Basics of Chiral HPLC and High Throughput Chiral Methods Development Screening

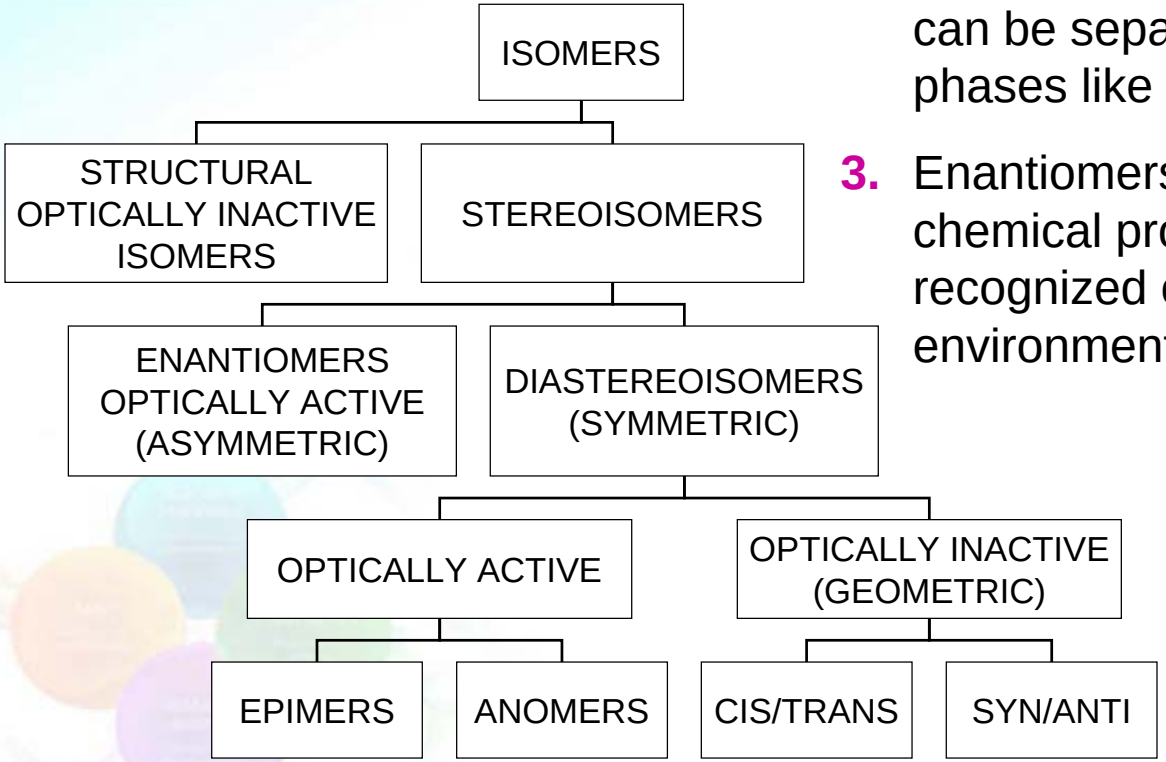
Presented by Tom Beesley

Sigma-Aldrich/Supelco

T408174

The Field of Stereochemistry

1. All isomers have 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_{18}.
3. Enantiomers have same physical and chemical properties and can be recognized or separated only in chiral environment.

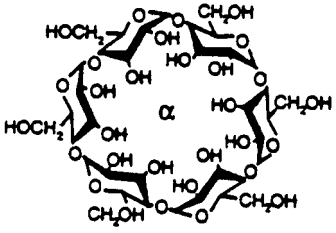


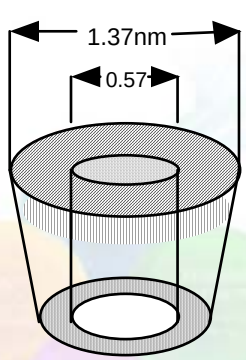
The Inclusion Complex

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.

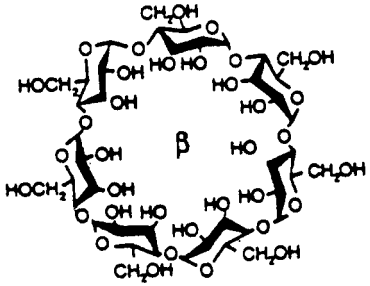
Structure and dimensions of the most common cyclodextrin molecules.

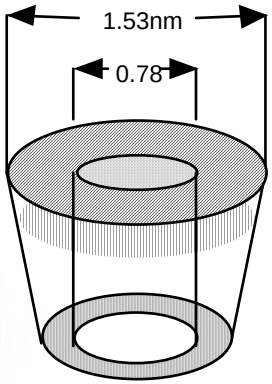
α-cyclodextrin



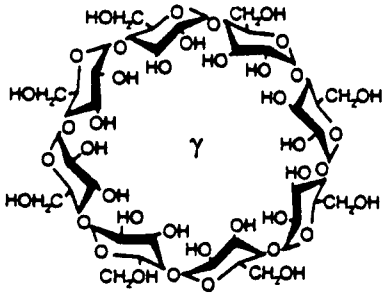


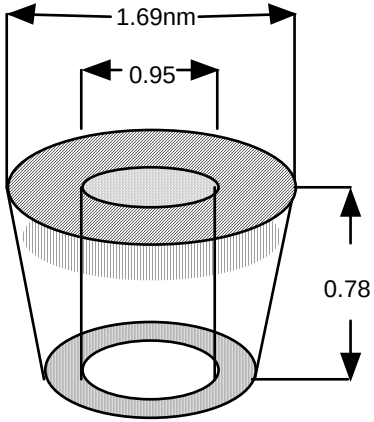
β-cyclodextrin



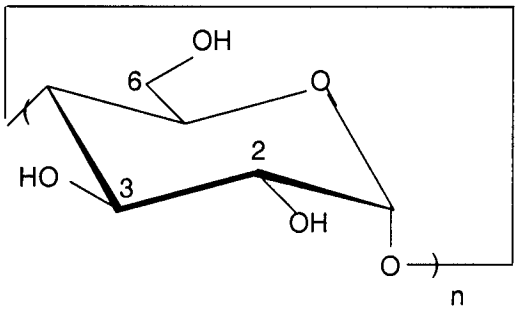


γ-cyclodextrin

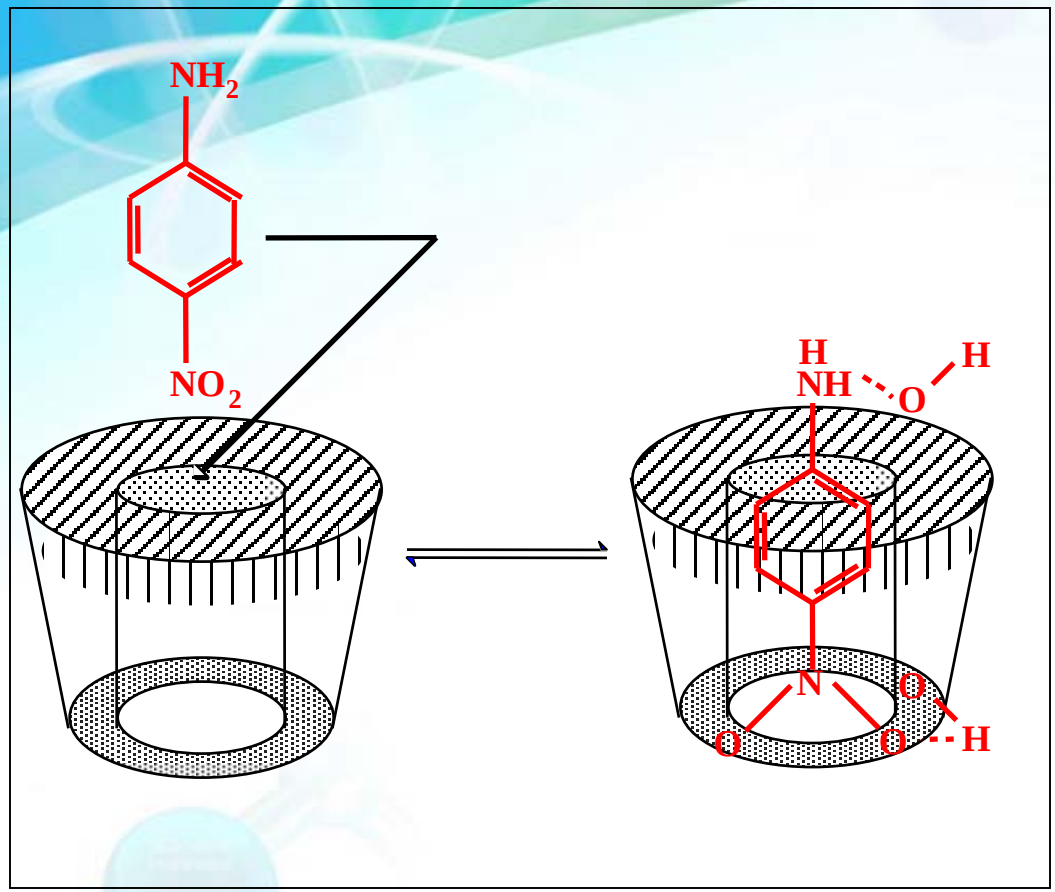




Schematic of one glucose unit in a cyclodextrin molecule



Inclusion Complex Schematic

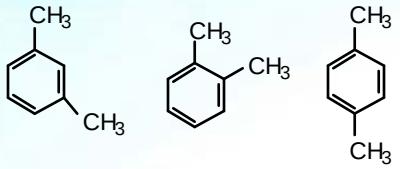


Inclusion complexing effectiveness is dependent upon the position and strength of substituents on benzene or fused ring analytes. The elution order is typically meta<ortho<para. The bulky character of the meta and ortho limits full inclusion. The linear nature of the para structure results in full inclusion.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

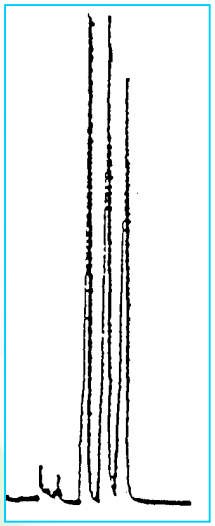
Acetonitrile accelerates release from the cavity. Methanol reduces hydrogen bonding effects to the cyclodextrin hydroxyl groups. Combinations of acetonitrile and methanol in water are useful in optimizing a separation.

Examples of Inclusion Phenomena
Positional Isomers

Xylenes

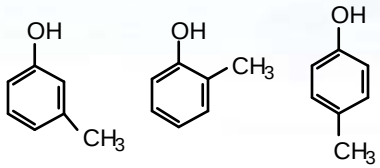


Peak 1 - 5.74 min. (ortho)
Peak 2 - 6.38 min. (meta)
Peak 3 - 6.83 min. (para)

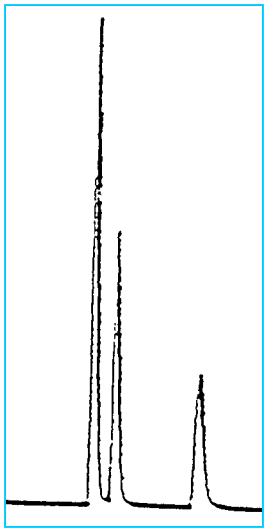


CYCLOBOND I *2000*
Mobile Phase: 30/70: CH_3CN/H_2O

Cresols

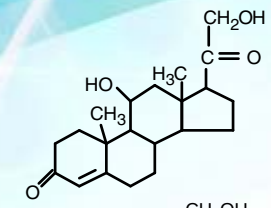


Peak 1 - 6.62 min. (ortho)
Peak 2 - 7.31 min. (meta)
Peak 3 - 9.82 min. (para)

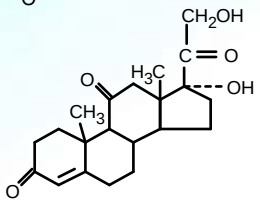


CYCLOBOND I *2000*
Mobile Phase: 40/60: CH_3OH/H_2O

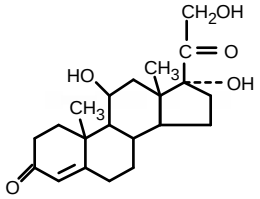
Structural Isomers



Corticosterone

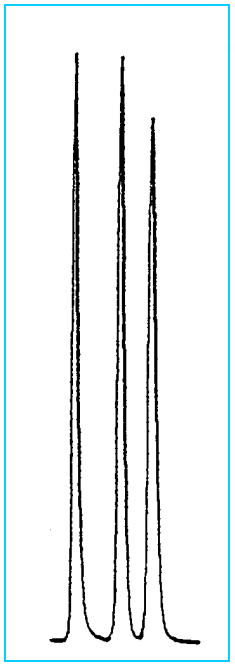


Cortisone



Hydrocortisone

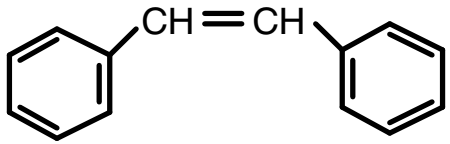
Peak 1 - 6.29 min.
Peak 2 - 8.02 min.
Peak 3 - 9.18 min.



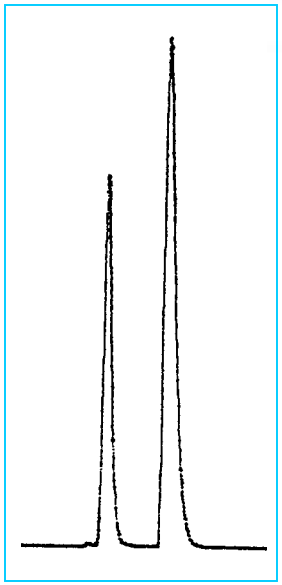
CYCLOBOND I *2000*
Mobile Phase:40/60: CH_3CN/H_2O

Geometric Isomers

cis/trans Stilbene



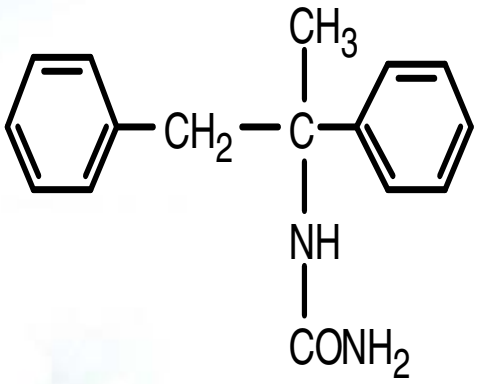
Peak 1 - 5.60 min.
Peak 2 - 7.14 min.



