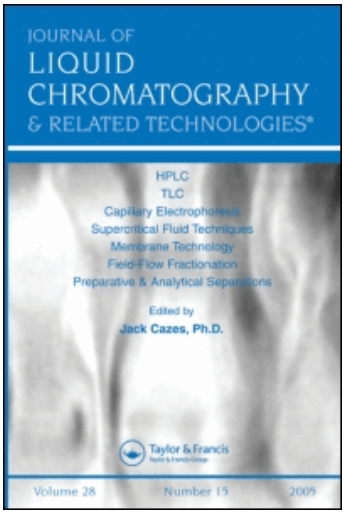

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Daniel W. Armstrong[a]; Randy Menges[a]; Irving W. Wainer[b]
[a] Department of Chemistry, University of Missouri-Rolla, Rolla, Missouri [b] Department of Onocology, McGill University, Montreal, Canada

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USE OF CENTRIFUGAL PARTITION CHROMATOGRAPHY AND PROTEINS IN THE PREPARATIVE SEPARATION OF AMINO ACID ENANTIOMERS

DANIEL W. ARMSTRONG*, RANDY MENGES, AND IRVING W. WAINER†
Department of Chemistry
University of Missouri-Rolla
Rolla, Missouri 65401

ABSTRACT

The growth of analytical methodologies for the separation of enantiomers has been impressive. Attention is now turning to the large scale separation of enantiomers. Often scaling-up sensitive analytical separations is ineffective and inefficient. Centrifugal partition chromatography (CPC) may be a viable alternative for the preparative separation of racemic mixtures in some cases. The use of proteins as chiral selectors in CPC is examined. Attention was focused on proteins that previously were used as bonded phases in analytical LC columns. The enzymatic properties of α-chymotrypsin allowed it to be used as a bioreactor in conjunction with CPC. When proteins are used as components of the stationary or mobile phase there can be problems with denaturation. However, when used in external incubation processes or as column bioreactors coupled with CPC, effective gram-scale separations can be performed. Tryptophan methyl ester was used as a model compound to evaluate this approach.

†Current address Department of Onocology, McGill University, Montreal, Canada H3G1Y6

INTRODUCTION

In the last few years there has been a tremendous increase in the scope and availability of chromatographic methods to separate enantiomers. These include over fifty different commercial varieties of liquid chromatographic chiral stationary phases (CSPs) and over ten different gas chromatographic CSPs (1-4). Indeed, the analytical separation of most enantiomers may be considered routine in the near future. However, the preparative-scale separation of enantiomers has not progressed as rapidly. Often there are severe limitations in scaling up sensitive analytical scale separations. These involve the loss of resolution with modest increases in the concentration of injected analyte, the unavailability of many CSPs for preparative-size columns, the high cost of available CSPs for preparative-scale columns, poor loadability of many CSPs and so forth.

Centrifugal Partition Chromatography (CPC), a variant of countercurrent chromatography, can be used in preparative scale separations (5-7). Since there is a liquid rather than a solid stationary phase, strongly retained solutes can be recovered by flushing the system. The large volume of liquid stationary phase makes CPC well suited for preparative separations since a greater amount of solute can be accommodated. Also, separation efficiencies improve with increased flow rate (8). This behavior is opposite to that observed for other chromatographic techniques. Despite the potential advantages of CPC in large-scale separation there have been no reports, to our knowledge, on its use in enantiomeric separations.

In this work we report the use of CPC in conjunction with proteins as chiral selectors to effect the resolution of amino acid esters. The use of protein selectors in CPC is potentially advantageous since a number of

protein-based HPLC chiral stationary phases have been reported (9-11). Hence, there is the possibility of using the analytical protein CSPs for rapid screening and determining optimum separation conditions. Subsequently, scaled-up separations could be done via CPC with the same chiral selector.

The enzyme, α-chymotrypsin, was chosen as the chiral selector because it was available in quantity at a reasonable cost and because data was published on its use as an HPLC chiral stationary phase. The immobilized α-chymotrypsin HPLC column was first described by Wainer and co-workers (11-13). This particular column had the unusual property that it could achieve the resolution of enantiomers via two completely different routes (12). Some enantiomeric solutes are substrates for the α-chymotrypsin enzyme (e.g., aromatic amino acid esters or amides). If one of these racemic substrates was injected on the α-chymotrypsin column, the L-enantiomer would bind to the active site of the immobilized enzyme and be hydrolyzed while the D-enantiomer would not. Hence, the column acts as both a reaction bed and a separating media for the L-amino acid and D-amino acid ester or amide. However, the α-chymotrypsin column also could act as a typical unreactive CSP support by forming transient diastereomeric complexes of different free energy with the two enantiomers. Either of these two means of achieving a separation could be useful in CPC.

