



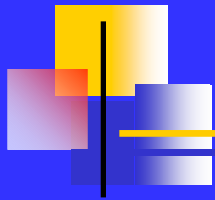
Chiral Method Development Techniques for Analytical & Prep HPLC

Advanced Separation Technologies

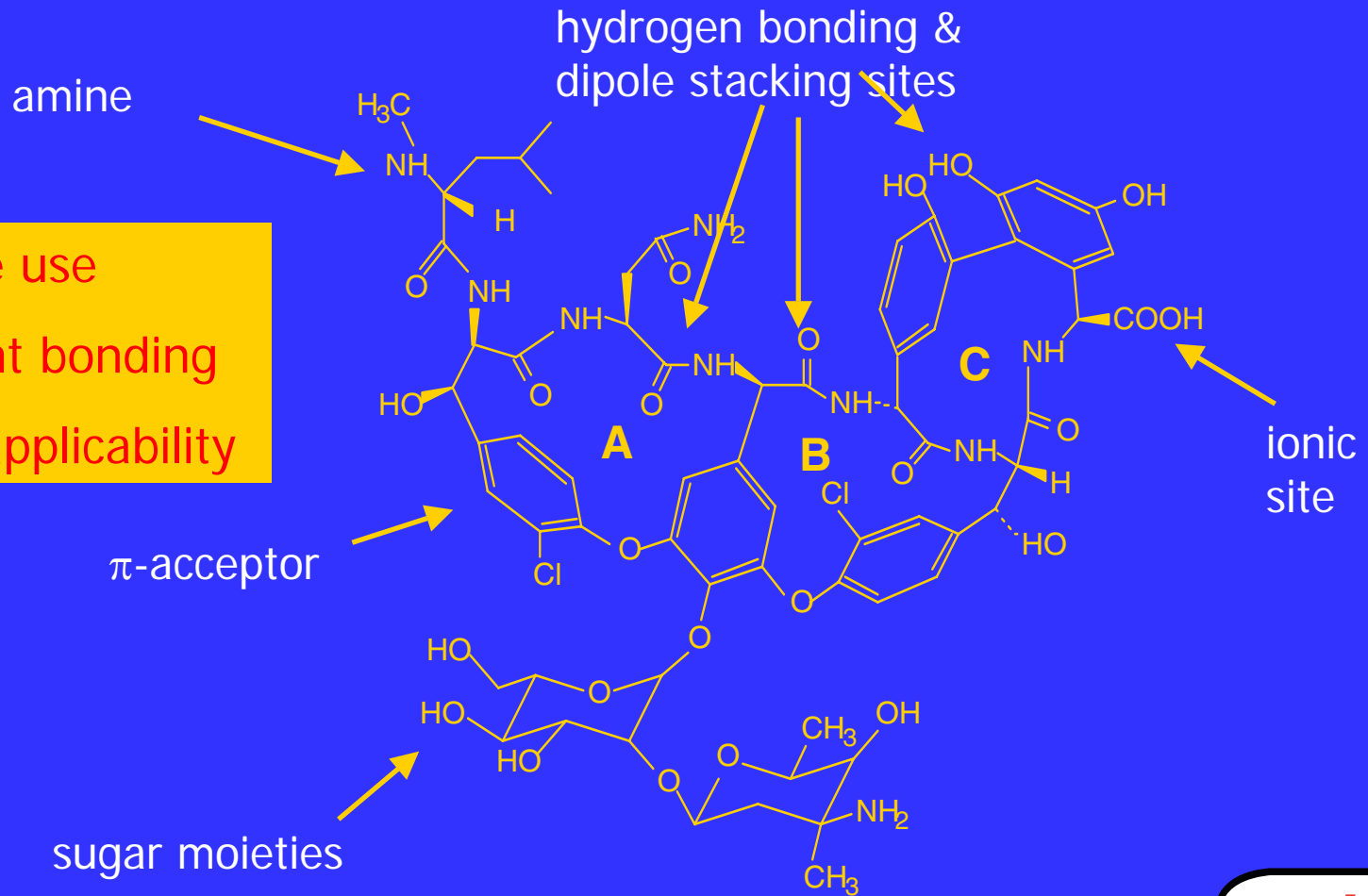
AGENDA:

- Method development techniques for the CHIROBIOTIC series of chiral LC columns
- Techniques for chiral LC/MS/MS
- Advances in preparative chiral LC

Proposed Structure of Vancomycin



- Multiple use
- Covalent bonding
- Broad applicability

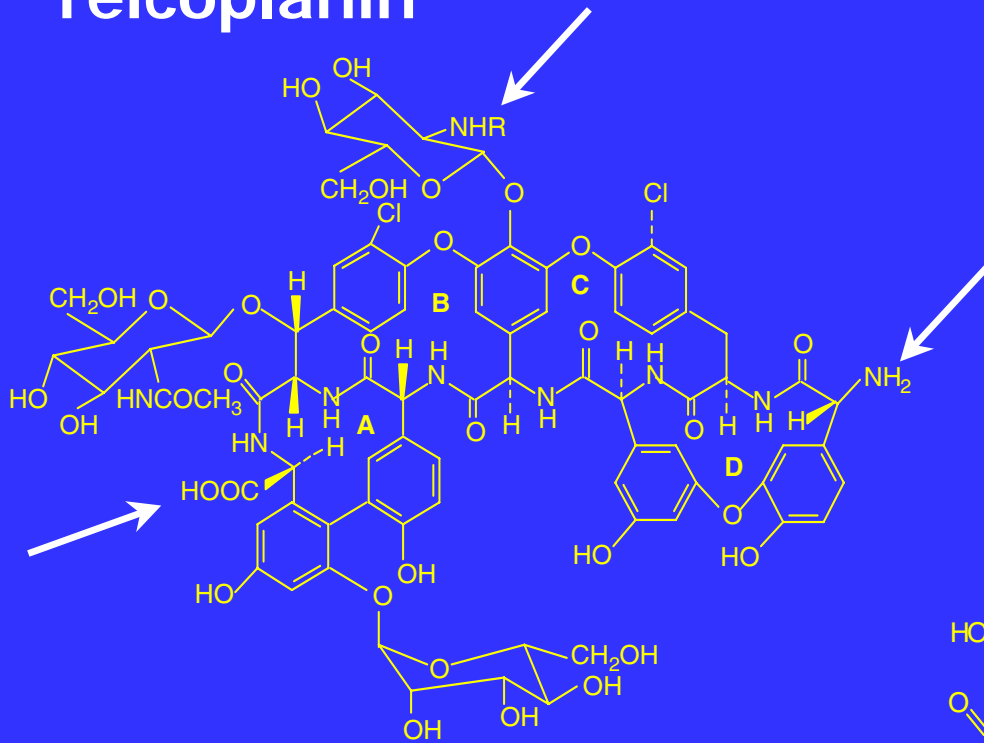


A, B, C are inclusion pockets (weak)

