

# Strategies for In-House Development of Chiral Separations for Routine Analytical Applications



EAS 2008  
Thomas E. Beesley  
Dr. J. T. Lee  
Dave Bell

T408180

**Strategy: Use generic screening to develop options for a variety of potential applications**

- Quick analytical method (possibly suitable for later optimization and validation); sample solubility options
- Suitable opportunities for possible small scale prep
- Applicable to trace analysis of unwanted isomer: reversal of elution order possibilities
- Provides impurity profiling
- Applicable to LC-MS methodologies

# Tactics

Choose a set of CSPs:

- that are broad-based to increase chances of success for a wide range of molecular types
- that offer selectivity in a wide range of mobile phases for increased selectivity possibilities and sample solubilities
- that are complementary to each other in order to increase overall hit rate
- where hits (ie. partial separations) can be quickly and easily optimized to baseline separation.



# Modern Chiral Stationary Phases

## Polymeric

### Synthetic

- Methacrylate
- Polycyclic amine-2

### Natural

- Cellulose
- Amylose
- Proteins

## Astec-Supelco Phases

## Small molecule ligands

- Copper complex-2
- $\pi$ -complex
- Crown ether
- Cyclodextrin-12
- Macrocyclic glycopeptides-6

# Published Statistics – 53 Chiral Compounds

% Positive	CSP	Mobile Phases	No. Operating Parameters
87%	AS, AD, OD, OJ	5	20
65%	V, T, R	2	6
96%	Combined	7	26

\* If HP-RSP had been added, % would have gone to 74% due to antifungal agents.

\* If DNP had been added in the POM, % would have gone to 90%.

Ref: Evaluation of Generic Liquid Chromatography Screens for Pharmaceutical Analysis, Andersson, M.E., Aslan, D., Clarke, A., Roeraade, J. Hagman, G., Journal of Chromatography A, 1005 (2003) 83-101.

CHIRALCEL and CHIRALPAK are registered trademarks of Daicel Chemical Industries Ltd.

## 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 must be ionized or 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*

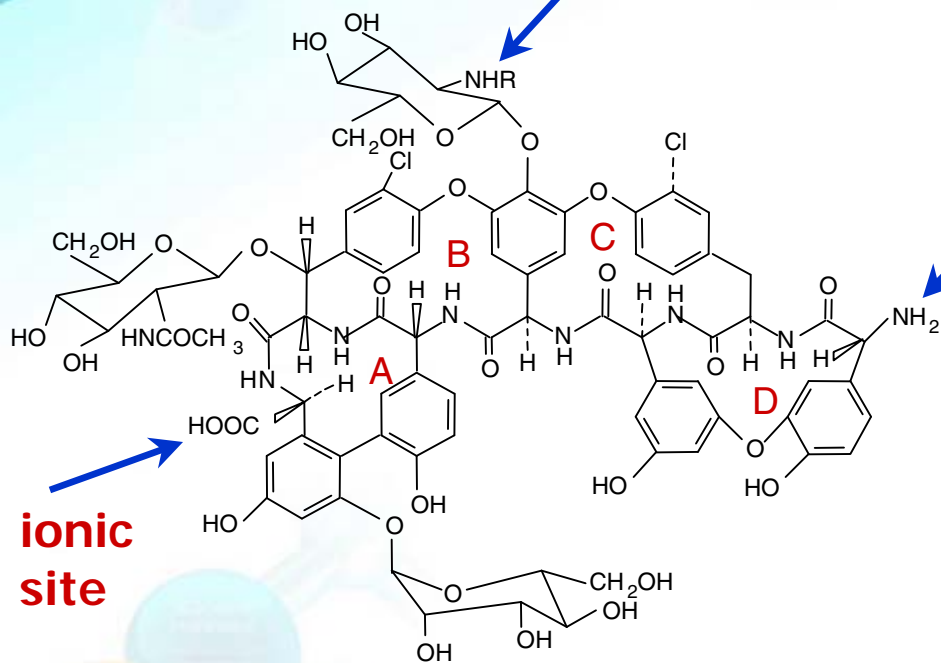
**\* Trademarks of Daicel/Chiral Technologies, West Chester, PA**

# Structure of Teicoplanin CSP

**Teicoplanin,  
CHIROBIOTIC T**

sugar and alkyl chain

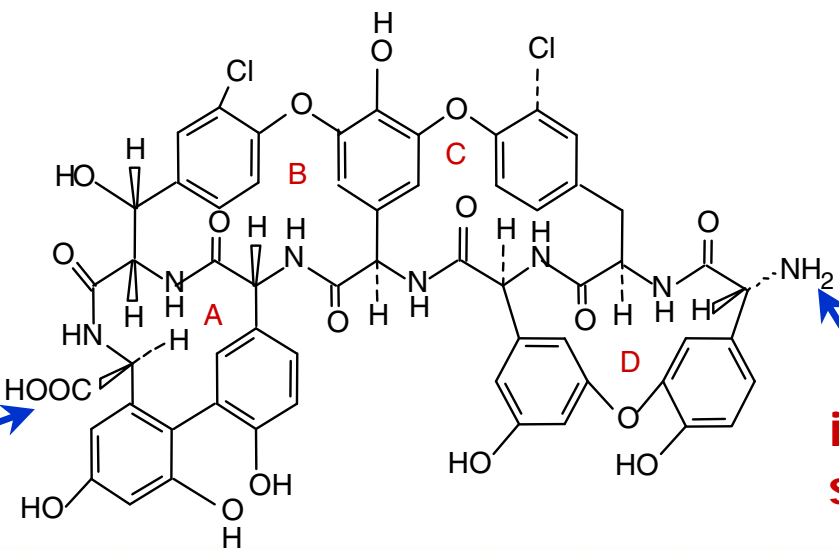
→ **Key sites**



ionic site

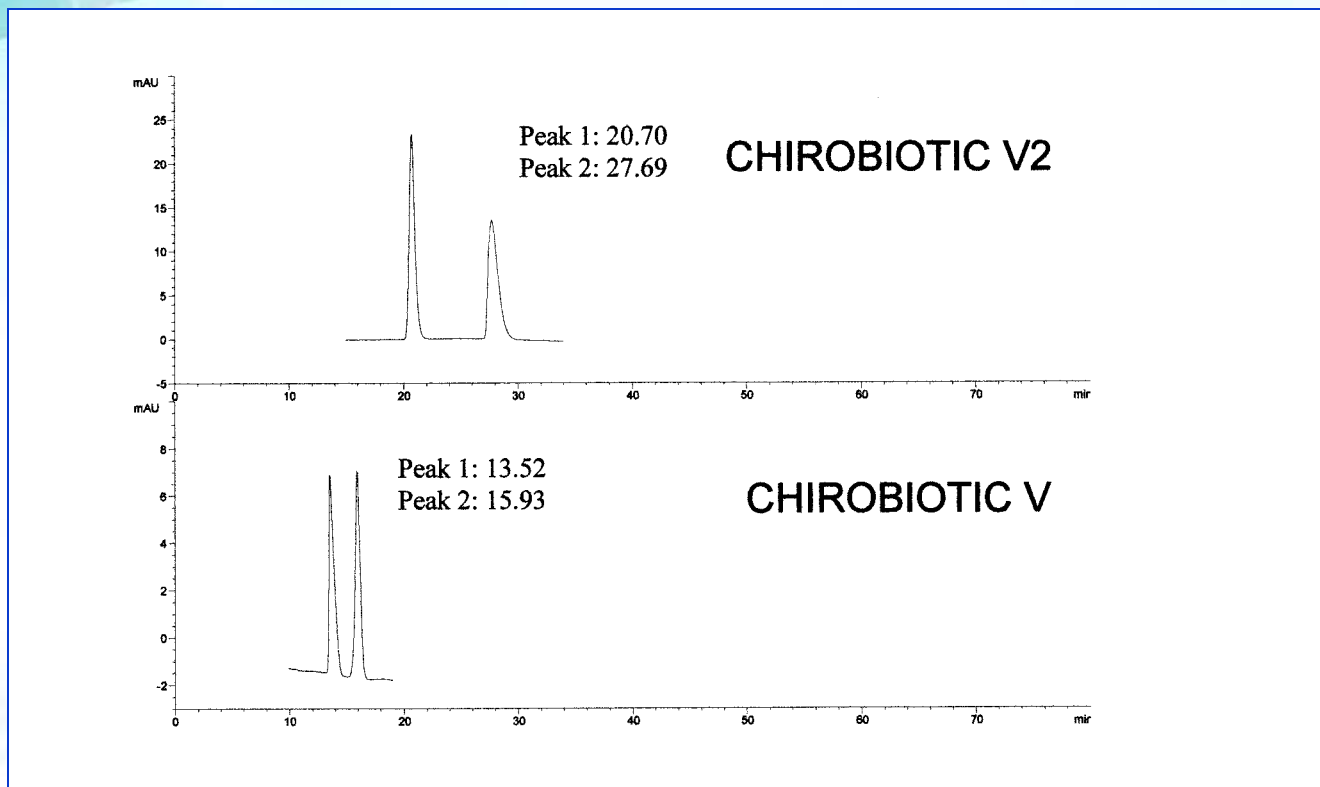
**Teicoplanin Aglycone,  
CHIROBIOTIC TAG**

ionic site



ionic site

# Comparison CHIROBIOTIC V2 vs. V



Enantio-separation of Ritalin by CHIROBIOTIC V2 (250x4.6mm) (top) and CHIROBIOTIC V (bottom), respectively. Mobile phase is 95/5, MeOH/20mM NH<sub>4</sub>OAc, pH 4.1 and the flow rate is 1 mL/min (ambient temperature).

