Basics of Chiral HPLC

Definitions
Principles
Available CSPs
Mobile phase types
The Field of Stereochemistry

1. All isomers have the same chemical formula but differ in the arrangement of certain chemical groups in space.

2. Many types of isomers have different physical and chemical properties and can be separated by conventional phases like C\textsubscript{18}.

3. Enantiomers have the same physical and chemical properties and can be recognized or separated only in chiral environment.
The Inclusion Complex

The basis for many chiral separations, especially in the reversed phase mode is a phenomena called inclusion complexing. First described for the polyglucose structures, cyclodextrins, it has been identified as a mechanism for the macrocyclic glycopeptides as well as the cellulose and amylose CSPs. An understanding of this phenomena is then imperative to an understanding of how these phases separate.

Structure and dimensions of the most common cyclodextrin molecules.

- **α-cyclodextrin**
- **β-cyclodextrin**
- **γ-cyclodextrin**

Schematic of one glucose unit in a cyclodextrin molecule.
Inclusion complexing is accomplished in reversed phase systems. Its effectiveness is dependent upon the position and strength of substituents on benzene or fused ring analytes. The elution order is typically meta<ortho<para. The bulky character of the meta and ortho limits full inclusion. The linear nature of the para structure results in full inclusion. Selectivity for this isomer is generally highest when strong hydrogen bonding groups are present.

Acetonitrile accelerates release from the cavity. Methanol reduces hydrogen bonding effects to the cyclodextrin hydroxyl groups. Combinations of acetonitrile and methanol in water are useful in optimizing a separation.
Examples of Inclusion Phenomena
Positional Isomers

**Xylenes**

![Chemical structure of xylenes](image)

- Peak 1 - 5.74 min. (ortho)
- Peak 2 - 6.38 min. (meta)
- Peak 3 - 6.83 min. (para)

**Cresols**

![Chemical structure of cresols](image)

- Peak 1 - 6.62 min. (ortho)
- Peak 2 - 7.31 min. (meta)
- Peak 3 - 9.82 min. (para)

CYCLOBOND I 2000
Mobile Phase: 30/70: CH₃CN/H₂O

CYCLOBOND I 2000
Mobile Phase: 40/60: CH₃OH/H₂O
Structural Isomers

Corticosterone

Cortisone

Hydrocortisone

Peak 1 - 6.29 min.
Peak 2 - 8.02 min.
Peak 3 - 9.18 min.

CYCLOBOND I 2000
Mobile Phase:40/60: CH₃CN/H₂O
Geometric Isomers

cis/trans Stilbene

Peak 1 - 5.60 min.
Peak 2 - 7.14 min.

CYCLOBOND I 2000
Mobile Phase: 70/30: MeOH/H₂O
Enantiomers

Remacemide

- Peak 1 - 5.74 min.
- Peak 2 - 6.79 min.

CYCLOBOND I 2000
Mobile Phase:
3.5/96.5: CH₃CN/PO₄(0.1M) pH 3.5
Diastereoisomers

2,2,3a,4,9,9a-Hexahydro-14-Pyrrolo[2,3g]quinoline-1-carboxylic (1-phenylethyl) amide

Peak 1 - 8.01 min.
Peak 2 - 11.66 min.

CYCLOBOND I 2000
Mobile Phase:
40/60: MeOH/0.1% TEAA, pH 6.0
**CHIRALITY** deals with steroisomers which are compounds with the same molecular formula and structure but different spacial orientations around a stereogenic center or axis commonly defined as a plane of symmetry. The molecular dissymmetry is based on the geometric nature of atoms like **carbon**, **sulfur**, **phosphorous** and **nitrogen**.
Models for Identification of Potential Racemates

A. Enantiomers based on **CARBON**

Type 1: Stereogenic Center

$$\begin{align*}
X-C^*Z & \quad Y \\
Z-C^*X & \quad Y \\
\end{align*}$$

**Ibuprofen**

- Peak 1: 5.77 min.
- Peak 2: 6.47 min.

CHIROBIOTIC V
10/90: THF/20mM Na Citrate, pH 6.3
Models for Identification of Potential Racemates

Type 2: Axis of Symmetry

1,1’-Binaphthyl-2,2’-diylhydrogenphosphate

Peak 1 - 9.12 min.
Peak 2 - 10.03 min.

CHIROBIOTIC V
30/70: ACN/0.1% TEAA, pH 4.1
Models for Identification of Potential Racemates

B. Enantiomers based on SULFUR
Possibilities include sulfoxide, sulfoximide, sulfinate and sulfonium ion.

Methyl phenyl sulfoxide

Peak 1 - 10.86 min.
Peak 2 - 14.49 min.
Models for Identification of Potential Racemates

C. Enantiomers based on **PHOSPHORUS**
Possibilities include phosphine, phosphine oxide, phosphinate and phosphonium ion.

Peak 1 - 7.53 min.
Peak 2 - 8.48 min.

Ifosfamide

CHIROBIOTIC T 10/90: THF/H₂O
Models for Identification of Potential Racemates

D. Enantiomers based on **NITROGEN**
Possibilities include amine oxide and ammonium ion.

![Chemical structures](image)

(+)- and (-)-Methylallylphenylbenzyl ammonium iodide

Peak 1 - 8.31 min.
Peak 2 - 11.40 min.

CHIROBIOTIC V
100/0.02/0.01: MeOH/HOAc/TEA
Definitions

R,S-CONFIGURATIONS:

Term used to describe the absolute conformation of a chiral compound (by Cahn, Ingold and Prelog) according to their sequence rules. “R” is the abbreviation for rectus (Latin, meaning right or clockwise), while “S” is the abbreviation for sinister (Latin, meaning left or counterclockwise).


THE 2^n RULE:

The maximum number of stereoisomers that can exist for a compound containing more than one chiral center is $2^n$, where $n$ is the number of chiral centers.

