Separation of Pharmaceuticals by SFC using Mono- and Di-Hydroxy Substituted Phenyl Stationary Phases

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Supercritical Fluid Chromatography (SFC) has gained significant momentum over the past 10 years as the technique of choice for analytical and preparative separation of small organic molecules. One of the main contributors to its success is the selectivity enhancements derived from multifunctional stationary phases above and beyond that achieved through mobile phase tuning. While the pyridine column has become a standard column in SFC by expanding the range of compounds amenable to SFC, the monofunctional pyridine phase may be too polar to be broadly applicable. Likewise, the C18 and phenyl columns may be too non-polar for many compounds. Therefore, in order to offer enhanced selectivity in SFC, these phases are often substituted. For instance, adding polar functional groups to the pyridine phase have produced alternatives phases such as hydroxylamino dipyridinyl column. In a similar approach, more polar functional groups can be added to the phenyl phase to increase its overall polarity and thus improve its selectivity and applicability. In this application note, we highlight selectivity changes observed when introducing mono- and di-hydroxy polar groups onto phenyl phases and comparing them to their unsubstituted counterpart.

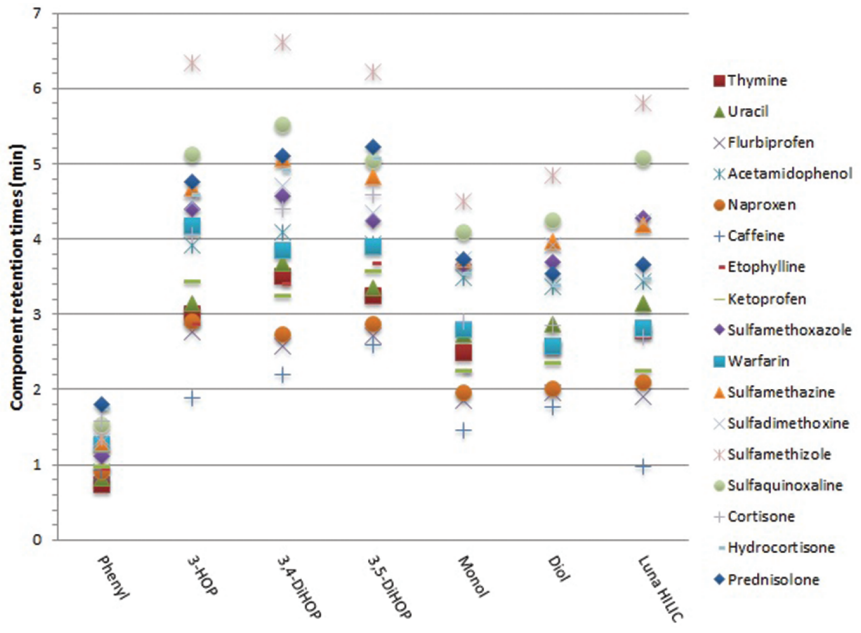

Figure 1: Elution profile for all 17 components of the mixture for each of the columns tested.

Introduction

SFC is a versatile technique for both chiral and achiral separations of small organic molecules due to the lower viscosity of supercritical fluids compared to liquids. This results in a lower pressure drop across a column, allowing for longer columns to be used at flow rates 3-5 times higher than HPLC [1,2]. The higher diffusivity of analytes in SFC produces higher selectivity with sharper peak shapes and wider resolution. Additionally, the shorter analysis times in SFC require less energy, use environmentally benign solvents, and generate considerably less volume of waste, all of which support the green chemistry principles. [3] Since SFC typically uses a simple mobile phase system consisting of CO_2 and an alcohol, separation optimisation can be achieved quickly through mobile phase tuning or by using multifunctional stationary phases. In SFC, selectivity is usually driven mainly by changing the surface chemistry or polarity of existing stationary phases, either through the addition or substitution of various functional groups (e.g. hydroxyl group) on polar bonded phases. The diversity of phases for SFC can range from non-polar (C18) to very polar (pyridine) [4,5] while offering a wide range of possible interactions. The monofunctional pyridine phase can be too polar for some compounds, such that very little solvent is required to obtain adequate retention, and detection (poor ionisation) [6] and fraction collection can become challenging. In those cases, the use of a less polar phase such as the C18 or phenyl columns may be beneficial, especially for very lipophilic compounds. Phenyl-bonded phases, due to the strong pi-pi and partitioning interactions of the aromatic groups, provide complementary selectivity to the alkyl chain C18 phases for more polar molecules. However conventional phenyl phases offer only moderate retention for hydrophobic compounds compared to a C18 column. This is problematic for samples that contain a variety of polar/non-polar moieties. In order to increase hydrophobic retention, different variations of phenyl columns such as those bonded to silica with longer alkyl chain spacers (e.g. phenyl-hexyl) were employed. However, these phases often do not offer enough selectivity for structurally related compounds, and thus fail to significantly impact achiral SFC applicability

