HILIC Flash Purification – Separation of Polar Pharmaceuticals on Silica

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Polar pharmaceuticals are notoriously difficult to separate by analytical and semi-prep HPLC and this research demonstrates how we have generated reproducible separations of polar pharmaceuticals using low-pressure flash purification in 'HILIC' mode. These separations are not trivial by HPLC, and often reversed-phase purification is the 'go-to' method but can be challenging. We demonstrate the feasibility using standard silica flash columns to open up new possibilities for amino acid, nucleoside and nucleotide separations. Flash chromatography is a common practice in medicinal chemistry laboratories and the ability to switch to 'HILIC' mode is a simple operation.

Introduction

Hydrophilic Interaction Chromatography (HILIC) was initially reported by Alpert in 1990 (1). The mechanism being that polar analytes interact with a hydrophilic stationary phase and the elution generated by mixing a predominantly aprotic solvent (usually acetonitrile) with a strong eluting solvent (usually water). It is thought analytes partition into the water-rich layer that is partially immobilised onto the stationary phase, and elute with increasing polar solvent [1]. This mechanism has been scrutinised in detail by an analytical evaluation with many different stationary phases in a comprehensive analysis [2], the conclusion being that HILIC retention is a combination of electrostatic adsorption and hydrogen bonding interactions [2]. An increase in water content in the elution solvent reduces multipoint hydrogen bonding interactions, whilst an increase in electrolyte concentration decreases selectivity of various columns [2]. HILIC has advantages over conventional reversed phase or normal phase liquid chromatography as retention and separation of many polar analytes has proven to be a challenge as often unretained or poorly retained on most conventional reversed-phase stationary phases. Other advantages of HILIC are that

the polarity of samples usually aids solubility into aqueous mobile phases and secondly there is also no requirement for ion pair reagents which is advantageous for coupling with mass spectrometry techniques. There are many bioanalytical examples of using gradient HILIC LC-MS to retain polar analytes such as studies of urine [3] in a 2D-LC-MS approach with RP-LC for pharmaceutical analysis in plasma [4] monoamine neurotransmitters [5] and metabonomic/metabolomic studies [6]. A comparison study was published which demonstrated the advantages of HILIC over reversed-phase for the separation of polar ephedrine's [7]. Many advantages for this specific analysis were noted for Heaton's study, mainly improved peak shape, faster analysis and lower viscosity of mobile phases [7], although in a later study it was noted that it's critically important to choose optimal sample solvent and lower injection volumes for HILIC to optimise peak efficiency for this methodology for analytical applications [8]. Although there are many analytical evaluations of utilising HILIC there are very few articles which describe reproducible preparative methods. One recent article describes the use of hydrophilic solid phase extraction of glycyrrhizin (GA) in liquorice coupled with reversed-phase liquid chromatography purification, and although this method improved enrichment of GA, HILIC was only employed as a crude extraction procedure [9]. Recently a method describes the use of HILIC for large-scale preparative isolation of capreomycin impurities [10] and highlights the advantages of this method over an ion-pair mechanism. Also, recently, an at-column dilution mechanism was utilised for preparative HILIC and demonstrates the advantages in compound loadability, whilst preserving peak shape [11]. Our group has previously demonstrated how to successfully develop flash C18 purifications from analytical methods [12] so we were eager to determine whether HILIC silica flash purifications could be predicted using the same approach. To our knowledge, this is the first publication which demonstrates the use of HILC purification using flash chromatography with silica columns in a reproducible and cost effective approach to separate polar pharmaceuticals.

