Advantages of Polar, Reversed-Phase HPLC Columns for the Analysis of Drug Metabolites

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Abstract

Small, polar molecules present formidable challenges for liquid chromatographic analyses on traditional reversed-phase columns. These analytes are often poorly retained, necessitating the addition of ion-pair modifiers to the mobile phase. Ion-pair methods tend to lack reproducibility and are often difficult to transfer to quality control laboratories. In addition, the nonvolatile and ion-suppressing nature of the common ion-pair reagents make them less amenable to liquid chromatography-mass spectrometry (LC/MS) experiments.

Drug metabolites, in general, fall into this class of small, polar molecules. Due to the complex nature of biological samples and the low levels of metabolites that are often present, LC/MS has become the dominant tool for analysis in this area. It is therefore important to develop tools to adequately retain drug metabolites without the need for detrimental ion-pair reagents.

Abstract (contd)

In this study several polar, reversed-phase HPLC columns are investigated for their effectiveness at retaining and resolving common types of drug metabolites. These include desmethylated, hydroxylated, sulfated, and glucuronide metabolites. Results regarding specific interactions between stationary phase chemistries and particular classes of metabolites will be discussed.

Introduction

Problems associated with metabolite analysis:

- Poor retention of polar analytes
- May require ion-pairing
- LC/MS compatibility
- General elution problem
- Too little or too much selectivity

A variety of functionalized, reversed-phase columns are available.

The problem: Which is the best phase to use for a particular class of metabolites?

Introduction (contd)

The goal of this work was to determine what phase or phases provide the best retention and separation for a given class of metabolites.

Classes of metabolites studied were:

- **Hydroxy metabolites**
- **Desmethyl metabolites**
- **Glucuronide metabolites**
- **Sulfated metabolites**

Experimental
Compounds Used in This Study

Hydroxy Metabolites	
Parent	**Metabolite**
Chloroquine	Hydroxychloroquine
Coumarin	7-Hydroxycoumarin
Propranolol	4-Hydroxypropranolol
Midazolam	α-Hydroxymidazolam
Chlorzoxazone	6-Hydroxychlorzoxazone
Metoprolol	4-Hydroxymetoprolol
Desmethyl Metabolites	
Parent	**Metabolite**
Diazepam	Nordazepam
Clozapine	Norclozapine
Clomipramine	Norclomipramine
Deprenyl	Nordeprenyl
Doxepin	Nordoxepin
Fluoxetine	Norfluoxetine

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Compounds Used in This Study (contd)

Glucuronide Metabolites	
Parent	**Metabolite**
3'-Azido-3'-deoxythymidine	3'-Azido-3'-deoxythymidine β-D-glucuronide
Codeine	Codeine β-D-glucuronide
Nicotine	Nicotine N-β-glucuronide
Morphine	Morphine 6-β-D-glucuronide
Estriol	Estriol β-D-glucuronide
Acetaminophen	p-Acetamidophenyl-β-D-glucuronide
Sulfated Metabolites	
Parent	**Metabolite**
Estriol	Estriol 3-sulfate
Estrone	Estrone 3-sulfate
β-Estradiol	β-Estradiol 3-sulfate

Phases Studied and Experimental Conditions

Phases Studied

- **Discovery C18**
- **Discovery HS F5**
- **Discovery Cyano**
- **Discovery HS PEG**

Conditions

Columns:	**5cm x 4.6mm ID, 5µm particles**
Mobile Phase:	Various ratios of 10mM ammonium acetate, pH 6.98 (unadjusted) and CH_3CN
Temperature:	35°C
Flow Rate:	1.0mL/min, split to mass spectrometer
Detection:	MS, ESI (+) or (-) in Single Ion Recording (SIR) mode
Injection Volume:	10µL
Sample:	1 or 5µg/mL (in 50:50 10mM ammonium acetate, pH 6.98 :CH_3CN)

Procedure

- Mobile phase composition adjusted such that parent k' values were between 3 and 6.
- Metabolite subsequently run under same conditions as the parent.

