Comparison of HILIC and Fluorinated Columns Using LC and SFC for the Separation of Polar Basic Compounds in Drug Development

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Introduction

PURPOSE

- **Analysis of highly polar and very basic compounds remains a challenge in pharmaceutical development. Typical reversed-phase liquid chromatography (RPLC) methods require high aqueous buffer content which can cause:**
  - low separation efficiency
  - poor or broad peak shape
  - impair the MS sensitivity
- **Some approaches to enhance retention of polar and basic analytes include**:
  - Adjust pH>8 or <2
  - Polar-embedded or polar-endcapped column
  - **Fluorinated column**
  - **HILIC column (including using NP columns)**
  - Graphite column
  - Alkyl C30 phases
  - Type-C silica
  - Mix-mode (ion-exchange plus RPLC)
  - *Ion-pair*
  - **SFC**
- **This study compares the separation of seven highly polar compounds using Fluorinated and HILIC columns under LC and SFC modes**

CHARACTERISTICS OF FLUORINATED COLUMNS

- Provide different elution orders - can lead to enhanced selectivity
- Simpler mobile phases can be used - extreme pH conditions unnecessary
- C-F bond has a greater dipole than C-H bond - greater retention of polar and halogenated compounds compared to a traditional phase
- Most commercially available columns are stable under highly aqueous conditions
- Fluorinated phases tend to work best when fluorinated compounds are encountered
- Studies still need to be done to determine the actual chromatographic interaction mechanism of the fluorinated phases.

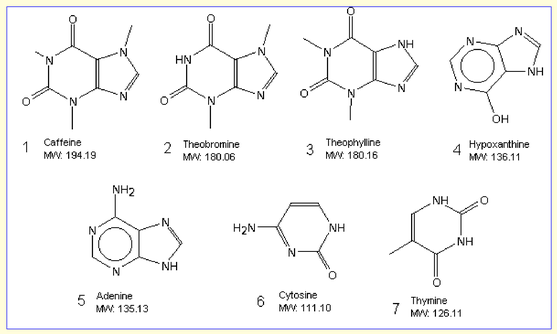
CHARACTERISTICS OF HILIC COLUMNS

- Uses an aqueous-organic mixture - makes HILIC more MS friendly
- Runs on polar stationary phases such as silica, amino, diol, cyano, amide, aspartamide, and cyclodextrin-based packings
- Elution order is least to most polar - opposite that in RPLC
- Retention is proportional to the amount of organic solvent in the mobile phase - opposite of RPLC
- HILIC mechanisms on Silica*
- Polar analyte moves in and out of the adsorbed water layer
- Charged analyte interacts with the charged silanol groups
- Combination of these mechanisms increases retention

*E.S. Grumbach, D.M.Diehl, B.Alden, P. Iraneta, U. Neue, H. Mazzeo, Waters Corporation, **Presented at HPLC, Nice, France, 15th-19th June 2003**

Experimental

POLAR AND BASIC COMPOUNDS AS PROBE



Experimental

COLUMNS

Product Name	Supplier	Dimensions
Fluophase RP (Perfluorohexyl)	Thermo Electron	100x3 mm, 5µm
Inertsil HILIC Diol	GL Sciences Inc.	150x3 mm, 5µm
EXP Diol	Phenomenex	150x3 mm, 5µm

LC: Agilent 1100 Series LC/MSD

SFC: Agilent 1100 Series LC/MSD with Dual Pump Fluid Control Module from Berger Instruments

Results & Discussion

POLAR-EMBEDED VS. HILIC COLUMN VS. FLUOPHASE RP

- Polar-embedded column shows good separation except for cytosine (Fig.1A compound#6); while HILIC column achieves good separation and peak shape for all compounds within 10min using high content of ACN (Fig.1B). However, the elution order is very different from the C18 column.