Instrumentation

Waters Acquity Binary system, PDA detector, Waters ZQ Mass Spec

Columns: Waters Atlantis 5µm 4.6x10mm, Kromasil 100-5-Sil 5µm 4.6x10mm, YMC-Pack CN 5µm 4.6x10mm, YMC-Pack NH2 5µm 4.6x10mm, YMC-Pack Diol-120-NP 5µm 4.6x10mm

Flow: 1.0 mlmin-1

UV Diode Array

Reverse Phase Flash Purification columns:

Teledyne ISCO SILICA 12g GOLD (20-40µm) Redisep column 30 mlmin-1

Teledyne ISCO 15g GOLD C18 (20-40µm) Redisep column 30 mlmin-1

UV detection 220 nm and 254 nm collection

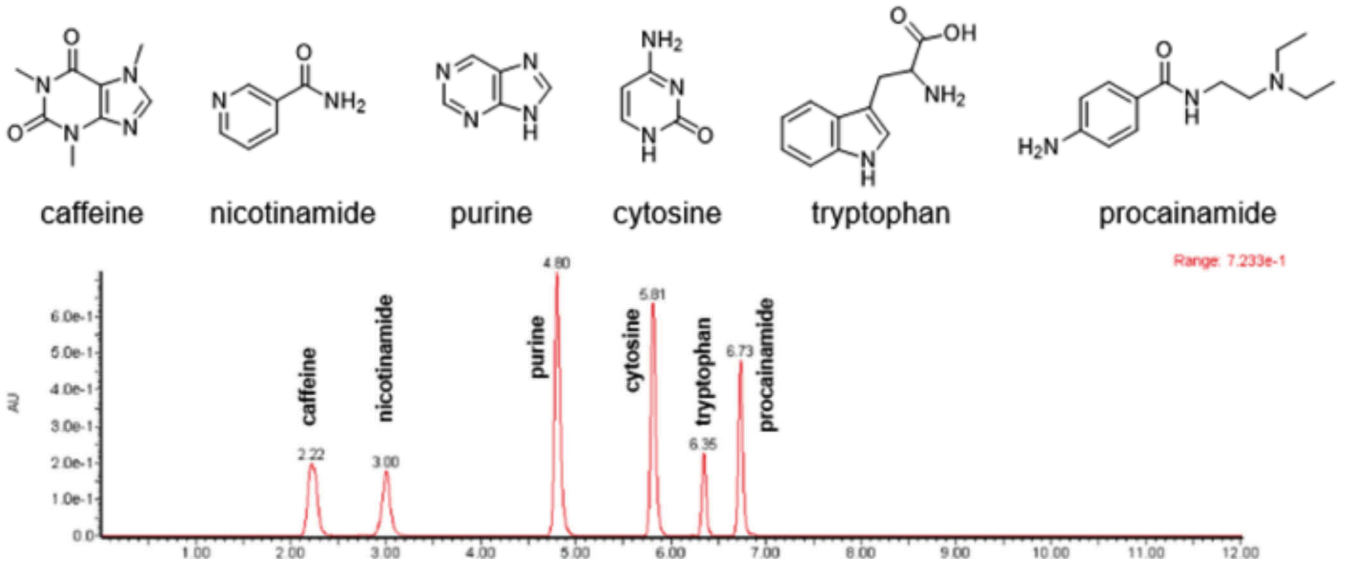

Figure 1. HILIC Analytical Polar Test Mix on 100-60% of B in A for 6 min gradient – Solvent A: water with 10 mM ammonium formate; Solvent B: 95% ACN in water with 10mM ammonium formate; Atlantis HILIC Silica column 4.6x100mm, 5µm.

HPLC-MS as a "Scout" for HILIC Flash Purification

A similar approach was undertaken to the reversed-phase methodology previously published [12] where we demonstrated the efficiency of predicting flash chromatography purification conditions from an analytical HPLC scout run. For HILIC we now selected a polar test mix using small molecule polar compounds, nicotinamide, nucleic bases purine and cytosine, amino acid tryptophan and small basic molecules caffeine and procainamide.

We decided to use this polar test mix separation to determine whether we can scale up to HILIC silica flash chromatography. We utilised a 12g Teledyne ISCO Gold silica cartridge (20-40 µm) and observed the separation in Figure 2 for a 20 mg total loading. We realised we needed to extend the gradient time out to 9 minutes from the 6 minutes employed on the analytical HPLC column to achieve baseline separation on the flash column.

