressure, redissolved in 40 μL 40 mM lithium carbonate buffer, pH 9.5, and dansylated. After drying the

derivatization mixture under reduced pressure, 100 μL constant boiling HCl were added to the tube. The material was incubated at 110 °C for 18 h, evaporated over solid NaOH and dissolved in 100 μL solvent A. A 50 μL aliquot of the hydrolyzate was used for the HPLC analysis of dansyl-amino acids. Control tubes in which hPSTI, Ahx or both substituted for water were processed in parallel.

RESULTS AND DISCUSSION

HPLC Separation of Dansyl-Amino Acids

Fig. 1 shows the resolution, within 35 min, of a mixture of Dns-amino acids, including dansyl sulfonic acid and the derivatives of: ammonia, the rection quencher ethylamine,[9] and the internal standard nor-leucine. The k's for the dansyl derivatives of: cysteic acid, Lys at the e-amino group (eK), Nor-valine (Avl, 2-aminovaleric acid), Nor-leucine, Trp, methylamine, ethylamine and n-propyl-amine were: 1.8, 11.5, 12.9, 15.5, 15.6, 14.7, 16.6 and 18.7, respectively.

Under the derivatization conditions used here Trp, His, Lys, cystine and Tyr are converted to their di-Dns derivatives.[5,9] Besides ethylamine, either methyl- or n-propyl-amine can be used as a reaction quencher. The quencher has been selected such that its Dns-derivative does not interfere in the chromatogram. Avl is an alternative internal standard to Ahx.

The limit of sensitivity attained here was about 2 pmol, for a signal-to-noise ratio of 5:1 .

When the guard column was changed after 100-120 analyses, Dns-amino acids were well separated for at least 600 analyses per column.

Effect of the Concentration of Sodium Phosphate Buffer, pH 7.4, on Resolution

Capacity factor lines of Dns-amino acids did not cross, except those of cysteic acid, Arg and ammonia (Fig. 2). Thus, for example, at a sodium phosphate buffer concentration of 10 mM in solvent A, Arg coeluted with Ala and after Ser, Thr and Gly. At a buffer concentration of 30 mM, Arg eluted between Ser and Thr and was well resolved from them (see also Fig. 1).

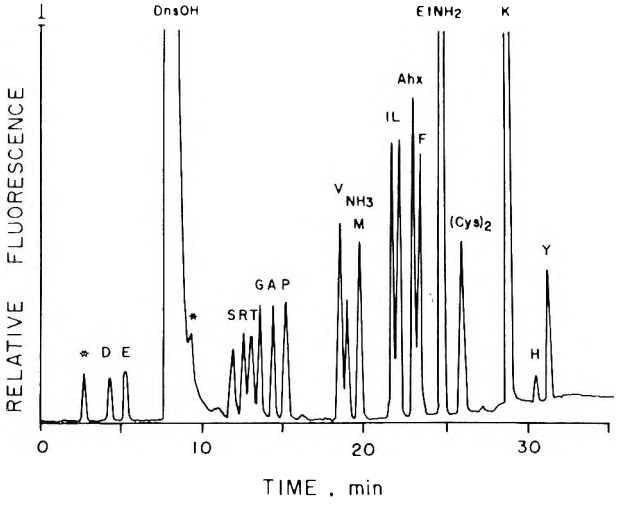

Figure 1. Reverse phase HPLC of a mixture of dansyl-amino acids. A sample containing: amino acid standard H (1 nmol of ammonia and of each amino acid, except L-cystine whose amount was 0.5 nmol) and norleucine (1 nmol) was derivatized and *ca.* 60 pmol of each dansyl-derivative was injected. Chromatographic conditions were as in Methods. Fluorometer gain, 64. Amino acids are abbreviated by the one-letter system. Ahx, norleucine; DnsOH, dansyl sulfonic acid; $EtNH_2$, ethyl-amine; *, unidentified peak.

Dns-derivatives of Asp, Arg and ammonia are usually difficult to resolve from those of cysteic acid/Glu, Ser/Thr/Gly and Met/Val, respectively.[3,4] The changes in sodium phosphate buffer concentration shown here have provided a way to optimize the separation of these Dns-amino acids.

Effect of Tetrahydrofuran Concentration on Resolution

The capacity factors of the Dns-amino acids decreased with increasing THF concentration in solvent A (Fig. 3). As expected, this effect was more intense for the earlier eluting derivatives, whereas those eluting by the end of the run were less affected. The differential effect of THF concentration on the separation of the Dns derivatives of: Arg, Thr and Gly; Ala and Pro; Val, ammonia and Met; Leu and Trp, was usefull for the separation of these compounds. Taken together, the data in Figs. 2 and 3 exemplify a systematic

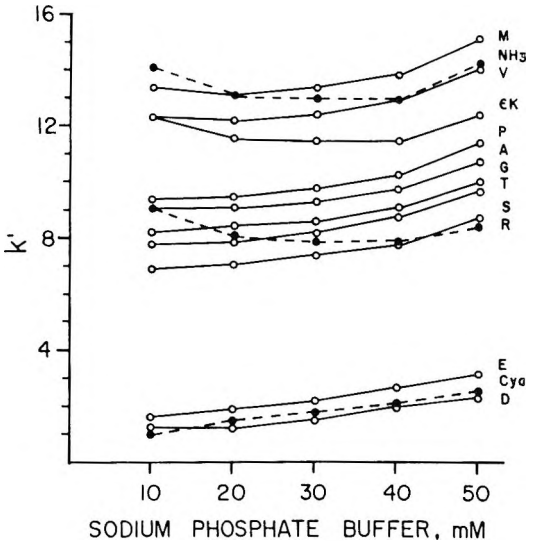

Figure 2. Changes in k' as a function of the concentration of sodium phosphate buffer, pH 7.4, in solvent A. A series of gradient runs using as solvent A: 10 to 50 mM sodium phosphate buffer, pH 7.4, containing 5 mL methanol and 6.5 mL tetrahydrofuran per 100 mL solvent A, and as solvent B: metanol and water, 70 : 30 (v/v), were performed. k's are the averages of three independent determinations, which did not differ by more than 2.2 %. Dashed lines are for: Cya, R and NH_3.

approach for the adjustment of the elution protocol proposed here to each new column to be used for the separation of Dns-amino acids.

Determination of the N-terminal Amino Acid Residue of Human Pancreatic Secretory Trypsin Inhibitor

The N-terminal amino acid identifyied here (Fig. 4), Asx, agreed with the known N-terminal of hPSTI, Asp.[10] Free Asp and Asn have not been detected in unhydrolyzed control samples of hPSTI. The ε-Dns-Lys derivative was also formed and did not interfere in the HPLC analysis.

In conclusion, we have proposed here a new method for the separation of Dns-amino acids that is fast, sensitive, and applicable to the determination of the N-terminal amino acid residue of small samples of polypeptides. When using new columns, the separation of Dns-amino acids can be conveniently

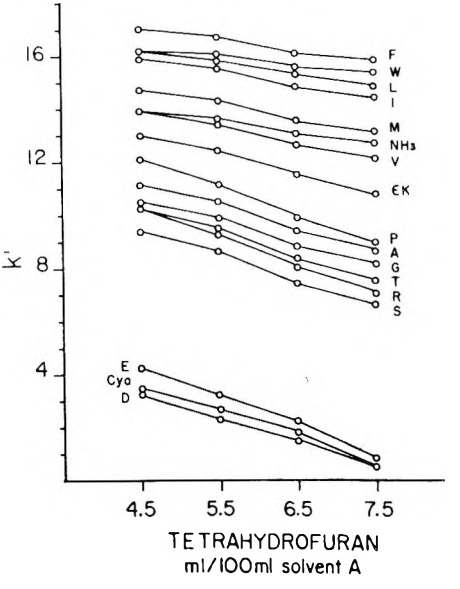

Figure 3. Changes in k' as a function of tetrahydrofuran concentration in solvent A. A series of gradient runs using as solvent A: 30 mM sodium phosphate buffer, pH 7.4, containing 5 mL methanol and 4.5 to 7.5 mL tetrahydrofuran per 100 mL solvent A, and as solvent B: metanol and water, 70 : 30 (v/v), were performed. k's are the averages of three independent determinations, which did not differ by more than 2.5 %.