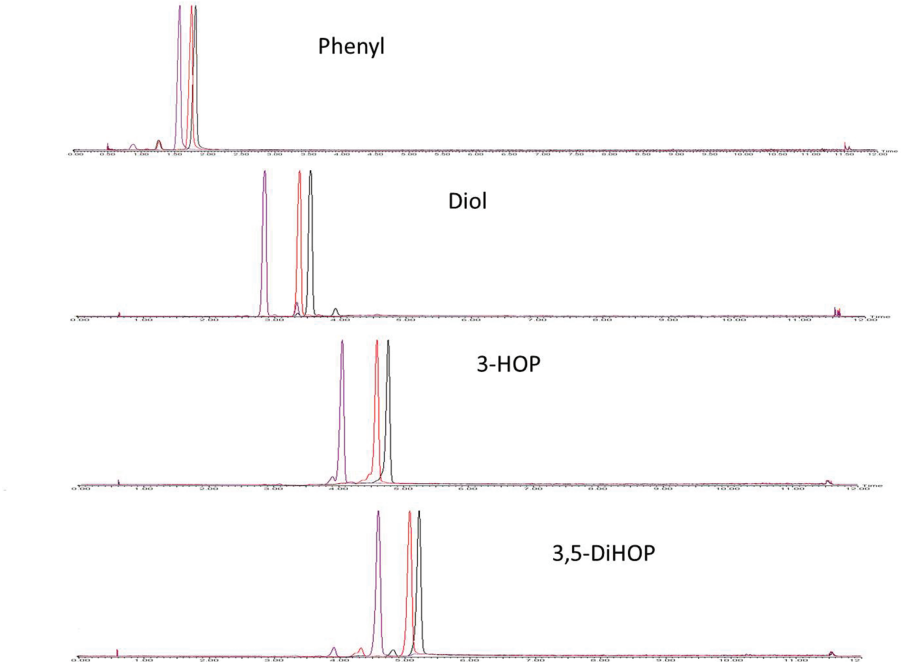

Figure 2. Separation of corticosteroids on the phenyl, diol, 3-HOP and 3,5-DiHOP phases. Elution order on all columns are as follows: cortisone, hydrocortisone, and prednisolone. Retention increased with each addition of a hydroxyl group to the phenyl phase.