EXPERIMENTAL

Materials

The CPC experiments were done using a centrifugal countercurrent chromatograph, model CPC-NMF, from Sanki Laboratories, Inc., Sharon Hill, PA. This instrument can be operated with up to 12 cartridges which have been described previously (5). The Sanki command module, model

CPC-FCU, was used to allow injection of the sample and selection of the ascending or descending mode. A LC pump, Shimadzu LC-5A, and a UV spectrophotometric detector, Shimadzu SPD-6A, were used with the CPC. A Linear, model 1200, recorder was used to record the CPC chromatograms.

All water used was distilled and passed through a Barnstead D8922 cartridge to trap organics and filtered through a 0.45 μm Alltech nylon 66 membrane to remove particles. HPLC grade 1-butanol and acetonitrile were purchased from Fisher, Fairlawn, NJ, and passed through a 0.45 μm filter prior to use. α-Chymotrypsin, bovine serum albumin and ovomucoid were purchased from Sigma Chemical Company, St. Louis, MO and used without further purification. Both racemic and enantiomerically pure phenylalanine, tryptophan and tyrosine and the racemic methyl ester derivatives of phenylalanine, tryptophan and tyrosine were purchased from Sigma Chemical Company, St. Louis, MO, and were used without further purification.

The enzymatic hydrolysis was monitored with HPLC. A Shimadzu LC-6A was used in the isocratic mode. The hydrolysis of aromatic amino acid methyl ester to the amino acid was monitored with a C_{18} column (ASTEC). 20% Acetonitrile and 80% buffer (1% triethylammonium acetate, pH = 5.1) and a flow rate of 1 ml/min were the mobile phase conditions to separate the methyl ester from the free amino acid. To ensure that enzymatic hydrolysis and not simple acid hydrolysis was taking place, the enantiomeric purity of the amino acid was monitored. This was done by using a Cyclobond III column (ASTEC) and the aforementioned HPLC. The mobile phase conditions were 100% buffer (1% triethylammonium acetate, pH = 5.1) and 0.75 ml/min.

Method

The enzymatic reaction was done in a vial in a water bath at 35°C. The methyl ester derivative of phenylalanine, tryptophan or tyrosine was dissolved in 1.5 ml phosphate buffer pH 7.0 with an ionic strength of 0.1. 0.5 ml of α-chymotrypsin solution (7.0×10^{-4} M) in the same buffer was added and stirred constantly. The reaction was monitored until a constant ratio of the amino acid to the starting methyl ester derivative was obtained. The reaction was always enantioselective, which indicates that no simple acid hydrolysis occurred. Portions of these reaction mixtures were injected directly into the CPC.

RESULTS AND DISCUSSION

Three different operational configurations were considered for the "CPC/protein chiral selector" experiments. Figure 1 shows the simplest set-up. The protein is dissolved in a buffered, aqueous, stationary phase that is evenly distributed throughout the engraved channels within the CPC rotor (for a complete description of the instrument see reference 5). An immiscible organic mobile phase is then pumped through the unit. It should be noted that the opposite configuration (e.g. an organic stationary phase containing protein and an aqueous mobile phase) can be used when working with lipophilic, membrane proteins. In the case of solutes that are substrates for α-chymotrypsin, the CPC unit acts both as a bioreactor and as a separation device. The main trouble with the Figure 1 configuration is that the protein-selector tends to denature during the experiment. This can occur at the aqueous-organic interface and in the bulk aqueous phase if the organic solvent is partially soluble in the aqueous phase (butanol for example). Of the three proteins tested (Figure 1), α-chymotrypsin and bovine serum

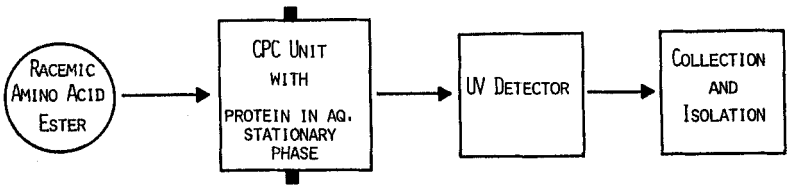

Figure 1. This diagram outlines the simplest set-up for a CPC separation of enantiomers using a protein chiral selector. In this case the protein is a component of the aqueous stationary phase. When α-chymotryposin was included, the stationary phase also contained pH=7.1 phosphate buffer (0.1 M) and 0.5 M NaCl. Hexane or butanol were both used as mobile phases. The disadvantage of this approach is that the protein selectors tend to denature unless they are very stable (see text).

albumin tended to denature during the experiment while ovomucoid appeared to be stable.