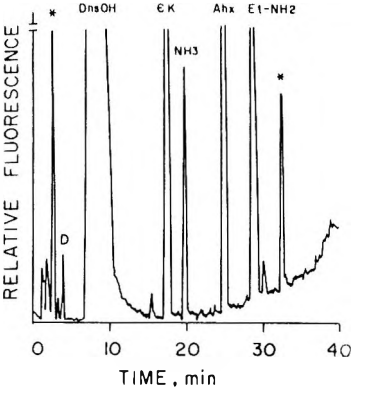

Figure 4. Identification of the N-terminal amino acid residue of a 200 pmol sample of hPSTI by the present HPLC method. Chromatographic conditions were as in Methods except gradient time (35 min) and the use of a new NovaPak C_{18} column. *, unidentified peak. Fluorometer gain, 128.

tailored by changes in the concentration of sodium phosphate buffer and THF, and by changes in gradient time.

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DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY HPLC WITH SPECTROFLUORIMETRIC DETECTION AND WAVELENGTH PROGRAMMING

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ABSTRACT

The sixteen polycyclic aromatic hydrocarbons (PAH) classified as priority pollutants by the EPA have been determined by HPLC, in isocratic conditions, with spectrofluorimetric detection and wavelength programming. The possibility of selecting the optimum wavelengths for each PAH gives the advantages of increased sensitivity - reaching the low pg level - and of improved selectivity, because it is possible to carry out an independent determination of coeluting compounds, provided that their excitation and emission spectra are different enough.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are of anthropogenic and natural origin and can be found in a wide range of concentrations in all kinds of environmental samples.[1] As their carcinogenic and mutagenic properties are well known, it is of the utmost importance to develop fast, selective and sensitive procedures for their identification and detection in all kinds of samples.

The most widely used procedures for their analysis are gas chromatography with either FID or MS detection[2-3] and high performance liquid chromatography with UV-Visible or fluorimetric detection.[4-7] One of the methods offering better promises is that of HPLC with fluorescent detection (HPLC-Fl). The possibility of selecting both the excitation and emission wavelengths significantly increases the selectivity and sensitivity of this technique over those obtained by UV-Visible detection. Moreover, its already good performances can be widely improved by the use of the so-called wavelength-programming method, in which, instead of recording the whole chromatogram at fixed settings, the excitation and emission wavelengths are changed as a function of time, in order to use the optimum conditions for each compound to be determined.[8-10]

According to the data available in the literature, separation and determination of PAH is better carried out making use of C_{18} columns with a high polymerization degree[9] and with an acetonitrile-water mobile phase. In most cases results are improved by the use of an elution gradient.[11-12]

In the present paper an isocratic elution procedure has been used, because of the limitations of the chromatographic system. In these conditions separation is not perfect, as two of the PAH (benzo[a]pyrene and crysene) coelute and others (acenaphthene, anthracene, phenanthrene and fluorene, or benzo[ghi]perylene and indene [1,2,3-cd]pyrene) have closely similar retention times. However, the individual determination of all compounds studied - with the exception of acenaphthylene, whose fluorescence is virtually nil - is made possible by the use of wavelength programming and, eventually, the recording of two consecutive chromatograms. Moreover, the use of the optimum excitation and emission wavelengths for each compound has led to a significant improvement of the detection limits, which have been lowered to the low-pg level.

This method has been used for the determination of PAH in a reference material (marine sediment HS-3, NRCC) with satisfactory results.

MATERIALS

Stock standard solutions (about 200 $\mu g.mL^{-1}$) of acenaphthene, anthracene, bez[a]anthracene, benzo[a]pyrene, crysene, dibenzo[a,h]anthracene, phenanthrene, fluoranthene, fluorene, naphthalene and pyrene were prepared by dissolving the pure solid (Supelco) in either methanol or acetonitrile, depending on its solubility. Solutions of benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene and indene[1,2,3-cd]pyrene in either acetonitrile or methylene chloride (all at about 200 $\mu g.mL^{-1}$), as well as a standard solution containing the sixteen PAH classified as

primary pollutants by the EPA, were purchased from Supelco. Working standards were prepared by dilution of the stock solutions with acetonitrile.

A certified reference material (marine sediment HS-3, National Research Council of Canada) was used to test the validity of the method.

Acetonitrile, methanol and methylene chloride were of HPLC quality (Merck).

Doubly distilled water (Culligan Ultrapure GS) was used in the mobile phase.

The mobile phase consisted of 80/20 acetonitrile/water and, before use, it was filtered through a 0.22 μm membrane filter and degasified with an helium stream.

The chromatographic system consisted of a twin-piston Gynkotek 480 HPLC pump, a Gynkotek MSV6 automatic injector, with a 20 μL injection loop, and a 125 mm LiChrospher 100 RP-18 column (Merck, Darmstadt), with 4 mm internal diameter and 5 μm particle size. An isocratic elution procedure was used throughout.

An Aminco Bowman Series 2 spectrofluorimeter, equipped with a 25 μl flow cell (Hellma 176.752) was used for detection.

RESULTS AND DISCUSSION

Standard solutions of all the individual compounds were used to determine the optimum excitation and emission wavelengths and the actual retention time for all of them in the experimental conditions used. Results are given in Table 1.

The standard solutions containing all 16 PAH was then injected. As the isocratic elution procedure used did not allow a complete separation of all compounds, two consecutive chromatograms were recorded. The spectrofluorimeter was programmed to shift excitation and emission wavelengths in order to detect alternatively eluting compounds in their optimum conditions. Therefore, the first chromatogram was used for the determination of naphthalene, fluorene, phenanthrene, fluoranthene, crysene, benzo[a]pyrene, benzo[k]fluoranthene and indene [1,2,3-cd]pyrene, while the second was used for the determination of acenaphthene, anthracene, pyrene, benzo[a]anthracene, dibenzo[a,h]anthracene, benzo[b]fluoranthene and benzo[ghi]perylene.

