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Liquid chromatographic separation of radiopharmaceutical ligand enantiomers

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ABSTRACT

A unique cyclodextrin-based chiral separation is presented for enantiomers which do not fit the commonly held selection criteria for chiral separations. The enantiomers of N,N′-1,2-ethylenediylbis(cysteine), diethyl ester are separated as the analogous rhenium(V)oxo complexes using a Cyclobond II column and a methanol-water mobile phase. The rigidity added to the molecule via the formation of the metal complex is sufficient to replace the normal requirement for an aromatic ring in the molecule for successful separation.

INTRODUCTION

There is great interest in determining the chiral purity of pharmaceuticals because of the profound pharmacological differences which may exist between optical isomers. An example of these differences are the enantiomers of penicillamine [1]. The *R* enantiomer of penicillamine is an antiarthrytic while the *S* enantiomer is highly toxic. It is this type of pharmacological difference which has led regulatory agencies and pharmaceutical manufacturers to reevaluate the strategy of developing racemic *versus* single-enantiomer drugs. In 1989, the US Food and Drugs Administration (FDA) approved 23 new drugs, 9 non-chiral, 6 racemic, and 8 single enantiomers. Justification for developing a racemic mixture rather than a pure isomer drug is now expected. The same concerns over the chiral purity of therapeutic drugs are valid for diagnostic drugs, where enantiomers may exhibit large differences in distribution. This is the case for radiopharmaceuticals such as Neurolite (DuPont Merck, North Billerica, MA, USA), the active ingredient of which is the topic of this paper.

Historically, optical rotation measurements have been used to determine optical purity. However optical rotation measurements do not always provide adequate sensitivity [2] and they are not well suited to mixtures which contain more than one optically active component. For these reasons a more specific method such as chiral chromatography is often preferred.

The determination of chiral purity has blossomed in recent years with the development of chromatographic techniques which can be used to separate the enantiomers of a wide variety of compounds. There are several general approaches to the separation of enantiomers using commercially available chiral bonded phases for high-performance liquid chromatography. The first to be introduced were the Pirkle columns [3] which used derivatized silica columns in a normal-phase mode. Separations were achieved for compounds which had at least three modes of interaction with the stationary phase — π–π interactions, hydrogen bonding, dipole interac-

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tions, etc. Cyciodextrin columns were the first chiral phases intended for use in the reversed-phase mode [4]. These columns facilitate chiral separations through the formation of analyte–bonded phase inclusion complexes. A variety of modified cyclodextrin columns are now available for normal- and reversed-phase separations. A number of polymeric phases have also been used to achieve separation of enantiomers, among them the cellulosic [5], protein [6] and methacrylate [7] columns. A fourth type of chiral stationary phase are the ligand-exchange columns [8] which achieve enantiomeric separations via formation of metal-diastereomeric complexes.

Each of the four modes of chiral bonded phase chromatography requires specific functional group interactions in order to separate enantiomers. With the exception of ligand-exchange separations, some cellulosic columns (for example Chiralcel columns by Diacel), and recent reports using β-[9] and derivatized β-cyclodextrin columns [10], these techniques generally require that an aromatic ring be present in the molecule. This obviously restricts separations to those compounds containing such a group or those which may be derivatized. In the case of N,N′-1,2-ethylenediylbis(cysteine), diethyl ester, no ring is present, and the conditions required for derivatization close to the chiral center (high pH) are such that oxidation and racemization occur. Attempts at separating the enantiomers without derivatization were not successful. Attempts at separating the enantiomers by ligand exchange were likewise unsuccessful.

We first reported the separation of non-aromatic enantiomers using cyclodextrin columns in 1990 [11]. In this paper, we present a review of the development of a chiral separation for the enantiomers of N,N′-1,2-ethylenediylbis-(cysteine), diethyl ester (also referred to as ECD). N,N′-1,2-Ethylenediylbis-L-cysteine, diethyl ester, the single L,L-ECD enantiomer, is the active ingredient in Neurolite, a technetium based agent for single-photon emission computed tomography (SPECT) brain imaging. Upon radiolabelling of racemic ECD with technetium (or rhenium, as described here) there are four possible isomers; two enantiomers and two *meso* compounds. The separation of these enantiomers is unique in that it was accomplished for molecules which do not contain an aromatic ring. This successful separation suggests the need for an aromatic ring for selectivity in cyclodextrin based separations may in some cases be based on the rigidity it adds to the molecule rather than direct chemical interaction with a source of aromaticity.