Criteria

- Respective k' of metabolite at least 1.5
- Little or no peak tailing (qualitatively evaluated)
- If above criteria met for a given metabolite set, this phase was classified as a good column for this analysis.
- Data was collected for each phase with respect to each class of metabolite and reported as % acceptable.

Results: Hydroxy Metabolites

Table 1. Parent Compound and Hydroxy Metabolite - Discovery C18					
Compound	**% Organic**	**k'**	**α**	**Comments on Peak Shape**	**Overall Acceptability**
Chloroquine	20	2.74	1.66	tailing	No
Hydroxychloroquine		1.65			
Coumarin	20	4.42	2.53	acceptable	Yes
Hydroxycoumarin		1.75			
Propranolol	25	4.07	4.73	tailing	No
Hydroxypropranolol		0.86			
Midazolam	40	3.1	1.88	tailing	No
Hydroxymidazolam		1.65			
Chlorzoxazone	25	4.12	4.85	acceptable	No
Hydroxychlorzoxazone		0.85			
Metoprolol	15	4.83	8.19	tailing	No
Hydroxymetoprolol		0.59			

Overall Acceptability = 16%

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Results: Hydroxy Metabolites (contd)

Table 2. Parent Compound and Hydroxy Metabolite - Discovery HS F5					
Compound	**% Organic**	**k'**	**α**	**Comments on Peak Shape**	**Overall Acceptability**
Chloroquine	10mM AA in 10% water/ 90% CH_3CN	15.82	2.25	tailing	No
Hydroxychloroquine		7.02			
Coumarin	75	5.67	1.89	acceptable	Yes
Hydroxycourmarin		3.00			
Propranolol	10mM AA in 10% water/ 90% CH_3CN	4.06	1.50	acceptable	Yes
Hydroxypropranolol		2.71			
Midazolam	45	2.68	1.75	acceptable	Yes
Hydroxymidazolam		1.53			
Chlorzoxazone	30	4.47	55.88	acceptable	No
Hydroxychlorzoxazone		0.08			
Metoprolol	10mM AA in 10% water/ 90% CH_3CN	3.17	1.31	acceptable	Yes
Hydroxymetoprolol		2.42			

Overall Acceptability = 66%

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Results: Hydroxy Metabolites (contd)

Table 3. Parent Compound and Hydroxy Metabolite - Discovery Cyano					
Compound	**% Organic**	**k'**	**α**	**Comments on Peak Shape**	**Overall Acceptability**
Chloroquine	20	3.73	1.42	tailing	No
Hydroxychloroquine		2.62			
Coumarin	5	2.06	0.81	acceptable	Yes
Hydroxycoumarin		2.53			
Propranolol	20	4.02	2.10	tailing	No
Hydroxypropranolol		1.91			
Midazolam	25	3.23	1.89	acceptable	Yes
Hydroxymidazolam		1.71			
Chlorzoxazone	5	3.04	1.45	acceptable	Yes
Hydroxychlorzoxazone		2.09			
Metoprolol	5	2.67	3.71	tailing	No
Hydroxymetoprolol		0.719			

Overall Acceptability = 50%

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Results: Hydroxy Metabolites (contd)

Table 4. Parent Compound and Hydroxy Metabolite - Discovery HS PEG					
Compound	**% Organic**	**k'**	**α**	**Comments on Peak Shape**	**Overall Acceptability**
Chloroquine	45	4.54	1.21	tailing	No
Hydroxychloroquine		3.74			
Coumarin		no elution	no elution	no elution	No
Hydroxycoumarin		no retention	no retention	no retention	
Propranolol	25	4.79	1.35	tailing	No
Hydroxypropranolol		3.55			
Midazolam	25	3.18	1.59	tailing	No
Hydroxymidazolam		2			
Chlorzoxazone	10	5.74	1.37	acceptable	Yes
Hydroxychlorzoxazone		4.2			
Metoprolol	0	2.13	3.48	tailing	No
Hydroxymetoprolol		0.612			

