PREPARING YOUR OWN CHIRAL COLUMN

via immobilization onto epoxy-modified silica monoliths

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MERCK

Introduction

For the separation of chiral substances there are several different stationary phases available on the market. However, for each racemic separation issue often many different columns have to be purchased and tested to identify necessary selectivity.

As a new tool, the Chromolith WP 300 Epoxy column is the first ready-to-use column, for the immobilization of ligands for the use in various applications, such as chiral separation. It comes as a single piece of silica in a completely bioinert column hardware, offering high matrix stability and very low column backpressure. Mesopores of 30nm allow the separation of larger biomolecules of about 100kDa, while also antibodies and other large molecules can enter the 2µm macropores for separation (Fig.1).

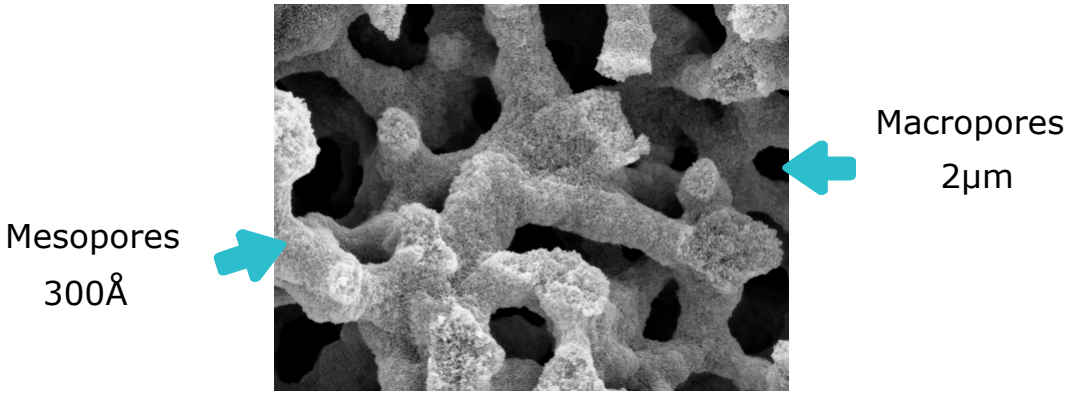


Figure 1: SEM of widepore silica monolith

Immobilization technique

There are many well described techniques for the immobilization of ligands onto various supports. One of the most simple and reliable method is the direct coupling of ligands carrying an amino-group to epoxide groups on the stationary phase.

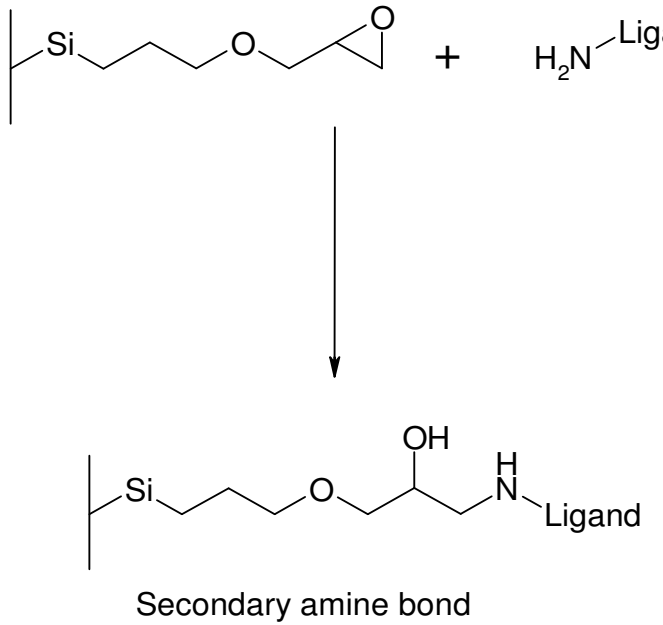


Figure 2: Schematic reaction of ligand to epoxy

- Epoxy ring system enables a nucleophilic attack yielding in a ring opening process
- Nucleophilic group form a covalent bond with the primary carbon atom and lead to the formation of hydroxyl groups at the adjacent secondary carbon atom
- Use of high salt concentrations (> 1.5 M) increases the interaction of hydrophobic molecules in aqueous solutions promoting ligand adsorption onto the support
- Lyotropic salts drive the soluble ligands toward the surface of the support by a salting out effect enhancing the covalent reaction of epoxide and amino groups

