Operating Guidelines for ChromTech CHIRAL-AGP, CHIRAL-HSA, and CHIRAL-CBH HPLC Columns

About Protein-Based Chiral Stationary Phases (CSPs)

Hermansson described the use of natural proteins immobilized onto a silica support for chiral separations in 1983 (1). Proteins contain a large number of chiral centers of one configuration, and many other sites that contribute to the general retention process. We offer three ChromTech CSPs with proteins as the chiral selectors, CHIRAL-AGP (α_1-acid glycoprotein), CHIRAL-CBH (cellobiohydrolase) and CHIRAL-HSA (human serum albumin). They are typically used in reversed-phase mode, and perform a wide variety of chiral separations. CHIRAL-HSA is also used for drug-binding studies. Solutes are retained by three types of interactions: ionic (for charged solutes), hydrophobic and hydrogen bonding. The relative contribution of the different forces to solute retention depends on the nature of the analyte.

CHIRAL-AGP: Extremely broad applicability. First choice when developing methods on protein-CSPs. Separates all types of compounds, including amines, acids, non-protic compounds (amides, esters, alcohols, sulphoxides, etc.)

CHIRAL-HSA: Analytes are typically very hydrophilic acids. Separates preferably compounds containing one or more nitrogens together with one or more hydrogen-accepting or hydrogen-donating groups (alcohols, phenols, carbonyls, amides, esters, ethers, etc.)

CHIRAL-CBH: Analytes are typically very hydrophilic amines and amino alcohols. Separates preferably weak and strong acids, zwitterionic and non-protic compounds.

Protein-Based CSP Features:

- Direct reversed-phase resolution of chiral molecules
- Stable in a variety of organic modifiers – typically acetonitrile or an alcohol
- Available in analytical and semi-preparative sizes
- CHIRAL-HSA is also used for drug-binding studies

Reference

1. Hermansson, J. Direct liquid chromatographic resolution of racemic drugs using α1-acid glycoprotein as the chiral stationary phase. J. Chromatogr. A, 1983, 269, 71-80.

Precautions:

- Maximum Pressure: 2000 psi
- Flow Rates: Low enough to remain within pressure restrictions
- Maximum Organic Modifier: 20%
- pH Range: AGP, pH 4 – 7; CBH, pH 3 – 7; HSA, pH 5 – 7
- Maximum Temperature: 40 °C (normal operating range is 20 - 25 °C)

Special Mobile Phase Precautions with CHIRAL-CBH:

- Do not use charged organic modifiers
- Mobile phase should contain 50 µM disodium EDTA to complex metal ions

We strongly recommend using a guard column to protect your analytical column investment.

Column Installation and Initial Preparation

The columns are shipped in a 10-15% aqueous solution of 2-propanol. Before use, flush the column for a few minutes with water at a low flow rate (0.1 mL/min. for columns of 4 mm I.D. and less). Gradually increase the flow rate to the recommended range and continue to flush with water for 10 minutes before switching to the operating mobile phase. See flow rate by column I.D. guidelines.

Flow Rate by Column I.D.

High flow rates can damage protein-based CSPs. It is important to follow these guidelines to maximize column performance and lifetime.

I.D.	Flow Rate Range	Maximum Pressure (psi)
2 mm	0.2 – 0.22 mL/min.	2000
3 mm	0.45 – 0.51 mL/min.	2000
4 mm	0.8 – 0.9 mL/min.	2000
10 mm	5 – 5.6 mL/min.	2000

Mobile Phase Composition

The mobile phases are mixtures of phosphate or acetate buffers and organic solvents such as 2-propanol or acetonitrile. The enantioselectivity and retention can easily be regulated by mobile phase pH and ionic strength, and the nature and concentration of the organic modifier. The most important tool in method development is the mobile phase pH, which affects the ionization of both solutes and the protein stationary phase. AGP has a low isoelectric point (pI) of 2.7. This means at pH 2.7 the column has a net zero charge. From pH 2.7 to 7, the net negative charge on the AGP molecule increases, providing increased retention of positively-charged analytes, like amines. These compounds are also retained by hydrophobic and hydrogen bonding interactions.

Use fresh mobile phases. Buffered mobile phases containing no organic modifier or low concentrations of organic modifier can rapidly become contaminated with microbes. They should be prepared fresh daily and filtered before use.