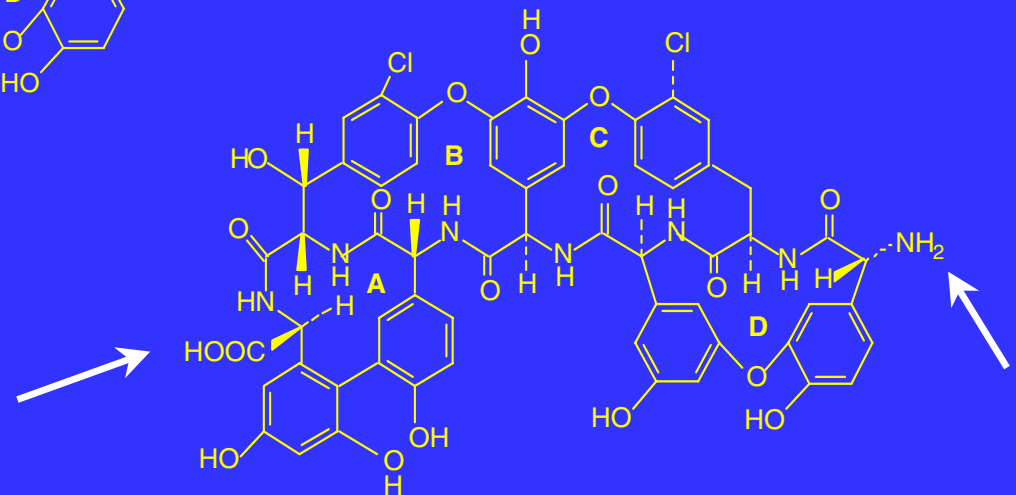
astec

Proposed Structures of Glycopeptide CSPs

Teicoplanin

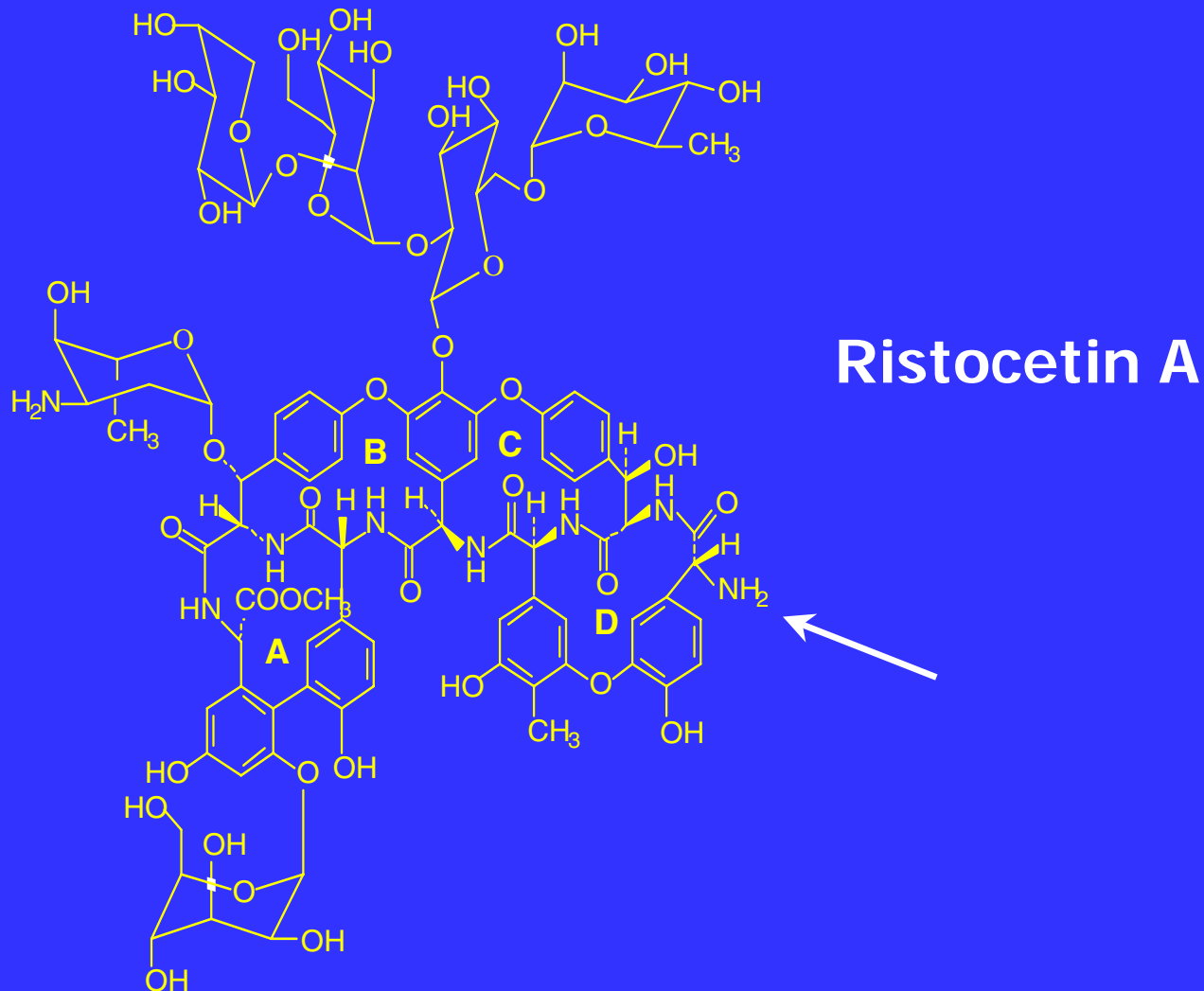


Teicoplanin Aglycone



→ Key sites

Proposed Structures of Glycopeptide CSPs





Features of CHIROBIOTIC Phases

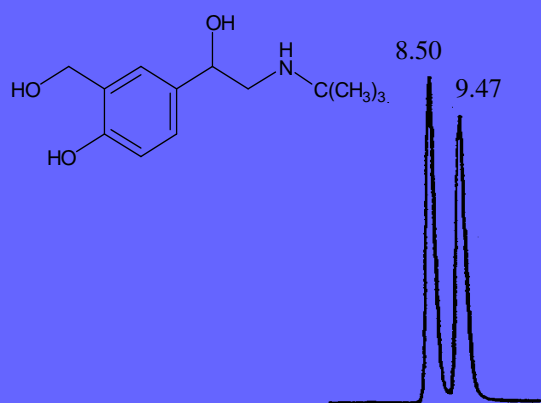
- Reversed phase
 - Largest and most versatile number of separations
- Polar Organic phase
 - Fast kinetics, second major group of separations
 - Best suited for LC/MS systems
- Normal phase
 - Generally provides ca 10-15% of the total methods
- Column coupling possible
 - Rapid, broad screening protocols

Each column can be used in all 3 modes

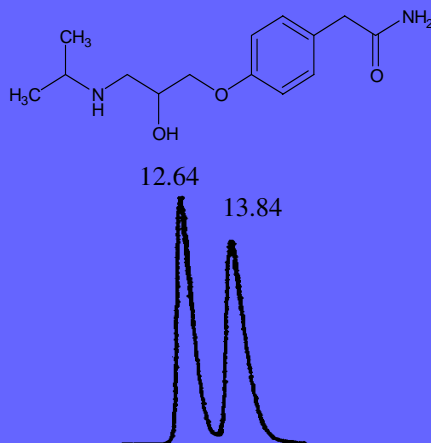
Broad Selectivity Based on the Same Stereogenic Center: CHIROBIOTIC T: Amino Alcohols



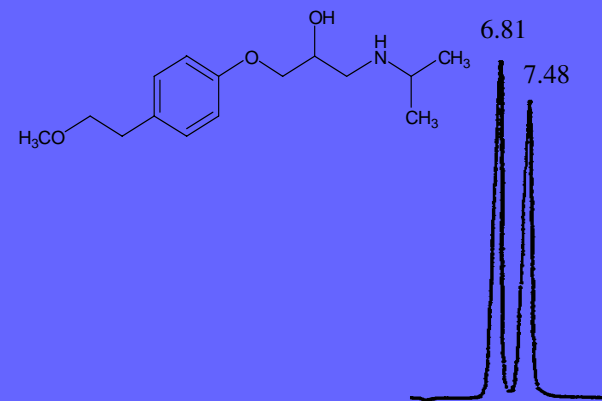
Albuterol



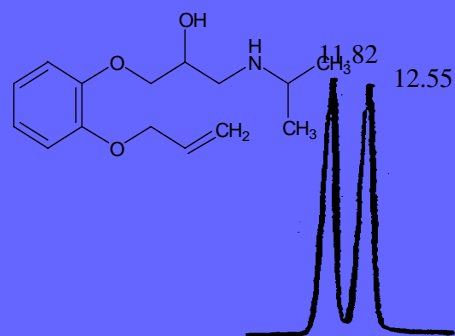
Atenolol



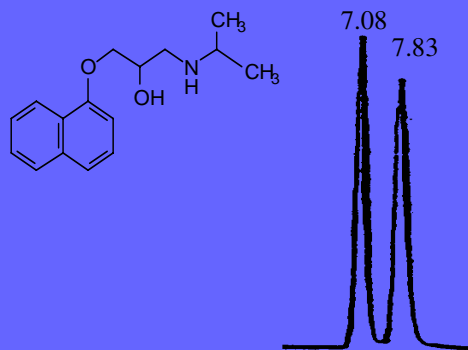
Metoprolol



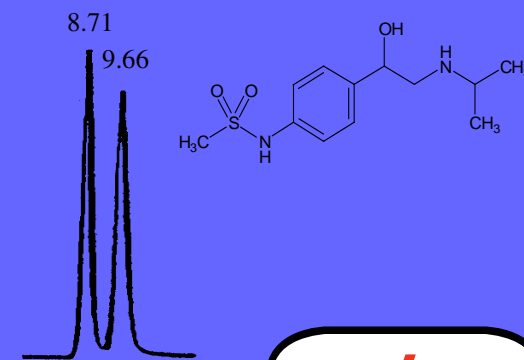
Oxprenolol



Propranolol



Sotalol



Mobile Phase: 100/0.1/0.1:MeOH/HOAc/TEA @ 2.0 mL/minute

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What is the Polar Organic Mode?

- The Polar Organic Mode was developed originally by Dr D W Armstrong and Astec in '85 for use with CYCLOBOND cyclodextrin technology
- 100 % MeOH with added acid and base (typically 0.1%, each with a range of 1.0 to 0.001%), or equivalent salts
- Method development is very simple and fast

Ionic

What is the Polar Organic Mode?

- The Polar Organic Mode terminology is now frequently applied to the Daicel phases, but in this case it means 100% organic only, usually MeOH, ACN or MeOH/EtOH
- The mechanism (predominantly hydrogen bonding) for the POM on Daicel is *different* from that on the CHIROBIOTIC phases and the optimization process is *different*

Ionic

What is the Polar Organic Mode?

- The mechanism for the POM on the CHIROBIOTIC phases is predominantly ionic, making it essential for acid and base to be added. Thus, Polar *Ionic* Mode[©]



Advantages of Polar Ionic Mode[©] on CHIROBIOTIC V, T and R

- No problem with salt containing compounds
- Compatible with column switching from C18 column to chiral column
- Scale-up is easy due to the volatility of mobile phase components
- Complementary to hexane/ethanol separations on polysaccharide CSPs
- For LC/MS, can use ammonium trifluoroacetate, acetate or formate (0.001 to 0.5% w/v in methanol)
- Applicable to the use of gradients for method development



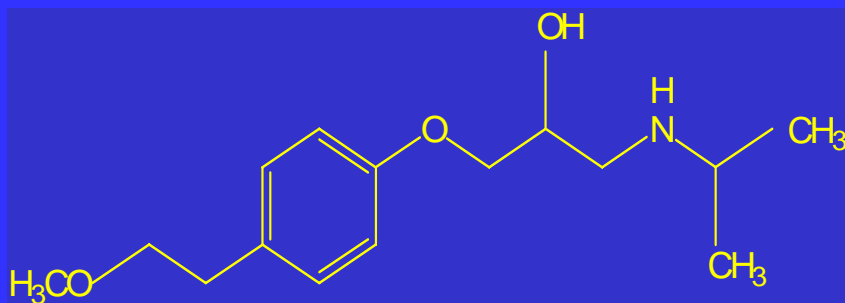
Normal phase vs Polar Ionic[©] Phase

- CHIROBIOTIC columns appear to be *complementary* to cellulosic and amylosic phases
 - Many separations that are done on Chiracel in normal phase can also be accomplished in the polar ionic[©] mode on CHIROBIOTIC columns
 - If a compound is polar, solubility may be better in the PIM rather than normal phase
 - The PIM can be advantageous and safer for prep

NOTE:

If using CHIROBIOTIC columns in the Normal Phase, they need much higher EtOH levels than Daicel columns for most chiral separations – typically 20-50% [EtOH]

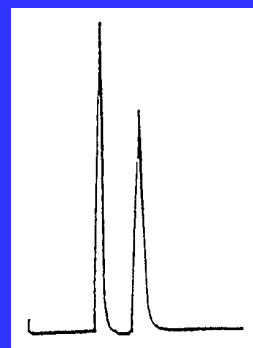
Metoprolol



CHIRACEL OD

Peak 1 – 11.9 min.
Peak 2 – 18.2 min.