# 3-Point Chiral Interactions on Multi-modal CHIROBIOTIC™ Phases

The most plausible interaction forces in descending order of strength under different mobile phase conditions:

1. **Polar Ionic Mode (ionizable compounds only)**
  - A. Ionic
  - B. Hydrogen Bonding
  - C. Steric/ $\pi$ - $\pi$
2. **Polar Organic/Normal Phase Mode (neutral compounds)**
  - A. Hydrogen Bonding
  - B.  $\pi$ - $\pi$
  - C. Steric/Dipole
3. **Reversed Phase Mode (all compounds)**
  - A. Ionic
  - B. Hydrogen Bonding
  - C. Steric/Inclusion/Hydrophobic

# Comparison Four Basic Mobile Phase Types for CHIROBIOTIC CSPs on same column

Compound is:

Ionizable

## **Polar Ionic mode:**

Methanol + Acid + Base (100+0.1+0.1, v/v/v)  
or Methanol + Volatile Ammonium Salt (100+0.1% v/w)

Neutral

## **Normal Phase mode:**

Polar + Nonpolar: EtOH+Heptane; 70/30;v/v

Neutral/Polar

## **Polar organic mode:**

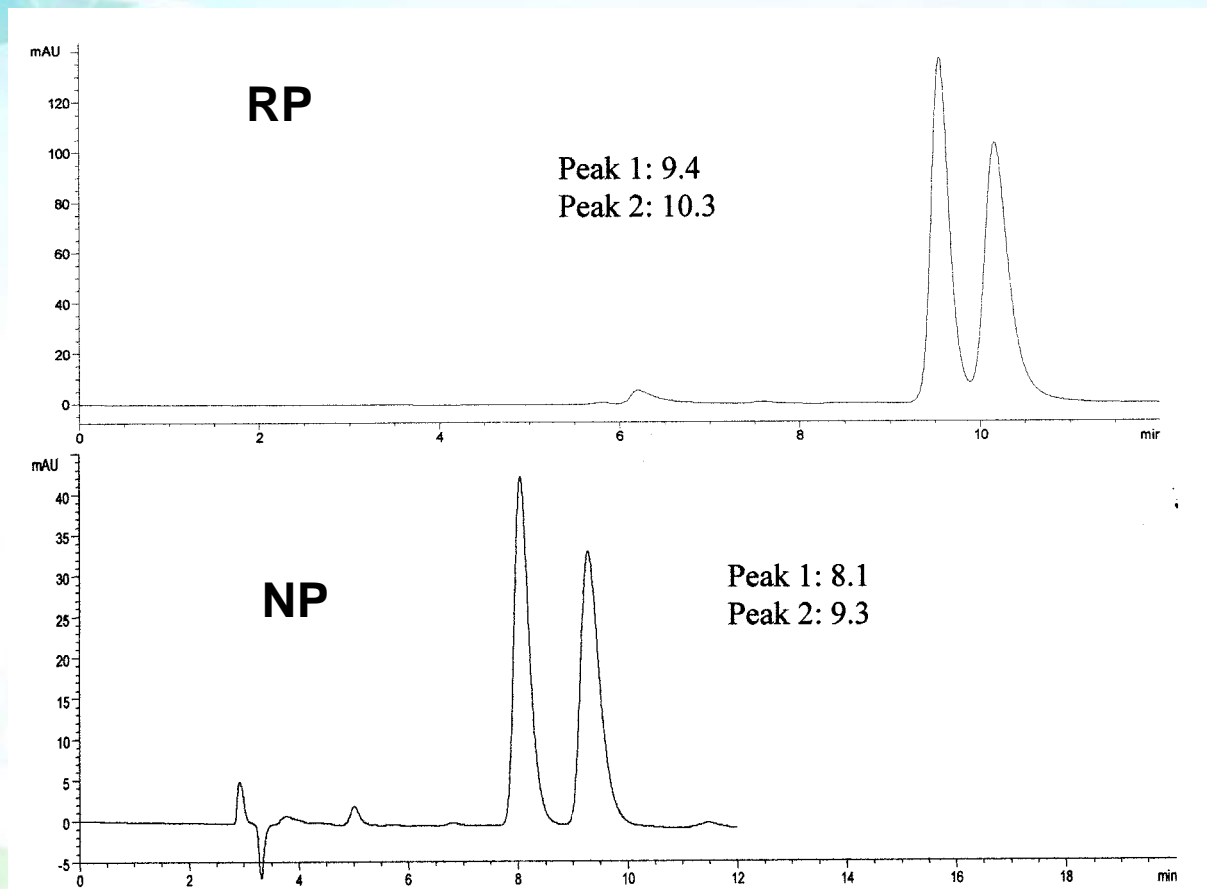
Polar/Nonpolar: MeOH; EtOH or ACN or combinations,  
eg MeOH/ACN

All types

## **Reversed Phase mode:**

Organic + Aqueous Buffer: ACN+TEAA or NH<sub>4</sub>OAc

# Conversion Reversed Phase to Normal Phase Using MtBE

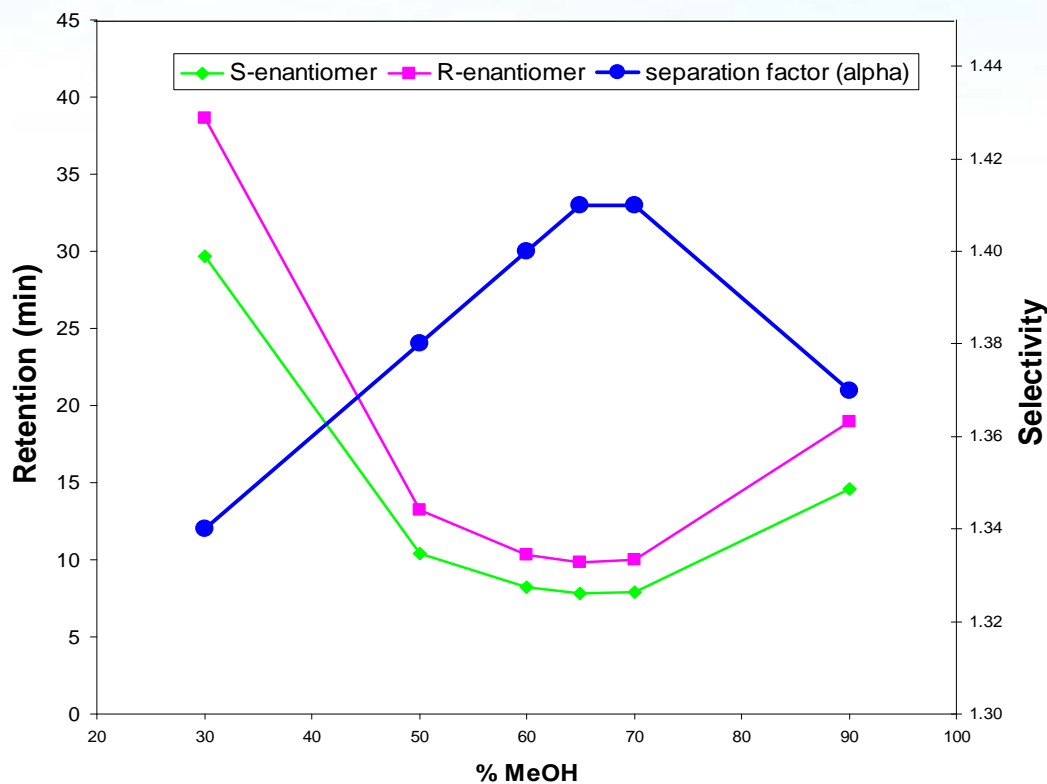


Methyl phenyl sulfoxide separation by CHIROBIOTIC V (250x4.6mm).  
Top: 20/80, THF/20 mM NH<sub>4</sub>NO<sub>3</sub>. Bottom: 97/2/1, MtBE/ACN/MeOH.

# Method Optimization in Reversed-Phase Mode

- Organic modifier type and composition can dramatically effect retention and selectivity
- Other parameters to consider:
  - Temperature
  - Flow rate
  - Organic type
  - pH
  - Buffer

## Fluoxetine in Reversed-Phase Mode



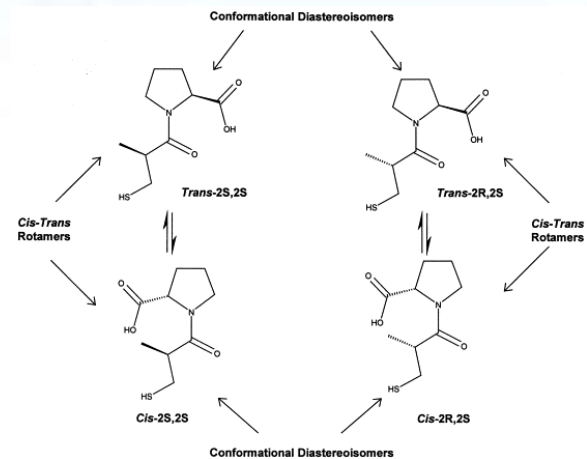
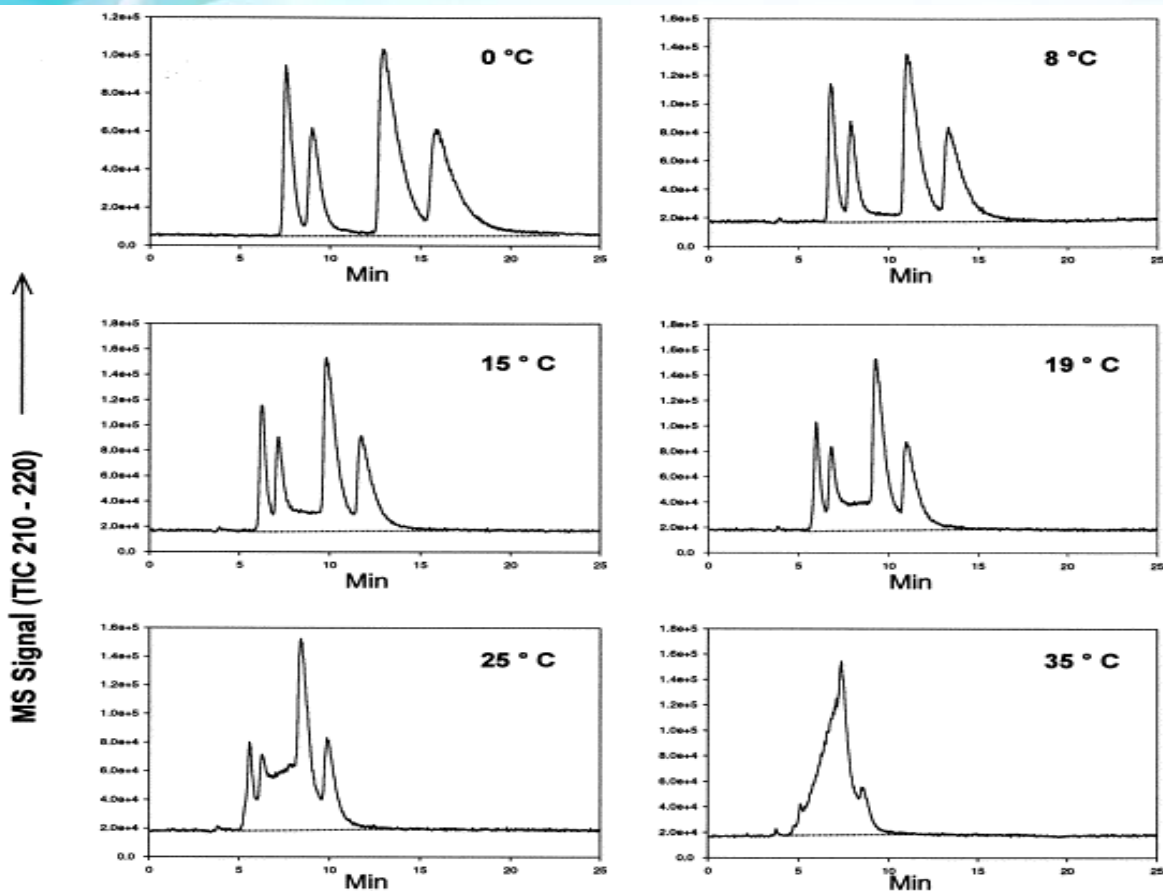
- The graph shows both reversed-phase and normal-phase behavior for fluoxetine on CHIROBIOTIC V2 as a function of methanol composition
  - selectivity is optimal between 65% and 70% methanol
  - Studies showed that methanol acted as a better organic modifier than ACN