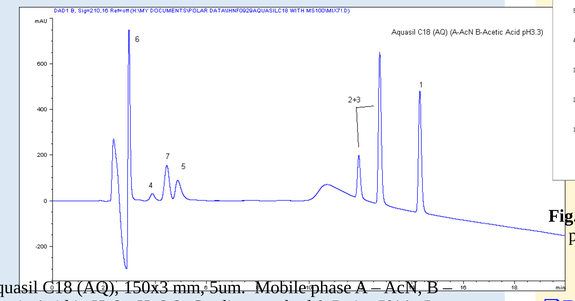


Fig. 1A Aquasil C18 (AQ), 150x3 mm, 5um. Mobile phase A – AcN, B – 0.1% Acetic Acid in H_2O pH~3.3, Gradient method 0-5min: 5%A, 5-25min: 5-95%A, 25-30min: 95%A

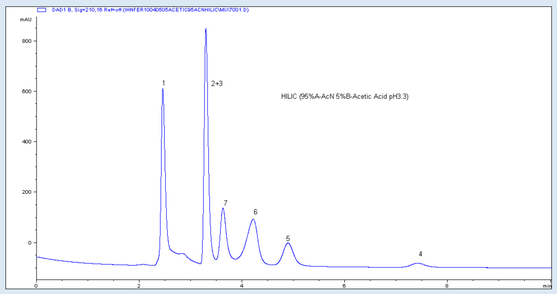


Fig 1B Inertsil HILIC Mobile phase A – AcN, B – 0.1% Acetic Acid in H_2O pH~3.3. Isocratic method 0-20min: 95%A.

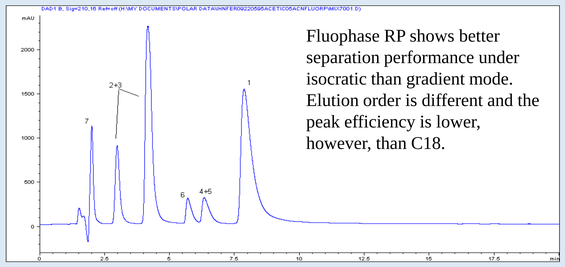


Fig. 1C Fluophase RP Mobile phase A – AcN, B – 0.1% Acetic Acid in H_2O pH~3.3. Isocratic method 0-20min: 5%A.

Results & Discussion

EFFECT OF BUFFER TYPES UNDER SAME pH CONDITIONS

- It is highly recommended to use buffers with bigger cationic strength, e.g. ammonium acetate, which will result in much better peak shapes than using acids at the same pH condition, e.g. acetic acid. (Fig 2).
- The stronger counterionic strength and ion-pair effect allows the faster displacement of the analytes from the stationary phases.
- This phenomena was observed on all HILIC type columns.

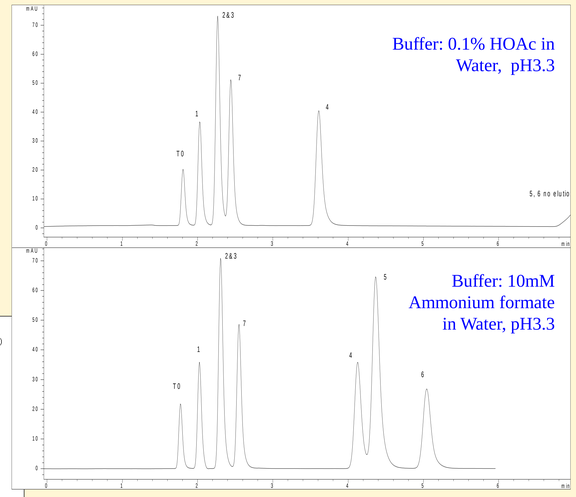


Fig. 2 Phenomenex EXP Diol, Mobile phase A – AcN, B – Buffer in water, pH 3.3. Isocratic 90%A

RETENTION VS. CONTENT OF ORGANIC MODIFIER

- HILIC columns generally give rise to longer retention for polar compounds at much higher organic content. So an opposite gradient to RPLC mode (starting with high % organic) is recommended to start the method development.
- Majority of compounds show increase in retention as concentration of organic modifier in mobile phase increases.
- It is also observed that HILIC runs better under isocratic mode for reproducible performance