$\begin{align*}
\text{NH}_2 & \\
\text{OH} & \\
\end{align*}$

$N=2$

Number isomers $\therefore (2^2)=4$
例:
如果比旋光度
e(B) = +60°
e(B) = +30°

求%(光纯度)和(对映体富集度)。

解:
1) 光学纯度 = \frac{+30°}{+60°} \times 100 = 50%

2) 对映体百分比
设 x = (+)对映体百分比
设 y = (-)对映体百分比

x + y = 100%
x - y = 50%

2x = 150%
x = 75% (+)

75 + y = 100
y = 100 - 75
y = 25% (-)
MESO COMPOUND:

A compound whose functional groups are superimposable on their mirror images even though they contain chiral centers. It is optically inactive.

2,6-bis-(1-phenyl-ethyl)-4-methylaniline

Peaks:
- Peak 1 – 6.19 (meso)
- Peak 2 – 7.96
- Peak 3 – 8.47

CYCLOBOND I 2000 RSP
250x4.6mm
30/70: CH$_3$CN/0.1% TEAA, pH 6.5
1.0 mL/min.
Note: Conventional reversed phase would separate this analyte into 2 peaks (diasteromers). Only a chiral stationary phase would resolve all 4 enantiomers.

Peak 1 – 11.54 min.
Peak 2 – 12.29 min.
Peak 3 – 16.57 min.
Peak 4 – 19.35 min.
RACEMATES OR RACEMIC MIXTURE:
A 50:50 mixture of enantiomers. This mixture is optically inactive due to the rotation of one molecule exactly cancelling the opposite rotation of its enantiomer.

OPTICAL ACTIVITY:
Rotation of the plane of polarized light caused by the presence of a stereogenic center or axis in a compound.

NOMENCLATURE:
Amino acids, sugars and related compounds still refer to the D,L designation. This system, invented by Emil Fisher, refers to the configuration of the glyceraldehyde. He arbitrarily assigned the + isomer of glyceraldehyde as the D isomer.

D-(+)-Glyceraldehyde

The d and l designation refers to the rotation of plane polarized light (Sodium d-line). If this light is rotated to the right, the designation is “d” or (+). This assignment has problems as D-glutamic acid is actually l or (-) for the rotation of polarized light. Care must be exercised in using D,L or d,l.
Principles Governing Chiral Separation

**Concept:** formation of a diastereomeric complex in a chromatographic equilibrium such that the nonchiral interactions are at minimum strength and the differential chiral interaction is at maximum strength. Identifying those points of interaction between the stationary phase and the racemate guides you in the choice of CSPs and the best conditions under which to operate.

Non-chiral interactions generally anchor a molecule and, therefore, assist in the formation of the diastereometric complex. Both $\pi-\pi$ interactions driven in normal phase phase solvents and inclusion complexation driven in reversed phase modes are the first significant areas to address the potential of an appropriate chiral stationary phase.
All forces in the chiral interaction process **do not** have to be attractive, they can be attractive as well as repulsive. Commonly described forces for chiral recognition are listed as follows:

- $\pi-\pi$
- **Hydrogen bonding**
  - a. Hydrogen donor site
  - b. Hydrogen acceptor site
- **Inclusion complexation**
- **Steric hindrance**
- **Dipole-dipole**
- **Ionic interaction**
Modern Chiral Stationary Phases

Polymeric

Synthetic
- Methacrylate
- Polycyclic amine-3

Natural
- Cellulose
- Amylose
- Proteins

Small molecule ligands
- Copper complex-2
- π-complex
- Crown ether
- Cyclodextrin-12
- Macrocyclic glycopeptides-6

Focus – normal phase, low cost, reproducible, high capacity

Astec-Supelco Phases

These 3 cover applications that are possible on 140 CSPs that have been created worldwide in this area.
Astec-Supelco Chiral Phases

Polycyclic Amine Phases
P-CAP, P-CAP-DP, P-CAP-EV

Copper Complex Phases
CLC-D, CLC-L

Macrocyclic Glycopeptide Phases
CHIROBIOTIC V, CHIROBIOTIC V2, CHIROBIOTIC T, CHIROBIOTIC T2, CHIROBIOTIC TAG, CHIROBIOTIC R
Astec-Supelco Chiral Phases con’t

Cyclodextrin Phases

CYCLOBOND I 2000

CYCLOBOND I 2000 AC, CYCLOBOND I 2000 SP,
CYCLOBOND I 2000 RSP, CYCLOBOND I 2000 HP-RSP,
CYCLOBOND I 2000 SN, CYCLOBOND I 2000 RN,
CYCLOBOND I 2000 DM, CYCLOBOND I 2000 DMP,
CYCLOBOND I 2000 DNP,
CYCLOBOND II, CYCLOBOND II AC
Structure of Vancomycin CSP

CHIROBIOTIC V

- Ionic site
- $\pi$-acceptor
- Sugar moieties
- Hydrogen bonding and dipole stacking sites

A, B, C are inclusion pockets (weak)
Structure of Teicoplanin CSP

Teicoplanin, CHIROBIOTIC T

Teicoplanin Aglycone, CHIROBIOTIC TAG

Key sites

sugar and alkyl chain

ionic site

ionic site

ionic site

ionic site

ionic site
CHIROBIOTIC V2 and CHIROBIOTIC T2

- Extensions of the CHIROBIOTIC V2 and T2 created by changing the position of several linkages and the chain length used to anchor the ligand.