Immobilization - protocol

Step I - Equilibration

- Column equilibration with 50 ml 50mM sodium phosphate + 1.9M ammonium sulfate pH8.0
- 2.0 ml/min flow rate at room temperature

Step II - Immobilization

- Dissolving of ligand in 25 ml 50mM sodium phosphate + 1.9M ammonium sulfate pH8.0
- Connection of ligand solution to pump
- Immobilization in circles with 0.2 ml/min flow rate at room temperature for 24 h

Step III - Quenching & washing

- Quenching of remaining epoxide functions with 150mM phosphoric acid or 1M glycine (optional)
- Washing of the immobilized column with 100mM sodium phosphate pH7.4

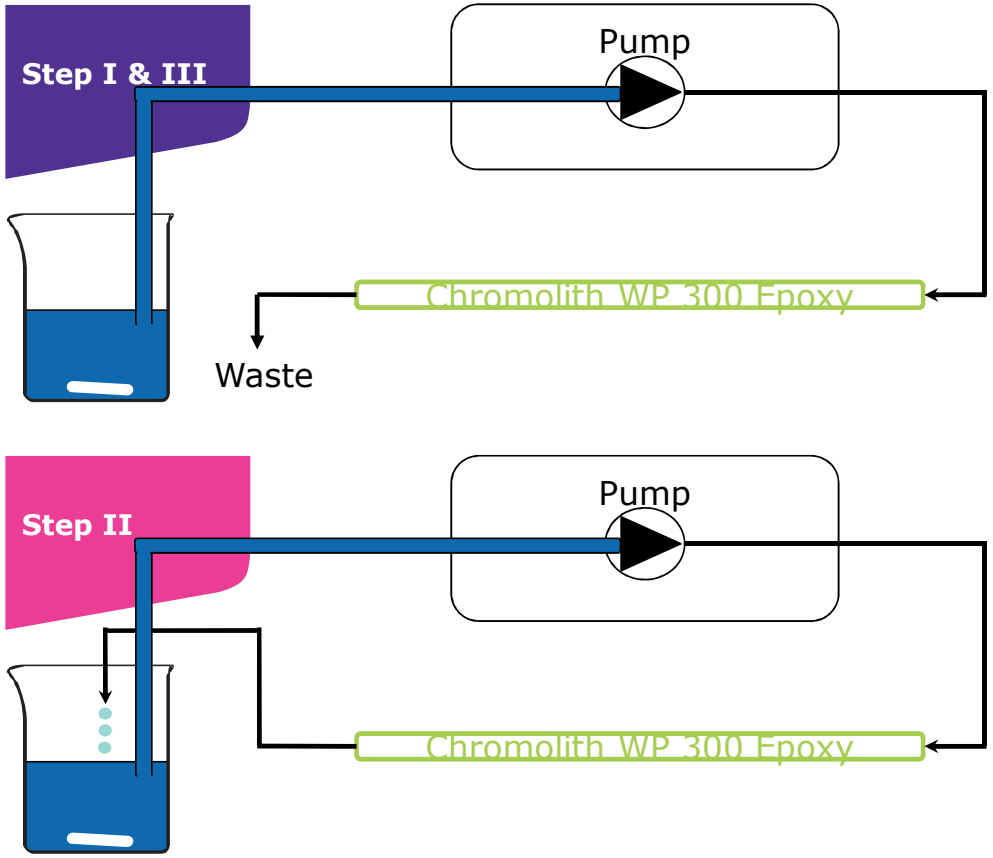


Figure 3: Immobilization and washing

Material & Method

Column:

Chromolith WP 300 Epoxy 100x4.6mm (Merck, 1.52250)

Ligands:

Bovine serum albumin fraction V BSA (Merck, 1.12018), Human serum albumin HSA (Sigma, A1653), Cellulase (Sigma, C2730), Vancomycin (Sigma, V8138)

Eluents/buffer:

Na_2HPO_4(Merck, 1.06580), acetonitrile gradient grade (Merck, 1.00030)

Samples:

DL-Tryptophane (Sigma, T3300), ibuprofen (Sigma, I4883), terbutaline (Sigma, T2528), Thalidomide(Sigma, T144)

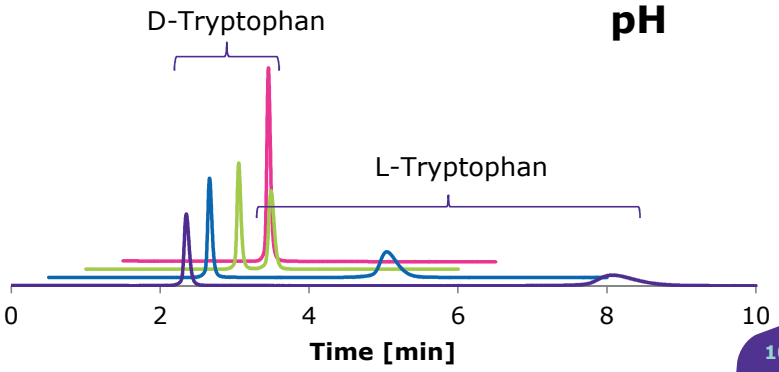
Immobilization:

Na_2HPO_4(Merck, 1.06580), $(NH_4)_2SO_4$(Merck, 1.01211), MilliQ water

- 250 mg BSA dissolved in 25 ml 50mM sodium phosphate + 1.9M ammonium sulfate pH8.0
- 250 mg HSA dissolved in 25 ml 50mM sodium phosphate + 1.9M ammonium sulfate pH8.0
- 5 ml cellulase solution dissolved in 20 ml 50mM sodium phosphate + 1.9M ammonium sulfate pH8.0
- 250 mg vancomycin dissolved in 25 ml 50mM sodium phosphate + 1.9M ammonium sulfate pH8.0