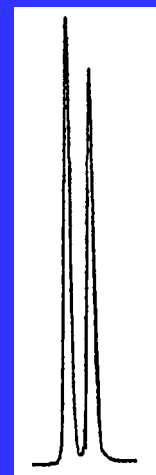
20/80/0.1: IPA/Hex/DEA



CHIROBIOTIC T

Peak 1 – 6.8 min
Peak 2 – 7.5 min

100/0.2/0.1: MeOH/HOAc/TEA



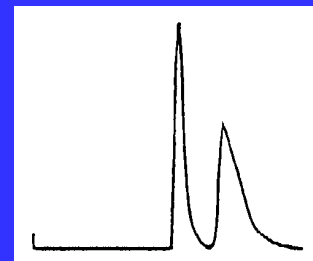
Alprenolol



CHIRACEL OD

Peak 1- 12.4 min
Peak 2 - 16.4 min

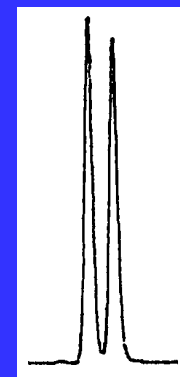
20/80/0.1: IPA/Hex/TFA



CHIROBIOTIC V

Peak 1 – 7.69 min.
Peak 2 – 8.33 min.

100/0.01/0.01: MeOH/HOAc/TEA



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CHIROBIOTIC TAG

- Selectivity on the CHIROBIOTIC T can be enhanced with CHIROBIOTIC TAG (aglycone from teicoplanin) for certain molecular types
- Enhanced resolution for α , β , γ and cyclic amino acids, neutrals, acids, sulfur containing molecules and certain primary amines



CHIROBIOTIC TAG

Mobile phase possibilities:

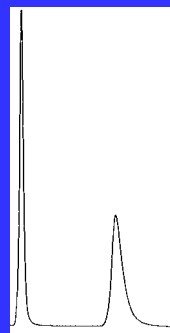
- Reversed phase – methanol as the organic modifier
- Polar ionic[©] phase – as for the CHIROBIOTIC T
- Neutral molecules use single solvent: 100% MeOH, EtOH, IPA, ACN. No acid or base required
- Normal Phase – good for large number of chiral sulfoxides, coumarins

Selectivity of Methanol for Neutral Molecules on CHIROBIOTIC TAG

5-Methyl-5-phenylhydantoin

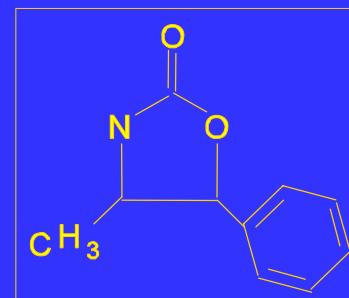


1. 5.08 min
2. 9.62 min

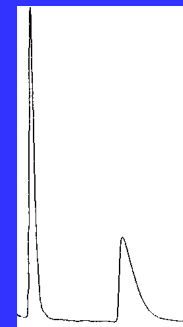


100% MeOH @ 0.8 mL/min

4-Methyl-5-phenyl-2-oxazolidinone

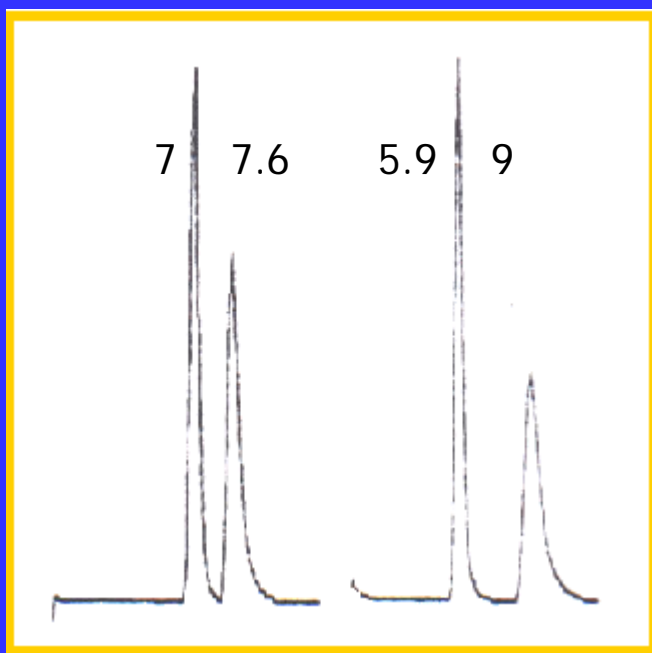
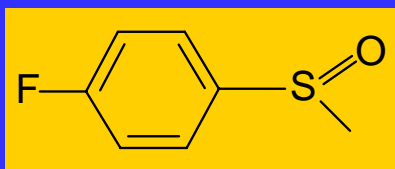


1. 5.35 min
2. 8.21 min



100% MeOH @ 0.8 mL/min

Chiral Sulfoxides on CHIROBIOTIC TAG



Teicoplanin

TAG

p-Fluorophenyl
methyl
sulfoxide

Mobile phase:
Hexane/EtOH, 50/50

Flow rate: 2 mL/min

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Method Development Techniques Using Column Coupling

- Generic screening methods for fast chiral method development
- Column coupling utilized to enable simultaneous multi-column screening



Method Development Techniques Using Coupled Columns

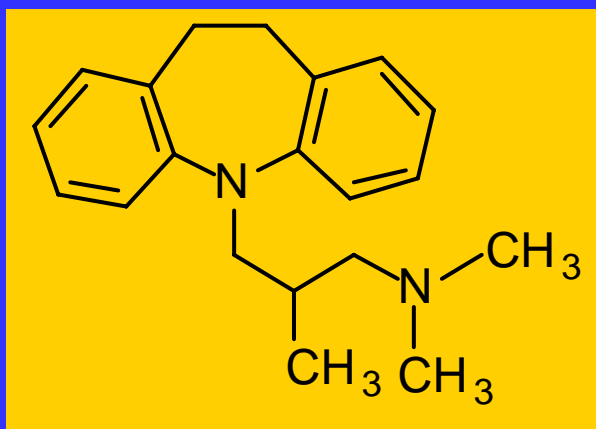
- 3 solvents systems, *plus* 3 different CHIROBIOTIC columns (close coupled kit) provides 9 screens in 105 minutes

SCREENING MOBILE PHASES:

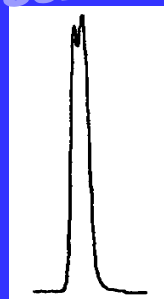
1. **Polar ionic mode**[©] (MeOH/AcOH/TEA, 100/0.02/0.01, 2.0 mL/min)
2. **Reversed phase mode** (MeOH/TEAA (0.1%, pH 6.0), 25/75, 1.0 mL/min)
3. **Normal phase mode** (Hex/EtOH, 40/60, 1.5 mL/min)

Optimization in the Polar Ionic Mode[©] from Coupled Column Screen for LC/MS

Trimipramine



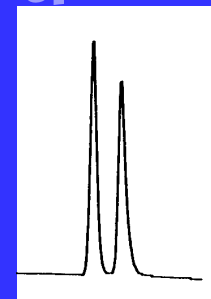
Screen



t₁: 11.27
t₂: 11.54

R+V+T (10cm)
MeOH/HOAc/TEA:
100/0.02/0.01
2 mL/min.

Optimised



t₁: 20.7
t₂: 22.8

V (25cm)
100/0.02 v/w:
MeOH/AFTA
0.8 mL/min.

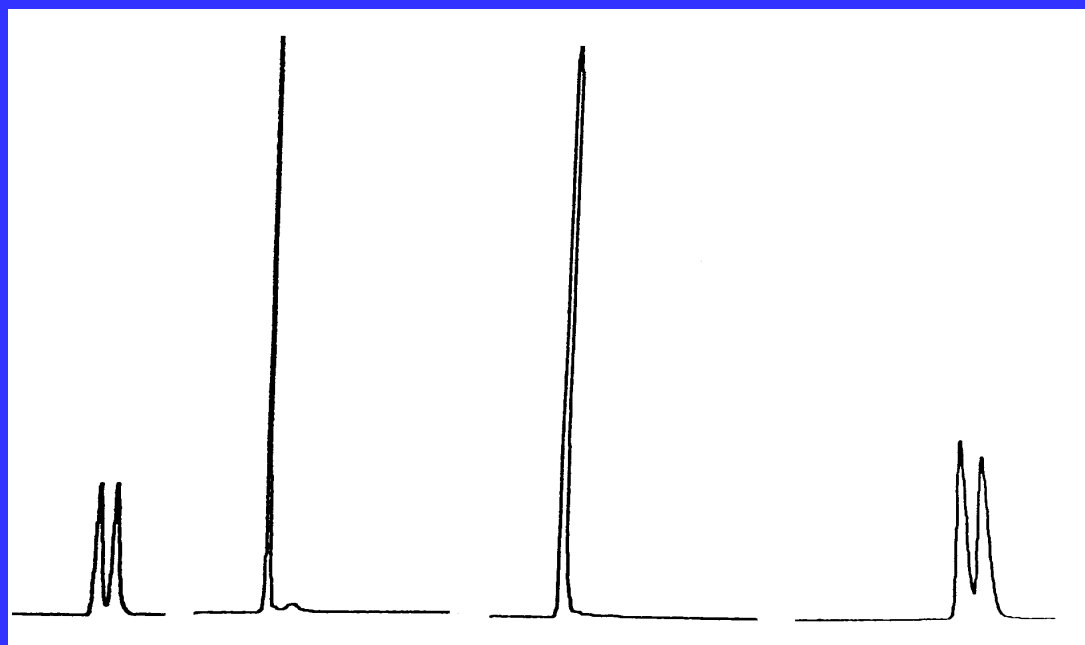
CASE HISTORY: Generic Method Development Screen: 4 Unknowns

Polar Ionic Mode, Run 1

Columns: CHIROBIOTIC R+V+T (100 x 4.6mm each)

Mobile Phase: 100/0.1/0.05, MeOH/AcOH/TEA

Results:



Sample A

Sample B

Sample C

Sample D

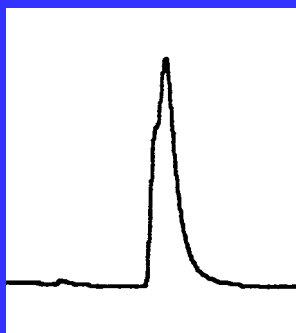
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Run 2 (on Sample B and C): Reversed Phase Screen

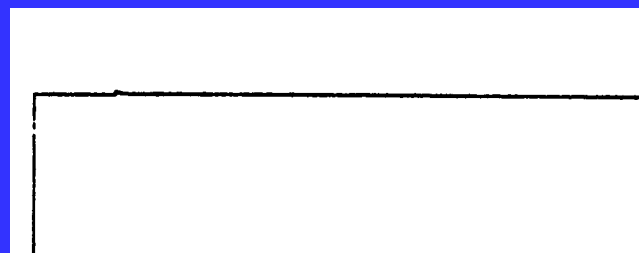
Columns: CHIROBIOTIC R+V+T (100x4.6mm each)

Mobile Phase: 20/80: MeOH/0.1% TEAA, pH 6.0

Results:



Sample B

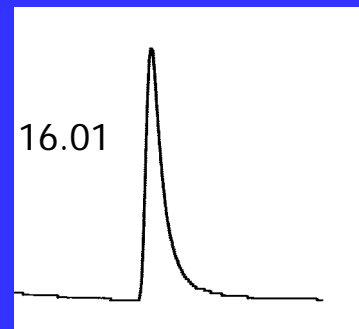


Sample C

Mobile Phase: 40/60: MeOH/0.1% TEAA, pH 6.0

Results: No selectivity observed

Sample C



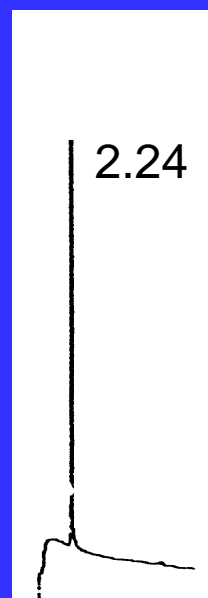
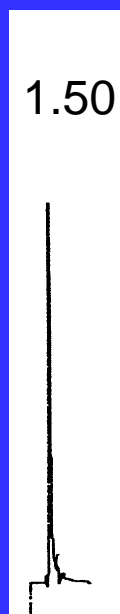
Run 3 (on Sample C): Normal Phase

Column: CHIROBIOTIC R+V+T (100 x 4.6mm each)

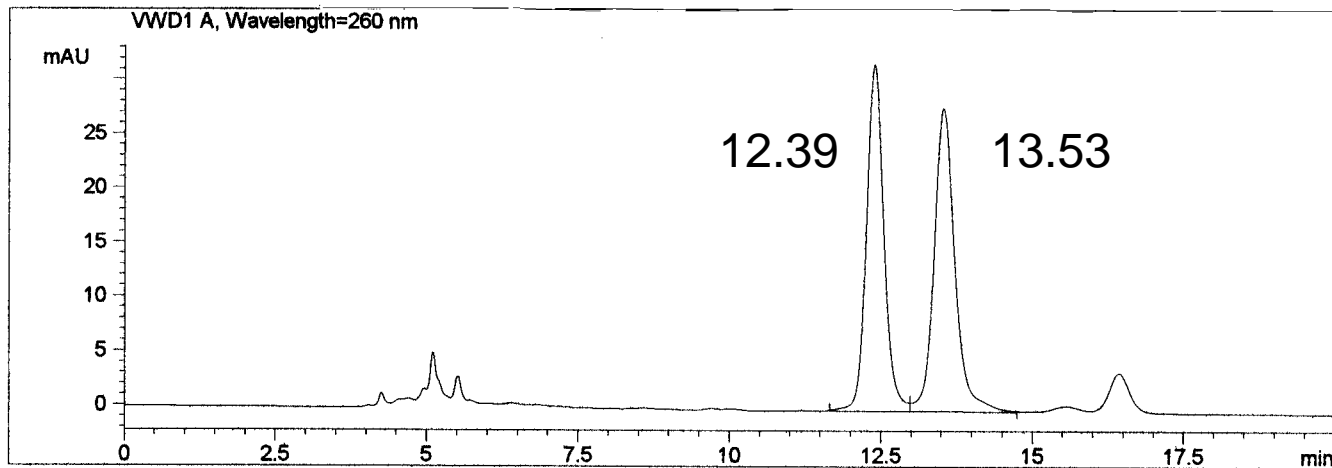
Mobile Phase:

1. 40/60: EtOH/Hexane
2. 5/95: EtOH/Hexane

Results: No selectivity (retention) observed



Optimized Results: Sample A



Column:

CHIROBIOTIC V

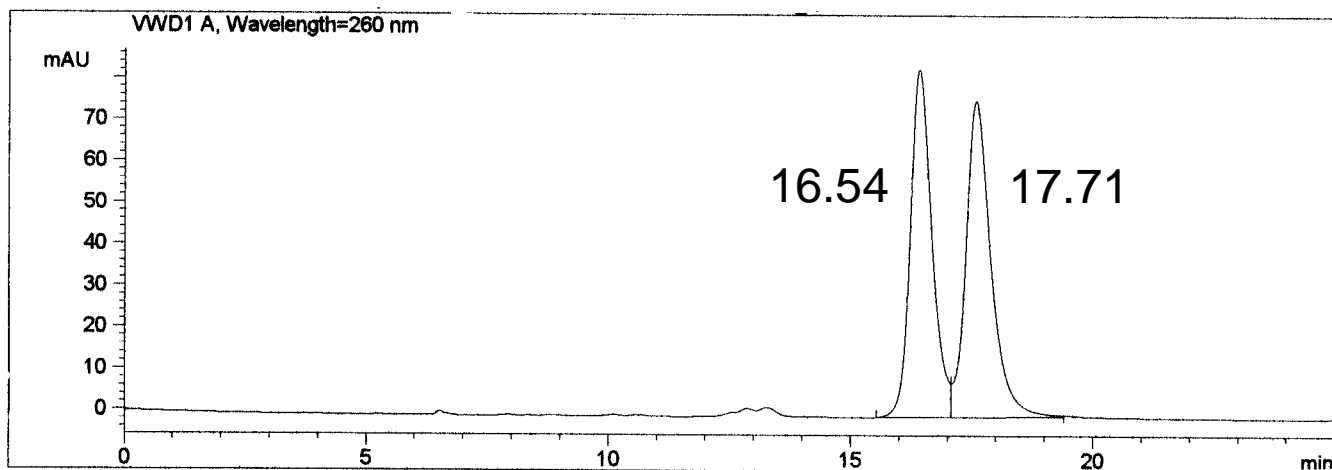
Mobile Phase:

100/0.2/0.1: MeOH/AcOH/TEA

Flow Rate:

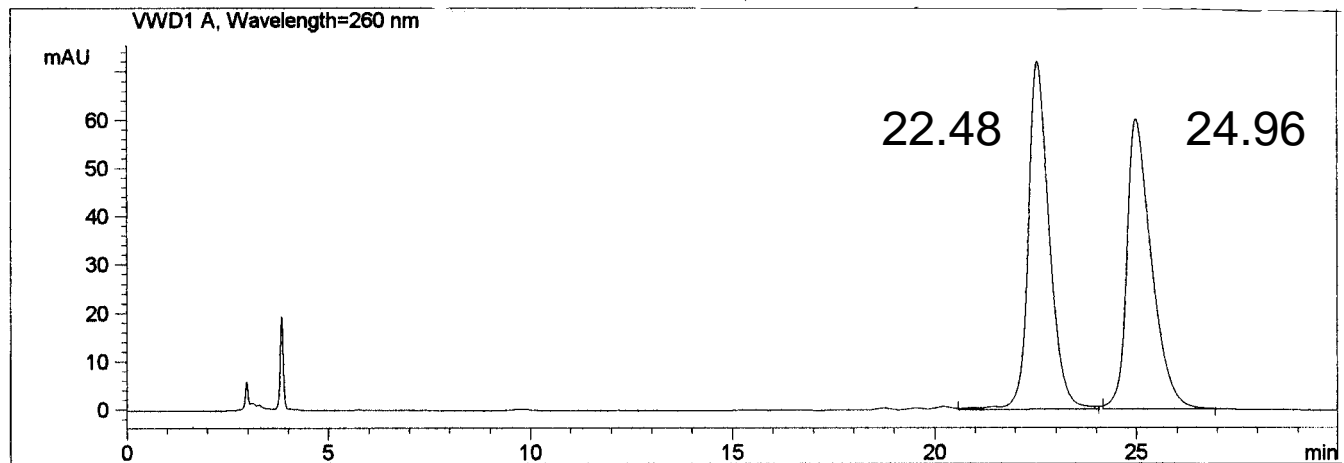
0.7 mL/min

Optimized Results: Sample B



Column: CHIROBIOTIC T
Mobile Phase: 40/60: MeOH/0.1% TEAA, pH 4.1
Flow Rate: 0.6 mL/min

Optimized Results: Sample D



Column: CHIROBIOTIC T
Mobile Phase: 100/0.2/0.1: MeOH/AcOH/TEA
Flow Rate: 1.0 mL/min