- These changes can enhance the selectivity and capacity of these phases mainly in the polar ionic and polar organic modes, and sometimes in reversed phase.
Proposed Structure of Ristocetin A CSP

CHIROBIOTIC R

- Key ionic site
- Complex sugar
- Inclusion cavities
Bonded Derivatized Cyclodextrins

<table>
<thead>
<tr>
<th>R =</th>
<th>Suffix</th>
<th>CD Type</th>
</tr>
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<tbody>
<tr>
<td>- OCH₃</td>
<td>DM</td>
<td>β-CD</td>
</tr>
<tr>
<td></td>
<td>(methylated)</td>
<td></td>
</tr>
<tr>
<td>- COCH₃</td>
<td>AC</td>
<td>β-CD</td>
</tr>
<tr>
<td></td>
<td>(acetylated)</td>
<td>γ-CD</td>
</tr>
<tr>
<td>OH</td>
<td>SP or RSP/HP-RSP</td>
<td>β-CD</td>
</tr>
<tr>
<td>- CH₂CHCH₃</td>
<td>(hydroxypropyl ether)</td>
<td></td>
</tr>
<tr>
<td>CH₃</td>
<td>RN or SN</td>
<td>β-CD</td>
</tr>
<tr>
<td></td>
<td>(naphthylethyl carbamate)</td>
<td></td>
</tr>
<tr>
<td>CH₃</td>
<td>DMP</td>
<td>β-CD</td>
</tr>
<tr>
<td></td>
<td>(3,5-dimethylphenyl carbamate)</td>
<td></td>
</tr>
<tr>
<td>O₂N</td>
<td>DNP</td>
<td>β-CD</td>
</tr>
<tr>
<td>- CF₃</td>
<td>(2,6-dinitro-4-trifluoromethyl phenyl ether)</td>
<td></td>
</tr>
</tbody>
</table>

* Signifies stereogenic center
Most Productive CYCLOBOND Phases

- CYCLOBOND I 2000
- CYCLOBOND I 2000 HP-RSP (highest hit rate)
  Primarily basic chiral compounds have been resolved on the RSP and to a lesser extent both neutals and acidics. All successful separations have been in the reversed phase mode with the organic component in the range of 5-40%.
- CYCLOBOND I 2000 DMP (second most productive CSP in this line) This is a π-basic phase.
- CYCLOBOND I 2000 DNP (new addition)
  This is a π-acidic phase that has demonstrated separations not previously possible on any cyclodextrin phase.
CYCLOBOND

two different enantioselective retention mechanisms

A: Polar organic mode (surface)

B: Reversed phase mode (inclusion)
Polar Organic: Structural/Functional Group Requirements

- **Structural Requirements:**
  
  2 functional groups capable of interacting with the stationary phase

- **Types of Functional Groups:**
  
  Halogen:  \( I > Br > Cl > F \)
  
  Amine:  \( 3° > 2° > 1° \)
  
  Carbonyl:  \(-COOH, -CHO, -C=O, -COOR\)
  
  Sulfo-, phospho- and hydroxyl groups

  One of these functional groups must be on or alpha to the stereogenic center
Mobile phases types and mechanisms

- Normal Phase
- Reversed Phase
- Polar Organic
- Polar Ionic
Normal phase and types of interaction

**Composition:** Hydrocarbon solvent: hexane or heptane + polar alcohol: IPA or EtOH

**Dominant interactions:** $\pi-\pi$ interaction, hydrogen bonding

**Type of CSPs:** CYCLOBOND I 2000 DMP, SN, RN, CHIROBIOTIC V, T, R and TAG
Normal Phase Solvents Mephenytoin

Armstrong, Tang, S. Chen, Zhou, Bagwill and J.R. Chen.
Gradient Separation in the Normal Phase

Derivative: 3,4-Dinitrobenzoyl
CYCLOBOND I 2000 SN
Hexane/EtOH Gradient

α-Methylbenzylamine
2-Aminoheptane
Tryptophan methyl ester
Reversed Phase

**Composition:** Organic solvent: ACN, MeOH or THF + aqueous buffer: TEAA, NH$_4$OAc

**Dominant interactions:** inclusion, hydrogen bonding

**Type of CSPs:** CYCLOBOND I 2000, RSP,AC, DMP,SN, RN, II, III, CHIROBIOTIC V, T, R and TAG
Reversed Phase Separation
5-Methyl-5-phenyl hydantoin

Ref: Anal. Chem.,
Reversed Phase Solvents
Factors Influencing a Separation

- pH
- Organic modifier
- Buffer type and concentration
- Flow rate
- Temperature
Polar Organic Phase

**Composition:** ACN + MeOH + HOAc + TEA

**Dominant interactions:** Hydrogen bonding, dipole-dipole

**Type of CSPs:** *Cyclodextrins only* – CYCLOBOND I 2000, RSP, AC, DMP, SN, RN
Polar Organic Solvents

Factors Influencing Retention and Selectivity

**CYCLOBOND Phases Only**

There are four components to the original polar organic mode (v/v/v/v):

- Acetonitrile 50-100 parts
- Methanol 0-50 parts
- Anhydrous, Glacial Acetic Acid 0.1 to 1.0 parts
- Anhydrous Triethylamine 0.1 to 1.0 parts

This composition is primarily used for cyclodextrins and cyclodextrin derivatives.