Overall Acceptability = 16 %

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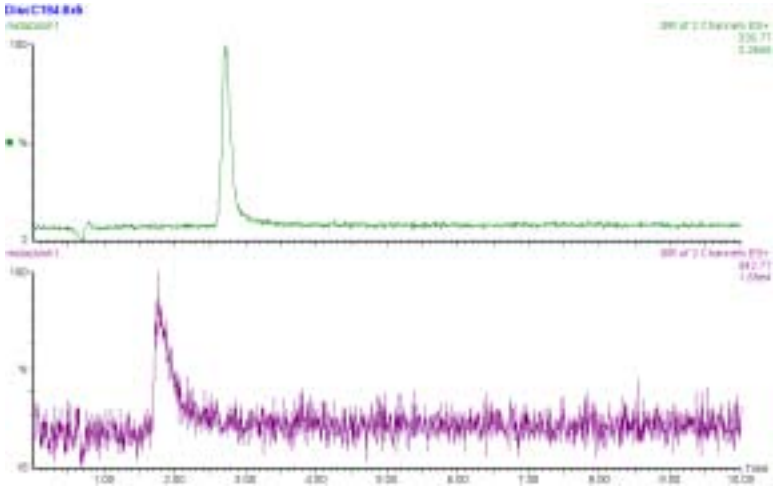
Summary of Results

Table 5. Summary of Results		
Metabolite Class	**Phase**	**% Acceptable for Separation**
Hydroxy	C18	16
	HS F5	66
	Cyano	50
	HS PEG	16
Desmethyl	C18	83
	HS F5	83
	Cyano	0
	HS PEG	16
Glucuronide	C18	33
	HS F5	0
	Cyano	0
	HS PEG	0
Sulfated	C18	0
	HS F5	0
	Cyano	0
	HS PEG	0

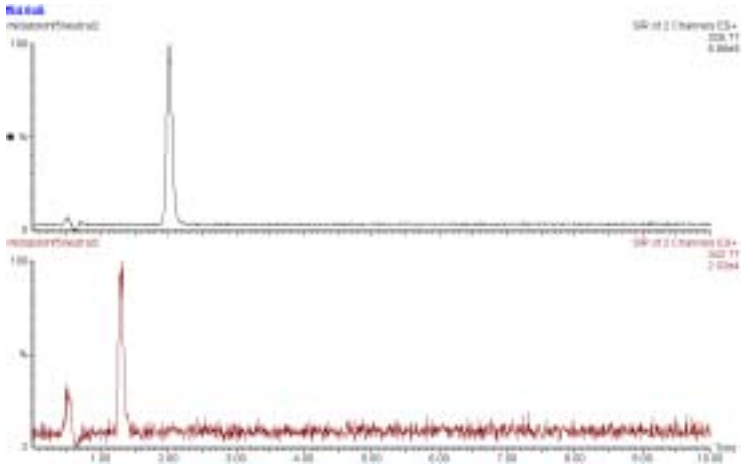
Hydroxy Metabolite Example

Midazolam and α-Hydroxymidazolam

C18



HS F5

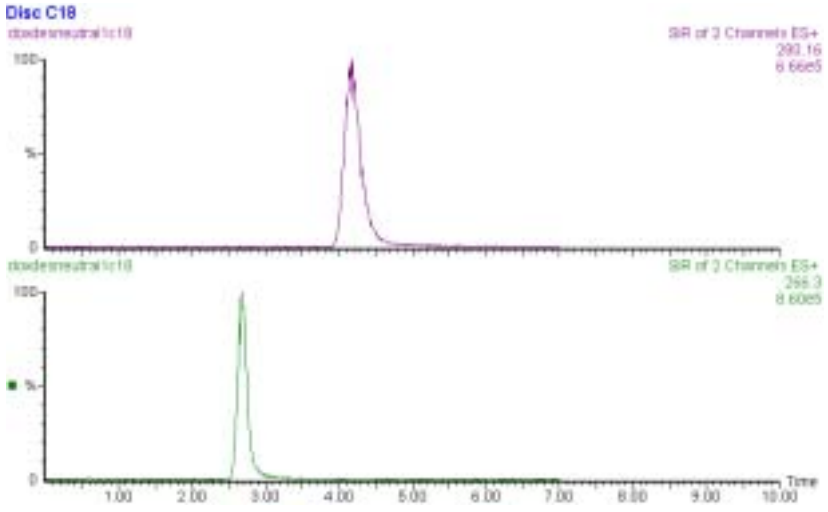


Conditions in Tables 1and 2. Note the improved peak shape on the HS F5. Note also the sensitivity increase on the HS F5 column due to the improved peak shape.