The wavelength programming allowed the independent determination of

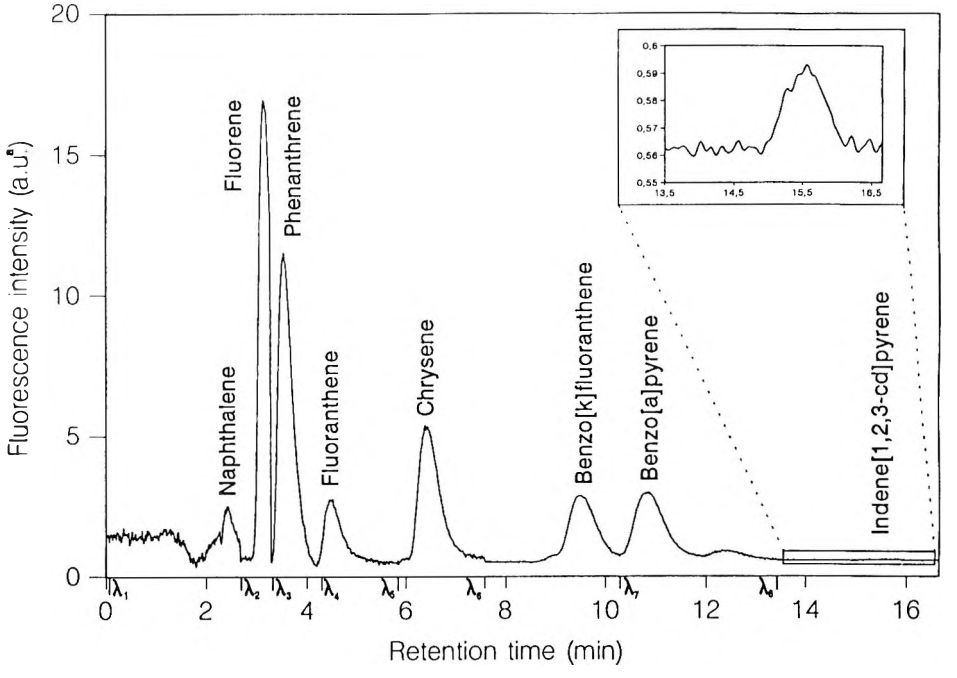

Figure 1. Chromatogram of a solution containing sixteen PAHs obtained with detector conditions set to detect eight of them. [a] Arbitrary units.

compounds which, in fact, could not be separated in the elution conditions used. This is shown in Figure 1 and Figure 2. Some overlapping can still be observed in some cases, but this did not affect the results, as the retention times were extremely reproducible and the wavelengths were shifted before the maximum of each peak was reached. This fact allowed the peak height to be measured, even if in some cases the whole peak could not be recorded.

The fact that wavelengths programming virtually eliminated the interference from coeluting compounds is shown in Fig. 3. In Fig. 3a, the detector was set at the optimum conditions for acenaphthene, and only fluorene did show a low fluorescence intensity, which did not interfere. In Fig. 3b and 3c the detector was set at the optimum conditions for anthracene and fluorene, respectively, and at these conditions the intensities of the other compounds were virtually nil. Only in the case of phenanthrene (Fig. 3d) had other compounds (acenaphthene and fluorene, concretely) significant fluorescence, but their retention times were different enough to allow the peak height of phenanthrene to be measured.

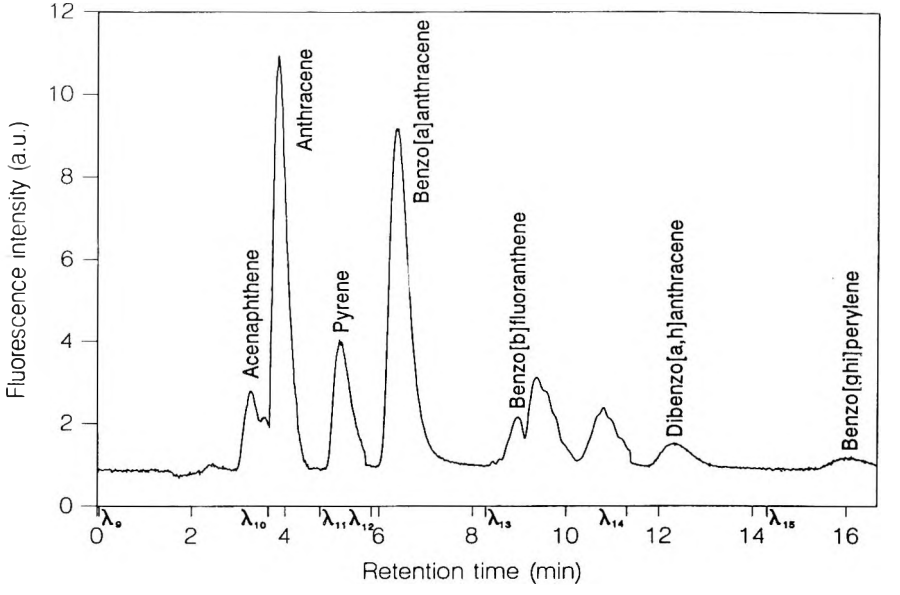

Figure 2. Chromatogram of a solution containing sixteen PAHs obtained with detector conditions set to detect the remaining seven of them (Acenaphthylene is not fluorescent).

After testing the correctness of the proposed method, the quality parameters were determined. In Table 2, detection limits (calculated as three times the standard deviation of the blank), the linear range and the precision, both of the peak height and of the retention time, for each compound are shown. The precision was determined at an amount of PAHs equal to 10-fold its detection limit. It can be observed that the possibility of always using the optimum excitation and emission wavelengths has led to very good detection limits, better, in some cases, than the lowest given in the literature.

The linear range given for each compound was found at the experimental conditions used to determine its detection limit. This means that the detector is set to detect very low amounts of the compound and that it can be easily saturated if the PAH is very fluorescent. Even with this difficulty, the linear ranges go from a minimum level of between 3 and 170 pg of injected PAH to a maximum level of between 500 and 5000 pg (Table 2). These limits could be increased by changing the detection conditions.

Precision for retention times ranged between 0.5 % and 1.5 % RSD, while for

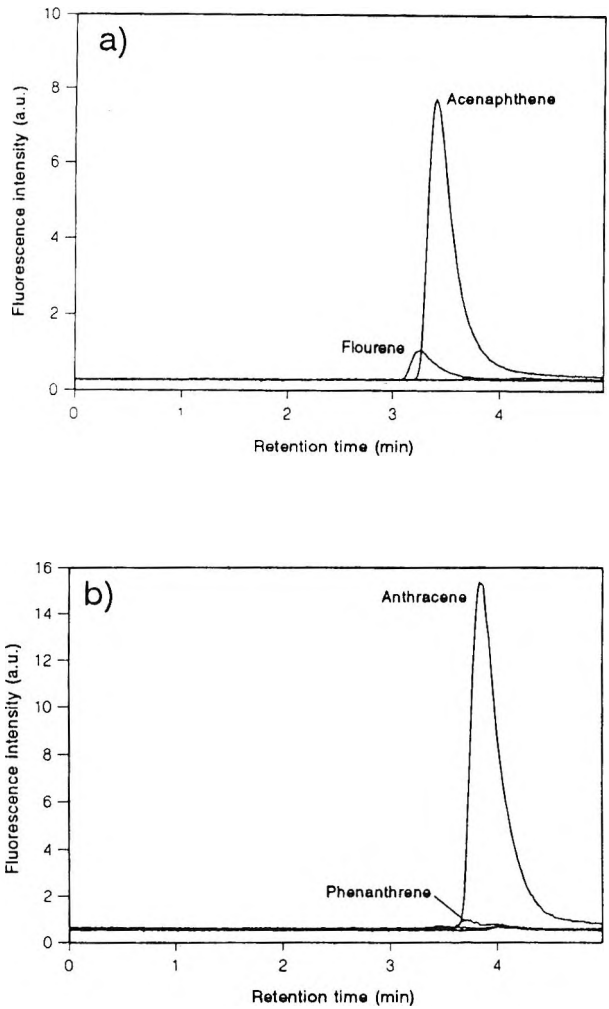

Figure 3. Determination of co-eluting PAHs (acenaphthene, anthracene, fluorene and phenanthrene) with wavelength programming.
a) Detector set at the conditions for acenaphthene. b) Detector set at the conditions for anthracene. c) Detector set at the conditions for fluorene. d) Detector set at the conditions for phenanthrene.

peak heights RSD increased from 1.9 % to 10% as the retention time increased.