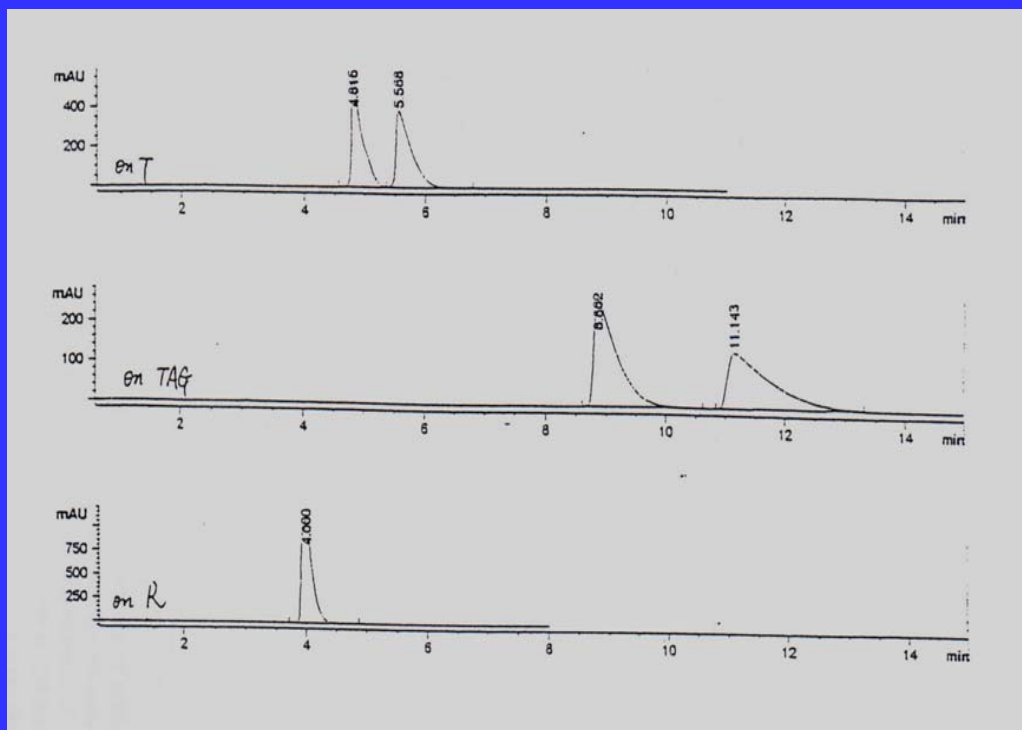


Using CHIROBIOTIC Phases for SFC

A recent (subcritical) study:

- TAG, $T > R$ for series of 110 compounds including neutral ketones, sulfoxides, underivatized amino acids, carboxylic acids and β -blockers
- Baseline separation achieved within 15 minutes on at least one CHIROBIOTIC CSP

Using CHIROBIOTIC Phases for SFC



Sulfoxide D6

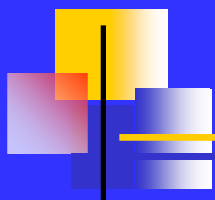
Mobile phases:

7% MeOH in CO₂

A: CHIROBIOTIC T

B: CHIROBIOTIC TAG

C: CHIROBIOTIC R



Chiral LC/MS/MS in Bioanalysis



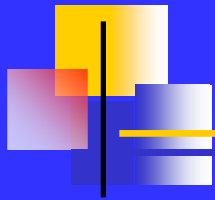
Challenges for Chiral LC/MS/MS in Bioanalysis

- Adaptability of spectrophotometric methods to LC/MS/MS with minimization of ion suppression
 - Limited use of inorganic buffers
 - Separations in higher organic, lower aqueous mobile phases preferred
 - Methods using flammable solvents such as hexane may require additional safety measures (eg, N₂ and/or post column dilution with organic/aq)
- Possible adaptability to cassette dosing and multiplexing LC/MS/MS techniques

Considerations for Using CHIROBIOTIC Chiral LC Columns in LC/MS/MS

- High enantioselectivity in 100% MeOH (polar ionic mode®) with added acid/base or salts
 - Less toxic and lower boiling point than hexane
 - Non-corrosive
 - Works especially well with ESI
- Columns are also compatible with ammonium acetate or formate (aqueous mode)
 - and with all principle solvents used in APCI (from alcohols to hydrocarbons, DMSO, DMF)

High Throughput Bioanalytical Chiral by LC/APCI/MS/MS: Examples



Compound	Column	Column size	Mobile phase	Flow rate	t_R enantiomers
Ritalinic acid ¹	CHIROBIOTIC T	50x4.6mm	100 MeOH/0.03% ATFA	1.2 ml/min	5.0, 10.4 min
Methylphenidate ² (2500 samples)	CHIROBIOTIC V	150x4.6mm	100 MeOH/0.03% ATFA	1.0 ml/min	6.1, 7.2 min
Fluoxetine ¹	CHIROBIOTIC V	50x4.6mm	100 MeOH/0.09% ATFA	1.0 ml/min	6.2, 6.8 min
Nicardipine ¹	CHIROBIOTIC V	50x4.6mm	100 MeOH/0.05% ATFA	1.0 ml/min	1.1, 1.6 min
Metoprolol ¹	CHIROBIOTIC T	150x4.6mm	100 MeOH/0.025% ATFA	1.2 ml/min	7.0, 7.8 min
Salbutamol ³ (4000 samples)	CHIROBIOTIC T	250x4.6mm	100/0.5/0.1 MeOH/AcOH/NH ₄ OH	2.0 ml/min	3.2, 3.7 min (metab at 2.0)

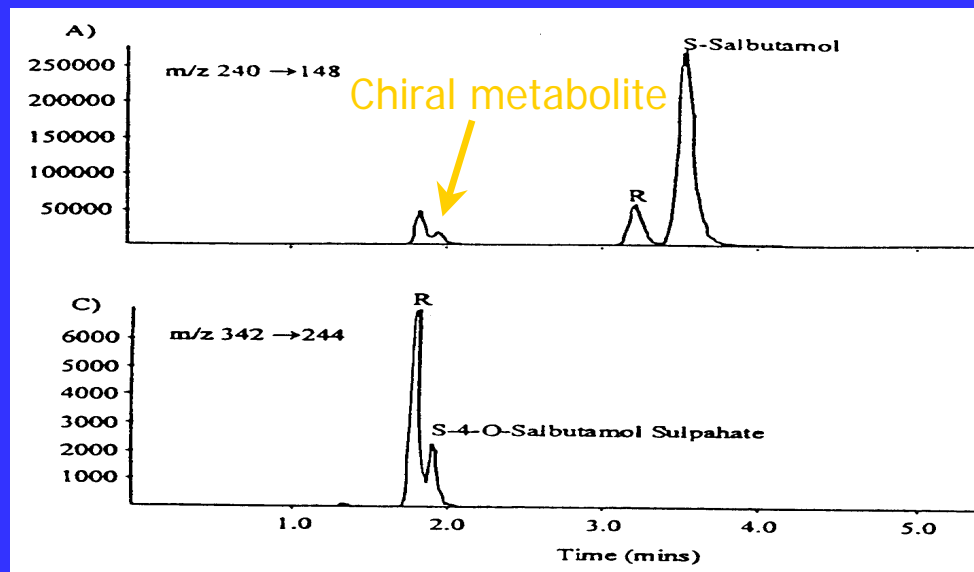
1. R Bakhtiar & F L S Lee, Rapid Commun in MS, 14, 1128-1135 (2000)

2. L Ramos, R Bakhtiar, T Majumdar, M Hayes & F Tse, Rapid Commun MS, 13, 2054-2062 (1999)

3. K Joyce, A E Jones, R J Scott, R A Biddlecombe & S Pleasance, Rapid Commun MS, 12, 1899-1910 (1998)

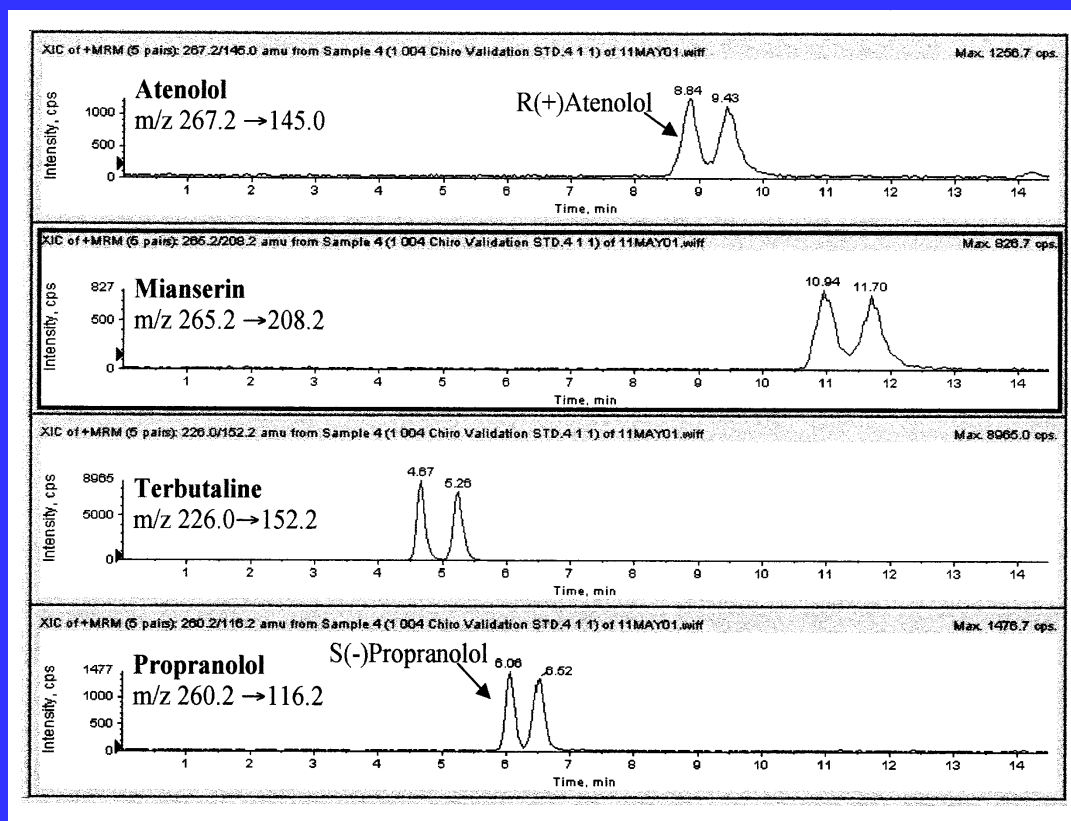
Application for Chiral LC/APCI/MS/MS in Pharmacokinetics: Salbutamol and its 4-O-sulfate Metabolite*

- CHIROBIOTIC T (Teicoplanin), 250 x 4.6mm
- MeOH/AcOH/NH₄OH: 100/0.5/0.1 @ 2 mL/min
- 3 minute assay/96 well SPE
- 100 pg/mL LOQ for parent compound, 5 ng/mL for sulphate metabolism;
- 25 pg/mL LOQ for 80 µL injection
- **4000** sample clinical study