EXPERIMENTAL

Reagents and chemicals

The L,L, D,D, and D,L isomers of N,N′-1,2-ethylenediylbis cysteine, diethyl ester were synthesized using modifications of the procedure by Blondeau et *al.* [12]. The structure of L,L-ECD · 2HCl is shown in Fig. 1. The conformations of the purified Re(O)ECD (hereafter referred to as ReECD) enantiomers were confirmed via X-ray crystallography [13]. The structures of the ReECD stereoisomers are shown in Fig. 2. Ammonium perrhenate (99+%) was obtained from Aldrich (Milwaukee, WI, USA). Sodium dithionite (Sodium hydrosulfite) was obtained from Mallinkrodt (Paris, KY, USA). Ultrapure water (Milli-Q, Millipore, Bedford, MA, USA) was used in preparation of the

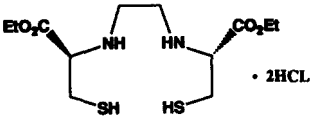

Fig. 1. Structure of L,L-ECD · 2HCl.

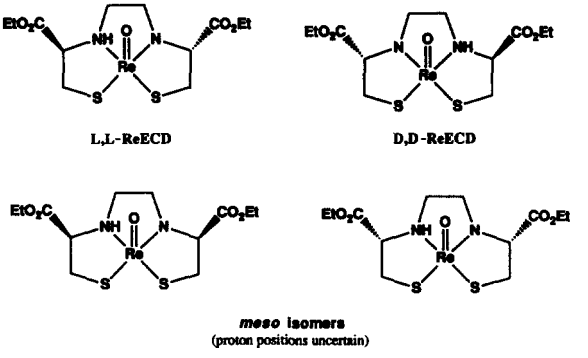

Fig. 2. Structures of stereoisomers of ReECD.

mobile phase. ACS-Grade sodium hydrogencarbonate, HPLC-grade methanol, and HPLC-grade chloroform were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Chromatographic system

The HPLC consisted of a Hewlett-Packard 1090M system (Hewlett-Packard, Avondale, PA, USA), equipped with a diode array UV-Vis detector monitoring at a wavelength of 210 nm. The analytical columns, Cyclobond I, I 3,5-dimethylphenyl carbamate (DMP), II and III were obtained from ASTEC Scientific (Whippany, NJ, USA). The majority of the work was performed using 500 mm x 4.6 mm Cyclobond II (γ-cyclodextrin) columns. Mixtures of methanol and Milli-Q water were prepared weekly and degassed by purging with helium. The flow-rate was varied during this study, but unless otherwise noted was set at 0.2 ml/min.

Preparation of rhenium complexes

Low-oxygen water (LOW) was prepared by purging Milli-Q water with low-oxygen nitrogen (LON). Ammonium perrhenate (169 mg), ECD · 2HCl (25.0 mg), and sodium hydrogencarbonate (10.6 mg) were added to a 30-ml vial with a crimp-seal top (the reaction vial). A 10-ml volume of LOW was added to the reaction vial, the vial was sealed and the contents dissolved through sonication. The reaction vial solution was then purged with LON for approximately 15 min via a syringe needle through the septum of the crimp seal.

A sodium dithionite solution was prepared at a concentration of 44 mg/ml in LOW. Immediately after dissolution, 5.0 ml of the solution were added to the 30-ml reaction vial. The contents of the vial were then allowed to react for a minimum of 3 h.

After the 3-h reaction time, the reaction vial solution is dark yellow in color and contains a dark precipitate. The contents of the reaction vial were added to a separatory funnel and extracted three times with 5-ml aliquots of chloroform, each time collecting the chloroform layer. The 15-ml extract solution was then taken to dryness. At the time of analysis, the sample was reconstituted with 10.0 ml methanol. A portion of this sample was then diluted with Milli-Q water to match the mobile phase composition.