- To increase selectivity alter ratio of acid to base
- To decrease retention without affecting selectivity:
  - Increase acid/base concentration at same ratio
  - Increase methanol concentration
- To increase retention:
  - Reduce or eliminate methanol
  - Decrease acid/base concentration at same ratio
New Polar Ionic Mode

**Composition:** MeOH + HOAc + TEA

**Dominant interactions:** Ionic interaction, hydrogen bonding

**Type of CSPs:** Macrocyclic glycopeptides only - CHIROBIOTIC V, T, R and TAG
Polar Ionic Mode: CHIROBIOTIC Phases Only

**BENEFITS:**

- Faster, more efficient separations, low pressure, long column life
- Faster method development, simple optimization, broad selectivity
- Best use ammonia and formic acid or acetic acid modifiers (0.01-1.0 parts) in 100 parts methanol for LC/MS/MS compatibility.
- Analyte salts easily disassociated
- Complimentary to normal phase separations on polysaccharide CSPs
- Very useful in preparative purifications to replace hexane/ethanol.
  - Lower boiling point than heptane or hexane, higher evaporation rate
  - Less toxic
  - Higher evaporation rate
POLAR IONIC MODE

Mobile Phase Components

- Methanol/Acid/Base Components:
  - Methanol, anhydrous
  - Acid: anhydrous TFA, acetic acid, formic acid
  - Base: TEA, DEA, NH3
  - Alternatives: ammonium formate or acetate

- Ratio of Acid to Base
  - Controls only selectivity
  - Rate: 4:1 to 1:4, most typical 2:1

- Concentration of Acid + Base
  - Controls retention and selectivity
  - Concentration range: 0.001 to 1.0 part per 100, most typical 0.10.
Conversion from Old Polar Organic Mode to New Polar IONIC Mode

For CHIROBIOTIC Phases Only

Albuterol

Peak 1 - 17.04 min.
Peak 2 - 18.37 min.
Peak 1 - 11.28 min.
Peak 2 - 12.45 min.

70/30/0.5/0.2: CH₃CN/CH₃OH/HOAc/TEA
100/0.01/0.01: CH₃OH/TFA/NH₄OH
Acid-Base Effects

With the new polar organic mode different acids and bases could be used, unlike the situation with the cyclodextrin, which demonstrates selectivity only employing acetic acid and triethylamine.

Terbutaline

\[
\begin{align*}
\alpha &= 1.38 \\
\text{CH}_3\text{OH}/\text{HOAc}/\text{TEA} &\quad 100/0.3/0.2 \\
\alpha &= 1.30 \\
\text{CH}_3\text{OH}/20\text{mM NH}_4\text{Ac} \\
\alpha &= 1.30 \\
\text{CH}_3\text{OH}/\text{TFA}/\text{NH}_4\text{OH} &\quad 100/0.05/0.05
\end{align*}
\]
Comparison Using HOAc/TEA vs ATFA in the Polar IONIC Mode

N-Benzyl-a-methylbenzylamine
CHIROBIOTIC V, 250x4.6mm

Peak 1 – 17.09 min.
Peak 2 – 18.82 min.

Peak 1 – 15.21 min.
Peak 2 – 16.66 min.

HOAc/TEA in Mobile Phase
100/0.02/0.01: MeOH/HOAc/TEA

ATFA* in Mobile Phase
100/0.02 v/w: MeOH/NH₄OOC CF₃

*Works best with CHIROBIOTIC V.
Comparison of Four Basic Mobile Phase Types for CHIROBIOTIC CSPs

- **Polar IONIC mode composition:**
  Methanol + Acid + Base (100+0.1+0.1, v/v/v)
  or Methanol + Volatile Ammonium Salt (100+0.1% v/w)

- **Normal Phase mode composition:**
  Polar + Nonpolar (EtOH+Heptane)

- **Polar organic mode composition:**
  Polar/Nonpolar (MeOH or EtOH or ACN or combinations, eg MeOH/ACN)

- **Reversed Phase composition:**
  Organic + Aqueous Buffer (ACN+TEAA; ACN & NH4OAc)
Temperature Effects

- Shorter analysis time
- Higher efficiency

**Arginine**

- CHIROBIOTIC T, 25°C
- 30/70 MeOH/ 10 mM NH₄OAc (pH4.0)
- 1.0 mL/ min, ELSD

**Diacetyl-cysteine (N¹⁵)**

- CHIROBIOTIC T, 25°C
- 100/ 0.1% (v/ w) MeOH/ NH₄OAc
- 1.0 mL/ min, ELSD

**Arginine**

- CHIROBIOTIC T, 45°C
- 30/ 70 MeOH/ 10 mM NH₄OAc (pH4.0)
- 1.0 mL/ min, ELSD

**Diacetyl-cysteine (N¹⁵)**

- CHIROBIOTIC T, 55°C
- 100/ 0.1% (v/ w) MeOH/ NH₄OAc
- 1.0 mL/ min, ELSD
Application of Temperature Effect in Loading Study of Diacetyl-Cysteine (N\textsuperscript{15})

CHI ROBI OTI C T, 250x4.6mm
Mobile Phase: MeOH/ NH\textsubscript{4}OAc (0.1% v/ w)
Detection: UV@230nm

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Flow Rate</th>
<th>Loading</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>23°C</td>
<td>2.0 mL/ min</td>
<td>15 mg</td>
<td>Less Time!</td>
</tr>
<tr>
<td>23°C</td>
<td>1.0 mL/ min</td>
<td>15 mg</td>
<td>Less mobile phase additive!</td>
</tr>
<tr>
<td>35°C</td>
<td>1.0 mL/ min</td>
<td>15 mg</td>
<td></td>
</tr>
</tbody>
</table>

Semi-Prep

Higher Temp

Flow Rate: 2.0 mL/ min
Loading: 15 mg

Flow Rate: 1.0 mL/ min
Loading: 15 mg

sigma-aldrich.com
Practical Guides for CSP Screening
Historical Chiral Screening Approaches

- Emphasized molecular structure and analysis of analyte-CSP surface interaction as a predictive tool

- Utilized databases and literature searches for similar molecules [but minor structural differences often resulted in a loss of selectivity for some CSPs]

- Screen all CSPs!!
Aim Method Development Process

- To quickly identify a suitable column for selectivity, in the minimum number of experiments
Method Development Approaches

- Single column with multi-mobile phases (mainly CHIROBIOTIC phases)

- Multi-column switching with minimal number of simple mobile phases
Choose a small set of CSPs that:

- are broad-based to increase chances of success for a wide range of molecular types
- offer selectivity in a wide range of mobile phases for increased selectivity possibilities and sample solubility
- are complementary to each other, minimum overlap in selectivity
Complementary

Defined as:

Offering separation potential in areas not possible with a given CSP, i.e., CHIROBIOTIC phases handle more polar molecules better than amylose or cellulose.