This result was extremely encouraging as this demonstrates we can enable chemists to separate extremely polar analytes on flash instead of utilising semi-prep HPLC. Technology advancements have led to flash purification being a relatively cost effective solution where inexpensive plastic cartridge columns are used to often purify larger batches of material quickly in one purification run. A further advantage is that the capital and running costs for a standard flash chromatography system make this an attractive financial solution for many laboratories for scaled up separations, and reduced fraction volume in comparison to HPLC due to sample loading increase is a real benefit for dry-down time of samples [12]. This type of HILIC gradient flash separation has yet to be utilised as a method for purification in medicinal chemistry laboratories, but is advantageous due to better theoretical sample loading than reversed phase HPLC. The Gold silica flash columns are re-useable and similar separation is achieved even after 10 injections. Using a stepwise gradient approach can also be beneficial to achieve further retention and provide a separation profile as seen in Figure 3.

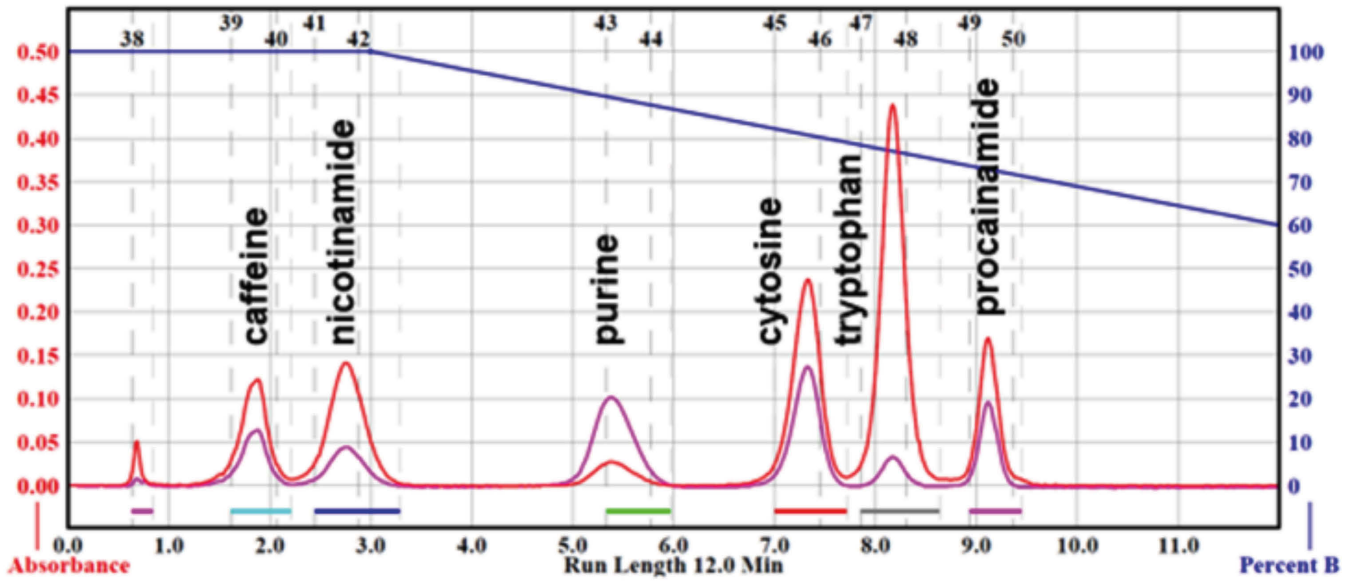

Figure 2. Flash Chromatography Polar Test Mix on 100-60% of B in A for 9 min gradient – Solvent A: water with 10 mM ammonium formate; Solvent B: 95% ACN in water with 10 mM ammonium formate; Teledyne ISCO 12g GOLD Silica (20-40µm) Redisep, 30 mlmin-1, 220 nm and 254 nm.