When the detection limits were determined, the calculated values did not agree

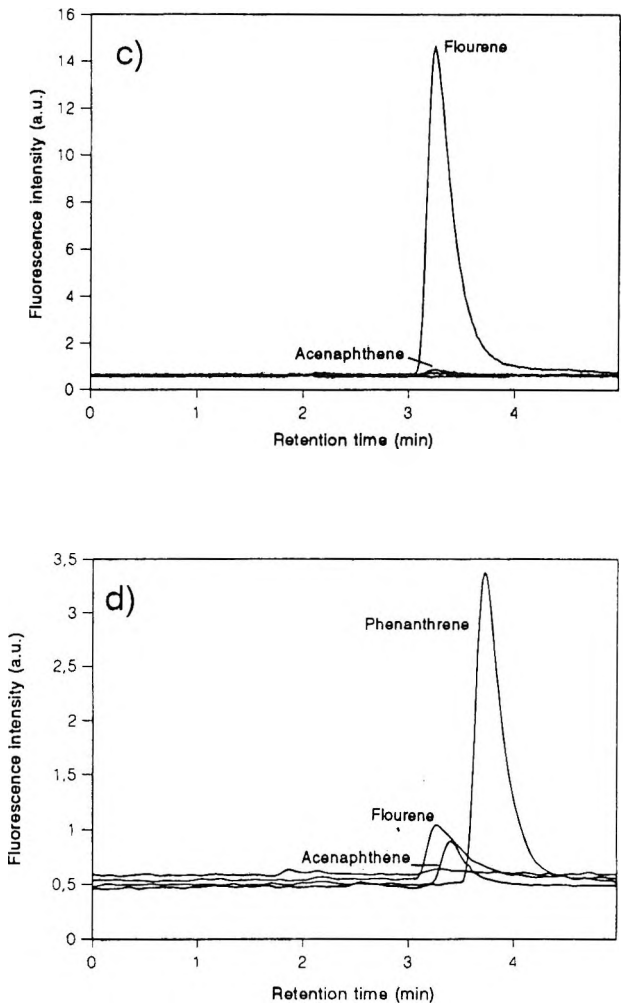

Figure 3. (continued). See previous page for details.

with those that could be observed from the experimental data. The difficulty lay in the fact that an increase in the voltage applied to the photomultiplier meant an increased signal, but also an increased background noise. In order to improve the signal-to-noise ratio, the chromatograms were smoothed by the fast Fourier transform algorithm (11 points).[13] This method significantly reduced the background noise but left the peak unchanged. In Table 3 the detection limits found

Table 1

Optimal Wavelengths and Retention Times for Each PAH

PAH	λ_{ex} (nm)	λ_{em} (nm)	t_R (min)
Acenaphthene	290	323	3.35
Anthracene	251	402	3.83
Benz[*a*]anthracene	287	388	6.32
Benzo[*b*]fluoranthene	254	437	9.97
Benzo[*k*]fluoranthene	304	411	9.60
Benzo[*ghi*]perylene	291	409	16.15
Benzo[*a*]pyrene	295	406	10.65
Chrysene	267	363	6.28
Dibenzo[*a,h*]anthracene	297	396	12.28
Fluoranthene	286	464	4.40
Fluorene	265	304	3.13
Indene[1,2,3-*cd*]pyrene	295	496	15.67
Naphthalene	221	323	2.42
Phenanthrene	250	348	3.50
Pyrene	275	374	5.08

by this procedure are shown. These limits, moreover, coincide with those that can be observed in practice.

This method was used to determine the PAHs of a certified reference material (marine sediment HS-3, NRCC). Two extraction procedures (sonication and Soxhlet), two solvents (acetonitrile and methylene chloride) and several extraction times were tested.

In the sonication method, about 1 g of sample was extracted in an ultrasonic bath with 25 mL of the solvent. No increase in recovery was detected with extraction times longer than 45 minutes. A second extraction increased the recovery only slightly (about 3%). Therefore, a single extraction of 45 minutes was used in all further research. The solution was filtered through a membrane filter before use. If methylene chloride was used for extraction, acetonitrile was then added and the methylene chloride was evaporated in a nitrogen stream. In Table 4 the recovery values are given.

In the Soxhlet extraction method, about 2 g of sample were extracted with 250

Table 2

Figures of Merit for the Method

PAH	Detection limit (pg)	Linear Range (pg)[a]	RSD(%)[b] t_R	RSD(%)[b] Peak Ht
Acenaphthene	12.4	41 - 2000	1.0	3.4
Anthracene	3.7	12 - 2000	1.8	1.9
Benz[*a*]anthracene	2.5	8 - 1000	1.2	3.5
Benzo[*b*]fluoranthene	16.0	53 - 2000	0.7	7.8
Benzo[*k*]fluoranthene	0.8	3 - 500	0.7	8.8
Benzo[*ghi*]perylene	42.5	142 - 4500	0.6	3.1
Benzo[*a*]pyrene	2.3	8 - 1000	1.3	6.6
Chrysene	7.7	26 - 4000	1.2	5.8
Dibenzo[*a,h*]anthracene	3.2	11 - 2500	1.1	7.0
Fluoranthene	41.3	138 - 3000	0.9	3.7
Fluorene	5.0	17 - 1250	0.6	2.9
Indene[1,2,3-*cd*]pyrene	28.0	93 - 5000	1.0	3.0
Naphthalene	51.0	170 - 5000	0.7	3.8
Phenanthrene	21.7	72 - 5000	1.1	4.4
Pyrene	29.1	97 - 5000	1.1	4.9

[a]At the same conditions used for the detection limits
[b]Mean of six independent replicates at a concentration ten times the detection limit

mL of methylene chloride for 12 hours at 4-6 cycles per hour. The extract was filtered and the solvent evaporated as before. As shown in Table 4, no significant differences in recoveries were found between this method and the sonication procedure, in concordance with literature data.[14]

Recoveries, calculated from the certified mean values, are of about 60%. It must be noticed, however, that in some cases the certified values have very wide confidence intervals.

It was decided not to spike the sample, because, according to literature data,[15] there are significant differences in the recoveries of native and spiked PAHs.

Table 3

Detection Limits after FFT Smoothing of the Chromatograms

PAH	Detection Limit (pg) After FFT Smoothing
Acenaphthene	1.7
Anthracene	1.7
Benz[*a*]anthracene	0.8
Benzo[*b*]fluoranthene	4.2
Benzo[*k*]fluoranthene	0.2
Benzo[*ghi*]perylene	3.4
Benzo[*a*]pyrene	0.7
Chrysene	2.4
Dibenzo[*a,h*]anthracene	0.7
Fluoranthene	9.9
Fluorene	1.0
Indene[1,2,3-*cd*]pyrene	8.4
Naphthalene	22.9
Phenanthrene	1.8
Pyrene	6.8

Table 4

Recoveries of PAHs in a Marine Sediment Sample (HS-3, NRCC)