Offer increased selectivity in same mobile phases conditions, i.e., substituting one CHIROBIOTIC phase for another.
Complementary Separations

CHIROBIOTIC R versus CHIROBIOTIC V

4-Benzyl-2-oxazolidinone

![Chemical Structure](image)

**Peak 1 (S)** 6.01  
**Peak 2 (R)** 6.91  
**Peak 1 (R)** 6.54  
**Peak 2 (S)** 7.15

CHIROBIOTIC R  
CHIROBIOTIC V

Mobile Phase: 50/50: Hex/EtOH
Complementary Separations

CHIROBIOTIC T versus CHIROBIOTIC R

Naproxen

Mobile Phase: 30/70: MeOH/0.1% TEAA, pH 4.1
Screening Strategy

Current industry trend is for generic screening methods with:

- a simple set of columns combining CHIROBIOITIC and Chiralcel/pak* CSP’s
- a minimum number of solvents covering all five mobile phase types

*Chiracel and Chiralpak are the trademarks of Daicel.
Distinct Mobile Phase Choices

- Normal phase

- Polar mobile phases
  - polar ionic mode: CHIROBIOTIC phases only
  - polar organic mode: CYCLOBOND phases only
  - Polar mode: Chiralcel/pak and CHIROBIOTIC phases

- Reversed phase
Normal Phase vs Polar Ionic Mode

**Metoprolol**

![Chemical Structure of Metoprolol]

**CHIRACEL OD®**
- Peak 1 – 11.9 min.
- Peak 2 – 18.2 min.

| Normal Phase | 20/80/0.1: IPA/Hex/DEA |

**CHIROBIOTIC T®**
- Peak 1 – 15.36 min
- Peak 2 – 17.11 min

| Polar Ionic Mode | MeOH/0.1% ATFA |
# Normal Phase vs Polar Ionic Mode

## Alprenolol

| CHIRACEL OD | Peak 1 – 12.4 min.  
|             | Peak 2 – 16.4 min. |
| Normal Phase | 20/80/0.1: IPA/Hex/TFA |
| CHIROBIOTIC V | Peak 1 – 7.69 min.  
|              | Peak 2 – 8.33 min.  |
| Polar Ionic Mode | 100/0.01/0.01: MeOH/HOAc/TEA |
Cellulosic vs CHIROBIOTIC CSP’s: RP vs PIM

**Tolperisone**

**CHIRALPAK® AD-RH**
- Peak 1 – 5.23 min
- Peak 2 – 6.06 min

*Reversed Phase* 60/40: ACN/20mM Borate

**CHIROBIOTIC V2**
- Peak 1 – 8.80 min
- Peak 2 – 10.69 min

*Polar Ionic Mode* 100/0.1%: MeOH/ATFA

CHIRALPAK® is the registered trademark of Daicel Chemical Industries, Ltd
Broad Selectivity Based on the Same Stereogenic Center with CHIROBIOTIC T Example: Amino Alcohols

Mobile Phase: 100/0.1/0.1:MeOH/HOAc/TEA @ 2.0 mL/minute
Broad Selectivity Based on the Same Stereogenic Center with CHIROBIOTIC V: Example: Profens

**Fenoprofen Methyl Ester**

![Fenoprofen Methyl Ester structure]

1. 8.31
2. 9.72

**Fenoprofen**

![Fenoprofen structure]

1. 8.10
2. 9.56

**Ketoprofen**

![Ketoprofen structure]

1. 8.16
2. 9.91

**Ibuprofen**

![Ibuprofen structure]

1. 5.77
2. 6.47

**Flurbiprofen**

![Flurbiprofen structure]

1. 8.53
2. 11.19

**Naproxen**

![Naproxen structure]

1. 8.13
2. 9.98

Mobile Phase: 10/90: THF/10mM Na Citrate, pH 6.3 @ 1.0 mL/min.
Purpose of Assay

Generic screening develops options for a variety of applications

- Quick analytical method (possibly suitable for later optimization and validation)
- Suitable for possible small scale prep
- Trace analysis of unwanted isomer
- Impurity profiling
- LC-MS method
## Complementary Method Development

<table>
<thead>
<tr>
<th><strong>Cellulose &amp; Amylose (CHIRALCEL/CHIRALPAK)</strong></th>
<th><strong>Macrocyclic Glycopeptides (CHIROBIOTIC)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad applicability over wide range of compound types</td>
<td>Broad applicability over wide range of compound types</td>
</tr>
<tr>
<td>Traditionally NP, but now also RP, POM (different columns)</td>
<td>Designed to provide selectivity in PIM, RP, NP (same column)</td>
</tr>
<tr>
<td>Chiral interaction sites via different chemistries &amp; helical structure</td>
<td>Large number of chiral interaction sites</td>
</tr>
<tr>
<td>Most useful are AD, OD, AS, OJ</td>
<td>Most useful are V2, T, R, TAG</td>
</tr>
<tr>
<td>Coated phases</td>
<td>Chemically bonded</td>
</tr>
</tbody>
</table>
Complementary Method Development

CHIRALCEL/PAK:
- Compound must be in neutral form - interaction always non-ionic
- Separate samples into acids, bases and neutrals (neutrals can be screened with either acids or bases)

CHIROBIOTIC:
- Compound can be ionised, or can be a salt – ionic interactions are a key mechanism
- Same mobile phase screens are used for all samples, but can choose selective screening for acid, bases or neutrals

Note: Functional group on or near stereogenic center dictates whether analyte is acid or base
CHIRAL METHOD DEVELOPMENT SCREEN
CHIROBIOTIC & CYCLOBOND PHASES

1. COLUMN INSTALLATION
CHIROBIOTIC™ columns are shipped in methanol. Before starting to use a new column, wash with 20 mL HPLC grade methanol at 1 mL/min. The column test standard, 5-methyl-5-phenylhydantoin, can be injected at this stage.

