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THE MEASUREMENT OF PORE SIZE DISTRIBUTIONS, SURFACE AREAS, AND PORE VOLUMES OF ZIRCONIA AND ZIRCONIA-SILICA MIXED OXIDE STATIONARY PHASES USING SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Size exclusion chromatography was used to evaluate the surface areas, pore volumes, and pore size distributions of a variety of zirconias and silica-zirconia composites. In addition to the size exclusion results presented, the zirconias had been previously analysed using nitrogen sorption experiments making comparisons to established methods feasible. Agreement between the nitrogen sorption and size exclusion techniques was reasonably good with the size exclusion method serving to illustrate the chromatographic significance of the pore structure.

Due to the stabilisation of the amorphous silica-zirconia composites the surface areas were shown to be much higher than the surface areas of the zirconias. However, after crystallisation the surface area decreased very rapidly. The results also illustrate that the inclusion of salts during calcination allows the widening of the pores for amorphous materials and increases the pore volume of both amorphous and crystalline materials.

INTRODUCTION

Recent studies on the development of zirconia as a new stationary phase support for liquid chromatography have yielded interesting results.[1-6] Zirconia has been shown to be a highly chemical resistant support that often exhibits, often complex, elution mechanisms due to the heterogeneous nature of the surface. However, because of the chemical stability of zirconia, a wide choice of eluent modifiers may be employed and this allows control of elution mechanisms and some excellent separations have been obtained. Nawrocki and coworkers[6] have recently reviewed the use of zirconia in chromatographic systems and described the changes in surface area, pore size, and pore volume obtained as a result of thermal treatment. Because of microcrystallite growth and intercrystallite sintering during thermal treatment, samples treated above 500°C generally have specific surface areas less than 100 m^2/g and the surface area further decreases as the calcination temperature increases. If the thermal treatment is less than 400°C the resulting zirconia is usually microporous. The crystalline phase of zirconia has also been shown to influence the surface area. Mercera and coworkers[7] for instance, have shown that the monoclinic phase has a higher thermal stability than the tetragonal phase and shows a slower decrease in surface area as the calcination temperature increases. Even so, because microporosity dominates, surface areas of tetragonal zirconia may remain as high as 90 m^2/g even after heat treatment at 800°C. The same authors showed that the doping of zirconia with either yttrium or lanthanum stabilises the tetragonal zirconia, and in particular, lanthanum-stabilised tetragonal zirconia was found to be more thermally stable than the corresponding monoclinic zirconia due to the inhibition of the processes of crystallite growth.

Recently Kaneko and coworkers[8] combined silica with each of zirconia, alumina, magnesia, and titania as stationary phase materials. The preparation of their composite supports involved the coprecipitation of silica with each of the corresponding oxides. The resulting particles were irregular and had broad size distributions that required milling followed by size separation to obtain a suitable particle size and distribution for chromatographic applications. The

composite stationary phase materials were dried at 110°C and the zirconia-silica stationary phase was predominately microporous with an average pore size of 2.1 nm.

In a previous communication we described[9] the preparation of spherical zirconia-silica composites for chromatography. These composites crystallised into a tetragonal crystal structure after calcination at a temperature dependent on the concentration of silica. Thermal treatment of the composites at temperatures as high as 1300°C produced zircon ($ZrSiO_4$) when the atomic ratio of silicon to zirconium approached unity. The tetragonal phase was less stable when low concentrations of silica were present and this allowed the formation of a monoclinic phase at high temperatures. Such transitions in crystalline state with varying degrees of silica content may produce a stationary phase that has a variety of porous states, offering varying and possibly novel selectivities. However, to date, very little chromatographic behaviour has been studied on these supports.

Studies on the porous structure of zirconia[10] have shown that the inclusion of sodium chloride during the calcination process changes the pore shapes from restricted 'ink bottle' shapes to more open cylindrical pores. Inclusion of sodium chloride during the calcination process was shown to increase the pore volume, surface area and pore diameter when compared to a zirconia calcined under the exact same conditions but without the sodium chloride. The method of salt inclusion for increasing pore sizes of narrow pore size silica gel was first introduced by Krebs *et al.*[11] Novák and Belek[12] showed that the inclusion of sodium chloride during calcination often caused bimodal pore size distributions or cracks to develop in the particles and suggested that improvements in the mixing of the salt and silica may improve the pore size homogeneity. Very little information regarding the nature of the effects of salt inclusion during calcination has been published since and no publications have been reported for zirconia or silica-doped zirconia composites.

The usual method of pore size determinations typically involves nitrogen sorption experiments, according to the theory of Brunauer, Emmet and Teller, or mercury porosimetry. However, Halász and Martin[13] described a method for determining pore size distributions of porous materials using size exclusion chromatography. The method relies solely on the elution of polystyrene standards when the chromatographic column is used in a size exclusion mode. Mobile phases must have a high solvent strength in order to avoid any adsorption of the polymer onto the stationary phase. The method assumes that, for a given column geometry, elution of the polystyrene is a function of the molecular size of the polymer. Hence, the stationary phase must be rigid so that the pore geometry does not undergo variations in size during elution.

Simply, the method is the reverse of classical size exclusion chromatography, where the porous structure of the chromatography column is known and the molecular weight of the polymer is unknown. Calibration of the molecular size of polystyrene standards was achieved by analysing the polystyrene standards on silica gel that had a narrow pore size distribution, which had been previously evaluated by classical methods.

The current work utilises size exclusion chromatography to study the changes in pore structure that occur for zirconia and a zirconia-silica composite, as the thermal treatment of the material is varied. In our laboratory, a large number of stationary phase supports are routinely prepared and size exclusion chromatography is an excellent method that allows a rapid identification of stationary phases suitable for more detailed investigations. An important advantage of the size exclusion method of pore analysis is that it allows the evaluation of the chromatographically available pore structure, hence, the results are directly applicable to chromatographic systems.

EXPERIMENTAL

The surfactants Span 80, Brij 35 and Tween 85 were supplied by the Sigma Chemical Company Inc (St. Louis, MO. USA). Sodium metasilicate pentahydrate was obtained from BDH Chemicals Ltd (Poole, England). Zirconyl chloride (99%), urea and hexamethylenetetramine were supplied by the Aldrich Chemical Company Inc (Milwaukee Wis. USA). Polystyrene standards with molecular weights $8.50x10^5$, $4.50x10^5$, $1.85x10^5$, $8.7x10^4$, $2.8x10^4$, $1.02x10^4$, $3.55x10^3$, and $1.35x10^3$ were obtained from Shodex. HPLC grade methanol was obtained from BDH Chemicals (Poole, England) and AR grade dichloromethane was supplied by Ajax Chemicals (N.S.W., Australia). All chromatographic solvents were filtered through a Millipore 0.45 μm filter prior to use.

The preparation of zirconia and the zirconia-silica composites was described in previous publications.[9,10] In the present work a composite material was prepared containing a silica concentration that would allow a change in the crystal structure from the amorphous state to the tetragonal phase at a temperature corresponding approximately to the melting point of sodium chloride, namely 804°C. This specifically involved the hydrolysis of zirconyl chloride octahydrate (80 g) in water (60 mL) and the formation of zirconia microspheres with particle sizes of approximately 2 μm. Sodium metasilicate pentahydrate (52.7 g) was hydrolysed in water (80 mL), which was then added to the zirconia microspheres in a total volume of 500 mL. The reaction was allowed to proceed for 48 hours allowing the water to evaporate until a thick

paste was obtained. The resulting composite was washed and calcined at either 500°C, 655°C or 810°C for two hours. Two samples of the zirconia-silica composite were calcined in the presence of either sodium iodide or sodium chloride. In both instances the amorphous zirconia-silica composite was stirred overnight in water containing one part composite to one part salt (%w/w). The total volume of water was such that the concentration was approximately 5% w/v with respect to the composite. The water was allowed to evaporate and the composite/salt mixture dried overnight at 110°C. The composite/salt mixture was then calcined for two hours at either 655°C (in the case of sodium iodide impregnation) or 810°C (for sodium chloride impregnation). The calcined composite materials were then washed in copious quantities of water to remove the salts. Previous studies have shown that the resulting zirconia-silica materials are in fact composites after calcination and not a mixture of silica and zirconia particles.[9]

The zirconia stationary phases were calcined at 450°C, 600°C and 810°C as described previously.[10] Additionally, the zirconia was calcined in the presence of sodium chloride at 810°C. This involved stirring one part amorphous zirconia with one part sodium chloride in water (% w/w), the initial concentration of zirconia being 5% w/v. The mixture was stirred overnight, after which the water was slowly evaporated with heat and the solid dried overnight at 110°C. The zirconia was then calcined at 810°C for one hour. The remaining sodium chloride was removed by washing with copious quantities of water.

Particle sizes and particle size distributions were determined using a Jeol 35CF JSM scanning electron microscope (SEM)(Jeol Ltd., Tokyo, Japan). Particle size distributions were determined by measuring the diameters of at least 300 spheres. Xray elemental analysis was performed by firstly mounting the samples in resin and grinding the surface to a highly polished, flat finish. The sample plug was then coated lightly with carbon and analysed using the Tracor Xray facilities on the SEM at 25 KeV.

Differential thermal analysis (DTA) was used to determine the temperature of transformation from the amorphous state to a crystalline phase.[14] The zirconia and zirconia-silica composites were subjected to thermogravimetric analysis (TGA) and DTA using a Setaram TGA 92 at heating rates of 5°C/min (Setaram, Saint-Cloud, France).

Raman spectra were obtained using a Perkin Elmer System 2000 FT-Raman spectrometer (Perkin Elmer, Cal., USA). The FT-Raman spectrometer was equipped with a quartz beamsplitter and a continuous-wave Spectron Laser Systems SL301 Nd:YAG laser emitting at 1064 nm and an InGaAs detector operated at

ambient temperature. The mirror velocity was 0.2 cm s^{-1}, and strong Beer-Norton apodisation was used. A notch type Rayleigh filter was installed to provide an extended scan range from 3600 - 100 cm^{-1}. Typically, 200 scans were recorded at a resolution of 4 cm^{-1} and laser power of 400 mW. All FT-Raman spectra were corrected for instrumental effects.

Chromatographic columns were prepared in 5 cm x 0.46 cm stainless steel column blanks fitted with 0.5 μm stainless steel end frits. A Haskel air driven fluid pump (Haskel Engineering and Supply Co. Burbank, CA.USA.) was used as a packing pump and columns were packed in a downward slurry using a methanol packing solvent, a methanol slurry solvent and a dichloromethane displacement solvent. Columns were packed until the flow rate became constant.

Chromatographic analysis was achieved using a Varian 5000 chromatographic system fitted with a 10 μL Valco injection port and a variable wavelength UV detector set at 254 nm. Size exclusion experiments were performed by injecting polystyrene standards of various molecular weights (0.5 mg mL^{-1}) into a dichloromethane mobile phase. Flowrates were approximately 0.3 mL min^{-1} as indicated in the text. The exact flowrate during the elution of each polystyrene standard was recorded during elution and elution volumes were corrected for actual flowrates. All chromatographic experiments were carried out at 23°C ± 1°C and were replicated.

For ease of discussion, the stationary phases will be referred to using abbreviations such as Zr810NaCl, where Zr refers to a zirconia stationary phase, 810 refers to the temperature of calcination and NaCl indicates that calcination was carried out in the presence of sodium chloride. Hence, Zr-Si655, refers to a composite stationary phase of zirconia and silica calcined at 655°C without salt, and Zr-Si655NaI refers to the same material calcined in the presence of sodium iodide.

RESULTS AND DISCUSSION

Using electron microscopy, it was determined that the particle sizes of each of the zirconia-silica composite stationary phases were within the range 2.0 μm ± 0.6 μm. No significant difference in particle size was apparent between composites calcined in the presence of salts compared to those calcined without salts. The zirconias used for stationary phases in this work were identical to the zirconia used in a previous publication that described the particle size as 1.3 μm ± 0.5 μm.

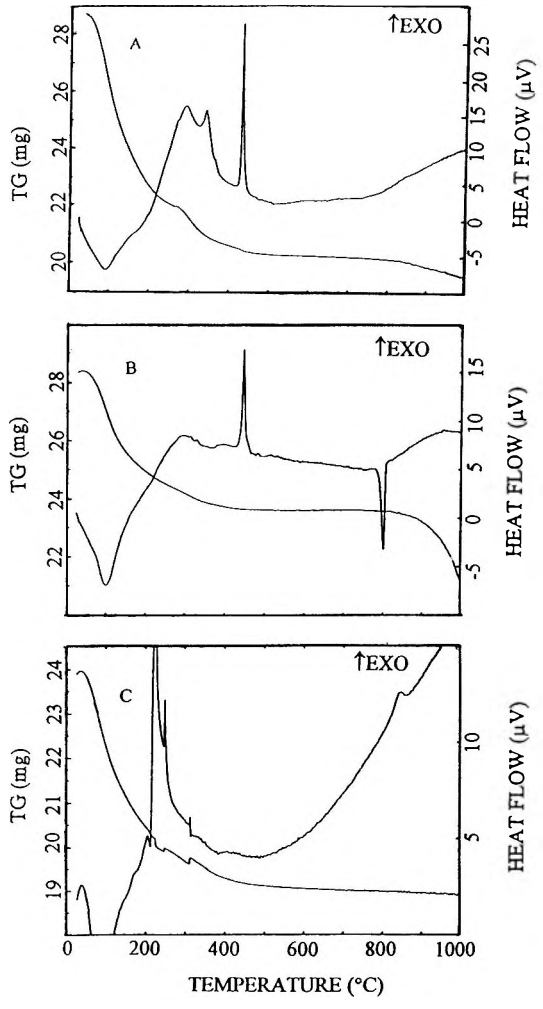

Figure 1. Differential thermal analysis and thermal gravimetric analysis of (A) zirconia and (B) sodium chloride impregnated zirconia at heating rates of 5°C min^{-1}, and (C) zirconia-silica composite at a heating rate of 2°C min^{-1}.

Differential thermal analysis (DTA) of the zirconia (Figure 1a) and the sodium chloride impregnated zirconia (Figure 1b) show the presence of a sharp exothermic 'glow' peak at 467°C and 450°C respectively. Previous workers have assigned this peak to a phase change from amorphous zirconia[14-18] to a metastable tetragonal phase, after which the zirconia undergoes a further phase change to a monoclinic form. Previously we reported Raman spectral results

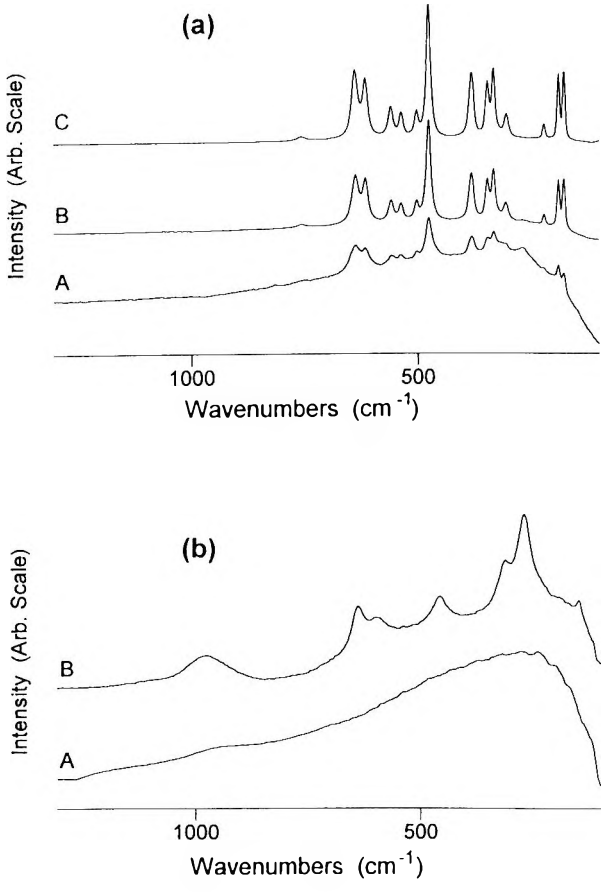

Figure 2. (a) Raman spectrum of the zirconia calcined at (A) 450°C, (B) 600°C, and (C) 810°C. (b) Raman spectrum of the zirconia-silica composites calcined at (A) 655°C and (B) 810°C.

that showed zirconia calcined at a temperature slightly above the crystallisation temperature according to DTA, transformed to a tetragonal phase, which when left at room temperature for two weeks further crystallised into the monoclinic phase[9]. Figure 1b also shows the presence of a large endothermic peak at 804°C corresponding to the melting of sodium chloride. Figure 1c illustrates the DTA and TGA of the zirconia-silica composite. A small exothermic 'glow' peak with a maximum at 844°C is apparent. This is associated with a phase change from the amorphous state to a tetragonal phase.[9] All DTA results showed a broad endothermic peak at around 100°C due to the loss of water, and

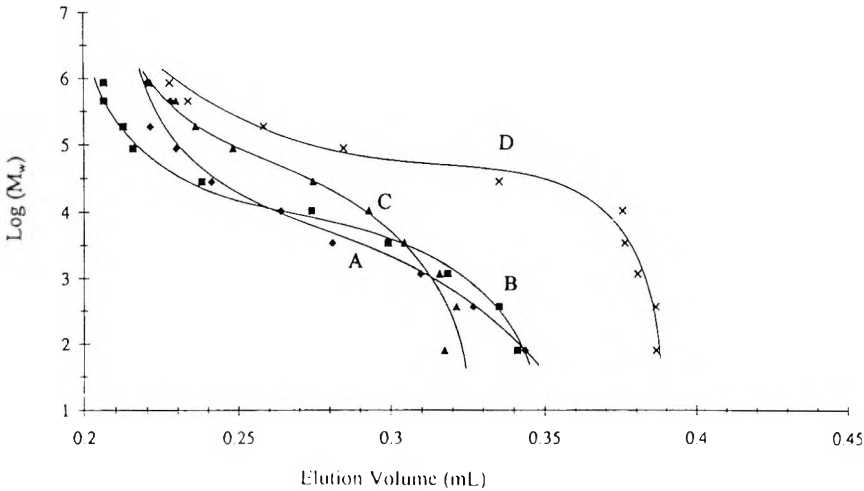

Figure 3. Size exclusion curves for the zirconia columns (A) Zr450, (B) Zr600, (C) Zr810 and (D) Zr810NaCl. Flow rate 0.4 mL min^{-1}, temperature 23°C ± 1°C.

an exothermic region at approximately 300°C due to the combustion of residual organics. Previous workers[17] determined that no phase change occurred within this temperature range. TGA shows a large mass loss within this temperature range, supporting the loss of water and organics.

Raman spectroscopy has been shown to be a useful method for determining phase compositions of zirconia.[19] Raman spectral analysis of the zirconia stationary phases showed that each sample contained varying degrees of monoclinic zirconia. The sharpness and the peak intensity, indicative of the monoclinic crystalline phase, was much greater for the zirconias calcined at 810°C, and 600°C than that of the zirconia calcined at 450°C, which was mainly amorphous with slight monoclinic crystallinity (Figure 2a). The Raman spectrum of the zirconia-silica composite (Figure 2b) calcined at 810°C showed the sample contained some tetragonal zirconia, with much of the material remaining amorphous. All samples calcined below the crystallisation temperature according to the DTA remained amorphous (for example ZrSi655, Figure 2b).

The size exclusion elution behaviour of polystyrene standards with narrow molecular weight distributions was examined in a dichloromethane mobile phase on each of the zirconia stationary phases. The size exclusion curves for each of the zirconia columns are illustrated in Figure 3. Pore size distributions

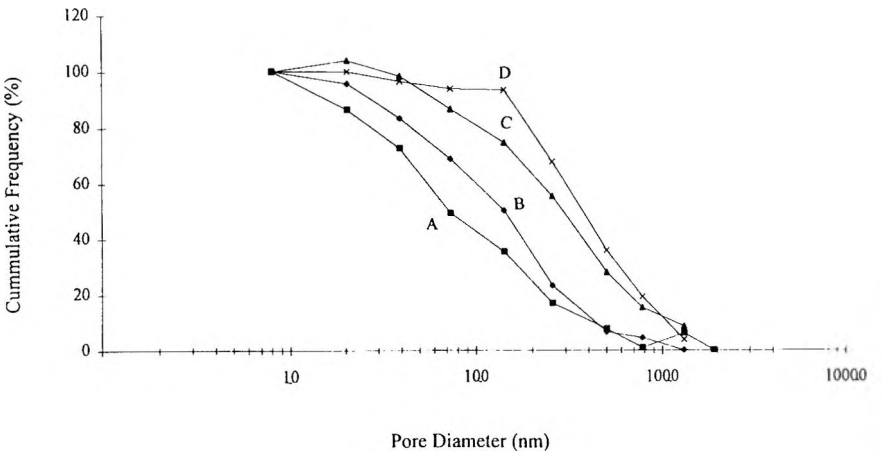

Figure 4. Cumulative pore size frequency distribution curves for the zirconia stationary phases (A) Zr450, (B) Zr600, (C) Zr810 and (D) Zr810NaCl columns.

were calculated from the size exclusion data for each of the stationary phases. Figure 4 illustrates the cumulative frequency of pores versus the pore size for each of the zirconia stationary phases and Figure 5 illustrates the pore size distribution curves for zirconia samples Zr 600 and Zr810NaCl as examples. The mean pore diameter and the standard deviation for each of the stationary phases were calculated by plotting the sum of residues versus the log of the pore diameter on probability paper.[13] The probability plots (not shown) for each of the zirconia samples resulted in a scatter of points along a straight line with some variation at the extremes, indicating the pore size distribution was approximately Gaussian. The mean pore diameter determined from these Gaussian curves are presented in Table 1, together with the standard deviation (log σ). Specific surface area estimates were determined using the Guassian mean pore diameter for each of the zirconia stationary phase supports and these results are given in Table 1. The specific pore volumes are also included in Table 1.

The size exclusion results illustrate that qualitatively two types of zirconia packing materials resulted after calcination. The first type is derived from the zirconia materials calcined at 450°C (curve A), 600°C (curve B) and 810°C (curve C). As the calcination temperature increased the exclusion limit and the inclusion limit of these stationary phases increased. Hence an increase in pore size occurred as illustrated by the pore size frequency distributions in Figure 4. This result is supported by nitrogen adsorption measurements previously

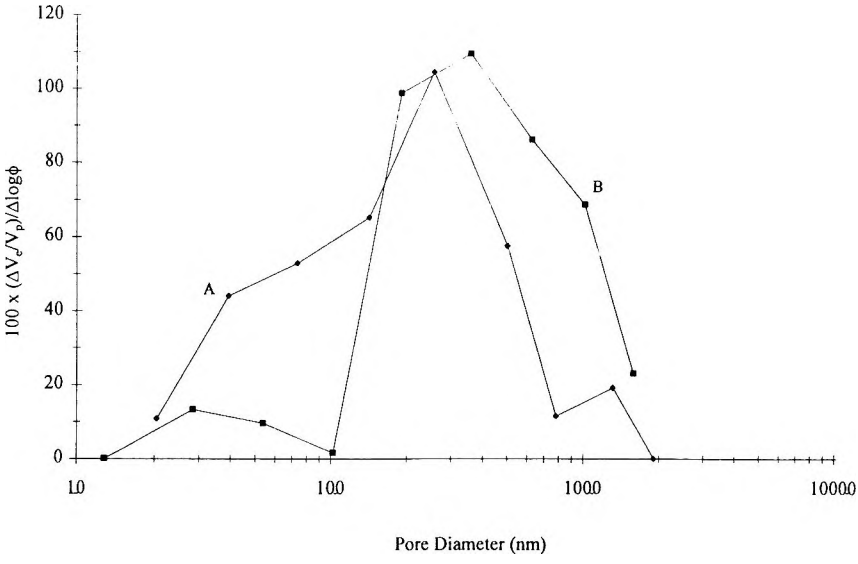

Figure 5. Pore size distribution curves for the zirconia stationary phases (A) Zr600, and (B) Zr810NaCl columns.

Table 1

Pore Size, Surface Areas, and Pore Volumes of the Zirconia Stationary Phases Determined from Size Exclusion Chromatography

Stationary Phase	Pore Size [nm] (Gussian)	Pore Size Std. Dev. (logσ)	Specific Surface Area [m^2/g]	Specific Pore Volume [mL]
Zr450	7.9	0.54	46	0.0912
Zr600	13.5	0.51	27.5	0.0930
Zr810	28.2	0.50	8.5	0.0600
Zr810Na	35.5	0.49	14.9	0.1324

conducted[10] on these samples. The Zr450 sample produced a poor quality pore size distribution curve, which reflected the limit of the size exclusion method for determining pore sizes as the microporous region is approached, particularly if the size distribution is broad. Note that this sample had an average pore size of 7.9 nm determined by size exclusion and 5.7 nm

determined by nitrogen adsorption measurements.[10] The size exclusion curve for Zr450 shows that the inclusion limit of the column was indistinct, illustrating the broad pore size distribution of smaller diameter pores. To a lesser extent, the size exclusion curve of the Zr600 stationary phase also showed that there was a broad size distribution of the smaller diameter pores as the inclusion region of the column was also not distinct. Less resolution is observed for the low molecular weights on the Zr810 stationary phase indicating fewer smaller mesopores and micropores. This is further illustrated by the pore size frequency plots in Figure 4 where the percentage of small pores decreased significantly as the temperature of calcination increased. For instance, examination of the Zr450 phase shows that 50% of the pores were less than 7.0 nm in diameter with approximately 14% of the pores being microporous. In comparison, for the Zr810 support, 50% of the pores were less than 30.0 nm and the microporosity was essentially zero percent.

The second type of zirconia packing material is illustrated by the size exclusion curve of the Zr810NaCl stationary phase (Figure 3, curve D), which was clearly different from the group of size exclusion curves obtained on the Zr450, Zr600, and Zr810 stationary phases. While the exclusion limit was similar to the exclusion limit of the Zr810 stationary phase, the inclusion region of the Zr810NaCl stationary phase was markedly different. Within the inclusion region, all polystyrenes with molecular weights up to 10 kdaltons co-eluted, and this indicated a significant change had occurred in the smaller mesoporous/microporous structure of the stationary phase compared to the zirconia calcined at the same temperature without the sodium chloride. The cumulative frequency plot for the sample Zr810NaCl in Figure 4 illustrates this effect, with only 7% of the pores being between 2.0 nm and 7.0 nm and 50% of the pores were greater than 40.0 nm and no microporosity was apparent. However, the most significant difference between the sodium chloride impregnated zirconia and the zirconias calcined without sodium chloride was the specific pore volumes. The Zr810NaCl material produced a specific pore volume that was slightly over twice the specific pore volume of the Zr810 stationary phase. This improved the linear region of the size exclusion curve (indicated by the slope of the linear portion of curve D). The specific pore volume of the Zr810NaCl stationary phase was a third greater than the specific pore volume of the Zr450 stationary phase, which contained a significant degree of microporosity. However, no microporosity was apparent on the Zr810NaCl stationary phase (see Figure 4).

Size exclusion curves for each of the zirconia-silica columns are illustrated in Figure 6. Pore size distributions were calculated from the size exclusion data for each of the stationary phases. Cumulative pore size frequencies are shown in Figure 7. Pore size distributions of the zirconia-silica

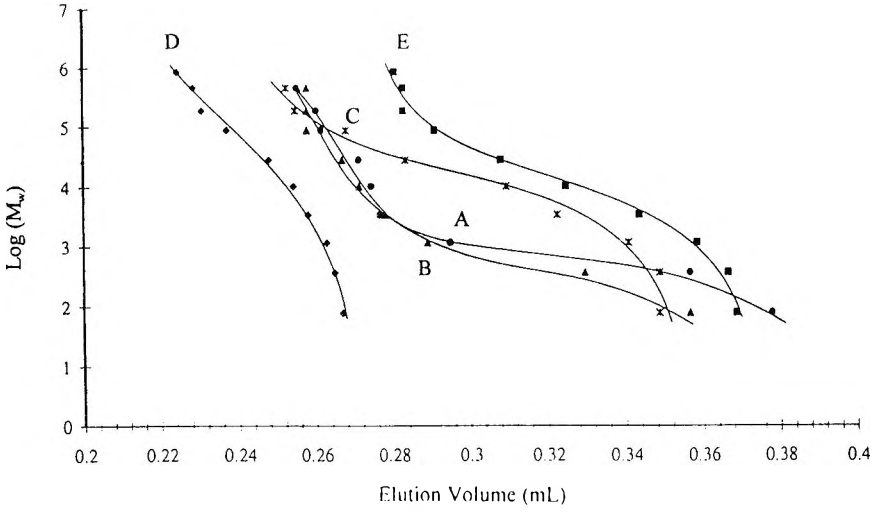

Figure 6. Size exclusion curves for the zirconia-silica columns (A) Zr-Si500, (B) Zr-Si655, (C) Zr-Si655NaI, (D) Zr-Si810 and (E) Zr-Si810NaCl. Flow rate 0.3 mL/min, temperature 23°C ± 1°C.

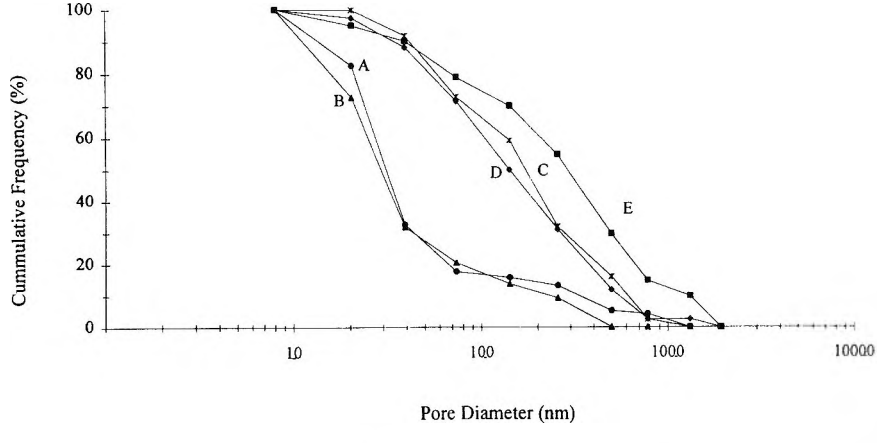

Figure 7. Cumulative pore size frequency distribution curves for the zirconia-silica stationary phases (A) Zr-Si500, (B) Zr-Si655, (C) Zr-Si655NaI, (D) Zr-Si810 and (E) Zr-Si810NaCl.

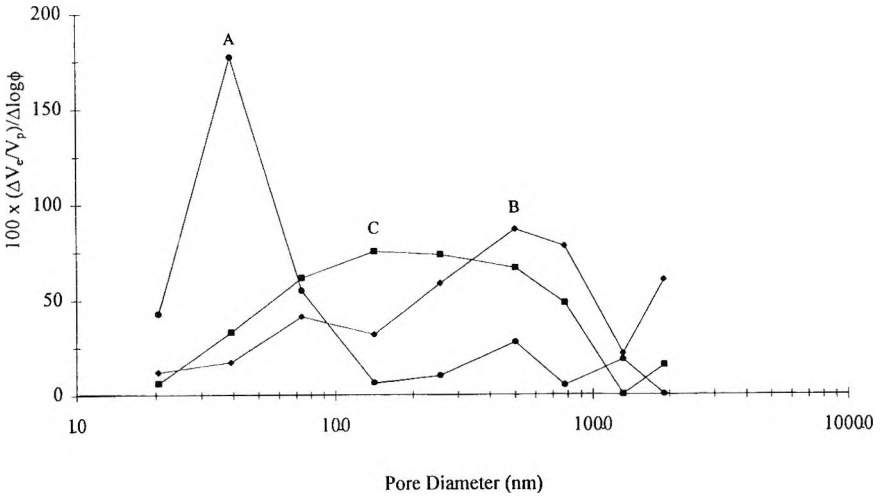

Figure 8. Pore size distribution curves for the zirconia-silica stationary phases (A) Zr-Si500, (B) Zr-Si810 and (C) Zr-Si810NaCl columns.

composites Zr-Si500, Zr-Si810NaCl and Zr-Si810 are shown as examples in Figure 8. The mean pore diameter and the standard deviation for each of the zirconia-silica stationary phases were calculated by plotting the sum of residues versus the log of the pore diameter on probability paper.[3] These probability plots resulted in a scatter of points along a straight line for the Zr-Si810NaCl, Zr-Si810 and Zr-Si655NaI stationary phases indicating the pore size distribution was approximately Gaussian. The probability plots of the Zr-Si655 and the Zr-Si500 stationary phase indicated that the pore size distribution may be bimodal, but further experiments using nitrogen sorption will be required to confirm this. Nevertheless, because the support was dominated by micropores, an averaged unimodal pore size distribution was assumed. The mean pore diameter determined from the probability plots for each of the stationary phases are presented in Table 2, together with the standard deviation (log σ). Surface area estimates and specific pore volumes are also included in Table 2.

Increasing the calcination temperature of the composite materials in the absence of salt increased the exclusion molecular weight and increased the pore size, but only after the calcination temperature exceeded the crystallisation temperature of the support. Despite a difference in calcination temperature of 155°C, both the Zr-Si500 and Zr-Si655 stationary phases produced very similar size exclusion calibration curves that indicated a microporous support. Both of

Table 2

Pore Size, Surface Areas, and Pore Volumes of the Zirconia-Silica Composites Determined from Size Exclusion Chromatography

Stationary Phase	Pore Size (Gussian) [nm]	Pore Size Std. Dev. [logσ]	Specific Surface Area [m^2/g]	Specific Pore Volume [mL]
Zr-Si500	2.0	0.80	368	0.1482
Zr-Si655	1.25	1.00	296	0.1198
Zr-Si655NaI	16.6	0.55	2.49	0.1035
Zr-Si810NaCl	15.8	0.50	2.27	0.0898

these phases remained amorphous at their respective calcination temperatures. A further increase in calcination temperature of 155°C to 810°C increased the exclusion limit of the stationary phase from $3.55x10^3$ daltons (Zr-Si600 and Zr-Si500) to $1.85x10^5$ daltons (Zr-Si810). The mean pore size of the Zr-Si810 stationary phase was 25.1 nm and the specific pore volume was less than 25% of the specific pore volume of the Zr-Si500 stationary phase. The Zr-Si810 stationary phase was essentially free of microporosity as shown by the pore size frequency plots in Figure 7.

Calcination of both the zirconia-silica composites and the zirconia in the presence of sodium salts significantly altered the pore structure of the support. The most significant effect was the increase in specific pore volume of the stationary phase as a result of the inclusion of the sodium salts. The specific pore volume of the Zr-Si810NaCl stationary phase was 2.8 times that of the Zr-Si810 stationary phase for which no salt was added and this resulted in an extension of the linear region of the size exclusion curve for the salt impregnated stationary phases as shown in Figure 6. Additionally the inclusion limit of the sodium chloride impregnated composite was more distinct and occurred at a slightly lower molecular weight. Previous studies on zirconia have shown that the inclusion of salt during calcination and the subsequent increase in specific pore volume leads to a more distinct pore structure where pores change from the 'ink bottle' shape to the more favourable cylindrical shape.[10]

The size exclusion curve of the zirconia-silica stationary phase calcined in the presence of sodium iodide at 655°C (curve C) is very different from the size exclusion curve of the same material without the salt. The exclusion region of the Zr-Si655NaI stationary phase was almost $1.85x10^5$ daltons and the mean

pore diameter was 16.6 nm compared to the microporous Zr-Si655 stationary phase that had an exclusion limit approximately $3.55x10^3$ daltons. Both the Zr-Si655 and Zr-Si655NaI stationary phases had similar specific pore volumes, but this is clearly a result of microporosity in the Zr-Si655 material. The linear region of the size exclusion curve of the Zr-Si655NaI stationary phase is more defined. In fact, the size exclusion curve is almost the same as the size exclusion curve for Zr-Si810NaCl stationary phase, yet the Zr-Si655NaI stationary phase is totally amorphous and the Zr-Si810NaCl is at least partially tetragonal after calcination at a temperature 155°C higher. Hence, crystallisation of the composite inorganic oxide is not solely responsible for the increase in pore size. The pore size distribution curves and cumulative pore size frequency plots clearly indicate that the addition of the salt has almost eliminated the microporosity.

In the current study, the inclusion of salts has been shown to be beneficial in the preparation of materials that yield more uniform pore size distributions, having a narrow pore size range, larger pore volumes and higher surface areas than identical supports prepared without the salt. The increase in pore volume due to the inclusion of the salts during calcination is significant, as large pore volumes are particularly useful for size exclusion chromatography where separation is based on a molecular sieving mechanism carried out within the column pore volume. A large pore volume equates to better separations as illustrated by the improvement in the linear region of the size exclusion curves for material calcined in the presence of the salts. Additional benefits may also be sought in liquid-liquid partition chromatography and interactive liquid chromatography.[20]

These results show that significant differences in pore sizes exist between the zirconia and the zirconia-silica composites for similar temperatures of calcination. For instance, the zirconia-silica material remained microporous when calcined at 655°C compared to a zirconia stationary phase that had a mean pore size of 13.5 nm calcined at 600°C. Surface areas of the amorphous zirconia-silica composites were much higher than the monoclinic/amorphous zirconia. However, the rate of decrease in the surface area of the zirconia-silica composite as a result of thermal treatment was much more rapid than for the zirconia. For example, the specific surface area for the zirconia-silica composites decreased from approximately 300 m^2/g to 0.5 m^2/g with a change in calcination temperature from 500°C to 810°C. In comparison, the specific surface area of the zirconia decreased from approximately 40 m^2/g at 450°C to 8.5 m^2/g at 810°C. These results illustrate that the monoclinic zirconia is more thermally stable than the amorphous-tetragonal composites and this supports the study be Mercera *et. al.*[15] where they found the monoclinic zirconia to be more stable than the tetragonal form.