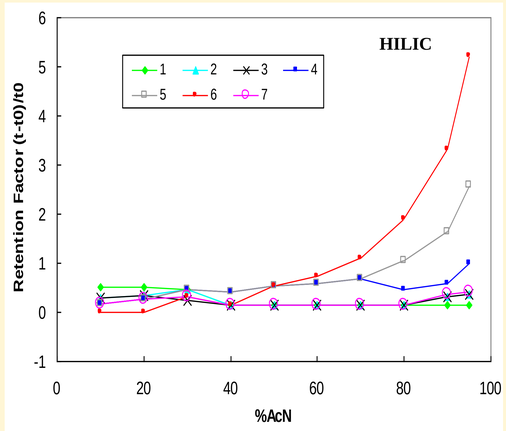


Fig. 3A Inertsil HILIC Mobile phase A – 0.1% Acetic Acid in AcN, B – 0.1% Acetic Acid in H_2O pH~3.3. Isocratic method 0-20min: X%A.

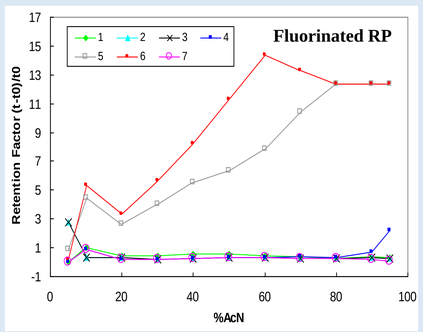


Fig. 3B Fluorinated RP column, mobile phase A – 0.1% HOAc in ACN, B – 0.1% HOAc in H_2O pH~3.3. Isocratic method 0-20min: X%A.

- For Fluorinated columns, most compounds show a kind of "U-shape" retention behavior, which indicates that at a certain organic content, the retention of analytes will switch from RPLC-like to a HILIC-like mechanism.
- Good retention of compounds 5, 6 - retention increased as organic content increased. Compound 4 only retained with >90% ACN. Compounds 1, 2 and 3 only retained with <10% ACN.

SFC SEPARATION

- SFC provides fast separation, good selectivity and peak shape for most of the analytes. This makes method development much simpler and the final method is more generic for general application and further validation.

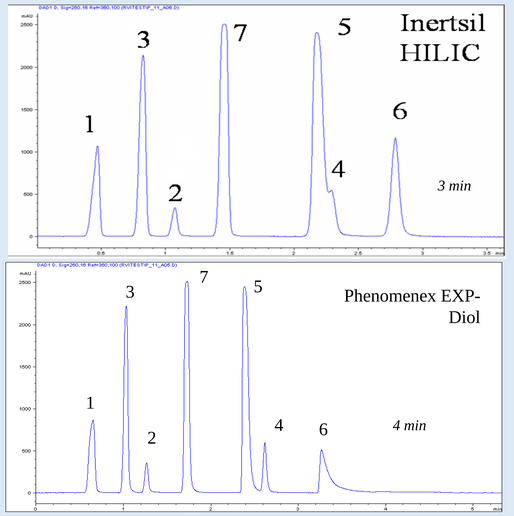


Fig. 4 SFC outlet Pressure 140 bar, flowrate 5.0 ml/min, Mobile phase: A; CO_2, B: MeOH w/ 0.1% Acetic Acid. Gradient Method 5-40% B at 10%/min. Columns are the same ones as used in LC mode

Conclusions

- In LC mode, none of the columns were able to separate all the compounds, while SFC was able to separate all seven polar compounds in the least amount of time - this new technology will be very useful for polar basic compound analysis.
- In LC mode, most HILIC columns generally follow the anticipated retention behavior, i.e. the retention increases with the increase of the organic content unless the compound cannot be retained at any condition. Strong counterionic strength buffers are recommended over acids.
- Fluophase columns give more complicated retention behavior (i.e. the "U-shape" retention relationship against % organic content). This causes additional difficulty in developing methods or predicting the retention/elution pattern.
- Further studies need to be done on SFC to find the most stable and robust Diol columns.

Acknowledgements

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