CYCLOBOND™ columns are shipped in IPA and should be washed with 30 mL HPLC grade water at 0.8 mL/min before starting the method development screen.

2. MOBILE PHASE CHOICE

<table>
<thead>
<tr>
<th>No.</th>
<th>Mobile Phase</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MeOH/20mM NH₄OAc, pH 4.0</td>
<td>20/80</td>
</tr>
<tr>
<td>2</td>
<td>MeOH/20mM NH₄OAc, pH 6.0</td>
<td>20/80</td>
</tr>
<tr>
<td>3</td>
<td>ACN/20mM NH₄OAc, pH 4.0</td>
<td>30/70</td>
</tr>
<tr>
<td>4</td>
<td>ACN/20mM NH₄OAc, pH 6.0</td>
<td>30/70</td>
</tr>
<tr>
<td>5</td>
<td>MeOH/10%Ac/TEA*</td>
<td>100/0/1/0.1</td>
</tr>
<tr>
<td>6</td>
<td>ACN/MeOH/10%Ac/TEA</td>
<td>95/5/0/3/0.2</td>
</tr>
<tr>
<td>7</td>
<td>ETOH/Hexane (or heptane, isohexane)</td>
<td>30/70</td>
</tr>
<tr>
<td>8</td>
<td>Washing cycle</td>
<td>100% ETOH</td>
</tr>
<tr>
<td>9</td>
<td>Column storage: CHIROBIOTIC</td>
<td>100% MeOH</td>
</tr>
<tr>
<td></td>
<td>CYCLOBOND</td>
<td>100% IPA</td>
</tr>
</tbody>
</table>

* Use salts (NH₄OAc,CF₃COO, or NaCl) for bases, NH₄OAc for acids when developing methods for prep.

3. COLUMN CHOICE AND RUN TABLE
Select your choice of columns from the list below. For a 6-column switching system, we recommend CHIROBIOTIC V2, R, and CYCLOBOND I 2000, DNP, and HP-RSP.

<table>
<thead>
<tr>
<th>No.</th>
<th>Column Type (250x4.6mm)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CHIROBIOTIC V2</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>II</td>
<td>CHIROBIOTIC R</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>III</td>
<td>CHIROBIOTIC R</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>IV</td>
<td>CHIROBIOTIC TAG</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>V</td>
<td>CYCLOBOND I 2000</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>VI</td>
<td>CYCLOBOND I 2000 DNP</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>VII</td>
<td>CYCLOBOND I 2000 DMP</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>VIII</td>
<td>CYCLOBOND I 2000 HP-RSP</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
</tr>
</tbody>
</table>

4. RUN CONDITIONS

- Flow Rate: 1.0 mL/min.
- Equilibration Time: 25 minutes
- Run Time: 25 minutes
- Temperature: Ambient
- Detector: UV - 230nm
- Sample: 1 mg/mL in MeOH

5. OPTIMIZATION PROCEDURES

- Polar ionic mode (CHIROBIOTIC phases only)
  - Test alternative acid/base ratios (generally higher acid for basic molecules, higher base for acidic molecules)
  - To change buffer to a volatile salt, use ammonium trifluoroacetate for basic compounds and ammonium acetate for acidic compounds at a concentration of 0.1%, adjust accordingly. Ammonium formate may be used as a compromise for both acidic and basic compounds.

- Normal phase mode
  - Change pH
  - Change buffer and buffer concentration
  - Change temperature

- Reversed phase mode
  - Test alternative acid/base ratios
  - Change organic to THF, ACN, MeOH
  - Change buffer type and concentration

6. OPTIMIZING FOR MS DETECTION

CHIROBIOTIC: Use salts, as in Step 5, when using the polar ionic or polar organic modes.

CYCLOBOND: Use NH₄OH to replace TEA in polar organic mode, lower concentration by 50 to 75%.

Both Phases: Use ammonium acetate or ammonium formate when using in reversed phase.

7. RETESTING YOUR METHOD DEVELOPMENT COLUMNS

To ensure the selectivity performance of CHIROBIOTIC columns, periodically test with 5-methyl-5-phenylhydantoin in 100% MeOH. For testing CYCLOBOND columns, please refer to your CYCLOBOND Handbook.
Typical Screening Results

Optimized CHIROBIOTIC V

MeOH/NH4TFA; 100/0.02 w%
Best Positive Screening Results

Column: CHIROBIOTIC V2, 250x4.6mm
Mobile Phase: 40/60, ACN/10 mM NH₄OAc, pH 3.8
Flow Rate: 0.8mL/min
UV: 380 nm

Column: CHIROBIOTIC V2, 150x4.6mm
Mobile Phase: 50/50, MeOH/5 mM NH₄OAc, pH 3.5
Flow Rate: 0.8mL/min
UV: 380 nm

Optimized Method for LC/MS application
Optimization Step: Polar Ionic Mode
Enhanced Selectivity T → T2

Terbutaline

Peak 1: 9.72
Peak 2: 10.92

CHIROBIOTIC T,
100/0.2/0.1; MeOH/AcOH/TEA

Peak 1: 9.70
Peak 2: 16.06

CHIROBIOTIC T2,
100/0.1w%; MeOH/NH₄TFA
Method Development Screen

- Monitor column performance *regularly*
- Store columns in correct solvents (free of additives):
  - CHIROBIOTIC: 100% MeOH
  - CYCLOBOND: 100% 2-PrOH
  - CHIRALCEL/PAK: Hexane/2-PrOH, 90/10
Performance Tests for CHIROBIOTIC Phases

To ensure the selectivity and performance of all CHIROBIOTIC LC columns, periodically test your columns. This can now be accomplished with a single compound for all the CHIROBIOTIC phases in a very simple mobile phase of 100% MeOH.