* Karina B. Joyce, Anne E. Jones, Rebecca J. Scott, Robert A. Biddlecombe, Stephen Pleasance, Drug Metabolism and Bioanalysis, Glaxo Wellcome R&D, Ware, UK, Rapid Communications MS, 12, 1899-1910 (1998)

Using the CHIROBIOTIC R,V,T Kit for Drug Metabolism: LC/APCI/MRM Chromatograms



Neat solution at 10 ng/mL of: Atenolol (m/z 267.2 → 145.0), Mianserin (m/z 265.2 → 208.2), Terbutaline (m/z 226.0 → 152.2), Propranolol (m/z 260.2 → 116.2). [Ref: R Bakhtiar, in press]

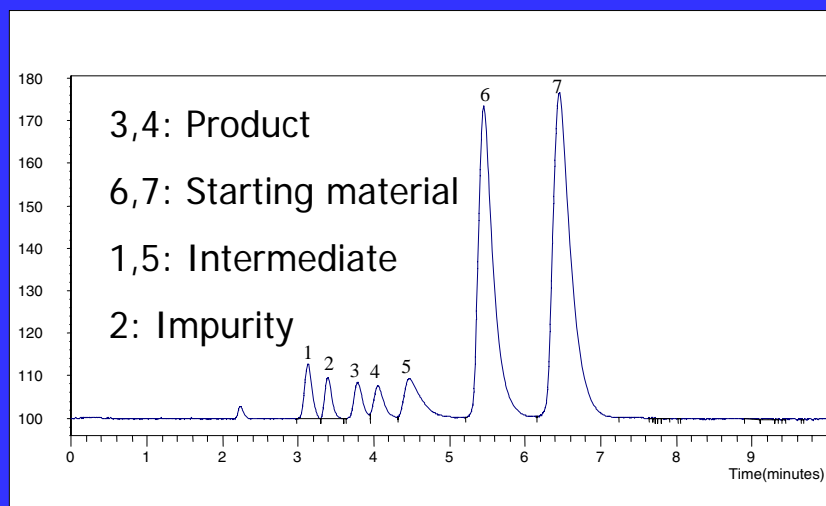
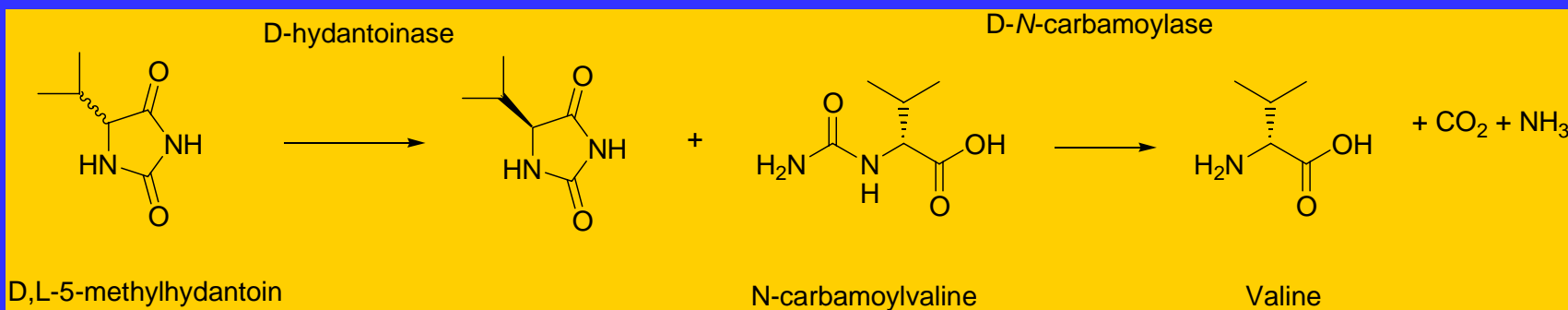


LC/MS – Conclusions

- The use of the polar ionic mode[©] provides the fastest methods for chiral LC/MS/MS with widest applicability
- The same column can be used for reversed phase separations in high organic with NH₄Ac buffer
- Simultaneous chiral separations offer the possibility of parallel loading, multiplexing and cassette dosing
- Column coupling provides fast, simple generic screening

Biocatalysis

Determination of the conversion and enantiomeric excess of substrate / reaction products in a D-hydantoinase / D-carbamoylase reaction



Column: CHIROBIOTIC T (250x4.6 mm, 5 μ m)

Eluent: 80/20 15mM NH₄Ac pH 4.1/MeOH

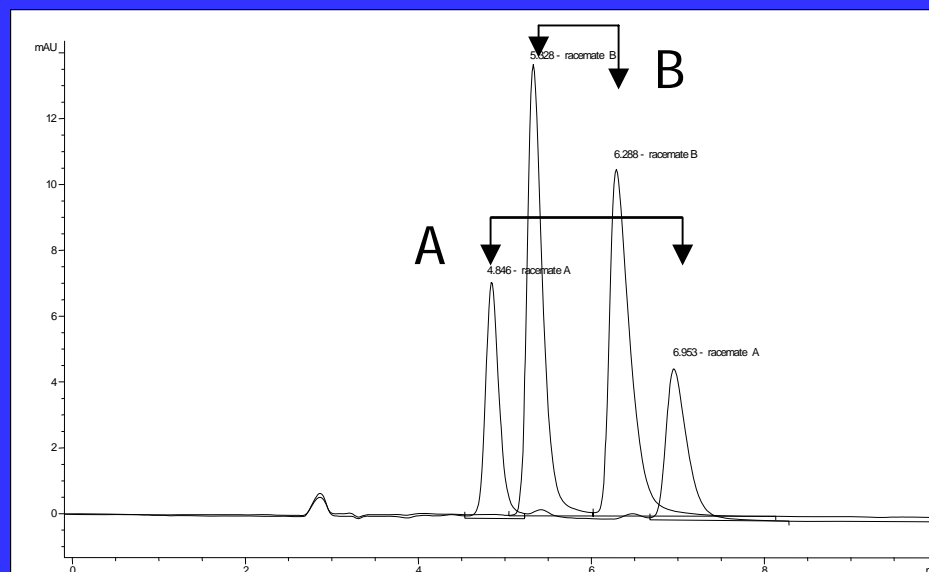
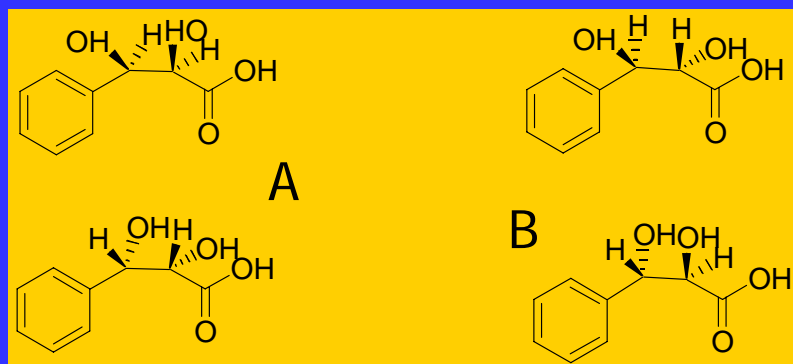
Flow: 1.0 mL/min

Courtesy of DSM Fine
Chemicals



Biocatalysis

Determination of the enantiomeric excess of *cis*- and *trans*-diol reaction products from an epoxyhydrolase reaction

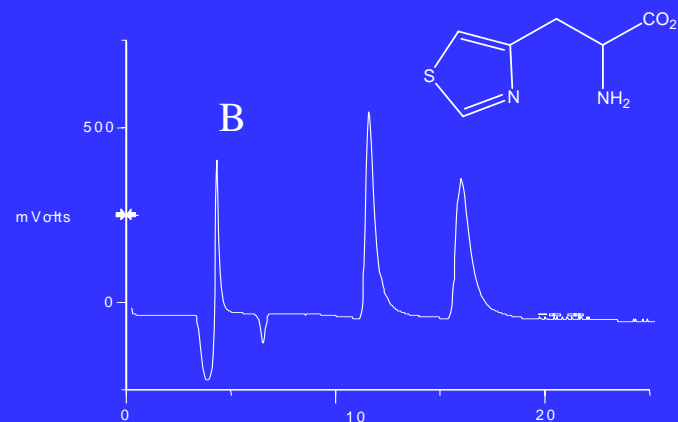
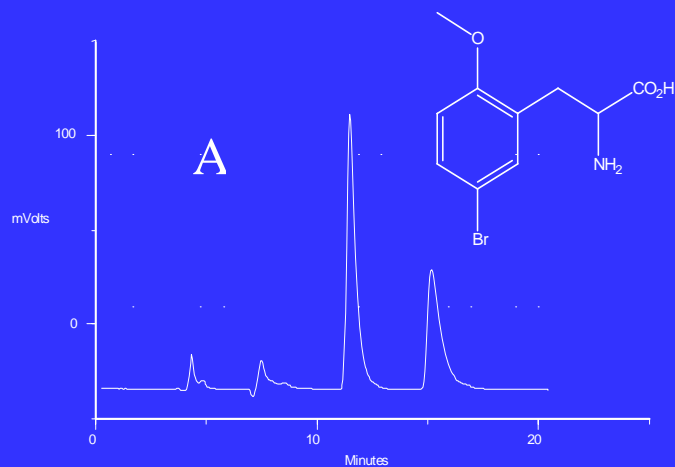


Column: CHIROBIOTIC R (250x4.6 mm, 5 μ m)

Eluent: 0.1% ammonia, pH 4.1 with formic acid / MeOH (50/50 %v/v)