	HS-3 Certified Value ($mg \cdot kg^{-1}$)	Recovery[a] Sonication With Acetonitrile		Recovery[a] Sonication With Dichloromethane		Recovery[a] Soxhlet With Dichloromethane	
		$mg \cdot kg^{-1}$[b]	%[c]	$mg \cdot kg^{-1}$[b]	%[c]	$mg \cdot kg^{-1}$[b]	%[c]
Acenaphthene	4.5 ± 1.5	2.6 ± 0.2	57.8 (2.2)	3.3 ± 0.7	73.3 (6.7)	3.6 ± 0.2	80.0 (2.2)
Anthracene	13.4 ± 0.5	3.9 ± 0.2	29.1 (0.7)	4.0 ± 0.5	29.9 (1.5)	3.4 ± 1.2	25.4 (3.7)
Benz[*a*]-anthracene	14.6 ± 2	10.9 ± 2.5	74.7 (6.8)	8.6 ± 1.2	58.9 (3.4)	10.5 ± 0.2	71.9 (0.7)
Benzo[*b*]-fluoranthene	7.7 ± 1.2	9.4± 1.7	122.1 (9.1)	11.1 ± 0.5	144.2 (2.6)	10.8 ± 0.7	140.3 (2.8)
Benzo[*k*]-fluoranthene	2..8 ± 2	3.8 ± 0.2	135.7 (3.6)	3.6 ± 0.5	135.7 (3.6)	4.0 ± 0.2	142.9 (3.6)
Benzo[*ghi*]-perylene	5 ± 2	3.0 ± 0.7	60.0 (6.0)	3.4 ± 0.5	68.0 (4.0)	2.9 ± 0.2	58.0 (2.0)
Benzo[*a*]pyrene	7.4 ± 3.6	5.4 ± 1.2	73.0 (6.8)	4.2 ± 0.5	56.8 (2.7)	3.3 ± 2.2	44.6 (12.1)
Chrysene	14.1 ± 2	8.8 ± 2.2	62.4 (6.4)	8.1 ± 0.7	57.4 (2.1)	6.4 ± 1.5	45.4 (4.3)
Dibenzo[a,h]-anthracene	1.3 ± 0.5	1.0 ± 0.2	76.9 (7.7)	1.0 ± 0.2	76.9 (7.7)	0.8 ± 0.2	61.5 (7.7)

Table 4. (Continued)

Recoveries of PAHs in a Marine Sediment Sample (HS-3, NRCC)

	HS-3 Certified Value ($mg \cdot kg^{-1}$)	Recovery[a] Sonication With Acetonitrile		Recovery[a] Sonication With Dichloromethane		Recovery[a] Soxhlet With Dichloromethane	
		$mg \cdot kg^{-1}$[b]	%[c]	$mg \cdot kg^{-1}$[b]	%[c]	$mg \cdot kg^{-1}$[b]	%[c]
Fluoranthene	60 ± 9	54.5 ± 3.2	90.8 (2.2)	77.0 ± 8.7	128.3 (5.8)	60.9 ± 4.0	101.5 (2.7)
Fluorene	13.3 ± 3.1	7.3 ± 0.2	54.8 (0.8)	7.5 ± 1.0	56.4 (3.0)	7.4 ± 0.7	55.6 (2.2)
Indene[1,2,3-*cd*]-pyrene	5.4 ± 1.3	3.9 ± 1.2	72.2 (9.3)	6.0 ± 0.5	111.1 (3.7)	3.5 ± 0.5	64.8 (3.7)
Naphthalene	9.0 ± 0.7	8.0 ± 0.7	80.8 (3.0)	9.9 ± 0.7	110.3 (3.0)	4.7 ± 0.5	52.2 (2.2)
Phenanthrene	85 ± 20	53.6 ± 5.7	63.1 (2.7)	57.9 ± 14.1	68.1 (6.7)	50.7 ± 6.9	59.6 (3.3)
Pyrene	39 ± 9	27.3 ± 1.7	70.0 (1.8)	33.7 ± 5.5	86.4 (5.6)	33.8 ± 3.5	86.7 (3.6)

[a]Mean of three independent determinations
[b]Confidence intervals at 0.05 significance level
[c]The relative standard deviation of recoveries are given in parentheses

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AN INVESTIGATION OF FACTORS INFLUENCING THE RESOLUTION OF CHIRAL ALKYL AROMATIC AMINES ON A DINITROBENZOYL-(S)-LEUCINE HPLC COLUMN

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ABSTRACT

Normal phase HPLC methodology was utilised to obtain separation of (±)-1-(4-bromophenyl)ethylamine and (±)-1-(1-Naphthyl)ethylamine on a 3,5- dinitrobenzoyl-(S)-Leucine Pirkle type chiral stationary phase (CSP). A proposed retention mechanism (based on a three point interaction model) is outlined where the key interaction is steric attraction/repulsion between aryl groups on the solutes and the (S) Leucine group on the CSP.

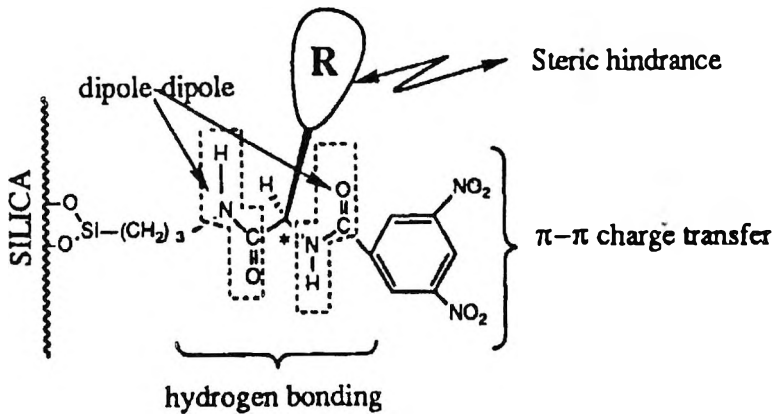


Figure 1. Interaction sites on π acidic Pirkle type chiral stationary phases.

INTRODUCTION

Over the last 15 years, the technique of choice for the determination of the enantiomeric composition in mixtures of enantiomers has been HPLC. There are many types of chiral stationary phases (CSP's) available and perhaps the most versatile is the brush type CSP, pioneered by the Pirkle group.[1] The brush type (also known as Pirkle type) stationary phases rely on a series of designed multiple interactions between the CSP and the solute enantiomers. Chiral separation occurs when one enantiomer can interact simultaneously at three points along the CSP. This three point interaction can occur by a number of different interaction modes including (a) π-π stacking, (b) dipole–dipole interactions, (c) hydrogen bonding, (d) steric attraction/repulsion (see Figure 1).

The principal interaction with brush-type columns is the interaction between a π acidic functionality and a π basic functionality, one of which is present on the CSP, the other being present or introduced onto the chiral analytes. The π acidic Pirkle bases usually consist of 3,5–dinitrobenzamides of α–amino acids, the most common examples being 3,5–dinitrobenzoyl-(D)-phenylglycine[2a] and (S)–N–3, 5–dinitrobenzoylleucine.[2b] The π acidic stationery phases have proved extremely useful in the monitoring of asymmetric synthesis[3] and in the analysis of commercial drugs such as Naproxen[4] and Nadolol.[5]

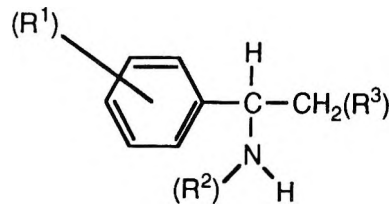


Figure 2. General structure of the alkyl aromatic amines.

The basis of chiral discrimination with brush–type columns is to generate diasteriomeric complexes between the CSP and the enantiomers. The stability of the diasterioisomers is influenced by attractive as well as repulsive interactions between the enantiomers and the CSP. The extent of the enantioselectivity on Pirkle type CSP's will be determined by the differences in stability between the diasterioisomers with the most stable diasterioisomeric complex being retained the longest.