CHIROBIOTIC V

- Peak 1: 3.70 min.
- Peak 2: 4.21 min.

Conditions for all columns:
- Sample: 5-Methyl-5-Phenylhydantoin (Aldrich 18,082-3)
- Column size: 250x4.6mm
- Mobile phase: 100% MeOH
- Flow rate: 1 ml/min.
- UV: 220nm

CHIROBIOTIC T

- Peak 1: 3.83 min.
- Peak 2: 5.05 min.

CHIROBIOTIC R

- Peak 1: 4.15 min.
- Peak 2: 4.63 min.

CHIROBIOTIC TAG

- Peak 1: 3.98 min.
- Peak 2: 7.22 min.
# Performance Tests for CYCLOBOND I 2000

## CYCLOBOND I 2000

<table>
<thead>
<tr>
<th>Compound</th>
<th>Additional Info</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin (Sigma # A2250)</td>
<td></td>
<td>6.89 min.</td>
<td>7.90 min.</td>
</tr>
<tr>
<td>Chlorthalidone (Sigma # C2775)</td>
<td></td>
<td>12.04 min.</td>
<td>13.73 min.</td>
</tr>
</tbody>
</table>

| Mobile Phase: | 100/0.3/0.2: ACN/HOAc/TEA | Flow Rate: 1.0 mL/min. | Injection Vol.: 5 μL | Sample Conc: 5 mg/mL | Detection: 254 nm
|---------------|----------------|----------------------|---------------------|---------------------|---------------------|

## CYCLOBOND I 2000 RSP

<table>
<thead>
<tr>
<th>Compound</th>
<th>Additional Info</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin (Sigma # A2250)</td>
<td></td>
<td>6.89 min.</td>
<td>7.90 min.</td>
</tr>
<tr>
<td>Chlorthalidone (Sigma # C2775)</td>
<td></td>
<td>12.04 min.</td>
<td>13.73 min.</td>
</tr>
</tbody>
</table>

| Mobile Phase: | 10/90: ACN/0.1% TEA, pH 4.1 | Flow Rate: 1.5 mL/min. | Injection Vol.: 3 μL | Sample Conc: 5 mg/mL | Detection: 230 nm
|---------------|----------------|----------------------|---------------------|---------------------|---------------------|

## CYCLOBOND I 2000 AC

<table>
<thead>
<tr>
<th>Compound</th>
<th>Additional Info</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norphenylephrine (Aldrich #11,372-7)</td>
<td></td>
<td>7.50 min.</td>
<td>8.77 min.</td>
</tr>
</tbody>
</table>

| Mobile Phase: | 10/90: MeOH/0.1% NaAc, pH 5.5 | Flow Rate: 1.0 mL/min. | Injection Vol.: 3 μL | Sample Conc: 5 mg/mL | Detection: 230 nm
|---------------|----------------|----------------------|---------------------|---------------------|---------------------|

## CYCLOBOND I 2000 SN

<table>
<thead>
<tr>
<th>Compound</th>
<th>Additional Info</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bendroflumethiazide (Sigma # B5775)</td>
<td></td>
<td>6.80 min.</td>
<td>7.40 min.</td>
</tr>
</tbody>
</table>

| Mobile Phase: | 50/50: ACN/0.1% TEA, pH 4.1 | Flow Rate: 1.0 mL/min. | Injection Vol.: 5 μL | Sample Conc: 5 mg/mL | Detection: 254 nm
|---------------|----------------|----------------------|---------------------|---------------------|---------------------|
Isocratic or Gradient?

- Gradients suitable for NP, unsuitable for RP

- Recent papers suggest that gradient method development not much faster and has no greater success rate

- In PIM, ammonium formate gradient possible from 0.01% to 0.05% in methanol to replace HOAc which has a very broad window
Two recent studies showed:

- Screening* four polysaccharides over 5 (NP, POM) mobile phases provided selectivity for 87% of a set of 53 compounds: the same study tested three CHIROBIOTIC phases over just 2 (RP, PIM) mobile phases with selectivities of 65%.

- It was noted that, together, they provided a 96% success rate, with increased selectivity for certain families of compounds on the CHIROBIOTIC CSPs confirming the complementary effect.

Sales Tools Available

- Product Guide
- Handbooks (4)
- New Product Bulletins
- New Applications will be available soon on web
- Seminar Program (CD)
- Method Development program
- Website: [www.astecusa.com](http://www.astecusa.com)
- E newsletter
CONCLUSIONS

• Chirobiotic phases are complimentary to cellulose and amylose CSPs
• Polar ionic mode is best mobile phase for ionic racemates
• Reversed phase can be run on all columns for any type of analyte
• Cyclobond phases have solve chiral separation problems not possible on any other CSP especially the Cyclobond HPRSP.
• Generic screening has proven to be the most efficient methodology for chiral selectivity
The Supelco Chiralyser

Optical rotation is a very useful tool when dealing with chiral entities. Up to now the major problem has been the sensitivity and ease of use of the commercially available devices. The Astec Chiralyser has come a long way in solving those problems. Two available scientific tools have made this a reality.

1. The availability of single wavelength LED’s which allowed the choice of the best, most sensitive wavelength (430 nm) as a light source. In addition, from the LED technology you get long life > 100,000 hours.

2. Use of the Faraday effect that allows nulling of the parallel magnetic field electronically so that the entire sample in the cell can be read instantaneously.
What are the benefits of a Chiralyser in the area of chiral separations?