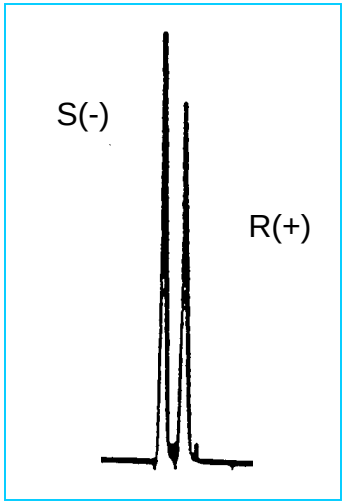
CYCLOBOND I *2000*
Mobile Phase: 70/30: MeOH/H_2O

Enantiomers

Remacemide



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

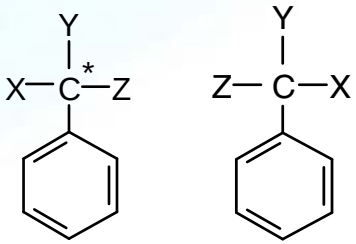


CYCLOBOND I *2000*
Mobile Phase:
3.5/96.5: CH_3CN/PO_4(0.1M) pH 3.5

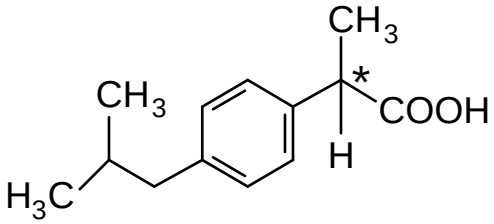
Models for Identification of Potential Racemates

A. Enantiomers based on **CARBON**

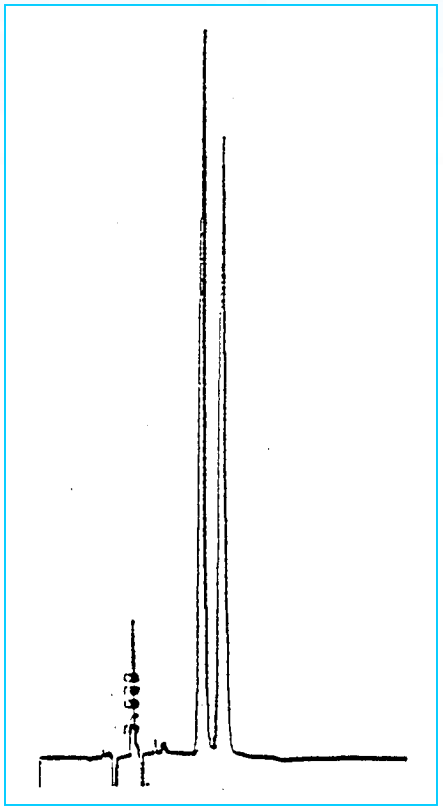
Type 1: **Stereogenic Center**



Ibuprofen



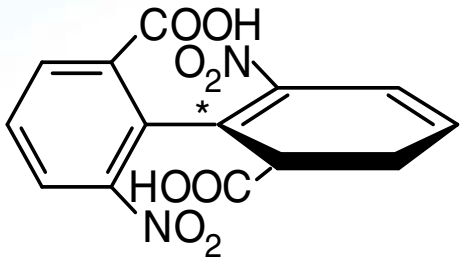
Peak 1 - 5.77 min.
Peak 2 - 6.47 min.



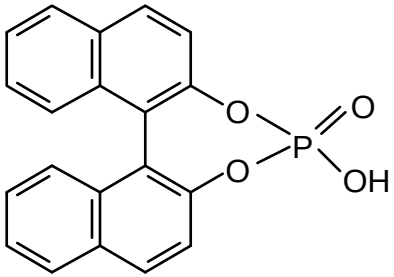
CHIROBIOTIC V
10/90: THF/20mM Na Citrate, pH 6.3

Models for Identification of Potential Racemates

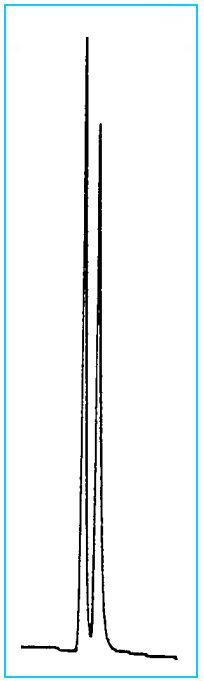
Type 2: Axis of Symmetry



1,1′-Binaphthyl-2,2′-diylhydrogenphosphate



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



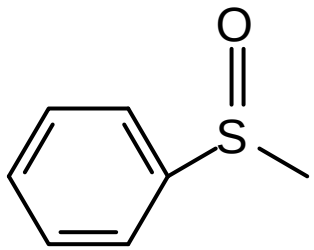
CHIROBIOTIC V
30/70: ACN/0.1% TEAA, pH 4.1

Models for Identification of Potential Racemates

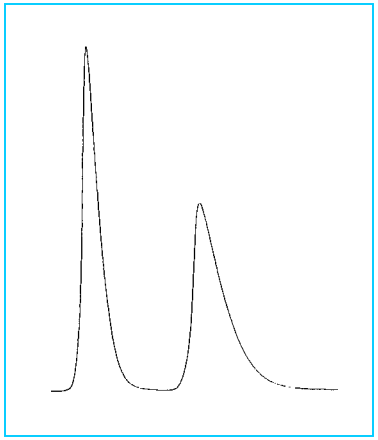
B. Enantiomers based on **SULFUR**

Possibilities include sulfoxide, sulfoximide, sulfinate and sulfonium ion.

Methyl phenyl sulfoxide



Peak 1 - 10.86 min.
Peak 2 - 14.49 min.



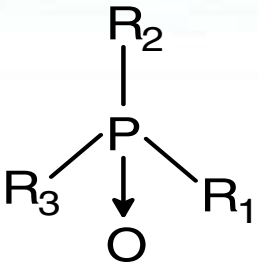
CHIROBIOTIC TAG
40/60: EtOH/Hex

Models for Identification of Potential Racemates

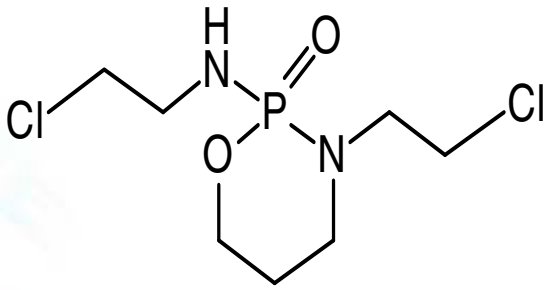
C. Enantiomers based on **PHOSPHORUS**

Possibilities include phosphine, phosphine oxide, phosphinate and phosphonium ion.

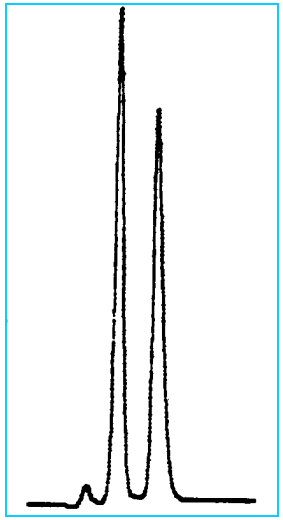
Phosphine Oxide



Ifosfamide



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

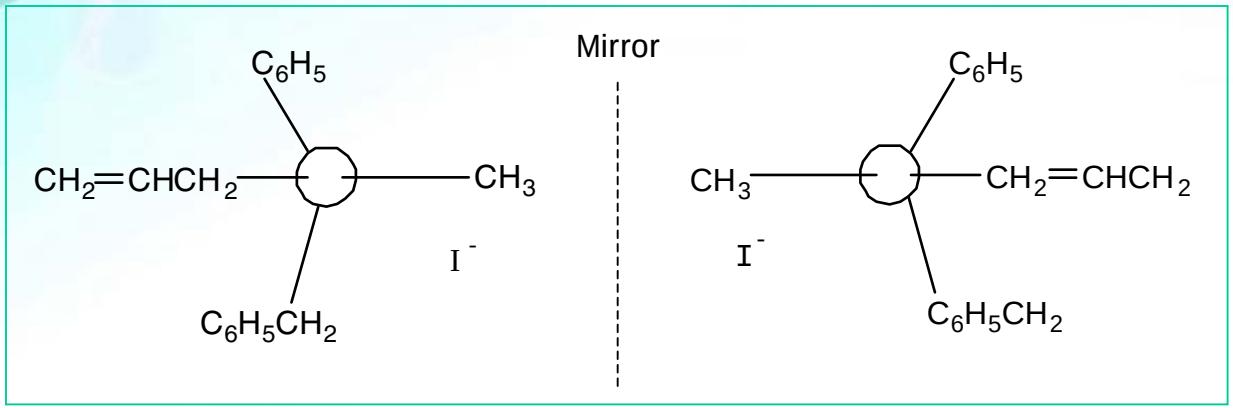


CHIROBIOTIC T
10/90: THF/H_2O

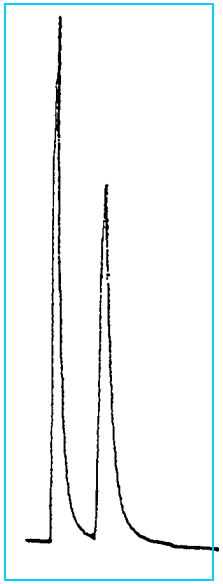
Models for Identification of Potential Racemates

D. Enantiomers based on **NITROGEN**

Possibilities include amine oxide and ammonium ion.



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



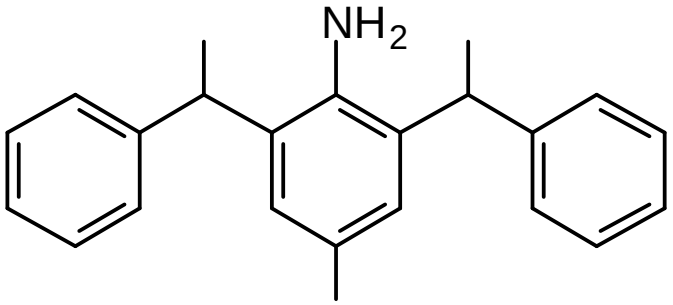
(+) and (-)-Methylallylphenylbenzyl ammonium iodide

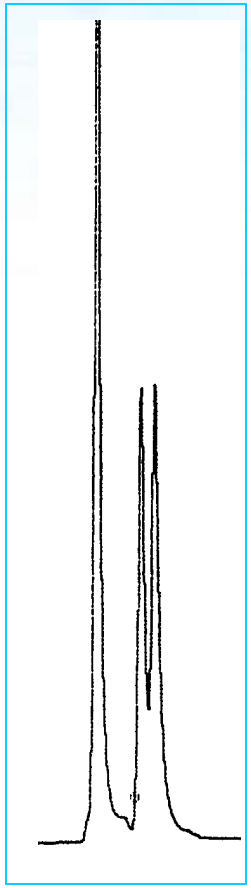
CHIROBIOTIC V
100/0.02/0.01: MeOH/HOAc/TEA

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

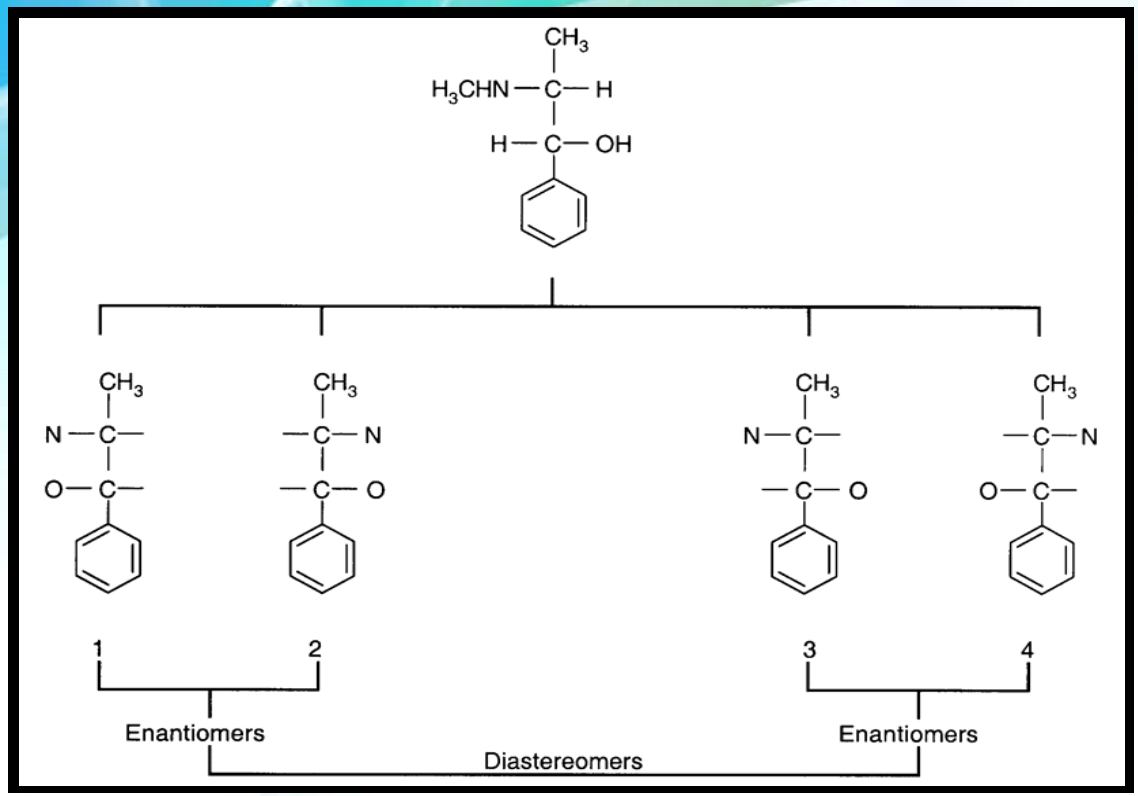




Peak 1 – 6.19 (meso)
Peak 2 – 7.96
Peak 3 – 8.47

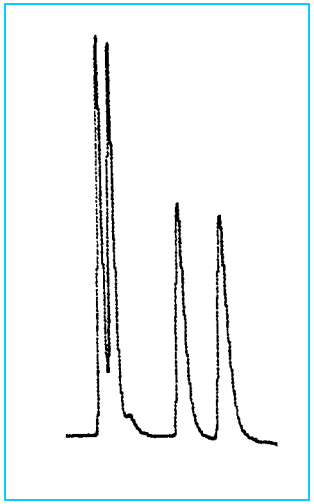
CYCLOBOND I *2000* RSP
250x4.6mm
30/70: CH_3CN/0.1% TEAA, pH 6.5
1.0 mL/min.