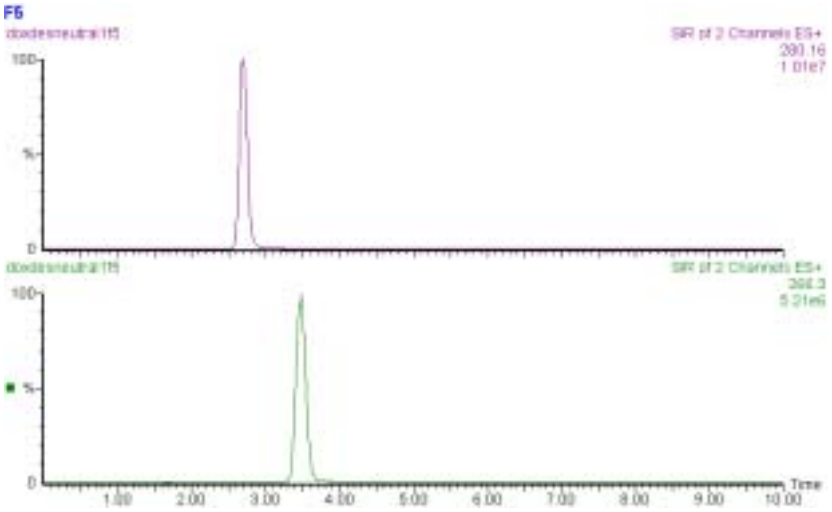
Desmethyl Metabolite Example

Doxepin and Nordoxepin

C18



HS F5



Conditions in Tables 1 and 2. Note the improved peak shape on the HS F5. Also note the elution order change on the HS F5 due to the ability of the nordoxepin to interact more strongly with the HS F5 phase as a result of the loss of a methyl group (secondary amine interacts more strongly than a tertiary amine).

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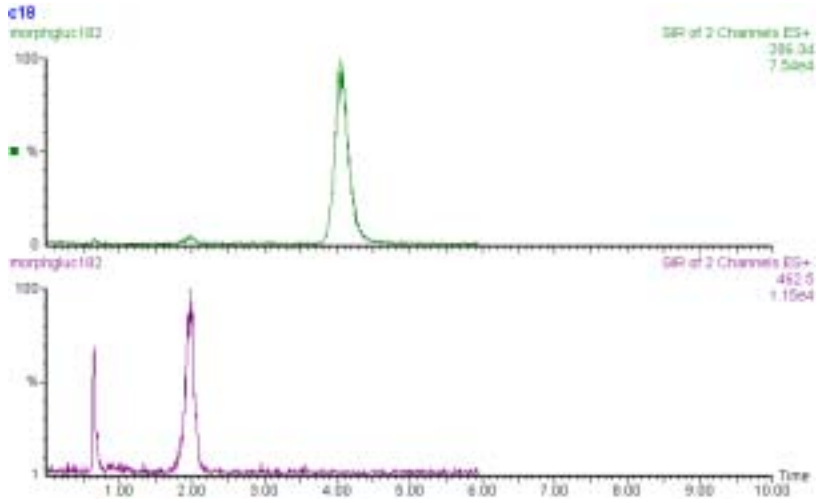
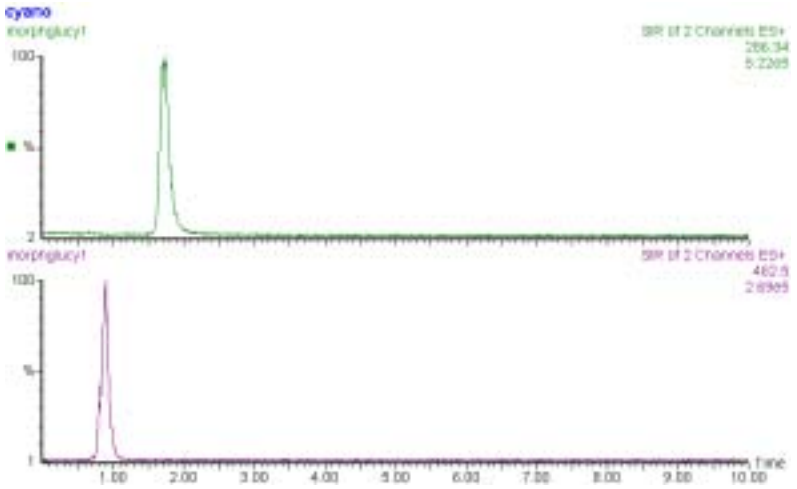
Glucuronide Metabolite Example