RESULTS AND DISCUSSION

It would appear that the separation of ECD enantiomers was unlikely by the currently available separation modes without derivatixation, given the commonly held selection criteria. We did attempt the direct use of a Pirkle type column (covalent L-leucine, Regis), a β-cyclodextrin column (Cyclobond I, Astec), and ligand-exchange TLC plates (Chiralplate, Macherey-Nagel) and obtained no separation of the ECD enantiomers. Based on our knowledge of ReECD chemistry, it was felt that a separation of the enantiomers as metal complexes via formation of inclusion complexes with cyclodextrins was feasible. For successful separations using cyclodextrins it is necessary that the molecules contain a relatively rigid hydrophobic section that fits tightly into the cyclodextrin cavity. Interaction of analytes with the hydroxyls at the rim of the cyclodextrin cone serves to further orient the molecules within the cyclodextrin cavity. The Re complex of ECD is easily formed, is stable and is less susceptible to oxidation than free ECD. The rigidity added to ECD by forming the Re complex seemed a likely substitute for the presence of an aromatic ring. The Re oxo group also provides an additional point of interaction.

The preparation of ReECD, described earlier, is performed at room temperature to minimize possible racemixation. Furthermore, the choice of a non-chiral derivatixation precludes the problems inherent in chiral derivations used to produce diastereomers [2]. Typical yields from the reaction were approximately 60% (at 3 h) and the complex was stable for at least several weeks, even after dissolution in mobile phase.

Cyclodextrin columns are available in α, β and γ forms which have cones composed of 6, 7 and 8 glycopyranose units, respectively. Thus different cone diameters (approximately 7, 8 and 10 A, respectively) are available for optimizing the fit between analyte and cyclodextrin. It is not necessary for the entire molecule to fit inside the cavity. A screening study was performed using a

sample of racemic ReECD and four different cyclodextrin bonded phase columns (α, β, γ and β-DMP), using a methanol water mobile phase. Successful chiral separations using the α-cyclodextrins have typically involved molecules which contain a single aromatic ring, and it was anticipated that ReECD (approximately 13 x 8 x 5 Å) would be too large to enter the α-cyclodextrin structure. No separation of the enantiomers was achieved with the a-cyclodextrin, as the two enantiomers and one *meso* complex coeluted (Fig. 3). The retention of each isomer was determined using separate solutions of each enantiomer and a solution containing both meso isomers. The chromatography achieved with the β, derivatized β and γ phases (Fig. 3) shows at least a slight separation of the enantiomer peaks under the conditions of the screening experiments. These columns have been most successful in separating enantiomers containing two or more rings, and therefore molecules more similar in size to ReECD. Although the nature of the inclusion complex has not been determined, it is likely that ReECD is oriented with the thiolate groups pointed into the cyclodextrin cavity and that the ethyl ester and Re oxo groups interact with the external hydrogen bonding groups.

Optimization of the enantiomeric separation was continued using the y-cyclodextrin column. For our purposes we were interested in detecting the presence of small amounts of D,D-ECD in the presence of L,L-ECD, the desired enantiomer. The column length was doubled to 50 cm to increase efficiency and counter the effects of injecting larger masses of sample onto the column. The most common-mobile phases used