DIASTEREOMERIC/ENANTIOMERIC STRUCTURES:



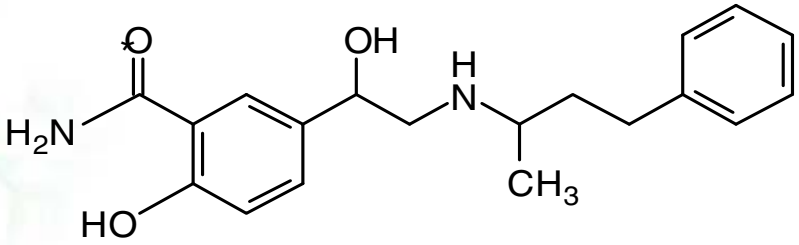
Note: Conventional reversed phase would separate this analyte into 2 peaks (diasteromers). Only a chiral stationary phase would resolve all 4 enantiomers.

Peak 1 – 11.54 min.
Peak 2 – 12.29 min.
Peak 3 – 16.57 min.
Peak 4 – 19.35 min.



CHIROBIOTIC V
100%: MeOH/0.1% ATFA, v/w

Labetalol



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 dipole interactions driven in normal phase and polar organic solvents; inclusion complexation driven in reversed phase modes and ion exchange driven in polar ionic mode are the first significant areas to address the potential of an appropriate chiral stationary phase.

Types of Interaction

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:

- π–π
- 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

Copper Complex Phases

CLC-D, CLC-L

Macrocyclic Glycopeptide Phases

CHIROBIOTIC V2, CHIROBIOTIC V,
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,

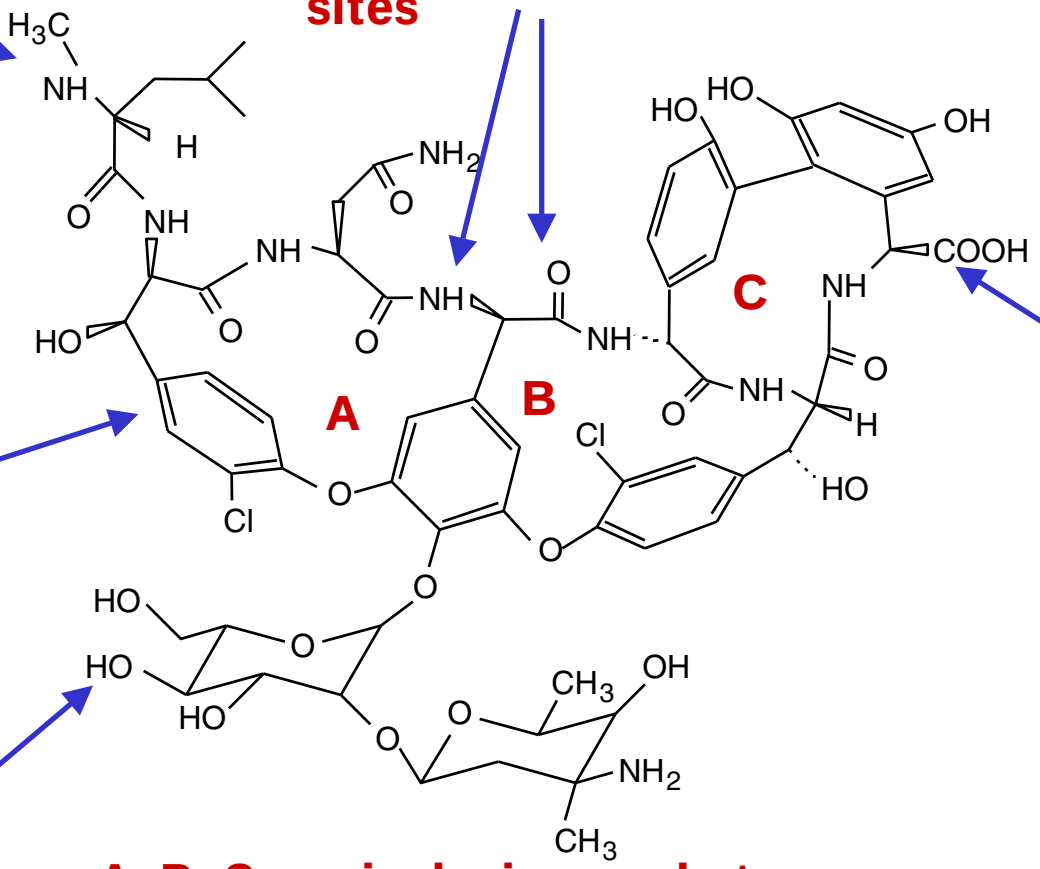
CYCLOBOND II, CYCLOBOND II AC

Structure of Vancomycin CSP

CHIROBIOTIC V

ionic site

hydrogen bonding and dipole stacking sites



ionic site

π-acceptor

sugar moieties

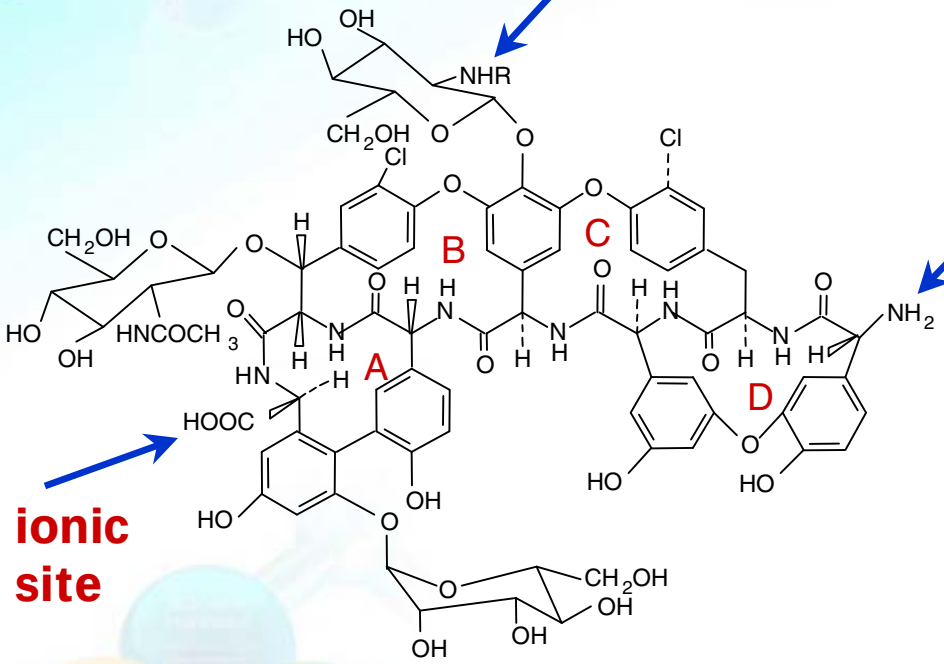
A, B, C are inclusion pockets

Structure of Teicoplanin CSP

Teicoplanin, CHIROBIOTIC T

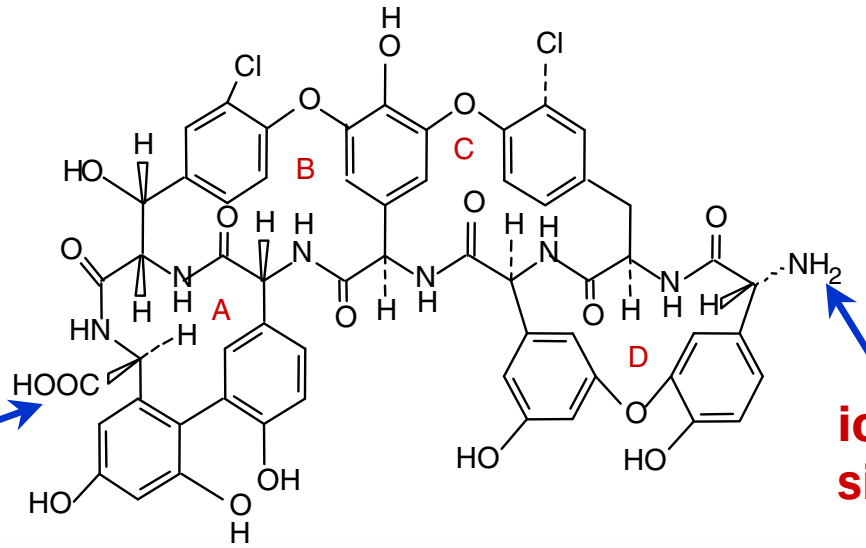
sugar and alkyl chain

Key sites



ionic site

Teicoplanin Aglycone, CHIROBIOTIC TAG



CHIROBIOTIC V2 and CHIROBIOTIC T2

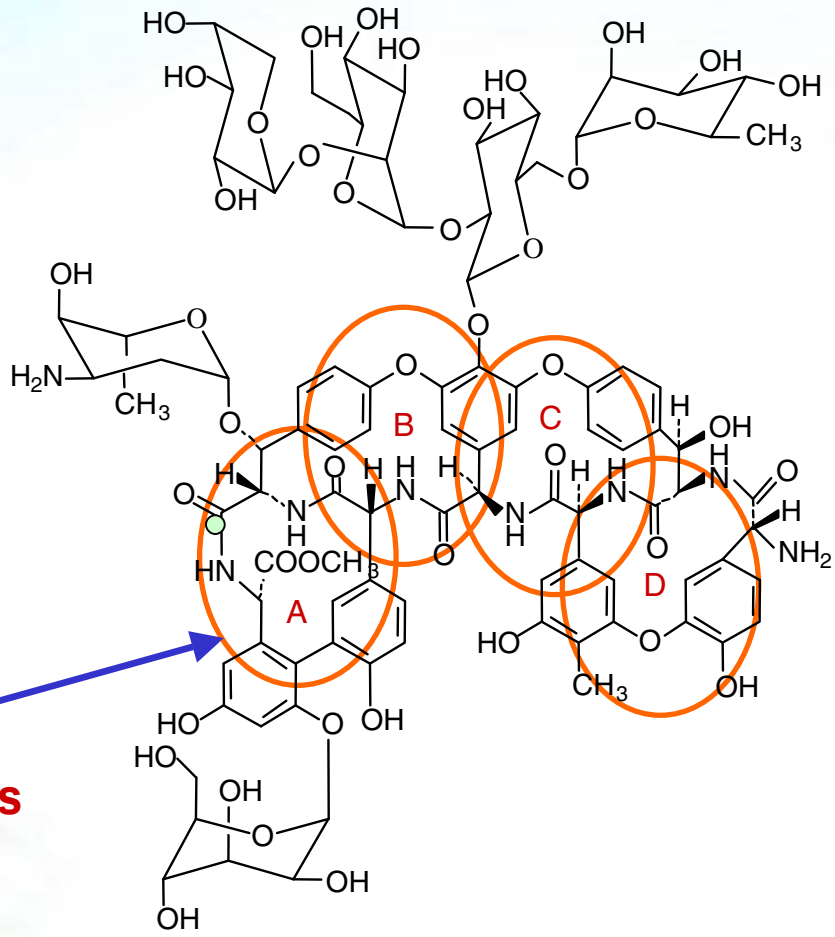
- Extensions of the CHIROBIOTIC V2 and T2 created by changing the silica porosity; position and type of several linkages and the chain length used to anchor the ligand.

- These changes have enhanced the selectivity and capacity of these phases for a large number of analyte classes

- The CHIROBIOTIC V2 has statistically a greater number of chiral separations than the V while the CHIROBIOTIC T performs better than the T2.