The size exclusion method of analysis has proved a useful tool for a comparative study on pore structures of various supports. The results for the zirconia supports were very similar to those obtained by nitrogen adsorption measurements previously determined[10] and the method allows a large number of supports to be examined quickly to determine which stationary phases warrant further detailed examination. The largest difference in surface area was 14% and the greatest difference in pore size was 30%, which was for the Zr450 stationary phase where the average pore size using nitrogen adsorption BET measurements was 5.7 nm compared to 7.9 nm using the size exclusion method. Hence, a high degree of confidence may be placed in the size exclusion results, particularly when used as a comparative method to examine differences between mesoporous stationary phases.

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ENANTIOSEPARATION OF D,L-PHENYLALANINE BY MOLECULARLY IMPRINTED POLYMER PARTICLES FILLED CAPILLARY ELECTROCHROMATOGRAPHY

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ABSTRACT

The chromatographic properties of molecularly imprinted polymer filled capillary electrochromatography (CEC) have been studied. The network polymers were prepared using L-phenylalanine anilide (L-pheAN) or L-phenylalanine as print molecules. Methacrylic acid was used as functional monomer, such that the acid function of monomer interacts ionically with the amine function and via hydrogen bonding with the carboxyl group of the print molecules. Ethylene glycol dimethacrylate was used as cross-linker and the reaction initiator was α, α′-azobis(isobutyronitrile). The obtained polymers were ground and sieved to particles<10 μm for filling into capillary for CEC and <30μm for packing into high performance liquid chromatographic columns. The separations of D,L-phenylalanine by using the molecularly imprinted polymers against L-pheAN and L-phenylalanine were compared. It was

interesting to find that the resolution of D,L-phenylalanine using L-pheAN printed polymer was higher than that using L-phenylalanine printed polymer. The selected polymers obtained were tested in CEC. Some aromatic amino acids, such as D,L-phenylalycine, D,L-tyrosine etc., could be separated to some extent. This method demonstrated that the molecularly imprinted polymer can also be used in CEC for the separation of enantiomers. The peak shape and the resolution of D,L-phenylalanine by this proposed method were better than those by the HPLC method.

INTRODUCTION

High resolution enantiomer separation by chromatography has been developed into an indispensable tool in contemporary chiral analysis. Specifically, the separation of amino acid enantiomers has been widely investigated because of their commercial significance and ease of availability. Several chromatographic techniques, such as HPLC,[1,2] GC,[3,4] and capillary electrophoresis[5,6] have been employed for the enantioseparation. Most of these techniques used chiral selectors and were largely empirical, and the mechanism of chiral recognition is not fully understood. Therefore, it is often difficult to predict the elution order of enantiomers. Molecular imprinting technique,[7-9] as a method to prepare support materials for molecular separation and concentration, has attracted much attention. Mosbach and his co-workers have made a lot of excellent contributions to this research field.[10] This technique has been demonstrated to be effective for amino acid derivatives,[11,12] metal ions,[13,14] saccharides,[15,16] organic compounds of low molecular weight,[17,18] and so on. The molecular imprinting method has been successfully used in HPLC for the separation of amino acid derivatives and some racemates.[19,20] In recent years, this technique has also been applied in CEC[21] and some interesting results have been obtained. Although, template polymerization is conceptually attractive for the preparation of highly selective adsorbents, successful demonstrations of chromatographic separation involving these materials are still relatively few. To our knowledge, although there are a lot of investigations concerned with chiral separation by using molecularly imprinted polymer, most all of them were based on HPLC method. There are almost no publications about chiral separation using molecularly imprinted polymer filled CEC. The aim of this study was to try to use molecularly imprinted polymer particles filled capillary for the enantioseparation by electrochromatography. The polymers printed with L-pheAN and L-phenylalanine were compared each other for the separation of D,L-phenylalanine, and the results from CE and HPLC are also discussed.

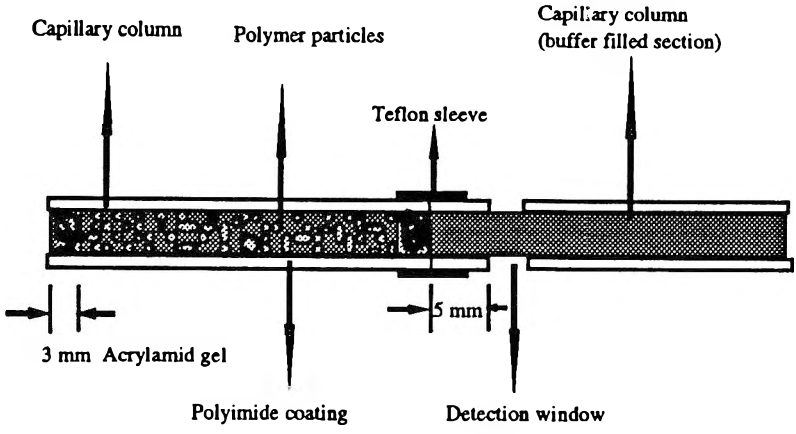

Figure 1. Schematic diagram of the connection of polymer filled capillary with an open tubular capillary.

EXPERIMENTAL

Apparatus

For capillary electrophoresis (CE) measurements, a laboratory made CE set[22] was used, which consisted of a UV-8011 detector (Tosoh, Japan), a HEL5-30P2-TTu high voltage power supply (Matsusada Precision Devices, Inc., Japan), and a C-R6A Chromatopac Recorder (Shimadzu, Japan). Separations were carried out in fused-silica capillaries (75-μm I.D. and 375-μm O.D.). The capillary was composed of two sections. One section is packed with molecularly imprinted polymer particles. The polymer particle slurry was pumped into the capillary at 200 kg cm^{-2} using a liquid chromatographic pump (Shimadzu, Japan). Another part has a 0.3 cm narrow segment detector window and is filled with the buffer. As shown in Figure 1, the total length of the capillary is 40 cm (20 + 20), and length to the detector is 20.7 cm. Before preparation, the capillary was washed with 1.0 mol L^{-1} potassium hydroxide solution, water and electrolyte buffer solution for 30 minutes, respectively.

The HPLC equipment consisted of a JASCO 88-PU pump (Japan Spectroscope Co. Ltd.), a Shimadzu SPD-6AV UV-VIS sepectrophotometric detector, and a Shimadzu C-R5R Chromatopac (Shimadzu, Japan). A Model 7125 Syringe Loading Sample Injector (Rheodyne Incorporated, California,

USA) with a 10-μL sample loop was employed. HPLC columns (150 mm x 2.1 mm I.D., GL Sciences Inc. Japan) were packed by the conventional slurry method using a Shimadzu LC-6A pump. The detection wavelength used was 254 nm or 225 nm depending on the samples. The eluent was a mixture of acetonitrile, water and acetic acid (90:5:5 v/v). The column was first purged with acetonitrile, and then with the eluent at a flow-rate of 0.2 mL min^{-1} until a stable baseline was observed.

Scanning electron micrographs were carried out in a JSM-6100 Scanning Electron Microscope (JEOL, Tokyo, Japan) operated at a voltage of 20 kV. The samples for SEM were coated with platinum in a Model 1BZ SEM coating unit (Eiko Engineering Co. Ltd, Japan). The metallic fine mesh filter series with the caves from 2-μm to 25-μm were purchased from Toyo Roshi Kaisha (Tokyo, Japan). The temperature of the capillary outside wall was measured by an IT202 Infrared Thermometer from Keyence Corporation (Japan).

Chemicals

All samples were prepared using deionized water that was double distilled. α,α'-Azobis(isobutyronitrile) (AIBN) and methacrylic acid (MAA) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Ethylene glycol dimethacrylate (EDMA) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Acetonitrile (MeCN), acetic acid (HAc) and chloroform are chromatographic grade and all of them were purchased from Kanto Chemicals Co. Inc. (Tokyo, Japan). D,L-Phenylalanine, L-phenyalalanine, L-pheAN and other amino acids were purchased from Sigma Chemical Company (St. Louis, Mo. USA.) and used as received. 90:5:5 (v/v) of acetonitrile, water and acetic acid mixed solution was used as the electrolyte for CEC and for the eluent of HPLC.

All solutions were passed through a 0.1μm syringe filter (Advantec, Tokyo, Japan) and then degassed.

Polymer Preparation

Polymers were synthesized by the method of Sellergren et al,[23] and the procedure is simply demonstrated in Scheme 1. Print molecule, cross-linking monomer, and the appropriate amount of functional monomer were weighed in a 20-mL borosilicate glass test tube and dissolved with 16 mL of chloroform or

Scheme 1

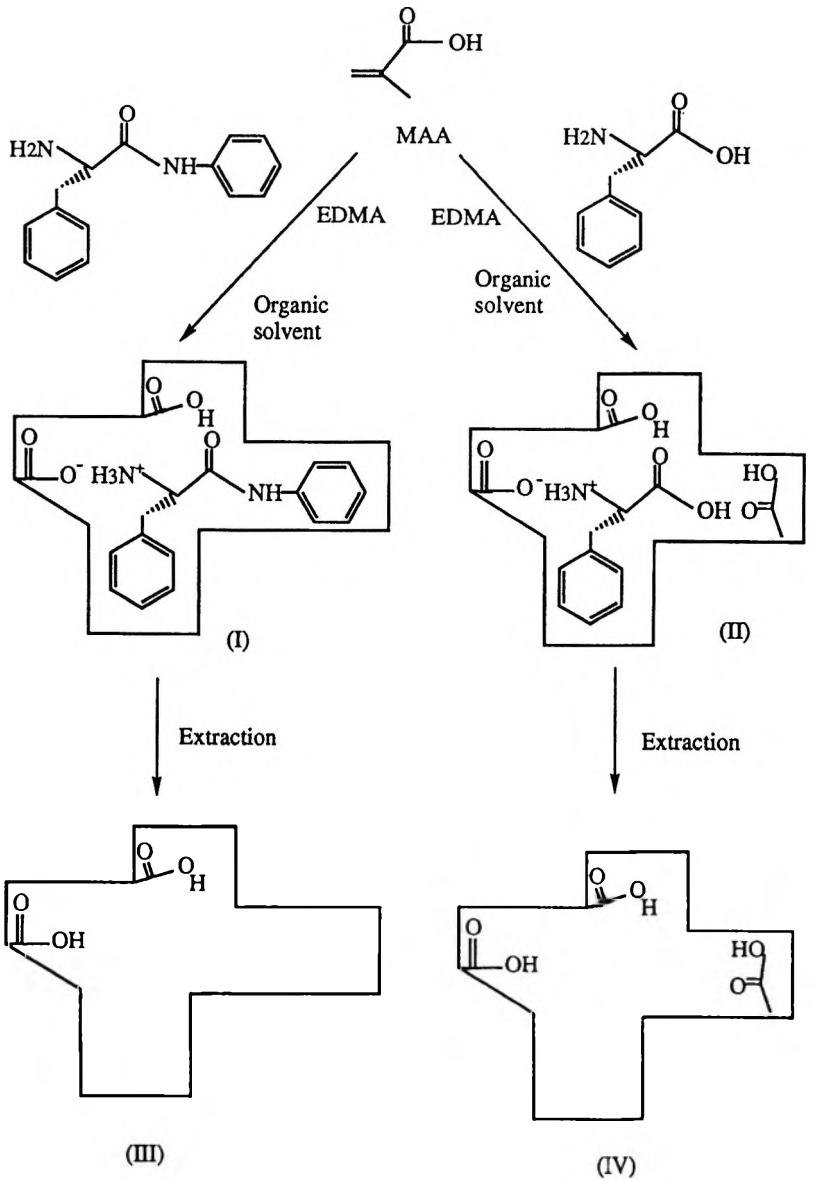
MAA
EDMA
EDMA
Organic solvent
Organic solvent
(I)
(II)
Extraction
Extraction
(III)
(IV)

Table 1

Composition of Polymerization Mixtures

	Composition of Monomer Mixture						
Polymer	Print Molec.	Monomer	Crosslinker	Initiator	Solvent	Temp. (°C)	Time (H)
1	L-PheAN	MAA	EDMA	AIBN	Chloroform	60	24
2	L-PheAN	MAA	EDMA	AIBN	MeCN+HAc	60	24
3	L-Phe	MAA	EDMA	AIBN	MeCN+HAc	60	24
4	L-Phe	MAA	EDMA	AIBN	Chloroform	60	24

The molar ratio for EDMA:MMA:L-pheAN is 20:5:1, and for EDMA:MMA:L-phe is 20:5:0.1.

acetonitrile-acetic acid (95:5, v/v) depending on the solubility of the print molecule. The molar ratio of cross-linking monomer to functional monomer to print molecule was 20:5:0.5 (or 20:5:1). The resulting polymers were ground to particles, sieved into 2-10 μm for CEC and 20-25 μm for HPLC.

RESULTS AND DISCUSSION

Preparation of Molecularly Imprinted Polymers

The aim of this study was to try to use molecularly imprinted polymers for the separation of phenylalanine by CEC. As shown in Scheme 1, print molecule, L-pheAN or L-phenylalanine, is present at low concentrations in a mixture of MAA monomer, EDMA cross-linker, AIBN initiator and organic solvent. In this mixture, it is envisaged that print molecules would interact preferentially with carboxyl-containing vinyl monomers due to coulombic forces between positively charged amino groups of print molecules and opposite charged carboxylates of carboxyl-containing vinyl monomers. During polymerization, specific combination of molecule and vinyl monomer could be formed due to electrostatic interactions and hydrogen bond. After polymerization, loosely bound print molecules were washed from the polymer under very mild condition by extracting the polymer with an organic solvent. The obtained polymers contained imprints of the added print molecule, and the formed cavities, shaped after the print structure. These polymers were equipped with carboxyl groups that could interact specifically with the amino function of rebound phenylalanine. In this work, four kinds of polymers (Table 1) using different print molecules or organic solvents were prepared. A high concentration of cross-linking agent was present in the polymerization mixture

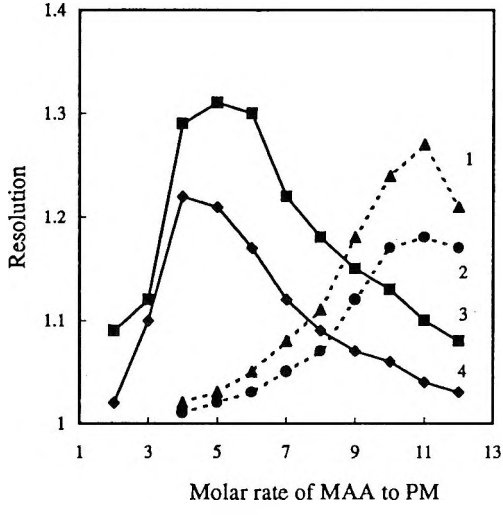

Figure 2. Effects of molar ratios of MAA to print molecule on the separation of phenylalanine. (——) presents that print molecule is L-pheAN and (-----) is for L-phenylalanine as print molecule. Curves 1 and 3 are the results from CEC under the conditions: capillary, 40cm x 75-μm I.D. x 375-μm O.D.; effective length, 20 cm; electrolyte solution, 90% MeCN-% 5 HAc-5% water; applied voltage, 350 V cm^{-1}; electroinjection, 300 V cm^{-1}, 5 s; detection, 254 nm. Curves 2 and 4 resulted from HPLC under the conditions: column, 150 mm in length and 2.1 mm I.D.; eluent, 95% MeCN-5% HAc; flow rate, 0.20 mL min^{-1}; detection, UV 254 nm.

in order to produce microporous polymers of high rigidity. The molar ratio of cross-linker (EDMA) to functional monomer was fixed at 5:1 according to the previous study.[23] The effect of molar ratio of functional monomer to print molecule on the resolution of phenylalanine was determined. As shown in Figure 2, the results indicated that the molar ratio of MAA to L-pheAN in the range of 5:1~4:1 is the most suitable for the separation of D,L-phenylalanine with both CEC and HPLC. When the polymer was imprinted with L-phenylalanine, the molar ratio of MAA to L-phenylalanine is high. The acceptable molar ratio of MAA to L-phenylalanine was 5:0.5. This may be due to phenylalanine being dissolved, a little, in acetonitrile-acetic acid or chloroform. Combined, with a previous study[23] and our results, polymer 1 and 2 were prepared from 2.510^{-3} mole of L-pheAN, 1.25×10^{-2} mole of MAA, 5.0×10^{-2} mole of EDMA and 32.0 mg AIBN in 16 mL of chloroform or 95:5 (v/v) MeCN-HAc solvent. Polymer 3 and 4 were prepared by the same method as Polymer 1 and 2, except the amount of print molecule (L-phenylalanine) was 2.5×10^{-4} mole.

The influence of solvents on the microstructure of polymer was investigated in the polymerization of methacrylic acid by Schroder.[24] The structure of the polymer prepared at 60 was found to depend on the solvent. In our experiment, we also noticed that the polymer prepared by using MeCN-HAc as solvent was harder than that using chloroform as solvent. At the same conditions, the polymer particles of Polymer 2 and 3 were more stable when MeCN-HAc was used as extraction solvent or electrolyte. This means that the usages of the same organic solvent in polymerization and eluent are beneficial to the separation. Therefore, polymer 2 and 3 were used for the following experiment.

Extraction of Print Molecule from Polymers

The extraction of print molecule from polymers is an important process, in principle, it releases the print molecules and creates the cavities capable of specific resorption of the template. It may also move other materials from the polymer, for example, residual monomers and initiator fragments. As ionic and hydrogen-bonding interactions between the amino groups of L-type amino acid and the carboxylic groups of a polymer were expected, the extraction procedure should not only facilitate the transport of print molecule from the polymer to outside, but also break the chemical interaction efficiently. This might be achieved by the use of an acid whose acidity is stronger than the template amino groups, e.g. acetic acid.

The amino group of the amino acid was more basic than that of the carboxyl group of the polymer when MAA was used as monomer. Therefore, in order to get polymer cavities with acidic properties, a mixture of acetonitrile (95 v%) and acetic acid (5 v%) was used in the extraction procedure.

Scanning Electron Microscopy

The molecularly imprinted polymer particles were examined by scanning electron microscopy (SEM) to compare the morphologies of the polymer particles. As shown in Figure 3, the polymer particles are irregular-shaped materials. The shapes of the blank polymer (without print molecule) particles and the molecularly imprinted polymer particles before and after extraction were compared. The shapes of them were almost the same. These results indicated that the polymer skeleton was stable when acetonitrile-HAc was used as extracting medium.

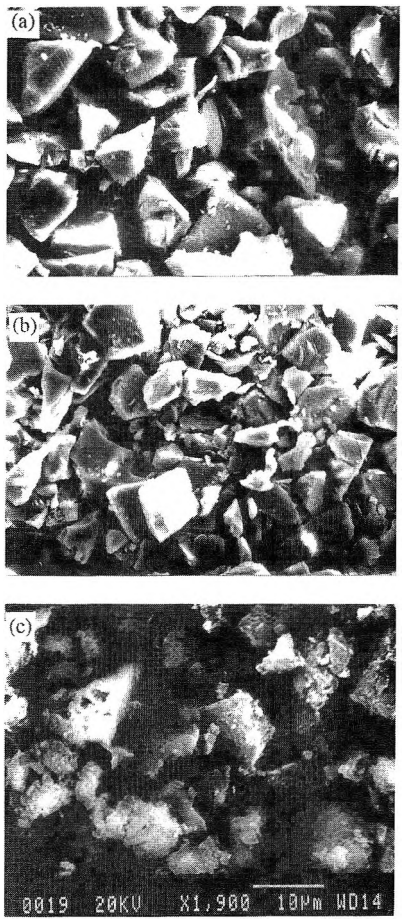

Figure 3. Scanning electron micrograph of the molecularly imprinted polymer particles. (a). the blank polymer particles which synthesized without any print molecules, (b). molecularly imprinted polymer particles without acetonitrile/HAc washing, and (c). particles washed with 95% acetonitrile-5% HAc 3 h.

The CEC resolution of D,L-phenylalanine using molecularly imprinted polymer was affected by the particle size. As shown in Figure 4, small particles are beneficial to the separation, but too small particles suffered some damage when the voltage was applied to the capillary. This influence became serious when the applied field strength was over 300 V cm^{-1}. This phenomenon can be explained by the fact that, although the polymer particles almost cannot be dissolved in acetonitrile-acetic acid solution, at high electrolyte voltage the

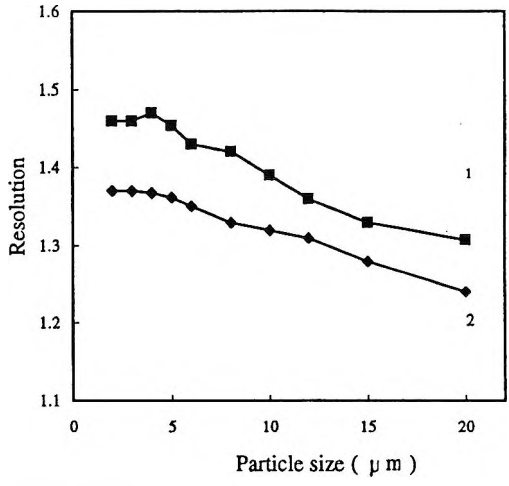

Figure 4. Effect of polymer particle size on the separation of phenylalanine by CEC. 1. polymer imprinted with L-pheAN, 2. polymer imprinted with L-phenylalanine. Separation conditions are the same as in Fig. 2.

capillary will be heated as a result of the high electric current, which will make the loss of some residual functional groups from the cavity surfaces, and may also remove some materials from the polymer. The lifetime of the capillary column will be shorter, even though a higher resolution can be achieved. On the other hand, if the polymer particle size is too large, it is difficult to fill them into the capillary and the resolution decreases. Because the effective surface area of the polymer become smaller when the particle size increases, the interaction activity between the polymer inner-surface and analytes decreased. As shown in Figure 4, the resolution gets a plateau when the polymer particle size is smaller than 5-μm. Considering the other conditions, including the fact that a longer time is needed to slurry the small polymer particle, we chose a size smaller than 10-μm for this experiment. The polymer particle sizes for HPLC were controlled in the range of 20-25 μm according to the Kempe's results,[20] and not discussed here.

A Practical Approach to Electrochromatography

The same as capillary electrophoresis, separation of phenylalanine using CEC method was also affected by several factors, such as variations of the applied voltage, buffer solution and temperature. With the exception of polymer

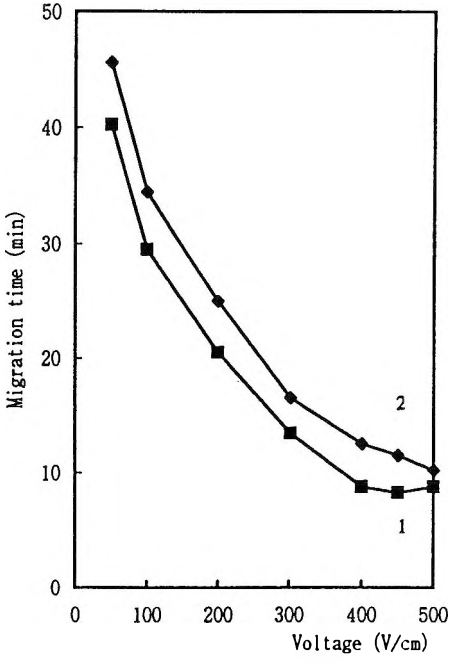

Figure 5. Migration time as a function of applied potential strength. 1. Capillary filled with L-pheAN printed polymer particles and 2. Capillary filled with L-phenylalanine printed polymer particles. Sample: D,L-phenylalanine. Other conditions are the same as in Fig.2.

properties, we investigated these factors for the determination of stability and reproducibility of the electrochromatographic performance. As illustrated in Figure 5, when the applied voltage is increased, the migration times decrease more rapidly than those predicted. Because the higher applied voltage caused the temperature increase in capillary, the interaction between carboxyl groups of molecularly imprinted polymers and re-added substrates will be affected. That is, the strength of the hydrogen bond and the ionic combination between polymer and analyte depended not only on the structure of the polymer and sample, but also on the temperature. The relationship between the separation factor with the temperature of the capillary outside wall, is shown in Figure 6. Although the temperatures between the capillary center and the outside are different, the temperature gradients from the capillary center to the outside is about 3.14 for the capillary of 75-μm I.D. and 375-μm O.D.[25]. The data show that the separation factor is lower at the high temperature which corresponds to the high applied voltage. We also determined the separation factor of the

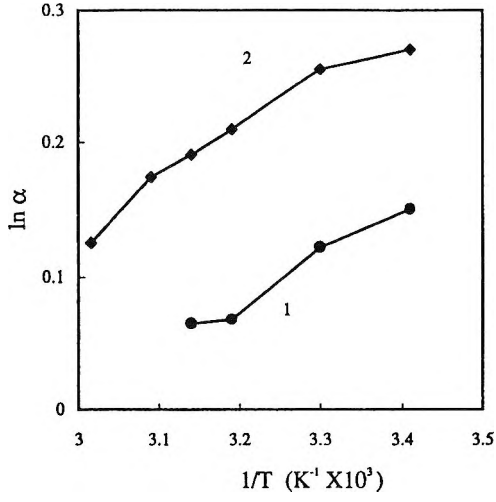

Figure 6. The relationship between column temperature and separation factor. 1, Results from CEC method and 2, data from HPLC. Polymer was imprinted by L-pheAN (Ploymer 2). Particle size for CEC and HPLC were 5-μm and 25-μm respectively. Other conditions are the same as in Fig. 2.

HPLC method when the column temperature was varied. As illustrated in Figure 6, curve 1 and 2 show some similarity. Although both of these methods were affected by temperature, the results indicated that separation factors from the HPLC method are more strongly dependent on the temperature. The reason for this, is that the recognition ability of CEC is concerned not only with the hydrogen bond between the carboxyl groups of polymer and the carboxyl groups of amino acid, but also the ionic bond between amine groups ($-NH^{+}_{3}$) of amino acid with the part of ($-COO^{-}$) groups in the polymer (see the Scheme 1 compound (I) and compound (II)), when a acidic eluent was used.

The enthalpy difference ($\Delta\Delta S^0$) and the entropy difference ($\Delta\Delta H^0$) were also calculated based on the following equation:[26]

$$\ln \alpha = -\Delta(\Delta H^0)/RT + \Delta(\Delta S^0)/R \qquad (1)$$

Where α is the separation factor, T is the absolute temperature, R is the gas constant. ΔH^0 and ΔS^0 are the different entropy and different enthalpy of the enantioselective energy, respectively.

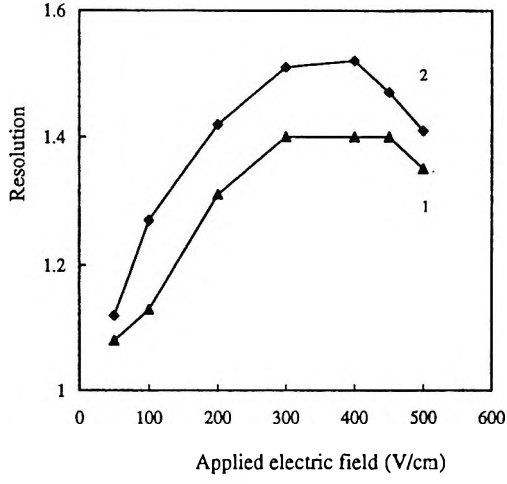

Figure 7. Effect of applied voltage on the resolution of phenylalanine by CEC. 1, using polymer 2 and 2, using polymer 3. Other conditions are the same as in Fig.2.

For CEC, the enthalpy differences and entropy differences were -8.75 J mol^{-1} K^{-1} and -2.96 KJ mol^{-1} , respectively. Similarly, ($\Delta\Delta S^0$) and ($\Delta\Delta H^0$) for the HPLC method were equal to -8.44 J mol^{-1} K^{-1} and -3.21 KJ mol^{-1}. From these results, we think that the interaction of analytes with the polymer is a diffusion-controlled process, and sufficient separation time for the recognition of chiral analytes is required. Increased thermal effect resulting from higher applied potentials can reduce the migration time, but the resolution will be decreased. In order to keep the capillary temperature constant, the environmental temperature of the CE was set at 20°C and a micro fan was also used.

Figure 7 shows the separation of D,L-phenylalanine using L-pheAN and L-phenylalanine imprinted polymers by changing the applied voltage. The applied voltage was changed from 2 to 20 kV corresponding to 50 100, 200, 300, 400, 450 and 500 V cm^{-1}, respectively. The resolution showed that the optimum is at 400 V cm^{-1}. Considering the Joule heating, peak shape, and migration time, a voltage of 350 V cm^{-1} was used in this work .

To achieve the most selective interaction between polymer and analyte, it was advantageous to use the same electrolyte solvent for CEC as that which was used for polymerization. Changes in solvent composition can not only change the strength of the interactions between substrate and interacting units in the polymer, but also, obviously, cause swelling or shrinking of the polymer.

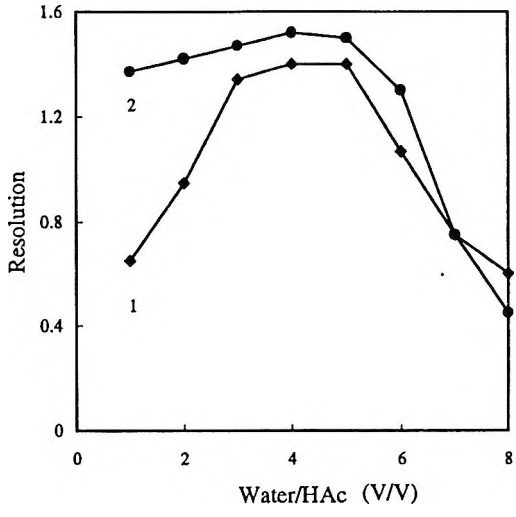

Figure 8. Effect of the eluent composition on the separation of phenylalanine by CEC and HPLC. 1, The results from HPLC and 2, results from CEC. The concentration of MeCN was 90% (v%). Total amount of water and acetic acid in the eluent was 10% (v%). Polymer 2 was used. Other conditions are the same as in Fig. 2.

We compared the solvent compositions, especially the concentration of acetic acid in the eluent and the electrolyte solution. In Figure 8, a suitable composition of solvent mixture for the electrolyte solution was chosen to be 90% acetonitrile -5% acetic acid -5% water (v%). Although this composition was slightly different from the solvent of polymerization, an appropriate amount of water in buffer solution was necessary for CEC, even if the resolution was decreased in some extent. On the other hand, increasing the concentration of acetic acid in mixed solvent is likely to result in weaker hydrophobic interaction and stronger hydrogen bond effect. The higher water content will cause the most substance strongly adsorbed on the polymer. The migration time of phenylalanine in electrochromatography was longer in the high water content electrolyte solution than, in the low one and broad peaks also appeared in the high water concentration. The concentration of water in electrolyte solution was controlled between 3%-6%. We also found that if the electrolyte contained only water and acetonitrile, separation of the analyte could not be done. Both acetic acid and water took an important role in the electrochromatography.

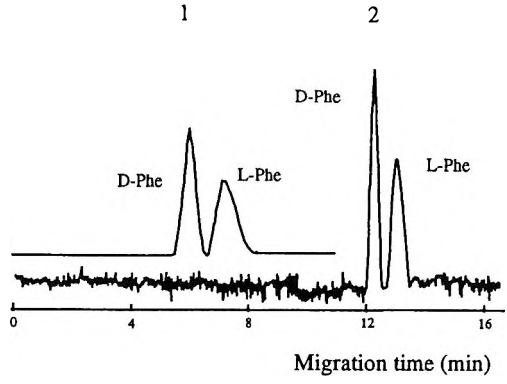

Figure 9. Typical signals of CEC and HPLC. Signal 1 got from HPLC method and Signal 2 obtained from CEC. Other conditions are the same as in Fig. 8 except the eluent (or electrolyte) is 90:5:5 MeCN/HAc/water (v/v).

Similar to most of capillary electrophoresis, degassing of the electrolyte solvent proved to be useful for the separation. Insufficient degassing led to increased baseline drift, unstable current and finally breakdown of electroosmotic flow as a result of bubble formation. These symptoms could be overcome by thoroughly degassing the eluent. The best result was obtained by a combination of purging with helium and applying vacuum under ultrasonification about 10 minute.

The typical electrochromatogram and high performance liquid chromatogram under the optimum conditions are illustrated in Figure 9. The electrochromatographic signals are much sharper than that of the liquid chromatogram. The resolution of phenylalanine by CEC was higher than that from the HPLC method. This means that the proposed method has potential for the chiral separation, and a more detailed investigation will be continued.

Resolutions of Other Amino Acids by CEC

We considered, that a polymer prepared using pheAN or phenylalanine as print molecule may also be able to separate the enantiomers of other aromatic amino acids. From the experiments, it was interesting to find that the proposed method was also a preliminary to resolving some aromatic amino acids, including D,L-phenylglycine, D,L-3-(3,4-dihydroxyphenyl)alanine, D,L-tyrosine and p-fluoro-D,L-phenylalanine. The results are summarized in Table

Table 2

Enantioseparations of Some Aromatic Amino Acids Using CEC Method

Compound	Resolution			
	Polymer 1	Polymer 2	Polymer 3	Polymeer 4
Phenylalanine	1.36	1.43	1.20	0.57
Tyrosine	0.92	0.88	0.67	0.53
p-Fluorophenylalanine	1.04	0.95	0.78	0.67
Phenylglycine	1.10	1.13	0.89	0.50
Phenulalanine anide	1.45	1.32	0	0
DOPA	0.54	0.64	0.21	0.24

2. These compounds have similar construction to phenylalanine. Although, these aromatic amino acids can be separated to some extent, the peak shapes and resolutions are not satisfactory enough for the practice application. This is a problem that needs our continued study, especially the real understanding of the separation mechanism. The molecules without the aromatic ring, for example, D,L-serine, D,L-leucine, D,L-alanine and D,L-valine, can not be recognized at all. D,L-Tryptophan and Dansyl-D,L-phenylalanine were also examined, the results proved that both of them could not be separated. The results indicated that the molecularly imprinted polymer has its special selection for the samples. The polymers printed by L-pheAN or L-phenylalanine seem to be highly specific for D,L-phenylalanine and its similar constructed samples, non-aromatic samples could not be distinguished at all. We think this advantage may be developed for other types of amino acids or chiral samples by using the CEC method.

CONCLUSION

This primary investigation successfully demonstrates that molecular imprinting method can be used for the separation of amino acid enantiomers by CEC. The polymers imprinted by L-pheAN and L-phenylalanine have different recognition characteristics. The former seems to give high resolution for D,L-phenylalanine although the structure of print molecule, L-pheAN is different from the analyte. Comparison of the results between HPLC and CEC, show peak shape and resolution by CEC were better than those of HPLC. Our present study is still in a preliminary stage and resolution improvements have not yet been addressed. Further investigation in detail will be necessary to find

a real understanding of the structure relation of molecularly imprinted polymers with analytes. We believe that the proposed technique will provide a significantly simple procedure for chiral separation. Potential applications of this technique to other template systems are also being explored.

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ENANTIOMERIC SEPARATION OF AROMATIC AMINO ALCOHOL DRUGS BY CHIRAL ION-PAIR CHROMATOGRAPHY ON A SILICA GEL PLATE

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ABSTRACT

Four chiral aromatic amino alcohol drugs were separated by TLC on a general-purpose silica gel plate with ammonium-D-10-camphorsulfonate (CSA) as chiral ion-interaction agent. The chiral aromatic amino alcohols are all of pharmacological importance as ß-adrenergic blockers. The influence of eluent composition, temperature and concentration of CSA on the chiral separation is discussed. It is found that the temperature is also one of the important parameters to be varied for optimum separation in ion-pair chiral resolution. Among four drugs, three enantiomeric drugs were resolved at lower temperature (5°C). In this study, analytical reagent grade methanol and dichlormethane can be directly used as mobile phase without using molecular sieve before use.

INTRODUCTION

Chiral stationary phases (CSP's) or chiral mobile phase additives (CMA's) have been used for chiral separations by thin layer chromatography (TLC). In general, the CSP's have been used less, probably because the commercially available TLC plates were too small and relatively expensive. ß-Cyclodextrin (ß-CD) and its derivatives as CMA's have been most widely used in the chiral TLC separation.[1-5]

Ion pair chromatography is also an important chromatographic technique commonly used for separation of ionized compounds in liquid chromatography. This separation technique is based on the fact that a chiral counter-ion can bind an enantiomeric substrate as diastereomeric ion pairs which will have different distribution properties if the counter-ion has a suitable structure.

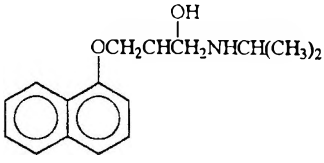
Pettersson et al.[6-7] separated a variety of enantiomers of organic amine derivatives, organic acids, and aromatic amino alcohols by ion-pair chromatography. They used camphorsulfonic acid (CSA), quinine, albumin, n-dibutyl tartrate, N-benzoxycarbonyl-glycyl-L-proline (ZGP) as chiral counter-ions in HPLC.

Recently, Duncan et al.[8] reported the separation of several racemic aromatic amino alcohols by TLC on diol or high performance silica gel plates using a mobile phase containing (IR)-(-)-ammonium-10-camphorsulfonate (CSA) or ZGP. In this work, based on reference 8, we reported on the enantiomeric separation of four aromatic amino alcohol drugs on silica gel plates, using a solution containing ammonium-D-10-camphorsulfonate as a mobile phase. The dependence of eluent composition, temperature and concentration of the counter ion on the separation was described. Some separations occurred only at lower temperature (5°C).