Mobile phase pH: Do not operate above pH 7. The most important tool in method development is the mobile phase pH, which affects the ionization of both solutes and the protein stationary phase. AGP has a low isoelectric point (pI) of 2.7. This means at pH 2.7 the column has a net zero charge. From pH 2.7 to 7, the net negative charge on the AGP molecule increases, providing increased retention of positively-charged analytes, like amines. These compounds are also retained by hydrophobic and hydrogen bonding interactions. Use of the columns outside the target pH range (AGP, pH 4 – 7; CBH, pH 3 – 7; HSA, pH 5 – 7), can reduce column lifetime.

Organic modifier: Keep organic composition of the mobile phase below 20%. Do not use charged modifiers on CHIRAL-CBH. Typical uncharged organic modifiers include 2-propanol, acetonitrile, methanol, ethanol and 1-propanol. High organic modifier concentration generally reduces the retention and enantioselectivity, although the opposite is true in some cases (*e.g.* warfarin). The type of organic modifier affects hydrogen-bonding interactions. The separation factor can be strongly affected by the type and the concentration of the uncharged modifier. For example, 1-propanol, 2-propanol and acetonitrile can give large differences in enantioselectivity. Aliphatic carboxylic acids like octanoic acid have also been used to control retention and resolution on CHIRAL-AGP and CHIRAL-HSA (**not on CHIRAL-CBH**) in concentrations between 1 - 5 mM. If the column is exposed to octanoic acid, it should be dedicated to that mobile phase because of the difficulty in completely removing it from the column. For best results, use one column for uncharged modifiers and another column for charged modifiers.

Buffers: Typical HPLC buffers are used. The buffer type can be selected to obtain the best separation. Typical buffering systems are phosphate and citrate (sodium or potassium salts), acetate and formate (ammonium or sodium salts). Ionic strength should be between 0.01 and 0.1 M (typically 10 –20 mM). **CHIRAL-CBH:** Use 10-50 mM phosphate or acetate buffers.

Disodium EDTA with CHIRAL-CBH: We recommend using 50 µM disodium EDTA in CHIRAL-CBH mobile phases in order to complex metal ions that can deteriorate the column performance. EDTA in the mobile phase can cause system peaks when injecting samples dissolved in buffers that do not contain EDTA.

Charged organic modifiers (ion pair agents): CHIRAL-AGP and CHIRAL-HSA columns are compatible with conventional ion-pair agents, like aliphatic carboxylic acids. Typical concentrations are 1 - 5 mM. However, they are very difficult to completely remove from the column. Once the column has been exposed to ion-pair reagents we recommend it be dedicated to those mobile phases. Do not use charged organic modifiers on CHIRAL-CBH.

Samples

The recommended sample concentration is less than 0.10 mg/mL with an injection volume of 10-20 µL. If possible, dissolve the sample in the mobile phase. If the sample is insoluble in the mobile phase, add a higher concentration of the organic modifier. However, be aware that too high of an organic modifier concentration might precipitate the buffer salts. Avoid dissolving the sample in pure solvents. Do not inject unclear sample solutions or samples containing undissolved compounds. In bioanalytical work, use an isolation procedure that produces clear sample solutions, free from emulsions of fatty compounds. Exchange the guard column regularly.

Cleaning the Column

If the column has been contaminated with hydrophobic material, run the column in reverse and disconnected from the detector. Flush the column overnight with a mobile phase of 20% 2-propanol in pure water. Increasing the temperature to 30 °C will facilitate cleaning. Do not exceed the pressure maximum during the flushing procedure.

Storing the Column

When in use, the column may be stored at room temperature in the operating mobile phase. However, if the column is not in operation for more than a few days, we recommend flushing the column with water then a solution of 15% 2-propanol in pure water for storage. This will remove buffer salts and discourage microbial growth.

Method Development Protocols for CHIRAL-AGP

1. Characterize your sample and find the starting mobile phase

Compound Type	Starting Mobile Phase (UV)	Starting Mobile Phase (MS)
Amines, hydrophobic	10 mM ammonium or sodium acetate, pH 4.5	10 mM ammonium acetate, pH 4.5
Amines, hydrophilic	5% 2-propanol in 10 mM sodium phosphate, pH 7	5% 2-propanol in 10 mM ammonium acetate, pH 7
Acids, weak (e.g. phenols)	5% 2-propanol in 10 mM sodium phosphate, pH 7	5% 2-propanol in 10 mM ammonium acetate, pH 7
Acids, strong (e.g. carboxylic acids)	10 mM sodium phosphate, pH 7	10 mM ammonium acetate, pH 7
Non-protic (e.g. amides, esters, alcohols)	5% 2-propanol in 10 mM sodium phosphate, pH 7	5% 2-propanol in 10 mM ammonium acetate, pH 7