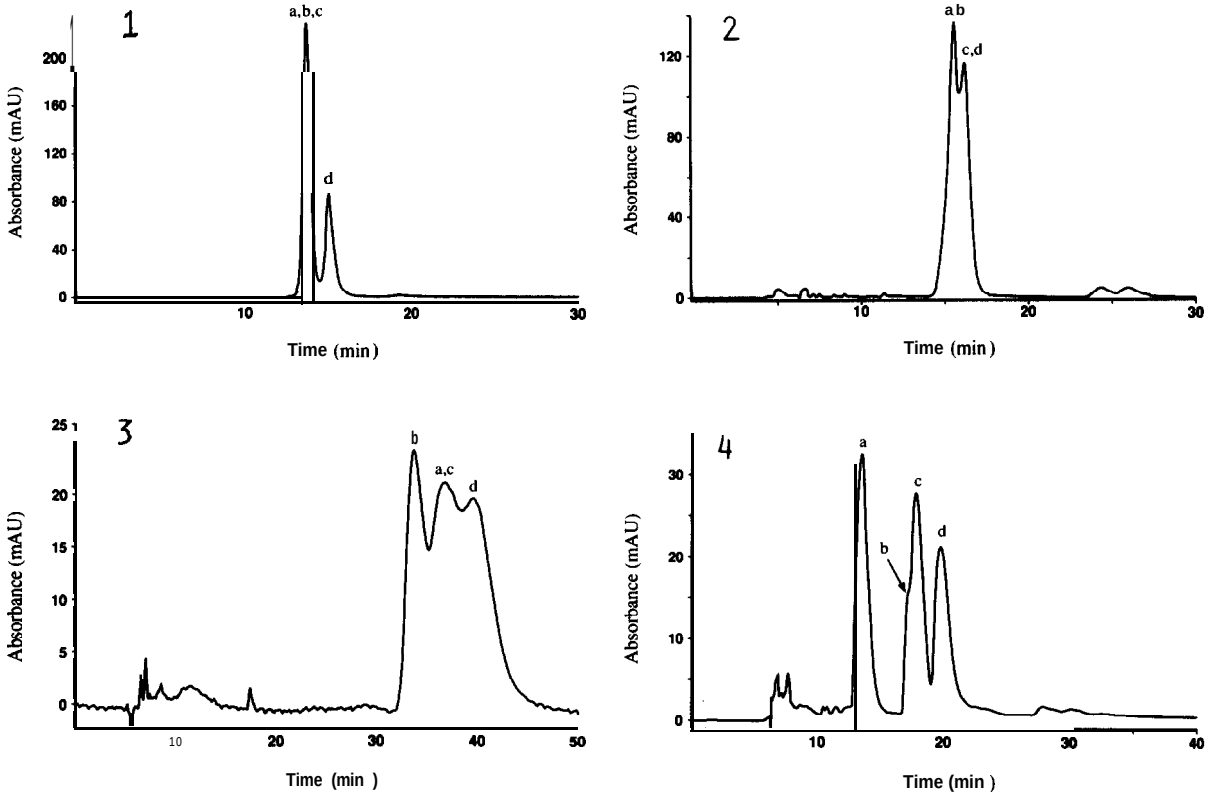

Fig. 3. Chromatograms of racemic ReECD using a (1) α-cyclodextrin column. [Cyclobond III, mobile phase methanol-water (25:75), flow-rate 0.5 ml/min], (2) β-cyclodextrin column [Cyclobond I, mobile phase methanol-water (20:80), flow-rate 0.5 ml/min], (3) derivatixed β-cyclodextrin column [Cyclobond I DMP, mobile phase methanol-water (55:45), flow-rate 0.2 ml/min], and (4) y-cyclodextrin column [Cyclobond II, mobile phase methanol-water (15:85), flow-rate 0.5 ml/min]. All columns were 25 cm x 4.6 mm. Peaks: a,d = *meso*-ReECD; b = D,D-ReECD; c = L,L-ReECD.

with cyclodextrin columns are mixtures of water and methanol or acetonitrile, a buffer, and an organic modifier such as triethylamine acetate (TEAA). These were evaluated using the γ-cyclodextrin column and it was determined that the modifier TEAA did not play a role in the separation and could be eliminated. Methanol, the lowest-strength solvent, was found to be sufficient to accomplish the separation. A plot of capacity factor versus percent methanol is shown in Fig. 4. This plot shows typical reversed-phase behavior -as the solvent strength increases the capacity factor decreases. This is in contrast to many cyclodextrin separations where at high organic mobile phase concentration increased retention occurs, presumably due to the increased strength of hydrogen bonding between the analyte and the cyclodextrin.

Reversed-phase separations using cyclodextrin columns often require lower flow-rates than commonly used in other types of reversed phase separations. We found that the best resolution was obtained using a 0.2 ml/min flow-rate. Also in contrast to typical reversed-phase separations where an increase in temperature quite often results in increased efficiency, the opposite effect is often seen for separations -based on formation of inclusion complexes. The stability of cyclodextrin complexes are, in general, much greater at lower temperatures. In our case however we found only a small increase in resolution (1.02 vs. 1.10) by lowering the temperature from room temperature to 4°C. Greater benefits may be obtained using acetonitrile instead of methanol at lower temperatures since it exhibits less viscosity change as a function of temperature.

As the separation conditions were optimized, we discovered that the sample capacity of the gamma column was quite low relative to typical reversed phase columns. The maximum sample mass injected before acceptable resolution was lost was approximately 3 μg. As a result of this mass limitation, the detection limit for the D,D enantiomer was determined to be 1%. Chromatograms showing a typical L,L-ECD sample (no D,D or *meso* isomers present) and a L,L-ECD sample spiked with 1% D,D-ECD are shown in Figs. 5 and 6. The optimized separation of a racemic mixture of ECD is shown in Fig. 7.