Proposed Structure of Ristocetin A CSP

CHIROBIOTIC R

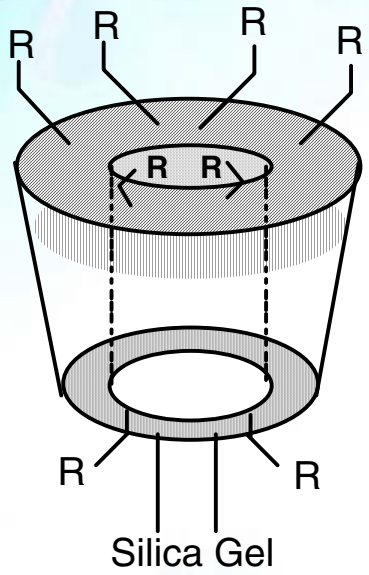


complex sugar

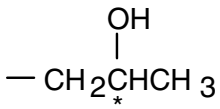
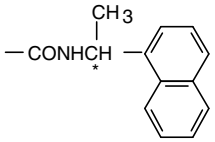
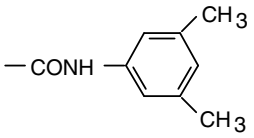
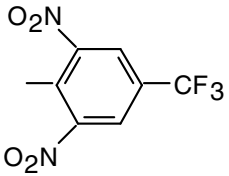
key ionic site

inclusion cavities

Bonded Derivatized Cyclodextrins



* Signifies stereogenic center

R =	Suffix	CD Type
- OCH_3	DM (methylated)	β-CD
- $COCH_3$	AC (acetylated)	β-CD γ-CD
	SP or RSP/HP-RSP (hydroxypropyl ether)	β-CD
	RN or SN (naphthylethyl carbamate)	β-CD
	**DMP** (3,5-dimethylphenyl carbamate)	β-CD
	**DNP** (2,6-dinitro-4-trifluoromethyl phenyl ether)	β-CD

Most Productive CYCLOBOND Phases

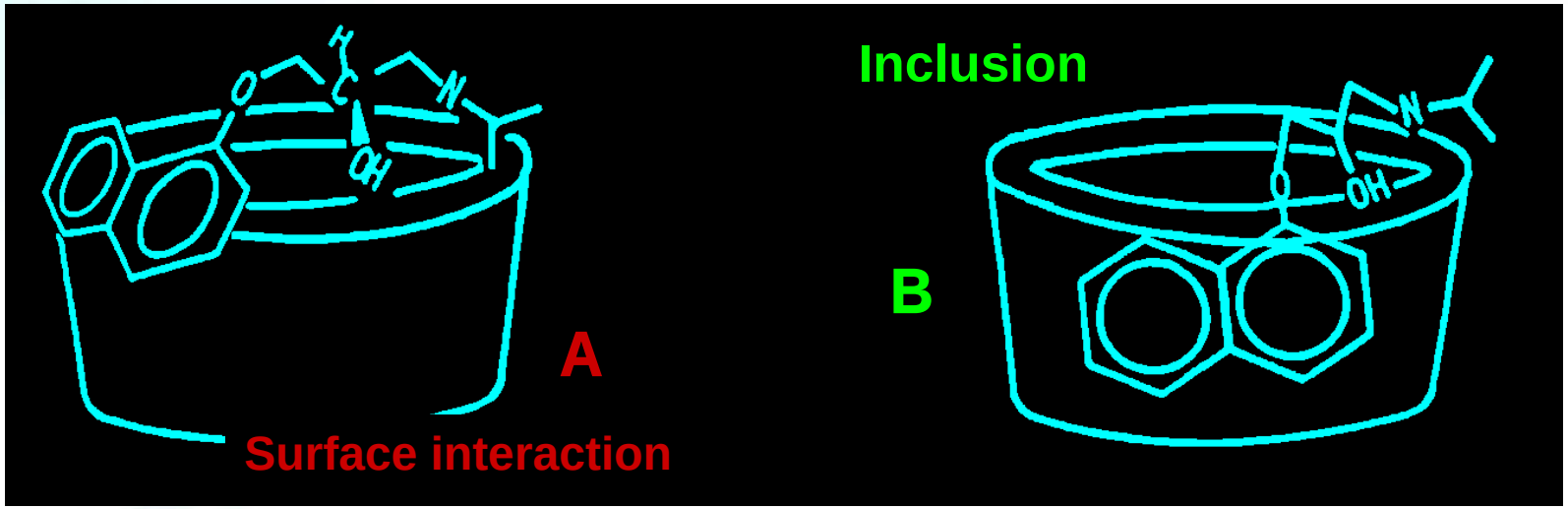
- CYCLOBOND I 2000
- CYCLOBOND I 2000 HP-RSP (highest hit rate)

  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)

Mobile phases types and mechanisms

Normal Phase

Reversed Phase

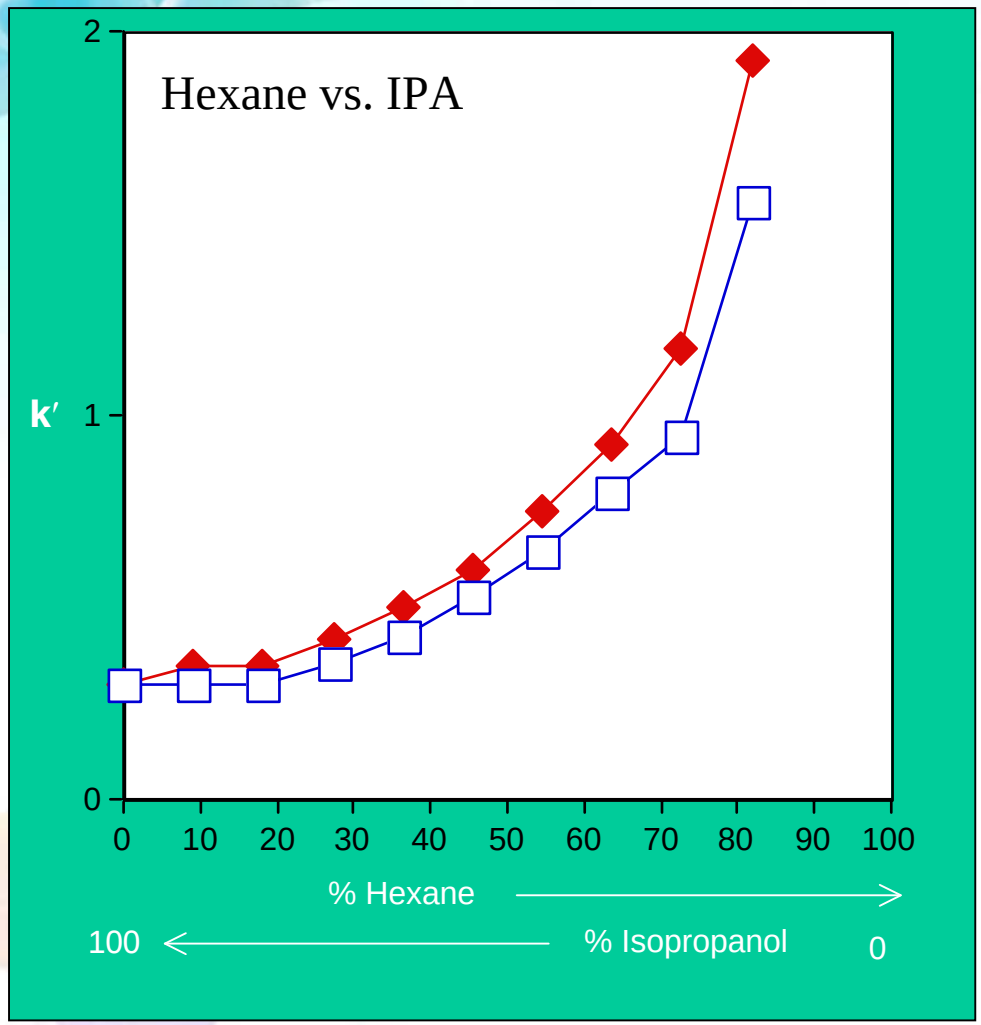
Polar Organic

Polar Ionic

Normal phase and types of interaction

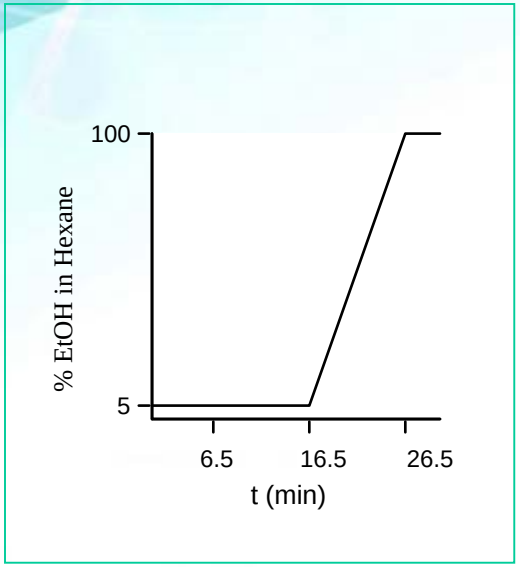
Composition:	Hydrocarbon solvent: heptane + polar alcohol: IPA or EtOH. Addition of MtBE a useful additive to enhance steric effects
Dominant interactions:	π–π interaction, hydrogen bonding
Type of CSPs:	CYCLOBOND I *2000* DMP, SN, RN, CHIROBIOTIC V2, T, R and TAG

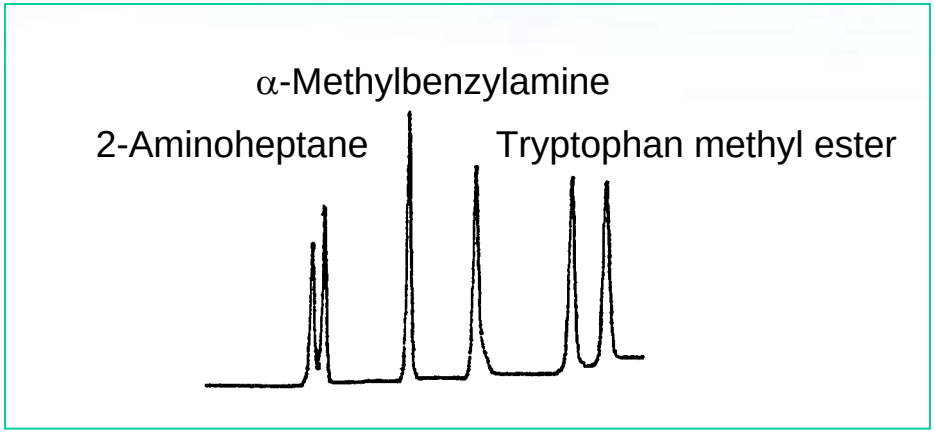
Normal Phase Solvents Mephenytoin



Ref: Anal. Chem., Vol. 66 (9), p 1473-184, May 1994. Armstrong, Tang, S. Chen, Zhou, Bagwill and J.R. Chen.

Gradient Separation in the Normal Phase



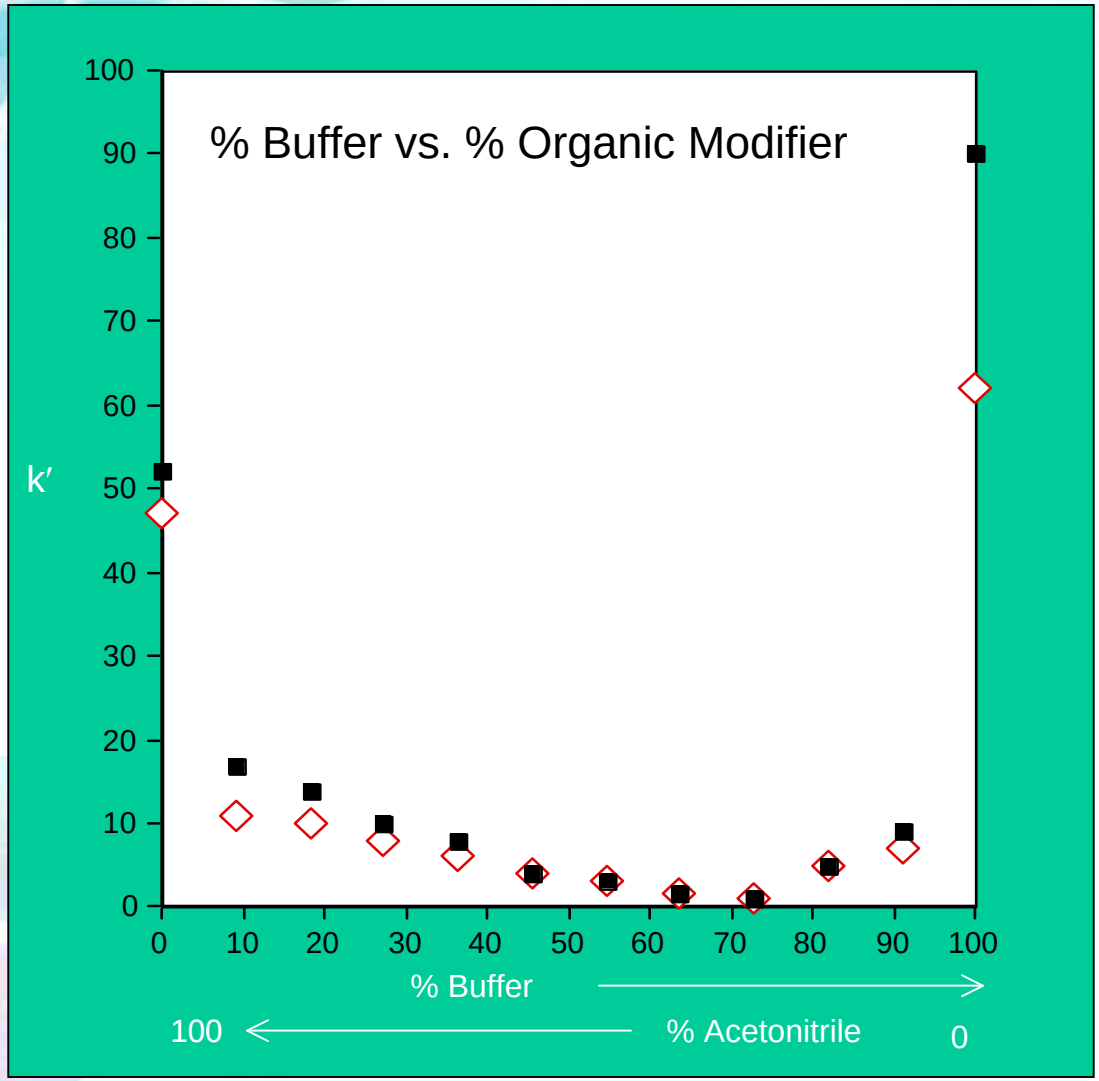


Derivative: 3,4-Dinitrobenzoyl
CYCLOBOND I *2000* SN
Hexane/EtOH Gradient

Reversed Phase

Composition:	Organic solvent: ACN, MeOH or THF + aqueous buffer: TEAA, NH_4OAc
Dominant interactions:	Inclusion complexation, hydrogen bonding
Type of CSPs:	CYCLOBOND I *2000*, RSP,AC, DMP, DNP CHIROBIOTIC V2, T, R and TAG

Reversed Phase Separation
5-Methyl-5-phenyl hydantoin



Ref: Anal. Chem., Vol.66(9), p. 1473-1484, May 1994.