Immobilization of BSA

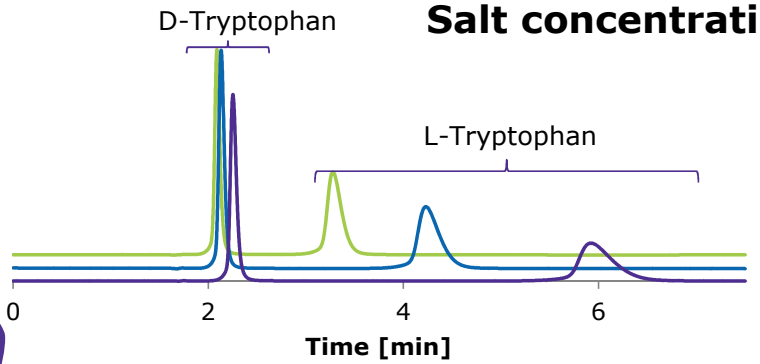
pH



pH 3,0
pH 5,0
pH 6,0
pH 6,5

Salt concentration

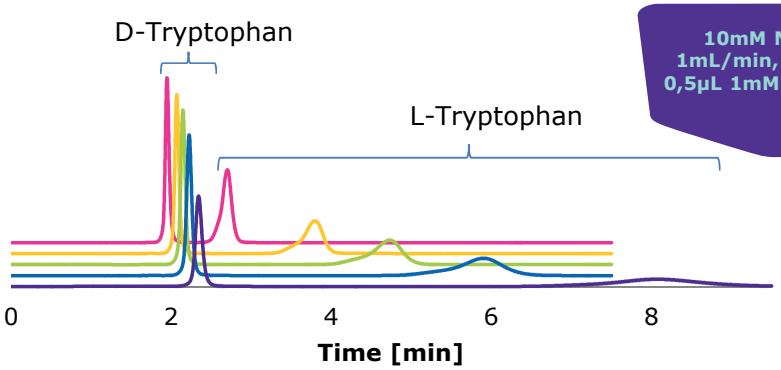
100mM
25mM
5mM



10mM Na_2HPO_4 pH3.0-7.0 1mL/min, 214nm, 25°C 0,5µL 1mM DL-Tryptophan in H_2O

5-100mM Na_2HPO_4 pH6.0 1mL/min, 214nm, 25°C 0,5µL 1mM DL-Tryptophan in H_2O

Temperature



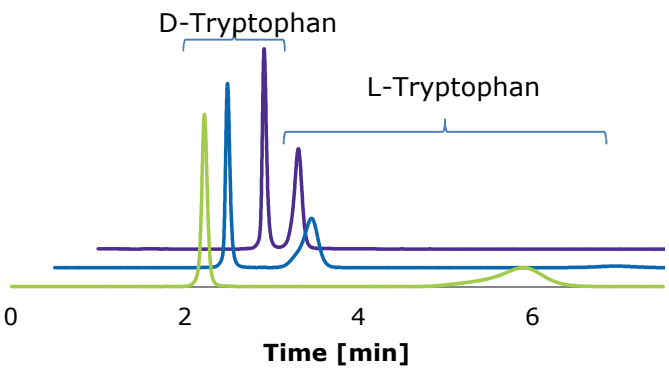
10mM Na_2HPO_4 pH6.0 1mL/min, 214nm, 10-60°C 0,5µL 1mM DL-Tryptophan in H_2O