# 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

# Temperature Study During Method Development



## Compound: Captopril diastereoisomers

Column: Chirobiotic T (4.6 mm x 250 mm)

Mobile phase: 0.05% TEAA (pH3.8) buffer

Flow rate: 1.0 ml/min

Detection: mass spectrometer in negative ion mode scanning between 210 and 220 mass units

P.K. Owens, *et. al.* J.Pharm.Bomed. Anal. 2001, 25, 453-464. Courtesy of Elsevier Science.

# Polar Ionic Mode: Ionizable analytes

Composition:

**MeOH + HOAc + TEA**

Dominant interactions:

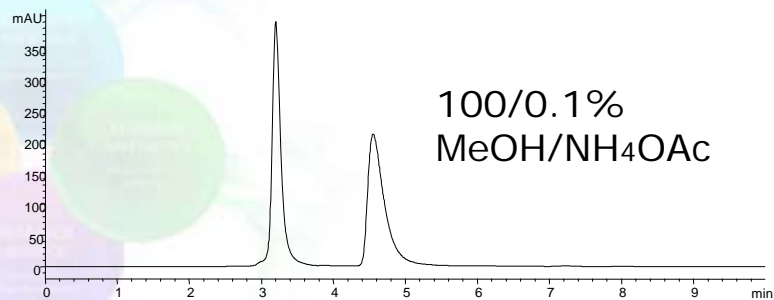
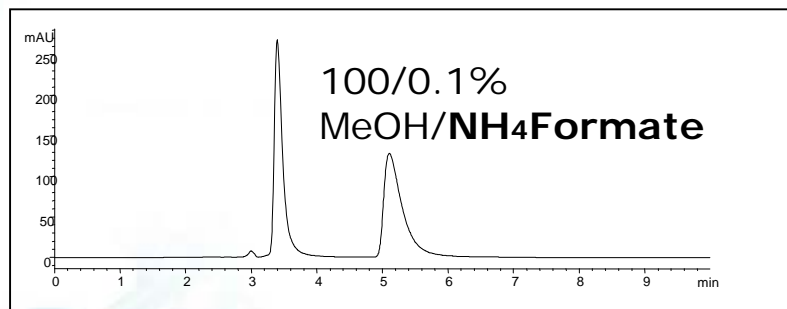
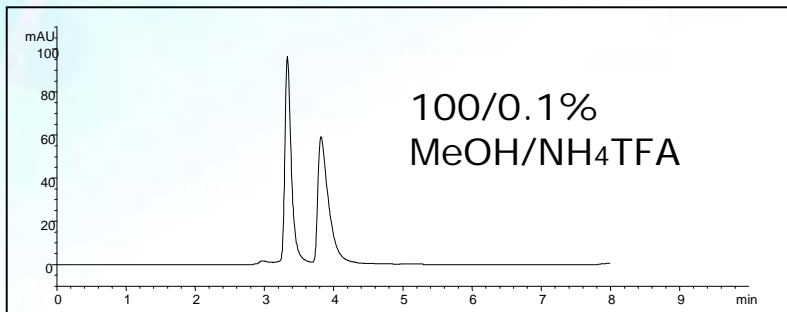
**Ionic interaction, hydrogen bonding**

Type of CSPs:

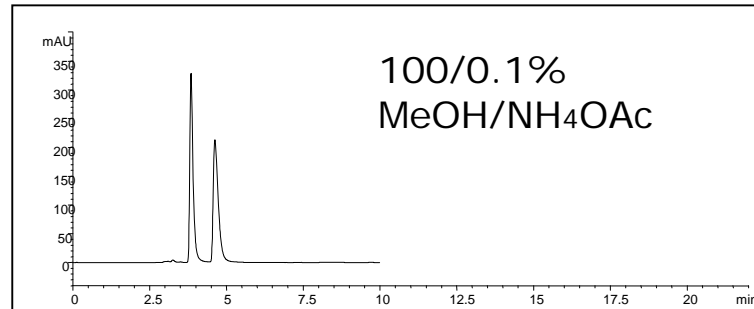
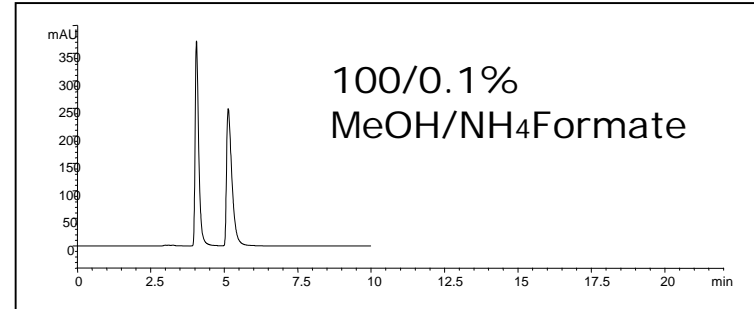
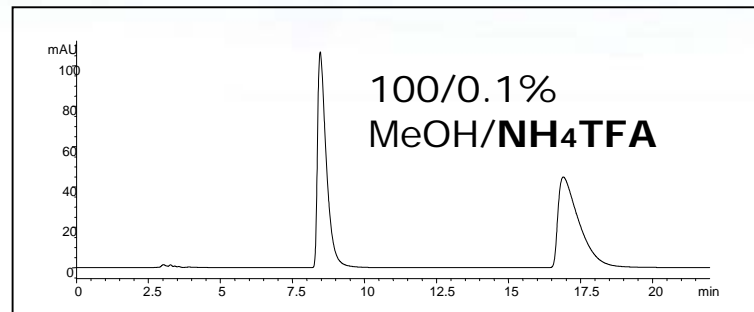
**Macrocyclic glycopeptides only -  
CHIROBIOTIC V2, T, R and TAG**

# Salt Effects in the Polar Ionic Mode

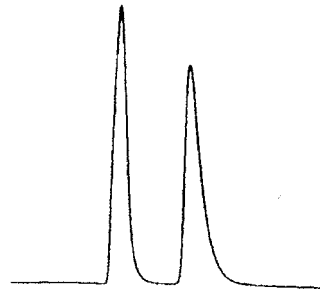
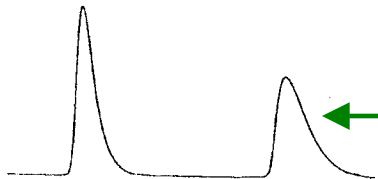
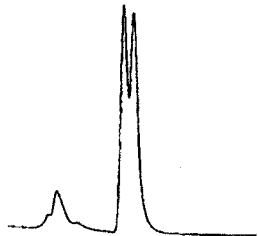
**Acid: Atrolactic acid**  
**CHIROBIOTIC T2**



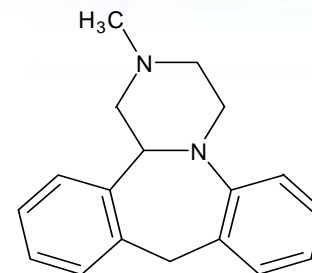
**Base: Mianserin**  
**CHIROBIOTIC V2**



# Importance of Acid/Base Ratios In PIM

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

## 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

Note:  
short  
retention  
times

**New Updated Version**

# 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)
<b>REVERSED PHASE MODE:</b>		
1	MeOH/20mM NH <sub>4</sub> OAc, pH 4.0	20/80
2	MeOH/20mM NH <sub>4</sub> OAc, pH 6.0	20/80
3	ACN/20mM NH <sub>4</sub> OAc, pH 4.0	30/70
4	ACN/20mM NH <sub>4</sub> OAc, pH 6.0	30/70
<b>POLAR IONIC MODE®:</b>		
5	MeOH/HOAc/TEA*	100/0.1/0.1
<b>POLAR ORGANIC MODE:</b>		
6	ACN/MeOH/HOAc/TEA	95/5/0.3/0.2
<small>CHIROBIOTIC PHASES ONLY</small> If not progressing to normal phase, wash with MeOH at this stage to test and store the column.		
<b>NORMAL PHASE MODE:</b>		
7	EtOH/Hexane (or heptane, isohexane)	30/70
8	Washing cycle	100% EtOH
9	Column storage: CHIROBIOTIC CYCLOBOND	100% MeOH 100% IPA

\* Use salts (NH<sub>4</sub>O<sub>2</sub>CCF<sub>3</sub> for bases, NH<sub>4</sub>OAc 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