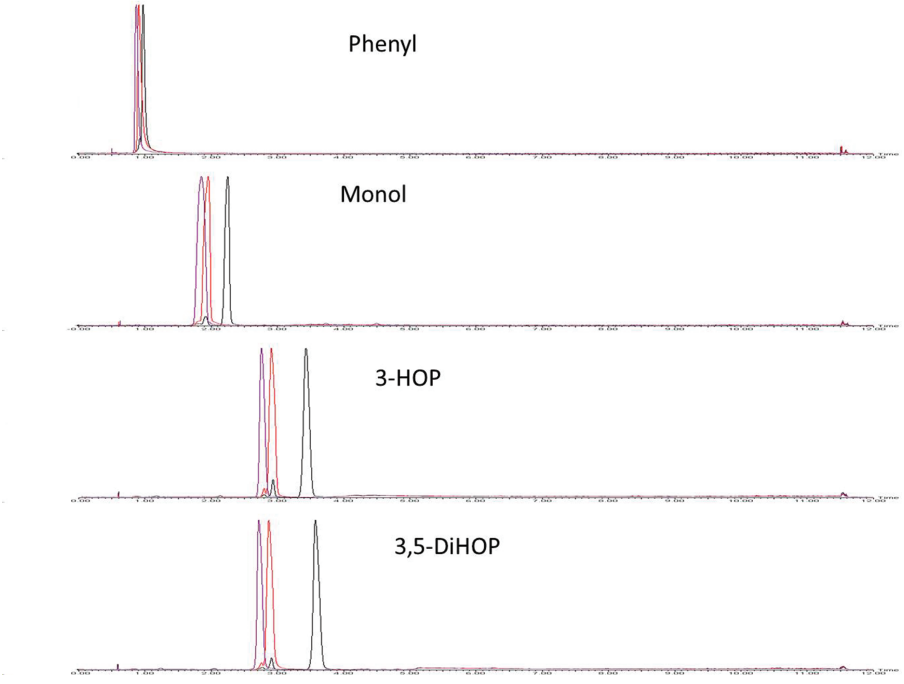

Figure 3. Separation of NSAIDs on the phenyl, monol, 3-HOP and 3,5-DiHOP phases. Elution order on all columns: 1-flurbiprofen, 2-naproxen, and 3-ketoprofen.The additional functional groups aid in increased retention, and the additional hydroxyl group on the 3-HOP and 3,5-DiHOP significantly improved retention and resolution.

across the compound diversity typically found in the pharmaceutical industry.

Resurgence in SFC use over the past few years can be attributed to the influx of new stationary phase chemistries, many of which include polar functional groups. For instance, the 2-ethylpyridine has become the de facto standard column since it often provides good retention, selectivity and peak shapes including those for more basic and polar compounds, thereby expanding the range of compounds amenable to SFC [7-9]. de la Puente et al. [10] determined that 84% of a diverse range of standards had less retention and better peak shapes on the 2-ethylpyridine when compared to the amino and diethylaminopropyl columns. Another group described a greater than 70% success rate, (Resolution > 1.5) using SFC with both a Polar RP (phenyl) and 2-ethylpyridine phase for diverse chemical libraries [11], while Mich et al. [12] utilised 2-ethylpyridine phases, among other columns, for combinatorial chemistry/library analysis.

The monofunctional 2-ethylpyridine has enabled successful SFC separations without the use of basic additives, which were often required to improve retention and peak shapes for polar, basic compounds [13]. However, complex solute chemistry may produce highly polar analytes which not only necessitates a mobile phase additive for selectivity tuning, but requires a more suitable column chemistry. In this application note, we highlight selectivity changes observed by introducing mono- and di-hydroxy polar groups onto phenyl phases and comparing them to their native counterparts.

Experimental

HPLC grade methanol (J.T. Baker, Phillipsburg, NJ, USA); ammonium formate, 99% (Fisher Scientific, Pittsburgh, PA, USA); and bulk grade carbon dioxide (AirGas West (Escondido, California, USA) were used in this study. The CO_2 was purified and pressurised to 1500 psig using a custom booster and purifier system from Va-Tran Systems, Inc. (Chula Vista, CA, USA).

The compounds used in this 17-component test mixture were purchased from Sigma-Aldrich (St. Louis, MO, USA) and are listed in Table 1. Samples were prepared to a concentration of ~1 mg/mL in methanol and injection volume was 0.5 µL.

Instrumentation

Analyses were performed on a Waters Acquity UPC2 system (Waters, Milford MA, USA) consisting of a convergence manager, sample manager, binary solvent manager, PDA detector, column manager with 6 positions, and an Acquity QDa mass detector. Data analysis was performed using Waters MassLynx 4.1 software. Table 2 shows the columns used in this study. All runs were performed with an SFC gradient consisting of 5-50% methanol with 20 mM ammonium formate over 12 minutes, with a flow rate of 3.5 mL/min and outlet pressure of 150 bar. Column temperature was maintained at 40°C, and UV detection of 230 nm was employed.

Results & Discussion

The chemistry and different functionality of the stationary phase can have a dramatic effect on SFC separations, since interactions

Table 1. Compounds in 17-component test mixture, their compound classes and physicochemical data*