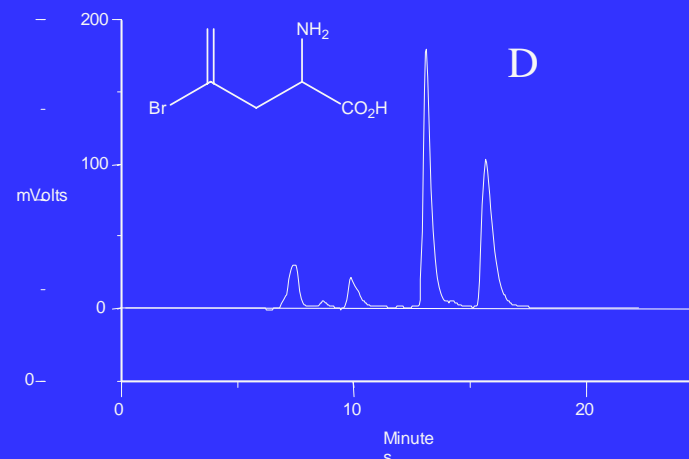
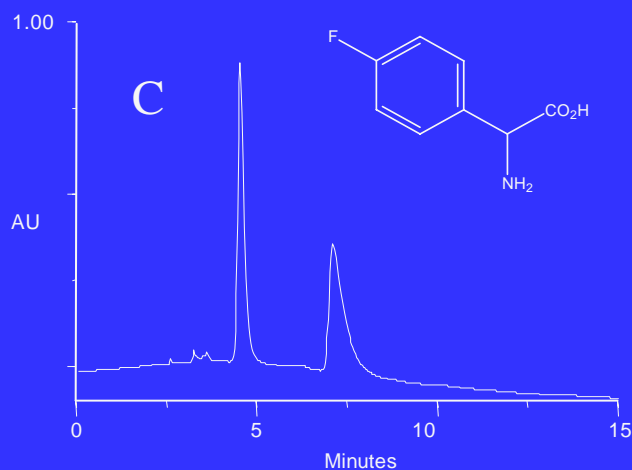
Flow: 1.0 mL/min

Unnatural Amino Acids as Chiral Synthesis Intermediates

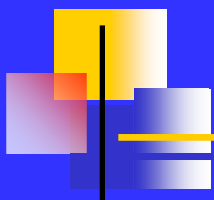


Column:	CHIROBIOTIC T column (250 x 4.6mm)
Temperature:	Ambient
Detection:	UV at 210nm
Mobile phase:	A – 70% methanol, 30% water, 0.5 mL/min B – 50% methanol, 50% water, 0.5 mL/min

Unnatural Amino Acids as Chiral Synthesis Intermediates



Column: CHIROBIOTIC T column (250 x 4.6mm)
Temperature: Ambient
Detection: UV at 210nm
Mobile phase: C – 15% methanol, 85% water, 1.0 mL/min
D – 50% methanol, 50% water, 0.5 mL/min



Using CHIROBIOTIC CSPs for Preparative LC Applications

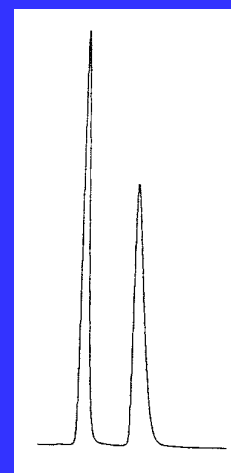
CASE STUDY 1: Nicardipine

Column: CHIROBIOTIC V
(250x4.6mm, 5 μ)

Mobile phase: 100/0.2/0.1, MeOH/AcOH/TEA

UV: 230 nm

Flow rate: 1mL/min



Peak 1: 4.66

Peak 2: 5.57

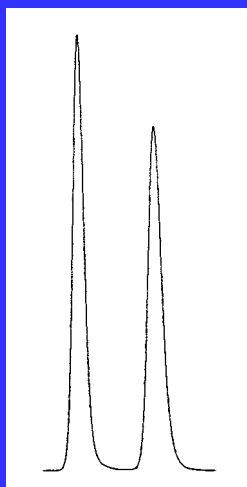
$\alpha = 1.50$

CASE STUDY 1: Nicardipine

Optimization for prep

Column: CHIROBIOTIC V
Flow rate: 1mL/min

100/0.1w%, MeOH/NH₄TFA

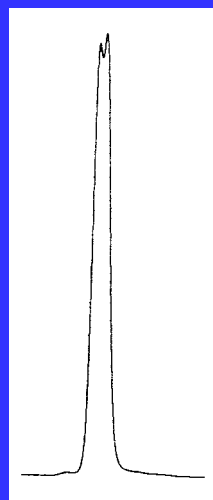


Peak 1: 4.07

Peak 2: 4.69

$\alpha = 1.50$

100/0.1w%, MeOH/NH₄OAc



Peak 1: 3.41

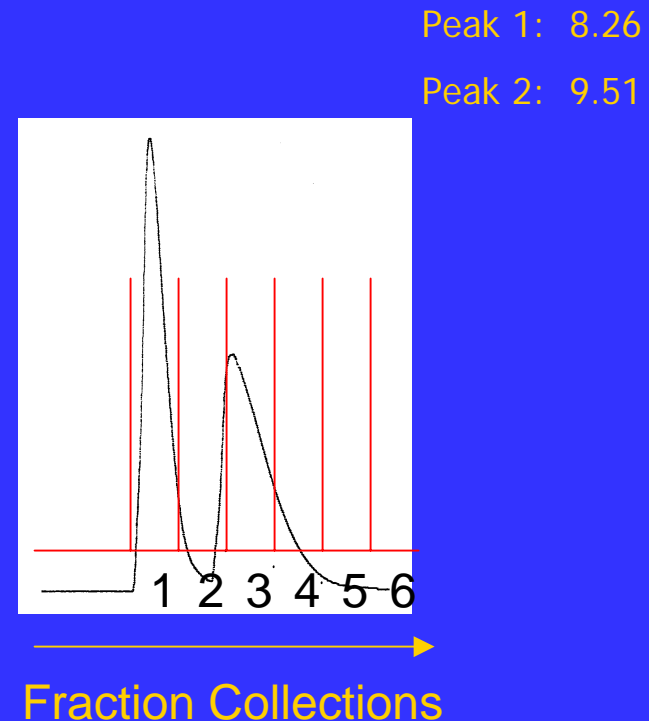
Peak 2: 3.47

$\alpha = 1.09$

CASE STUDY 1: Nicardipine

Column: CHIROBIOTIC V
(250 x 22.1 mm), 5 μ m)
Load: 20mg in 4mL
Mobile phase: 100/0.1w%,MeOH/NH₄TFA
UV: 230 nm
Flow rate: 12 mL/min
Throughput: 1.2 mg/g CSP/hr

Purity Peak 1: 99.67%
Peak 2: 99.46%



CASE STUDY 2: Polar Ionic Mode[©]

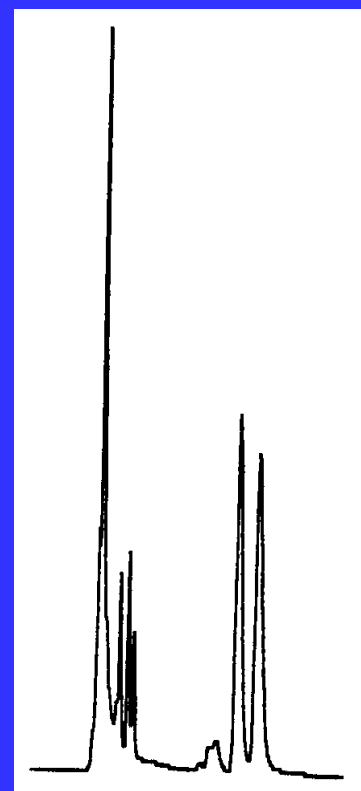
BASIC COMPOUND

Column:	CHIROBIOTIC V, 5 μ m 250 x 4.6mm
Mobile phase:	100/0.2/0.1, MeOH/AcOH/TEA
Flow rate:	0.9 mL/min
UV:	254 nm
Inj:	2 μ L