Reversed Phase Solvents
Factors Influencing a Separation

- pH: CHIROBIOTICS 3.0-7.0
- Organic modifier: MeOH, ACN
- Buffer type and concentration
- Flow rate: 0.2 to 1.0 mL/min
- Temperature: 0 to 50˚C

Polar Organic Phase

Composition: ACN + MeOH + HOAc + TEA

Dominant interactions: Hydrogen bonding, dipole-dipole

Type of CSPs: **Cyclodextrins only –**
CYCLOBOND I *2000*, HP-RSP, AC, DMP,DNP

New Polar Ionic Mode: Ionizable analytes; CHIROBIOTIC phases only!

Composition:	MeOH + HOAc + TEA
Dominant interactions:	Ionic interaction, hydrogen bonding
Type of CSPs:	Macrocyclic glycopeptides only - CHIROBIOTIC V2, 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**

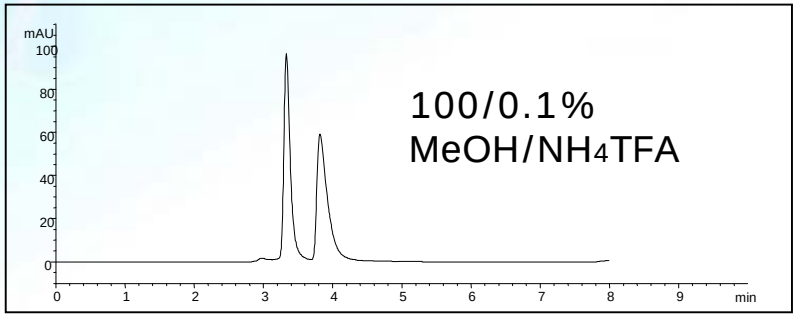
POLAR IONIC MODE

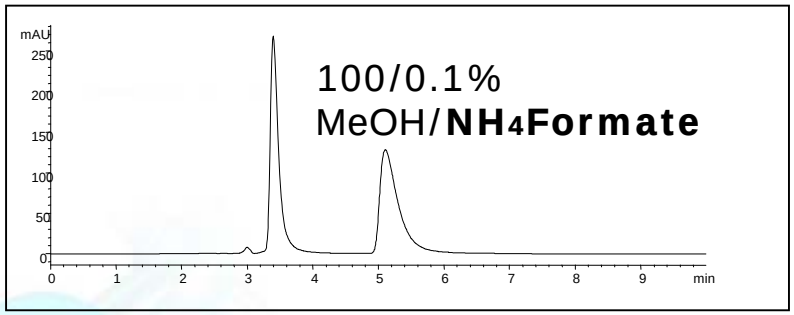
Mobile Phase Components

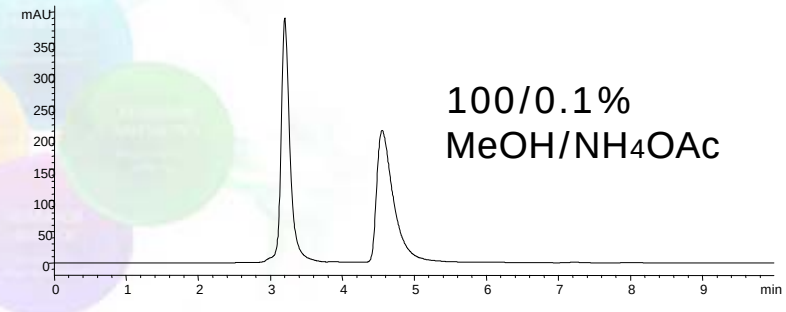
- **Methanol/Acid/Base Components:**
  - **Methanol, anhydrous**
  - **Acid: anhydrous TFA, acetic acid, formic acid**
  - **Base: TEA, DEA, NH3**
  - **Alternatives: ammonium formate or acetate**
- **Ratio of Acid to Base**
  - **Controls only selectivity**
  - **Rate: 4:1 to 1:4, most typical 2:1**
- **Concentration of Acid + Base**
  - **Controls retention and selectivity**
  - **Concentration range: 0.001 to 1.0 part per 100, most typical 0.10.**

Salt Effects in the Polar Ionic Mode

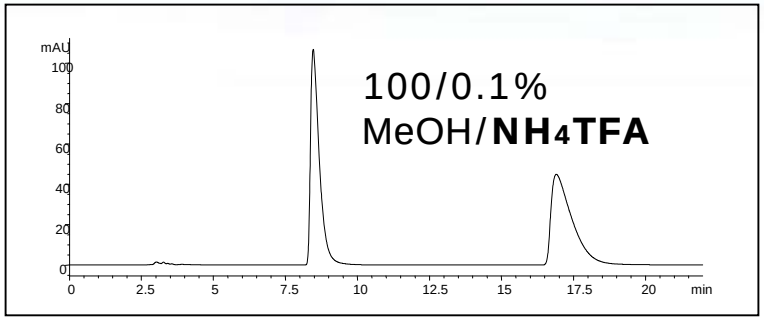
Atrolactic acid, CHIROBIOTIC T2

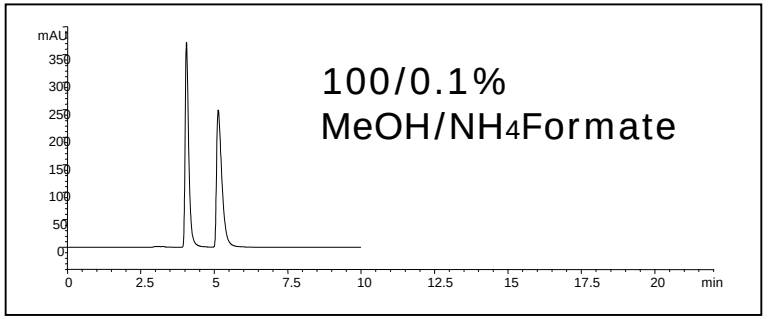


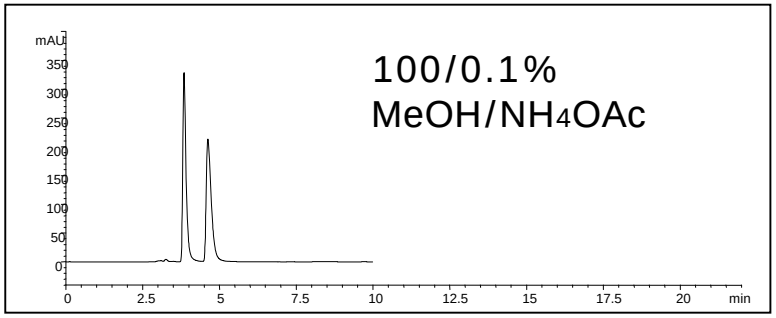




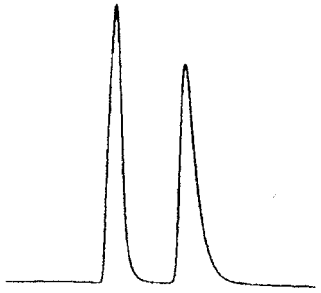
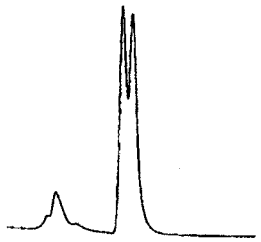
Mianserin CHIROBIOTIC V2





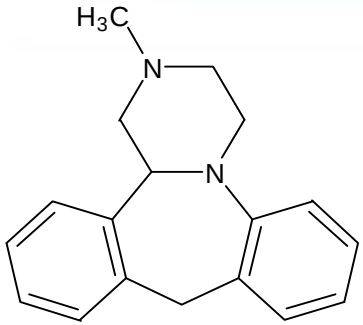


Importance of Acid/Base Ratios In PIM

Example	CHIROBIOTIC V
Mobile Phase	MeOH/Acid/Base
100/0.1/0.1 Peak 1 – 6.2 min. Peak 2 – 7.4 min. Ratio: 1:1	
100/0.15/0.05 Peak 1 – 10.4 min. Peak 2 – 14.5 min. Ratio: 3:1	
100/0.05/0.15 Peak 1 – 3.4 min. Peak 2 – 3.6 min. Ratio: 1:3	

Note: short retention times

Mianserin



Nitrogen on Mianserin group is NH(+), COOH on V is (-)

Nitrogen on Mianserin group as free amine, but COOH on V is fully charged: weak ionic interaction

Comparison of Four Basic Mobile Phase Types for CHIROBIOTIC CSPs

Compound is:

Ionizable

Neutral

Neutral/Polar

All types

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)

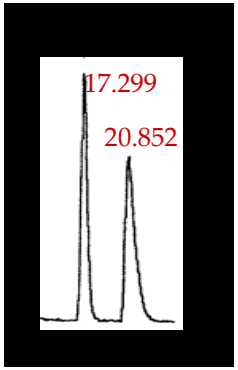
Reversed Phase composition:

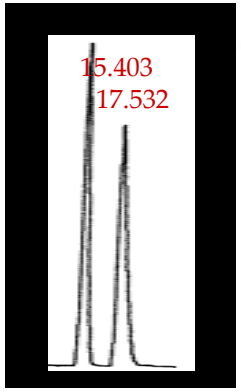
Organic + Aqueous Buffer (ACN+TEAA; ACN & NH4OAc)

Temperature Effects

- **Higher efficiency**
- **Shorter analysis time**

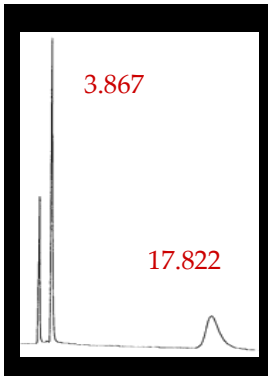
Arginine
CHIROBIOTIC T,
25°C
30/70 MeOH/10 mM NH_4OAc (pH4.0)
1.0 mL/min, ELSD

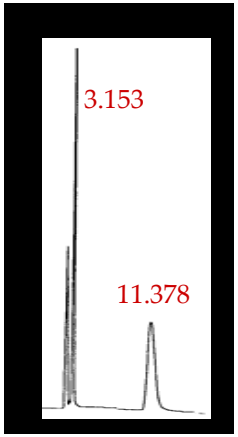




Arginine
CHIROBIOTIC T,
45°C
30/70 MeOH/10 mM NH_4OAc (pH4.0)
1.0 mL/min, ELSD

Diacetyl-cysteine (N^{15})
CHIROBIOTIC T,
25°C
100/0.1% (v/w)
MeOH/NH_4OAc
1.0 mL/min, ELSD



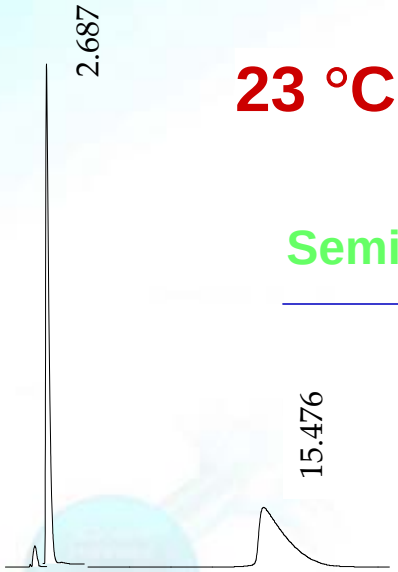


Diacetyl-cysteine (N^{15})
CHIROBIOTIC T,
55°C
100/0.1% (v/w)
MeOH/NH_4OAc
1.0 mL/min, ELSD

Application of Temperature Effect in Loading Study of Diacetyl-Cysteine (N^{15})

CHIROBIOTIC T, 250x4.6mm
Mobiile Phase: MeOH/NH_4OAc (0.1% v/w)
Detection: UV@230nm

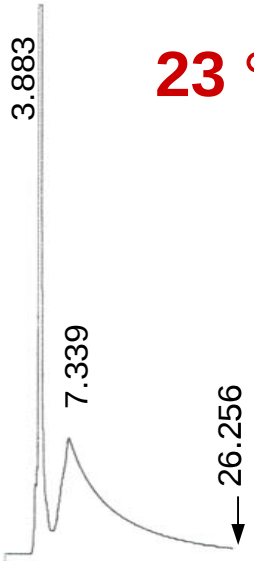
Less Time!
Less mobile phase additive!



23 °C

Semi-Prep

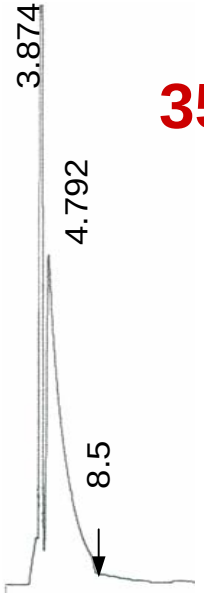
Flow Rate: 2.0 mL/min



23 °C

Higher Temp

Loading: 15 mg
Flow Rate: 1.0 mL/min



35 °C

Loading: 15 mg
Flow Rate: 1.0 mL/min

Practical Guides for CSP Screening

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

Screening Strategy

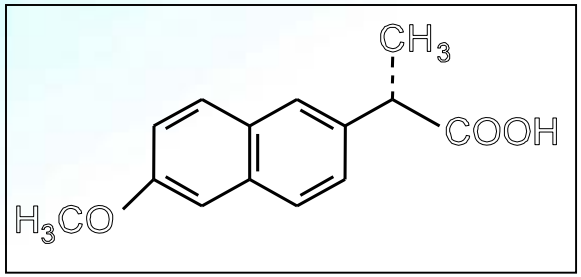
Choose a small set of CSPs that:

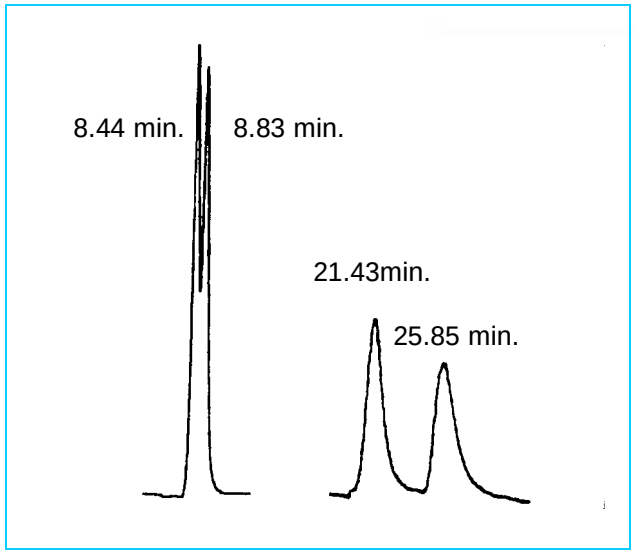
- are broad-based to increase chances of success for a wide range of molecular types
- offer selectivity in a wide range of mobile phases for increased selectivity possibilities and sample solubility
- are complementary to each other, minimum overlap in selectivity

Complementary Separations

CHIROBIOTIC T versus CHIROBIOTIC R

Naproxen





T **R**

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

Screening Strategy

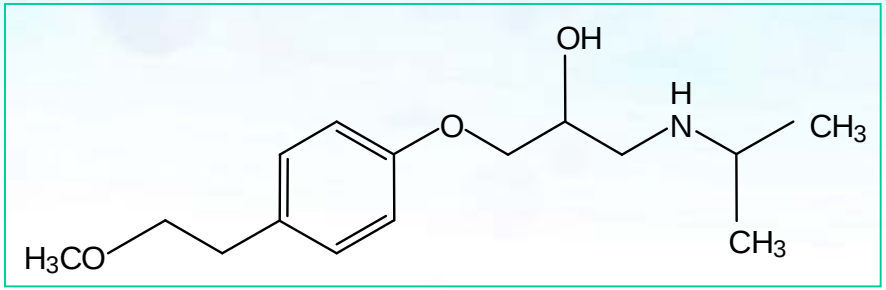
Current industry trend is for generic screening methods with:

- a simple set of columns combining CHIROBIOTIC and Chiralcel/pak* CSP's or CHIROBIOTICS and CYCLOBOND
- a minimum number of solvents covering all five mobile phase types

*Chiracel and Chiralpak are the trademarks of Daicel.