1. Validates peaks as (-) and (+). Eliminates peaks that are achiral contaminants.

2. Identifies a reversal of elution order. A common occurrence between CSP’s and utilizing different mobile phase conditions.

3. Identifies enantiomeric pairs. A useful determination as the introduction of an achiral guard column can easily complete resolution if diasteriosomers are overlapping.

4. Detects analytes with no UV unlike circular dichroism.

5. System is easy to set up and economical to use, no complicated software.
Below is a list of compounds that have been run in our lab. It demonstrates the reversal of elution order problem and the variety of chiral molecules that have been assayed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>First peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,5 Hydantoin</td>
<td>All Chirobiotics</td>
<td>MeOH</td>
<td>(-)</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>V/T/R</td>
<td>MeOH</td>
<td>(+)</td>
</tr>
<tr>
<td>*N-Amine</td>
<td>V/V2</td>
<td>100/0.1w%, MeOH/ATFA</td>
<td>R(+)</td>
</tr>
<tr>
<td>*N-Amine</td>
<td>V</td>
<td>30/70, MeOH/TEAA, 4.1</td>
<td>S(-)</td>
</tr>
<tr>
<td>Methadone</td>
<td>V2</td>
<td>100/0.1w% MeOH/ATFA</td>
<td>R(-)</td>
</tr>
<tr>
<td>Methadone</td>
<td>HP-RSP</td>
<td>20/80, ACN/NH4OAc, 3.6</td>
<td>R(-)</td>
</tr>
<tr>
<td>*Propranolol</td>
<td>T/T2</td>
<td>100/0.1w% MeOH/ATFA</td>
<td>S(-)</td>
</tr>
<tr>
<td>*Propranolol</td>
<td>TAG</td>
<td>100/0.1w% MeOH/ATFA</td>
<td>R(+)</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>T/T2/TAG/V</td>
<td>100/0.1w% MeOH/ATFA</td>
<td>(-)</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>T</td>
<td>100/0.1w%, MeOH/ATFA</td>
<td>R(+)</td>
</tr>
<tr>
<td>*Bendrofluomethiazide</td>
<td>V</td>
<td>10/90, THF/NH4NO3</td>
<td>(+)</td>
</tr>
<tr>
<td>*Bendrofluomethiazide</td>
<td>T</td>
<td>30/70, MeOH/H2O</td>
<td>(-)</td>
</tr>
<tr>
<td>Naproxen</td>
<td>V</td>
<td>10/90, THF/NaCitrate</td>
<td>S(+)</td>
</tr>
<tr>
<td>Naproxen</td>
<td>R</td>
<td>20/80, MeOH/TEAA, 5.5</td>
<td>S(+)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>V</td>
<td>10/90, THF/NaCitrate</td>
<td>R(-)</td>
</tr>
<tr>
<td>Albuterol</td>
<td>V/T</td>
<td>100/0.1w%, MeOH/ATFA</td>
<td>(-)</td>
</tr>
</tbody>
</table>

* Reversed elution order
<table>
<thead>
<tr>
<th>Substance</th>
<th>Modifier</th>
<th>Mobile Phase</th>
<th>Composition</th>
<th>Elution Order</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mianserin</em></td>
<td>V</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td><em>Mianserin</em></td>
<td>T</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Mandelic acid</td>
<td>T/R</td>
<td>30/70, MeOH/pH 4.5</td>
<td>S(+)</td>
<td></td>
</tr>
<tr>
<td>Mandelic acid</td>
<td>T/R</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>S(+)</td>
<td></td>
</tr>
<tr>
<td>Bupvacaine</td>
<td>V/V2</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>S(-)</td>
<td></td>
</tr>
<tr>
<td>Nicardipine</td>
<td>V/V2/T</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td><em>Ritalin</em></td>
<td>V/V2</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td><em>Ritalin</em></td>
<td>T2</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Omeprazole</td>
<td>R</td>
<td>40/60, EtOH/Heptane</td>
<td>R(+)</td>
<td></td>
</tr>
<tr>
<td>Omeprazole</td>
<td>R</td>
<td>30/70, MeOH/HOAc,4.1</td>
<td>R(+)</td>
<td></td>
</tr>
<tr>
<td>Amphetamine</td>
<td>V2</td>
<td>100/0.05w%,MeOH/ATFA</td>
<td>S(+)</td>
<td></td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>V2</td>
<td>100/0.05w%,MeOH/ATFA</td>
<td>S(+)</td>
<td></td>
</tr>
<tr>
<td>Dextro/Levorphanol</td>
<td>V/V2</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>D(+)</td>
<td></td>
</tr>
<tr>
<td>Dextro/Levo methorphan</td>
<td>V/V2</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>D(+)</td>
<td></td>
</tr>
<tr>
<td>Butorphanol</td>
<td>T/T2</td>
<td>100/0.1w%,MeOH/ATFA</td>
<td>D(+)</td>
<td></td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>R</td>
<td>100/0.02w%,MeOH/HOAc</td>
<td>S(+)</td>
<td></td>
</tr>
<tr>
<td>*a-Me a-Ph succinimide</td>
<td>R</td>
<td>20/80,EtOH/Hex</td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td>*a-Me a-Ph succinimide</td>
<td>TAG/T/V</td>
<td>20/80,EtOH/Hex</td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td><em>Warfarin</em></td>
<td>B-CD</td>
<td>100/0.3/0.2, ACN/HOAc/TEA</td>
<td>S(-)</td>
<td></td>
</tr>
<tr>
<td><em>Warfarin</em></td>
<td>V</td>
<td>30/70, ACN/NH₂OAc,4.1</td>
<td>R(+)</td>
<td></td>
</tr>
</tbody>
</table>

* Reversed elution order