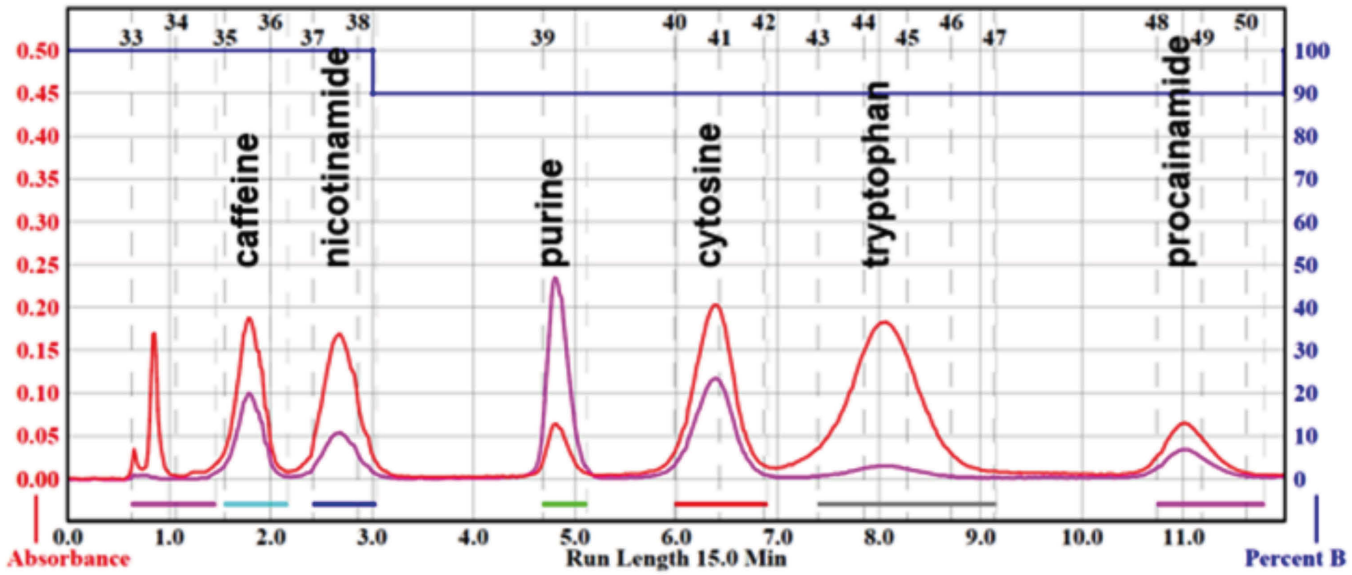

Figure 3. Flash Chromatography Polar Test Mix on 100-90% of B in A 3 min step gradient – Solvent A: Water with 10 mM ammonium formate; Solvent B: 95% ACN in water with 10mM ammonium formate; Teledyne ISCO 12g GOLD Silica (20-40 µm) Redisep, 30 mlmin-1, 220 nm and 254 nm.

We have invested a lot of time in developing methods for HILIC semi-prep core separations however this is a core purification service and this study has demonstrated the ability for chemists to use their own flash chromatography systems. In order to compare the silica flash column separation with that of a C18 reversed phase flash column separation, we injected the same 20mg of test mix on a RP gradient (5 to 20% ACN with 10 mM ammonium formate) as shown in Figure 4. Cytosine was not retained under these condition and purine and nicotinamide co-elute as do procainamide and tryptophan. The elution order for caffeine on the C18 flash column is totally reversed when compared to the HILIC silica flash column. This demonstrates the utility of employing HILIC flash for these types of polar samples.

Another example of separation of an extremely polar test mix (Adenosine CLogP -1.2, Cytidine CLogP -2.2, 5'-adenylic acid CLogP -3.1) on HILIC silica (nucleoside and nucleotide) is demonstrated analytically in Figure 5, and a successful purification by flash HILIC is performed in Figure 6. This result being performed without the need for ion-pairing reagents, which is the normal approach using tetrabutylammonium hydroxide [13].

Finally, we investigated and compared different manufacturer's analytical HILIC silica columns and alternative analytical HILIC stationary phases which are also available in flash chromatography cartridge versions (namely Cyano, Amino and Diol). We found that Kromasil silica was virtually identical to Waters Atlantis for our test mix separation whilst we observed lower selectivity and retention on cyano and differing selectivity and retention order on amino and diol columns. This experiment demonstrates that HILIC separation may be achieved on alternative phases other than silica. The results are shown in Figure 7.