10mM Na_2HPO_4 pH3.0-7.0 1mL/min, 214nm, 25°C 0,5µL 1mM DL-Tryptophan in H_2O

60°C
45°C
35°C
25°C
10°C

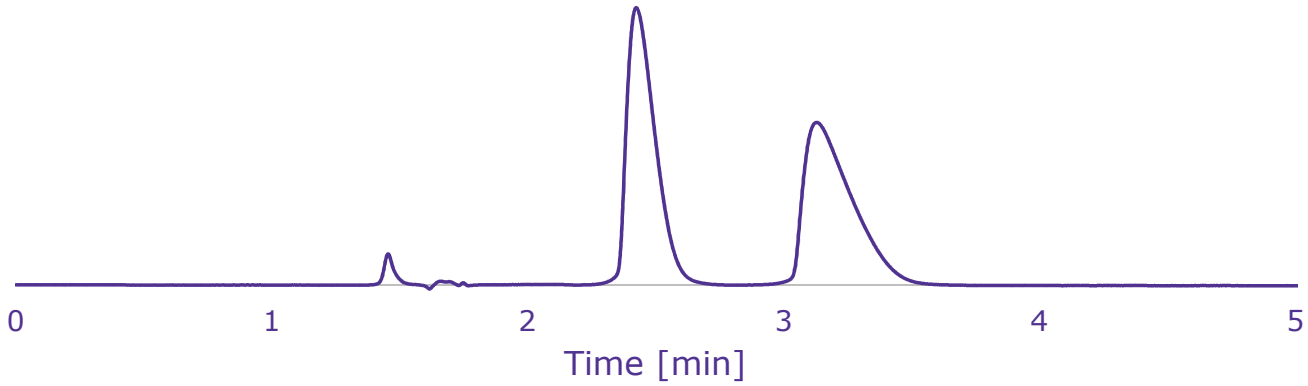
Organic modifier

5% Acetonitril
2% Acetonitril
0% Acetonitril



Immobilization of HSA

Separation of racemic Ibuprofen



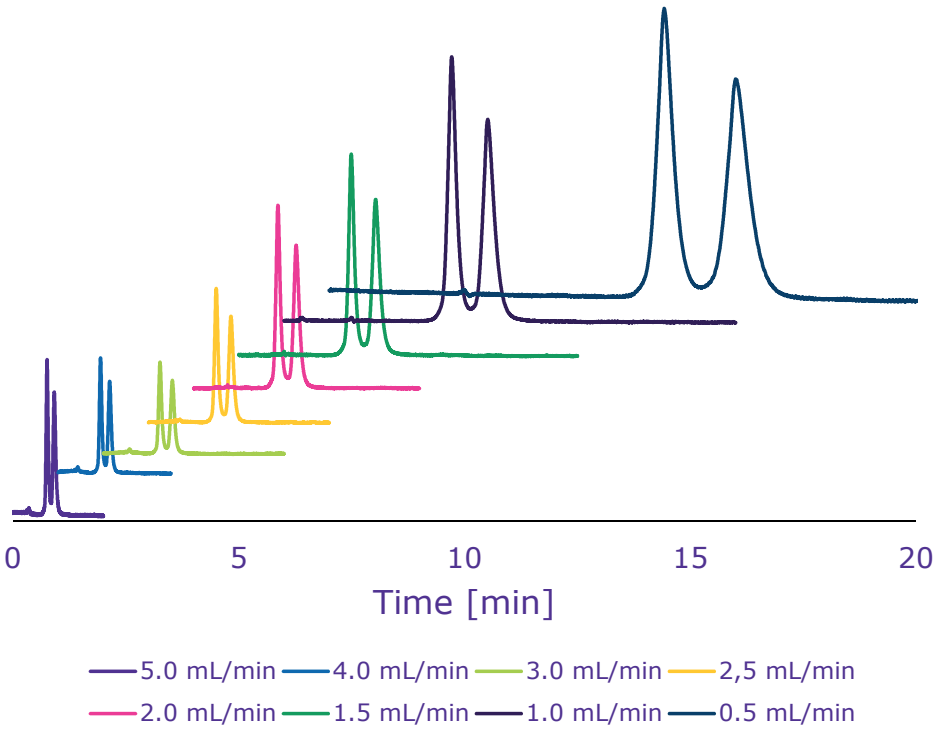
Ibuprofen (1) Retention factor, k	Ibuprofen (2) Retention factor, k	Selectivity factor	Resolution
0.67	1.15	1.72	2.51

Conditions

10 mM sodium phosphate pH6.0 / Acetonitrile 30/70
1.0 mL/min
25°C
214 nm
1.0 µL 1mM Ibuprofen in Acetonitrile