2. Optimize based on particular analyte class

Hydrophobic amines

Result:	No or Low Enantioselectivity and Low Retention	Enantioselectivity and too High Retention	No Enantioselectivity and too High Retention
Optimize with pH and/or modifiers	1) Increase pH stepwise to pH 7 and adjust retention with 2-propanol (lower conc. gives higher enantioselectivity.)	1) Decrease pH to 4 and/or add 2-propanol	1) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)
	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)	2) Test low conc. of a charged modifier (e.g. 1-20 mM octanoic, hexanoic or heptanoic acids; 1-5 mM tetraethyl- or tetrapropylammonium bromide (note: columns must be dedicated to this system) **LC-MS: Use triethylamine (TEA) as modifier**
	3) Test low conc. of a charged modifier (e.g. 1-20 mM octanoic, hexanoic or heptanoic acids; 1-5 mM tetraethyl- or tetrapropylammonium bromide (note: columns must be dedicated to this system) **LC-MS: Use triethylamine (TEA) as modifier**		

Hydrophilic amines, weak acids and non-protic compounds

Acceptable Result	No or Low Enantioselectivity and Low Retention	Enantioselectivity and too High Retention	No Enantioselectivity and too High Retention
Optimize with pH and/or modifiers	1) Decrease the 2-propanol conc.	1) Decrease pH stepwise to pH 4 and/or increase the 2-propanol conc.	1) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)
	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)	2) Test low conc. of a charged modifier (e.g. 1-20 mM octanoic, hexanoic or heptanoic acids; 1-5 mM tetraethyl- or tetrapropylammonium bromide (note: columns must be dedicated to this system) **LC-MS: Use triethylamine (TEA) as modifier**
	3) Amines: Test low conc. of a charged modifier (e.g. 1-20 mM octanoic, hexanoic or heptanoic acids; 1-5 mM tetraethyl- or tetrapropylammonium bromide (note: columns must be dedicated to this system) **LC-MS: Use triethylamine (TEA) as modifier**		

Strong acids (e.g. carboxylic acids)

Acceptable Result	No or Low Enantioselectivity and Low Retention	Enantioselectivity and too High Retention	No Enantioselectivity and too High Retention
Optimize with pH and/or modifiers	1) Decrease the pH to 4 and/or increase ionic strength up to 75 mM (max. 100 mM)	1) Add 2-propanol	1) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)
	2) Try low conc. (1-5 mM) of DMOA (N,N-dimethyloctylamine) (note: columns must be dedicated to this system) **LC-MS: Use a volatile modifier**	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)	2) Try low conc. (1-5 mM) of DMOA (N,N-dimethyloctylamine) (note: columns must be dedicated to this system) **LC-MS: Use a volatile modifier**

Method Development Protocols for CHIRAL-HSA

1. Characterize your sample and find the starting mobile phase

Compound Type	Starting Mobile Phase (UV)	Starting Mobile Phase (MS)
Acids and non-protic compounds	5% 2-propanol in 10 mM sodium phosphate, pH 7	5% 2-propanol in 10 mM ammonium acetate, pH 7

2. Optimize based on particular analyte class

Acids

Acceptable Result	No or Low Enantioselectivity and Low Retention	Enantioselectivity and too High Retention	No Enantioselectivity and too High Retention
Optimize with pH and/or uncharged modifiers and/or ionic strength	1) Decrease pH stepwise to pH 5 and/or decrease modifier conc.	1) Increase the 2-propanol conc. stepwise up to 10%	1) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol) and/or increase the ionic strength
	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol), or remove the modifier altogether	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)	2) UV: Add 1 mM octanoic acid, increase, if necessary, up to 5 mM (note: columns must be dedicated to this system)

Non-protic compounds

Acceptable Result	No or low Enantioselectivity and Low Retention	Enantioselectivity and too High Retention	No Enantioselectivity and too High Retention
Optimize with uncharged modifiers and/or ionic strength	1) Decrease the modifier conc.	1) Increase the 2-propanol conc. stepwise up to 10%, and/or increase ionic strength	1) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol) or increase the ionic strength
	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol), or remove the modifier	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol), or remove the modifier	

Using CHIRAL-HSA for Fast Drug/Protein Binding Studies

The primary use of CHIRAL-HSA is for fast drug/protein binding studies (1). To calculate the % protein binding, measure the retention time of an unretained compound (t_0) and the compound of interest (t_r) on the CHIRAL-HSA column. Then use the capacity factor equation:

$k = (t_r - t_0)/t_r$

to calculate the % protein binding (P):

$P = 100k/(k+1)$

Different types of mobile phases can be used. A mobile phase consisting of 6% 2-propanol in 20 mM potassium phosphate buffer, pH 7.0 gives data in good agreement with literature. The mobile phase conditions should be chosen to suit the drugs to be tested, i.e., for high protein binding drugs a mobile phase with higher eluting strength might be needed in order to reduce retention times.