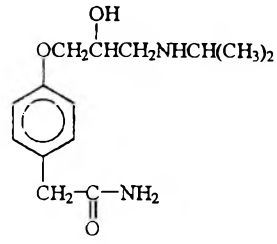
EXPERIMENTAL

Materials

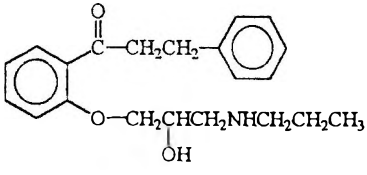
Silica gel GF_{254} (TLC, d_p = 10-40 μm, Qingdao Haiyong Chemical Factory, China), propranolol, pindolol and propafenone (Shanghai Huanghe Pharmacological Factory, China) and atenolol (Tianzhong Junyang Pharmacological Factory, China), D-10-camphorsulfonic acid (J. T. Baker Chemical Co.), methanol and dichloromethane (CH_2Cl_2) were analytical reagent grade and were used as received, without further purification.



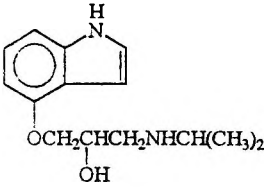
Propranolol



Atenolol



Propafenoni



Pindolol

Figure 1. The structure of test compounds.

Methods

Silica gel plates (2.5 x 10 cm) were prepared with 300-400 μm layer thickness and were placed in a drying oven at 120-130°C for one hour, then stored in a desiccator. All developments were carried out at 5°C (in a refrigerator) or at room temperature (25-30°C) in small glass jars of 250 mL volume. The distance of development was 8 cm. It took approximately 30-40 min. to completely develop the plate. The spot visualization was performed by use of a fixed wavelength (254 nm) UV lamp.

All samples were dissolved in the methanol. Counter-ion was added to the mobile phase in the ammonium form.

RESULTS AND DISCUSSION

The structures of the four test drugs are shown in Figure 1. Note that all of the compounds have three functionalities in common: an aromatic ring, an α-hydroxyl group and a ß-amino group. Figure 2 shows the TLC

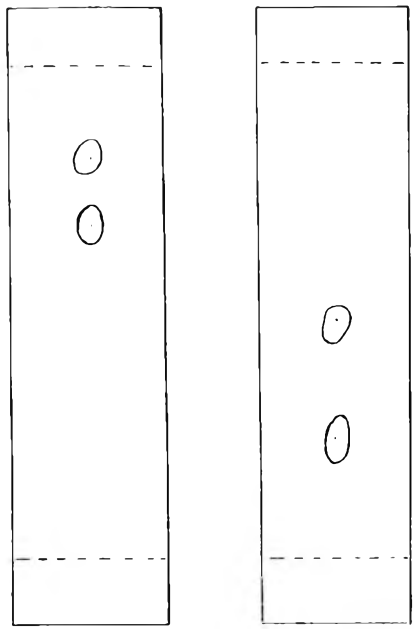


Figure 2. TLC chromatogram showing the resolution of the racemates. A: propafenone, B: atenolol. Mobile phase: A: methanol-dichloromethane = 50:50; B:methanol-dichloromethane = 70:30. Other conditions, see Fig. 3.

Table 1

Enantiomers Separated using CSA as Chiral Mobile Phase Additive with a Silica Gel Plate

Compound	R_{f1}	R_{f2}	α	Mobile Phase * (v/v) CH_2Cl_2 : MeOH
Propranolol	0.62	0.81	1.31	50 : 50
Propafenone	0.69	0.84	1.22	50 : 50
Pindolol	0.61	0.79	1.30	60 : 40
Atenolol	0.24	0.48	2.00	70 : 30

* 6.8 mM CSA in the mobile phase. Development was done at 5°C.

chromatogram of the chiral separation of propafenone and atenolol. The data for the enantiomeric separations are listed in Table 1. As can be seen in Table 1, it was possible to achieve very efficient separations using CSA as chiral mobile phase additive on the silica gel plate.

Figure 3 shows the influence of varying the ratio of dichloromethane and methanol in the mobile phase on the chiral separation. The separation occurs over a range of 30 % to 60 % dichloromethane content in the mobile phase (v/v) for propranolol (Fig. 3A). The ΔR_f value is the largest when the content of dichlormethane in the mobile phase is 30 % (v/v), but a poorer separation is obtained because of the tailing spots. The optimum resolution is achieved when the content of dichloromethane in the mobile phase is 50 %. The curves and the optimum eluent composition for chiral separation of propafenone are very similar to propranolol, probably due to similar molecular structure, and the optimum eluent composition for chiral separation of propafenone is 50 % dichloromethane in the mobile phase.

The chiral separation of pindolol and atenolol is also similar. An analogous curve to that shown in Fig. 3B is generated for the effect of the content of dichloromethane in the mobile phase on the chiral separation of atenolol. The optimum enantiomeric resolution occurs at approximately 60 % dichloromethane in the mobile phase for pindolol and at 70 % dichloromethane in the mobile phase for atenolol. The aforementioned results indicate that the eluent composition plays an important role in the enantiomeric separation.

In gas chromatography, temperature is the main parameter to be varied for optimizing separation. Recently, the effect of temperature has also been recognized as an important parameter in improving and optimizing chiral resolution by HPLC[9-12]. Duncan et al.[8] reported the enantiomeric separation of pindolol on a diol TLC plate using ZGP as chiral ion interaction agent and the enantiomeric separation of propranolol on a high performance silica gel plate using ZGP or CSA as chiral ion interaction agent. No chiral separation occurred on the silica gel plate. In this work, the pindolol and propranolol were also not separated on the silica gel plate using CSA as chiral ion interaction agent at room temperature (25-30°C), but the chiral separations of these two drugs and propafenone were achieved at 5°C in the refrigerator. Among the four drugs, only atenolol can be separated at 30°C, $R_{f1} = 0.33$, $R_{f2} = 0.53$, $\alpha = 1.61$. The R_f value at 30°C was larger than that at 5°C, and the separation at 30°C was poorer than that at 5°C due to slightly tailing spots and smaller R_f value. The reason for this (separation at room temperature for atenolol) probably is due to including a higher polarity amide group in the

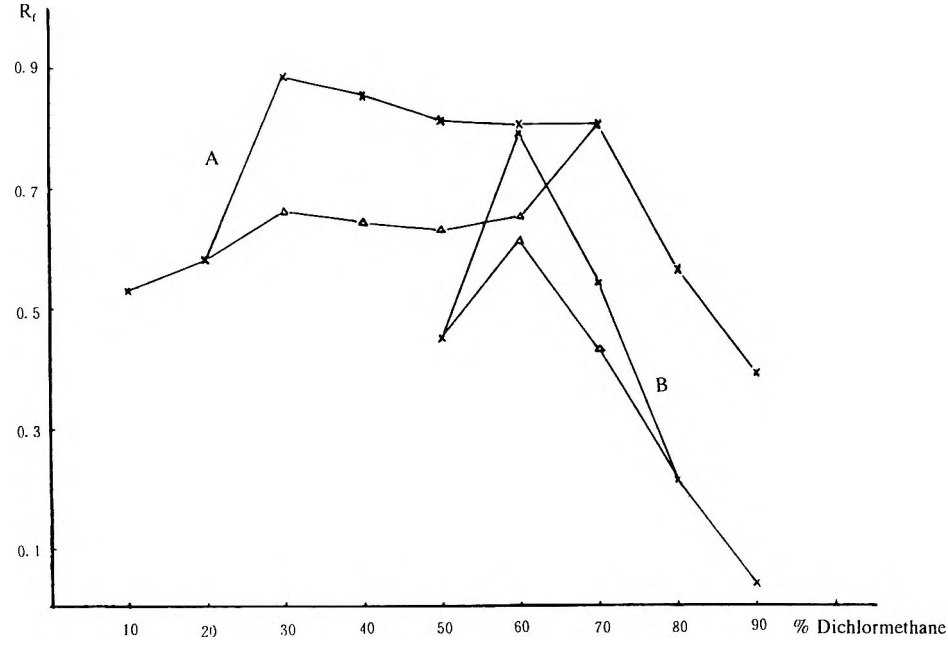


Figure 3. Plot showing the effect of the content of the dichloromethane in the mobile phase on chiral TLC separation. –x–: first eluted enantiomer, –Δ–: second eluted enantiomer. The concentration of CSA is 0.68 mM. Stationary phase: silica gel plate, temperature: 5°C. A: propranolol, B: pindolol.

molecular structure of the atenolol and more strength in the binding to the absorbent. The R_f value of atenolol in Table 1 was minimum (the largest retention) and the content of dichloromethane in the mobile phase was the largest.

From the results above, it is obvious that the temperature is also one of the main parameters to be varied for optimizing separation in the ion-pair TLC chiral resolution, besides the stationary phase, eluent composition and counter ion.

According to the report by Duncan et al., originally, molecular sieve (Type 5A) was dried for 24 hours at 350°C and then was used to dry solvent. The plates were placed in a drying oven at 125°C for 3-4 hours. Later it was found that one hour heating for the plates is necessary and adequate. If the plates were heated a longer time, the plates were easily broken during use. This is probably due to fact that the particle diameter of silica gel is ununiform

(10-40°) in this work. It was also found that the separation at 5°C can be achieved when the mobile phase solvents were not dried with the molecular sieve before use. The content of water in the methanol and dichloromethane was determined by GC and was about 1000-2000 ppm. Therefore, the methanol and dichloromethane were directly used. In addition, the R_f value was hardly changed with a decrease of the concentration (8.8-4.8 mM) of CSA. The concentration of CSA in the mobile phase was kept at 6.8 mM in this study because the resultant spot was smaller (or more concentrated) and there was no tailing.

The shapes of the spots on TLC plate were visualized with a UV lamp, then the spots were scraped out and dissolved in methanol. The methanolic solution, containing sample, was scanned from 190 nm to 600 nm with a Model UV 240 (Shimadzu, Kyoto, Japan). The UV spectrum of the two spots are identical, thereby indicating they are enantiomers.

CONCLUSION

The chiral separation can be achieved with a general purpose silica gel plate using CSA as a mobile phase additive. Besides eluent composition, stationary phase and counter-ion, it is found that the temperature is also an important parameter to be adjusted for optimizing a separation in this work. The chiral separations of propranolol, pindolol and propafenone only are achieved at 5°C. It appears that the presence of 1000-2000 ppm water in the analytical system does not influence chiral separation. Once the separation was optimized, it was possible to attain enantiomeric separation with good α values. These separation conditions were easily obtained. This method is relatively inexpensive and attractive.

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ANALYSIS OF CONFORMATIONAL CHANGES OF CHOLERA TOXIN BY SIZE EXCLUSION, HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Cholera toxin (CT) consists of the A-subunit (ACT) and B-subunit (BCT) consisting of 5 identical beta-peptides. The dynamics of denaturation and subsequent renaturation of CT and BCT was investigated by SE HPLC. The denaturation was performed in the 6M urea at different temperature. The decomposition of CT to ACT and BCT and subsequent breaking of BCT to the beta-peptide is clearly seen at CT denaturation. The proteins were denatured completely and rapidly only when heating urea solution up to 50°C. The renaturation performed with the help of dialysis is more effective than that by chromatography. The quantitative correlation pentamer/monomer of renatured BCT is higher than a fresh solved protein. Thus, a shift of the fluorescence maximum of BCTmight be explained by the presence of some denatured BCT in the solution.

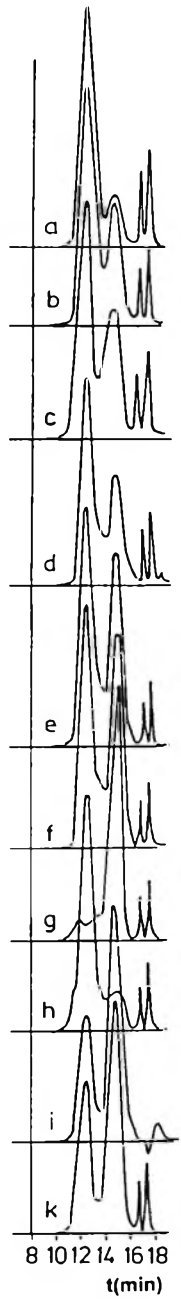
INTRODUCTION

Cholera toxin (CT) is a protein consisting of two different subunits: B-subunit (BCT) (56 kD), A-subunit (ACT) (28 kD), and bound noncovalently.[1] BCT properly consists of 5 identical beta-peptides and is responsible for CT binding to ganglioside GM1 on the surface of the cell-target membrane.[2,3] Pentamer of BCT has a cylinder form with the diameter 60-64 Å and height 40 Å, with the pore 30 Å long and changing the diameter - 11-15 Å.[4] The pentamer is stable because of the high ratio of the beta-peptide surface area, bound with the surface of neighboring beta-peptides, to the complete surface area of beta-monomer: 0.39.[5] ACT consists of two different peptides A1 - (22 kD) and A2 - (5kD) bound by a disulfide bridge.[6] The C-terminus of A2-peptide is bound to the BCT surface pore by electrostatic interactions. A strong interaction between N-terminal of A2-peptide and A1-peptide consists of Wan-der-Waals and hydrogen interaction. So, A2-peptide joins the component between BCT and A1-peptide.[4]

As known, each of the beta-peptides contains single, and A1-peptide 3 triytophane residues. Fluorescent spectras of renatured CT and BCT are practically identical (292 nm). At the bonding of CT or BCT to the carbohydrate fragment of ganglioside GM1, the fluorescent spectrum of BCT triytophiles changes.[7,8] However, in real investigations, the shift of the fluorescence maximum (up to 5 mN) of triytophane residues of BCT in the 0.05M NaCl, Tris-HCl, (pH 7,4) solution, and in liposome suspension in the same buffer was observed. Consequently, a small part of the protein in solution may be partially or completely denatured and forms beta-peptide.

To verify the above assumption, the dynamics of CT and BCT denaturation by urea at different temperatures, and the following chromatographic renaturation or dialysis, were researched by size exclusion high performance liquid chromatography (SE HPLC).

Figure 1. **(right)** Dynamics of BCT denaturation and renaturation under different conditions (λ=280). The BCT sample was treated properly: a) dissolved in 0.05 M Tris-HCl (pH 7.4), b) 6 M urea, 1 h, T=20°C, c) 6 M urea, 2 h, T=20°C, d) 6 M urea, 3 h, T=20°C, e) 6 M urea, 19 h, T=20°C, f) 6 M urea, 17 h, T=4°C, g) 6 M urea, 20 h, T=20°C, + 20 min 50°C, h) 6 M urea, 2 h, T=20°C, then renaturation by dialysis, i) 6 M urea, 19 h, T=20°C, then beta-peptide was isolated by SE HPLC and injected to the column in 1 h. k) 6 M urea, 19 h, T=20 °C, then beta-peptide was isolated by SE HPLC and injected to the column in 48 h.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MATERIALS AND METHODS

Chromatographic analysis was performed by a SE HPLC Protein Pack column (30 x 0,75) (Waters, Millipore, USA) in 0.1 M Na-phosphate buffer (pH 7.4); elution was performed at the rate of 0.5 mL/min. Chromatography was carried out on a Waters 510 pump with a Waters U6K injector, a Waters 490E spectrophotometer and Waters 740 integrator (Millipore, USA). For the separation we used the specimens of insulin, proinsulin, denatured proinsulin, proinsulin-S-sulphonate, fusion protein, (obtained in our laboratory)[9], bovine serum albumin, ovalbumin and lysozime. The following reagents were used: water, purified on Milli-Q equipment (Millipore, USA), sodium hydroxide, sodium phosphate, phosphoric acid, urea (Serva, Germany). Before chromatography, the eluents were filtered through nitrocellulose filters, pore diameter 0.45 μM, (Millipore, USA) and degassed for 20 min.

Denaturation was performed in 6M urea under different temperature conditions. Renaturation was performed by dialysis and SE HPLC. At chromatographic renaturation of CT, whole proteins were separated from salts after 6M urea treatment (18 h) and then injected to the SE column for different periods of time for analysis. In the case of BCT, only beta-peptide was isolated and then analyzed, like above. BCT was renatured by dialysis against 0.05M Tris-HCl (pH 7.4) in 24h.

For picture performance, software for the IBM PC Surfer and Grapher (Golden Software Inc., USA) was used.

RESULTS AND DISCUSSION

According to SE HPLC, analysis of the initial sample of BCT, dissolved in 0.05 M Tris-HCl (pH 7.4), has two forms - pentameric and monomeric and its pentamer/monomer correlation is about 5/1 (Fig.1a). The 6M urea treatment of BCT at room temperature revealed that denaturation occurs only

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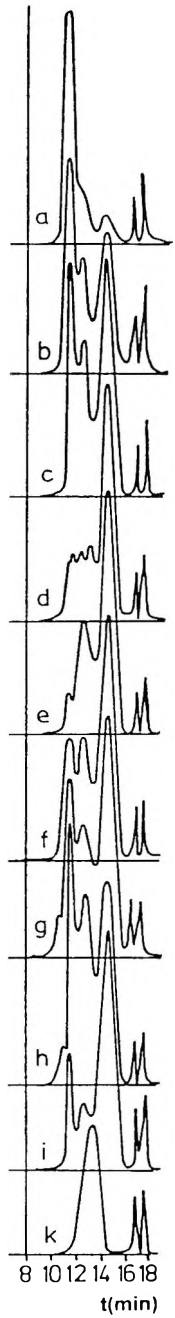
Figure 2. (right) Dynamics of CT denaturation and renaturation under different conditions(λ=280). The CT sample was treated properly: a) dissolved in 0.05 M Tris-HCl (pH 7.4), b) 6 M urea, 25 min, T=20°C, c) 6 M urea, 75 min, T=20°C, d) 6 M urea, 18 h, T=20°C, e) 6 M urea, 18 h, T=4°C, f) 6 M urea, 18 h, T=20°C, than CT sample was desalted by SE HPLC and injection to the column in 0.5h, g) 6 M urea, 18 h, T=20°C, then CT sample was desalted by SE HPLC and injected into the column in 3h, h) 6 M urea, 18 h, T=20°C, then CT sample was desalted by SE HPLC and injected into the column in 5h, i) 6 M urea, 20 min, T=50°C, k) ACT dissolved in 0.05 M Tris-HCl (pH 7.4).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partially (about 25-30%), and that practically there is no difference in the time denaturation for 1, 2 or 3 hours (Fig.1 b-d). Even at long urea treatment (19 h), the protein is denatured less than 50% (Fig.1e). Essential differences in denaturation at room temperature and at 4° was not observed (Fig 1f). Thermotreatment with urea of the protein (50°C) during 20 min (Fig.1g), results in almost complete denaturation. It is clear, that practically the whole pentamer is transformed, and has a monomeric form.

Renaturation by dialysis, with gradual decreasing of the urea concentration, is more effective than renaturation by chromatographic desalting (85 and 30% renatured protein respectively) (Fig.1 h-k). The quantitative ratio pentamer/monomer of dialysis renatured BCT is higher than fresh dissolved protein (6/1 - 5/1 accordingly) (Fig.1 a,h). However, a small amount of high molecular weight component formation is observed here. One can suppose it is an oligomer of the beta-peptide (a small left shoulder of the first peak) (Fig.1 h). At the chromatographic purification and isolation the beta-peptide is only renatured for 35-40%, even in 2 days (Fig.1 i, k).

The initial CT sample contains some free beta-peptide (second peak) and probably BCT and ACT (right shoulder of first peak) (Fig.2 a). At the denaturation, the visible transformation of CT to BCT and ACT, and following decomposition of BCT into beta-peptide (Fig.2 b-d), is observed. The rate of CT decomposition is higher than BCT under the same conditions. As for BCT, there is no big difference in the denaturation rate at room temperature and 4°C (Fig.2 d,e). The CT denaturation occurs rapidly at temperature 50°C (Fig.2 i). It is difficult to identify the peak of ACT (Fig.2 k) at denaturing CT, because it is fused partially with BCT. However, in some cases, all components of denaturation are identified - CT, BCT, ACT, and beta-peptide (Fig.2 d). At the CT renaturation by chromatographic desalting, the increase of CT and BCT peaks (Fig.2 f) is observed in half an hour. However, even in 3-5 h, renaturation is not completed (Fig.2 g,h). Besides, high molecular weight impurities are formed (left shoulder of the first peak).

CONCLUSION

The present work, thus investigates the dynamics of denaturation at different temperature and subsequent renaturation of CT and BCT by SE HPLC and dialysis. The resultant data explains the shift of fluorescence maximum by the presence of the small amount of denatured BCT or monomeric beta-peptide, which may transfer into a pentamer after dialysis. The data may also be used for the calculation of equilibrium parameters of bonding (constant of bonding, stoichiometry) of the B-subunit of cholera toxin to GM1 ganglioside.

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COMPARISON OF FILTER AND TUNABLE FLUORESCENCE DETECTION FOR THE HPLC SIMULTANEOUS QUANTITATION OF LACTONE AND CARBOXYLATE FORMS OF TOPOTECAN IN PLASMA

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ABSTRACT

The hydrolysis of the α-hydroxy-δ-lactone ring moiety in topotecan is routinely monitored using high performance liquid chromatography (HPLC) with fluorescence detection. While both tunable and filter fluorescence detectors are commercially available, only the tunable detector has been studied for clinical and in vitro applications of topotecan. In the present study we have developed a simple HPLC method for the simultaneous separation of the lactone and carboxylate forms of topotecan in plasma, which, can be utilized for both clinical and in vitro studies. Limits of detection, percent relative standard deviation, and linear range for both the lactone and carboxylate forms of the drug in plasma are presented and compared using a tunable and filter fluorescence detector. Limits of detection in plasma of 0.10 ng/mL for carboxylate and 0.26 ng/mL for lactone have been obtained using a tunable fluorescence detector. A filter

fluorescence detector produced limits of detection of 0.15 ng/mL for carboxylate and 0.30 ng/mL for lactone. Reproducible quantitation using a tunable fluorescence detector from 0.25 to 250 ng/mL for carboxylate and from 0.50 to 250 ng/mL for lactone was achieved, which is an improvement over existing methods. The filter detector, which has not been previously studied, provided reproducible detection from 0.50 to 250 ng/mL for carboxylate and from 0.75 to 250 ng/mL for lactone.

INTRODUCTION

Topotecan ((S)-9-dimethylaminomethyl-10-hydroxy-camptothecin, SK&F 104864, NSC609669) is the first DNA topoisomerase I inhibitor to gain approval by the Food and Drug Administration. Camptothecin analogues, such as topotecan, halt the growth of various human tumors[1-5] by stabilizing the DNA-topoisomerase I complex and preventing normal enzyme function.[6-9] Due to encouraging results in clinical trials, the general use of topotecan in the United States has recently been approved for patients with ovarian cancer.

As shown in Figure 1, topotecan (I) has an α-hydroxy-δ-lactone ring moiety which undergoes a pH dependent hydrolysis yielding the carboxylate form (II) of the drug.[10] Evidence has shown that an intact lactone ring is necessary for biological activity of camptothecin and camptothecin analogues.[9,11-13] Understanding the pharmacokinetic and pharmacodynamic parameters of lactone ring opening in topotecan requires methodologies which allow for reproducible quantitation of I and II at various concentrations in plasma. High performance liquid chromatography (HPLC) using fluorescence detection has been routinely used to monitor the lactone ring opening of topotecan in plasma for both clinical and *in vitro* studies. HPLC separations of topotecan have been described for both the lactone only,[14] and simultaneous separation of both I and II.[15,16,17] All of the separation schemes to date employ fluorescence detection using a tunable detector. Tunable fluorescence detection has been shown to provide reproducible quantitation from 0.50 to 20 ng/mL (15) and from 5.0 to 500 ng/mL[17] for both I and II. As yet, no quantitative studies have been published using a filter fluorescence detector for clinical applications of topotecan. To date, no one HPLC method has been studied over the wide concentration range capable of covering both clinical and *in vitro* studies.

Filter fluorescence detectors differ from tunable detectors in that filters of the appropriate wavelength range are employed as wavelength selectors instead of a monochromator. Because of the larger bandpass, the throughput of filters

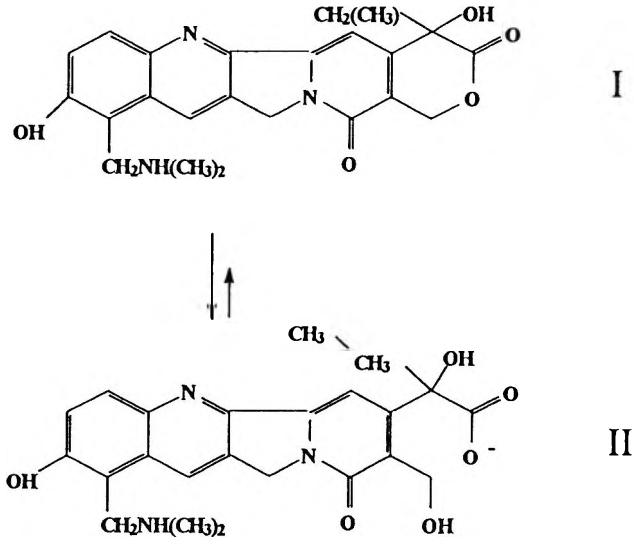

Figure 1. Chemical structure and hydrolysis of the lactone (I) and carboxylate (II) forms of topotecan.

are generally much greater than that of a monochromator. This allows for a very inexpensive lamp such as quartz halogen to be used in filter detectors, rather than, much more expensive lamps such as xenon, which are common in tunable detectors. For adequate results, lamps must be changed after 1000 hours of use in either detector, which correlates to twice per year for routine analysis. This represents a yearly maintenance cost of approximately $3000 for tunable detectors, as compared to $100 per year for filter detectors. Because of low maintenance costs, filter detectors have been utilized in analyses similar to that of topotecan in plasma, which have low background fluorescence matrices over a large wavelength range. In these instances, filter detectors can provide similar reproducibility and limits of detection as found with tunable detectors.

In the present study we have employed a simple HPLC methodology utilizing fluorescence detection for the simultaneous separation I and II in plasma. Limits of detection, percent deviations, and linear range, using both a tunable and filter fluorescence detector, are determined for I and II. Analyte concentrations are then back-calculated from peak using a log-log plot. A comparison of filter versus tunable detection in terms of the above-mentioned parameters is presented.

EXPERIMENTAL

Chemicals and HPLC Reagents

Samples of topotecan were graciously provided by the National Cancer Institute, Division of Cancer Treatment (Bethesda, MD). HPLC grade acetonitrile and triethylamine were purchased from Fisher Scientific (Fair Lawn, NJ). High purity water was provided by a Milli-Q UV PLUS purification system (Millipore, Bedford, MA). Unless noted, all other chemicals were reagent grade and used without further purification. Stock solutions of topotecan were prepared in A.C.S. spectrophotometric grade dimethylsulfoxide (Aldrich Chemical Company, Milwaukee, WI) at a concentration of 100 μM and stored in the dark at 4°C until use. Phosphate buffered saline (PBS) refers to an aqueous solution of 8 mM dibasic sodium phosphate (Na_2HPO_4), 1 mM potassium phosphate monobasic crystals (KH_2PO_4), 137 mM sodium chloride (NaCl) and 3 mM potassium chloride (KCl). Outdated plasma was obtained from Central Kentucky Blood Center (Lexington, KY) and stored at -20°C until use.

HPLC Apparatus

The isocratic HPLC system utilized consisted of a Waters 501 HPLC pump (Millipore Corp., Milford, MA), a Rheodyne injector (Rainin Instrument Co., Woburn, MA) fitted with a 200 μL sample loop and one of the following detectors. A Gilson Model 121 Fluorescence Detector (Gilson Medical Electronics, Middleton, WI) equipped with filters of 350 to 470 nm for excitation and 510 to 650 nm for emission was used as the filter detector. A Waters Model 470 Scanning Fluorescence Detector (Millipore Corp., Milford, MA) with 16 μL flow cell and excitation wavelength of 390 nm and emission of 520 nm (18 nm bandwidth) was used as the tunable detector. Separations were carried out at ambient temperature using a Waters NovaPak-C_{18} 4 μm particle-size reversed-phase 3.9 x 150 mm column preceded by a Waters Guard-Pak C_{18} Nova-Pak precolumn insert (Millipore Corp., Milford, MA). The mobile phase consisted of 88% triethylamine acetate buffer (3% triethylamine in water (v/v) adjusted to pH 5.5 with glacial acetic acid) and 12% acetonitrile. All mobile phases were filtered and vacuum degassed using sonication prior to use. In all instances, a flow rate of 1.0 mL/min was employed. Fluorescence output signal was monitored and integrated using Millennium 2010 Chromatography Manager (Millipore Corp., Milford, MA) on a 386 IBM computer.

Sample Preparation

To determine limit of detection, linear range and percent relative standard deviation of topotecan solutions in plasma, the following sample preparation procedure was used. A stock solution containing 100 μM of the drug of interest in DMSO was prepared and stored at -20°C. A 50 μL aliquot of this stock solution was added to 407.5 μL PBS pH 2.0 for lactone or PBS pH 11.0 for carboxylate in order to form 5000 ng/mL PBS solutions of I and II. These 5000 ng/mL solutions were allowed to stand at room temperature for 30 min to ensure equilibration. Serial dilutions with PBS of the appropriate pH were performed to make 2.5-2500 ng/mL solutions of I and II. To make 0.25-250 ng/mL solutions of I and II in plasma, 100 μL of the appropriate PBS solution was added to 900 μL plasma. This solution was then vortexed for 20 sec. A 200μL aliquot was added to 800 μL cold methanol (-40 °C), vortexed for 20 seconds and then centrifuged for 2 min. A 500 μL aliquot of supernate was then added to 500 μL PBS of the appropriate pH. A 200 μL aliquot of the resulting solution was then directly injected.

For percent recovery from plasma studies, 0.25 and 0.50 ng/mL solutions of I and II in PBS were diluted by adding 100 μL of the desired concentration to 900 μL PBS of the appropriate pH. A 200 μL aliquot of this solution was directly injected. This provided the same on column concentration as the 2.5 and 5.0 ng/mL plasma samples after extraction and dilution. Percent recovery was then determined by comparing drug concentrations in PBS with drug concentrations after precipitation from plasma.

Validation

Validation was performed for the detection of I and II in plasma. In all instances, three replicate samples of each concentration were used to determine limit of detection, linear range, between run deviation, and percent recovery for I and II in plasma. In addition, three injections of each sample was made to determine within run deviation. Limit of detection (LOD) was calculated according to Equation 1, with the standard deviation of the blank being determined by six injections of the sample blank.

$$\text{limit of detection} = \frac{3\ (\text{standard deviation of blank})}{\text{slope of calibration curve}} \qquad (1)$$

The linear range of each calibration curve was determined by including the lowest concentration which gave a correlation coefficient greater than 0.9950 for the linear regression equation and log-log regression equation. Between run and within run deviation was determined at those concentrations of I and II in plasma as shown in Equation 2.

$$\text{percent deviation} = 100^* \frac{\text{standard deviation of peak area}}{\text{average peak area}} \quad (2)$$

An upper limit of 15% was set as the maximum between and within run percent deviation which was tolerated. Only those concentrations which had between and within run percent deviations below 15% were considered reproducible. Recovery is calculated for I and II in plasma at 2.5 and 5.0 ng/mL as shown in Equation 3.

$$\text{percent recovery} = 100^* \frac{\text{average peak area in plasma}}{\text{average peak area in PBS}} \quad (3)$$

In addition to the above mentioned validation parameters, the peak areas corresponding to each theoretical concentration were used to back-calculate the measured concentration. This was performed by producing a log/log plot of the average peak areas versus concentration for each concentration used in the calibration curve. The regression equation from this plot was then used to back-calculate the measured concentration from each peak area. The average measured concentration and percent deviation from the theoretical concentration are presented for each concentration.

RESULTS AND DISCUSSION

Chromatography

The chromatographic parameters which were employed for this assay were chosen due to the dynamic nature of topotecan. The mobile phase consists of only two components, namely triethylamine acetate (TEA) buffer (3%) and acetonitrile. TEA buffer was used as the aqueous component of the mobile phase for multiple reasons. TEA is known to reduce peak tailing of amino containing compounds such as topotecan on C_{18} columns. Reducing peak tailing improves peak shape which generally lowers the limit of detection.

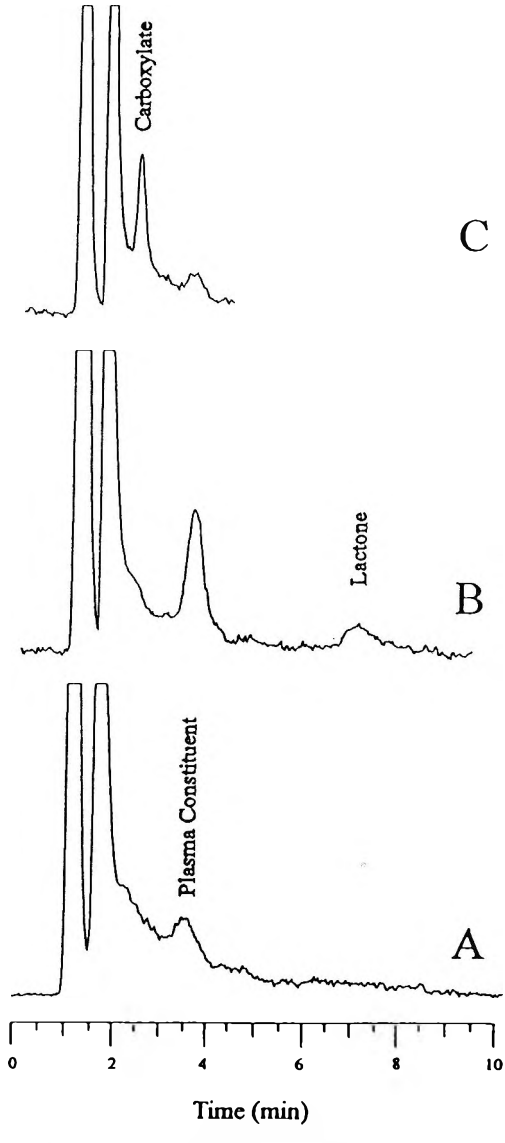

Figure 2. Chromatograms of plasma blank sample (A), plasma sample spiked with 2.5 ng/mL topotecan lactone (B) and plasma sample spiked with 2.5 ng/mL topotecan carboxylate (C) using a tunable fluorescence detector.

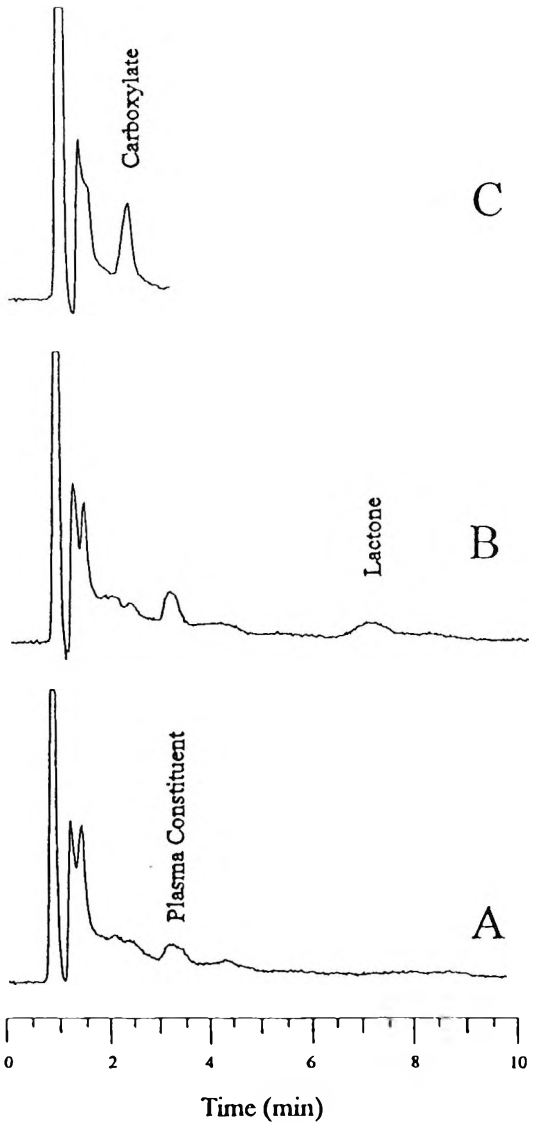

Figure 3. Chromatograms of plasma blank sample (A), plasma sample spiked with 2.5 ng/mL topotecan lactone (B) and plasma sample spiked with 2.5 ng/mL topotecan carboxylate (C) using a filter fluorescence detector.

TEA buffer also has the desired buffering capability at pH 5.5, which minimizes the hydrolysis of topotecan on column. Most importantly, TEA is an effective ion-pairing agent, which allows the carboxylate form of topotecan to be adequately retained on the C_{18} column.

Past HPLC methodologies, utilizing fluorescence detection for the simultaneous separation of I and II in plasma, have not been able to obtain adequate resolution of the carboxylate peak from the plasma or blood matrix peaks.[15,17] Figure 2 and 3 shows chromatograms of plasma and plasma samples spiked with 2.5 ng/mL of I and II using both the tunable and filter detectors, respectively. The plasma blank has only one major constituent with a retention time of 3.2 min and does not interfere with the quantitation of the topotecan samples. Topotecan carboxylate has a retention time of 2.3 min, whereas the lactone species has a retention time of 7.3 min. As is evident from Figures 2 and 3, the use of a filter detector rather than a tunable detector does not degrade the detection of the chromatographic separation.

Figures of Merit

This analytical method has been validated in terms of limit of detection, linear range, percent deviation, percent recovery and back-calculation of concentration, with all results being reported for both the tunable and filter detector.

The limit of detection was determined to be 0.26 ng/mL for I and 0.10 ng/mL for II in plasma using the tunable detector (Table 1). These LOD values are comparable to reported lower limit of quantitation (LLQ) values of 0.1 ng/mL and 0.2 ng/mL for topotecan using a tunable detector (15,17). Although the terms LOD and LLQ differ in that LOD is defined as three times the standard deviation of the baseline and LLQ is an empirical value, they tend to be somewhat comparable. The filter detector yielded limits of detection of 0.30 ng/mL for I and 0.15 ng/mL for II in plasma (Table 1).