- 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.
- 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.  
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.
- If a separation occurs in the polar ionic mode®, for a neutral molecule, change to 100% organic solvent (i.e. MeOH, EtOH or ACN).
- 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)	<ul style="list-style-type: none"> <li>● Test alternative acid/base ratios (generally higher acid for basic molecules, higher base for acidic molecules)</li> <li>● 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.</li> </ul>
Polar organic mode	<ul style="list-style-type: none"> <li>● Eliminate MeOH</li> <li>● Test alternative acid/base ratios</li> </ul>
Reversed phase mode	<ul style="list-style-type: none"> <li>● Test smaller pH changes</li> <li>● Change organic to THF, ACN, MeOH</li> <li>● Change buffer type and buffer concentration</li> <li>● Change temperature</li> </ul>
Normal phase mode	<ul style="list-style-type: none"> <li>● Change EtOH concentration</li> </ul>

## 6.

### OPTIMIZING FOR MS DETECTION

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

**CYCLOBOND:** Use NH<sub>4</sub>OH 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@astecuro.com www.astecuro.com

# Test Range of Racemic Switches- Representatives of Popular Chiral Drugs

- **Basic racemates:**

- **Analgesic:** Methadone; Nefopam; Tramadol
- **CNS stimulant:** Methylphenidate
- **Gastroprokinetic:** Mosapride
- **Anthelmintic:** Oxamniquine
- **Decongestant:** Pseudoephedrine
- **Antipsychotic:** Thioridazine
- **Muscle relaxant:** Tolperisone
- **Sedative:** Zopiclone

# Test Range of Racemic Switches- Representatives of Popular Chiral Drugs

- **Basic racemates continued:**

- **Bronchodilator:** Albuterol; Clenbuterol; Epinephrine; Formoterol; Isoproterenol; Terbutaline
- **Antihypertensive:** Amlodipine; Lercanidipine; Metoprolol; Nicardipine; Propranolol; Sotalol
- **Antifungal:** Miconazole
- **Anesthetic:** Bupivacaine
- **Antihistaminic:** Chlorpheniramine
- **Antidepressant:** Citalopram; Fluoxetine; Mianserin; Nefopam; Sertraline; Trimipramine

# Test Range of Racemic Switches- Representatives of Popular Chiral Drugs

- **Acidic racemates:**

- Anti-inflammatory: Ibuprofen; Ketoprofen;  
Naproxen

- **Neutral racemates:**

- Antiulcerative: Omeprazole
- Anxiolytic: Lorazepam; Oxazepam
- Pediculicide: cis-Permethrin
- Anticoagulant: Warfarin

# Racemic Switches: Optimized Conditions

## CHIROBIOTIC Phases

**Final Results**

<b>Compounds</b>	<b>Mobile Phase</b>	<b>Column</b>	<b>k1</b>	<b><math>\alpha</math></b>	<b>Rs</b>
Albuterol	100/0.1w%, MeOH/NH <sub>4</sub> TFA	T2	2.5	1.33	2.8
Amlodipine	100/0.1w%, MeOH/NH <sub>4</sub> TFA	V2	3.2	1.11	1.5
Bupivacaine	100/0.1w%, MeOH/NH <sub>4</sub> TFA	V2	0.8	1.40	2.5
Citalopram	100/0.1w%, MeOH/NH <sub>4</sub> TFA	V	4.8	1.12	1.5
Clenbuterol	100/0.1w%, MeOH/NH <sub>4</sub> TFA	T2	2.1	1.26	2.5
Fluoxetine	100/0.1w%, MeOH/NH <sub>4</sub> TFA	V2	1.5	1.27	2.5
Formoterol	100/0.6/0.4, MeOH/HOAc/TEA	T2	2.6	1.20	1.5

# Racemic Switches: Optimized Conditions

## CHIROBIOTIC Phases con't

**Final Results**

<b>Compounds</b>	<b>Mobile Phase</b>	<b>Column</b>	<b>k<sub>1</sub></b>	<b>α</b>	<b>Rs</b>
Ibuprofen	10/90, THF/20mM NaCitate, 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 <sub>4</sub> TFA	V	1.0	1.18	1.5
Lorazepam	100 % MeOH	T	0.4	4.01	11
Methylphenidate	100/0.1w%, MeOH/NH <sub>4</sub> TFA	V2	2.4	1.53	4.0
Metoprolol	100/0.1w%, MeOH/NH <sub>4</sub> TFA	T	4.5	1.14	2.0
Mianserin	100/0.1w%, MeOH/NH <sub>4</sub> Formate	V2	0.5	1.86	2.8

# Racemic Switches: Optimized Conditions

## CHIROBIOTIC Phases con't

**Final Results**

<b>Compounds</b>	<b>Mobile Phase</b>	<b>Column</b>	<b>k<sub>1</sub></b>	<b>α</b>	<b>Rs</b>
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 <sub>4</sub> TFA	V	0.7	1.74	4.5
Oxamniquine	100/0.1w%, MeOH/NH <sub>4</sub> TFA	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 <sub>4</sub> TFA	T	4.2	1.14	1.9
Pseudo-ephedrine	100/0.1w%, MeOH/NH <sub>4</sub> TFA	T2	2.7	1.13	1.7

# Racemic Switches: Optimized Conditions CHIROBIOTIC Phases con't

**Final Results**

<b>Compounds</b>	<b>Mobile Phase</b>	<b>Column</b>	<b>k<sub>1</sub></b>	<b>α</b>	<b>Rs</b>
Sotalol	100/0.2/0.1, MeOH/HOAc/TEA	T	3.5	1.12	1.5
Terbutaline	100/0.1w%, MeOH/NH <sub>4</sub> TFA	T2	2.2	1.92	7.0
Thalidomide	100% MeOH	V	0.7	3.44	7.0
Tolperisone	100/0.1w%, MeOH/NH <sub>4</sub> TFA	V2	1.8	2.33	2.7
Trimipramine	100/0.1w%, MeOH/NH <sub>4</sub> TFA	V2	1.7	1.28	2.3
Warfarin	40/60, EtOH/0.1% TEAA, pH 4.1	V	1.3	1.45	3.0

Final Results

# Racemic Switches: Optimized Conditions CYCLOBOND Phases

Compounds	Mobile Phase	Column	k <sub>1</sub> '	$\alpha$	Rs
Chlorpheniramine	10/90, ACN/0.1% TEAA, pH4.1	$\beta$ -CD	4.7	1.14	1.5
Chlorthalidone	15/85, ACN/10mM NH <sub>4</sub> OAc, pH4.0	HP-RSP	1.4	1.22	1.6
Epinephrine	25/75, ACN/20mM NH <sub>4</sub> OAc, pH4.1	HP-RSP	7.1	1.16	2.0
Methadone	20/80, ACN/ 10mM NH <sub>4</sub> OAc, pH 3.6	HP-RSP	1.5	1.24	1.7
Miconazole	25/75, ACN/10mM NH <sub>4</sub> OAc, pH 4.0	HP-RSP	4.1	1.18	1.6
Nefopam	25/75, ACN/20mMNH <sub>4</sub> OAc,pH 4.1	DNP	0.5	1.52	2.2
Omeprazole	100/0.4/0.1, ACN/HOAc/NH <sub>4</sub> OH	DMP	3.3	1.41	2.4
Sertraline	30/70, ACN/20mM NH <sub>4</sub> OAc, pH4.1	HP-RSP	5.4	1.19	1.7
Thioridazine	20/80, ACN/0.1% TEAA, pH4.1	$\beta$ -CD	6.9	1.21	2.5
Tramadol	20/80, ACN/20mM NH <sub>4</sub> OAc, pH5.5	DMP	1.6	2.20	2.8
Zopiclone	95/5/0.3/0.2,ACN/MeOH/HOAc/TEA	$\beta$ -CD	1.0	1.13	1.5

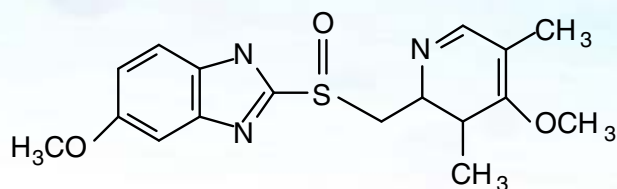
# Statistics

- 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:

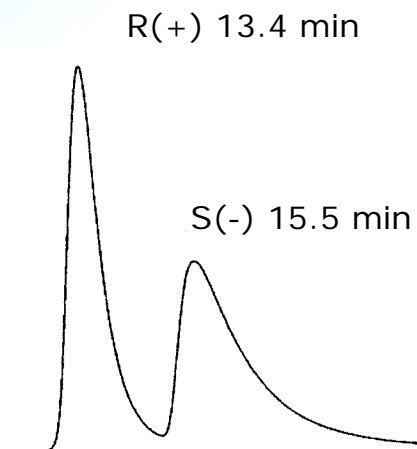
CYCLOBOND Phases:	1.5 to 2.8
CHIROBIOTIC Phases:	1.5 to 11.0