Normal Phase vs Polar Ionic Mode©

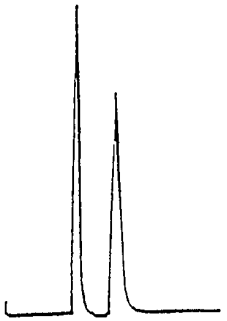
Metoprolol



CHIRACEL OD®

Peak 1 – 11.9 min.
Peak 2 – 18.2 min.

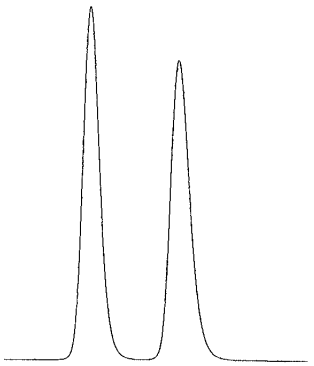
Normal Phase 20/80/0.1: IPA/Hex/DEA



CHIROBIOTIC T®

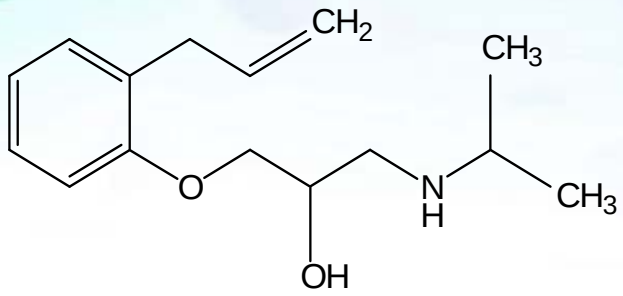
Peak 1 – 15.36 min
Peak 2 – 17.11 min

Polar Ionic Mode MeOH/0.1% ATFA



Normal Phase vs Polar Ionic Mode©

Alprenolol

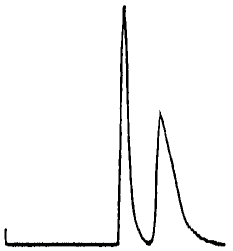


CHIRACEL OD

Peak 1- 12.4 min.
Peak 2 - 16.4 min.

Normal Phase

20/80/0.1: IPA/Hex/TFA

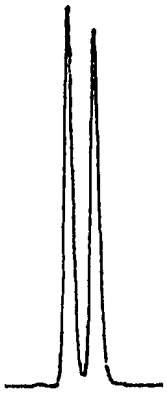


CHIROBIOTIC V

Peak 1 – 7.69 min.
Peak 2 – 8.33 min.

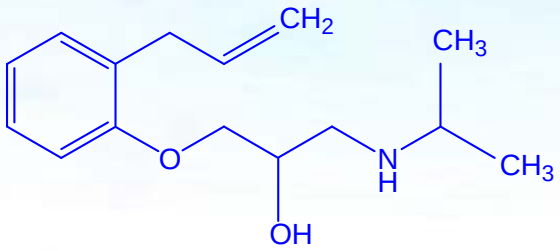
Polar Ionic Mode

100/0.01/0.01: MeOH/HOAc/TEA



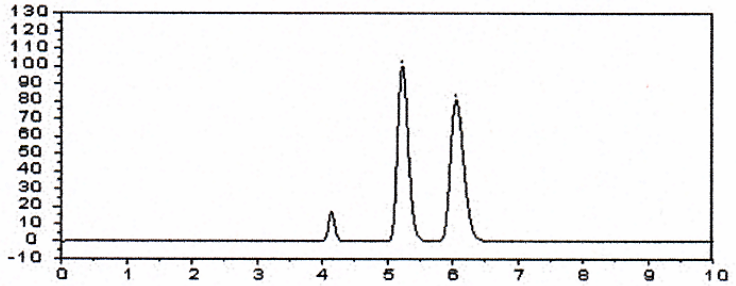
Cellulosic vs CHIROBIOTIC CSP's : RP vs PIM

Tolperisone



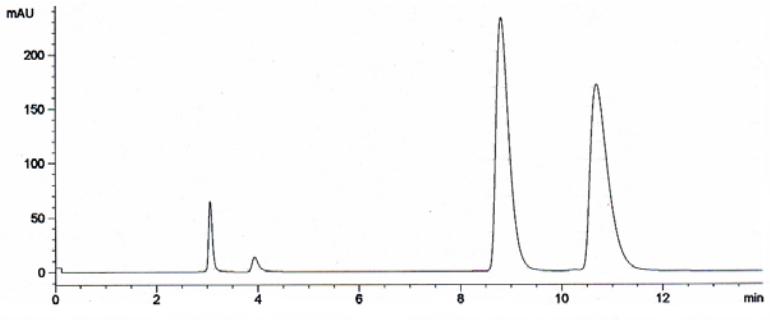
CHIRALPAK® AD-RH Peak 1 – 5.23 min
Peak 2 – 6.06 min

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

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

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

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

astec 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

No.	Mobile Phase	Composition (% v)
	REVERSED PHASE MODE:	
1	MeOH/20mM NH_4OAc, pH 4.0	20/80
2	MeOH/20mM NH_4OAc, pH 6.0	20/80
3	ACN/20mM NH_4OAc, pH 4.0	30/70
4	ACN/20mM NH_4OAc, pH 6.0	30/70
	POLAR IONIC MODE©:	
5	MeOH/HOAc/TEA*	100/0.1/0.1
	POLAR ORGANIC MODE:	
6	ACN/MeOH/HOAc/TEA	95/5/0.3/0.2
CHIROBIOTIC PHASES ONLY	If not progressing to normal phase, wash with MeOH at this stage to test and store the column.	100% MeOH
	NORMAL PHASE MODE:	
7	EtOH/Hexane (or heptane, isohexane)	30/70
8	Washing cycle	100% EtOH
9	Column storage: CHIROBIOTIC	100% MeOH
	CYCLOBOND	100% IPA

* Use salts ($NH_4O_2CCF_3$ for bases, NH_4OAc 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, T, R and CYCLOBOND I *2000*, DNP and HP-RSP.

No.	Column Type (250x4.6mm)	1	2	3	4	5	6	7	8
I	CHIROBIOTIC V2	y		y		y		y	y
II	CHIROBIOTIC T	y	y		y	y		y	y
III	CHIROBIOTIC R		y		y	y		y	y
IV	CHIROBIOTIC TAG	y	y		y	y		y	y
V	CYCLOBOND I *2000*	y		y			y		y
VI	CYCLOBOND I *2000* DNP	y		y			y	y	y
VII	CYCLOBOND I *2000* DMP	y		y			y	y	y
VIII	CYCLOBOND I *2000* HP-RSP	y		y	y		y		y

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

Notes

1. The recommended protocol assumes the use of 250 x 4.6mm columns. For 100 x 4.6mm columns, use the same conditions at 0.5 mL/min.
2. It is permissible to run straight from the reversed phase to the polar ionic mode©, and from the polar ionic mode to normal phase without an intermediate solvent wash.
3. If any screening run results in a retention time less than 5 minutes, reduce the strength of the mobile phase and re-run. Aim for retention times from 5 to 20 minutes. In reversed phase mode reduce organic component, in polar ionic mode© or polar organic mode reduce acid/base concentration. Retention times can be later reduced in the optimization process.
4. If a separation occurs in the polar ionic mode©, for a neutral molecule, change to 100% organic solvent (i.e. MeOH, EtOH or ACN).
5. If the compound does not elute in reversed phase, increase the organic content to 40%. In the polar ionic mode©, increase the acid/base concentration up to 1.0/1.0. In the polar organic mode for the CYCLOBOND columns, increase the MeOH concentration up to 10%.

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 acid/base to a volatile salt, use ammonium trifluoroacetate for basic compounds and ammonium acetate for acidic compounds at a concentration of 0.1wt%, adjust accordingly. Ammonium formate may be used as a compromise for both acidic and basic compounds.
Polar organic mode	● Eliminate MeOH ● Test alternative acid/base ratios
Reversed phase mode	● Test smaller pH changes ● Change organic to THF, ACN, MeOH ● Change buffer type and buffer concentration ● Change temperature
Normal phase mode	● Change EtOH concentration

6. OPTIMIZING FOR MS DETECTION

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

CYCLOBOND: Use NH_4OH 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.

ADVANCED SEPARATION TECHNOLOGIES
World Headquarters: 37 Leslie Court, Post Office Box 297, Whippany, NJ 07981 USA Tel:(973) 428-9080 Fax:(973) 428-0152
E-mail:info@astecusa.com www.astecusa.com
UK and Ireland Sales Office: 1 Blake Street, Congleton, Cheshire CW12 4DS UK Tel:+44 (0) 1260 276276 Fax:+44 (0) 1260 290067
E-mail:info@asteceuro.com www.asteceuro.com
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Chiral Screening Protocol

1. Mobile Phase Composition:

No.	Mobile Phase	Composition (v/v)
1	MeOH/20mM NH_4OAc, pH 4.0	20/80
2	MeOH/20mM NH_4OAc, pH 6.0	20/80
3	ACN/20mM NH_4OAc, pH 4.0	30/70
4	ACN/20mM NH_4OAc, pH 6.0	30/70
5	MeOH/HOAc/TEA (PIM)*	100/0.1/0.1
6	ACN/MeOH/HOAc/TEA (POM)**	95/5/0.3/0.2
7	EtOH/Heptane	30/70
8	EtOH	100

***PIM: Polar Ionic Mode, CHIROBIOTIC**
**** POM: Polar Organic Mode, CYCLOBOND**

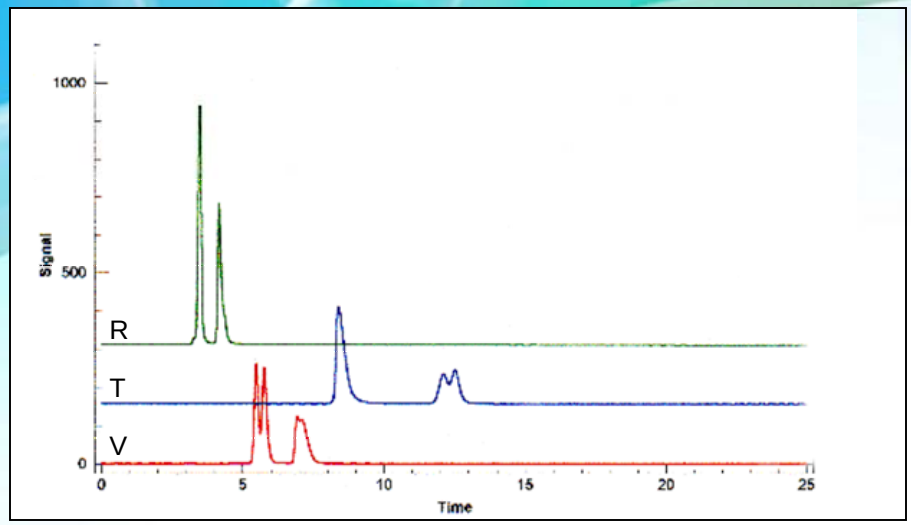
Chiral Screening Protocol

2. Run Table:

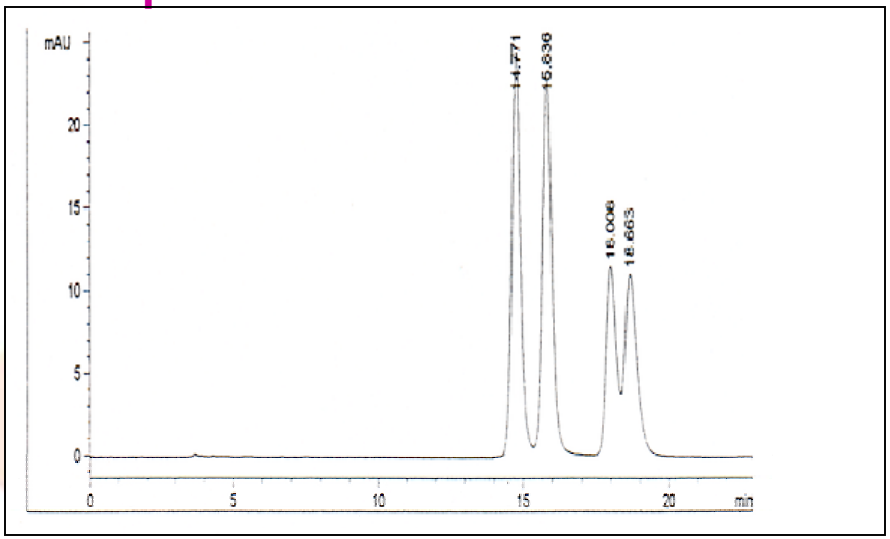
No	Column Type	1	2	3	4	5	6	7	8
I	CHIROBIOTIC V2	y		y		y		y	y
II	CHIROBIOTIC T	y	y		y	y		y	y
III	CHIROBIOTIC R		y		y	y		y	y
VI	CHIROBIOTIC TAG	y	y		y	y		y	y
V	CYCLOBOND I 2000	y		y			y		y
VI	CYCLOBOND DNP	y		y			y	y	y
VII	CYCLOBOND DMP	y		y			y	y	y
VIII	CYCLOBOND HP-RSP	y		y	y		y		y

Note: Each CSP favors certain molecular types.