Morphine and Morphine-6-β-D-glucuronide

C18



Cyano



Conditions in Tables 1and 3. C18 appears to offer the most retention of glucuronide metabolites, without the use of ion pairing.

Discussion

- Tables 1-4 show the “Overall Acceptability” for hydroxy metabolites.
- Those metabolites showing a k' >1.5 (1) would be separated well enough from the solvent front, giving acceptable quantitation.
- Discovery C18 gave a 16% acceptable rating in meeting the overall criteria.
    - Polar analytes, especially bases, show poor retention on C18, due to weak dispersive interactions. These analytes often exhibit tailing, even on the most base-deactivated C18 columns, due to silanophilic interactions.
    - Note that neutral species exhibited acceptable peak shapes, however, even neutral polar analytes (hydroxychlorzoxazone) may be difficult to retain.

1. B. Matuszewski, et al., Anal. Chem. 70 (1998) 882-889.

Discussion (contd)

- HS F5 gave a better (66%) rating in meeting the overall criteria.
  - Increase in k' can be explained in terms of the available ionic and polar interactions provided by the HS F5 phase.
  - Improved peak shape is seen for metoprolol, midazolam, propranolol and chloroquine on the F5 compared to C18.
  - Improved peak shape due to the availability of polar interactions designed into the fluorinated phase.
- Cyano gave a 50% rating in meeting the overall criteria.
  - High rating can be attributed to the greater availability of polar interactions that can aid in the retention of bases.
  - Compounds on cyano exhibited more tailing than HS F5, indicating that sufficient availability of ionized silanols may not be present under the conditions studied.

Discussion (contd)

- HS PEG phase gave a 16% rating in meeting the overall criteria.
    - Peaks slightly broader on HS PEG than for the other three phases studied, most likely due to the ion exchange character of this phase. The major retention mechanism appears to be hydrophobic in nature. Broad peaks are likely due to a minor contribution from surface silanols.

This same procedure was followed for all other compound sets, i.e., the desmethylated, glucuronide and sulfated metabolites.

Table 5 summarizes these data.

Summary

To rank the phases for hydroxy metabolites:

- **HS F5-best overall**
- **Cyano-second best- but peak shape slightly worse than HS F5**
- **C18 and PEG-third best**

- **C18 and HS F5 gave good results (83%) for desmethyl metabolites.**
- **HS F5 offers unique selectivity in some cases (basic analytes).**
- **C18 is the best choice for glucuronide metabolites.**
- **Sulfated metabolites are not retained well on any of the four phases studied.**

Conclusion

- Polar reversed phases can be advantageous for use in drug metabolite studies. C18 phases are not always the best choice.
- HS F5 shows enhanced retention of basic drug metabolites and, at times, alternate selectivity to traditional C18 phases.
- Cyano phase also shows enhanced retention of certain basic analyte metabolites relative to C18.
- Neutral drugs and their metabolites are generally more suited for C18 analysis.
- Glucuronide and sulfated metabolites studies show the need for a more suitable column chemistry.