# Options from Results of Dual Screen

## Omeprozole (Nexium)

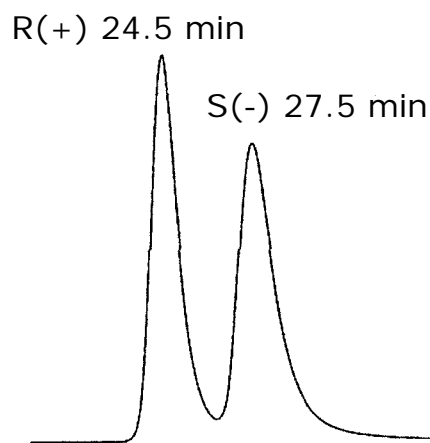


### CHIROBIOTIC R (NP)



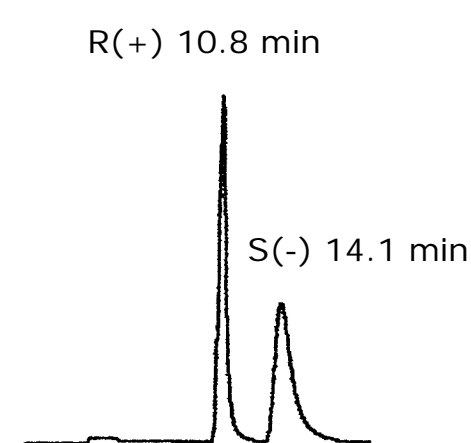
40/60:  
EtOH/Heptane

### CHIROBIOTIC R (RP)



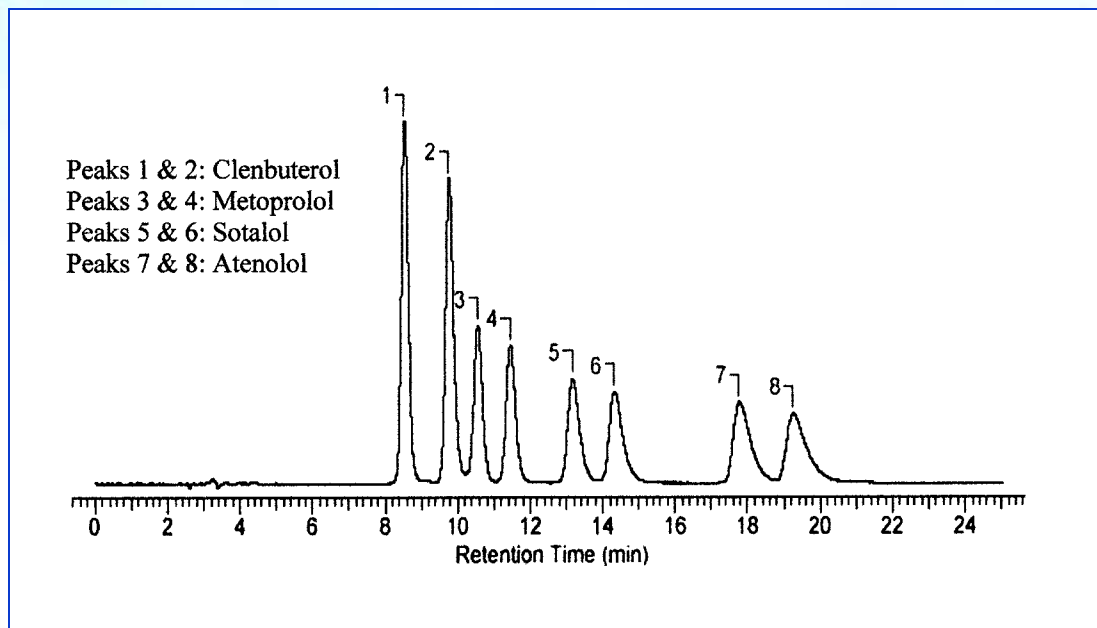
30/70: MeOH/10mM  
NH<sub>4</sub>OAc, pH 4.1

### CYCLOBOND I 2000 DMP (POM)



100/0.4/0.1:  
ACN/HOAc/NH<sub>4</sub>OH

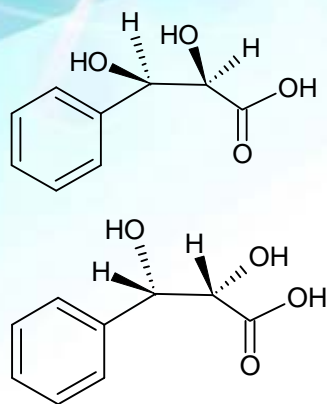
# Accommodation diverse structures with same chiral environment: Beta-blocker Separations



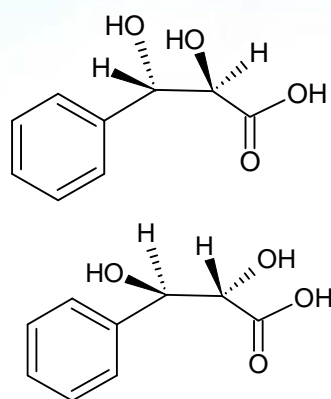
Simultaneous chiral separations of 4 beta blockers using CHIROBIOTIC T (250x4.6mm in polar ionic mode. The mobile phase is 15 mM Ammonium Formate in CH<sub>3</sub>OH, 1mL/min (ambient temperature).

Accommodates multiple chiral centers: Separation of 2,3-Dihydroxy-3-phenyl-propionic acid enantiomers on CHIROBIOTIC R

Racemate A



Racemate B



Column: CHIROBIOTIC R, 5  $\mu$ m, 25cm x 4.6mm

Mobile Phase : 50:50; MeOH/ .01% NH<sub>4</sub>HCO<sub>2</sub>, pH 4.1

Flow Rate: 1 mL/min.

Temperature: Ambient.

Detection: UV @258nm

Injection: 2,3-Dihydroxy-3-phenyl-propionic acid isomers:  
Racemate A (4.85 and 6.95 min.)  
Racemate B (5.33 and 6.29 min.)

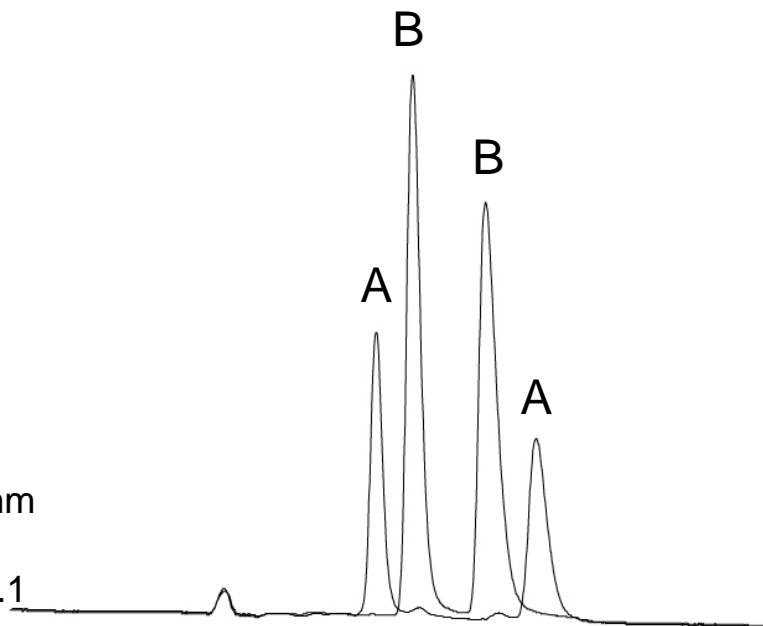
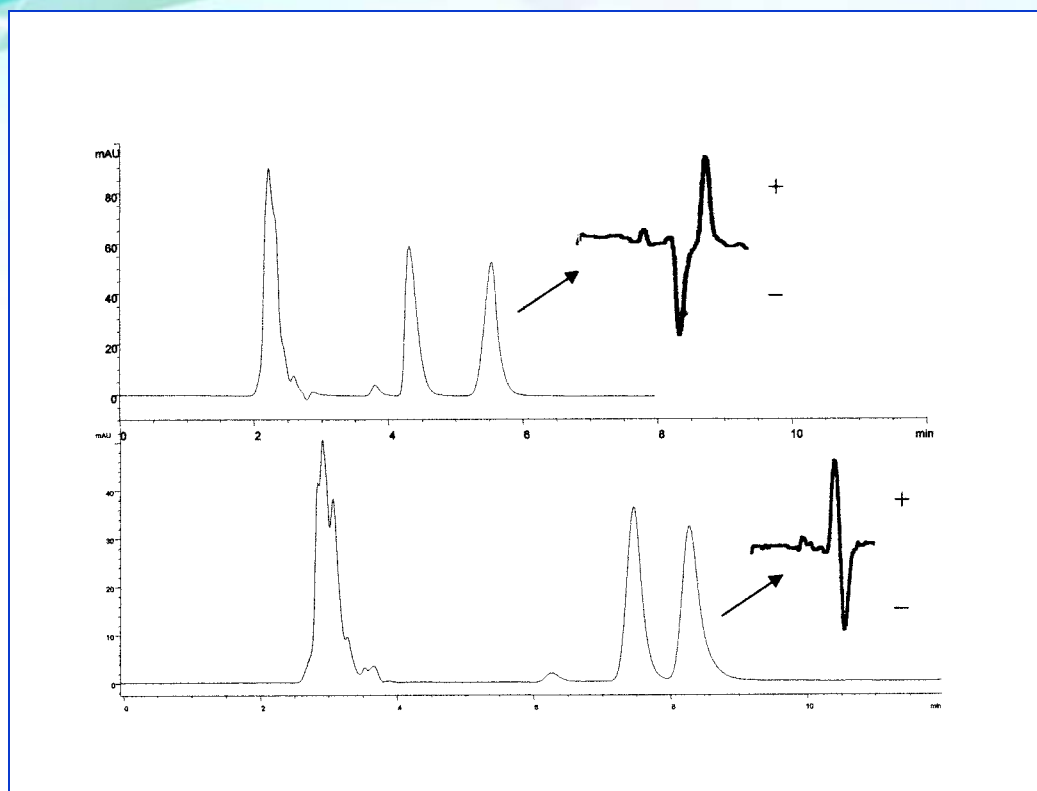