In order to utilize the catalytic properties of α-chymotrypsin and to avoid the possibility of denaturation, an external incubation step was added (Figure 2). When using an enzyme as a "bioreactor" there are a number of advantages in the external incubation approach (i.e., Figure 2 vs. Figure 1). First and foremost, enzyme deactivation through denaturation is avoided. Second, far less enzyme is used in an external incubation chamber than when it is used in the stationary phase of a CPC unit. Third, more efficient separations are achieved with preincubation. When the enzyme is present in the stationary phase, the first part of the CPC "column" is used as a bioreactor after which the separation of the L-amino acid and D-amino acid ester occurs on the remaining plates. When an external incubation step is used, all of the plates of the CPC are used for the separation.

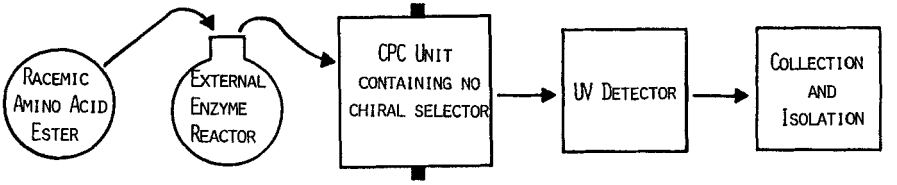

Figure 2. This diagram oulines a simple, efficient way to utilize labile enzymes as bioreactors in conjection with CPC. In a typical incubation step, 2 ml of aqueous solution containing ~ 90 mg of racemic tryptophan methyl ester was hydrolized by 0.4 μM α-chymotrypsin (pH = 7.1 phosphate buffer) for 5 hours.

Figure 3 shows a preparative CPC separation of D-tryptophan methyl ester from L-tryptophan after the injection sample was pretreated with α-chymotrypsin. In order to monitor the enzymatic digestion and the integrity of the CPC separation, both chiral and nonchiral analytical methodologies had to be employed. A reversed phase LC separation (Figure 4A) on a C_{18} column was employed to monitor the enzymatic production of tryptophan from the corresponding ester. When the peak ratios ceased to change, the reaction was assumed to be complete and the solution was injected into the CPC unit. However, the C_{18} separation gives no information on the enantiomeric purity of the tryptophan or the methyl ester. It is possible for nonenzymatic hydrolysis to occur either during the reaction step or during the CPC fractionation. Nonenzymatic hydrolysis would produce both D- and L- tryptophan resulting in a contaminated product. Therefore, an α-cyclodextrin bonded CSP (Cyclobond III) column was used to monitor the enantiomeric purity of the L-tryptophan. L-Tryptophan was found to be > 99% pure after CPC separation except within the small overlap area (Figure

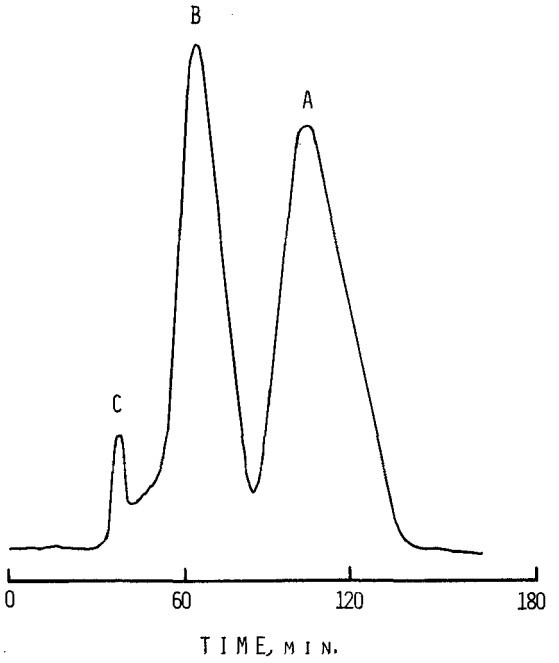

Figure 3. CPC chromatogram showing the separation of (A) L-tryptophan from (B) D-tryptophan methyl ester after incubation of ~ 90 mg of the racemic ester. Peak C consisted of impurities from the added enzyme. Experimental conditions are as described in Figure 2. The mobile phase was butanol saturated with water and the flow rate was 4 ml/min.