		H-Acceptors	H-Donors	Rotatable Bonds	Log P	Molar Refractivity	TPSA
Flurbiprofen	NSAIDs	2	1	3	3.25	61.79	37.3
Naproxen	NSAIDs	3	0	3	0.76	56.33	49.36
Ketoprofen	NSAIDs	3	1	4	2.94	62.56	54.37
Caffeine	Xanthine	6	0	0	0.34	49.21	34.14
Etophylline	Xanthine	7	1	2	-0.2	55.31	54.37
Uracil	Nucleobase	4	2	0	0.86	27.9	34.14
Thymine	Nucleobase	4	2	0	1.12	32.37	34.14
Cortisone	Corticosteroid	5	2	2	1.99	80.62	91.67
Hydrocortisone	Corticosteroid	5	3	2	1.78	86.13	94.83
Prednisilone	Corticosteroid	5	3	2	1.56	86.04	94.83
Sulfadimethoxine	Sulfonamide	8	2	5	0.16	74.55	99.03
Sulfamethazine	Sulfonamide	6	2	3	1.58	71.6	80.57
Sulfaquinoxaline	Sulfonamide	6	2	3	1.8	77.97	80.57
Sulfamethizole	Sulfonamide	6	2	3	0.38	64.07	80.57
Warfarin		4	1	4	3.51	79.99	54.37
Acetamidophenol		3	2	2	0.51	36.47	49.33

Values calculated using MolPrime+, Molecular Materials Informatics at http://molmatinf.com

Table 2: SFC Stationary Phases (150mm x 4.6mm i.d., 5µ)

ZymorSpher Phenyl	Zymor Inc., Wayne, NJ, USA
Cosmosil 3-hydroxyphenyl (3-HOP)	Nacalai USA, San Diego, CA USA
Cosmosil 3,4-dihydroxyphenyl (3,4-DiHOP)	Nacalai USA, San Diego, CA USA
Cosmosil 3,5-dihydroxyphenyl (3,5-DiHOP)	Nacalai USA, San Diego, CA USA
ZymorSpher Monol	Zymor Inc., Wayne, NJ ,USA
ZymorSpher Diol	Zymor Inc., Wayne, NJ, USA
Luna Hilic	Phenomenex Inc., Torrance, CA, USA

between the solute and stationary phase are not typically hindered by the solvent, or at least not to the same degree as in HPLC. In reversed phase HPLC, the stationary phase mechanism is primarily due to solute partitioning between the solvent and stationary phase, and based on the lipophilicity of the compound. Additional interactions can be influenced by changing the pH or solvent composition, but rarely involves modifying the stationary phase chemistry significantly. However, in SFC the dielectric constant for the mobile phases is very low compared to aqueous solvent systems [14]. This chromatographic environment allows not only for solute interactions due to lipophilicity, but also for hydrogen bonding and pi-pi stacking, resulting in unique chromatographic benefits in terms of retention and/or and selectivity.

Starting with columns that exhibit mostly either hydrogen bonding (monol, diol, etc.) or pi-pi interactions (phenyl), we compare the separations with phases that have characteristic of both types of interactions: 3-hydroxyphenyl (3-HOP), 3,4-dihydroxyphenyl (3,4-DiHOP) and 3,5-dihydroxyphenyl (3,5-DiHOP). Using the 17-component mixture under a generic set of SFC conditions, we compared the retention and selectivity (resolution and retention order) of the various phases to demonstrate any added advantages to SFC. Under these conditions, the phenyl phase exhibited the least retention and resolution compared to the other columns. This was not a surprise, because many of the compounds have aromaticity, but they also have significant potential for other interactions such that dramatic alterations of the standard SFC parameters would be required in order to obtain optimised separations. Nonetheless, the phenyl column still exhibited enough selectivity and retention differences to serve as a baseline for comparison to the other columns. For instance, the phenyl phase was the only column studied that had a different selectivity order of elution as well as higher retention for caffeine. Although the phenyl phase offers some benefit in its unsubstituted form, it provides only moderate lipophilicity and, therefore, it is still too non-polar to be of general use for diverse pharmaceuticals.

Increasing the hydrophilicity of the phenyl stationary phase is achieved through the addition of one or more functional hydroxyl groups, where other interactions such as hydrogen bonding can provide additional benefits of complementary selectivity for optimised separations. Figure 1 shows the differences in separation and retention for the 17-component mixture under the same SFC conditions. Clearly, a significant increase in retention and resolution of all the compounds is observed relative to the phenyl, with the 3,4-dihydroxyphenyl column demonstrating the best separation for this sample. Due to the complexity of the mixture, the differences are better demonstrated by breaking the 17-component mixture into several structural sub-classes or types, such as xanthines, nucleobases, NSAIDs, corticosteroids, and sulphonamides. The data for the other 2 remaining test compounds, warfarin and acetamidophenol, were not classified.