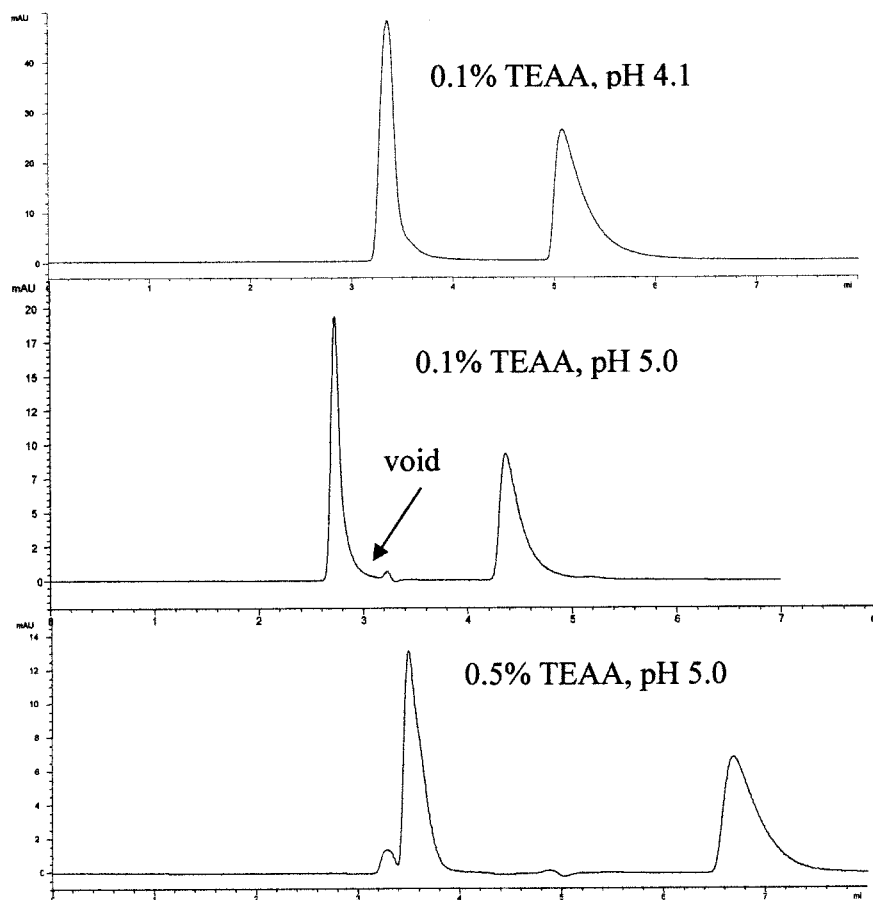
Figure courtesy  
DSM Fine Chemicals

# Reverse Elution Order



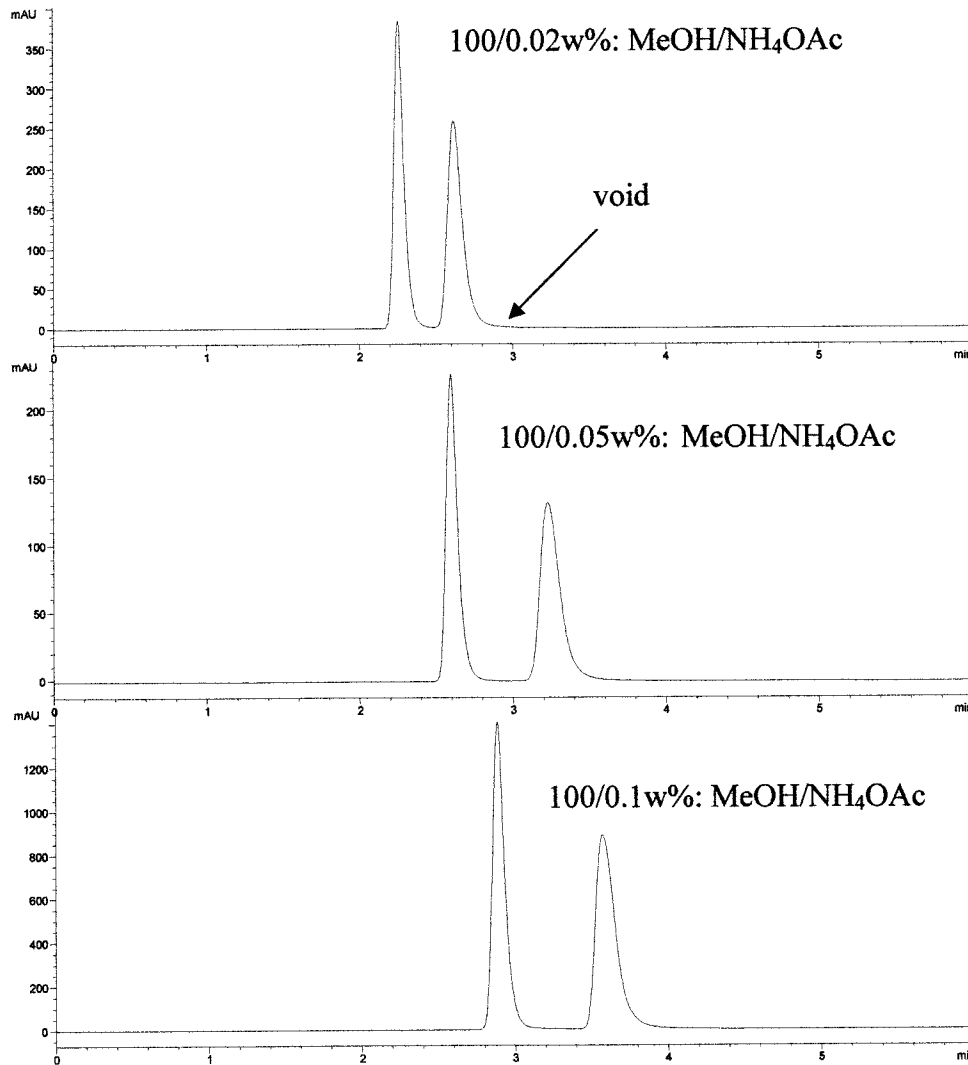
Reversal of elution order between CHIROBIOTIC V2 (top) and CHIROBIOTIC T2 (bottom) columns under the exact same condition. Mobile phase is 15mM NH<sub>4</sub> formate in MeOH and the flow rate is 1 mL/min. The polarimeter showed the first peak is (-) on the CHIROBIOTIC V2 while the first peaks is (+) on the CHIROBIOTIC T2.

# Ion Repulsion



Ionic effects showing the repulsion phenomenon between CSP (CHIROBIOTIC T, 250x4.6mm) and the analyte (mandelic acid) in 30/70, MeOH/TEAA. At pH 5 (0.1% TEAA), the first peak eluted before column void (3 mL). Higher concentrations (0.5% TEAA) of buffer can alleviate that effect.

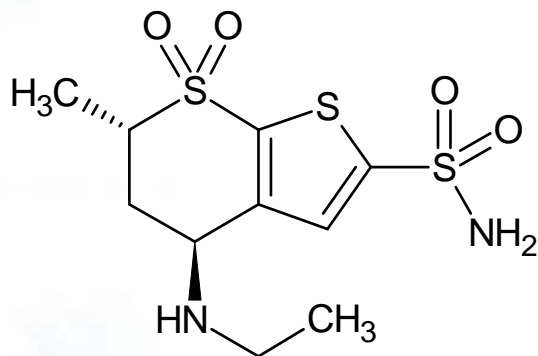
# Ion Repulsion



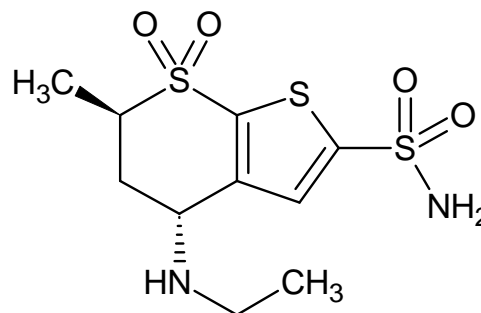
CHIROBIOTIC T (250x4.6mm)  
Dansyl methionine.  
Higher salt concentrations can  
reduce this response.  
The column void is 3.0 mL and  
the flow rate is 1 mL/min.

# Dorzolamide \*HCl Method Development



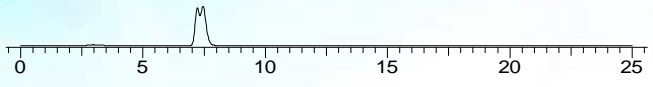
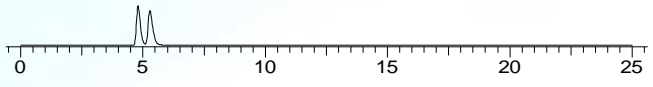
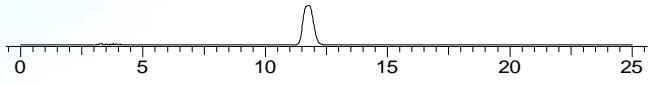
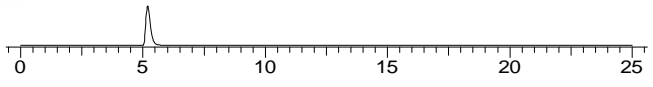
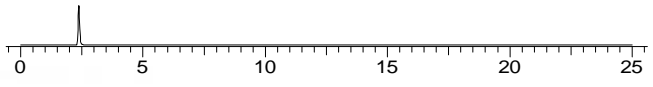
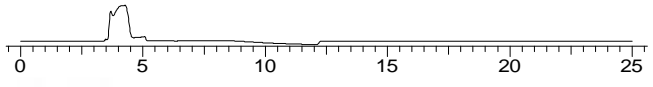

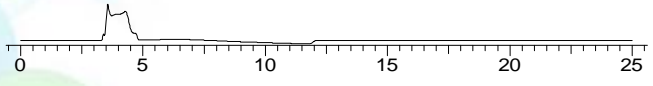

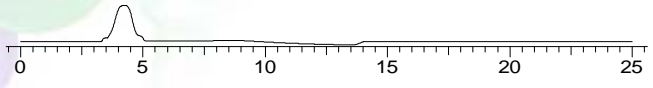
- Processed through method development screen:
- 3 CHIROBIOTIC phases: V2,T,TAG
- 3 CYCLOBOND phases: B-CD,DNP,HP-RSP.