3, between 80 and 100 minutes). Using the method discussed previously (7) it was determined that approximately 880 mg of D,L-tryptophan methyl ester can be fractionated into enantiomerically pure products per injection. The low solubility of tryptophan in the butanol mobile phase limited further increases in the amount separated.

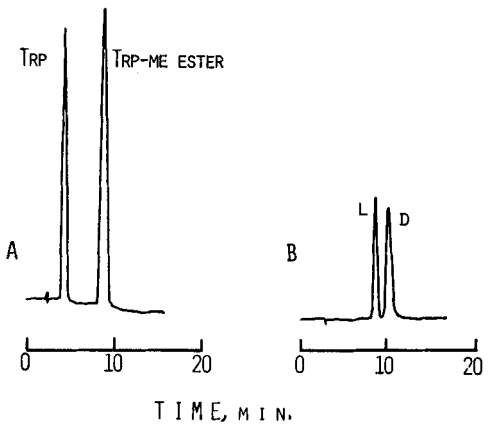

Figure 4. (A) Reversed phase LC separation of tryptophan (1st peak) and tryptophan methyl ester (2nd peak) on a 25 cm C_{18} column. The mobile phase was 80:20 (v:v) 1% triethylammonium acetate buffer, pH = 5.0/acetonitrile. The flow rate was 1.0 ml/min. (B) Enantiomeric separation of racemic tryptophan. A 25 cm α-cyclodextrin column was used. The mobile phase consisted of 1.0% triethylammonium acetate, pH = 7.0. The flow rate was 0.75 ml/min.

In a further extension of this technology, it is possible to couple an analytical protein column (which serves as a bioreactor) to the CPC thereby automating the system and preforming rapid successive purifications. This set-up is shown in Figure 5. The effluent from the protein column flows through a detector into the CPC injection vessel or loop. When the loop is full it is automatically injected into the CPC for separation. Immediately after injection the loop valve switches back to the fill position. The main parameter that must be controlled is the flow rate through the protein column and to the "injection-loop-reservoir". This flow rate must be sufficiently

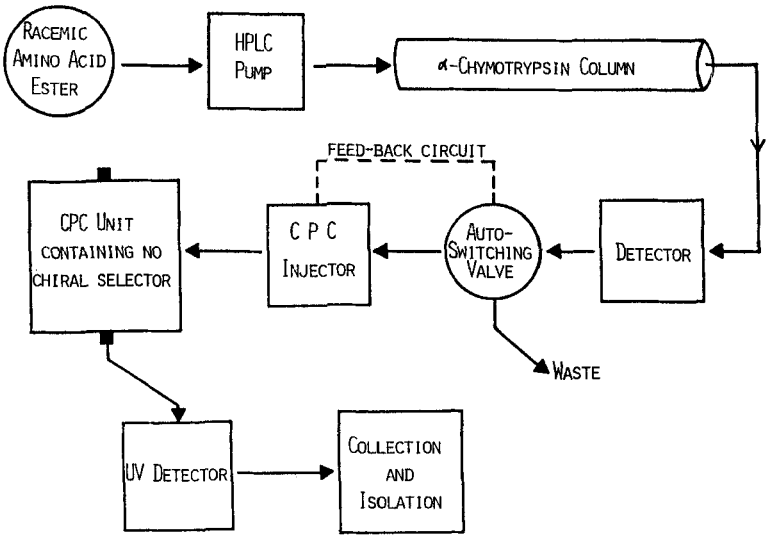

Figure 5. Diagram of an automated system for the gram-scale separation of amino acid enantiomers. In this approach the analytical-enzyme LC column is used as a bioreactor and is coupled to the CPC unit which completes the separation.

slow so that the subsequent injection produces no overlap between the first eluting peak of the second injection and the last eluting peak of the first injection. In the case of L-tryptophan and D-tryptophan methyl ester (Figure 3) the time between injections should be approximately two hours. Therefore, if 10 ml are being injected the flow rate through the α-chymotrypsin column must be 5 ml/hr. Approximately 10 grams of racemic tryptophan methyl ester can be resolved per day using this approach.

Future studies will continue to examine the use of proteins as chiral selectors in CPC. It is expected that the use of lipophilic proteins and highly

stable proteins, such as ovomucoid, will greatly expand the usefulness of this method.

ACKNOWLEDGEMENT

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