In order to introduce π acid / π base charge transfer between π acidic CSP's and analytes, it is necessary to derivatize the analytes (if they do not already possess a π basic functionality) and introduce an N-aryl π basic group. Numerous acyl chlorides can be used to achieve this goal, the most commonly used reagent being α– naphthoyl chloride,[6,7] partly because of its strong π basicity and partly because it possesses a strong chromophore in the UV region and thus eases detection. The π basic group stacks on top of the π acidic group on the chiral stationery phase and holds the enantiomers in place to allow the other chiral interactions (hydrogen bonding, dipole/dipole interactions, steric attraction/repulsion) to effect enantioseparation.

The nature of Pirkle columns is such that they perform best in the normal phase mode, usually utilising n-hexane modified by a polar alcohol. It has been observed that in general less polar mobile phase systems lead to greater resolution using Pirkle CSP's and conversely that increasing the mobile phase polarity decreases retention and chiral discrimination between enantiomers.[8] It is also found that the nature of the alcohol modifier is important in effecting resolution of enantiomers, with 2-propanol being the modifier of choice for most applications.[5]

The purpose of this study is to observe the effects of hydrogen bonding and steric attraction/repulsion on the enantioselectivity of the DNBLeucine CSP. We studied a range of alkyl aromatic amines of the general structure shown in Figure 2. By varying the nature of the R groups, we can observe the effect of changing the R groups and determine the importance of hydrogen bonding and steric attraction/repulsion in effecting enantioseparation. In order to ensure that a π acidic/π basic interaction is present, we derivatized the amine using 3-toluoyl chloride because the analytes were judged to be sufficiently chromophoric for UV detection.

MATERIALS AND METHODS

Apparatus

Chromatographic analysis was performed using a Shimadzu LC-4A HPLC system fitted with a Rheodyne model 7125 injector with a 20 μL sample loop, a Shimadzu SPD-2AS UV detector set at 254 nm and a Shimadzu CR3A Chromatopac integrating recorder.

Materials

All solvents were of HPLC grade. 4M sodium hydroxide was prepared using reagent grade sodium hydroxide pellets (BDH Chemicals, Poole, Dorset, England). 3-toluoyl chloride (> 99% purity) was obtained from Aldrich (Gillingham, Dorset, England). All chiral amines were obtained from Arran Chemical Co. Ltd.. The HPLC column used (Bakerbond Chiral Phase DNBLeucine (covalent) 5 μm, 4.6 x 250 mm) was obtained from J.T. Baker Inc. (Phillipsburg, NJ, USA).

Derivatization of Enantiomers

All enantiomers were derivatized as follows prior to HPLC analysis: Approximately 15 mg of the racemic analyte was weighed out accurately into a test tube. To this was added 1.5 mL of dichloromethane and 0.5 mL of 4M sodium hydroxide. The test tube was placed on a vortex mixer and vortexed for 30 seconds. To this was added 30 μL of 3–toluoyl chloride. Again the test tube

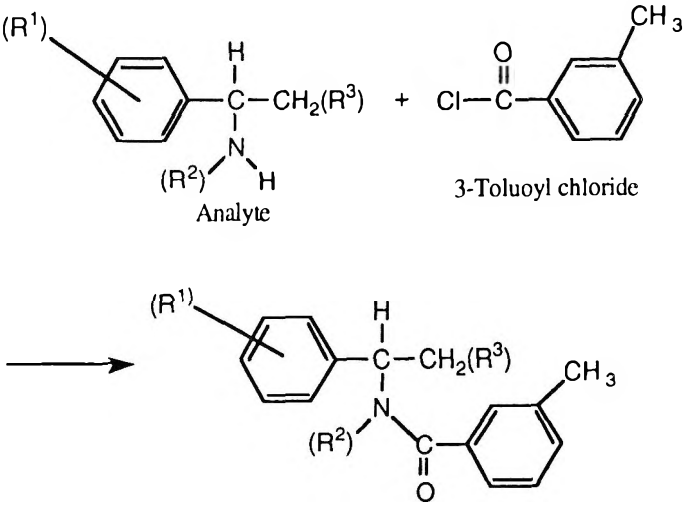


Figure 3. Derivatizing reaction between analytes and 3-toluoyl chloride.
was placed on the mixer and vortexed for at least 30 seconds (derivatization reaction is shown in Figure 3).

After 30 seconds two layers separated. 100 μL of the lower (organic) layer was transferred to a 25 mL volumetric flask. The flask was filled to volume using 2-propanol. This solution was filtered through a 0.45 μm membrane filter prior to analysis by HPLC. Linearity testing on (±)-1-(4-bromophenyl)ethylamine involved weighing different amounts of racemate and then following the derivatization procedure. Limits of detection involved looking at different weight ratios of (R)-(+) and (S)-(-)-1-(4-bromophenyl)ethylamine prior to carrying out derivatization procedure.

Important points to note for this procedure include;

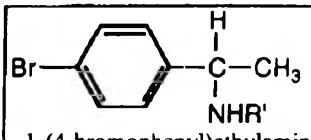
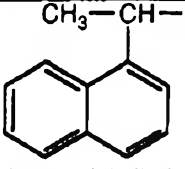
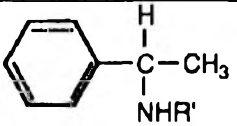
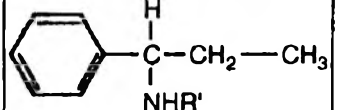
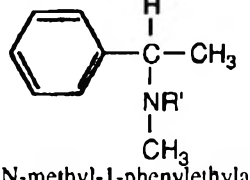
(i) All enantiomers were weighed out under nitrogen to prevent reaction with atmospheric carbon dioxide.

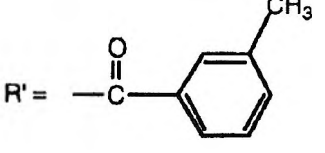
(ii) 3–Toluoyl chloride was also weighed out under nitrogen owing to its moisture sensitivity and its lacrymatory properties.

(iii) To ensure complete derivatization of the analyte, 30 seconds mixing at each stage of vortex mixing is essential.

Table 1

Summary of Results for the Range of Alkyl Aryl Amines

Compound	Capacity Factor (k)	Separation Factor (α)	Resolution
 1-(4-bromophenyl)ethylamine	4.17 (+)	1.21	1.81
 1-(1-naphthyl)ethylamine	5.06 (+)	1.2	2.05
 1-phenylethylamine	4.03 (±)	–	–
 1-phenylpropylamine	4.16 (±)	–	–
 N-methyl-1-phenylethylamine	2.31 (±)	–	–



Conditions: Flow rate = 0.5 mL / min; mobile phase 6% 2-propanol in hexane.

HPLC Conditions

The following HPLC conditions were used throughout . Column : DNBleucine (covalent); Mobile phase: 94 : 6 hexane - 2-propanol; Flow rate : 0.5 mL/min; Injection volume : 20μL; Detector : UV at 254 nm; Absorbance range : 1.0 AUFS; Attenuation : 6.

RESULTS

The chromatograms obtained were evaluated in terms of retention, separation (α values) and resolution. Retention is reported in terms of capacity factors (k) of the first eluted enantiomer. Where no separation has been effected, retention of the racemate is reported as the capacity factor for the racemate. Each sample was chromatographed under the same conditions of mobile phase, stationary phase and integrator conditions so that the data obtained could be evaluated in terms of enantiomeric structure and chromatographic behaviour under similar conditions. Table 1 gives a summary of results.