**Dorzolamide  
Hydrochloride  
4S,6S**

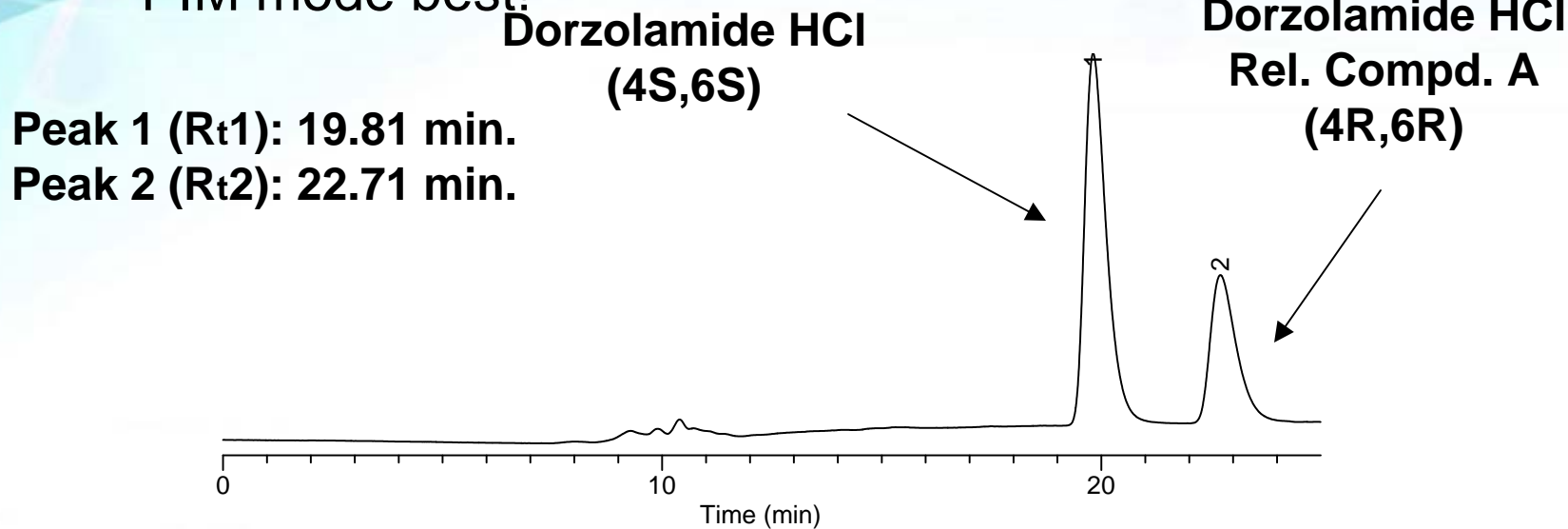


**Dorzolamide  
Hydrochloride  
Related Compound A  
4R,6R**

Spectrum	Column	mode	elution	File
	CHIROBIOTIC TAG	RP	No Retention	C:\ascii\AC D_Chrom1 373.cdf
	CHIROBIOTIC TAG	PIM	Separation	C:\ascii\AC D_Chrom1 384.cdf
	CHIROBIOTIC V2	RP	Separation	C:\ascii\AC D_Chrom1 397.cdf
	CHIROBIOTIC V2	PIM	Separation	C:\ascii\AC D_Chrom1 404.cdf
	CHIROBIOTIC T	RP	No Separation	C:\ascii\AC D_Chrom1 416.cdf
	CHIROBIOTIC T	PIM	No Separation	C:\ascii\AC D_Chrom1 423.cdf
	Cyclobond I 2000	RP	No Retention	C:\ascii\AC D_Chrom1 435.cdf
	Cyclobond I 2000	POM	Unknown	C:\ascii\AC D_Chrom1 442.cdf
	Cyclobond 2000 HP-RSP	RP	No Retention	C:\ascii\AC D_Chrom1 461.cdf
	Cyclobond 2000 HP-RSP	POM	Unknown	C:\ascii\AC D_Chrom1 485.cdf
	Cyclobond 2000 DNP	RP	No Separation	C:\ascii\AC D_Chrom1 497.cdf
	Cyclobond 2000 DNP	POM	Unknown	C:\ascii\AC D_Chrom1 504.cdf

# Chromatographic Results

- PIM mode best!



**CHIROBIOTIC V2, 250x4.6mm I.D., 5 $\mu$  particles (15024AST)**  
**100:5 MeOH:H<sub>2</sub>O, 3.81mM NH<sub>4</sub>TFA (or 0.05% NH<sub>4</sub>TFA)**  
**22°C, 0.3mL/min., UV-254nm**  
**Inj. Vol. 10  $\mu$ L @1.0 mg/mL in MeOH**

# CHIROBIOTIC LC Columns in LC-MS-MS

- PIM mode (nonaqueous): High enantioselectivity in 100% MeOH with acid/base or salts, short Rt times, works well with ESI

MeOH/HOAc/TEA; 100/0.1/0.1 or  
MeOH/0.1% NH<sub>4</sub>OAc, NH<sub>4</sub>OTFA or NH<sub>4</sub>HCO<sub>2</sub>

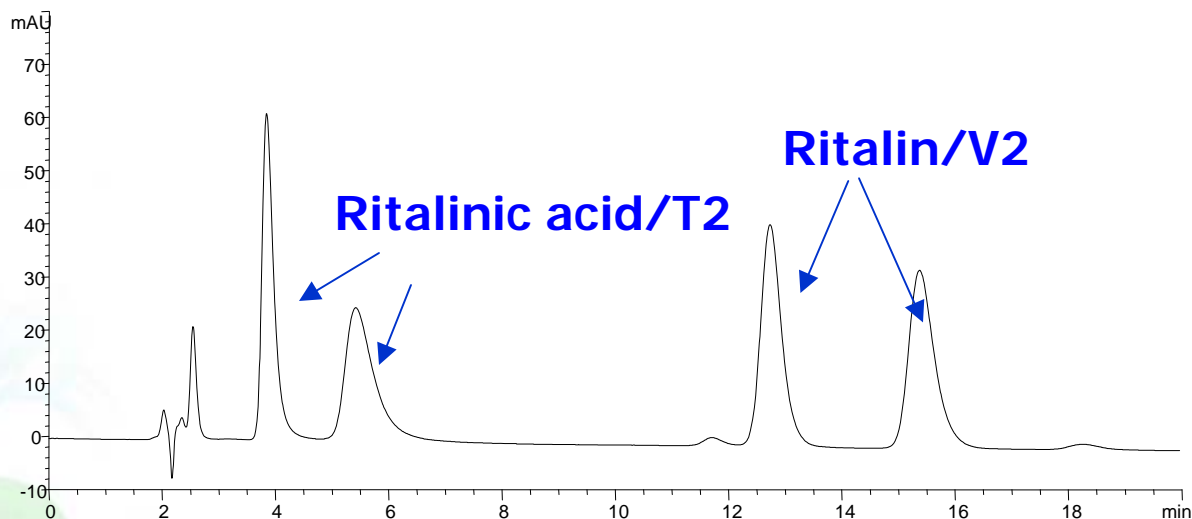
- RP mode (aqueous): Compatible with ammonium acetate or formate
  - No problem with principle solvents used in APCI (from alcohols to hydrocarbons, DMSO, DMF)

# Column Coupling: Separation of Ritalin and Its Metabolite

**Column:** **CHIROBIOTIC T2** (20x4.0mm) +  
**CHIROBIOTIC V2** (150x4.6mm)

**Mobile Phase:** 93/7: MeOH/20 mM NH<sub>4</sub>OAc, pH 4.1

**Flow Rate:** 1.0 mL/min



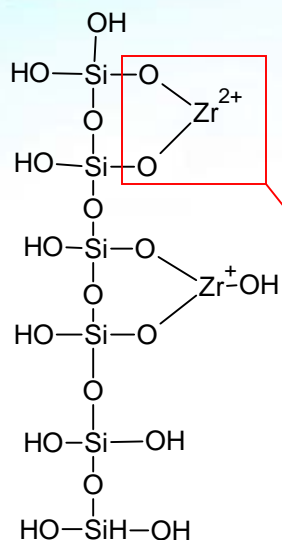
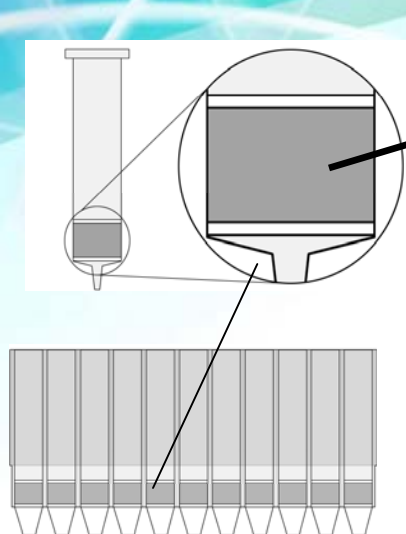
# Bioanalytical Sample Preparation

- Objective:
  - Concentrate analytes of interest
  - Selectively separate analytes of interest from **endogenous interferences** inherent with the sample
  - more compatible sample matrix for analytical system
- Available techniques: dialysis, monolithic chromatography, SFC extraction, protein precipitation, liquid/liquid extraction, SPE

# Phospholipid & Ion-Suppression

- Phospholipids: primary constituent of cell membranes & documented to be a major cause of ion-suppression in the positive ion electrospray mode (+ESI)
- Present in extremely high concentrations in biological matrixes & represent the second largest lipid component next to triglycerides and fats

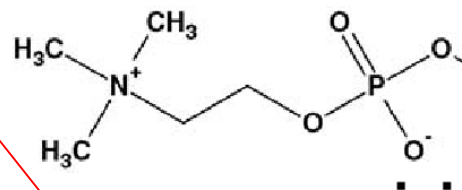
# How are Phospholipids Selectively Removed using HybridSPE?



## Proprietary Zirconia coated Silica

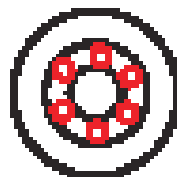
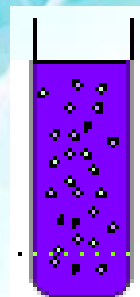
**Note:** The presence of  $\geq 1\%$  formic acid in the MeCN precipitation agent is critical because of: 1) formic acid is a stronger Lewis base than most carboxyl (-COOH) groups found in acidic pharmaceutical compounds but not as strong a Lewis base as the phosphate moiety found in phospholipids; and 2) the low pH environment neutralizes residual silanol activity on the silica surface thereby eliminating secondary cation-exchange interaction with basic compounds of interest.

The phosphate moiety of phospholipids is a strong Lewis base (electron donor) that interacts Zr atoms coated on the silica surface.

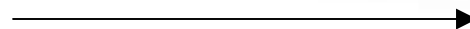


The Zr atom acts as a Lewis acid (electron acceptor) because it has empty d-orbitals.