Reference

1.Goodman, A.; Gilman, A.G. The Pharmacological Basis of Therapeutics, 9th Edition, McGraw-Hill: New York, 1996; pp 1712-1792.

Method Development Protocols for CHIRAL-CBH

1. Characterize your sample and find the starting mobile phase

Compound Type	Starting Mobile Phase (UV)	Starting Mobile Phase (MS)
Amines, hydrophobic or hydrophilic	5% 2-propanol in 10 mM sodium phosphate, 50 µM EDTA, pH 6	5% 2-propanol in 10 mM ammonium acetate, pH 6

2. Optimize

Acceptable Result	No or Low Enantioselectivity and Low Retention	Enantioselectivity and too High Retention	No Enantioselectivity and too High Retention
Optimize with pH and/or modifiers	1) Increase pH stepwise to pH 7	1) Decrease pH tepwise to pH 3 and/or increase the 2-propanol conc. and/or increase the ionic strength	1) Increase the modifier conc. and/or ionic strength
	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol), or remove the modifier	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol), or remove the modifier	2) Test another uncharged modifier (acetonitrile, methanol, 1-propanol, ethanol)

Product Listing

Guards for the CHIRAL-AGP, CHIRAL-HSA and CHIRAL-CBH columns are supplied in 1 cm length by 2.0, 3.0 or 4.0 mm I.D. cartridge format in packs of 2. They require a holder (58159AST) that is sold separately. The holder accommodates standard 1/16" O.D. tubing. You can couple the holder to the analytical column using a short piece of 1/16" tubing, or use the convenient column couplers.

Guard cartridges and holder, sold separately

CHIRAL-AGP
(α_1-Acid glycoprotein)

I.D. (mm)	L (cm)	Cat. No.
2	5	58129AST
2	10	58130AST
2	15	58131AST
3	5	58169AST
3	10	58170AST
3	15	58171AST
4	5	58149AST
4	10	58150AST
4	15	58151AST
10	10	58155AST
10	15	58157AST

CHIRAL-CBH
(Cellobiohydrolase)

I.D. (mm)	L (cm)	Cat. No.
2	5	58529AST
2	10	58530AST
2	15	58531AST
3	5	58569AST
3	10	58570AST
3	15	58571AST
4	5	58549AST
4	10	58550AST
4	15	58551AST
10	10	58555AST
10	15	58557AST

CHIRAL-HSA
(Human serum albumin)

I.D. (mm)	L (cm)	Cat. No.
2	5	58529AST
2	10	58530AST
2	15	58531AST
3	5	58569AST
3	10	58570AST
3	15	58571AST
4	5	58549AST
4	10	58550AST
4	15	58551AST
10	10	58555AST
10	15	58557AST

Guard columns for CHIRAL-AGP (pack of two cartridges)

I.D. (mm)	L (cm)	Cat. No.
2	1	58178AST
3	1	58158AST
4	1	58188AST

Guard columns for CHIRAL-CBH (pack of two cartridges)

I.D. (mm)	L (cm)	Cat. No.
2	1	58578AST
3	1	58558AST
4	1	58588AST

Guard columns for CHIRAL-HSA (pack of two cartridges)

I.D. (mm)	L (cm)	Cat. No.
2	1	58478AST
3	1	58458AST
4	1	58488AST

Guard Column Holders and Couplers

Cat. No.	Description
58159AST	Guard Column Holder for CHIRAL-AGP, -CBH and -HSA
58162AST	Column Coupler, PEEK (1 in. L x 0.007 in. I.D. x 1/16 in. O.D.)
54986	Column Coupler, PEEK (1 in. L x 0.01 in. I.D. x 1/16 in. O.D.)

For further questions, please contact your local Sigma-Aldrich office, or send an email to techservice@sial.com

View our complete Chiral Chemistry & Chromatography offering at sigma-aldrich.com/chiral