(±)-1-(4-Bromophenyl)ethylamine is resolved into its (R) and (S) forms under these chromatographic conditions (see Figure 4). The chromatogram shows two peaks with separation α =1.21, a resolution of 1.81 and for the first eluted isomer (R)-(+), a capacity factor of k = 4.17. The method is reproducible (Coefficient of Variation Area (R)-(+) = 3.01%, Coefficient of Variation Area (S)-(-) = 1.93%), linear in the range of 0 - 25 mg (see Figure 5) of sample derivatized as per procedure and has a limit of detection of 99.7 : 0.3 (R) : (S) using the method outlined.

Similarly resolution was obtained for (±)-1-(1-naphthyl)ethylamine which gave a capacity factor for the first eluted isomer (R)-(+) of k = 5.06. Separation (a =1.20) and resolution (= 2.05) were along the same magnitude of values for (±)-1-(4-bromophenyl)ethylamine.

However no resolution was obtained for (±)-1-phenylethylamine (k (±)= 4.03), (±)1-phenylpropylamine (k(±) = 4.16) or (±)-N-methyl-1-phenylethylamine (k(±) = 2.31) under the conditions stated.

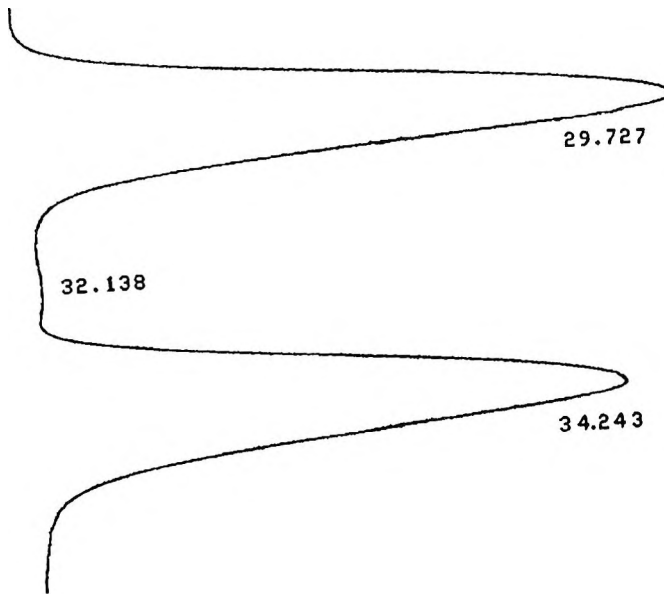


Figure 4. Chromatogram of resolved (±)-1-(4-bromophenyl)ethylamine, (R)-(+) isomer eluting first.

DISCUSSION

As can be seen from the results section, two out of the five racemic mixtures studied were resolved under the listed chromatographic conditions. Possible reasons for this are discussed below.

Separation of (±)-1-(4-bromophenyl)ethylamine and (±)-1-(1-naphthyl)-ethylamine

Working on the basis of the three point interaction model to enforce enantiomeric separation, the proposed interactions common to the separation of (±)-1-(4-bromophenyl)ethylamine and (±)-1-(1-naphthyl)ethylamine are shown in Figure 6. Figure 6 outlines the diasterioisomer formed between the CSP and (R)-(+)-1-(4-bromophenyl)ethylamine. As can be seen, the three points of interaction are (I) π-π stacking between the dinitrobenzoyl group on the

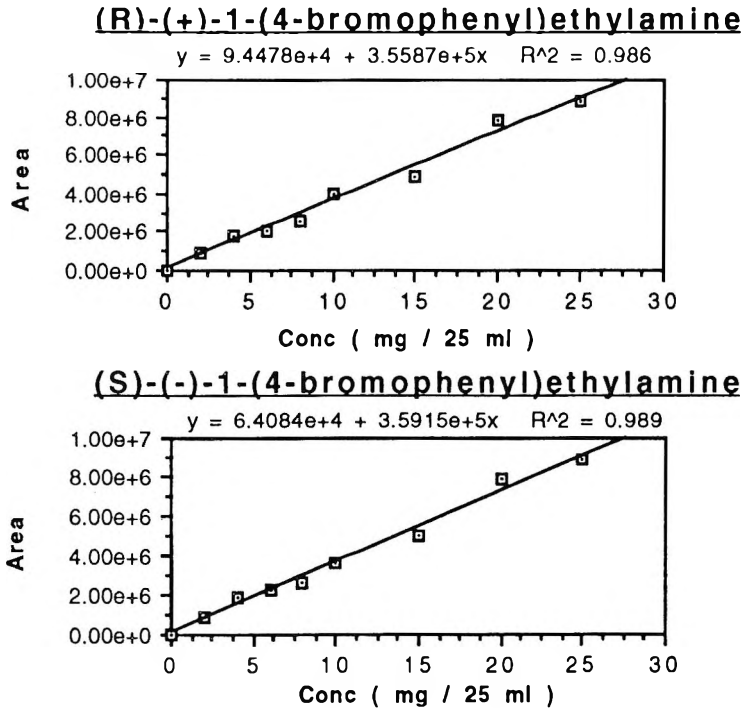


Figure 5. Linearity graphs for (±)-1-(4-bromophenyl)ethylamine.

stationary phase and the 3-toluoyl group on the analyte, (ii) Hydrogen bonding between the aminyl hydrogen on the analyte and the carboxyl oxygen on the CSP (with possible additional hydrogen bonding between the carboxyl oxygen on the analyte and the aminyl hydrogen on the CSP) and (iii) The steric interactions associated with the relative geometry and size of the methyl group and the 4-bromophenyl group on the chiral carbon of the analyte and the (S)-Leucine group on the chiral stationary phase. It can be clearly seen that if the geometry of the methyl and 4-bromophenyl groups are reversed (as is the case in (S)-(-)-1-(4-bromophenyl)ethylamine then different steric interactions will occur between the analyte and the (S)-Leucine group on the CSP.

A similar argument can be applied to (±)-1-(1-naphthyl)ethylamine, where the resolution of the (R)-(+) and (S)-(-) enantiomers is slightly greater.

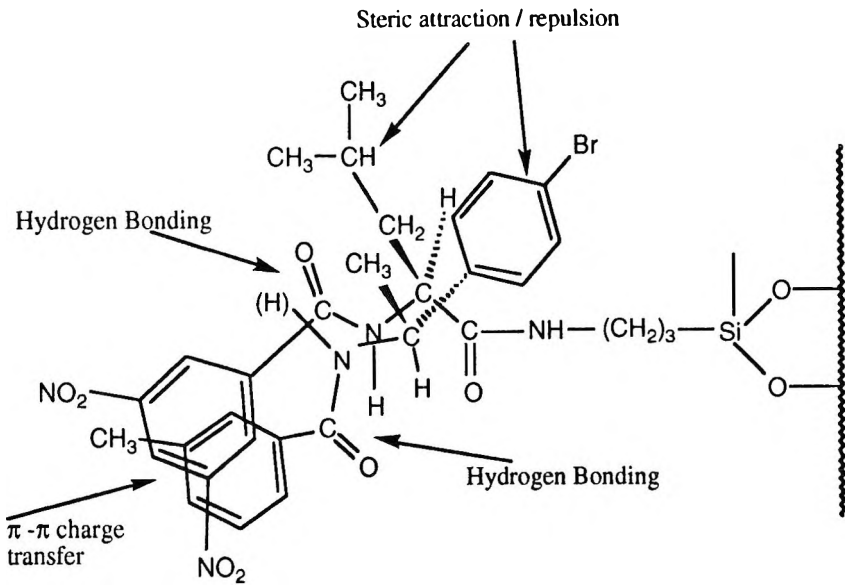


Figure 6. Resolution of (R)-(+)-1-(4-bromophenyl)ethylamine on DNB-(S)-Leucine. The key interactions between the stationary phase and analyte are highlighted.