Peak 1: 10.51 min

Peak 2: 11.53 min

$\alpha = 1.14$



CASE STUDY 2: Polar Ionic Mode ©

BASIC COMPOUND

Column: CHIROBIOTIC V 5 μ m
(Modified) 250 x 4.6mm

Mobile phase: 100/0.5/0.5,
MeOH/AcOH/TEA

Flow rate: 1 mL/min

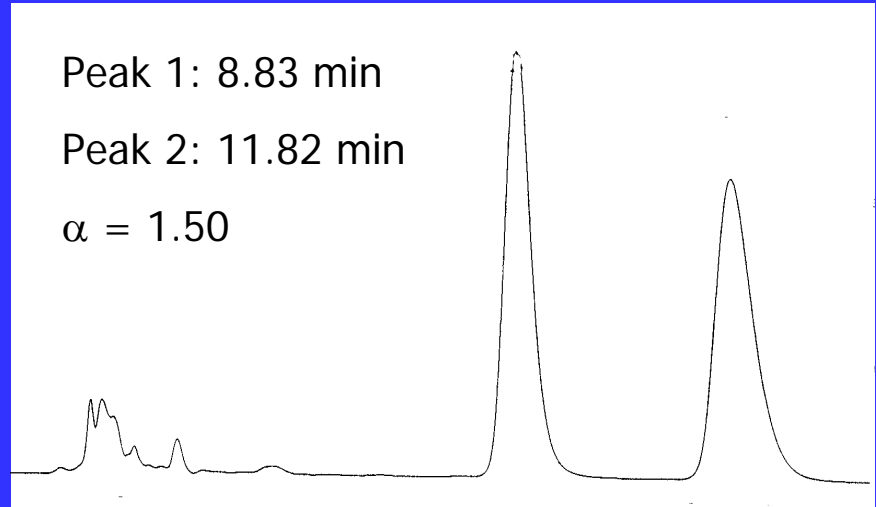
UV: 254 nm

Inj: 100 μ g

Peak 1: 8.83 min

Peak 2: 11.82 min

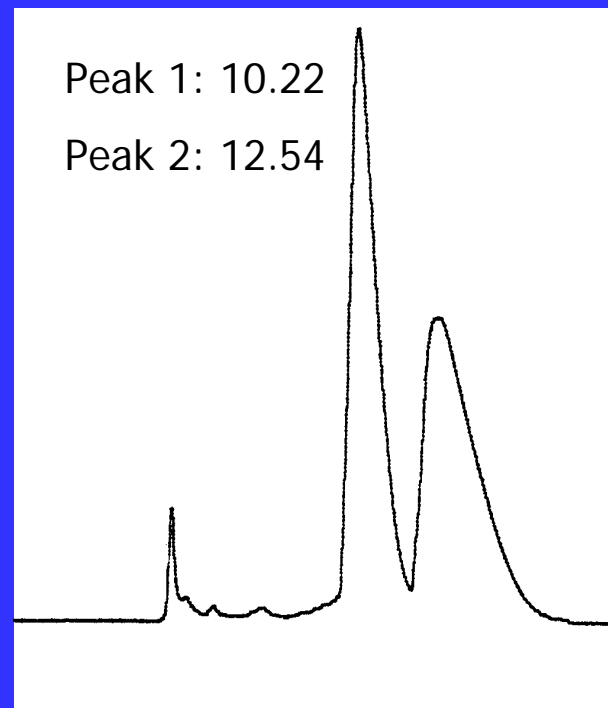
$\alpha = 1.50$



CASE STUDY 2: Polar Ionic Mode ©

BASIC COMPOUND

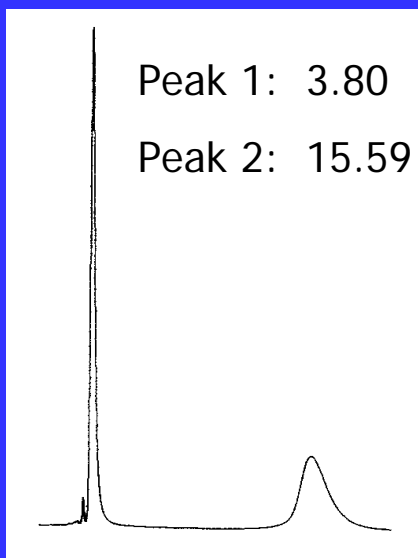
Column:	CHIROBIOTIC V, 5 μ m (Modified) 250 x 22.1mm
Mobile phase:	100/0.5/0.5, MeOH/AcOH/TEA
Flow rate:	15 mL/min
UV:	254nm
Inj.	140mg (in 2ml MeOH)



CASE STUDY 3: N-Acetyl Typtophan

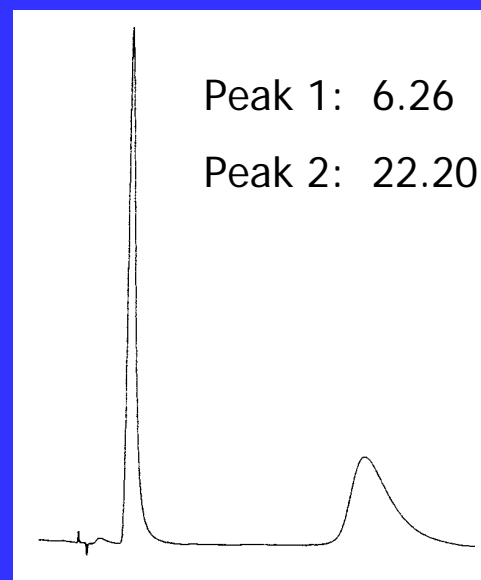
Column: CHIROBIOTIC TAG (250x4.6mm, 5 μ m)
UV: 254 nm
Flow rate: 1 mL/min

Mobile phase:
100/0.1w%, MeOH/NH₄OAc



$k_1 = 0.36$, $\alpha = 12.7$

Mobile phase:
40/60 MeOH/0.1% TEAA, pH4.1



$k_1 = 1.09$, $\alpha = 5.87$

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CASE STUDY 3: N-Acetyl Tryptophan

Column: CHIROBIOTIC TAG
(250x22.1mm, 5 μ m)

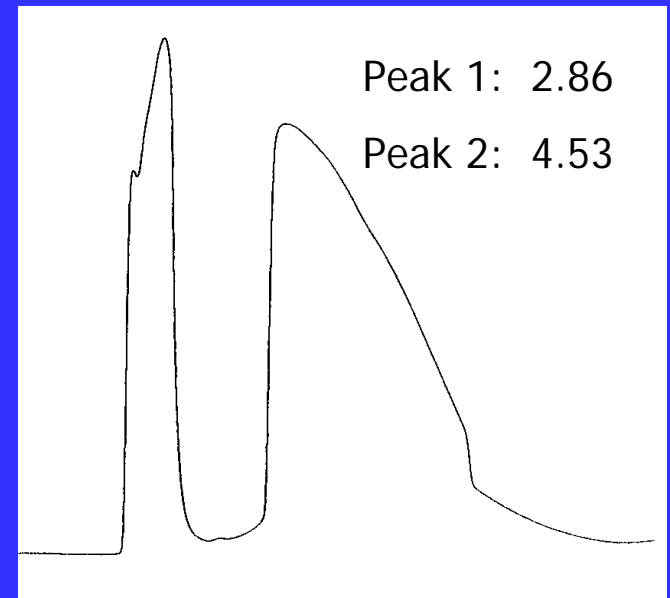
Load: 200mg in 6mL

Mobile phase: 100/0.1w%, MeOH/NH₄OAc

UV: 300 nm

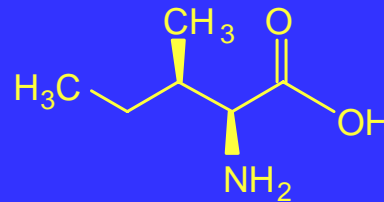
Flow rate: 35 mL/min

Throughput: 20 mg/g CSP/hr

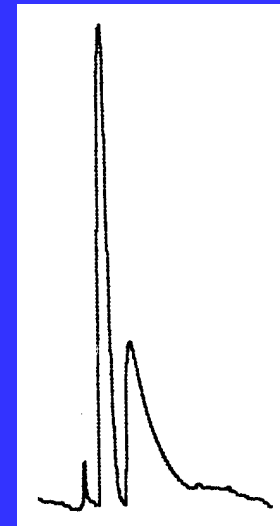


CASE STUDY 4: Purification of Isoleucine on CHIROBIOTIC TAG

ISOLEUCINE



Column:	CHIROBIOTIC TAG
Size:	250 x 22.1mm
Mobile Phase:	30/70: MeOH/H2O
Flow Rate:	20 mL/min.
Injection:	170 mg in 10 mL
Load:	24mg/gm CSP/hr

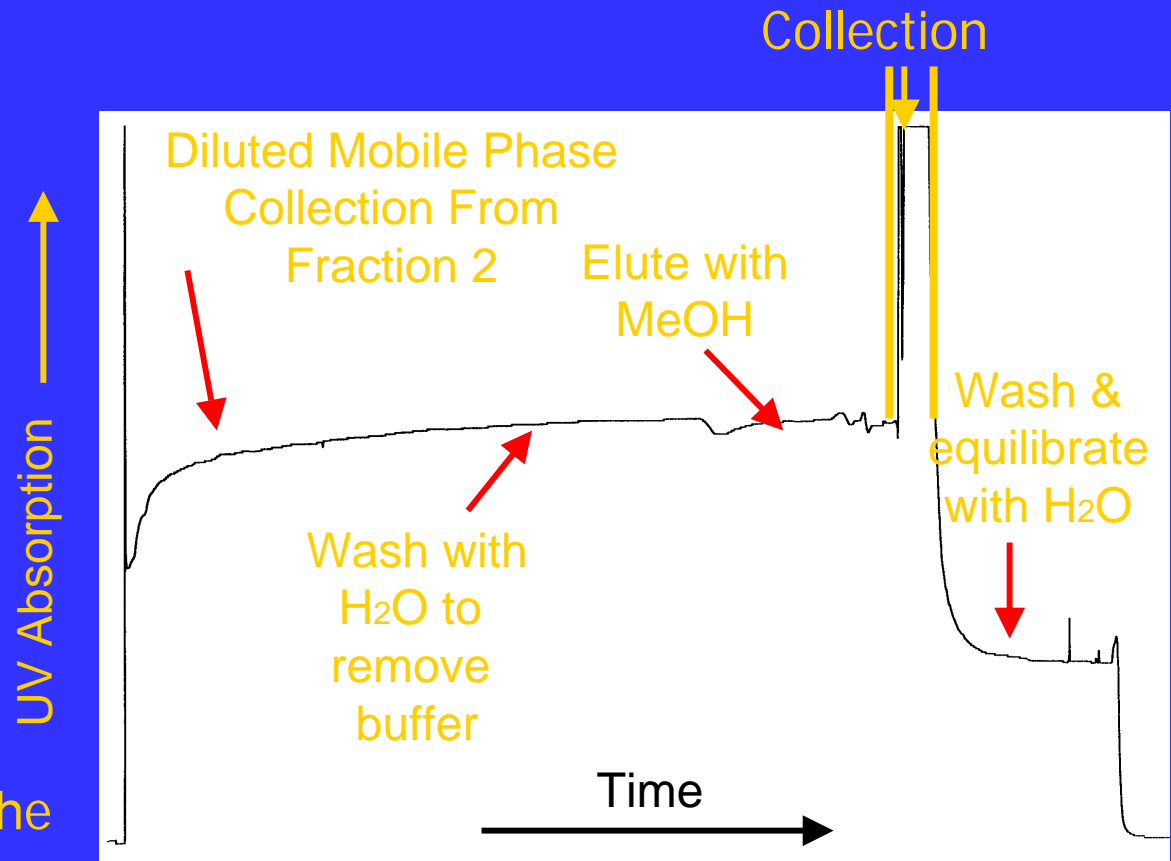


Recovery of Warfarin from Aqueous Mobile Phase

WARFARIN

C₁₈, 250 x 4.6mm
2x dilution of
Fraction 2 with H₂O
1.5 mL/min.
UV-220nm
Inj. 35mg

Refer to pages 34/5 of the
CHIROBIOTIC Handbook





Conclusions: Preparative LC on CHIROBIOTIC CSPs

Use of MeOH and the polar ionic mode[©] :

- Separations are fast and efficient
- High sample throughput
- Increases long term stability of CSPs
- Less toxic than traditional normal phase solvents
- Low volatility reduces product recovery time
- The *same* column can also be used for prep in reversed and normal phase



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