Conclusion

We have demonstrated that scaling to HILIC flash chromatography from HILIC LC-MS analytical methods is routinely achievable and can offer several advantages over generic semi-prep HPLC methods, being

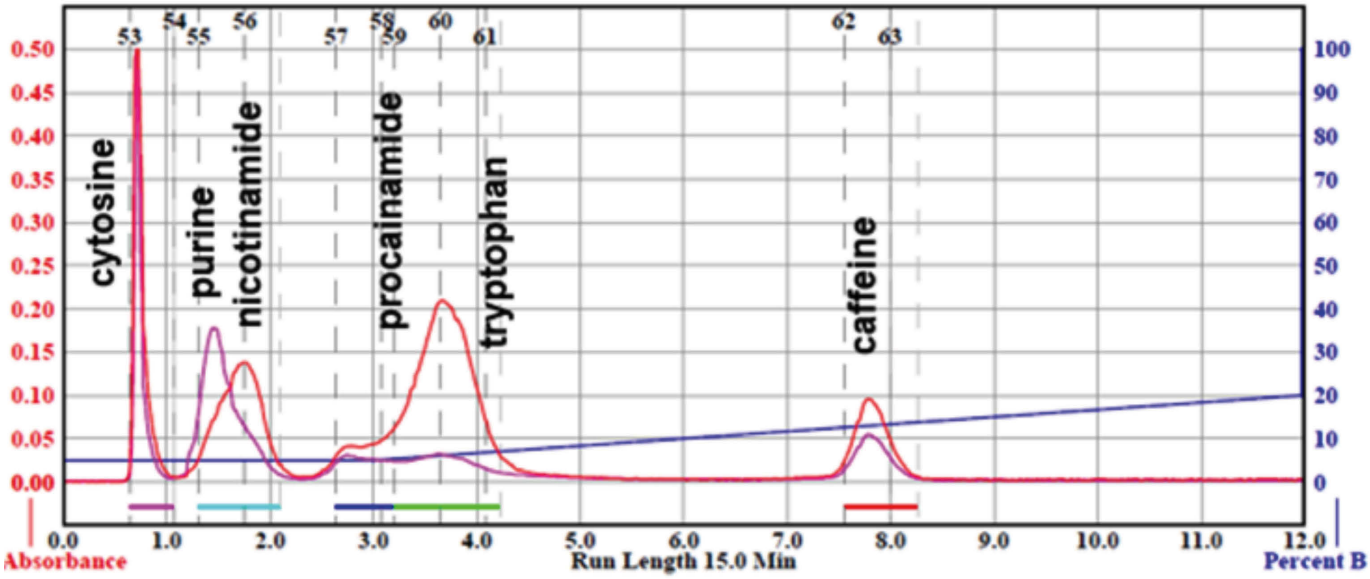

Figure 4. Flash Chromatography Polar Test Mix on 5-20% of B in A for 9 min gradient – Solvent A: Water with 10mM ammonium formate; Solvent B: 95% ACN in water with 10 mM ammonium formate; Teledyne ISCO 15.5g GOLD C18 (20-40µm) Redisep, 30 mlmin-1, 220 nm and 254 nm.