Again the principle interactions are (i) π - π stacking between the dinitrobenzoyl and 3-toluoyl groups, (ii) hydrogen bonding between aminyl hydrogens and carboxyl oxygens and (iii) steric attraction and repulsion relating to the stereochemical configuration of the (R)-(+) and (S)-(-) enantiomers. The increase in resolution relative to (±)-1-(4-bromophenyl)ethylamine seems to be due to the increased size of the naphthyl group over the 4-bromophenyl group. Thus it seems possible that the size of the aryl group is influencing separation, a point which is further discussed in later sections.

One problem which was initially encountered during the separation of (±)-1-(4-bromophenyl)ethylamine was a lack of reproducibility in relation to replicate injections. Complete baseline resolution between the enantiomers was achieved in moving from an initial mobile phase composition of 88 : 12 hexane / 2-propanol to 94 : 6 hexane / 2-propanol, which, although it lengthened retention times, made for good reproducibility for the separation method. This

point highlights the importance of achieving the correct balance between resolution and retention on CSP's, which will depend on the application of the method. The linear range (0 -25 mg) and limit of detection (99.7 : 0.3) for (±)-1-(4-bromophenyl)ethylamine make this separation method very applicable to quality control testing of the enantiomeric composition of 1-(4-bromophenyl)ethylamine in batch manufacturing.

(±)-1-Phenylethylamine and (±)-1-Phenylpropylamine

In order to investigate the magnitude of the steric attraction / repulsion interaction on the enantioselectivity of the DNBLeucine stationary phase, both of the above compounds were investigated under the same chromatographic conditions as used for (±)-1-(4-bromophenyl)ethylamine and (±)-1-(1-naphthyl)ethylamine. (±)-1-Phenylethylamine possesses the same functionality's as the resolved enantiomers except for the size of the aryl group. The fact that it lacks a bulky moiety on the phenyl ring, and the fact that it has not been resolved on DNBLeucine under the stated chromatographic conditions is further evidence to suggest that the size of the aryl group in the aryl alkyl amines could be the key to their separating ability on the DNBLeucine stationary phase. The fact that (±)-1-phenylpropylamine has not been resolved suggests that it is primarily the aryl group and not the alkyl group which is the discriminatory factor in enantioseparation on this stationary phase. It is interesting to note that the extent of retention of (±)-1-phenylethylamine and (±)-1-phenylpropylamine on this CSP is similar, which is in good correlation with their chemical structure. (±)-1-Phenylpropylamine is retained longer because of its extra methylene group. The results seem to suggest that the phenyl group on the amines is not sufficient alone to provide chiral discrimination. This situation can be highlighted in Figure 7.

Figure 7(a) shows the case where the phenyl group attached to the chiral centre of the analyte is not substituted. The two methyl groups attached to the methyne group on the end of the leucine side chain on the CSP have unrestricted rotation around the methyne group. This is the case both (a) where the alkyl group on the analyte is in the same plane of space as the (S)-leucine side chain on the CSP and (b) where the phenyl group on the analyte is in the configuration shown in Figure 7(a).

The situation where the aryl group on the analyte is larger (e.g. 4-bromophenyl) is shown in Figure 7(b). When the alkyl group on the analyte is

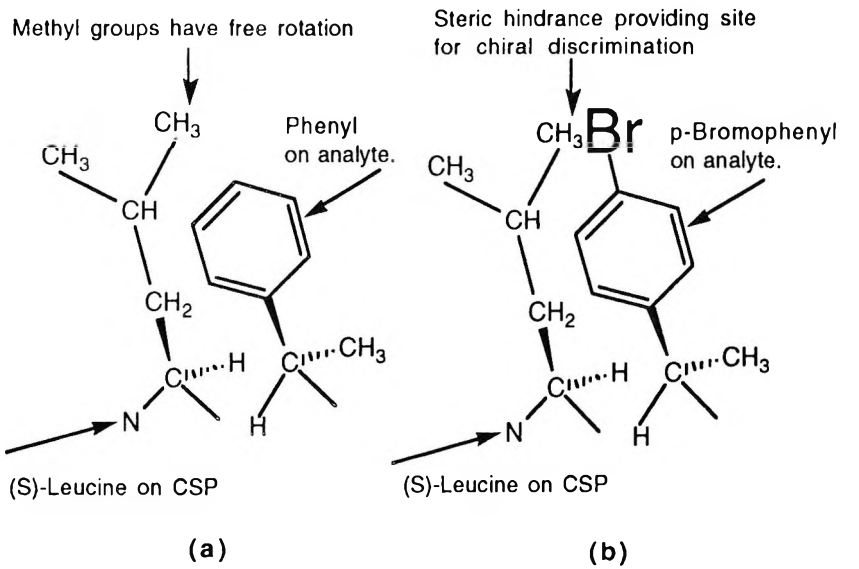


Figure 7. (a) Interaction when the phenyl group on the analyte is in the same plane as the (S)-Leucine on the CSP, (b) Interaction when the p-Bromophenyl group on the analyte is in the same plane as the (S)-Leucine group on the CSP.

in the same spatial plane as the (S)-leucine group, the methyl groups again have free rotation. However, in the case of the substituted phenyl and the (S)-leucine being in the same plane (as in Figure 7(b)), it can be clearly seen that the methyl groups have restricted rotation around the methyne group due to the bulky bromo group present on the phenyl ring of the analyte.

It is possible that here is the basis for chiral discrimination. The enantiomeric configuration for substituted aryl alkyl amines shown in Figure 7(b) will be the least retained enantiomer because it forms the least stable diasterioisomer with the DNBLeucine stationary phase.

The most retained enantiomer will be that which allows free rotation of the two methyl groups around the methyne group on the (S)-leucine portion of the CSP.

(±) N-Methyl-1-phenylethylamine

As seen, no separation of (±) N-methyl-1-phenylethylamine has occurred under the given chromatographic conditions. This is again most probably due to the lack of substitution on the phenyl ring. With this racemate there is limited hydrogen bonding capacity. The only site for hydrogen bonding is between the carboxyl oxygen on the analyte and the aminyl hydrogen on the chiral stationary phase. This limited hydrogen bonding capability is highlighted in the capacity factor obtained for this racemate (2.31) as compared to the capacity factor obtained for (±)-1-phenylethylamine (4.03), where hydrogen bonding occurs between the aminyl hydrogen on the analyte and the carboxyl oxygen on the stationary phase.

Overall, the experimental data highlights the three point interaction criteria which must be met in order to achieve enantioseparation. With regards to our choice of analytes and stationary phase, the three interactions are

(i) the π basic site on the analyte stacking on the π acidic site of the CSP,

(ii) hydrogen bonding between aminyl hydrogens and carboxyl oxygens and

(iii) steric interactions between substituted phenyls on the analyte and the (S) leucine group on the stationary phase.

The latter interaction is indicated as the key point in distinguishing between enantiomers.

ACKNOWLEDGEMENTS

This work has been supported by FORBAIRT and Arran Chemical Co. Ltd.

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