The calibration curves of topotecan in plasma are linear over 2.5 decades for I and 3.0 decades for II using the tunable detector and over 2.25 decades for I and 2.5 decades for II using the filter detector (Table 1). In all cases, the correlation coefficients (r^2) are above 0.9990. Using this analytical method, both detectors surpass other published linear ranges, which are generally less than two decades.[15,17] Therefore, this method is recommended for both clinical and *in vitro* studies of topotecan in plasma.

Table 1

Figures of Merit for Topotecan in Plasma Using Tunable and Filter Fluorescence Detectors

Detector	Species	LOD[1] (ng/mL)	Linear Range (decades)	r^2
Tunable	I	0.26	2.5	0.9990
	II	0.10	3.0	1.0000
Filter	I	0.30	2.25	0.9997
	II	0.15	2.5	1.000

[1] Limit of detection (LOD) is calculated as three times the standard deviation of the blank sample divided by the slope of the calibration curve.

Percent relative standard deviations for I and II using the tunable and filter detectors are shown in Tables 2 and 3. Using a tunable detector, within run deviations were less than 7.9%, while between run deviations were under 8.8%. The lowest concentration which produced reproducible quantitation was 0.25 ng/mL for I and 0.50 ng/mL for II. This chromatographic method provides a two-fold improvement for lower limit of reproducible quantitation for II and identical results for I when compared to existing methods.[17] The within run deviations below 13.3% and between run deviations of less than 14.4% were found for the filter detector. The lowest concentration which produced reproducible quantitation using the filter detector was 0.75 ng/mL for I and 0.50 ng/mL for II.

Back-calculation of concentration from peak area for both detectors with percent deviation is shown in Tables 4 and 5. Due to the large linear range of the assay, a log-log plot was utilized for back-calculation. All back-calculated concentrations were determining by using the linear regression equation of the log-log plot of all concentrations studied.

This method is an improvement over existing methods of determining regression equations over each individual decade for concentrations within that decade.[14,15] Deviations from the theoretical concentration ranged from 0.4-11.2% for the tunable detector and from 2.4-17.8% for the filter detector.

Table 2

Within Run and Between Run Deviation of Topotecan Samples in Plasma Using a Tunable Fluorescence Detector

Concentration (ng/ML)	Percent Deviation Within Run[1]		Between Run[2]	
	I	II	I	II
0.25	---	6.7	----	8.8
0.50	4.5	----	0.4	----
2.5	4.1	3.8	2.1	3.7
5.0	7.9	2.8	1.2	4.2
25	2.4	5.3	3.7	2.2
250	2.6	2.6	5.0	2.8

[1] Within run deviation was determined by three injections of each sample.
[2] Betweeen run deviation was determined by injections of three samples.

Table 3

Within Run and Between Run Deviation of Topotecan Samples in Plasma Using a Filter Fluorescence Detector

Concentration (ng/ML)	Percent Deviation Within Run[1]		Between Run[2]	
	I	II	I	II
0.50	----	11.1	----	14.4
0.75	13.3	----	9.8	----
2.5	4.5	7.1	2.0	7.2
5.0	6.5	3.8	5.3	2.9
25	3.9	2.7	2.6	6.3
250	1.8	1.9	5.1	6.9

[1] Within run deviation was determined by three injections of each sample.
[2] Between run deviation was determined by injections of three samples.

Table 4

Back-Calculation of Concentration from Peak Areas for Topotecan Lactone and Carboxylate Using a Tunable Fluorescence Detector

Species	Theoretical Conc. (ng/mL)	Measured Conc. (ng/mL)	Percent Deviation[1]
I	0.500	0.507	1.4
	2.50	2.28	8.8
	5.00	5.13	2.6
	25.0	27.8	11.2
	250	238	4.8
II	0.250	0.267	6.8
	2.50	2.26	9.6
	5.00	5.02	0.4
	25.0	25.4	1.6
	250	256	2.4

[1] Deviation calculated as theoretical concentration minus measured concentration divided by theoretical concentration.

Recovery of I and II using protein precipitation with methanol from plasma has been determined from three independent determinations at two concentrations (2.5 and 5.0 ng/mL) of I and II. Percent recoveries range from 80.3-82.2% for I and 86.6-104.9 % for II and are in accordance with other published values.[14]

Comparison of Filter versus Tunable Fluorescence Detector

The limit of detection of topotecan in plasma is similar using the tunable and filter detectors. Although the LOD for both I and II are slightly better using the tunable detector, the values for both detectors are below concentration levels found in clinical studies following drug infusion (1-5 ng/mL). The linear range for the tunable detector is 0.75 decades larger for I and 0.5 decades larger for II, but both exceed previously published values.[15,17]

Reproducible detection was achieved over the range of 0.50-250 ng/mL for I and 0.25-250 ng/mL for II using the tunable detector. For the filter detector the range is slightly smaller, 0.75-250 ng/mL and 0.50-250 ng/mL, but

Table 5

Back-Calculation of Concentration from Peak Areas for Topotecan Lactone and Carboxylate Using a Filter Fluorescence Detector

Species	Theoretical Conc. (ng/mL)	Measured Conc. (ng/mL)	Percent Deviation[1]
I	0.750	0.669	10.8
	2.50	2.86	14.4
	5.00	4.74	5.2
	25.0	27.7	10.8
	250	232	7.2
II	0.500	0.434	13.2
	2.50	2.63	5.2
	5.00	5.89	17.8
	25.0	24.4	2.4
	250	235	6.0

[1] Deviation calculated as theoretical concentration minus measured concentration divided by theoretical concentration.

appropriate for the concentration levels found in clinical trials and *in vitro* studies. At the lowest concentration studied, within run and between run deviations are somewhat better for the tunable detector as compared to the filter detector. At concentrations above 2.5 ng/mL, deviations are similar for both detectors.

Back-calculation of concentrations from peak areas yielded deviations below 11.2 % for the tunable detector. The filter detector produced deviations ranging from 2.4-17.8%, which is still adequate for most applications. This back-calculation is performed using the log-log linear regression over all concentrations studied.

If a smaller concentration range is appropriate, as with clinical samples, a more accurate linear regression equation can be produced for concentrations within that range. Therefore, it is expected that the percent deviations using back-calculation will be smaller in that case.

CONCLUSIONS

An HPLC method has been established for the simultaneous separation of topotecan lactone and carboxylate species in plasma using both a filter and tunable fluorescence detector. Both detection schemes have produced reproducible quantitation of topotecan lactone and carboxylate at concentrations appropriate for clinical and *in vitro* studies, which is an improvement over existing methods. Limits of detection for topotecan lactone and carboxylate in plasma are similar for both the tunable and filter detector. From these studies, it seems that the more cost efficient filter detector is adequate for quantitating topotecan lactone and carboxylate in plasma.

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PRACTICAL OPTIMIZATION OF AN ION-PAIR HPLC ASSAY FOR 1,3-DICHLORO-6,7,8,9,10,12-HEXAHYDROAZEPINO[2,1-B] QUINAZOLINE MONOHYDROCHLORIDE BULK PHARMACEUTICAL CHEMICAL

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ABSTRACT

An ion-pair HPLC method was optimized for the assay of the experimental Alzheimer's bulk pharmaceutical chemical CI-1002 Hydrochloride and ten potential impurities. The process of selecting the most robust chromatographic system (i.e., mobile phase composition, pH and column) was completed. A mobile phase consisting of aqueous sodium dodecyl sulphate (SDS) and methanol was utilized. Several commercially available base-deactivated C_{18} columns were evaluated and a Waters Symmetry 150 x 4.6 mm, 5 μm particle size column was selected. The Waters Symmetry column was used in subsequent experiments to optimize the mobile phase pH, the percent methanol, and the SDS content. The ruggedness of the HPLC method was evaluated by documenting slight modifications under controlled conditions. The ion-pairing method enables the assay and impurity profile for ten potential impurities to be carried out simultaneously. The method also provides baseline resolution for all components and a run time of approximately 30 minutes.

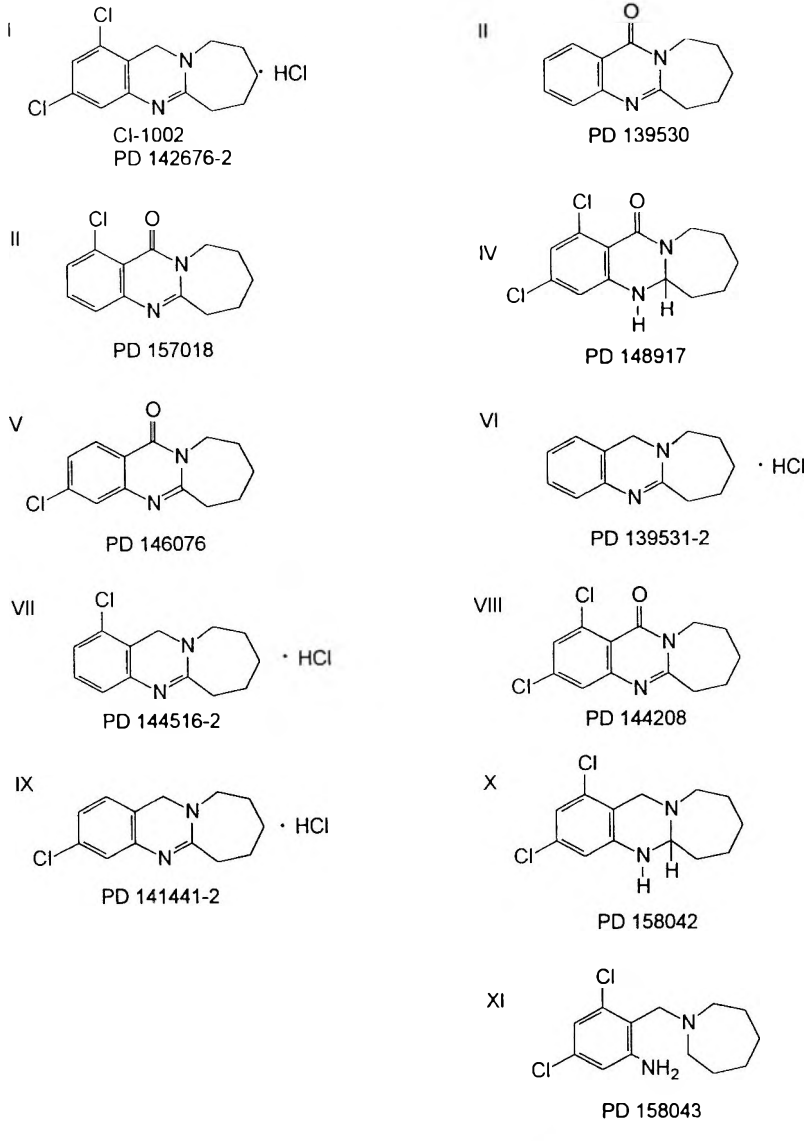


Figure 1. Chemical Structures for CI-1002 and Ten Potential BPC Impurities

INTRODUCTION

CI-1002 hydrochloride bulk pharmaceutical chemical (BPC) (PD 142676-2, compound I in Figure 1), 1,3-dichloro-6,7,8,9,10,12-hexahydroazepino[2,1-b] quinazoline monohydrochloride, is being developed as a cognition activator for the treatment of Alzheimer's. The original HPLC assay for the BPC was a reverse phase method which utilized a C_8 column. A 60-minute run time was required for this method, and it was not able to resolve several potential impurities from CI-1002 .

The chemical structures of CI-1002 and the ten potential impurities are shown in Figure 1.

A shorter run time, baseline resolution, and ruggedness were key factors considered in the development and optimization of a replacement HPLC method. Ion-pair theory[1-2] and investigative efforts suggested the use of the ion-pairing agent sodium dodecyl sulfate (SDS) as the mobile phase modifier and methanol as the organic solvent.

An ion-pair reverse phase HPLC method was optimized to reduce the run time to approximately 30 minutes, while maintaining baseline resolution of CI-1002 and the ten potential impurities. Specifically, several commercially available base-deactivated C_{18} columns were evaluated. Also, experiments to optimize the percent methanol, the SDS concentration, and the mobile phase pH were conducted to enable all of the potential BPC components to be baseline resolved.

EXPERIMENTAL

Reagents

Methanol (HPLC grade) and ammonium hydroxide (reagent grade) were purchased from EM Science, Gibbstown, NJ. Phosphoric acid (85%, reagent grade) and sodium dodecyl sulphate (electrophoresis grade) were obtained from Fisher Scientific, Fair Lawn, NJ. Water (HPLC grade) was obtained from a Waters, Milford, MA Milli-Q water purification system. The ten potential impurities and CI-1002 shown in Figure 1 were prepared at Parke-Davis Pharmaceutical Research Division, Holland, MI.

Chromatographic Columns

The following base-deactivated C_{18} , 5 μm particle size, 4.6 mm ID x 150 mm length columns were evaluated: a Waters Symmetry, (Waters, Milford, MA); a Mac Mod Zorbax RX-C18, (Mac Mod, Chadds Ford, PA); and an Alltech Alltima, (Alltech, Deerfield, IL).

Chromatographic and Other Equipment

Initially, chromatographic analysis was performed using a Hitachi L-6200A Intelligent Pump, a Micromeritics 728 autosampler, a Rheodyne 7010 injector with a 20 μL sampling loop, a Hitachi 655A variable wavelength UV detector, a Hitachi D-2500 Chromato-Integrator, and the three base-deactivated columns selected. The final chromatograms used the three columns and a chromatographic system from Waters (i.e., a 600E pump, a 700 WISP, and a 996 photo diode array detector). The system was controlled, and the data were collected and processed with Waters Millennium (version 2.1) software. The mobile phase pH was determined using an Orion Model 701A digital IonAnalyzer with a Orion 8103 ROSS combination pH electrode.

USP Plate Count, Tailing Factor, and Resolution Test Solution

Aliquots (1.0 mL) of individually prepared stock solutions of PD 158042 (compound X in Figure 1) and CI-1002 were added to a 25 mL volumetric flask and diluted to volume with mobile phase. Each stock solution concentration was 1.0 mg/mL in methanol.

Elution Optimization Test Mix Solution

Aliquots (0.5 mL) of individually prepared stock solutions of each compound were added to a 25 mL volumetric flask and diluted to volume with mobile phase (see Figure 1). Each impurity stock solution (II through XI) concentration was 1.0 mg/mL in methanol. The concentration of the BPC stock solution was 5.0 mg/mL in methanol.

RESULTS AND DISCUSSION

The original reverse phase HPLC method, which utilized an aqueous triethylamine buffer at pH 3.0 mixed with acetonitrile and methanol (3:1:1,

v/v), and a 4.6 x 250 mm C_8 column, eluted CI-1002 at approximately 10 minutes, and the penultimate (PD 144208, compound VIII in Figure 1) at about 51 minutes. Based on the basicity (tertiary vs. secondary vs. primary amine content) of CI-1002 and the potential impurities, it was decided that ion-pairing might be useful as an alternative HPLC approach. According to ion-pair theory, the relatively basic amine analytes would be retained on the column by acidic ion-pair reagents such as octane sulfonic acid or SDS.[1-2] SDS was used initially and found to provide a promising separation, with (in accordance with the basicity of the analytes) a resulting reversal in elution order of the penultimate (VIII) and CI-1002 (in comparison to the original reverse phase method). This reversal in elution order was significant since it gave a much sharper peak for the penultimate (VIII) and resulted in a lower limit of detection and quantitation for this BPC impurity. Next, experiments were run to compare the three base-deactivated C_{18} columns. The resolution and USP tailing factor was superior with the Waters column, and this column was selected for further optimization experiments.

The ion-pairing of the compounds with SDS was structurally dependent on the basicity of the various amines. The greater the ion-pairing, the longer the retention time of the compounds.[1-2] A number of parameters play a significant role in ion-pairing chromatography: mobile phase organic modifier and ion-pair reagent concentration, as well as the pH of the mobile phase.[1-2] These parameters were therefore systematically varied to optimize the final HPLC method.

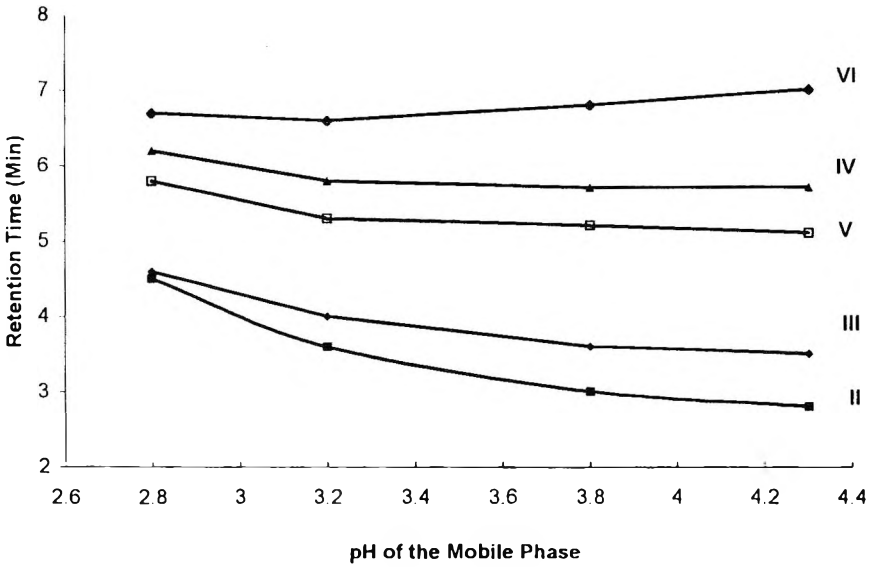
Optimization of Methanol Content

The elution time for the ion-pair HPLC method was greatly effected by the content of methanol in the mobile phase. For example, with all other parameters being equal, the increase of methanol content in the mobile phase from 67% to 69% (with the balance of the mobile phase being 0.03 M SDS, with a final apparent pH of 4.0) changed the retention time of the last eluting component (XI) by 9.3 minutes. However, the system suitability results remained essentially unchanged. A 68% methanol content (v/v) in the eluent was selected to achieve the desired 30 minute run time.

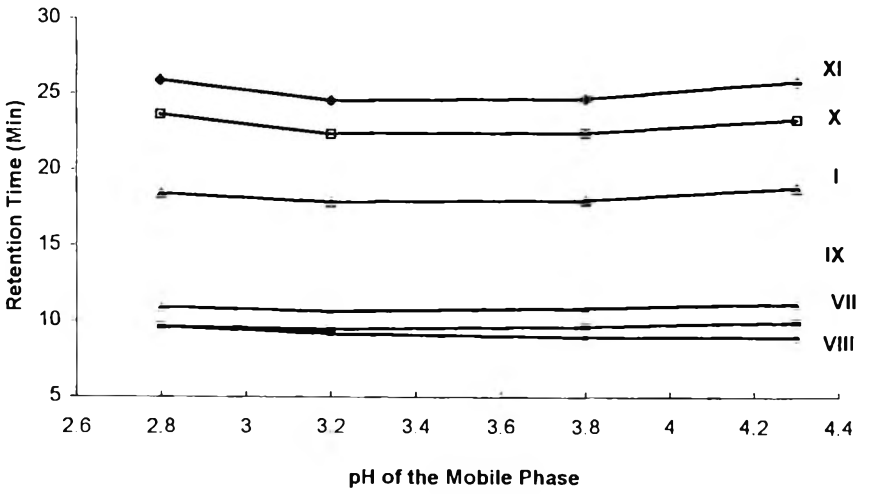
Optimization of SDS Concentration

A range of SDS concentrations from 0.006 M to 0.02 M (concentration per liter of mixed mobile phase) were evaluated. The concentration of the counterion altered analyte retention times, and subsequent resolution. The

Part A


Retention Time (Min)
8
7
6
5
4
3
2
VI
IV
V
III
II
2.6
2.8
3
3.2
3.4
3.6
3.8
4
4.2
4.4
pH of the Mobile Phase

Part B


Retention Time (Min)
30
25
20
15
10
5
XI
X
I
IX
VII
VIII
2.6
2.8
3
3.2
3.4
3.6
3.8
4
4.2
4.4
pH of the Mobile Phase

alterations were predictable based on the basicity of the analytes (refer to Figure 1). The 0.006 M counterion concentration reversed the elution order of VII and the penultimate (VIII), creating potential quantitation problems due to co-elution of the two compounds.

No significant advantages were found in the separation above 0.013 M SDS. The 0.01 M SDS concentration enabled the penultimate (VIII) to be quantitated without interference, at a relative retention time of approximately 0.46 vs 1.00 for CI-1002.

Based on these results, and in order to reduce the potential for poor system performance and improve ruggedness,[2] the optimum SDS concentration was determined to be 0.01 M. This SDS concentration was therefore used for subsequent experiments.

Optimization of pH

The final (apparent) pH of the mobile phase was evaluated and the results are shown in Figure 2A and 2B. The resolution of the impurities increased with increasing mobile phase pH. The resolution of VII, the penultimate (VIII) and IX showed the greatest increase. At pH 2.8, VII and the penultimate (VIII) co-eluted, but were resolved at pH 3.8. These effects were critical since the penultimate (VIII) in the CI-1002 Hydrochloride BPC synthetic scheme needed to be resolved and quantitated.

The system suitability resolution parameters were unchanged over the pH range of 2.8 to 4.3. As the pH was increased to 4.3, the retention time decreased for impurities II, III, and V. Similarly, as the pH was increased to 3.2 the retention time consistently decreased for all components, and then at pH values greater than 3.2, retention times increased or remained unchanged for I, IV, VI, VII, IX, X, and XI (see Figure 2A and 2B).

The effects of pH on resolution and retention time resulted in pH 4.0 being selected as the most desirable in terms of ruggedness.

Figure 2. (left) Effect of Mobile Phase pH on the Retention of Compounds I through XI as Shown in Figure 1. Part A shows Compounds II through VI, while Part B shows Compounds VII through XI and Compound I.

Table 1

System Suitability Measurements (Resolution, USP Plate Count and Tailing Factor) for the Three Columns Tested

Column	Resolution	Plate Count	Tailing Factor
Waters Symmetry	5.3	9184	1.03
Mac Mod Zorbax	4.2	8155	1.12
Alltech Altima	2.4	9885	1.25

Table 2

Waters Millennium Resolution Values for Each Compound Identified in Figure 1

Compound	Waters CD-2522	Waters IMP-MIX	Zorbax IMP-MIX	Alltima IMP-MIX
II	NA*	NA*	NA*	NA*
III	5.29	3.19	2.38	4.62
V	6.63	6.51	1.12	1.71
IV	NA*	2.20	4.61	5.60
VI	NA*	3.67	3.18	1.76
VIII	10.91	5.38	6.30	<1.0
VII	2.39	2.23	1.11	7.54
IX	3.00	2.78	1.78	>1.0
I	12.34	12.01	12.32	12.16
X	5.70	5.31	4.16	2.44
XI	NA*	2.45	3.61	3.07

*See the Results and Discussion Section for an explanation of the not-applicable (NA) values reported.
IMP-MIX refers to the Elution Optimization Test Mix Solution.

Column Comparison with Optimized Conditions

Column selection involved testing and evaluating the following C_{18} columns: a Waters Symmetry, a Mac Mod Zorbax RX-C18, and an Alltech Alltima. The columns were compared by running the elution optimization

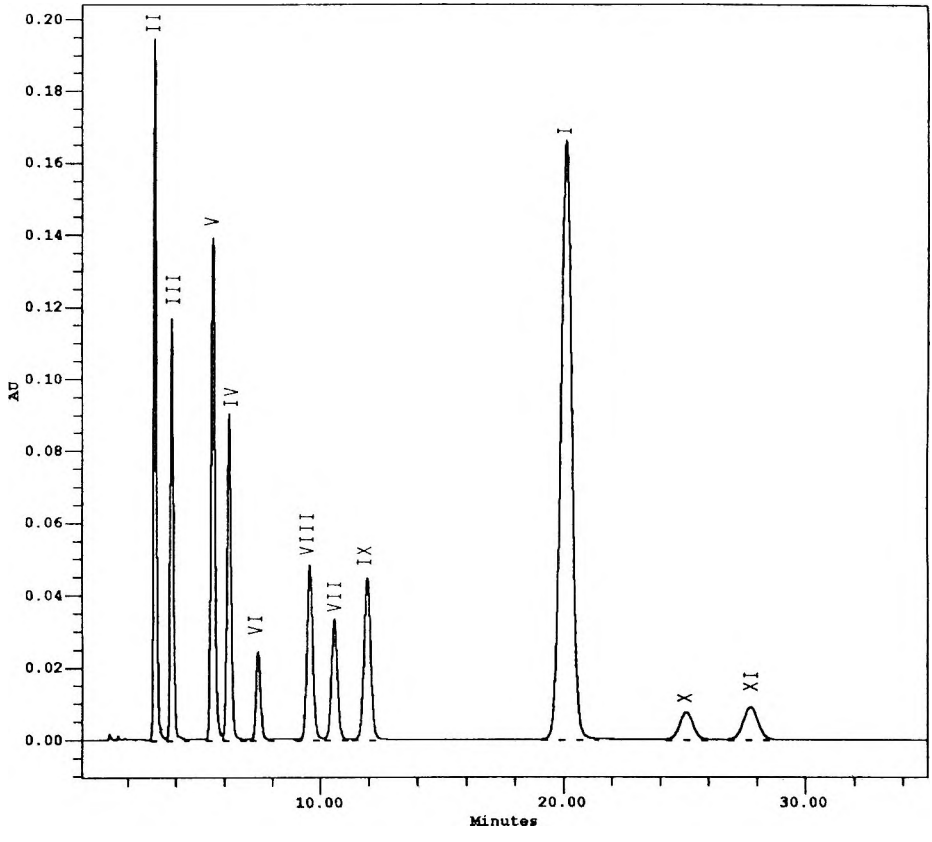


Figure 3. Waters Symmetry Chromatogram of the IMP-MIX. Experimental Conditions: Mobile Phase of 68 parts MeOH and 32 parts 0.03 M SDS with a final pH of 4.0, Flow Rate of 1.0 mL/min, Column Temperature of 27°C, Detection Wavelength of 228 nm, Injection Volume of 20 µL. Figure 1 shows the Structures of Compounds I through XI.

test mix at a concentration of 0.02 mg/mL for (I through XI), and 0.10 mg/mL for CI-1002. The run conditions for each system tested varied slightly in percent mobile phase composition in order to keep the total run time equal to approximately 30 minutes. System suitability results shown in Table 1 were used to evaluate overall column performance. The system suitability results showed: the Waters column with a higher plate count and resolution than the Zorbax column; the Alltech column with the highest plate count and lowest resolution.

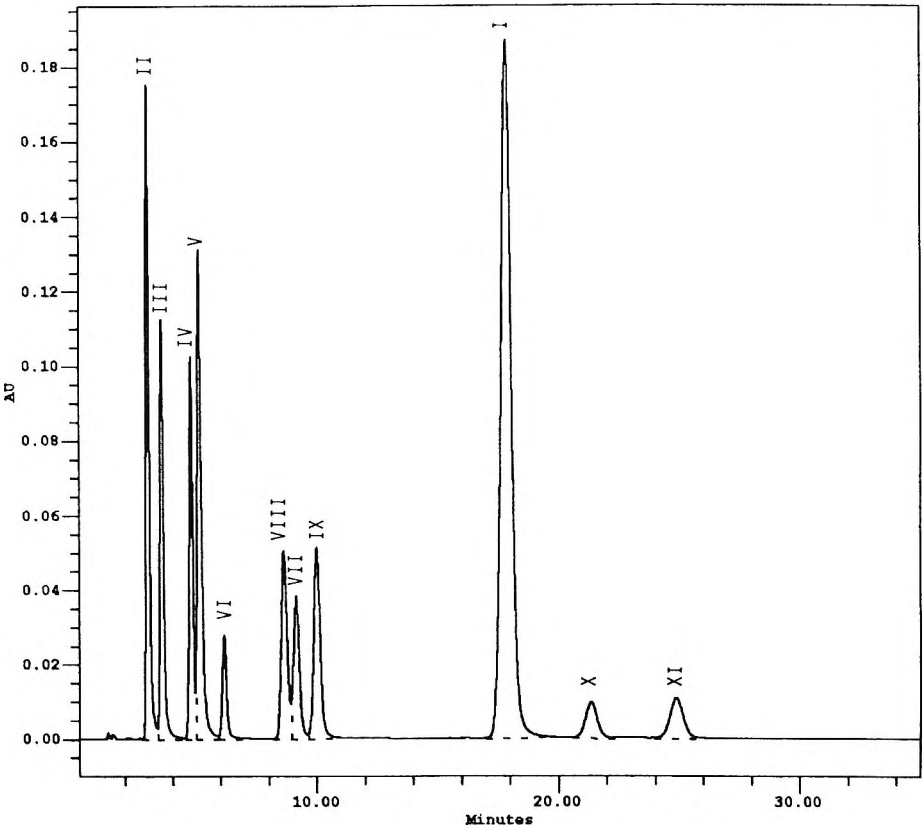


Figure 4. Zorbax Rx-C18 Chromatogram of the IMP-MIX. Experimental Conditions: Mobile Phase of 67 parts MeOH and 33 parts 0.03 M SDS with a final pH of 4.0, Flow Rate of 1.0 mL/min, Column Temperature of 27/C, Detection Wavelength of 228 nm, Injection Volume of 20 μL. Figure 1 shows the Structures of Compounds I through XI.

The tailing factors were all found to be below 1.5, with the highest being found for the Alltech column at 1.3. In summary, the Waters column was selected for routine use based on superior resolution and tailing factor results.

Tabulated resolution results using the Waters Millennium Software for CI-1002 and the ten impurities on each column tested can be found in Table 2. The resolution in Table 2 refers to the separation from the previous eluting impurity. Not applicable (NA) applied when the previous compound was not detected.

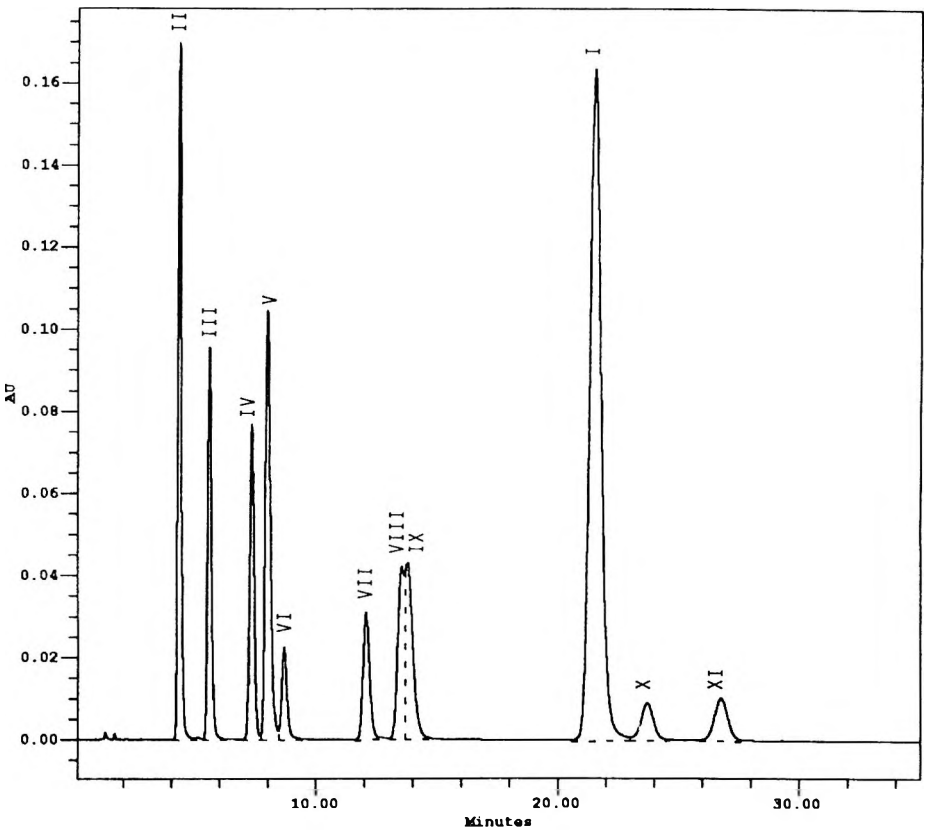


Figure 5. Alltech Alltima Chromatogram of the IMP-MIX. Experimental Conditions: Mobile Phase of 69 parts MeOH and 31 parts 0.03 M SDS with a final pH of 4.0, Flow Rate of 1.0 mL/min, Column Temperature of 27°C, Detection Wavelength of 228 nm, Injection Volume of 20 μL. Figure 1 shows the Structures of Compounds I through XI.

The Waters column did not exhibit a resolution factor less than 2.0 for any single compound. This resolution of at least 2.0 met recent FDA recommendations.[3] The Zorbax and Alltech columns exhibited some resolution factors less than 2.0 with the Alltech column unable to separate the penultimate (VIII) and IX. Recall that the penultimate compound in the process needed to be resolved for quantitation. It was interesting to note, however, that the Alltech column provided the best resolution for the early eluting potential

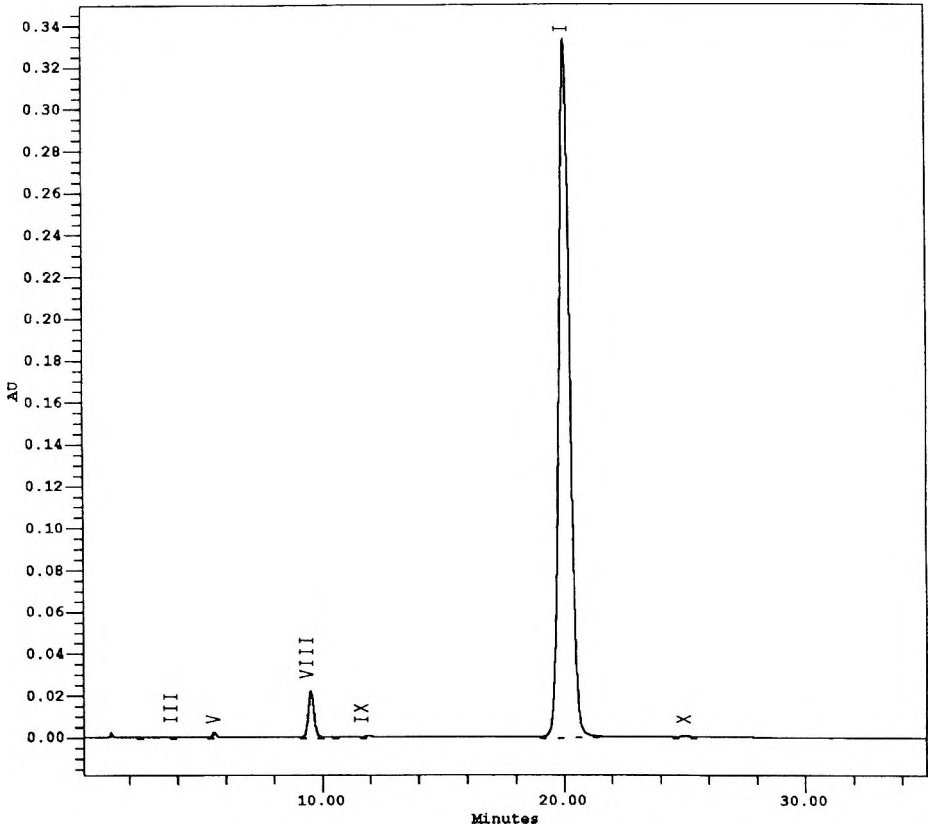


Figure 6. Chromatogram of a Typical Lot of CI-1002 Hydrochloride BPC. Experimental Conditions: Mobile Phase of 68 parts MeOH and 32 parts 0.03 M SDS with a final pH of 4.0, Flow Rate of 1.0 mL/min, Column Temperature of 27°C, Detection Wavelength of 228 nm, Injection Volume of 20 µL. Figure 1 shows the Structures of Compounds I through X.

impurities II and III. The HPLC chromatograms of the impurity mix for each column tested are shown in Figures 3-5. A typical production lot of BPC assayed using the Waters column showed excellent resolution of all the compounds identified, and the penultimate (VIII) was resolved from the nearest pre-eluting compound by a resolution factor of 10.9 (see Figure 6).

CONCLUSIONS

These experiments demonstrated that by careful optimization of mobile phase characteristics and column selection, it was possible to develop a rapid isocratic method for the BPC and numerous potential impurities. The selection of SDS as the counterion proved beneficial (i.e., the twelve carbon length) by providing the versatility necessary to achieve baseline resolution for the ten potential impurities and CI-1002, within approximately 30 minutes. The Waters Symmetry column selected has approximately 20 percent carbon loading, this feature helps explain the superior resolution and low tailing factor observed with this column. The hydroxyl groups of the silanol backbone are well protected and this protection benefits column robustness and column longevity in this application. The well protected silica surface of the Waters Symmetry column does not require silanol blockers in the mobile phase (e.g., triethylamine) to help control tailing and improve resolution.

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RAPID DERIVATIZATION OF ALCOHOLS WITH CARBOXYLIC-SULPHONIC MIXED ANHYDRIDES FOR HPLC-UV/FLUORESCENCE ANALYSIS: APPLICATION TO THE DETECTION OF DIHYDROQINGHAOSU (DQHS) AND ITS METABOLITES IN BIOLOGICAL SAMPLES

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ABSTRACT

The formation of ester derivatives of alcohols for the purpose of HPLC-ultraviolet/fluorescence analysis is achieved rapidly when a mixture of the alcohol and the triethylamine salt of the required acid in chloroform or dichloromethane is treated with a solution of either 2,4,6-triisopropylbenzenesulphonyl chloride or 2,4,6-trimethylbenzenesulphonyl chloride (mesitylenesulphonyl chloride) followed by 4-dimethylaminopyridine in the same solvent. Aromatic carboxylic acids give a quantitative yield of the esters while the yield is reduced for arylacetic acids.