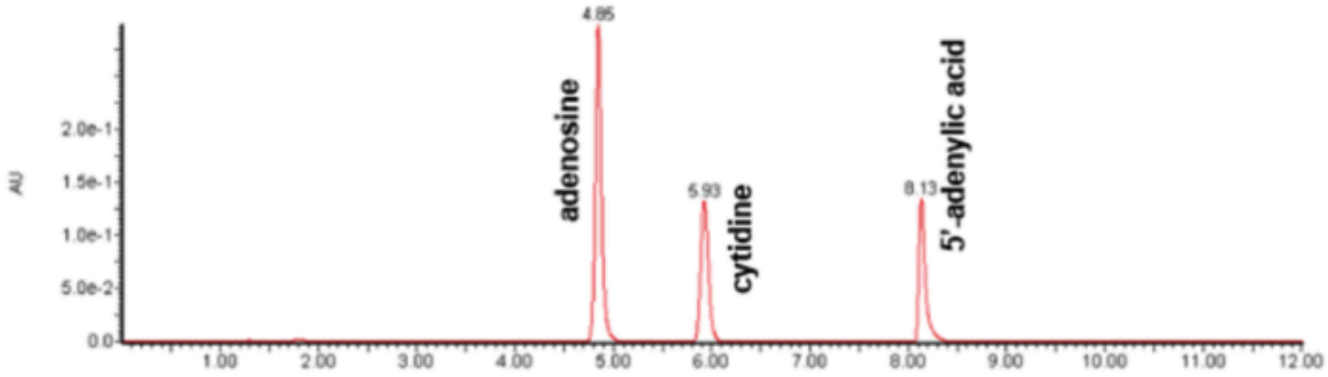

Figure 5. HILIC Analytical on 100-60% of B in A for 6 min gradient – Solvent A: Water with 10 mM ammonium formate; Solvent B: 95% ACN in water with 10mM ammonium formate; Atlantis HILIC Silica column 4.6x100mm, 5µm

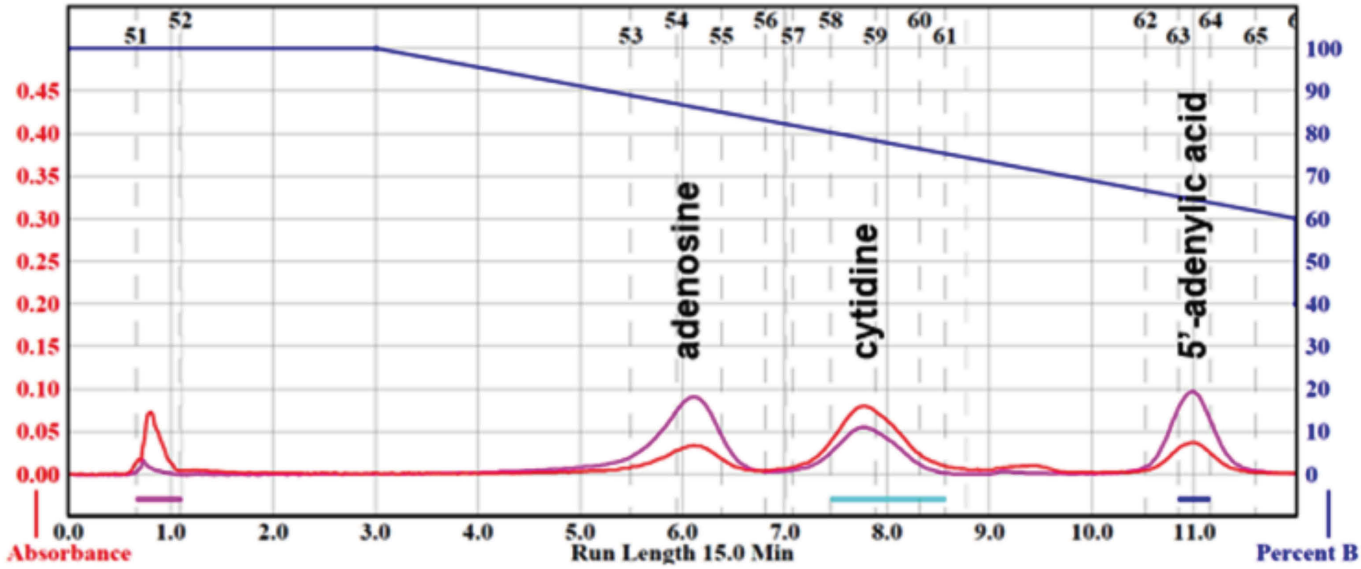

Figure 6. Flash Chromatography Polar Test Mix on 100-60% of B in A for 9 min gradient – Solvent A: Water with 10 mM ammonium formate; Solvent B: 95% ACN in water with 10mM ammonium formate; Teledyne ISCO 12g GOLD Silica (20-40 µm) Redisep, 30 mlmin-1, 220 nm and 254 nm.