# Off-Line Precipitation Method for HybridSPE 1 mL cartridge or 96-well format



2) Centrifuge



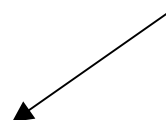
3) Transfer supernatant to HybridSPE cartridge or 96-well plate



Retained Phospholipids

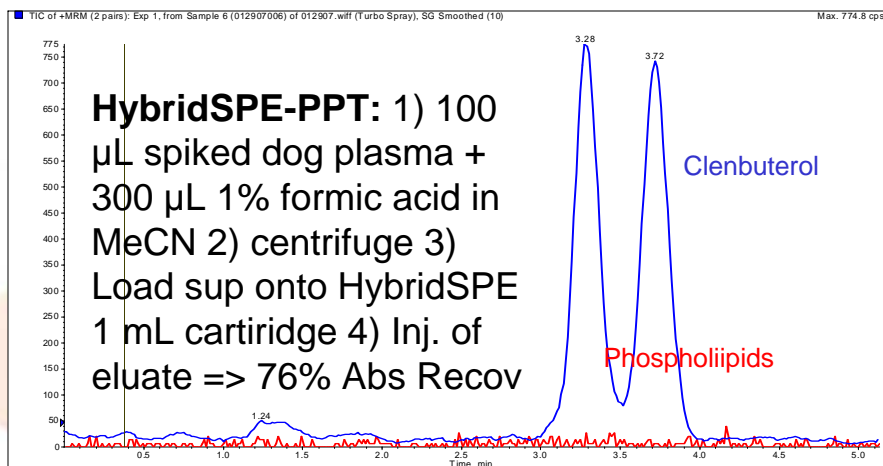
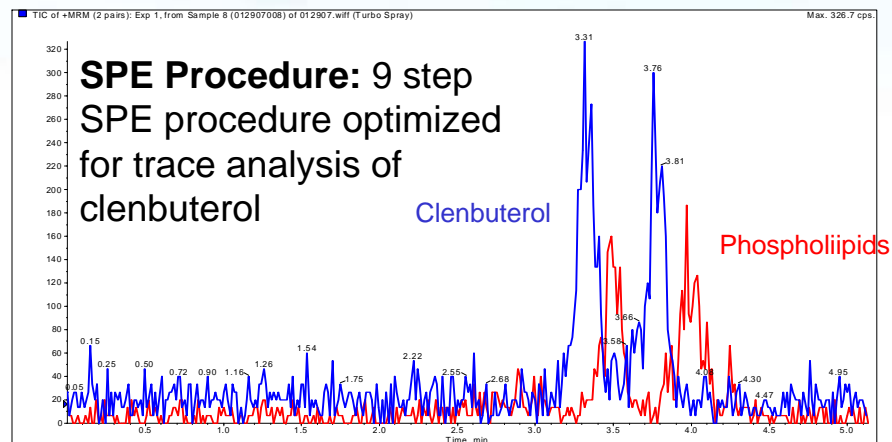
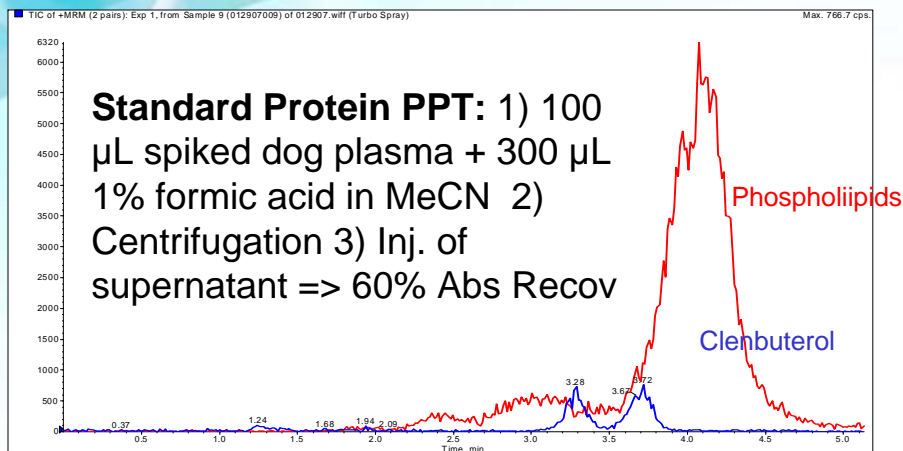
1) **Precipitate Proteins:** Combine 100  $\mu$ L plasma serum with 300  $\mu$ L 1% formic acid in acetonitrile. Add I.S. as necessary.

4) **Apply vacuum.** Phospholipids are retained on HybridSPE sorbent while small molecules (e.g., pharma compounds & metabolites) pass through un-retained.



5) **Resulting filtrate/eluante** is free of proteins and phospholipids and ready for immediate LC-MS/MS analysis; or it can be evaporated and reconstituted as necessary prior to analysis

# Extraction of 10 ng/mL Clenbuterol from Dog Plasma



Column: Chirobiotic T 10cm X 2.1mm, 5  $\mu$ m  
Mobile Phase: 10mM Ammonium Formate/Methanol  
Flow: 300  $\mu$ L/min  
Temp: 30  $^{\circ}$ C  
Detection: MS-MRM

# HybridSPE Recovery of Representative Pharma Compounds

## Summary of Abs. Recovery from Plasma:

Compound Name	Spike Conc.	Matrix	Abs. Recovery
Propranolol	100ng/ml	Rat plasma	62%
Ketoprofen	100ng/ml	Rat plasma	82%
Doxepin	7.5ng/ml	rat plasma	87%
Imipramine	7.5ng/ml	rat plasma	92%
Amitriptyline	7.5ng/ml	rat plasma	90%
Oxyphenbutazone	20ng/ml	rat plasma	104%
Ketoprofen	20ng/ml	rat plasma	92%
Naproxen	20ng/ml	rat plasma	93%
Phenylbutazone	20ng/ml	rat plasma	99%
Flunixin	20ng/ml	rat plasma	76%
Procainamide	12.5ng/ml	rat plasma	42%
Doxepin	12.5ng/ml	rat plasma	89%
Mirtazapine	12.5ng/ml	rat plasma	56%
Dextromethorphan	12.5ng/ml	rat plasma	88%
p-Coumaric Acid	12.5ng/ml	rat plasma	54%
Clenbuterol	10ng/ml	dog plasma	75%
Sulfamethizone	10ng/ml	dog plasma	101%
Sulfacetamide	10ng/ml	dog plasma	98%
Sulfamerazine	10ng/ml	dog plasma	94%
Sulfathiazole	10ng/ml	dog plasma	98%
Sulfanilamide	10ng/ml	dog plasma	96%
Sulfadiazine	10ng/ml	dog plasma	49%

## Summary of Abs. Recovery Solvent (no plasma):

During the course of development, 12 mixes containing 5-8 unique/representative pharma compounds were prepared at a concentration 600 ng/mL (diluted in 1% formic acid in MeCN:Water (75:25, v/v)) – total of **66 compounds**.

Each test mix was passed through a 1 mL HybridSPE cartridge and the eluate was collected for LC-MS analysis. Recovery was assessed by measuring the analyte response before and after HybridSPE processing.

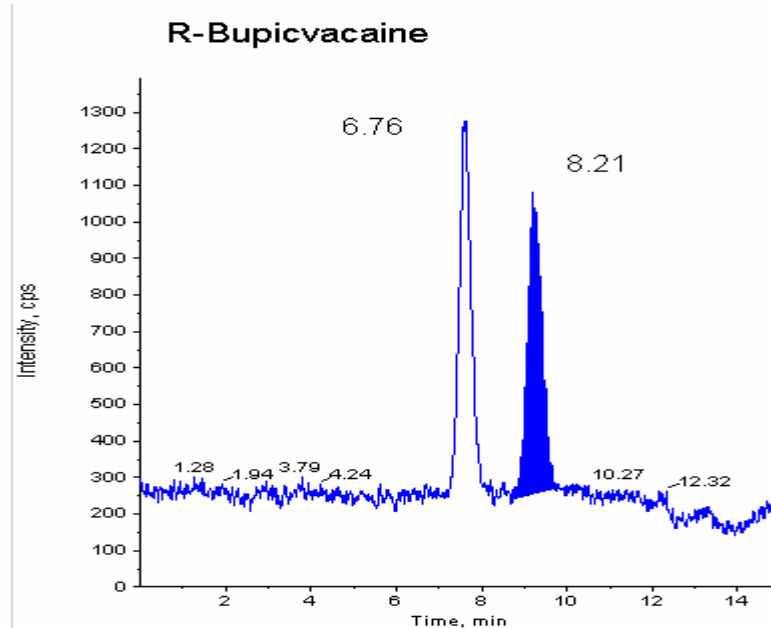
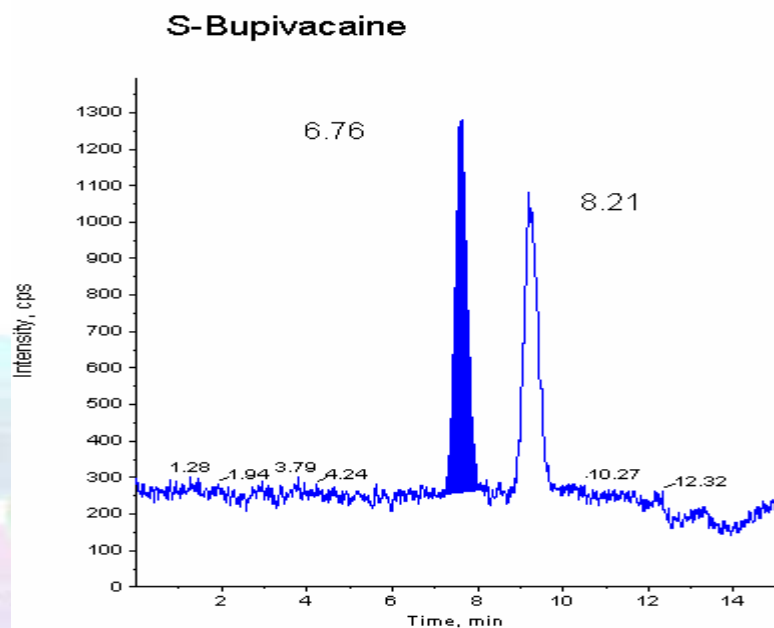
An **average absolute recovery of 83%** observed for the 66 compounds tested. The majority of the analytes tested achieved recoveries > 80%

# Bupivacaine in Plasma by LC/MS-ESI

**Column:** CHIROBIOTIC V2, 150x2.1 mm  
**Mobile Phase:** 90/10, MeOH/10mM NH<sub>4</sub>OAc, pH 4.1  
**Flow Rate:** 0.2 mL/min

**Excellent Correlation Coefficients** > 0.999

**Linear Range** = 0.15ng-50ng/mL



# Supelco Chiral Services Laboratory



# Conclusions

- **A successful high throughput screening system consisting of 4 CHIROBIOTIC phases and 4 CYCLOBOND phases has been devised employing 8 mobile phases.**
- **Polar ionic mode offers excellent opportunities for LC/MS and preparative applications.**
- **A 100% hit rate for 40 divergent racemic switches was obtained with a 20% overlap and resolutions from 1.5 to 11.0.**
- **LC-MS/MS technology offers excellent benefits in terms of throughput and sensitivity; but good sample prep is still required. Hybrid SPE aids speed by removal of phospholipid interferences.**