The procedure has been applied to the detection of dihydroqinghaosu (DQHS) and its metabolites in different types of biological samples. The general applicability of the method is also demonstrated by the ready esterification of the sterically hindered hydroxy groups of testosterone and 6β-hydroxy-testosterone.

This approach to analytical esterification of alcohols is more convenient and more efficient than previous methods which require the prior conversion of the carboxylic acids to the acyl chlorides, acyl nitriles, acyl azides or the symmetric anhydrides.

INTRODUCTION

Labeling of alcohols with UV-absorbing or fluorescent tags for the purpose of high performance liquid chromatography is usually based on acylation or esterification of the hydroxyl group of alcohols with UV-absorbing or fluorescent carboxylic acids. Because of the poor acylating ability of carboxylic acids, it is often necessary to activate a carboxylic acid for acylations. This is usually done by conversion of the carboxylic acid to the anhydride or acid chloride or pseudohalide such as the acyl nitrile.[1-3]

Analytical esterification with acyl chlorides, acyl nitriles or anhydrides is neither a satisfactory nor a convenient procedure because of the instability of these reagents towards moisture.

The derivatization of alcohols with fluorescent carbonyl azides has also been reported.[4] Although the carbonyl azides may be more stable than the acid chlorides they too are prepared initially from the acid chlorides. Furthermore, reaction of carbonyl azides with alcohols require extreme conditions for the thermal decomposition of the carbonyl azide to the isocyanate, which is the acylating species. Thermal decomposition of the carbonyl azide also gives rise to many other unknown compounds which may interfere in the chromatography of the desired carbamate derivative. There is, therefore, still a great need for versatile and facile procedures for the analytical esterification of alcohols based on stable, readily available reagents, which may be used under mild conditions.

During our study of the metabolism of dihydroqinghaosu (DQHS), a new antimalarial, there arose the need to derivatize the compound and its metabolites for the purpose of HPLC with UV or fluorescence detection. DQHS possesses a relatively unreactive, acid-sensitive hemiacetal hydroxy group. This prompted an investigation of the mixed anhydride method as a

mild and facile approach to the analytical derivatization of alcohols, with DQHS as a model compound for the study. Formation of peptide bonds which involves activation of the carboxylic group by conversion to a mixed anhydride was introduced over 40 years ago.[5-7]

The following is a report on the preparation of UV/fluorescent derivatives of DQHS and its metabolites by a mixed carboxylic-sulphonic anhydride method. 2,4,6-Trimethylbenzenesulfonyl chloride (mesitylenesulfonyl chloride) and 2,4,6-triisopropylbenzene sulfonyl chloride were investigated as the activating agents.

MATERIALS

Acids

Aromatic carboxylic acids

9-Anthracenecarboxylic acid (9ACA), 4-biphenylcarboxylic acid (BCA), 2-naphthoic acid, 9-phenanthrenecarboxylic acid (9PCA), 1-pyrenecarboxylic acid.

Arylalkanoic acids

4-Biphenylacetic acid, 9-fluoreneacetic acid (9FAA), 9-fluorenecarboxylic acid, 1-pyreneacetic acid, 1-naphthylacetic acid, 1-pyrenebutyric acid.

Coumarin acids

Coumarin-3-carboxylic acid, 7- (carboxymethoxy)-4-methylcoumarin.

Condensing Agents

2,4,6-Trimethylbenzenesulphonyl chloride (mesitylenesulphonyl chloride) (METS-chloride), 2,4,6-triisopropylbenzenesulphonyl chloride (TIPS-chloride), 2,4,6-trichorobenzoyl chloride (TCB-chloride).

Catalyst

4-Dimethylaminopyridine (DMAP).

Hydroxy Compounds

Dihydroqinghaosu (DQHS), testosterone, 6β-hydroxytestosterone, octanol, 3,4-dimethyl-2-hexanol.

All reagents and chemicals were obtained from Aldrich Chemical Co (Milwakee, USA) except testosterone and hydroxytestosterone (Sigma Chemical Co, St. Loius, USA), and DQHS (Walter Reed Inventory). 9-Phenanthrenecarboxylic acid was obtained by the alkaline hydrolysis (10 M NaOH; reflux for 28 h) of 9-cyanophenanthrene obtained from Aldrich.

Instrumentation

HPLC was performed on a Waters liquid chromatography system consisting of a Waters model 510 solvent delivery unit, a U6K injector, and a Waters model 440 UV detector set at 254 nm. A Beckman Ultrasphere C8 column (4.6 mm x 15 cm) was used with a mobile phase of acetonitrile:water (80:20 v/v) at a flow rate of 3 mL/min.

Mass spectrometric identification of the derivatives was performed using a HPLC-MS system consisting of a Hewlett Packard 1090 Liquid Chromatograph System linked with a Hewlett Packard HP 5989A Mass Spectrometer via a Hewlett Packard thermospray interface. A μBondapak C_{18} column (2.1 mm x 100 mm; 5 μ) was used, with a mobile phase consisting of 0.1M ammonium acetate (pH 4.5) and acetonitrile. Elution was done with a linear gradient of 95:5 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 10 min and then increasing to a 30:70 ratio at 60 min, and held at this ratio for a further 20 min (flow rate 0.4 mL/min). The thermospray interface was operated in the "fragmenter on" mode at a vaporiser temperature of 85-96°C (or 87-105°C) and a source temperature of 220°C.

METHODS

Esterification of DQHS Using Mesitylenesulphonyl Chloride (METS-Chloride) or Triisopropylbenzenesulphonyl Chloride (TIPS-Chloride) or Trichorobenzoyl Chloride (TCB-Chloride) as Condensing Agent and Prior Preparation of the Mixed Anhydrides

Esterification of DQHS involving prior formation of the mixed anhydride is illustrated by the reaction with 9-fluoreneacetic acid.

To a solution containing 40 mg (0.178 mmol) of 9-fluoreneacetic acid and 30 μL of triethylamine in 2 mL of dichloromethane (or chloroform) was added 2 mL of a solution of 39 mg (0.18 mmol) of mesitylene sulphonyl chloride in 2 mL of dichloromethane. The mixture was kept at room temperature for 30 min to allow formation of the carboxylic-sulphonic mixed anhydride reagent.

A 2 mL portion of the reagent was then added to a solution of 25 mg (0.088 mmol) of DQHS and 12 mg (0.098 mmol) of DMAP in 2.5 mL of dichloromethane. The remaining 2 mL of the reagent solution served as a blank.

At 30 min intervals, 20 μL aliquots of the reaction mixture were taken and evaporated under a stream of nitrogen, and the residue redissolved in 200 μL of methanol, followed by HPLC analysis of 10 μL of the solution. The blank reagent mixture was treated similarly.

After keeping at room temperature for 2.5 h, the reaction mixture was shaken successively with 2 mL each of 2M hydrochloric acid, water, 2M sodium hydroxide and water. The chloroform solution was then dried with anhydrous sodium sulphate and evaporated and the white solid obtained examined by HPLC-MS.

Semi-Preparative Esterification of DQHS Using Mesitylenesulphonyl Chloride (METS-Chloride) or Triisopropylbenzenesulphonyl Chloride (TIPS-Chloride) or Trichorobenzoyl Chloride (TCB-Chloride) as Condensing Agent and *in situ* Preparation of the Mixed Anhydrides

The general procedure involves the reaction of DQHS with an excess of the acid (relative to DQHS) together with an excess of the condensing agent (relative to the acid) and an excess of DMAP (relative to the condensing agent). This is illustrated by the following preparations:

Esterification of DQHS with 9-Fluoreneacetic Acid Using TIPS-Chloride as Condensing Agent

A solution of the triethylamine salt of 9-fluoreneacetic acid was prepared by dissolving 204 mg (0.91 mmol; equivalent to about 3.3 % molar excess relative to DQHS) of the acid in 2 mL of dichloromethane (or chloroform) and adding 200 μL of triethylamine. The solution of the acid was then mixed with a solution of 250 mg (0.88 mmol) of DQHS in 2 mL of dichloromethane.

A solution of 300 mg (0.99 mmol, equiv. to about 12.5 % molar excess relative to DQHS) of TIPS-chloride in 5 mL of chloroform was then added, followed immediately by a solution of 150 mg (1.23 mmol; equivalent to about 40 % molar excess relative to DQHS) of DMAP in 2 mL of dichloromethane.

After keeping the reaction mixture at room temperature for 3h, the solvent was evaporated under a steam of nitrogen. The residue was mixed with 5 mL of sodium carbonate buffer (pH 11) and allowed to stand at room temperature for 30 min. The mixture was then extracted with 15 mL of methyl t-butyl ether by shaking on a vortex mixer for 3 min. After removal of the aqueous layer, the organic extract was washed successively with 5 mL each of distilled water, hydrochloric acid (2M) and distilled water. The extract was then dried with anhydrous sodium sulfate and evaporated under a stream of nitrogen to obtain an oily residue which on warming briefly with 3 mL of methanol turned to a pure white powder, which was filtered and dried by suction. HPLC analysis of the white solid showed only one peak which was not that of 9-fluoreneacetic acid. Similarly, HPLC-MS showed that the product was pure and contained no unreacted DQHS.

Esterification of DQHS with 9-Anthracenecarboxylic Acid Using METS-Chloride as Condensing Agent

To a solution of the triethylamine salt of 9-anthracenecarboxylic acid, prepared by dissolving 400 mg (1.8 mmole) of the acid in 10 mL of dichloromethane and 300 μL of triethylamine, was added 250 mg (0.88 mmole) of DQHS and the mixture shaken well to dissolve the DQHS. A solution of METS-chloride was prepared by dissolving 400 mg (1.83 mmole) in 5 mL of dichloromethane with brief warming in a water bath (60°C). The solution of METS-chloride was added to the mixture of anthracenecarboxylic acid and DQHS, followed immediately by 250 mg (2.04 mmole) of DMAP. The intense yellow mixture was kept at room temperature overnight (15 h).

The mixture was then evaporated under a stream of nitrogen to obtain an oily residue which, on shaking with 20 mL sodium carbonate buffer, turned into a yellow powder. After allowing the yellow powder to settle, the aqueous layer was removed and the powder washed again with 2 x 20 mL of sodium carbonate buffer. The yellow powder was then washed with 3 x 20 mL of distilled water, filtered by suction, and washed successively on the filter paper with 50 mL of 2M hydrochloric acid and 100 mL of distlled water. On drying, a bright yellow powder was obtained. The excessive yield of 560 mg and the intensely yellow filtrate obtained when the powder was being washed with either alkaline buffer or acid indicated the presence of a substantial amount of

impurity, probably the anhydride of 9-anthracenecarboxylic acid, in the product. Heating with water in a boiling water bath was found to be only partially effective in removing this side-product. The yellow powder was, therefore, mixed with carbonate buffer and heated in a boiling water bath for 30 min to obtain a pale yellow solid and a dark yellow solution. The mixture was filtered and the solid washed with distilled water until the washing was colorless. On drying, a pale yellow powder (205 mg) was obtained, which was found to be pure by HPLC-MS.

Esterification of DQHS with 4-Biphenylcarboxylic Acid (BCA) Using TCB-Chloride as Condensing Agent

A solution of the triethylamine salt of 4-biphenyl- carboxylic acid was prepared by dissolving 202 mg (1.01 mmol) of the acid in 2 mL of dichloromethane and adding 200 μL of triethylamine. The solution of the acid was mixed with a solution of 250 mg (0.88 mmol) of DQHS in 2 mL of dichloromethane and a solution of 170 μL (1.1 mmole) of TCB-chloride in 5 mL of chloroform was then added, followed immediately by a solution of 150 mg (1.2278 mmol) of DMAP in 2 mL of dichloromethane.

After keeping the reaction mixture at room temperature for 3h, it was processed as described previously for the reaction of DQHS with 9-fluoreneacetic acid and TIPS-chloride.

Derivatization of Microgram Quantities of DQHS with Carboxylic Acids Using TIPS- or METS-Chloride as Condensing Agent

To 100 - 500 μg of DQHS (20 μL of 25 mg/mL solution in dichloromethane) was added 0.2 mL of a solution of the triethylamine salt of 9-fluoreneacetic acid prepared by dissolving 20 mg of the acid in 2 mL of dichloromethane and 20 μL of triethylamine. To the mixture of DQHS and 9-fluoreneacetic acid was added 2 mL of a 3 mg/mL solution of TIPS-chloride (or METS-chloride) followed by 2 mL of a 2.5 mg/mL solution of DMAP in dichloromethane.

After keeping the mixture at room temperature for 2 h, the solvent was evaporated under a stream of nitrogen. The residue was shaken with 2.5 mL of carbonate buffer and the mixture kept at room temperature for 15 min. The mixture was extracted with 4 mL of methyl tert-butyl ether by shaking on a vortex mixer for 2 min. The aqueous layer was removed and the ether extract washed successively with distilled water (4 mL), 2M hydrochloric acid (2.5

mL) and distilled water (4 mL). After removing the last aqueous layer, the ether extract was dried with anhydrous sodium sulfate, transferred to a clean test tube and evaporated with a stream of nitrogen. The residue was redissolved in 1 mL of methanol and 30 μL of the solution was analyzed.

The above procedure was also carried out using 2 mL of a solution of benzenesulphonyl chloride (prepared by mixing 15 μL of benzenesulphonyl chloride with 10 mL of dichloromethane) in place of TIPS-chloride.

Derivatization of Microgram Quantities of DQHS Following Extraction from Blood Plasma and Reaction with Carboxylic Acids, Using TIPS- or METS-Chloride as Condensing Agent

Solutions of DQHS (100 - 500 μg/mL) were prepared in sheep plasma and 1 mL aliquots were extracted with 5 mL of methyl tert-butyl ether by shaking on a vortex mixer for 3 min. After centrifuging at 2500 rpm for 15 min, the ether layer was removed and evaporated under a stream of nitrogen. The residue was reacted with the acid and the reaction mixture treated as described in the preceeding section for solutions of DQHS in dichloromethane.

Derivatization of DQHS and Its Metabolites Extracted from Rat Liver Microsomes

Rat liver microsomes were prepared from homogenized liver tissue by differential centrifugation as previously described,[8] and the microsomal suspensions were stored at -80°C in 0.10 M potassium phosphate buffer (pH 7.4) containing 20 % glycerol until needed.

Rat liver microsomes (980 μg of protein) were pre-incubated at 37°C in 0.1 M potassium phosphate buffer (pH 7.4) containing a NADPH regenerating system ($NADP^+$: 0.5 mM, glucose-6-phosphate: 10mM, glucose-6-phosphate dehydrogenase: 1.0 I.U./mL, $MgCl_2$: 5 mM). The final volume of incubation was 1 mL. The reaction was initiated by the addition of 353 μM of DQHS. After incubation for 90 or 180 min, the reaction was terminated by the addition of 100 μL of 0.05M sodium hydroxide and extracted with 5 mL of methyl tert-butyl ether as described above for plasma samples. The residue, obtained after evaporating the ether, was reacted with the chosen acid using TIPS- or METS- or TCB-chloride as condensing agent, as described previously for solutions of microgram levels DQHS in dichloromethane.

Derivatization of DQHS and Its Metabolites Extracted from Rat Bile After Incubation of Arteether with the Isolated Perfused Rat Liver (IPRL)

The livers were isolated using standard techniques and perfused in a constant flow (15 mL/min) recirculating system at a controlled temperature of 37°C as previously described.[9] The perfusate (100 mL) contained 20% washed sheep red blood cells, 1% (w:v) bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and 0.1% glucose in a standard Krebs Henseleit buffer. A 5 mg/kg bolus injection of arteether (n=4) was added directly into the perfusate reservoir as a 5 mg/mL solution in ethanol/H_20 (50/50). Bile was continuously collected into preweighed vials at the time intervals 0-30, 30-60, 60-90, 90-120, 120-180, and 180-240 min after dosing. Bile was extracted with methyl t-butyl ether and the extract derivatized with 9-fluoreneacetic acid or 9-phenanthrenecarboxylic acid as described previously for plasma extracts.

Esterification of Testosterone, 6β-Hydroxytestosterone and Other Hydroxy Compounds

Testosterone (500 μg) and 6β-hydroxytestosterone (500 μg) were respectively reacted with 9-fluoreneacetic acid using either METS-chloride or TIPS-chloride as the condensing agent, following the procedure described for derivatization of microgram quantities of DQHS with carboxylic acids, using these sulfonyl chlorides as condensing agents.

Similarly, octanol and 3,4-dimethyl-2-hexanol were each esterified by the same procedure. Esterification of these compounds were also carried out using TCB-chloride in place of the sulphonyl chlorides.

RESULTS AND DISCUSSION

The acylation reaction resulting in the formation of esters and amides is one of the most important reactions in organic synthesis and analysis. In particular, trace analysis of alcohols is based almost exclusively on the acylation of alcohols and subsequent chromatographic analysis of the ester derivatives. Because of the fundamental importance of the acylation reaction, new reagents and approaches are constantly being developed for the activation of carboxylic acids under mild conditions. However, most of the reagents which have been introduced for the activation of the carboxyl group, are costly and/or not readily accessible and have found no application in the analytical

derivatization of alcohols. The esterification of alcohols for the purpose of chromatographic trace analysis is, therefore, still based on reaction with acyl chlorides or acyl nitriles or acyl anhydrides, which are generally not satisfactory as analytical reagents.

Of the principles that may be adopted for the activation of carboxylic acids, conversion to the mixed carboxylic-sulphonic anhydride is one of the most attractive for analytical esterification, because the reagents are readily available and easy to handle.

The reagents we investigated for the formation of the mixed carboxylic-sulphonic anhydrides are the hindered benzenesulphonyl chlorides 2,4,6-trimethylbenzenesulphonyl chloride (mesitylenesulphonyl chloride) and 2,4,6-triisopropylbenzenesulphonyl chloride. These hindered sulphonyl chlorides have been widely used only as condensing agents in the formatiom of internucleotide bonds,[10,11] although, there are two reports on the use of mesitylenesulphonyl chloride in the cyclisation of amino acids to macrocyclic lactams.[12,13] For comparison, 2,4,6-trichlorobenzoyl chloride (TCB-chloride) was also investigated as a condensing agent in the esterification of DQHS. TCB-chloride, a hindered benzoyl chloride which may be considered analogous to the hindered benzenesulphonyl chlorides, has been applied to the synthesis of macrocyclic lactones.[14] TCB-chloride was also reported to be useful in the preparation of esters, but it has the disadvantage of reacting with some alcohols to form the trichlorobenzoyl ester as a side-product.

Esterification of DQHS Using Mesitylenesulphonyl Chloride (METS-Chloride) or Triisopropylbenzenesulphonyl Chloride (TIPS-Chloride) as Condensing Agent in Presence of 4-Dimethylaminopyridine (DMAP)

It was found that the relatively unreactive hydroxy group of DQHS was readily acylated when a mixture of the triethylamine salt of the acid and DQHS were treated with METS-chloride (or TIPS-chloride) followed by the powerful acylation catalyst 4-dimethylaminopyridine (DMAP).

2,4,6-Trichlorobenzoyl chloride (TCB-chloride) also served well in place of the benzenesulphonyl chlorides. However, with alcohols which are more reactive than DQHS, TCB-chloride was less satisfactory because it reacted with some of the alcohol to form the trichlorobenzoyl ester. Furthermore, being a moisture sensitive liquid, TCB-chloride is a little less convenient to use than the solid and more moisture-resistant benzenesulphonyl chlorides.

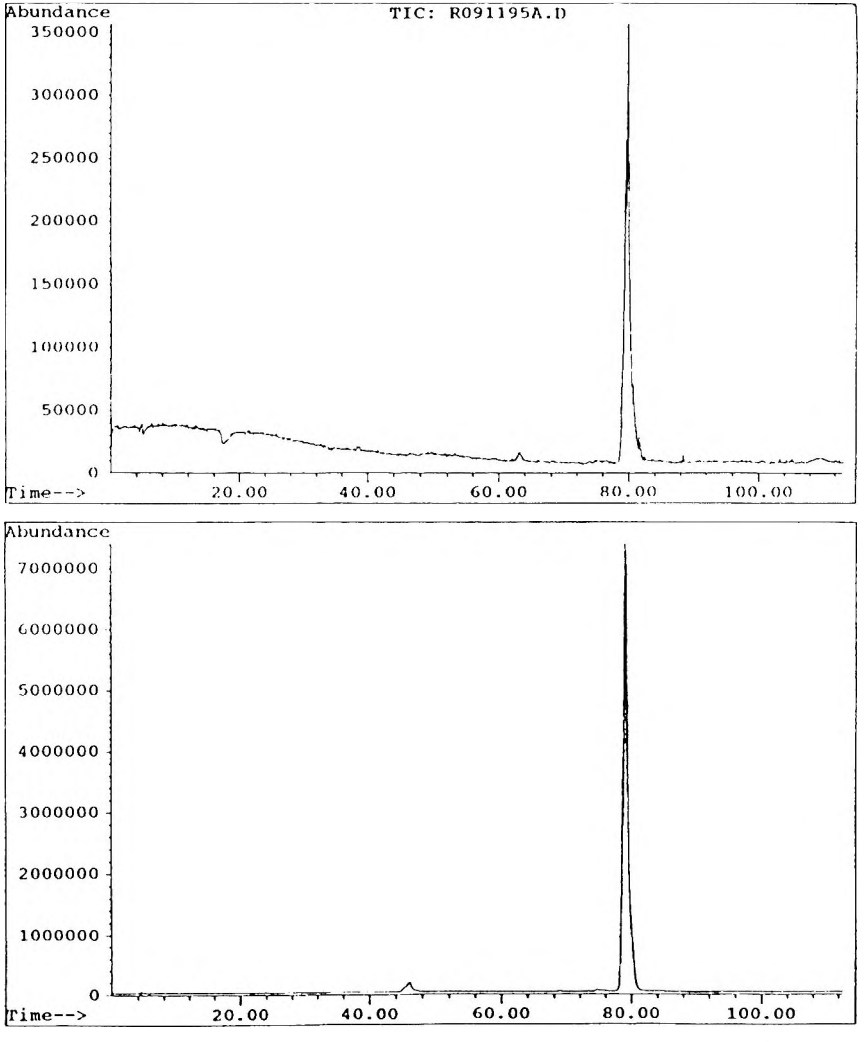


Figure 1. Total ion- and corresponding UV-chromatograms of the ester obtained from the semi-preparative reaction of DQHS with 4-biphenylcarboxylic acid /METS-chloride.

Benzenesulphonyl chloride itself, failed in this reaction, the symmetrical anhydride of the acid being the major product in most cases. Formation of the carboxylic anhydride instead of the ester, was observed in some cases, in an early report on the use of benzenesulphonyl chloride as the activating reagent in esterifications.[15]

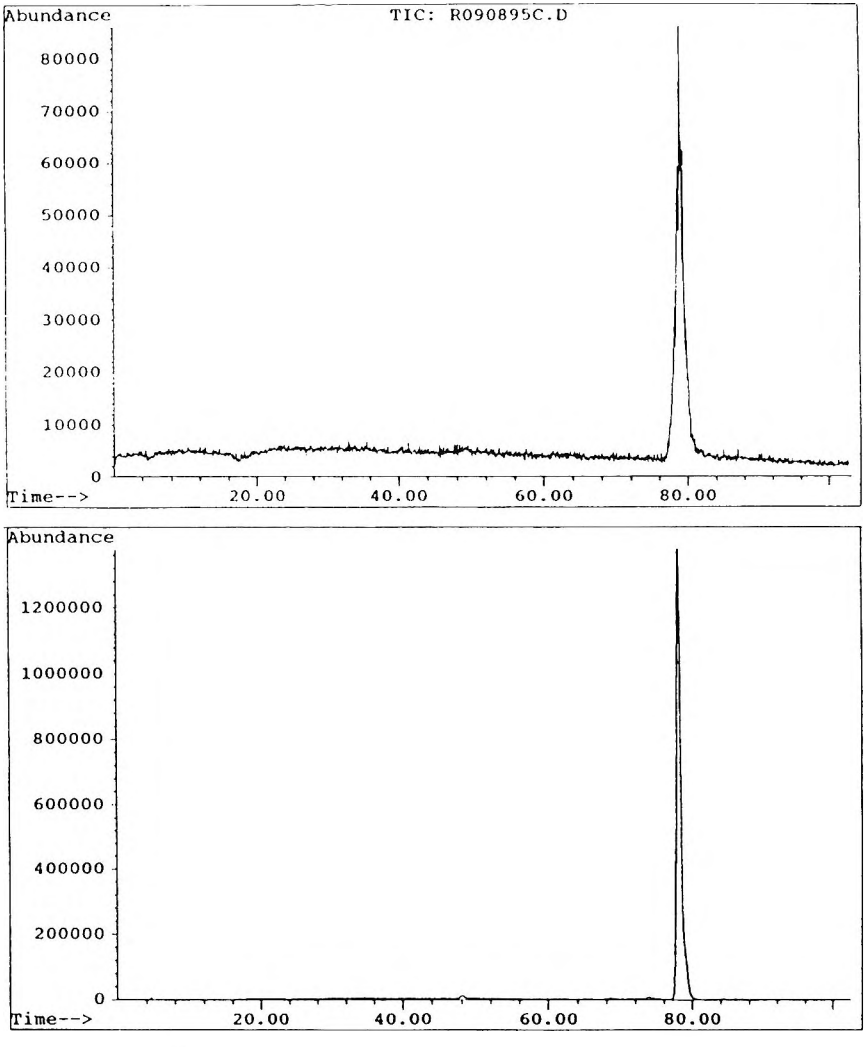
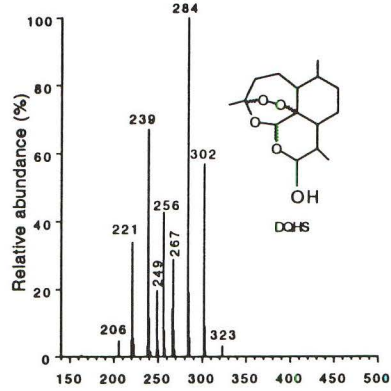
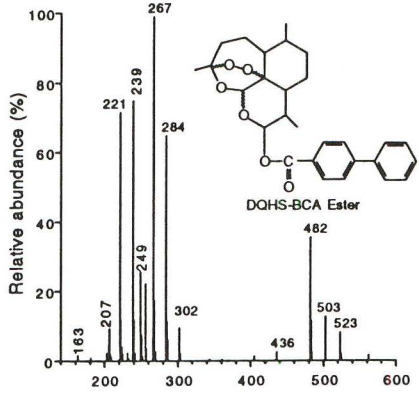
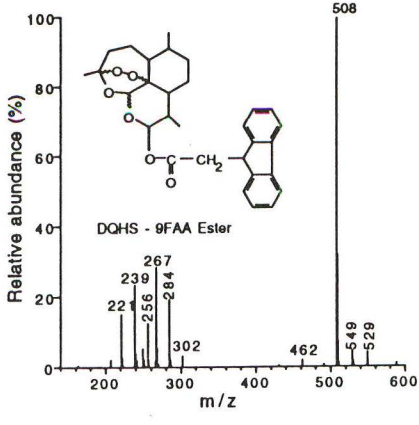


Figure 2. Total ion- and corresponding UV-chromatograms of the ester obtained from the semi-preparative reaction of DQHS with 9-fluoreneacetic acid/TIPS-chloride.

Figure 3. **(right)** Thermospray mass spectra of DQHS and its respective biphenylcarboxylic and 9-fluoreneacetic acid esters.


Relative abundance (%)
284
239
302
256
221
267
249
206
323
OH
DQHS
150 200 250 300 350 400 450 500


Relative abundance (%)
267
239
221
284
249
482
207
302
503
523
163
436
DQHS-BCA Ester
200 300 400 500 600


Relative abundance (%)
508
DQHS - 9FAA Ester
267
239
284
221
256
302
462
549
529
200 300 400 500 600
m/z

With the exception of 9-anthracenecarboxylic acid, the aromatic carboxylic acids (4-biphenylcarboxylic acid, 2-naphthoic acid, 9-phenanthrenecarboxylic acid and 1-pyrenecarboxylic acid) reacted with DQHS to give isolated yields of the pure DQHS ester of 70 % or above. The reaction was found to take place rapidly and there was no evidence of a time-dependent yield of the DQHS ester. HPLC-MS of the reaction products showed there was no unreacted DQHS, suggesting that the yields were quantitative. This is demonstrated, for example, by the total ion- and UV chromatograms of the DQHS esters of 4-biphenylcarboxylic acid and 9-fluoreneacetic acid shown in Figures 1 and 2 respectively.

The thermospray (TSP) mass spectra of the DQHS esters of the two acids are also shown in Figure 3 while the proposed fragmentation pattern is shown in Figure 4. The TSP mass spectra of the esters of DQHS are characterised by an abundance of the quasi-molecular, ammonium adduct ion ($[M + NH_4]^+$; M + 18). The spectra also exhibit M+39 and M+59 ions. The former is of obscure origin and has been observed only with these aromatic esters of DQHS, while the M+59 line is a known feature of the TSP mass spectra of DQHS and its ester and ether derivatives and is probably due to the $[M + NH_4 + CH_3CN]^+$ ion. The primary fragmentation of the [M+18] ion results in the loss of acyl group of the ester, and the rest of the spectrum is accounted for by the fragmentation pathways of the DQHS portion of the molecule. As illustrated in the spectra of the DQHS esters shown in Figure 3, the spectra of the different esters differ only in the molecular ion region while the rest of the spectra are identical to that of DQHS itself.

Using 4-biphenylcarboxylic acid as an example, the acylation of DQHS and other alcohol hydroxy groups with METS-chloride or TIPS-chloride as the carboxylic acid activating agent is illustrated in Figure 5. It is expected that when the hindered benzenesulphonyl chloride is added to a mixture of the alcohol and the triethylamine salt of the acid, the hindered benzenesulphonyl chloride would react preferentially with the carboxylic anion to form the mixed anhydride which then acylates the alcohol. The rapidity of the reaction is an indication that the mixed carboxylic-sulphonic anhydride generated *in situ* is the acylating species rather than the symmetric anhydride of the carboxylic acid.

In contrast, when the acid was first allowed to react with the hindered benzenesulphonyl chloride for about 1 h before the addition of DQHS and catalyst, there was formation of the acid anhydride and a reduced yield of the DQHS ester.

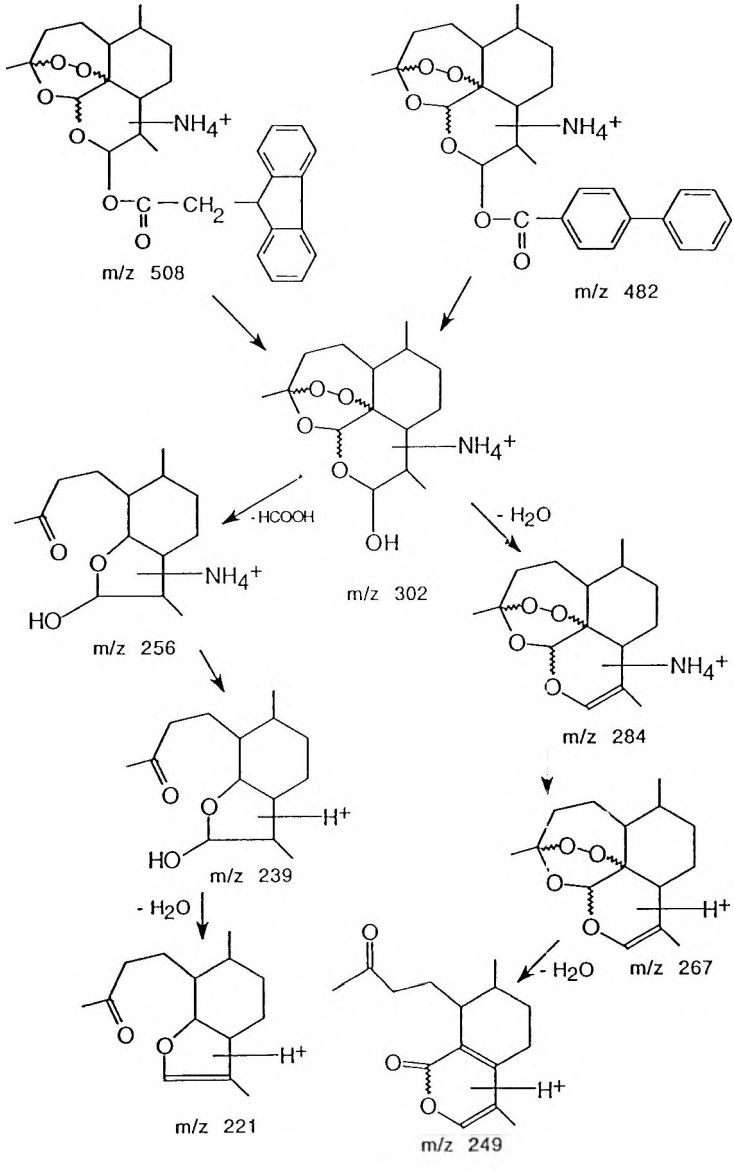


Figure 4. Thermospray mass spectra fragmentation pattern of DQHS and its respective biphenylcarboxylic and 9-fluoreneacetic acid esters.

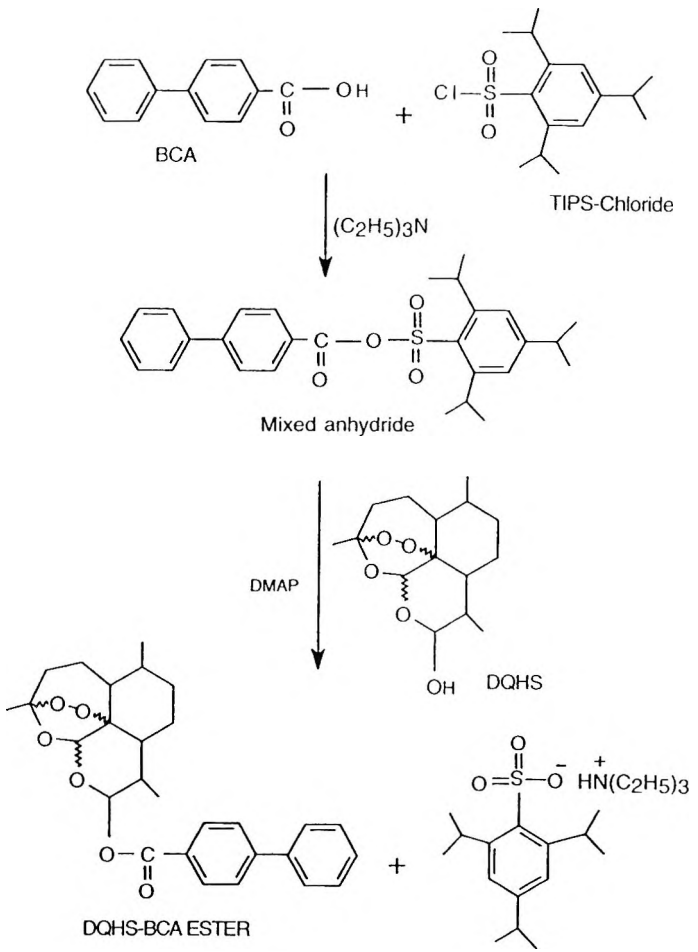


Figure 5. Illustration of the esterification of DQHS by the mixed anhydride method.

With 9-anthracenecarboxylic acid, the yield of the DQHS ester was poor in an initial attempt to react equivalent quantities of the acid with DQHS. It was found that the acid was converted, in good yield, to the symmetric anhydride, which in this case must have been the predominant acylating agent. When two equivalents of acid was used, the DQHS ester of 9-anthracenecarboxylic acid was obtained in an isolated yield of 48 %. Some of the ester must have been lost when the reaction product was warmed with alkali to destroy the unreacted 9-anthracenecarboxylic anhydride.

Similarly, the yields of the esters of DQHS with the arylacetic acids were poor. The poor performance of this class of acids in this reaction, may have been due to the instability of their mixed carboxylic-sulphonic anhydrides, probably brought about by the lability of their benzyl group. In particular, the reaction failed completely with 9-fluorenecarboxylic acid, which has the even more labile fluorenyl group adjacent to the carboxylic function. The instability of the mixed anhydrides of these acids also shows in their tendency to form the less reactive symmetric anhydrides which may be thought of as resulting from attack of the carboxylate anion released from the initial decomposition of the mixed anhydride with the intact mixed anhydride.

In contrast, 9-fluoreneacetic acid, in which the fluorenyl group is further removed from the carboxylic group, was found to be one of the best acids for the analytical esterification of DQHS by the present mixed anhydride approach.

7-(Carboxymethoxy)-4-methylcoumarin, which has been proposed as a fluorescent reagent for the precolumn derivatization of hydroxy compounds,[16] was also found to react readily with DQHS, using either TIPS- or METS-chloride as the carboxylic acid activating agent, rather than conversion of the acid to the acid chloride as originally reported.

Detection of DQHS and Its Metabolites Extracted from Biological Samples

To date, only the diacetyldihydrofluorescein (DADF) ester of DQHS has been proposed for the HPLC/UV analysis of the compound.[17,18] The derivatization of DQHS with diacetyldihydrofluorescein was carried out using dicylohexylcarbodiimide (DCC) as the carboxylic acid activating agent. The DADF ester was obtained from reaction with pure DQHS, but no attempt was made to apply this reaction to the detection of DQHS extracted from biological samples. Our attempt to apply this reaction to the derivatization of DQHS extracted from biological samples was unsuccessful. Irrespective of the acid used, we found the corresponding N-acyl dicyclohexylurea was formed as a major side-product which interfered seriously in subsequent chromatography of the DQHS ester derivative. Besides, dicylohexylurea which is also another side product normally formed during DCC-catalysed acylations, interfered with the recovery of the derivative because of its insolubility in common organic solvents.