Typical Screening Results

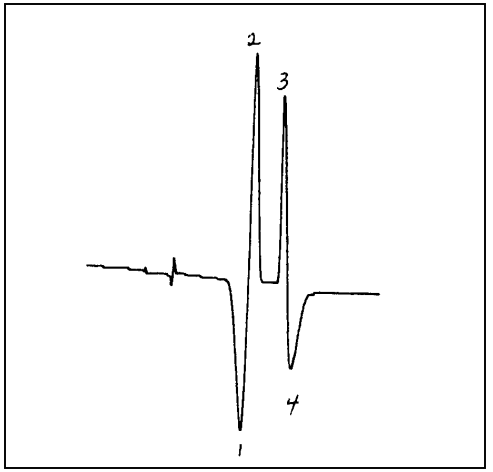


Optimized CHIROBIOTIC V



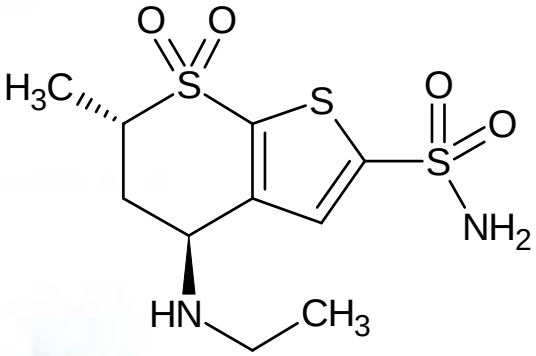
MeOH/NH_4TFA; 100/0.02 w%

Chiralyzer® Optical Rotation

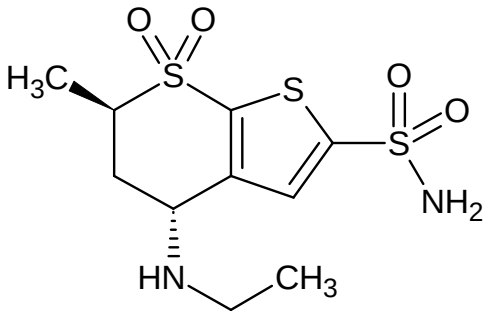


Dorzolamide *HCl Method Development

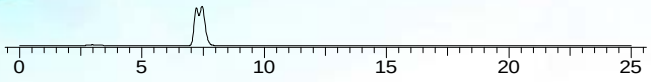
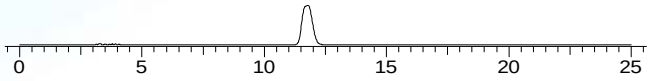
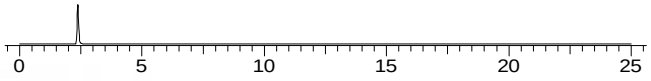
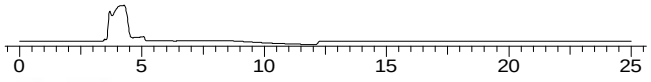
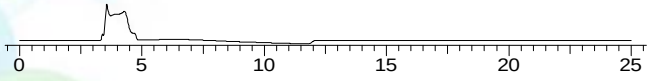
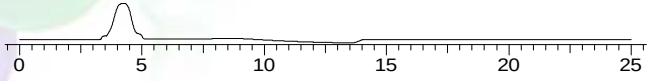
- Processed through method development screen:
- 3 CHIROBIOTIC phases: V2,T,TAG
- 3 CYCLOBOND phases: B-CD,DNP,HP-RSP.



Dorzolamide Hydrochloride 4S,6S



Dorzolamide Hydrochloride Related Compound A 4R,6R

Spectrum	Column	mode	elution	File
	CHIROBIOTIC TAG	RP	No Retention	C:\ascii\ACD_Chrom1373.cdf
	CHIROBIOTIC TAG	PIM	Separation	C:\ascii\ACD_Chrom1384.cdf
	CHIROBIOTIC V2	RP	Separation	C:\ascii\ACD_Chrom1397.cdf
	CHIROBIOTIC V2	PIM	Separation	C:\ascii\ACD_Chrom1404.cdf
	CHIROBIOTIC T	RP	No Separation	C:\ascii\ACD_Chrom1416.cdf
	CHIROBIOTIC T	PIM	No Separation	C:\ascii\ACD_Chrom1423.cdf
	Cyclobond I 2000	RP	No Retention	C:\ascii\ACD_Chrom1435.cdf
	Cyclobond I 2000	POM	Unknown	C:\ascii\ACD_Chrom1442.cdf
	Cyclobond 2000 HP-RSP	RP	No Retention	C:\ascii\ACD_Chrom1461.cdf
	Cyclobond 2000 HP-RSP	POM	Unknown	C:\ascii\ACD_Chrom1485.cdf
	Cyclobond 2000 DNP	RP	No Separation	C:\ascii\ACD_Chrom1497.cdf
	Cyclobond 2000 DNP	POM	Unknown	C:\ascii\ACD_Chrom1504.cdf

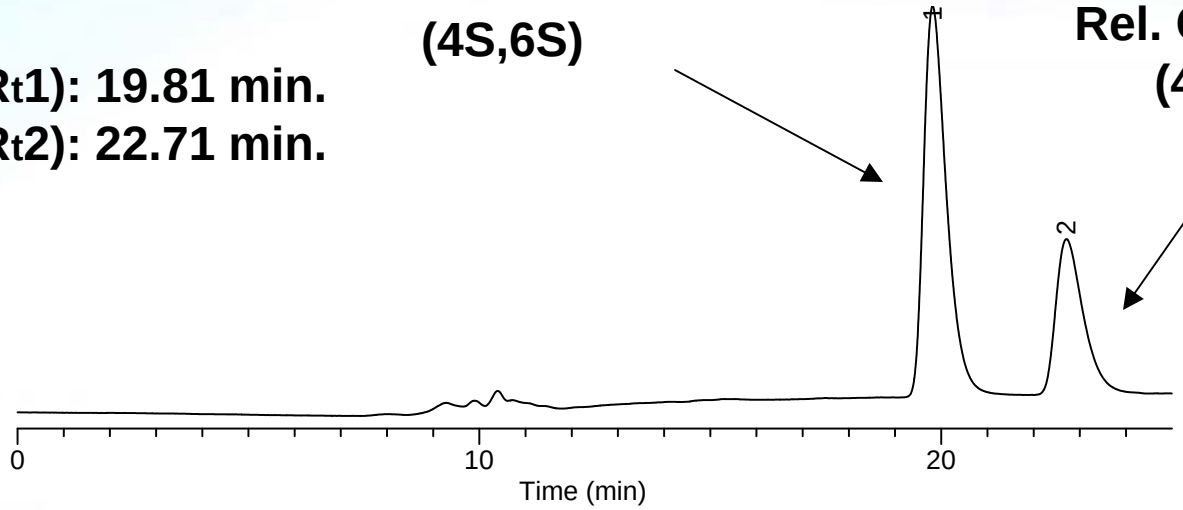
Chromatographic Results

- PIM mode best!

Dorzolamide HCl (4S,6S)

Dorzolamide HCl Rel. Compd. A (4R,6R)

Peak 1 (R_t1): 19.81 min.
Peak 2 (R_t2): 22.71 min.



CHIROBIOTIC V2, 250x4.6mm I.D., 5μ particles (15024AST)
100:5 MeOH:H_2O, 3.81mM NH_4TFA (or 0.05% NH_4TFA)
22°C, 0.3mL/min., UV-254nm
Inj. Vol. 10 μL @1.0 mg/mL in MeOH

Racemic Switches: Optimized Conditions CHIROBIOTIC Phases

Final Results

Compounds	Mobile Phase	Column	k_1'	α	Rs
Albuterol	100/0.1w%, MeOH/NH_4TFA	T2	2.5	1.3	2.8
Amlodipine	100/0.1w%, MeOH/NH_4TFA	V2	3.2	1.1	1.5
Bupivacaine	100/0.1w%, MeOH/NH_4TFA	V2	0.8	1.4	2.5
Citalopram	100/0.1w%, MeOH/NH_4TFA	V	4.8	1.1	1.5
Clenbuterol	100/0.1w%, MeOH/NH_4TFA	T2	2.1	1.2	2.5
Fluoxetine	100/0.1w%, MeOH/NH_4TFA	V2	1.5	1.2	2.5
Formoterol	100/0.6/0.4, MeOH/HOAc/TEA	T2	2.6	1.2	1.5

Racemic Switches: Optimized Conditions CHIROBIOTIC Phases con't

Final Results

Compounds	Mobile Phase	Column	k_1'	α	Rs
Ibuprofen	10/90, THF/20mM NaCitrate, pH6.3	V	0.8	1.24	1.5
Isoproterenol	100/0.1/0.1, MeOH/HOAc/TEA	T2	5.5	1.33	3.3
Ketoprofen	20/80, MeOH/0.1% TEAA, pH 6.0	R	2.6	1.60	3.0
Lercanidipine	100/0.02w%, MeOH/ NH_4TFA	V	1.0	1.18	1.5
Lorazepam	100 % MeOH	T	0.4	4.01	11
Methylphenidate	100/0.1w%, MeOH/NH_4TFA	V2	2.4	1.53	4.0
Metoprolol	100/0.1w%, MeOH/NH_4TFA	T	4.5	1.14	2.0
Mianserin	100/0.1w%, MeOH/NH_4Formate	V2	0.5	1.86	2.8

Racemic Switches: Optimized Conditions CHIROBIOTIC Phases con't

Final Results

Compounds	Mobile Phase	Column	k_1'	α	Rs
Mosapride	30/70, ACN/0.1% TEAA, pH 4.1	V	3.0	1.32	3.3
Naproxen	10/90, THF/NaCitrate,20mM,pH6.3	V	1.4	1.22	1.5
Nicardipine	100/0.02w%, MeOH/NH_4TFA	V	0.7	1.74	4.5
Oxamniquine	100/0.1w%, MeOH/NH_4TFA	V2	2.7	1.23	2.2
Oxazepam	100% MeOH	T	0.5	4.31	12
cis-Permethrin	99.5/0.5, Hex/EtOH	TAG	4.6	1.22	2.5
Propranolol	100/0.1w%, MeOH/NH_4TFA	T	4.2	1.14	1.9
Pseudo-ephedrine	100/0.1w%, MeOH/NH_4TFA	T2	2.7	1.13	1.7

Racemic Switches: Optimized Conditions CHIROBIOTIC Phases con't

Final Results

Compounds	Mobile Phase	Column	k_1'	α	Rs
Sotalol	100/0.2/0.1, MeOH/HOAc/TEA	T	3.5	1.12	1.5
Terbutaline	100/0.1w%, MeOH/NH_4TFA	T2	2.2	1.92	7.0
Thalidomide	100% MeOH	V	0.7	3.44	7.0
Tolperisone	100/0.1w%, MeOH/NH_4TFA	V2	1.8	2.33	2.7
Trimipramine	100/0.1w%, MeOH/NH_4TFA	V2	1.7	1.28	2.3
Warfarin	40/60, EtOH/0.1% TEAA, pH 4.1	V	1.3	1.45	3.0

CYCLOBOND Screening Hits Leading to Baseline Separations

Compounds	Columns*	Compounds	Columns*
Chlorpheniramine	β-CD	Sertraline	HP-RSP/β-CD
Chlorthalidone	HP-RSP/β-CD	Thioridazine	β-CD
Epinephrine	HP-RSP	Thalidomide	DNP
Methadone	HP-RSP	Tramadol	DMP
Miconazole	HP-RSP	Warfarin	β-CD
Nefopam	DNP/HP-RSP	Zopiclone	β-CD
Omeprazole	DMP		
Oxazepam	DNP		
Propranolol	β-CD		
Pseudo-ephedrine	β-CD		

***CYCLOBOND I 2000: β-CD/HP-RSP/DNP/DMP – 40% hit rate!**

Note: Compounds from racemic switch list.