Immobilization of cellulase

Separation of racemic terbutaline

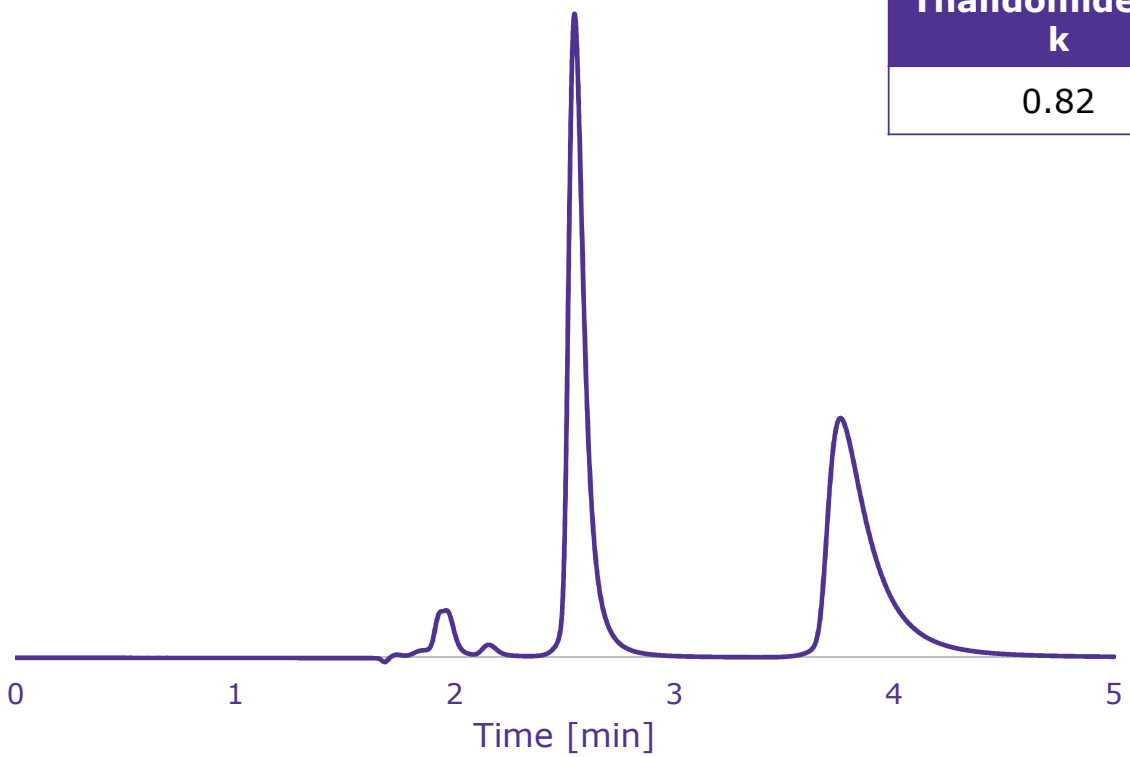


Conditions

10 mM Na_2HPO_4 pH6.0
0.5-5.0 mL/min
25°C
214 nm
0.5 µL terbutaline in water (0.33mg/mL)

Flow [mL/min]	Terbutaline (1) k	Terbutaline (2) k	Selectivity factor	Resolution
0.5	1.65	2.21	1.34	2.19
1.0	1.65	2.22	1.35	2.02
1.5	1.67	2.24	1.34	2.03
2.0	1.66	2.24	1.35	1.98
2.5	1.68	2.26	1.35	1.94
3.0	1.69	2.27	1.34	1.93
4.0	1.68	2.26	1.35	1.79
5.0	1.69	2.27	1.34	1.78

Immobilization of vancomycin – Chiral separation



Thalidomide (1) k	Thalidomide (2) k	Selectivity factor	Resolution
0.82	1.68	2.04	5.17

Separation of racemic thalidomide

Eluent:	10 mM Na_2HPO_4 pH 7.0
Flow:	1.0 mL/min
Temperature:	25°C
Detection:	214 nm
Injection:	1.0 µL 1 mM thalidomide in acetonitrile

Conclusion

BSA, HSA, Cellulase and Vancomycin could be immobilized successfully onto Chromolith WP 300 Epoxy columns. The columns were used for the separation of DL-tryptophan, ibuprofen, terbutaline and thalidomide. Separation conditions like pH, temperature, organic modifier, buffer concentration and flow rate were varied to receive best selectivity values and good separations.

It could be shown that the immobilization of ligands onto Chromolith WP 300 Epoxy is a simple way of preparing columns with unique selectivities.

Chromolith WP 300 column overview

Column	100-4.6mm	50-4.6mm	25-4.6mm	10-4.6mm	5-4.6mm
Chromolith WP 300 RP-18	1.52270.0001	1.52271.0001		1.52272.0001	1.52273.0001
Chromolith WP 300 RP-8	1.52265.0001	1.52266.0001		1.52267.0001	1.52268.0001
Chromolith WP 300 RP-4	1.52260.0001	1.52261.0001		1.52262.0001	1.52263.0001
Chromolith WP 300 Epoxy	1.52250.0001	1.52251.0001	1.52252.0001	1.52253.0001	1.52254.0001
Chromolith WP 300 Protein A			1.52258.0001		
Chromolith Guard Cartridge Holder Bioinert				1.52256.0001	1.52255.0001

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