In the present work, microgram to subnanogram levels of DQHS extracted from biological fluids were readily detected by HPLC-UV after acylation with aromatic carboxylic acids, using either TIPS- or METS-chloride as the condensing agent. A chromatogram of a derivatized plasma extract of DQHS

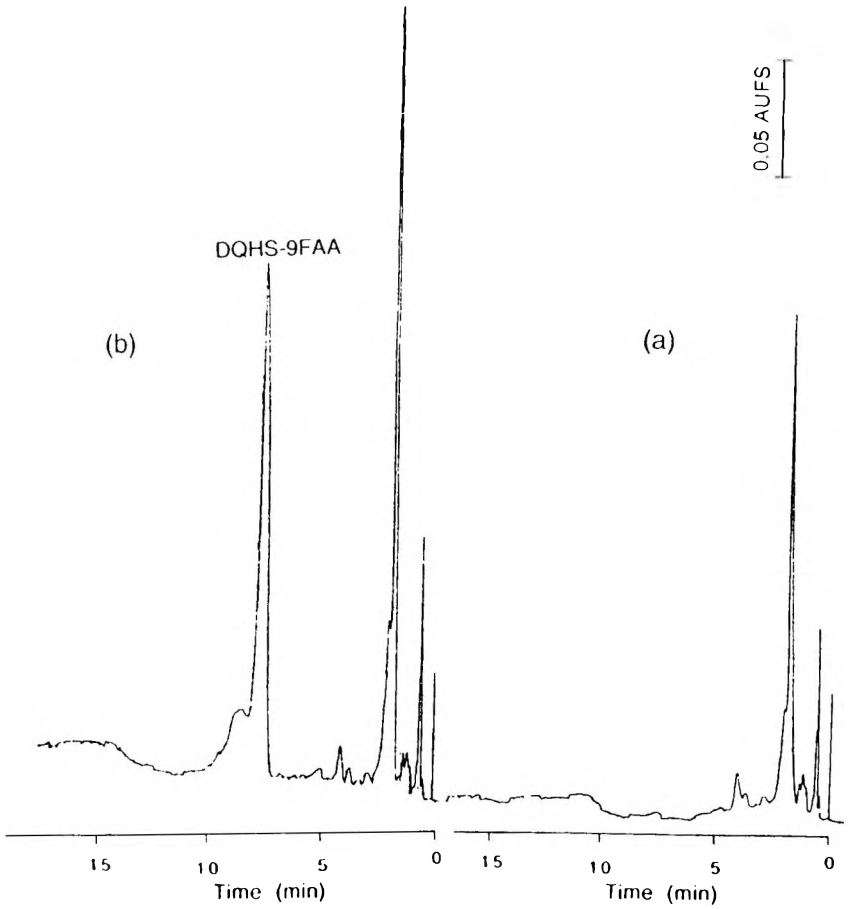


Figure 6. HPLC chromatograms of (a) blank plasma extract and (b) DQHS extracted from plasma and derivatized with 9-fluoreneacetic acid/TIPS-chloride.

is shown in Figure 6. Chromatograms of DQHS and its metabolites extracted from rat liver microsomes and then acylated by the mixed anhydride method are shown in Figures 7-9. Arteether, the ethyl ether analogue of DQHS, undergoes metabolic deethylation to DQHS. As shown in Figure 10, the present derivatization method has also been successfully applied to the HPLC-UV detection of DQHS and another hydroxylated metabolite (AEM) formed by the metabolism of arteether in the isolated perfused rat liver (IPRL).

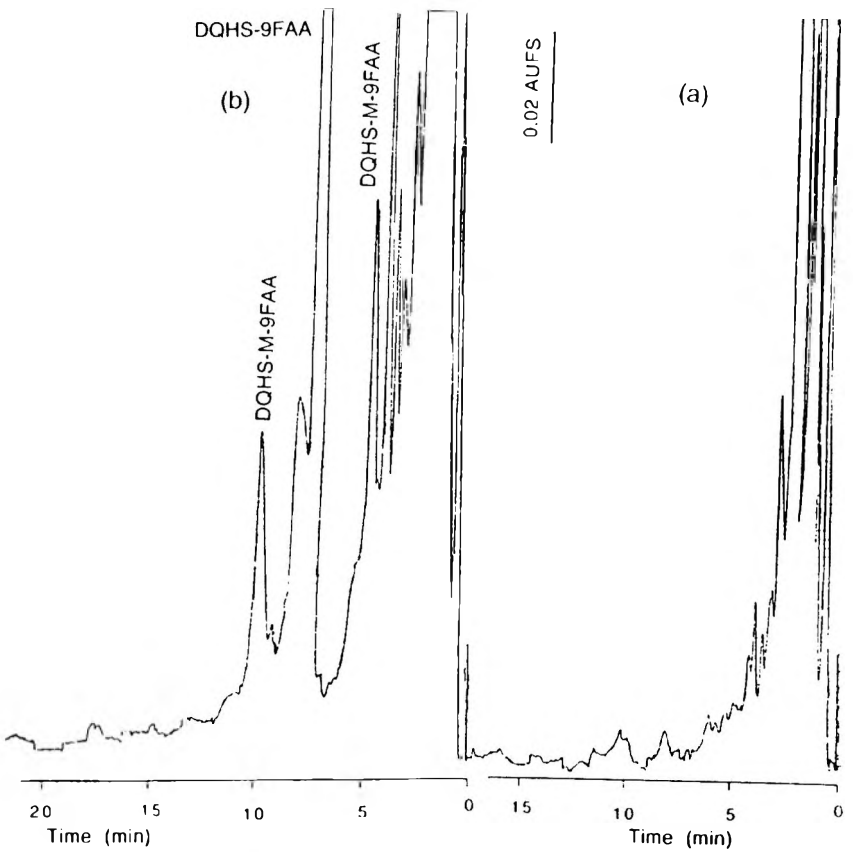


Figure 7. HPLC chromatograms of (a) extract of blank rat liver microsomes and (b) DQHS and its metabolites (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 180 min) and derivatized with 9-fluoreneacetic acid/METS-chloride.

Esterification of Testosterone, 6β-Hydroxy-Testosterone and Other Hydroxy Compounds

To further demonstrate the general applicability of the present derivatization method, the acylation of testosterone, and 6β-hydroxy-testosterone, by the mixed carboxylic-sulphonic anhydride method, was investigated. The best available approach to the derivatization of the hydroxy group of hydroxysteroids is based on acylation with acyl nitriles.[19,20,21] A serious drawback to acylation with acyl nitriles is the pronounced sensitivity of

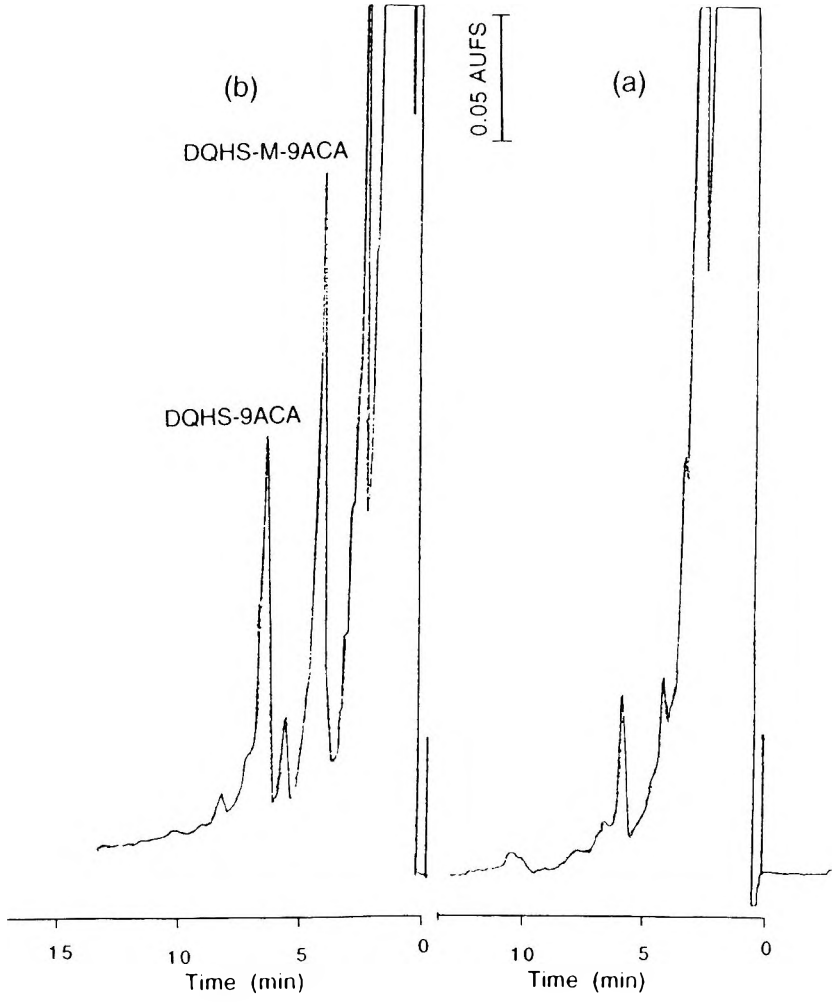


Figure 8. HPLC chromatograms of (a) extract of blank rat liver microsomes and (b) DQHS and its metabolite (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 90 min) and derivatized with 9-anthracenecarboxylic acid/TIPS-chloride.

the reaction to steric hindrance in the neighborhood of the hydroxy groups of hydroxysteroids.[22] For examples, testosterone, which possesses a quasi-equitorial hydroxy group reacts poorly with 4-dimethylamino-1-naphthoyl nitrile, while both the 11β and 17α hydroxy groups of cortisol are inert towards this reagent.

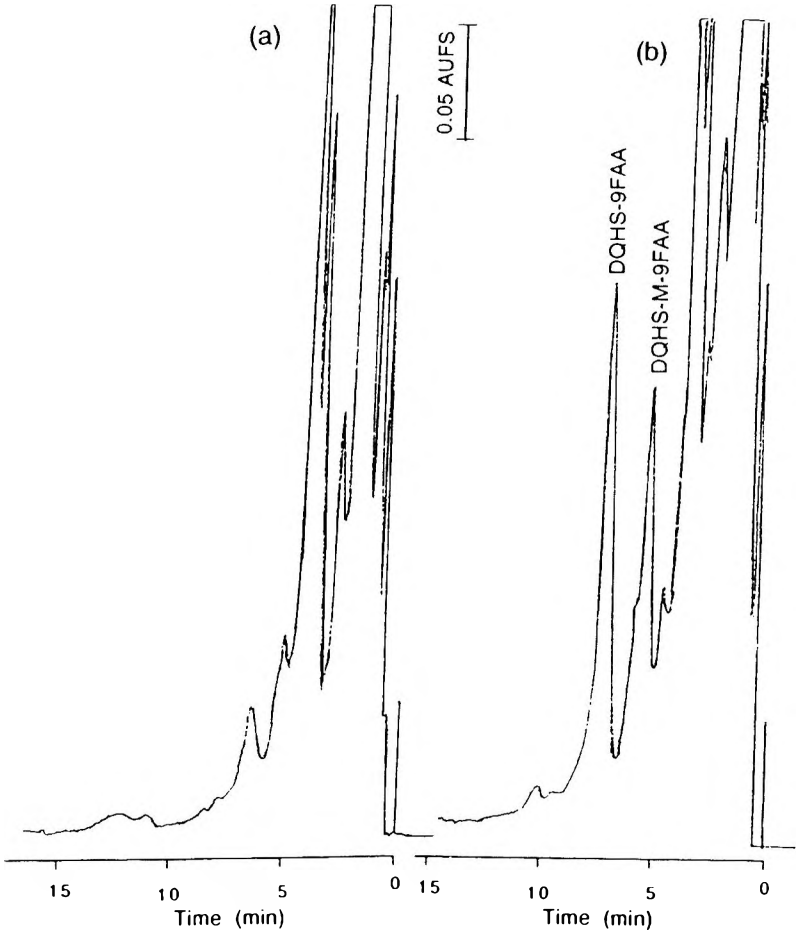


Figure 9. HPLC chromatograms: (a) extract of blank rat liver microsomes; (b) DQHS and its metabolite (DQHS-M) extracted from rat liver microsomes (after incubation with DQHS for 90 min) and derivatized with 9-fluoreneacetic acid/TCB-chloride.

Quantitative acylation with acyl nitriles is achieved only for primary hydroxy groups, while only yields of 30 % or less are achieved with secondary hydroxy groups, and teriary hydroxy groups are unreactive. Esterification of testosterone and 6β-hydroxytestosterone by the mixed anhydride method was, therefore, investigated because of the reported poor reactivity of the 6β- and 17β-hydroxy groups of these compounds with the acyl nitriles, such as benzoyl

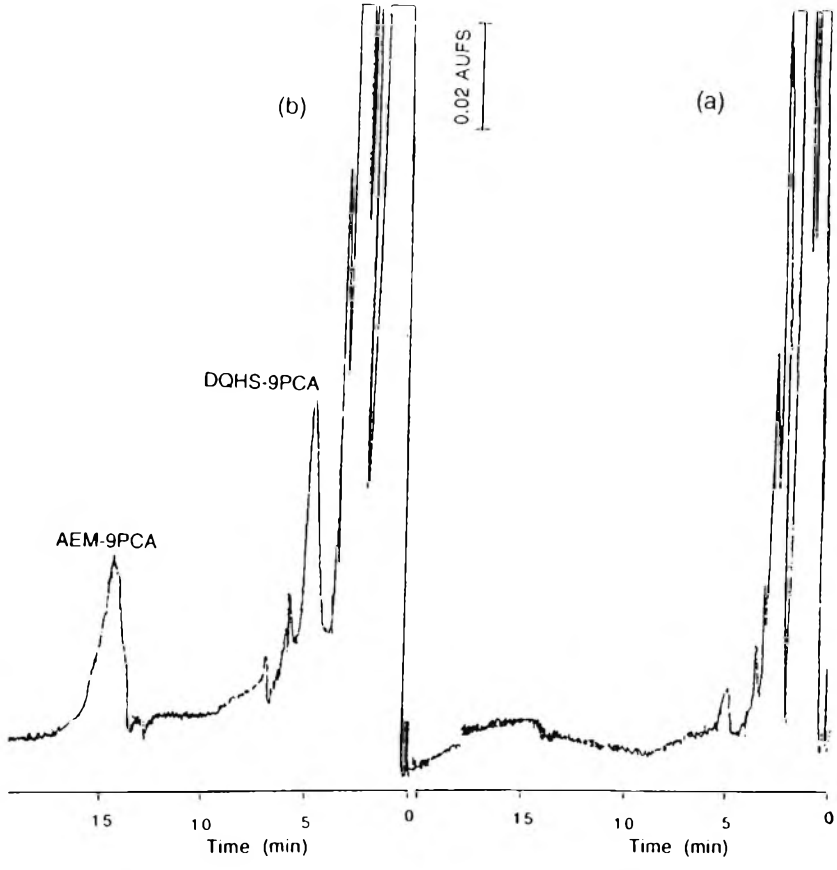


Figure 10. HPLC chromatograms of (a) extract of blank rat liver bile and (b) metabolites of arteether (DQHS and AEM) extracted from rat liver bile and derivatized with 9-phenanthrenecarboxylic acid/TIPS-chloride.

nitrile, pyrene-1-carbonitrile, anthracene-1- and 9-carbonitrile, which have been proposed for the derivatization of hydroxysteroids. Both testosterone and 6β-hydroxytestosterone were quantitatively acylated by the present mixed anhydride method. A chromatogram of the ester derivatives of testosterone and 6β-hydroxytestosterone are shown in Figure 11. Both of the 6β and 17β-hydroxy groups in 6β-hydroxytestosterone were quantitatively acylated. Similarly, the acylation of 1-octanol and the secondary hydroxy group of 3,4-dimethyl-2-hexanol by the carboxylic-sulphonic mixed anhydride method was quantitative.

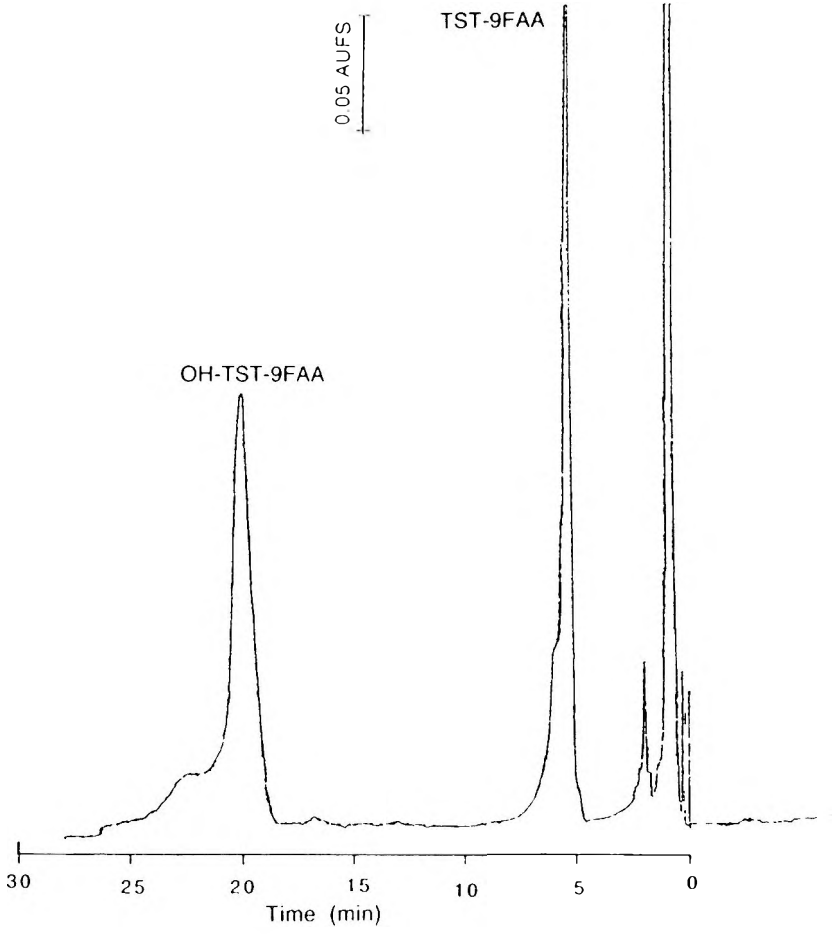


Figure 11. HPLC chromatogram of the 9-fluoreneacetic acid esters of testosterone (TST-9FAA) and hydroxytestosterone (OH-TST-9FAA).

CONCLUSION

Rapid, direct derivatization of alcohols with UV/fluorescent carboxylic acids is readily achieved when the hindered benzenesulphonyl chlorides (TIPS- or METS-chloride) are used as condensing agent in combination with DMAP as catalyst. This is a very convenient procedure which is superior to previous approaches that require the prior conversion of the carboxylic acid to the acid chloride or acyl nitrile or acyl azide or acid anhydride. The obvious lack of

reaction between alcohols and the hindered benzenesulphonyl chlorides, under the present conditions, suggests that this procedure may also be adapted for the derivatization of carboxylic acids with UV/fluorescent alcohols for HPLC-UV/fluorescence analysis.

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DETERMINATION OF ETHOSUXIMIDE IN PLASMA BY DERIVATIZATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and sensitive liquid chromatographic method is described for the determination of ethosuximide in plasma as a highly sensitive derivative. Ethosuximide in plasma, after separation with isopropanol extraction, was derivatized with strong chromophore reagent, 4-bromomethyl-7-methoxy-coumarin. The resulting derivatives were separated on a Nova-Pak C18 column with water-acetonitrile-methanol(60:20:20, v/v) as the mobile phase. The linear range for the determination of ethosuximide in spiked plasma was over 2-40 nmol. The limit of detection for ethosuximide was about 7.0 ± 1.2 pmol per 20 μL injection (S/N= 5). The intraday relative standard deviation (n = 6) and the interday relative standard deviation (n = 12) were all less than 2.5% for ethosuximide. The recovery for ethosuximide in plasma was greater than 96%.

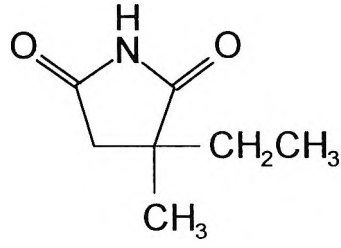

Figure 1. Chemical structure of ethosuximide.

INTRODUCTION

Ethosuximide (3 - ethyl - 3- methylpyrrolidine - 2,5-dione, ESM) (Fig. 1) is an antiepileptic drug and it is widely used for treatment of absence seizures.[1-3] ESM therapy is long term therapy of at least two years.[4] Physiologic functions may change with time, especially in the infant and child. The monitoring of ESM in plasma levels is required for clinic treatment to improve the seizure control and reduce the toxicity from overdosage. Therefore, a sensitive and specific method for trace analysis of ESM in plasma is very essential.

A variety of methods including immunoassay,[5-7] titration,[8] gas chromatography,[9-12] and high performance liquid chromatography,[13-15] have been applied to the analysis of ESM. That ESM has no strong chromophore or conjugated system, except that of dione group, as shown in Fig.1, made it necessary to choose the lower UV region at 204 nm or 207 nm for detection in previous HPLC methods,[13-14] and the strong background absorption of a mobile phase in the lower UV region ensued. In order to enhance the sensitivity, we have previously reported,[15] a detector-oriented derivatization HPLC method for determination of ESM in capsules as the derivative of 4-bromomethyl-7-methoxycoumarin (BrMmC). In this paper, a sensitive and specific HPLC method is described for the trace analysis of ESM in human plasma.

METHODS

Reagents and Solution

ESM (Sigma, St. Louis MO, USA), BrMmC and 2-2'-dinitrobiphenyl (Aldrich, Milwaukee, WI, USA), potassium carbonate and potassium

hydroxide (E. Merck, Darmstadt, Germany), acetonitrile , isopropanol and other reagents were of analytical-reagent grade. Solutions of ESM at various concentrations were prepared by dissolving a suitable amount of ESM in deionized water. Solutions of 2-2'-dinitrobiphenyl and BrMmC were prepared in acetonitrile.

HPLC Conditions

A Waters-Millipore LC system with a U6K injector and a Model 486 UV-VIS detector was used. A Nova-Pak C18 column(150 x 3.9 mm I.D., 4 μm) and a mixed solvent of water-acetonitrile-methanol (60:20:20, v/v) at a flow-rate of 1.3 mL/min were used. The column eluate was monitored at 320 nm. Pretreatment of the solvents with a vacuum filter for degassing was performed.

Sample Preparation Procedures

A 0.4-mL volume of plasma was pipetted into a 10-mL glass-stoppered test tube, and 100 μL containing various amount of ethosuximide aqueous solution was added to each tube. The tubes were mixed for 10s. 1.0 mL of isopropanol was added and mixed by vortexing for 30s. The tubes were centrifuged (1800g) for 5 min. The 1.0 mL of supernatant organic layer from each tube was transferred to a 10-mL glass-stoppered test tube. Then 0.3 mL of 10^{-2} M NaOH aqueous solution was added and evaporated to dryness under a slow stream of nitrogen by heating in a water bath at 50°C.

The dried residue was derivatized by adding 200 μL of BrMmC (2.5 mM) acetonitrile solution, 0.3 mL 2,2'-dinitrobiphenyl (I.S.), and 100 mg of potassium carbonate. The reaction mixture was shaken for 1.5 h at 70°C in a thermostated water bath.

At the end of the reaction, a 15μL aliquot of the solution was injected into the HPLC system.

Precision and Accuracy Test

The reproducibility and reliability tests of ESM from spiked plasma were determined by extracting 0.5 mL aliquots of plasma sample containing ESM at three different levels (5.0, 10.0 and 40.0 nmol).

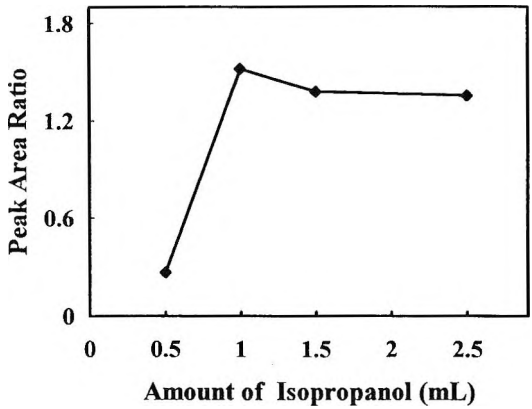

Figure 2. Effect of amount of isopropanol on the extraction of ESM from human plasma.

RESULTS AND DISCUSSION

For the optimization of the conditions for liquid extraction of ESM (40 nmol) from spiked plasma and also conditions for the derivatization of the extracted ESM, several related parameters, including the volume of extraction solvent, amount of derivatizing agent, base catalyst and reaction time were studied. The effect of the tested parameters on the extraction/derivatization of ESM were evaluated by the peak area ratio of the resulting derivative to the I.S.

Effect of the Volume of Extraction Solvent

Liquid extraction[13] and solid phase[12] methods were reported to extract ESM from human plasma, but the recoveries were not satisfied. This is likely due to its high water solubility. For increasing the extraction efficiency, the higher polar organic solvent, isopropranol, was used as an extraction solvent. The effect of various amounts of isopropanol (0.5, 1.0, 1.5 and 2.5 mL) on the liquid extraction of ESM from plasma was studied. Because of the low melting point (64-65°C) of ESM, the possible loss under purge nitrogen stream should be considered. 0.3 mL of 10^{-2} M NaOH solution was added to the isopropanol extract to make ESM a sodium salt before drying. The effect of the amounts of isopropanol on the extraction of ESM from human plasma is presented in Fig.2. 1 mL of isopropanol is enough for extraction of ESM from spiked plasma.

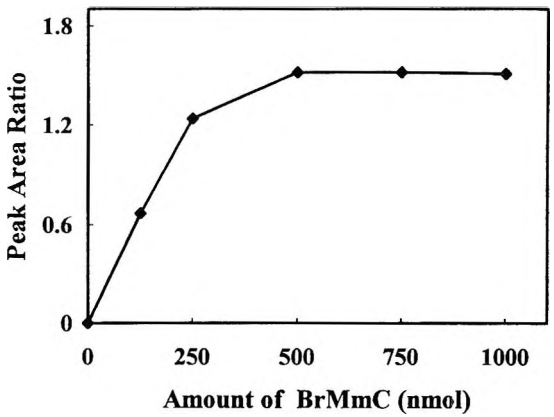

Figure 3. Effect of amount of BrMmC on the formation of the derivative of ESM extracted from human plasma.

Effect of Amount of Derivatizing Agent

The amount of BrMmC required for the derivatization of ESM isolated from spiked plasma (40 nmol ESM) to a plateau formation of the derivative is shown in Fig. 3. 500 nmol of BrMmC was needed for derivatization of the ESM extracted from plasma.

Effect of Amount of Catalyst

The effect of the amount of potassium carbonate(30-50 mesh) on derivatization is illustrated in Fig. 4. In the absence of the base catalyst in the reaction system, a small amount of ESM derivative was detected. Adding the potassium carbonate as base catalyst, the ESM derivative obviously increased. A suitable amount of potassium carbonate (about 100 mg) was selected for the derivatization of plasma ESM. After the derivatization reaction, the supernatant was directly introduced into the HPLC system.

Effect of Reaction Time

The effect of reaction time at 70°C was examined for derivatization of ESM under the optimum conditions. The results were presented in Fig. 5. 1.5 h was needed to reach the equilibrium.

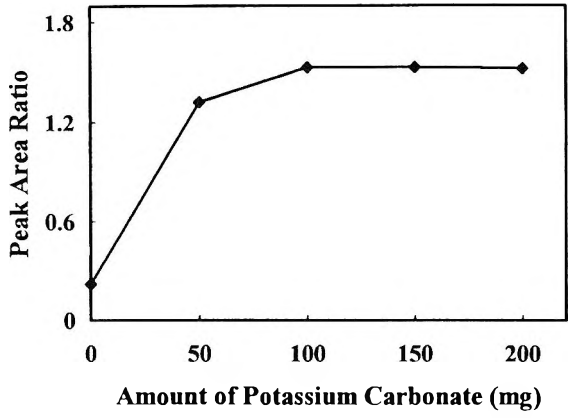

Figure 4. Effect of the amount of potassium carbonate on the formation of the derivative of ESM extracted from human plasma.

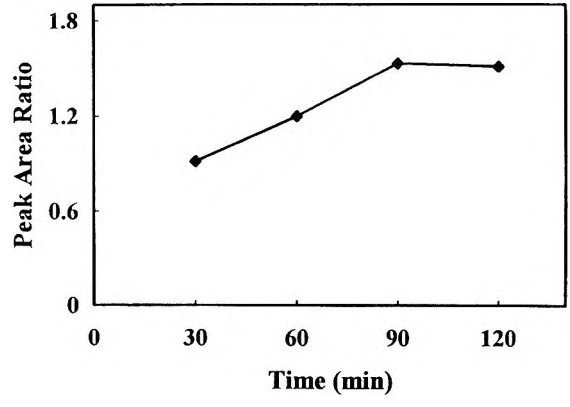

Figure 5. Effect of reaction time on the formation of the derivative of ESM extracted from human plasma.

Analytical Calibration

To evaluate the quantitative applicability of the method, five different amounts of ESM spiked in plasma over the range of 2-40 nmol were analyzed. The linear regression equation obtained were $y = (-0.052 \pm 0.003) + (0.040 \pm 0.001)\ x$ for intraday assay ($n = 6$, $r = 0.999$); $y = (-0.036 \pm 0.009) + (0.038$

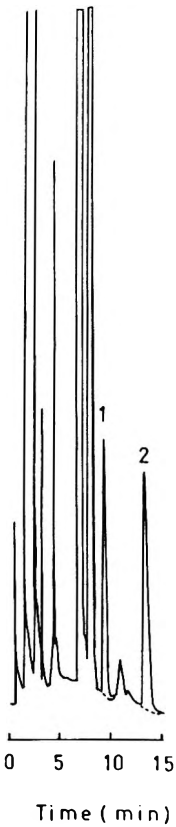

Figure 6. Composite liquid chromatogram of reagent blank (dotted line) and the derivative of ESM extracted from spiked human plasma (solid line).

± 0.002)x for interday assay (n = 12, r = 0.999); y is the peak-area ratio of ESM to I.S., x is the amount of ESM (in nanomoles), and r is the correlation coefficient. The data indicate good linearity of the method. The detection limit (signal to noise ratio = 5) of ESM was 7.0 ± 1.2 pmol in 20 μL of injection sample. A typical chromatogram for the analysis of ESM extracted from plasma is illustrated in Fig 6. Peak 1 and peak 2 represented the ESM derivative and the I.S., respectively. There was no interference in the resolution of the peak of ESM derivative from that of the reagent blank. The structure of the ESM derivative was performed by JEOL JMS-HX mass spectrometry with fast atom bombardment of xenon as the ionization mode and an acceleration energy of 10 kV . The quasi-molecular ion of the derivative of

Table 1

Precision and Accuracy of the Analysis of ESM Spiked in Human Plasma

Amount Range (nmol)	Amount Found (nmol)	R.S.D. (%)	Recovery (%)
Intraday*			
40	40.69 ± 0.34	0.85	101.7
10	9.94 ± 0.19	1.87	99.4
5	4.89 ± 0.09	1.84	97.8
Interday**			
40	40.11 ± 0.42	1.05	100.3
10	9.87 ± 0.23	2.32	98.7
5	4.95 ± 0.12	2.51	99.0

* Intraday assay variance was calculated from the assay values of prepared standards on a single day (n = 6).

**Interday assay variance was calculated from the assay values of prepared standards on twelve consecutive days (n = 12).

ESM was found at m/z = 330 (MH^+). Furthermore, by comparing the ^1H-NMR($CDCl_3$) spectrum of the ESM derivative with that of underivatized ESM, a chemical shift about δ 8.96 of imide proton disappears. It is due to the alkylation of ESM on imide nitrogen atom. The structure of the ESM derivative of peak 1 in Fig 6 is briefly identified as 4-[1-(3-ethyl-3-methyl 2,5-dioxo)pyrrolidinyl]methyl-7-methoxy coumarin.

Precision and Accuracy

The reproducibility and reliability of the method for determination of ESM were studied on the plasma spiked at three different levels of the ESM and evaluated as relative standard deviation (R.S.D.) and relative recovery, respectively. As shown in Table 1, the precision of the method for ESM is less than 3% (R.S.D.) for both intraday and interday analyses. Based on a calibration graph constructed from plasma spiked with different amounts of ESM over the range of 2-40 nmol, as shown in Table 2, the relative recovery of the method for ESM is more than 95%.

Table 2

The Recoveries of ESM Extracted from Human Plasma

Sample	Amount Spiked (nmol)	Amount Found* (nmol)	Recovery (%)
1	----	----	----
	5.00	4.85 ± 0.03	96.93
	15.00	14.95 ± 0.25	99.68
	30.00	30.01 ± 0.22	100.05
2	----	----	----
	5.00	4.83 ± 0.16	96.60
	15.00	14.59 ± 0.11	97.27
	30.00	29.18 ± 0.46	97.27
3	----	----	----
	5.00	4.86 ± 0.22	97.20
	15.00	14.41 ± 0.10	96.07
	30.00	29.05 ± 0.14	96.83

*Mean ± S.D. of triplicate analyses.

In our previous paper,[15] the specificity of the method was studied by spiking ESM with other anticonvulsants including valproic acid, carbamazepine, primidone, acetazolamide and phenobarbital. The ESM derivative could be resolved from those of the other drugs, indicating that other anticonvulsants did not interfere with the HPLC analysis of ESM. As a consequence, the proposed method is specific and feasible for the analysis of ESM in plasma for biological study or therapeutic drug monitor.

In conclusion, the present study showed that ESM extracted from plasma may be readily derivatized by 4-bromomethyl-7-methoxy coumarin. The proposed method is specific and feasible for analysis of ESM in plasma for biological or clinic interest.

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DETERMINATION OF 4-CHLOROANILINE AND 4-CHLOROPHEHYL UREA IN HONEY BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A liquid chromatographic technique with amperometric detection was developed for determining diflubenzuron metabolites, 4-chlorophenyl urea and 4-chloroaniline, using a Spherisorb C_{18} column 5 μm (4.1 x 250 mm). A mobile phase of acetonitrile / citrate-disodium hydrogenphosphate buffer, 30:70, at pH=7.0, was pumped at a flow rate of 1 mL min^{-1}. The amperometric detector, equipped with a glassy carbon working electrode was operated at 1100 mV vs Ag/AgCl. The method showed a limit of detection of 0.46 μg l^{-1} for 4-chlorophenyl urea and 0.20 μg l^{-1} for 4-chloroaniline. Reproducibility in terms of relative standard deviation ranged between 1.24 and 4.50 % for the 4-chlorophenyl urea and 1.60 and 3.93% for the 4-chloroaniline. The method was applied to the determination of both metabolites in honey. The prior extraction of the two substances from honey was established using different solid-liquid extraction phases.

INTRODUCTION

Diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea)], is a chemical used to control numerous forest and agricultural pests.[1-3] It acts through disruption of the synthesis of new cuticle at moults and is, therefore, arthropod specific.

Generally it only has short-term effects against sensitive nontargets and it is not persistent in habitats where it would be applied. However, it is degraded quickly in the environment to the metabolites 4-chlorophenyl urea and 4-chloroaniline. These compounds are classified as mutagens by the National Cancer Institute and the Cancer Assessment Group of the U. S. Environmental Protection Agency.

The environmental fate of diflubenzuron has been studied in the laboratory and in field.[4-7] Several of these studies have shown that diflubenzuron normally persists for only about 2 to 3 days. However, persistence is related to water quality and climatic conditions, and low concentrations can persist for 7 weeks or longer in pond waters under low pH and low temperatures. The major degradation products of diflubenzuron are 2,6-diflurobenzamide, 2,6-diflurobenzoic acid, 4-chlorophenyl urea and 4-chloroaniline.

Determination of phenylurea herbicides and their metabolites has been mainly performed by gas chromatography (GC) and liquid chromatography (LC). Some phenylureas and anilines cannot be analyzed by GC because of their thermolability and polar character.[8] In these cases the derivatization before injection has been used.[9-11] Bromination[12] or acylation with heptafluorobutyric anhydride,[13] both of which enable sensitive determination by electron capture detection (ECD), have been used in GC separation. More recently, attempts have been made to analyze substituted anilines by direct GC without derivatization: Boër et al.[14] used cold column injection for analysis of thirteen substituted anilines in environmental and drinking waters by direct capillary GC with nitrogen-phosphorus detection (NPD) on a DB-Wax column. Phenylurea herbicides interfered with the determination when present at relatively high levels.

For such reasons HPLC is the more frequently used method. Most authors[15-18] have employed reversed phase chromatography and UV detection but, better sensitivity and selectivity can be achieved with the electrochemical detector (ED).

Hatrik[19] used tandem UV and ED for the simultaneous determination of phenylurea herbicides which have been widely used in agriculture and for the determination of their corresponding degradation products in water samples.

Due to aerial treatments used to apply the diflubenzuron in big forest areas, some close apiaries could be affected. Studies with other pesticides like malathion and dimethoate have been made.[20] For this reason, quality control of the honey is necessary. Most authors use GC with different detection modes for determination of pesticides in honey.[21,22] However, some have used HPLC-UV for determination of these compounds. Thus, cymazole and residues were determined with limit of determination of 0.01 mg l^{-1}.[23]

However, the presence of diflubenzuron major metabolite, 4-chlorophenyl urea, was detected sporadically at minor levels.[24] In this work, electrochemical detection has been used for the simultaneous determination of this substance and its degradation product, 4-chloroaniline. Diflubenzuron and metabolites determination methods in honey were not found, even though different toxicology studies have been carried out.[25]

MATERIALS

Apparatus

The instruments used for the cyclic voltammetry studies were a Metrohm (Metrohm Ltd. Herisau, SW) E-506 Polarecord, a Model E-612 scanner, a VA-663 unit stand and an Yokogawa (Yokogawa Europe, BV, Amersfoort ND) X-Y recorder. A glassy carbon (GC) Metrohm was used as the working electrode, a platinum electrode as the auxiliary, and an Ag/AgCl electrode as the reference.