As part of our method ruggedness studies, we have evaluated this separation using five different Cyclobond II columns. These columns ranged from new, unused columns to columns on which hundreds of injections had been made. A plot of stereoisomer retention *versus* mobile phase methanol composition for a typical column is shown in Fig. 8. The chromatographic differences between the five columns is illustrated in Fig. 9, in which the L,L-ReECD retention for each column is plotted *versus* the mobile phase methanol composition. Adequate separation of the enantiomers and *meso* complexes were obtained with each column, but each column differed in the ratio of methanol and water required

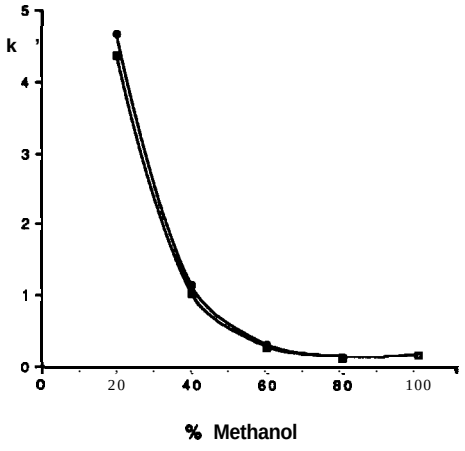

Fig. 4. Capacity factors for D,D-ReECD (■) and L,L-ReECD (0) as a function of % methanol in the mobile phase. Separation conditions, Cyclobond II column, 50 cm x 4.6 mm, flow-rate 0.2 ml/min.

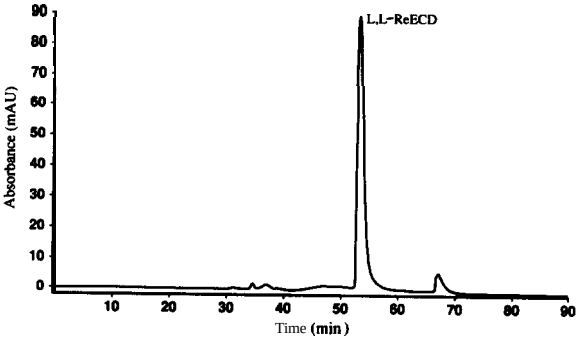

Fig. 5. Chromatogram of a typical L,L-ReECD sample. Column, Cyclobond II, 50 cm x 4.6 mm; mobile phase, methanol-water (50:50), flow-rate, 0.2 ml/min.

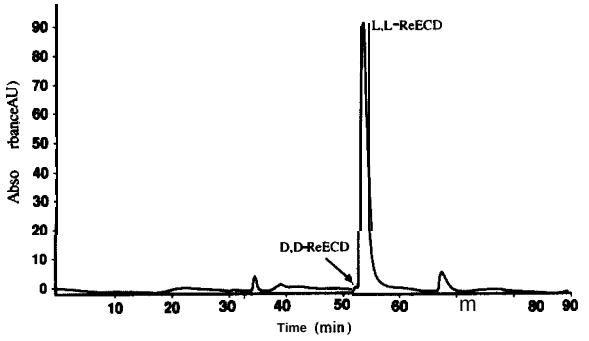

Fig. 6. Chromatogram showing detection of 1% D,D-ReECD in the presence of L,L-ReECD. Column, Cyclobond II, 50 cm × 4.6 mm; mobile phase, methanol-water (50:50), flow-rate, 0.2 ml/min.

for the separation. This is illustrated in Table I, where the % methanol required for a 65-min retention time for L,L-ReECD was calculated along with the retentions of the remaining three stereoisomers. Among the possible causes for these differences are column manufacturing variability (which may not be noticeable for the majority of separations performed with these columns), and changes in retentivity upon use due to accumulation of permanently retained sample components. In general we have found that a retention of between 60 and 70 min for the L,L-ReECD peak will result in adequate separation ($R_s \geq 1$) of the enantiomers.

This work has presented a new approach to the separation of non-aromatic enantiomers using cyclodextrin columns. The presence of a rigid non-aromatic ring can function in place of

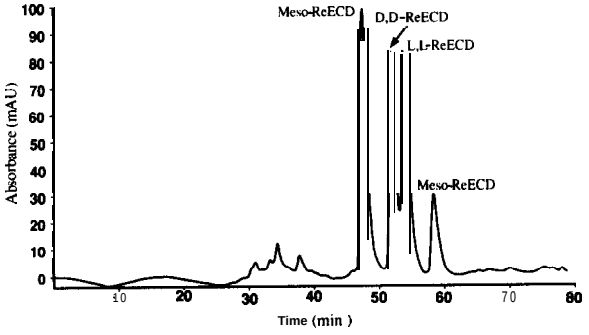

Fig. 7. Chromatogram of racemic ReECD using a γ-cyclodextrin column. Column, Cyclobond II, 50 cm × 4.6 mm; mobile phase, methanol:water (50:50), flow-rate, 0.2 ml/min.