Overall, similar selectivities were found across all columns for the nucleobases, corticosteroids, NSAIDs and xanthines. Only in the case of the phenyl and Luna HILIC columns did we observe different selectivities for the sulphonamide drugs as highlighted in Table 3. Despite the fast elution of all components from the phenyl column (in less than 2 minutes), selectivity still exists between compound types, as demonstrated by the elution differences between the sulpha drugs and corticosteroids.

The difference in selectivity provided by the Luna HILIC can be attributed to dissimilar hydrogen bonding mechanisms. While we included the Luna HILIC phase (cross-linked diol) in this study to compare separation capabilities to the diol, selectivities were different for several of the drugs, so we chose to consider only the diol column for the remainder of the study.

It was interesting to note that when a hydroxyl group is added to the phenyl phase, selectivity remained the same between the phenyl and the 3-HOP for acetamidophenol and warfarin. But when a second hydroxy group was added to the phenyl ring, elution order was reversed regardless of its position on the ring. In contrast, selectivity did not change with the addition of a second alcohol functional group (diol) when compared to the monol. While changing the position of one of the hydroxyl groups (e.g. 3,4-DiHOP vs. 3,5-DiHOP) did not produce any significant differences between the two phases, there were some slight differences in resolution between the two, especially for the structurally similar nucleobases. In this case, the 3,4-DiHOP demonstrated better resolution for those compounds. While both dihydroxyphenyl phases were more retentive than the hydroxyphenyl phase, overall the 3,4-DiHOP demonstrated the longest retentivity. A further example of this increase in retentivity of the dihydroxyphenyl columns is the separation of corticosteroids, which were chosen due to their modest polarity and structural similarity. As shown in Figure 2, under identical conditions, the addition of hydroxyl groups enhances the separation by introducing a hydrogen bonding mechanism. Therefore, in this case, not only did the number of hydroxyl substituents increase retention, resolution was also improved. Likewise, the additional alcohol functional group in the diol showed a difference in resolution of the xanthines, another group of structurally close compounds, when compared to the monol phase, with the diol phase having greater resolution.

Table 3. Elution order (from top to bottom) of sulfonamide drugs on all columns tested.

	Phenyl	3-HOP	3,4-DiHOP	3,5-DiHOP	Monol	Diol	Luna HILIC
↓	Sulfamethoxazole	Sulfamethoxazole	Sulfamethoxazole	Sulfamethoxazole	Sulfamethoxazole	Sulfamethoxazole	Sulfamethazine
	Sulfamethazine	Sulfadimethoxine	Sulfadimethoxine	Sulfadimethoxine	Sulfadimethoxine	Sulfadimethoxine	Sulfadimethoxine
	Sulfadimethoxine	Sulfamethazine	Sulfamethazine	Sulfamethazine	Sulfamethazine	Sulfamethazine	Sulfamethoxazole
	Sulfamethizole	Sulfaquinoxaline	Sulfaquinoxaline	Sulfaquinoxaline	Sulfaquinoxaline	Sulfaquinoxaline	Sulfaquinoxaline
	Sulfaquinoxaline	Sulfamethizole	Sulfamethizole	Sulfamethizole	Sulfamethizole	Sulfamethizole	Sulfamethizole

The NSAIDs also demonstrate selectivity and retention due primarily to hydrogen bonding given the resolution on the hydroxyl columns (Figure 3). Interestingly, the hydroxyl phenyl columns, although not distinguished between each other, show significant gains in retention and resolution, suggesting an additive effect of the pi-pi and hydrogen bonding mechanisms.

Conclusion

Our intent was to highlight whether or not the addition of a single hydroxyl (3-HOP) or two hydroxyl (3,4-DiHOP, 3,5-DiHOP) groups bonded to phenyl yielded any benefits over the alcohol phases (monol and diol). While overall retention of the 17-component mix did increase for the monol column, and the addition of a second alcohol substituent increased retention even further, resolution also improved when compared to the phenyl phase. But the most marked differences can be seen with the combination of the phenyl and hydroxyl groups in the same phase. Here, not only were the dihydroxyphenyl phases able to resolve the most peaks of the mixture, they were the most retentive among all columns. Therefore, while the position of the additional hydroxyl group on the hydroxyphenyl column did not provide significant benefits, the overall benefits in terms of resolution, retentivity and selectivity with the additional hydroxyl groups on a phenyl-bonded phase were realised.

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