The isocratic HPLC system used was a Hewlett Packard (Palo Alto, CA, USA) series 1050 pump, and a Waters (Waters Assoc. Milford M.A. USA) model 460 amperometric detector with a 2.5 μL microcell, a GC model 41215 electrode and a Ag/AgCl reference electrode. The chromatograms were recorded using a Waters Model 745 data module.

The column was a 25.0 cm x 4.0 mm, I.D. 5 mm Spherisorb C18 (Tracer Anal. Barcelona SP). A centrifuge Selecta (Barcelona SP) Model Centronic and a Selecta Model Vibromatic-384 were used in the extraction procedure.

A Vac-Elut sample preconcentration system (Varian, Harbor City CA. USA) for solid-phase extraction was used in the purification procedure. In the solid phase extraction three types of Sep-Pack cartridges (Waters Associates, Milford, MA., USA) were used: florisil, cyanopropyl and aminopropyl containing 500 mg of sorbent.

Chemicals and Solvents

The standards 4-chlorophenyl urea and 4-chloroaniline were supplied by the Laboratory of Dr. Ehrenstorfer GmbH (Angsburg, Germany) with a purity of 99.0 and 99.7% respectively. The internal standard todralazine was obtained from Sigma (St. Louis, MO, USA)

Standard stock solutions were prepared by dissolution of 10 mg of 4-chloroaniline and 4-chlorophenyl urea in 100 mL of acetonitrile.

All chemicals were of analytical-reagent grade. The solvents used in the mobile phase were of HPLC-grade and were obtained from BDH (Poole, Dorser, UK). Helium was of oxygen-free grade (Air Liquide, Madrid, SP).

HPLC-grade water was prepared by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA, USA). All solvents and samples were filtered through 0.22 μm Millipore membrane filters before injection.

METHODS

Chromatographic Conditions

The supporting electrolyte used in the mobile phase was prepared by mixing 5.0×10^{-3} mol l^{-1} citric acid and 1.0×10^{-2} mol l^{-1} disodium hydrogen phosphate to yield the desired pH. The resulting buffer solution was diluted with acetonitrile to obtain the desired concentrations.

The mobil phase was sonicated for 15 min and the eluent reservoir was purged with helium before use. The column head-pressure was 1732 psi at a flow rate of 1.0 mL min^{-1}. The system was operated at room temperature and injection volume was 20 μL.

Preparation of Spiked Samples

For recovery determinations, samples of honey, 10 g, were spiked by the addition of an appropriate amount of a standard stock solution (1 μg mL^{-1} in acetonitrile). The spiked samples were allowed to stand for a few minutes before extraction to allow the spike solution to penetrate the sample. The efficiency of the method was determined by fortifying control samples over a concentration of 10 to 60 mg Kg^{-1}.

Extraction and Clean-up

A 10 g sample of honey heated to 30°C was extracted in a capped centrifuge tube with 5 mL of acetonitrile shaken for 10 min. This mixture was centrifuged at 3000 rpm (1000 g) for 5 min and filtered by suction.

The three different solid-liquid extraction phases (aminopropyl, cyanopropyl and fluorisil) were washed with 10 mL of acetonitrile for activation of the sorbent. The sample extract was passed through the cartridge by negative pressure of 7 in of Hg with a Vac-Elut station.

A 200 μL of 1 μg mL^{-1} solution of internal standard todralazine was added to 1.0 mL of the liquid that passes through the cartridge and contains 4-chlorophenyl urea and 4-chloroaniline, and then aliquots of 20 μL were injected into the HPLC system.

RESULTS AND DISCUSSION

The presence of oxidizable amine groups on the glassy carbon electrode can be used for electroanalytical determination of 4-chloroaniline, 4-chlorophenyl urea and todralazine

Figure 1 shows the current-potential curves obtained for these substances with cyclic voltammetry. On the anodic scan at pH=7.0, the compounds exhibited broad irreversible oxidation waves at different potentials: 570, 438 and 462 mV, respectively. This showed that the three substances can be jointly determined by HPLC-ED chosing an appropriate working potential that assures the oxidation of all the groups.

Hydrodynamic voltammograms were used to determine the optimum pH, and potential conditions for the electrochemical detection. The graphs were derived by injecting 20 ng of 4-chlorophenil urea, 10 ng of 4-chloroaniline and

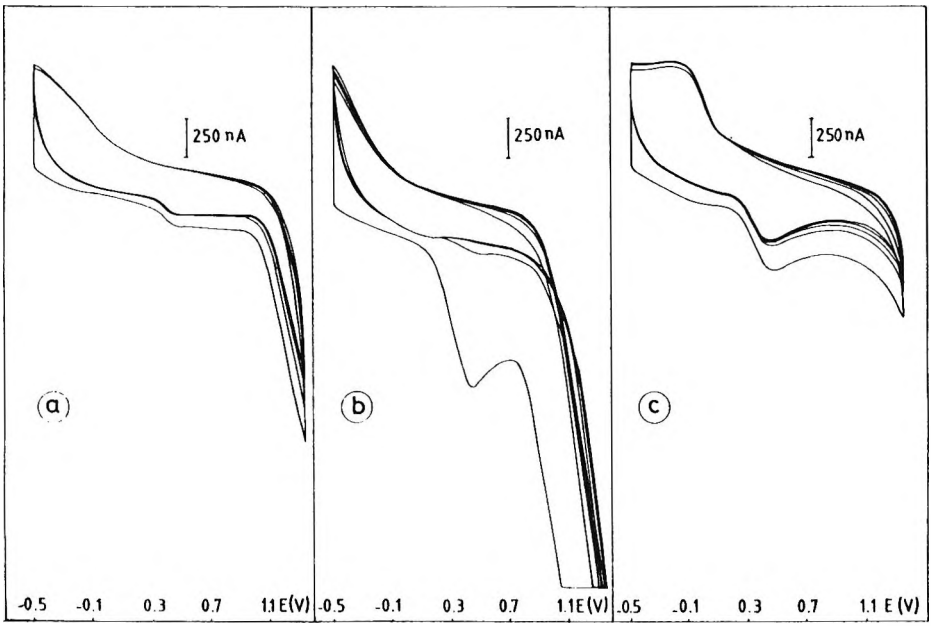

Figure 1. Cyclic voltammograms of (a) 4-chlorophenyl urea, (b) 4-chloroaniline, (c) todralazine, on glassy carbon electrode. Concentration: 1.0×10^{-4} mol l^{-1}. Scan rate: 400 mV s^{-1}. Solvent: Acetonitrile / citric acid - disodium hydrogenphosphate buffer (30:70), pH=7.0

20 ng of todralazine and varying the potential between 100 and 1200 mV and the pH between 4.0 and 7.6 (Figure 2). The optimum values of potential for each substance were: 1100 mV for 4-chlorophenyl urea, 900 mV for 4-chloroaniline and 700 mV for internal standard. A potential of 1100 mV was selected because lower potentials diminished the 4-chlorophenyl urea signal. On the other hand, an optimum value of 7.0 was selected for the analytical work.

The effect of the buffer molarity on the height of the peak was examined at pH=7.0 using phosphate/citrate buffer ranging from 0.005 to 0.05 mol l^{-1} in citric acid. It was observed that peak height increases when the ionic strength increases. Nevertheless, an increase of background was also observed. That is why an optimum value of the buffer molarity that guarantees a stable measure value, 0.005 mol l^{-1}, was selected.

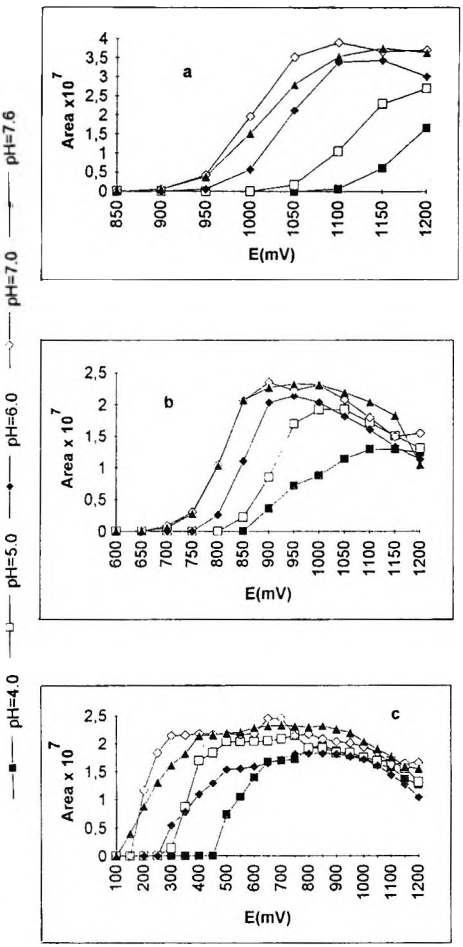

Figure 2. Hydrodynamic voltammograms. (a) 4-chlorophenyl urea 1.0 mg l^{-1}, (b) 4-chloroaniline 0.5 mg l^{-1}, (c) todralazine 1.0 mg l^{-1}. Injection volume: 20 μL. Flow rate: 1 mL min^{-1}.

Two organic modifiers, methanol and acetonitrile or, a mixture of them, were used to optimize the time of analysis, the peak width and the resolution. Table 1 shows the influence of the acetonitrile and methanol contents of the mobile phase in relation to the capacity factor K'.

Acetonitrile produced better defined chromatographic peaks than methanol, with less peak broadening. The best result for those three compounds was obtained when a mixture of acetonitrile-buffer (30:70) with a

Table 1

Influence of the Organic Modifiers Content of the Mobile Phase in Relation to K'

CH_3CN (%)	CH_3OH (%)	Citric Acid Phosphate Buffer (%)	K'_{tod}	$K_{chloroph}$	$K'_{chloraniline}$
20	0	80	4.32	6.16	12.70
20	10	70	3.14	4.70	8.53
25	0	75	1.98	2.93	6.89
25	5	70	2.46	3.50	6.88
25	10	65	1.87	2.71	5.15
28	0	72	1.95	2.90	6.82
30	0	70	1.44	2.43	5.80
30	5	65	1.41	2.21	4.57
32	0	68	1.32	2.19	5.27
35	0	65	0.42	1.74	4.54
0	40	60	3.80	5.77	8.94
0	50	50	1.90	2.84	5.47

Table 2

Mean Recoveries of 4-Chlorophenil Urea and 4-Chloroaniline in Different Solid Extraction Phases (n = 5)

Solid Phase	4-Chlorophenyl Urea	4-Chloroaniline
Aminopropyl	99.4 ± 1.9	95.5 ± 3.1
Cyanopropil	95.0 ± 2.1	90.8 ± 2.9
Florisil	95.3 ± 1.5	85.6 ± 1.1

flow rate of 1.0 mL min^{-1} was used. The retention times obtained for todralazine, 4-chlorophenyl urea and 4-chloroaniline were 2.11, 3.91 and 7.81 min, respectively (Figure 3). The detector used in this work is based on the principle of a thin-layer cell. Because the mass transport to the electrode is controlled by both diffusion and convection, the dependence of the detector response on flow rate must be studied. As was expected, the peak area decreased with an increase in flow rate, while the effect on the resolution was

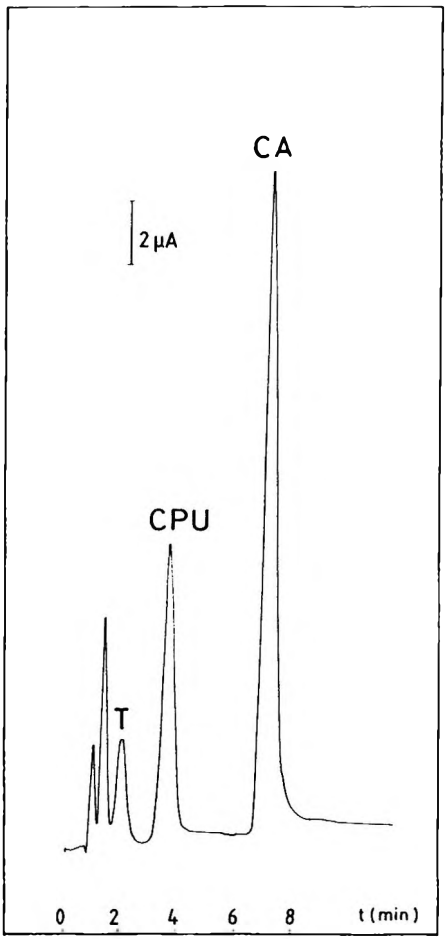

Figure 3. Chromatogram of 4-chlorophenyl urea (CPU) and 4-chloroaniline (CA) in optimum conditions: flow rate: 1.0 mL min-1, E=1100 mV, acetonitrile-citrate buffer (30:70), pH=7.0. Injection volume: 20 µL. Concentration: 200 µg l^{-1}. Internal standard: todralazine (T), 200 mg l^{-1}.

practically negiglible. A value of 1 mL min^{-1} was chosen as optimum. The determination of these compounds is based on the linear dependence of the relationship "$area_{compound}/area_{internal\ standard}$" on the concentration of the substance (in ng). A linear response was observed for concentrations ranging from 0.01 to 12.00 ng. The linear calibration plots correspond to the equations:

$A_{4\text{-chlorophen}} / A_{is} = 0.0785 + 0.0142$ [4-chlorophenyl urea],

$r = 0.9987$

$A_{4\text{-chloroanil}} / A_{is} = 0.0414 + 0.0325$ [4-chloroaniline],

$r = 0.9992$

The limits of detection based on 2N/m ratio (N is the noise and m is the slope of calibration graph) were found to be 9.2 pg for 4-chlorophenyl urea and 4.0 pg for 4-chloroaniline.

The relative standard deviations (n=10) at a concentration level of 0.16 ng (8 ppb) injected were 4.50 and 3.93%. These values decreased to 1.24 and 3.93% when 8.00 ng (400 ppb) of each substance were injected.

Analytical Applications

The validity of the proposed method was proven by spiking with 4-chlorophenyl urea and 4-chloroaniline in honey samples over the range of 10 to 60 mg Kg^{-1} and subjecting the samples to the described analytical procedure.

The prior extraction of the two substances from honey was established using different solid-liquid extraction polar phases: florisil, aminopropyl and cyanopropyl. The recovery data are reported in Table 2. The best resolution from extracts was obtained using aminopropyl cartridge in the clean-up stage. No significative differences in the extraction efficiency were observed when the concentration levels varied in the studied range.

Figure 4 illustrates the chromatograms for a blank sample honey and a spiked sample honey containing 25 $\mu g\ Kg^{-1}$ of 4-chlorophenyl urea and 4-chloroaniline. The high mean recovery obtained permitted the establishment of low detection limits.

The method can be applied to the determination of 4-chlorophenyl urea and 4-chloroaniline in honey at levels of 0.23 and 0.10 $\mu g\ Kg^{-1}$ respectively.

In conclusion, the HPLC-ED method developed has been used for the sensitive and reproducible determination of the major diflubenzuron metabolites, 4-chlorophenyl urea and 4-chloroaniline. The low detection limit achieved makes the monitoring of both compounds in honey samples possible.

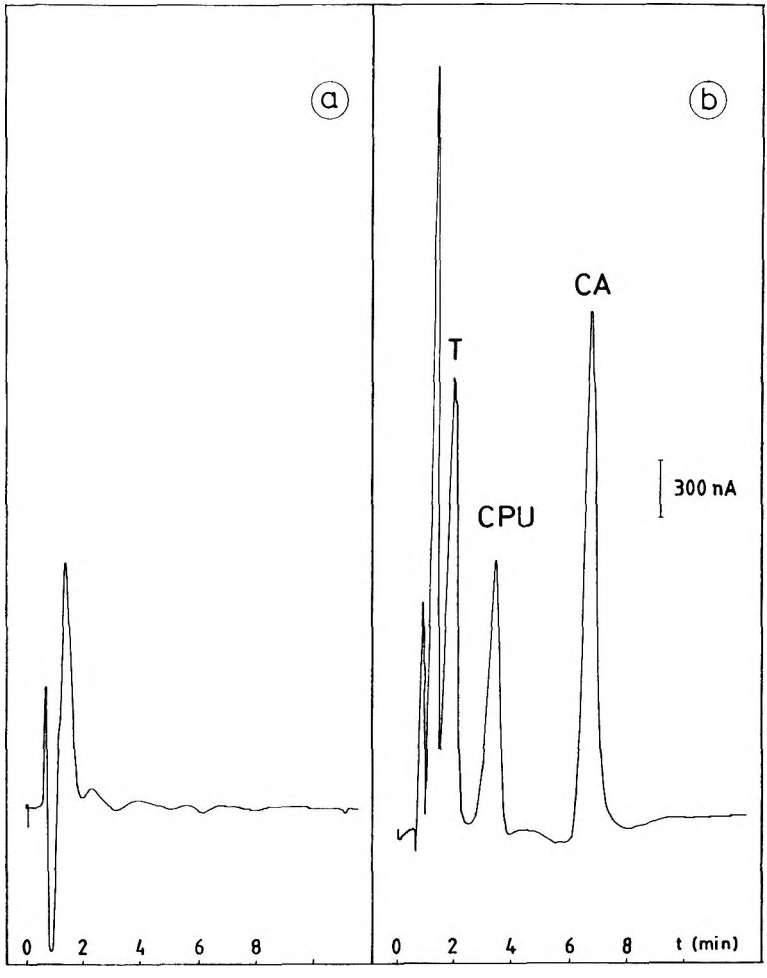

Figure 4. (a) Blank honey, (b) Chromatogram of fortified honey sample extract with the usual doses: 25 mg kg^{-1} of 4-chlorophenyl urea and 4-chloroaniline, internal standard injected: 200 μg l^{-1}. Other conditions in Figure 3.

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DETERMINATION OF FREE-FORM AMPHETAMINE IN RAT'S BRAIN BY *IN VIVO* MICRODIALYSIS AND LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

The determination of free amphetamine in rat's brain by *in vivo* microdialysis technique and HPLC/Fluorescence derivatization method is described. In vivo microdialysis was used to sample the extracellular amphetamine and its contents were analyzed by HPLC/Fluorescence derivatization assay. The effects of pH, temperature and reaction time on the derivatization were examined. The detection limit, linearity and precision associated with this assay were also evaluated. The stability of amphetamine-dansyl chloride in different storage conditions was determined. A single 3.0 mg/kg i.p. amphetamine administration in rats resulted in a maximum concentration of 654 ± 201 μM in the medial prefrontal cortex.

By using a non-linear curve fitting of two-compartment open model, the first-order rate constant for the appearance of amhetamine (k_1) was 0.0280 and the first-order rate constant for the disappearance of amhetamine (k_2) was 0.0281. These results document the utility of our method in pharmacokinetics studies.

INTRODUCTION

The number of illegal amphetamine users has increased tremendously in Taiwan. This has signaled many scientists in Taiwan to explore the euphoria mechanism of amphetamine in the human brain. Although, this research topic has been studied for many decades in other parts of world, it still is not clear. Understanding the pharmacokinetics of amphetamine in each key nuclei (e.g. medial prefrontal cortex; mPFC, nucleus accumbens, and striatum) is of interest to all scientists. As we know, only the free form of amphetamine, not the binding form, can have the pharmacological actions in the brain. Therefore, more importantly, monitoring of free amphetamine concentrations in the extracellular space is a preferable method for this study.

In vivo microdialysis has become a widely accepted sampling device for monitoring extracellular free drug concentrations in rat's brain.[1] Firstly, this method avoids exposure of the brain tissue to the perfusion medium and, therefore, minimizes tissue damage. The dialysis membrane, with a molecular weight cut-off range from 5,000 to 50,000 daltons based on the necessity of the study, eliminates the need of sample clean-up procedures before analytical quantitation. Additionally, samples will not be subjected to further metabolization after collection, since, the analytes can be separated from enzymes by the dialysis membrane.

Finally, the dialysis membrane prevents us to collect the binding form of amphetamine, only the free form. Therefore, using in vivo microdialysis as a sampling technique is a preferred technique to study the pharmacokinetics of the drug in various brain regions in rats.

In the past, several gas chromatography (GC) and GC/mass spectrometry (GC/MS) methods have been developed for the analysis of amphetamine in human urine and plasma.[2,3,4,5] In their methods, samples usually were of a large amount for pre-analysis preparation. For example, an extraction procedure is needed to transfer amphetamine into an organic solvent for GC or GC/MS analysis. Derivatization of amphetamine is always required to avoid decomposition of amphetamine in the GC injection port with high temperature and to enhance the signal to noise ratio by moving the molecular ion from low

100 to over mid 300. However, a relatively small amount of microdialysis sample (about 5 μL to 20 μL) make it difficult to apply extraction and derivatization procedure.

HPLC is an alternative method for the detemination of amphetamine in biological fluids. However, several other HPLC methods have been developed for the measurement of amphetamine in urine and plasma. Amphetamine was quantitated directly by UV,[6,7] photodiode array,[8] chemiluminescence or fluorescence detection.[9,10] However, all these methods are not suitable for small sample sizes of microdialysate samples.

Until recently, a gradient elution HPLC with fluorescence detection method has been developed for the analysis of microdialysate samples.[11] The relative long analysis time of gradient elution makes it less desirable for routine analysis, however. Also, no real application example was provided in their report.

This paper describes a simple isocratic elution HPLC method for the detemination of amphetamine in microdialysate samples. It includes addition of dansyl chloride for flourescence derivatization and HPLC fluorescence detection of amphetamine-dansyl chloride (AP-DanCl). More importantly, we apply this method to determine the concentration of free amphetamine in rat's medial prefrontal cortex (mPFC) after 3.0 mg/kg i.p. administration.

EXPERIMENTAL

Chemicals and Reagents

Purified water (> 18 MΩ from a NANOPure water purification system (Barnstead Corp., Dubuque, IA, USA) and HPLC grade acetonitrile and acetone (Labscan Limited, Dublin, Ireland) were used throughout. Sodium hydroxide, and amphetamine sulfate were purchased from Sigma (St. Louis, MO, USA). Dansyl chloride was obtained from Aldrich Corp. (Milwaukee, WI, USA). Sodium bicarbonate, sodium chloride, magnesium chloride, potassium chloride, calcium chloride, ascorbic acid, and glucose were purchased from Nakarai Chemical (Tokyo, Japan).

Pentobarbital sodium was obtained from Veterans General Hospital, Taipei, Taiwan. Lidocaine was obtained from the Department of Health, R.O.C.

Microdialysis

The design of a microdialysis method for conscious rats is different from that for anesthetized rats due to increased mobility of conscious rats after amphetamine administration. A piece of dialysis membrane tubing (220 μm i.d.; 6,000 dalton cut-off; Spectrum Medical Industries Inc., Houston, TX USA) was sealed at one end, then the two pieces of fused silica tubing (100 μm o.d., 40 μm i.d.; Polymicro Technologies, Inc., Phoenix, AZ, USA) were placed side by side and the ends of the inlet and outlet tubes were separated by 4 mm. Polyamide sealing resin (Alltech Associates Inc., Deerfield, IL, USA) was used to seal both ends of the dialysis membrane and to coat the excess portion of membrane. The probes were allowed to dry for 12 hours after being sealed by polyamide resin prior to implantation. The inlet and outlet fused silica tubes were protected by an elastic tubing (Baxter Scientific Products, Charlotte, NC, USA). The inlet was connected to a home-made single channel fluid swivel to allow the rat free movement without entanglement with the tubes.[12] The fluid swivel was then connected to a 500 μL syringe via PE-10 tubing. The perfusion syringe containing artificial cerebrospinal fluid (ACSF), was mounted on a microliter syringe pump (Model 22, Harvard Apparatus, S. Natick, MA, USA) at a flow rate of 1.19 μL/min. ACSF is composed of 0.13 M sodium chloride, 0.98 mM magnesium chloride, 2.65 mM potassium chloride, 1.2 mL calcium chloride, 0.25 mM ascorbic acid, and 10 mM glucose. The mixture was adjusted to a pH of 7.2 to 7.4 with 0.1 M sodium hydroxide.

Adult male Sprague-Dawley rats, weighing 250 ± 20 g on arrival, were supplied by the Animal Center of National Yang-Ming University (Taipei, Taiwan). They were housed in a 12 hour light/dark cycle room with free access to food and water. On the experimental day, animals were first anesthetized with 50 mg/kg i.p. sodium pentobarbital and placed on a stereotaxic apparatus (Koff models 1430 &1460). A local anesthetic (6% lidocaine) was used to relieve pain. A stainless steel guide cannula (C313GP, Plastics One, Inc.) was then lowered to 4 mm above the ventral surface of the medial prefrontal cortex (mPFC). The coordinates used, from bregma, were +3.1 AP, +0.8 ML, and -0.2 V below the skull. The guide cannula was secured to the skull with three mounting screws and dental cement. Intramuscular penicillin (60,000 I.U.) was administered immediately following surgery, then, the dialysis probe was inserted through the guide cannula.

Thereafter, the rat was placed in a behavioral box for at least an 18 hour recovery period prior to amphetamine administration (3.0 mg/kg i.p.). After the recovery period, the blank dialysate was assayed for ensuring no interference of amphetamine detection. After intraperitoneal injection of

amphetamine, dialysis samples were collected for 120 min. at 20 min. intervals. The dialysis fluid was collected in a 200 μL eppendroff tube wrapped with aluminum foil and stored in a -20°C refrigerator prior to analysis.

HPLC System

The HPLC system consisted of a model 750 piston pump from GL Science (Tokyo, Japan), a Waters 470 scanning fluorescence detector (Bedford, MA, USA), and a Rheodyne 7125 injector with a 20 μL injection loop. The excitation was set at 343 nm and the fluorescence was monitored at 500 nm. A SISC chromatography data system (Scientific Information Service Corp., Taipei, Taiwan) was used for data acquisition and processing. A Vercopak Inertsil 5-ODS-80A column, (3.2 x 250 mm, 5 μm, Vercotech Corp., Taipei, Taiwan) with an on-line filter (TFE exchange membrane, 82102, IRICA Instrument Inc., Kyoto, Japan) was used for separation. A mixture of 70 % acetonitrile and 30% water (v/v) was used as a mobile phase for chromatography analysis. The mixture was filtered through a 0.45 μm membrane (FP-450, Gelman Science, Michigan, USA) and sparged with helium gas for 30 minutes before use. The HPLC flow rate was 0.5 mL/min.

Standard Solution

For the examination of chromatography elution conditions, linearity, and detection limit associated with this method, 1 mM of amphetamine in ACSF was prepared and stored at 4°C in the dark. This stock solution was prepared weekly, but the working solutions were diluted with ACSF to appropriate concentration daily. For interday and intraday studies, the stock and working solutions were prepared daily.

For the quantitative analysis of microdialysate samples, standard solutions were prepared from the same solution that was used to inject the rats.

Derivatization of Samples

Into an eppendorf centrifuge tube, 15 μL of microdialysate was added to 8 μL of 0.1 M sodium bicarbonate/NaOH buffer (pH 9.0) and 15 μL of 0.1 mM dansyl chloride in acetone. The eppendorf tube was wrapped with aluminum foil, then the mixture was incubated at 45°C in a water bath for 1 hour. An aliquot of the solution (20 μL) was injected into the HPLC system for analysis.

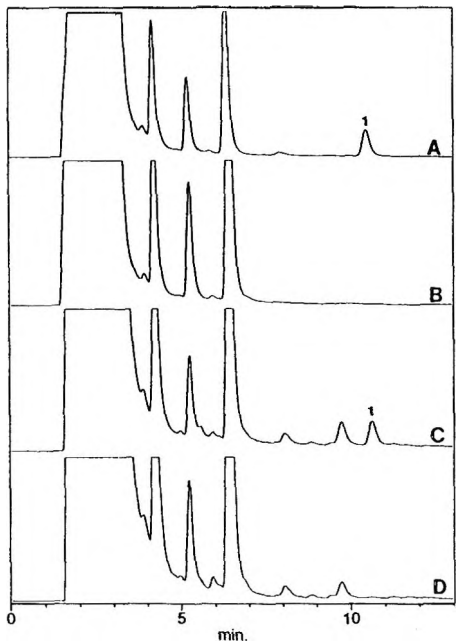

Figure 1. Typical chromatograms of a spiked amphetamine sample in ACSF (A), an ACSF only (B), a real microdialysis sample (C), and a microdialysis sample before amphetamine administration (D). Peak 1= amphetamine

RESULTS AND DISCUSSION

Figure 1 shows the typical chromatograms of ACSF blank, amphetamine spiked ACSF, and mice brain microdialysate samples. There are many substances in the ACSF and brain microdialysate, which reacted with the dansyl chloride; however, none of them interfered with AP-DanCl. AP-DanCl was eluted at 10.6 minutes, with the separation completed in less than 12 minutes.

The effects of pH, reaction temperature and reaction time on fluorescence derivatization reaction were evaluated by the method described in a previous report.[11] We found the most effective reaction condition for ACSF samples to be at pH 8.75 and a temperature of 45°C. For practical purposes, the fluorescence derivatization reaction condition was set at 45°C, pH 8.75 and 1 hour of reaction time throughout the remainder of this study.

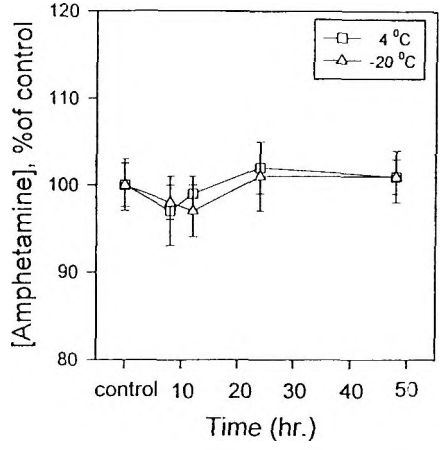

Figure 2. Stability of the spiked amphetamine samples in ACSF (0.1μM) versus time, which were stored at either 4°C or -20°C. The calculated concentration of a fresh prepared solution was used as 100%. Values are represented as the percentile changes of control (mean ± S.E.M.; n=3).

The effect of sample storage conditions on this assay was then evaluated. Amphetamine spiked ACSF samples (0.1μM) were stored in a freezer (-20°C) and a refrigerator (4°C). The samples were prepared by the procedures described in previous paragraphs and measured by HPLC for 24 hours. The results as shown in Figure 2 were examined by a one-tailed student-T test and $P<0.05$ was taken to indicate statistical significance. There was no significant decrease in response after 24 hours of storage in the refrigerator or freezer. The measurements of amphetamine in microdialysate for the remainder of this study were done within 24 hours after collection.

The stability of AP-DanCl in ACSF was evaluated and the results are summarized in Figure 3. The derivatized amphetamine spiked ACSF samples (0.1 μM) were stored either in a refrigerator at 4°C or at room temperature (about 26°C). Samples were withdrawn from both solutions hourly during a ten hour period by injection into HPLC for measurement to insure the stability of amphetamine. The 0 hour sample served as a control. The difference between the results of each of the 10 samples and the control was examined by a one-tail student-T test. $P<0.05$ was taken to indicate statistical significant. There was no significant decrease in response after storage in the refrigerator or freezer.

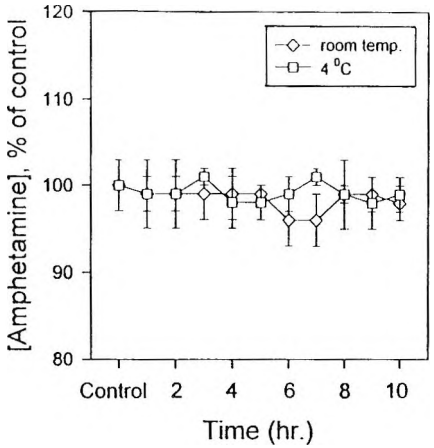

Figure 3. Stability of dansyl chloride derivatized amphetamine samples (0.1μM) versus time, which were stored at either room temperature (26°C), or 4°C. Values are represented as the percentile changes of control (mean ± S.E.M.; n=3).

Solutions of amphetamine spiked ACSF samples (5.0, 1.0, 0.5, 0.1, 0.05, 0.01 μM) were prepared, then each solution was derivatized throughout the procedure as described in a previous section. Each standard was analyzed six times. A calibration curve for amphetamine spiked ACSF samples was constructed and the linearity was good from 5 to 0.01 μM (r^2=0.999). The detection limit was 0.005 μM based on a signal-to-noise ratio of 3.

The analytical precision of this method was evaluated by replicated analysis of amphetamine spiked ACSF samples. A new calibration standard set was prepared and analyzed each day. A total of six series of samples were analyzed over a two week period and each sample was determined in triplicate. The results of interday and intraday studies are summarized in Table 1. The intraday precision showed a coefficient of variation (CV) ranging from 1.8% to 8.3%. The interday variation was similarly evaluated on several days and the CV varied from 0.2% to 10.0%.

The time course of amphetamine concentration (n=5) in the extracellular fluid in mPFC of rats which received 3 mg/kg amphetamine i.p. is shown in Figure 4. Amphetamine reached a maximum concentration of 654 ± 201μM during the 20-40 min collection interval. The time course of amphetamine change was rapid.

Table 1

Interday and Intraday Precision of Amphetamine in ACFS

Added Conc.		Intraday[1] Measured Conc. μM	Interday[2] Measured Conc. μM
5.0 μM	mean	4.99	4.98
	S.D.	0.089	0.010
	CV	1.8%	0.2%
1.0 μM	mean	1.01	1.04
	S.D.	0.040	0.030
	CV	4.0%	2.9%
0.5 μM	mean	0.54	0.51
	S.D.	0.014	0.028
	C.V.	2.6%	5.6%
0.1 μM	mean	0.09	0.10
	S.D.	0.007	0.006
	C.V.	7.8%	6.0%
0.05 μM	mean	0.046	0.049
	S.D.	0.004	0.005
	C.V.	8.7%	10.2%

[1]Mean and S.D. represent four different ACSF samples for each concentration.

[2]Interday reproducibility was determined from six different runs over a two week period.

A non-linear curve-fitting computer program, Minsq, written by MicroMath Scientific Software (Salt Lake City, Utah, U.S.A.) was used to fit an equation consisting of the difference of two first-order kinetic processes for the appearance and disappearance of amphetamine in the extracellular fluid of the mPFC to experimental amphetamine data:

$$\text{Amphetamine} = A\,(\exp(-k_1 t)-\exp(-k_2 t))$$

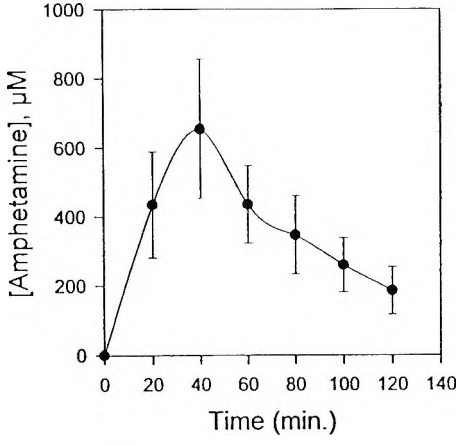

Figure 4. Time course of extracellular amphetamine concentration at the medial frontal cortex in rats (n=5) following a 3.0 mg/kg amphetamine i.p. injection.

Three parameters fit into the data: A, k_1, and k_2. A is a concentration and adsorption efficiency factor, k_1 is the first-order rate constant for the appearance of amphetamine, and k_2 is the first-order rate constant for the disappearance of amphetamine. Both rate constants have the units of min^{-1}. After this non-linear fit, the k_1 was 0.0280 and k_2 was 0.0281. The 95% non-linear confidence intervals for these rate constants were: $[0.0212 < k_1 < 0.0349]$ and $[0.0211 < k_2 < 0.0350]$.

In conclusion, we have successfully evaluated the concentration of free amphetamine in rat brain by microdiaysis technique and a simple, sensitive and accurate HPLC with fluorescence derivatization assay.

ACKNOWLEDGMENT

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DETERMINATION OF POLAR CONSTITUENTS OF *SCROPHULARIAE RADIX* IN TRADITIONAL CHINESE MEDICINAL PRESCRIPTIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatographic methods were established for the determination of 2-(3-hydroxy-4-methoxyphenyl) ethyl 1-O-[α-L-arabinopyranosyl (1→6)]-feruloyl (1→4)-α-L-rhamnopyranosyl (1→3)-β-D-gluco pyranoside (SN-A), harpagoside (SN-B) and cinnamic acid (SN-C) in four kinds of traditional Chinese medicinal prescriptions which contain Scrophulariae Radix: Tian-Uang-Bu-Shin-Dan, Bai-He-Gu-Jin-Tang, Bay-Du-San and Ching-Reh-Bu-Shiee-Tang. The samples were processed with a Cosmosil ODS reversed phase column by gradient elution with various ratios of 1 % (v/v) acetic acid, acetonitrile and methanol as the mobile phases and detected at UV 278 nm. Regression equations were derived showing linear relationships (correlation coefficients: 0.9918-0.9999) between the peak area ratios of each marker to an internal

standard (sulfadimethoxine, chlorzoxazone or dexamethasone) and concentration. The recoveries of SN-A, B, and C from the Chinese medicinal prescriptions were 99.3-103.0 %, 94.0-102.2 % and 97.0-104.7 %, respectively. The relative standard deviations of markers ranged between 0.96-5.91 % (intraday) and 0.34-4.83 % (interday). The contents of SN-A, B, and C from Chinese medicinal prescriptions were 2.35-3.30 mg/g, 0.19-1.72 mg/g and 0.21-0.99 mg/g, respectively.