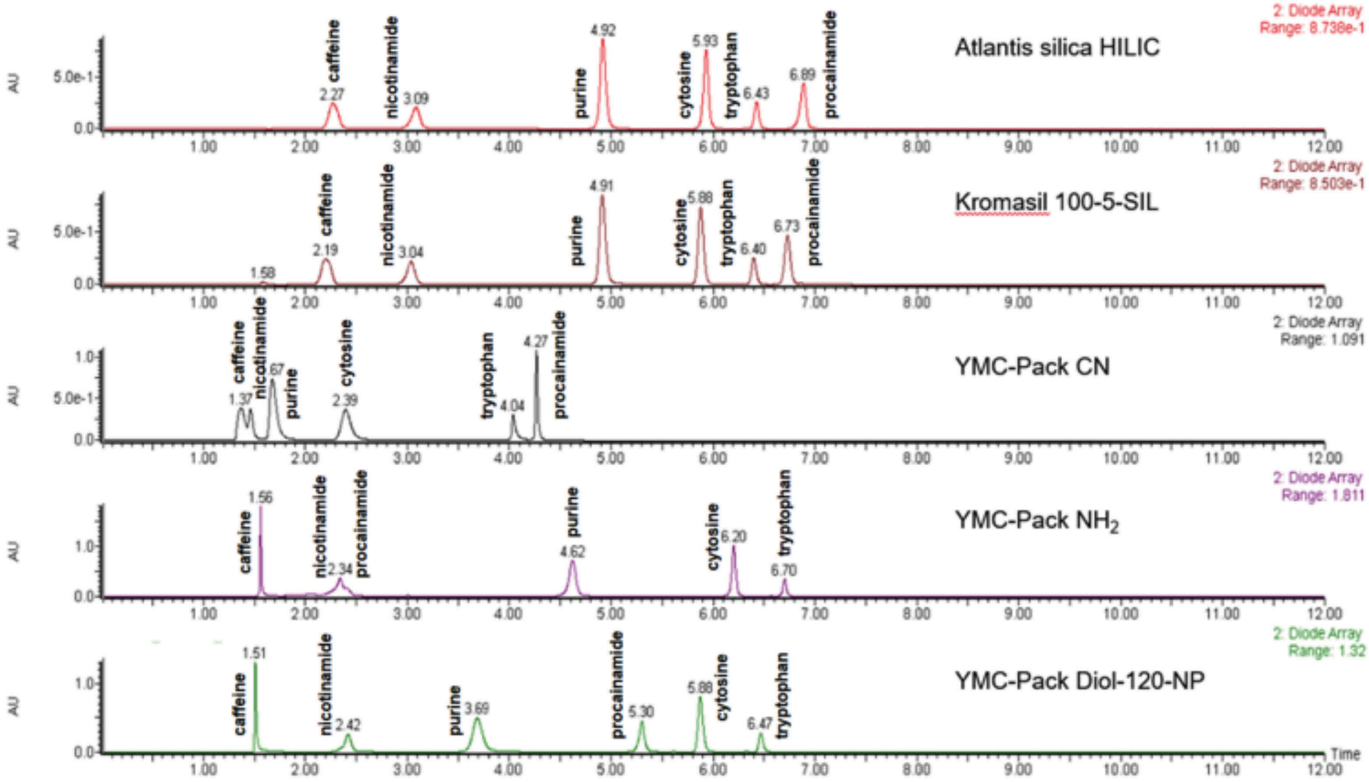

Figure 7. HILIC Analytical on 100-60% of B in A for 6 min gradient – Solvent A: Water with 10 mM ammonium formate; Solvent B: 95% ACN in water with 10mM ammonium formate;

All column dimensions 4.6x100mm, 5µm.

ease of use for medicinal chemists, reduced capital and consumable costs and reduced fraction volumes. The main advantage being that chemists now have the ability to purify extremely polar samples by use of HILIC flash which is readily available at their own fume hood, instead of having to submit samples for HILIC semi-prep or normal phase, SFC separations. HILIC chromatography on flash has been demonstrated to be reproducible. The ability to purify nucleosides and nucleotides is impressive and demonstrates how easy this methodology is to adapt to medicinal chemistry programs where polar compound separations is a bottleneck. Additionally, the use of alternative stationary phases other than bare silica has been discussed for analytical HILIC and these phases are also available in flash chromatography cartridge form.

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