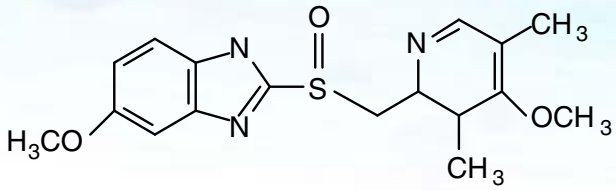
Racemic Switches: Optimized Conditions CYCLOBOND Phases

Final Results

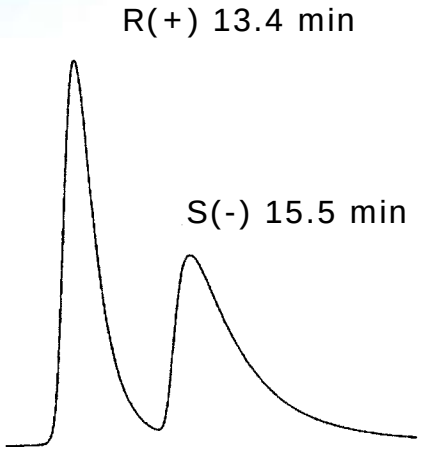
Compounds	Mobile Phase	Column	k1′	α	Rs
Chlorpheniramine	10/90, ACN/0.1% TEAA, pH4.1	β-CD	4.7	1.14	1.5
Chlorthalidone	15/85, ACN/10mM NH_4OAc, pH4.0	HP-RSP	1.4	1.22	1.6
Epinephrine	25/75, ACN/20mM NH_4OAc, pH4.1	HP-RSP	7.1	1.16	2.0
Methadone	20/80, ACN/ 10mM NH_4OAc, pH 3.6	HP-RSP	1.5	1.24	1.7
Miconazole	25/75, ACN/10mM NH_4OAc, pH 4.0	HP-RSP	4.1	1.18	1.6
Nefopam	25/75, ACN/20mMNH4OAc,pH 4.1	DNP	0.5	1.52	2.2
Omeprazole	100/0.4/0.1, ACN/HOAc/NH4OH	DMP	3.3	1.41	2.4
Sertraline	30/70, ACN/20mM NH_4OAc, pH4.1	HP-RSP	5.4	1.19	1.7
Thioridazine	20/80, ACN/0.1% TEAA, pH4.1	β-CD	6.9	1.21	2.5
Tramadol	20/80, ACN/20mM NH_4OAc, pH5.5	DMP	1.6	2.20	2.8
Zopiclone	95/5/0.3/0.2,ACN/MeOH/HOAc/TEA	β-CD	1.0	1.13	1.5

Options from Results of Dual Screen

Omeprozole (Nexium)

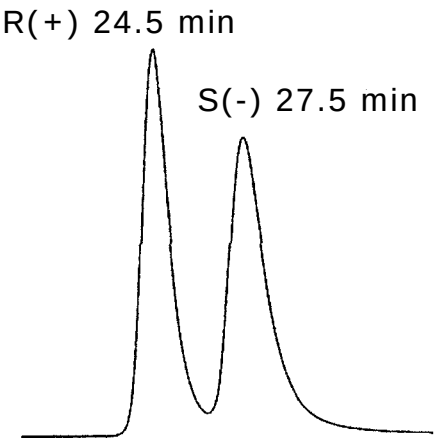


CHIROBIOTIC R (NP)



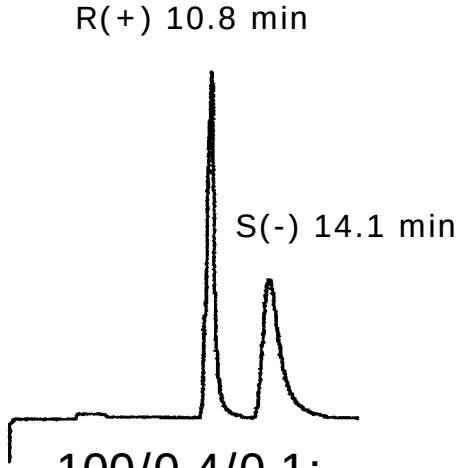
40/60: EtOH/Heptane

CHIROBIOTIC R (RP)



30/70: MeOH/10mM NH_4OAc, pH 4.1

CYCLOBOND I *2000* DMP (POM)



100/0.4/0.1: ACN/HOAc/NH_4OH

Statistics

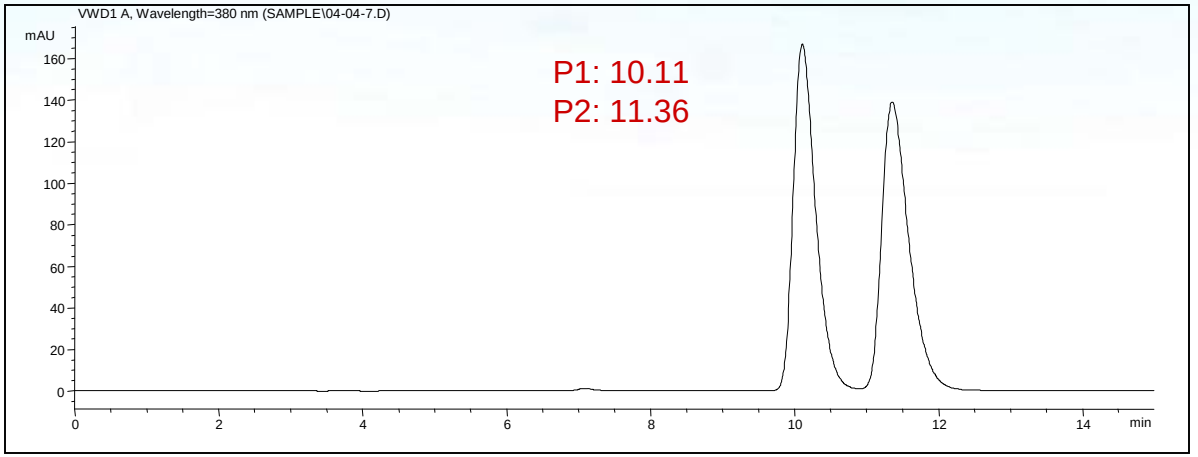
- Of forty racemic switch drugs tested in this study:
  - The success rate for CHIROBIOTIC phases is 80%.
  - The success rate for CYCLOBOND phases is 40%.
  - Eight compounds can be separated by both types (20%).
  - Resolution range:

    CYCLOBOND Phases: 1.5 to 2.8

    CHIROBIOTIC Phases: 1.5 to 11.0

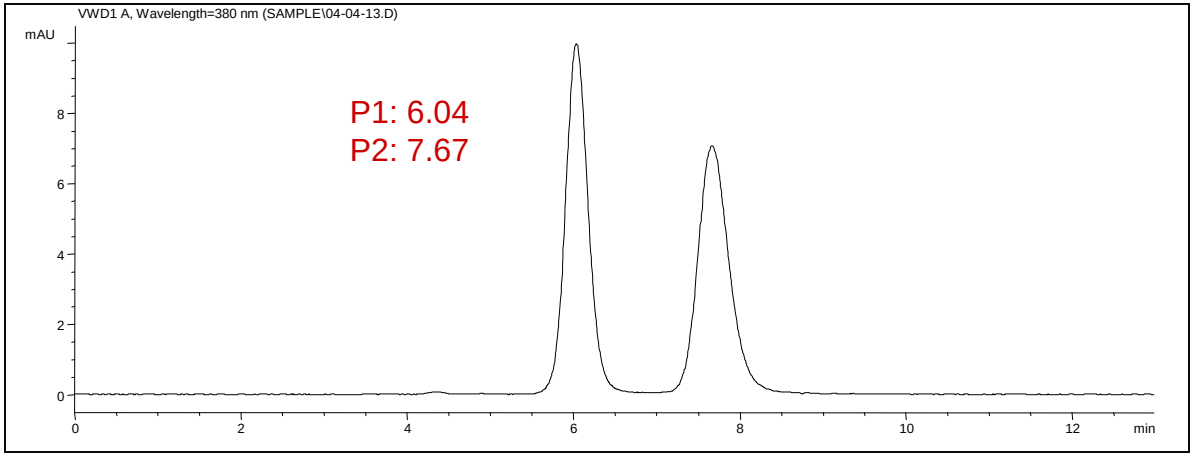
Best Positive Screening Results

Column: CHIROBIOTIC V2, 250x4.6mm
Mobile Phase: 40/60, ACN/10 mM NH_4OAc, 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



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

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 0.5% in methanol to replace HOAc which has a very broad window

Results of Complementary Generic Screening (1)

Previous studies reported:

- 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.

* M E Andersson et al, J Chrom A, 1005 (2003) 83

Sales Tools Available

- Product Guide
- Handbooks (4)
- New Product Bulletins
- New Applications now available
- Seminar Program (CD)
- Method Development program
- Website: **www.sigma-aldrich.com**
- E newsletter

Chiral Analytical Services From Supelco

- Chiral column screening; HPLC & GC
- Method optimization
- Small-scale purification
- Achiral method development services as well

Mission

- Provide fast, efficient and effective analytical & purification services
- Provide consistent, informative and friendly communication – before, during and following each study
- Provided timely assistance with legal and technical issues
- Present a streamlined, easy to use system
- Offer fair pricing structure

Supelco Chiral Services Laboratory

Supelco Chiral Services Laboratory

Key Features

- Fully confidential
- Fast service – goal is within 48 hours of sample receipt for screening projects, time variable for method development and purification
- Full report given upon completion of project
- Elution order determination by measurement of optical rotation
- Purified material to the customer’s specification

GC Chiral Column Screening

- Manual exploration of 3-4 GC column chemistries
- Samples that require derivatization are verified by GC-MS

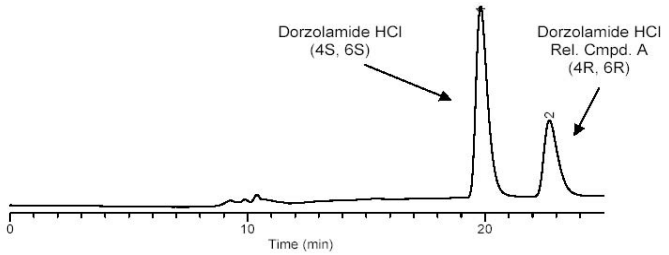
HPLC Method Optimization

- Certain customers may be interested in obtaining enantiomerically-pure materials
- Following successful chiral screening or successful reproduction of methods provided by customer, a method optimization and loading study is conducted to establish the most efficient means of chromatographically purifying the materials
- The output of a loading study is approximately 100 mg of purified material and the methodology utilized to obtain such material

Output

SUPELCO Analytical

595 North Harrison Rd.
Bellefonte, Pennsylvania 16823
(814) 359-3041



Conditions:

Column: CHIROBIOTIC V2, 25 cm x 4.6 mm I.D., 5 µm particles (15024AST)
Mobile Phase: 5:100, H2O:0.05w% NH4TFA in MeOH
Temperature: 22 °C
Flow Rate: 0.3 mL/min
Detection: UV at 254 nm
Injection Volume: 10 µL
Sample: 1.0 mg/mL in MeOH

Peak 1 retention time (R_{t1}): 19.81 min.
Peak 2 retention time (R_{t2}): 22.71 min.

Recommendation for Optimization/Conclusions:

1. The best resolution of the chiral enantiomers Dorzolamide Hydrochloride and Dorzolamide Hydrochloride Related Compound A was observed on the CHIROBIOTIC V2. The aforementioned method was used for confirmation and optimization purposes.
2. The buffer salt ammonium trifluoroacetate was employed to sharpen the peaks, and thus, increase resolution.

We are committed to the success of our Customers, Employees and Shareholders through leadership in Life Science, High Technology and Service.
The SIGMA-ALDRICH Family SIGMA ALDRICH Fluka SUPELCO ISOTEC

- Client specified information
- Detailed report
- Full conditions
- Recommendations

} Project-dependent recommendations by our experts for advice in optimization, scale-up, etc.

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
- Mobile phase can be run sequentially on all Supelco phases.
- Cyclobond phases have solved chiral separation problems not possible on any other CSP especially the Cyclobond HP-RSP.
- Generic screening has proven to be the most efficient methodology for chiral selectivity can be streamlined to six columns and 3-4 mobile phases.

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.

Compound	Column	Mobile Phase	First peak
5,5 Hydantoin	All Chirobiotics	MeOH	(-)
Oxazepam	V/T/R	MeOH	(+)
*N-Amine	V/V2	100/0.1w%,MeOH/ATFA	R(+)
*N-Amine	V	30/70,MeOH/TEAA,4.1	S(-)
Methodone	V2	100/0.1w% MeOH/ATFA	R(-)
Methodone	HP-RSP	20/80, ACN/NH_4OAc,3.6	R(-)
*Propranolol	T/T2	100/0.1w% MeOH/ATFA	S(-)
*Propranolol	TAG	100/0.1w% MeOH/ATFA	R(+)
Terbutaline	T/T2/TAG/V	100/0.1w% MeOH/ATFA	(-)
Metoprolol	T	100/0.1w%,MeOH/ATFA	R(+)
*Bendrofluomethiazide	V	10/90,THF/NH_4NO_3	(+)
*Bendrofluomethiazide	T	30/70, MeOH/H_2O	(-)
Naproxen	V	10/90, THF/NaCitrate	S(+)
Naproxen	R	20/80, MeOH/TEAA,5.5	S(+)
Ibuprofen	V	10/90, THF/NaCitrate	R(-)
Albuterol	V/T	100/0.1w%,MeOH/ATFA	(-)

* Reversed elution order

*Mianserin	V	100/0.1w%,MeOH/ATFA	(-)
*Mianserin	T	100/0.1w%,MeOH/ATFA	(+)
Mandelic acid	T/R	30/70, MeOH/pH 4.5	S(+)
Mandelic acid	T/R	100/0.1w%,MeOH/ATFA	S(+)
Bupvacaine	V/V2	100/0.1w%,MeOH/ATFA	S(-)
Nicardipine	V/V2/T	100/0.1w%,MeOH/ATFA	(-)
*Ritalin	V/V2	100/0.1w%,MeOH/ATFA	(-)
*Ritalin	T2	100/0.1w%,MeOH/ATFA	(+)
Omeprazole	R	40/60, EtOH/Heptane	R(+)
Omeprazole	R	30/70, MeOH/HOAc,4.1	R(+)
Amphetamine	V2	100/0.05w%,MeOH/ATFA	S(+)
Methamphetamine	V2	100/0.05w%,MeOH/ATFA	S(+)
Dextro/Levorphanol	V/V2	100/0.1w%,MeOH/ATFA	D(+)
Dextro/Levo methorphan	V/V2	100/0.1w%,MeOH/ATFA	D(+)
Butorphanol	T/T2	100/0.1w%,MeOH/ATFA	D(+)
Ketoprofen	R	100/0.02w%,MeOH/HOAc	S(+)
*a-Me a-Ph succinimide	R	20/80,EtOH/Hex	(-)
*a-Me a-Ph succinimide	TAG/T/V	20/80,EtOH/Hex	(+)
*Warfarin	B-CD	100/0.3/0.2, ACN/HOAc/TEA	S(-)
*Warfarin	V	30/70, ACN/NH_4OAc,4.1	R(+)

* Reversed elution order