INTRODUCTION

Scrophulariae Radix (Chinese name: Xuanshen) is the dried root of *Scrophularia ningpoensis* and *S. buergeriana* (Scrophulariaceae) and is a commonly used Chinese herb. It is administered to allay thirst in febrile disease, in macula, pharyngolaryngitis and constipation.[1] In our previous paper, the isolation and identification of three polar constituents, 2-(3-hydroxy-4-methoxyphenyl) ethyl 1-O-[α-L-arabinopyranosyl (1→6)]-feruloyl (1→4)-α-L-rhamnopyranosyl (1→3)-β-D-glucopyranoside (SN-A), harpagoside (SN-B) and cinnamic acid (SN-C) from the root of *Scrophularia ningpoensis* was reported.[2] For the determination of the above constituents in Scrophulariae Radix, an HPLC method using acetonitrile and 1.0% (v/v) acetic acid as mobile phase on an ODS column was also developed.[2]

Guillerault *et al.* reported the determination of harpagide, 8-para-coumaroyl harpagide, and harpagoside by HPLC in *Harpagophytum procumbens* using methanol and water as mobile phase on an RP18 column.[3] However, the assay for the markers from Chinese medicinal prescriptions containing Scrophulariae Radix by HPLC has not yet been described. For the purpose of quality control of traditional Chinese medicinal prescriptions, we used the three polar constituents mentioned above as marker constituents and studied HPLC methods for the assay of these markers in prescriptions containing Scrophulariae Radix .

In this study, we selected four kinds of Chinese medicinal prescriptions: Tian-Uang-Bu-Shin-Dan (hereafter abbreviated as P1), Bai-He-Gu-Jin-Tang (P2), Bay-Du-San (P3) and Ching-Reh-Bu-Shiee-Tang (P4) all of which contain Scrophulariae Radix. This paper deals with the HPLC methods for determining the contents of the marker constituents, SN-A, B and C, in each prescription. Validation methods are also reported.

EXPERIMENTAL

Materials

The materials used to prepare the traditional Chinese medicinal prescriptions were as follows:[4]

Tian-Uang-Bu-Shin-Dan (P1): Scrophulariae Radix, Polygalae Radix, Platycodi Radix, Ophiopogonis Tuber, Coptidis Rhizoma, Ginseng Radix, Biotae Semen, Zizyphi Spinosi Semen, Salviae Miltiorrhizae Radix, Hoelen, Asparagi Cochinchinensis Tuber, Angelicae Radix, Acori Graminei Rhizoma (1.2 g each) and Schizandrae Fructus (1.5 g).

Bai-He-Gu-Jin-Tang (P2): Scrophulariae Radix, Fritillariae Bulbus, Paeoniae Radix (3.0 g each), Lilii Bulbus, Angelicae Radix, Rehmanniae Radix (4.0 g each), Ophiopogonis Radix (6.0 g), Glycyrrhizae Radix (1.5 g) and Platycodi Radix (2.0g).

Bay-Du-San (P3): Scrophulariae Radix, Trichosanthis Radix, Platycodi Radix, Arctii Fructus, Forsythiae Fructus, Lonicerae Flos (2.5 g each), Moutan Cortex, Bupleuri Radix (2.0 g each), Phellodendri Cortex, Paeoniae Radix, Menthae Folium (1.5 g each), Rehmanniae Radix, Gypsum Fibrosum (4.5 g each) and Glycyrrhizae Radix (1.0 g).

Ching-Reh-Bu-Shiee-Tang (P4): Scrophulariae Radix, Phellodendri Cortex, Bupleuri Radix, Moutan Cortex (1.5 g each), Angelicae Radix, Rehmanniae Radix, Schizandrae Fructus, Paeoniae Radix, Ophiopogonis Radix and Ligustici Wallichii Rhizoma (3.0 g each).

All materials were obtained from retail outlets in Taipei and pulverized. Two different commercial brands of concentrated preparations of Tian-Uang-Bu-Shin-Dan and three of Bai-He-Gu-Jin-Tang were also purchased from retail outlets.

Chemicals and Reagents

2-(3-Hydroxy-4-methoxyphenyl) ethyl 1-O-[α-L-arabinopyranosyl (1→6)]-feruloyl (1→4)-α-L-rhamnopyranosyl (1→3)-β-D-glucopyranoside (SN-A) and harpagoside (SN-B) were isolated from the root of *Scrophularia ningpoensis*.[2] Chlorzoxazone and sulfadimethoxine were obtained from Sigma

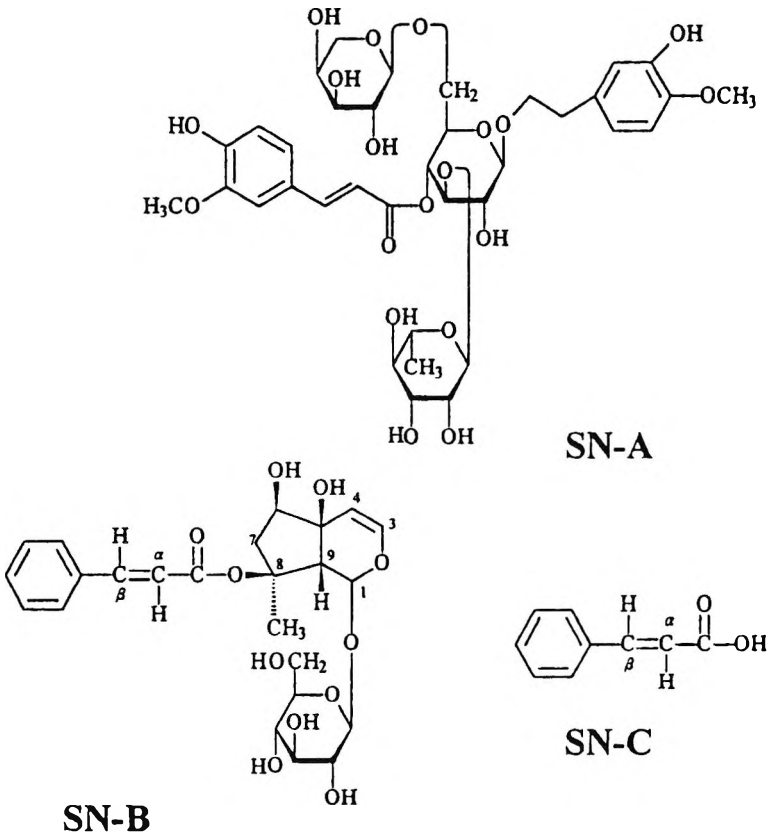

Figure 1. Structures of marker constituents.

Chemical Co. (St. Louis, U.S.A.). Cinnamic acid (SN-C) and dexamethasone were obtained from Nacalai Tesque (Kyoto, Japan). Acetonitrile and methanol were HPLC grade (purchased from Labscan, Dublin, Ireland) and acetic acid was analytical reagent grade. Ultrapure distilled water with a resistivity greater than 18 MΩ was used. The structures of the marker constituents are shown in Fig. 1.

Instruments

HPLC was conducted with a Waters 600E HPLC pump with a Waters 486 UV Detector and a Shimadzu SIL-9A autoinjector. Cosmosil 5C18-AR (5, 15 and 25 cm x 4.6 mm I.D.) reversed phase columns were used. Peak areas were calculated with a Shiunn Haw computing integrator.

Table 1

Gradient Conditions

Prescriptions	Internal Standard	Column Length (cm)	Gradient Conditions								
P1	SDM	5[a] + 25	**Time (Min)**:	0	25	26	36	42	50	53	55
			A/B/C:	12/88/0	25/75/0	15/75/10	18/72/10	20/70/10	19/71/10	18/72/10	17/73/10
P2	CZX	5[a] + 25	**Time (Min)**:	0	15	30	35				
			A/B/C:	20/80/0	20/80/0	30/70/0	20/80/0				
P3	DM	15	**Time (Min)**:	0	15	20	30	40			
			A/B/C:	15/85/0	19/81/0	19/76/5	22/73/5	28/67/5			
P4	CZX	15	**Time (Min)**:	0	40						
			A/B/C:	10/85/5	25/70/5						

[a] Connected as a guard column

Liquid Chromatography

The mobile phases which were used in these four prescriptions were mixtures of acetonitrile (A), 1.0 % (v/v) acetic acid (B) and methanol (C). Each mobile phase was filtered through a 0.45 μm Millipore filter and degassed prior to use. The flow rate was 1.0 mL/min and the detecting wavelength was 278 nm for each prescription. A constant operating temperature (room temperature) was maintained. The internal standards, sulfadimethoxine (SDM, 5.4 mg for P1), chlorzoxazone (CZX, 5.3 mg for P2 and P4), dexamethasone (DM, 5.3 mg for P3), were dissolved in 25mL of 70 % methanol respectively to give the internal standard solutions. The mobile phases, column and internal standard for each prescription are given in Table 1.

Preparation of Standard Solution

To prepare a standard solution containing SN-A, B and C, accurately weighed amounts of SN-A, B and C standard were dissolved in 70 % methanol after which an appropriate amount of internal standard solution was added to give various concentrations within the ranges 5.2-21.8, 1.04-15.60 and 1.0-20.0 μg/mL, respectively. Calibration graphs were plotted after linear regression analysis of the peak area ratios with concentrations.

Preparation of Sample Solution

Standard decoction

The individual crude drugs of each of the four kinds of Chinese medicinal prescriptions in amounts equivalent to a daily dose were weighed and pulverized separately, a twentyfold weight of water was added and the mixture of each prescription was boiled respectively for more than 30 min to half the original volume. The extract was filtered while hot. Finally it was diluted with 70 % methanol stock solution and then a suitable amount of internal standard was added to the solution to give a concentration of 10.6 μg/mL of CZX, 10.6 μg/mL of DM and 10.8 μg/mL of SDM.

Blank decoction

Amounts of individual crude drugs equivalent to a daily dose of the four kinds of Chinese medicinal prescriptions but without Scrophulariae Radix were processed as above.

Table 2

Inter-Day and Intra-Day Relative Standard Deviations (n = 5) of Marker Constituents for Chinese Medicinal Prescriptions

Prescriptions	Marker Constituents	Concentration (μg/mL)	Intraday R.S.D. (%)	Interday R.S.D. (%)
P1	SN-A	21.6	2.61	1.86
	SN-B	10.4	4.44	2.83
	SN-C	5.0	2.10	2.03
P2	SN-A	15.6	4.65	4.83
	SN-B	10.4	1.36	1.03
	SN-C	3.75	1.06	0.40
P3	SN-A	10.8	2.34	2.75
	SN-B	2.08	5.91	2.16
	SN-C	10.0	2.81	2.03
P4	SN-A	15.6	2.26	1.75
	SN-B	10.4	0.96	0.34
	SN-C	3.75	1.27	0.94

Concentrated Preparations (Tian-Uang-Bu-Shin-Dan and Bai-He-Gu-Jin-Tang) from Retail Outlets

An amount of the concentrated preparation equivalent to a daily dose was weighed accurately and extracted with 35 mL of 70 % methanol for 30 min in an ultrasonic bath. After filtration, the filtrate was diluted to 50.0 mL with 70 % methanol, and a suitable amount of internal standard was added to the solution to give a concentration of 10.8 μg/mL of SDM for Tian-Uang-Bu-Shin-Dan and 10.6 μg/mL of CZX for Bai-He-Gu-Jin-Tang. The solutions were filtered by 0.45 μm Millipore and analysed by HPLC.

Solutions for Recovery Study

Three different concentrations of markers were used for each prescription. For P1 and P3: 32.4, 54.0 and 108.0 μg/mL for SN-A; 31.2, 52.0 and 104.0 μg/mL for SN-B and 30.0, 50.0 and 100.0 μg/mL for SN-C, were added. For

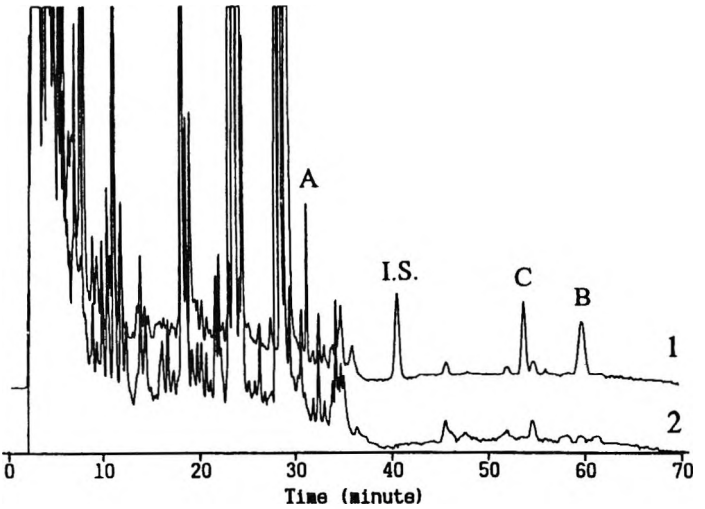

Figure 2. Chromatograms of SN-A, B and C in Tian-Uang-Bu-Shin-Dan (P1): (1) standard decoction; (2) blank decoction. Peaks: A = SN-A; B = SN-B; C = SN-C; I.S. = internal standard (sulfadimethoxine).

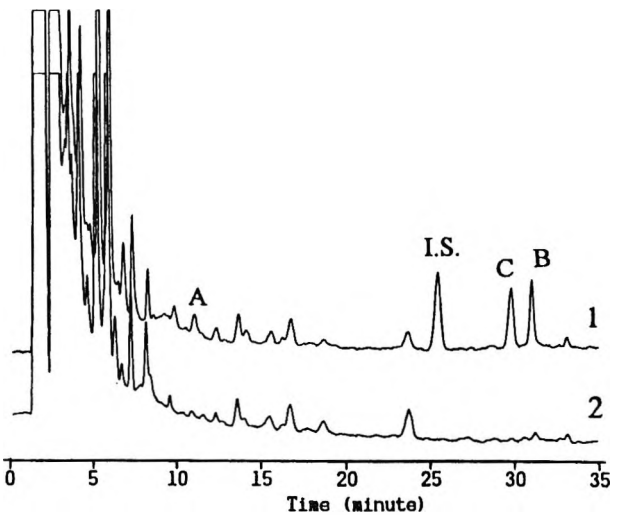

Figure 3. Chromatograms of SN-A, B and C in Bai-He-Gu-Jin-Tang (P2): (1) standard decoction; (2)blank decoction, I.S. = Internal Standard (chlorozoxazone). Marker peaks as in Figure 2.

Table 3

Recovery of SN-A, B, and C from Chinese Medicinal Prescriptions

Sample	Marker Constituents	Amount Added (μg/mL)	Amount Measured (μg/mL)	Recovery (%)	Mean ±S.D. (%)	R.S.D. (%)
		32.4	31.6	97.6		
	SN-A	54.0	56.9	105.3	100.9±3.2	3.2
		108.0	107.8	99.8		
		31.2	31.5	101.1		
P1	SN-B	52.0	46.8	90.0	98.5±0.2	0.2
		104.0	108.7	104.5		
		30.0	29.4	97.9		
	SN-C	50.0	51.9	103.8	102.8±3.6	3.5
		100.0	106.7	106.7		
		31.2	32.1	102.9		
	SN-A	52.0	48.8	93.9	99.3±3.9	3.9
		104.0	105.0	100.9		
		31.2	33.8	108.3		
P2	SN-B	52.0	50.3	96.7	101.9±4.8	4.7
		104.0	104.7	100.7		
		9.5	6.9	92.6		
	SN-C	12.5	12.0	96.1	97.0±4.0	4.1
		25.0	25.6	102.3		
		32.4	32.4	100.1		
	SN-A	54.0	56.2	104.1	101.1±2.2	2.2
		108.0	106.9	99.0		
		31.2	28.2	90.3		
P3	SN-B	52.0	47.8	92.0	94.0±4.1	4.4
		104.0	103.7	99.7		
		30.0	29.2	97.5		
	SN-C	50.0	49.8	99.6	102.1±5.2	5.1
		100.0	109.3	109.3		

(continued)

Table 3 (Continued)

Sample	Marker Constituents	Amount Added (μg/mL)	Amount Measured (μg/mL)	Recovery (%)	Mean ±S.D. (%)	R.S.D. (%)
		31.2	31.4	100.7		
	SN-A	52.0	57.3	110.2	103.0±5.1	5.0
		104.0	102.2	98.3		
		31.2	28.5	91.4		
P4	SN-B	52.0	54.9	105.5	102.2±7.8	7.6
		104.0	114.1	109.7		
		9.5	8.2	108.7		
	SN-C	12.5	12.7	101.2	104.7±3.1	3.0
		25.0	26.1	104.4		

for SN-B and 9.5, 12.5 and 25.0 μg/mL for SN-C, were added. To each solution a suitable amount (the peak height was similar to the marker peaks) of internal standard was added. All samples were filtered through a 0.45 μm Millipore filter and injected for HPLC analysis. The recoveries of SN-A, B and C were calculated from their calibration graphs.

RESULTS AND DISCUSSION

The detection wavelength of 278 nm was chosen because the absorbance of SN-B is the highest at this wavelength. The other constituents SN-A (UV $\lambda_{max}^{70\%MeOH}$: 287 nm) and C (267 nm) also show high absorbance.

The intra- and inter-assay precisions of these methods were examined with standard solutions of SN-A, B and C, five times on the first day and then once a day for a 5-day period (Table 2). The intra- and inter-assay relative standard deviations (R.S.D.s) indicate that the precision is acceptable.

To ensure the specificity and selectivity of the methods, we prepared four blank decoctions of these prescriptions for comparison. The chromatograms of P1-P4 are shown in Figs. 2-5. The retention times of the marker constituents, SN-A, B and C in P1, P2, P3 and P4, were 31.2, 59.2 and 53.6 min; 11.3, 31.1 and 29.9 min; 23.8, 45.6 and 37.6 min and 27.5, 40.8 and 34.1 min, respectively. No peaks were detected at these retention times in the four blank

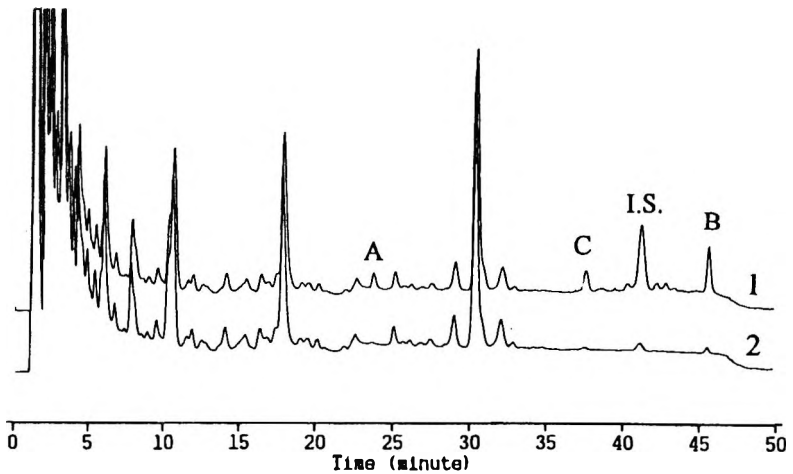

Figure 4. Chromatograms of SN-A, B and C in Bay-Du-San (P3) : (1) standard decoction; (2) blank decoction. I.S. = internal standard (dexamethasone). Marker peaks as in Figure 2.

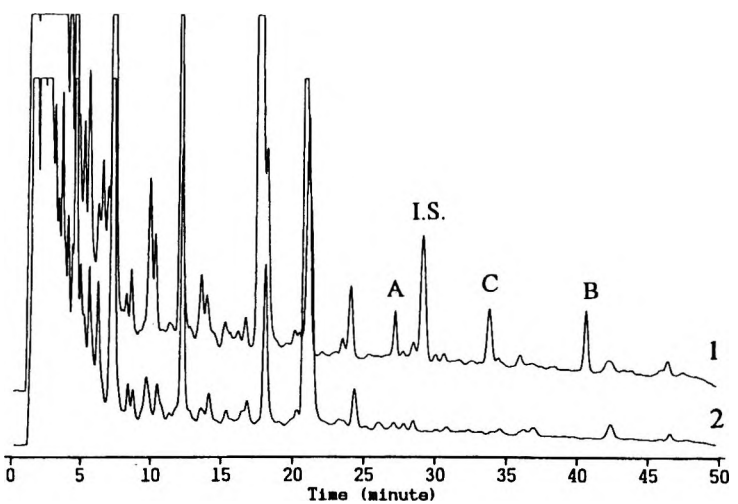

Figure 5. Chromatograms of SN-A, B and C in Ching-Reh-Bu-Shiee-Tang (P4): (1) standard decoction; (2) blank decoction. I.S. = internal standard (chlorzoxazone). Marker peaks as in Figure 2.

Table 4

The Regression Equations and Their Correlation Coefficients (r) of Marker Constituents for Chinese Medicinals Prescriptions

Prescriptions	Marker Constituents	Concentration Range (μg/mL)	Slope	Intercept	r
	SN-A	5.4-21.6	30.45	-1.28	0.9942
P1	SN-B	1.04-15.60	4.45	0.23	0.9997
	SN-C	1.0-10.0	1.61	0.01	0.9998
	SN-A	5.2-20.8	56.64	1.03	0.9943
P2	SN-B	2.08-15.60	7.65	-0.12	0.9998
	SN-C	1.25-5.00	2.78	-0.01	0.9996
	SN-A	5.4-21.6	14.09	-1.14	0.9950
P3	SN-B	1.04-15.6	2.48	0.01	0.9999
	SN-C	0.5-15.0	0.95	-0.18	0.9997
	SN-A	5.2-20.8	70.91	-1.34	0.9918
P4	SN-B	2.08-15.60	9.41	-0.70	0.9997
	SN-C	1.25-5.00	3.68	0.400	0.9992

decoctions. The recoveries of SN-A, B and C were 100.9, 98.5 and 102.8 % for P1; 99.3, 101.9 and 97.0 % for P2; 101.1, 94.0 and 102.1 % for P3; 103.0, 102.2 and 104.7 % for P4, respectively (Table 3). The R.S.D.s of SN-A, B and C ranged from 2.2 to 5.0 %, 0.2 to 7.6 % and 3.0 to 5.1 %, respectively. The regression equations and their coefficients of the marker constituents for the four Chinese medicinal prescriptions are shown in Table 4. Linear regression analysis showed good linear relationships between the peak area ratio and the concentration of marker constituents in the four prescriptions.

The contents of marker constituents in the standard decoctions are shown in Table 5. The contents of the three marker constituents, SN-A, B and C, corresponded to the contents in the crude drug (Scrophulariae Radix), 0.53-17.10, nondetected-1.75 and 0.58-5.35 mg/g, respectively.[2] The contents of marker constituents in commercial concentrated preparations of Tian-Uang-Bu-Shin-Dan (P1) and Bai-He-Gu-Jin-Tang (P2) are shown in Tables 6 and 7.

Table 5

The Content of Marker Constituents in Chinese Medicinal Prescriptions

Prescriptions	Marker Constituents		
	SN-A Mean±S.D.[a] (mg/g)	SN-B Mean±S.D.[a] (mg/g)	SN-C Mean±S.D.[a] (mg/g)
P1	2.58±0.03	0.19±0.01	0.68 ±0.02
P2	3.30±0.01	1.72±0.04	0.99±0.02
P3	2.35±0.01	0.54±0.01	0.21±0.04
P4	2.47±0.01	0.47±0.01	0.22±0.02

[a] n = 3.

Table 6

The Contents of Marker Constituents in Commercial Concentrated Herbal Preparation of Tain-Uang-Bu-Shin-Dan (P1)

Sample	Marker Constituents		
	SN-A Mean±S.D.[a] (mg/g)	SN-B Mean±S.D.[a] (mg/g)	SN-C Mean±S.D.[a] (mg/g)
1	0.86±0.002	0.15±0.01	none detected
2	0.66±0.02	0.19±0.01	0.07±0.02

[a] n = 3.

Their chromatograms are shown in Figs. 6 and 7. The contents of SN-A and C were lower than in the standard decoctions (0.30-0.86 mg/g versus 2.35-3.30mg/g and nondetected-0.12 mg/g versus 0.21-0.99 mg/g). These differences might have resulted from the manufacturing process.

Although the constituents of crude drugs are complex, and these four prescriptions were composed of at least nine kinds of crude drugs, in this study we developed HPLC methods for the determination of three markers, SN-A, B and C, in all four prescriptions. The methods diffrered only in the ratio of the

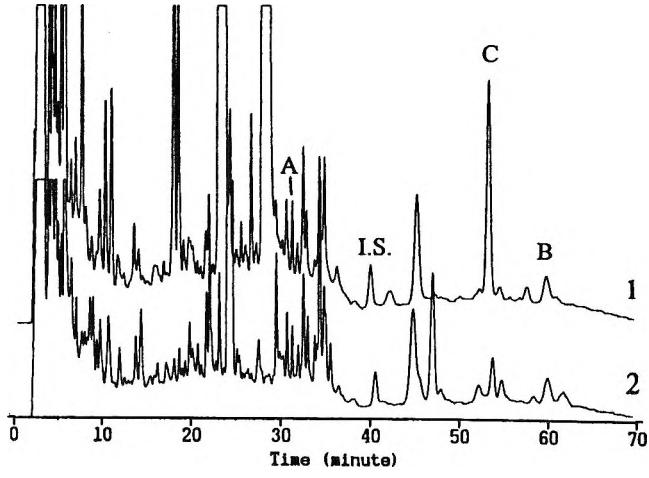

Figure 6. Chromatograms of SN-A, B and C in commercial preparations (1, 2) of Tian-Uang-Bu-Shin-Dan. Marker peaks as in Figure 2.

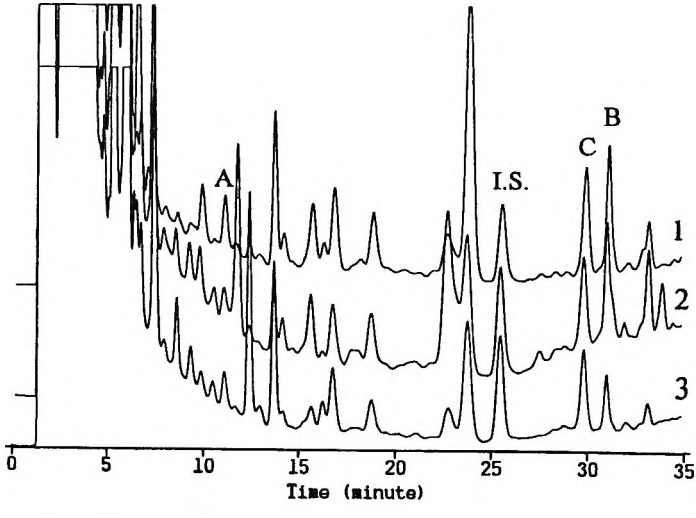

Figure 7. Chromatograms of SN-A, B and C in commercial preparations (1-3) of Bai-He-Gu-Jin-Tang. Marker peaks as in Figure 2.

Table 7

The Contents of Marker Constituents in Commercial Concentrated Herbal Preparation of Bai-He-Gu-Jin-Tang (P2)

Sample	Marker Constituents		
	SN-A Mean±S.D.[a] (mg/g)	SN-B Mean±S.D.[a] (mg/g)	SN-C Mean±S.D.[a] (mg/g)
1	0.43 ± 0.01	0.10 ± 0.01	0.08 ± 0.03
2	0.30 ± 0.01	0.21 ± 0.02	0.07 ± 0.03
3	0.84 ± 0.01	0.33 ± 0.01	0.12 ± 0.02

[a] n = 3.

solvents of the mobile phases and the internal standards. Therefore, these methods are economical and suitable for quality control of the Chinese medicinal prescriptions containing Scrophulariae Radix. For instance, Tian-Uang-Bu-Shin-Dan (P1) is composed of 14 kinds of crude drugs. The weight ratio of Scrophulariae Radix was 7.0 % in this prescription.

A 25 cm column with 5 cm of guard column was used and three solvents, acetonitrile (A), 1.0% (v/v) acetic acid (B) and methanol (C), were used as the mobile phase. Acetonitrile and acetic acid were used initially (12/88) and at 25 min (25/75) for eluting more interference peaks, then at 26 min methanol was added to the mobile phase (15/75/10) for separating SN-A. The ratio of the mixture was then changed by linear gradient: 36 min (18/72/10), 42 min (20/70/10), 50 min (19/71/10), 53 min (18/72/10) and 55 min (17/73/10) for separating SN-B and C. Bay-Du-San (P3) was complicated too, like Tian-Uang-Bu-Shin-Dan (P1). It was also separated by adding the third solvent. Nonetheless, the intensity of some of the marker peaks was weaker than that of the interference (Fig. 6).

ACKNOWLEDGMENT

The authors thank the National Health Research Institute, Republic of China, for support of this research under grant DOH 84-HR-307.

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J. LIQ. CHROM. & REL. TECHNOL., 20(10), 1633–1636 (1997)

PURIFICATION OF AZADIRACHTIN-B (3-TIGLOYLAZADIRACHTOL) BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY, USING THE RECYCLING MODE

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SPIC Science Foundation
110, Mount Road
Madras 600 032, India

ABSTRACT

Isolation of Azadirachtin-B in pure form suitable for crystallisation from neem seed kernel extract by a preparative high performance liquid chromatography followed by a subsequent preparative HPLC using the recycling mode is described.

INTRODUCTION

Of the numerous tetranortriterpenoids present in neem kernel extract or in neem oil, Azadirachtin-B (3-tigloylazadirachtol) is an important constituent with high biological activity and present in almost a third of the quantity of Azadirachtin-A, the principal anti-feedant and ecdysis inhibition principle.

The Azadirachtin-B of Rembold et al[1] and 3-tigloylazadirachtol of Klenk et al[2] are undoubtedly identical. However, the assignment of the tigloyl group to the 3-position is based entirely on NOE studies. Although Klenk et al[2] obtained this compound in a crystalline condition, no X-ray studies were carried out. Rembold could not crystallise Azadirachtin-B.[1] Herein, we report the purification of Azadirachtin-B suitable for crystallisation by direct preparative high performance liquid chromatography[3] of neem seed kernel extract.

MATERIALS AND METHODS

Preparative High Performance Liquid Chromatography was carried out using a Shimadzu LC8A HPLC system linked to a CR4A data processor and the peaks detected at 215 nm. Two Shimpack reverse phase (C_{18}) preparative columns (25 cm x 50 mm i.d.) and (25 cmx 20 mm i.d.) were used for preparative runs and Phenomenex reverse phase column (C_{18}) (25 cm x 4.6 mm) was used for analysis.

Neem kernel extract enriched to 25% Azadirachtin content as described earlier[3] was employed.

RESULTS AND DISCUSSION

The isolation of Azadirachtin-B by preparative high performance liquid chromatography from neem kernel extract has been reported from this laboratory,[3] but attempts to crystallise this material failed. It was surmised that material of higher purity would be needed for successful crystallisation. Hence it was decided to apply the recycling mode in preparative HPLC, which effectively increases the column length several fold, depending on the number of cycles. Also undesired accompanying impurities could be rejected using peak shaving techniques. Although recycling is a well-established procedure very little use has been made for purification of natural products.

Azadirachtin-B (500 mg) obtained as earlier[3] was subjected to one more preparative HPLC run under recycling mode using Shimadzu RP C_{18} column (25 cm x 20 mm i.d.) with 50:35:15 H_2O:MeOH: CH_3CN as eluent at 15 mL/min. flow rate (Figure 1). Peak eluting out at 16.6 min. (fraction from 11 min. to 26 min.) was recycled four times. The recycled portions are indicated by broken lines (Figure 1).

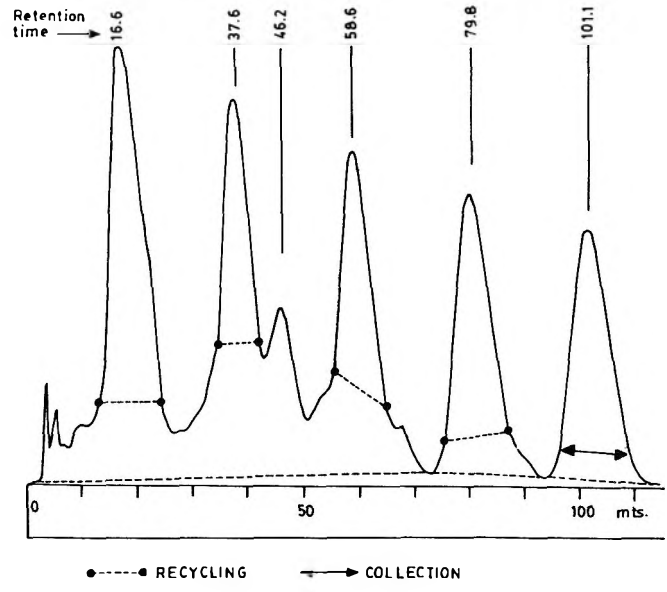

Figure 1. Preparative high performance liquid chromatogram of azadirachtin-B using recycling mode.

At the end of the fourth cycle, the peak eluting at 101.074 was symmetric and, hence, the fraction eluting out between 97 min. and 110 min. was collected and concentrated *in vacuo* to yield pure azadirachtin-B (172 mgs), which was subjected to crystallisation in various solvents (ethylacetate:hexane, methanol:water and ethanol:water), successively.

The characteristics of the crystals of azadirachtin B ($EtOH-H_2O$) are the following: m.pt 208°C; $[\alpha]_D$ = -76.9 (C=0.1, CH_2Cl_2); UV λ_{max} 208 nm (ε = 1.6688 x 10^4). Analysis C 59.80% H 6.38% $C_{33}H_{42}O_{14}$ requires C 59.79% H 6.39%.

The NMR data(proton and C-13) agreed completely with the data reported by Klenk et al.[1] On examination by X-ray diffraction it was found that the crystal belonged to the orthorhombic system, space group $P2_1\ 2_1\ 2_1$ with cell parameters , a = 7.646 (3) A, b = 18.599 (2) A and C = 44.671 (7) A. Determination of the structure of Azadirachtin B by X-ray diffraction is in progress and will be reported separately.

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J. LIQ. CHROM. & REL. TECHNOL., 20(10), 1637–1639 (1997)

THE BOOK CORNER

CAPILLARY ELECTROPHORESIS GUIDEBOOK, PRINCIPLES, OPERATION, AND APPLICATIONS, Edited by K. D. Altria, Methods in Molecular Biology Series, Volume 52, Humana Press, Totowa, New Jersey, 1996, 349 pp., Price: $74.50.

In the last few years, we have witnessed the publication of a series of books dealing with CE applications. *Capillary Electrophoresis Guidebook* is a welcome addition to the CE library which contains no less than 15 books on the subject. The editor writes in the preface of the book, "This book is intended to be a working guide to the operation of capillary electrophoresis (CE) instrumentation. Since CE is still a rapidly maturing technique, detailed validated protocols are not widely established. Therefore, extensive experimental procedures are not provided for individual analyses. The intention is to provide general guidelines on the principles and practice of CE and to give an overview of the specific technologies and important applications areas." We agree with Dr. Altria that there is a need for validated and reproducible protocols and hope that this book will serve such a purpose.

The book is divided into two parts. The first part (11 chapters, 122 pages) deals with general guidelines of CE methods and instrumentation. This part is written by Dr. Altria and provides operating instructions for standard commercially available instruments. Guidelines are included for activities such as changing capillaries, method development, quantitative procedures, optimization of precision and sensitivity, and the validation of methods, fraction collection, and troubleshooting, as well as a quick guide to performing a separation. The second part of the book deals with applications of CE and special technologies (9 chapters, 220 pages).

Part II is made up of review chapters written by acknowledged experts in their particular fields. Specific technology-related chapters include micellar electrokinetic capillary chromatography, capillary gel electrophoresis, advanced sampling techniques, and electrochromatography. Important applications areas are covered, such as the analysis of proteins, peptides, amino acids, pharmaceuticals, chiral compounds, and nucleic acids. A further applications chapter covers a variety of additional areas.

Overall, the book is well written and referenced. Few omissions were found in the book; for example, on page 34, there are two omissions (work that was not given a reference or credit). The first one deals with ionic strength, Figure 2, on page 35. This figure is the work of McLaughlin et al. which was published in the Journal of Liquid Chromatography. The other omission is our work on the effect of ionic radius of electrolyte on current generation, which was published in 1990 in the Journal of Liquid Chromatography. Also, on page 97, reference numbers 19 and 23 are the same. Otherwise, the book is well documented, organized, and easy to read and follow. The book is recommended to all those interested in CE separations and, as I mentioned earlier, is a welcomed addition.

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Part II. Applications of Capillary Electrophoresis and Specific Technologies

Reviewed by
Haleem J. Issaq, Ph.D.
Book Corner Editor

J. LIQ. CHROM. & REL. TECHNOL., 20(10), 1641 (1997)

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Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097. Tel.: (954) 570-9446; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(10), 1643–1649 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

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

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

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

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

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

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

AUGUST 11 - 13: 10th International Symposium on Polymer Analysis and Characterization (ISPAC-10), University of Toronto, Canada. Contact: Prof. S. T. Balke, Dept. of Chem. Engg & Appl. Chem., Univ. of Toronto, Toronto, Ont., Canada, M5S 1A4. Tel/FAX: (416) 978-7495; Email: balke@ecf.toronto.edu.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

SEPTEMBER 14 - 17: International Ion Chromatography Symposium, Westin Hotel, Santa Clara, California. Contact: Janet Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052-0493, USA. Tel: (508) 359-8777; FAX: (508) 359-8778; Email: century@ixl.net.

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

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

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

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

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

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

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

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

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

1998

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

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

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

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

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

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

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

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

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

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

1999

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

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

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

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

2000

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

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

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Seattle, Washington. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

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

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

2002

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

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

2003

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

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

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

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

2005

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

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

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Book:

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

Chapter in a Book:

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

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

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

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