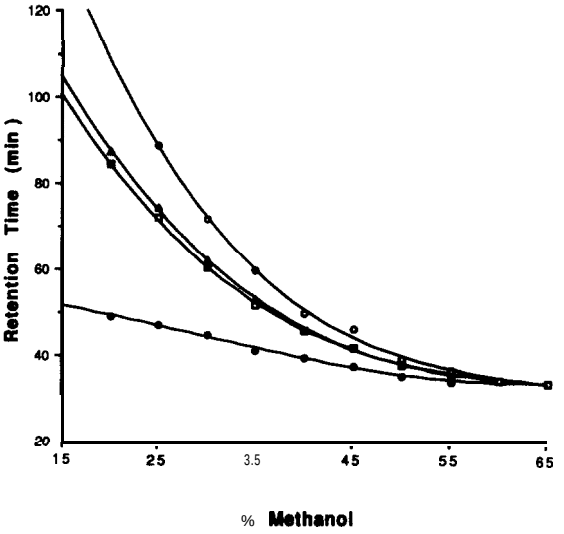

Fig. 8. Column ruggedness study showing retention changes as a function of mobile phase methanol concentration for a typical Cyclobond II column (50 cm × 4.6 mm). Symbols: ● = Re(*meso*ECD)1; □ = D,D-ReECD; A = L,L-ReECD; 0 = Re(*meso*ECD)2.

an aromatic ring when additional chemical interaction is not required. The formation of metal complexes may be desirable and generally applicable to the separation of the enantiomers of technetium labelled radiopharmaceuticals and other metal ligand complexes. If the thiolate

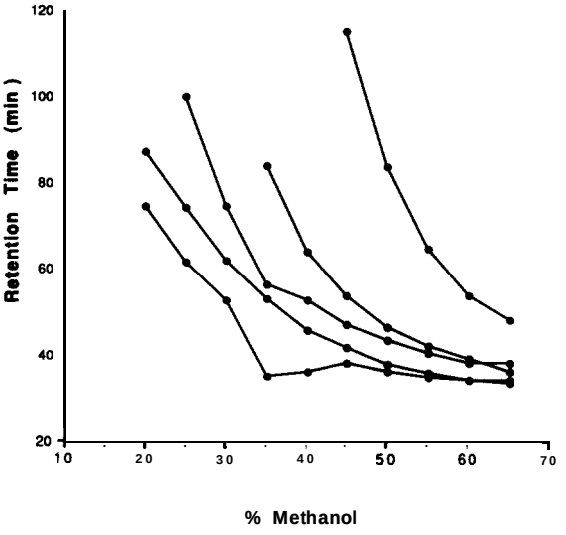

Fig. 9. Column ruggedness study showing L,L-ReECD retention changes as a function of mobile phase methanol concentration for five Cyclobond II columns (50 cm × 4.6 mm).

TABLE I

RETENTION TIMES FOR ReECD ISOMERS CALCULATED FROM FITTED CURVES (AS SHOWN IN FIG. 8) FOR EACH OF FIVE CYCLOBOND II COLUMNS

The % methanol is the percentage required to obtain a 65-min retention time for the L,L-ReECD peak.

Column no.	Retention time (min)				
	Meso 1	D,D-ReECD	L,L-ReECD	*Meso* 2	% MeOH
3818	51	63.5	65	70	23
4166	44	63.5	65	76	29
5460	59	62	65	71	55
5461	48	62	65	73	40
5462	49	62	65	73	33

groups are the key interaction with the interior of the cyclodextrin cavity, the separation of other cysteine related compounds may also be viable.

Additional studies are planned to evaluate the separation of ECD enantiomers by HPLC and capillary electrophoresis using cyclodextrin mobile phase additives. Recent studies have indicated that the use of cyclodextrins as mobile phase additives provides great flexibility in method development and yields efficiencies similar to that obtained with cyclodextrin bonded phase columns [14,15].

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