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Enhanced fluidity liquid chromatography for hydrophilic interaction separation of nucleosides

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ABSTRACT

The application of enhanced fluidity liquid (EFL) mobile phases to improving isocratic chromatographic separation of nucleosides in hydrophilic interaction liquid chromatography (HILIC) mode is described. The EFL mobile phase was created by adding carbon dioxide to a methanol/buffer solution. Previous work has shown that EFL mobile phases typically increase the efficiency and the speed of the separation. Herein, an increase in resolution with the addition of carbon dioxide is also observed. This increase in resolution was achieved through increased selectivity and retention with minimal change in separation efficiency. The addition of CO_2 to the mobile phase effectively decreases its polarity, thereby promoting retention in HILIC. Conventional organic solvents of similar nonpolar nature cannot be used to achieve similar results because they are not miscible with methanol and water. The separation of nucleosides with methanol/aqueous buffer/CO_2 mobile phases was also compared to that using acetonitrile/buffer mobile phases. A marked decrease in the necessary separation time was noted for methanol/aqueous buffer/CO_2 mobile phases compared to acetonitrile/buffer mobile phases. There was also an unusual reversal in the elution order of uridine and adenosine when CO_2 was included in the mobile phase.

1. Introduction

Hydrophilic interaction liquid chromatography (HILIC) [1] has found increased use for the separation of highly polar molecules that are often unretained under reversed-phase LC conditions. For example, HILIC is increasingly found to be much more effective for separating polar molecules that are often encountered in biological matrices, especially nonvolatile polar organic molecules such as amino acids [2], peptides [3], organic acids [4], and nucleosides [5]. Since many of these compounds are of prime importance in pharmaceutical applications [6], new means of improving their separation would be highly beneficial and this type of separation could also be beneficial to a range of analyte separations.

Enhanced-fluidity liquid (EFL) mobile phases are liquid mixtures to which high proportions of a liquefied gas have been added and were first developed by our research group in the early 90s [7]. These mixed mobile phase have been used to improve reversed-phase, normal-phase and size exclusion based liquid chromatography [8]. For example, previous studies showed that under reversed-phase conditions, the addition of 30 mol% carbon dioxide doubled the average efficiency and decreased the analysis time by 50% for the sixteen priority pollutant polyaromatic hydrocarbons [9]. The separation of five substituted benzoic acid compounds using methanol/buffer/CO_2 under reversed-phase conditions showed an analysis time decrease of nearly a factor of 10 [10]. Zhao and Olesik also illustrated the importance of buffering in the EFLC separation of tricyclic antidepressants and also noted the effective separation in half the amount of time using EFLC compared to that obtained using methanol/H_2O mobile phases [11]. Other work also illustrated improved efficiency for normal-phase separations by at least a factor of two when adding 50 mol% CO_2 to the mobile phase by the use of EFL mobile phases [12].

These valuable improvements in chromatographic performance are achieved as a result of the substantial increase in the solute's diffusion coefficient as well as the diminution of solvent viscosity with the increased proportion of carbon dioxide. Furthermore, these improvements are also typically gained without substantial loss of polarity from the mobile phase [13].

HILIC employs a hydrophilic stationary phase with a polar organic mobile phase with an aqueous cosolvent. While the mechanism of HILIC is not completely elucidated, it is currently postulated to involve the partitioning of a solute between the mixed mobile phase and a water-rich layer on the surface of the stationary phase. The resulting retention in HILIC is increased with increased solute polarity which is opposite to the retention order found in reversed-phase chromatography [14]. Methanol/H_2O mobile phases are typically not used in HILIC because of their inherent high viscosity and lower efficiency compared to acetonitrile/H_2O mixtures. However, methanol/H_2O mixtures are markedly more miscible with CO_2 than acetonitrile/H_2O mixtures [15] and were therefore used

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for these initial studies. Separations using acetonitrile/H_2O based mobile phases were investigated to characterize mobile phase selectivity differences.

The application of EFLC for the separation of the RNA nucleosides, adenosine, uridine, cytidine, and guanosine, is the focus of this study. These compounds are of interest to pharmaceutical chemistry since nucleoside levels can be used in metabolomics as disease markers [16], and in food chemistry for product analysis and identification [17]. Since such analyses are often done in complex biological matrices, efficient, selective separation techniques are required for analysis. Current techniques for analyzing nucleosides are capillary electrophoresis [16], gradient reversed-phase HPLC [17] and HILIC [5,18–20], but all these techniques require extended analysis times. Finally, as mentioned earlier, many HILIC techniques rely upon the use of acetonitrile-based mobile phases, which can be an issue from a cost and environmental prospective. Therefore alternative mobile phases would be beneficial.

2. Experimental

2.1. HPLC setup

The HPLC system was assembled from commercially available components. An ISCO 260 D syringe LC pump (Teledyne Isco, Inc. Lincoln, Nebraska, USA) was connected to a 6-port, 5000 psi injector equipped with a 2 μL injection loop (VICI Valco Instruments, Houston, TX, USA). The injector was connected to the HILIC column: a 4.6 mm × 150 mm Tosoh Amide-80 column packed with 3 μm particles (TOSOH Bioscience, King of Prussia, PA, USA). The outlet of the column was then connected to a Jasco UV-2075 UV–vis detector with a high-pressure 4 μL flowcell (Jasco Inc., Easton, MD, USA). The wavelength of the detector was set at 262 nm. The outlet of the flow cell was attached to a 30 μm internal diameter fused silica capillary (Polymicro Technologies, Inc., Phoenix, AZ, USA) of an adjusted length to control the mobile phase flow rate and maintain the system pressure.

2.2. Mobile phase preparation

The liquid mobile phase was either 90/10 (v/v) methanol/buffer (mole ratio methanol/H_2O = 4) or acetonitrile/buffer of varying proportions. Spectroscopy grade methanol (Fisher Scientific, Pittsburgh, PA, USA) and acetonitrile (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) were used as received, and the deionized water was purified on a Barnstead Nanopure Infinity system (Thermo Scientific, Asheville, NC, USA). A sodium acetate (Jenneile Enterprises, Cincinnati, OH, USA)/acetic acid (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) buffer with an ionic strength of 20 mM and aqueous pH of 4.4 was used as the water component of the mobile phase. The buffer was stored at 4 °C when not in use to enhance its stability. The buffer was filtered before making the mobile phase to ensure that residual particulates did not enter the chromatographic system. The final organic/buffer solution was degassed for 20 min before use using a Branson 2210 ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury, CT, USA). Supercritical fluid extraction grade carbon dioxide (Praxair, Inc., Danbury, CT, USA) was added to the LC mobile phase in a similar fashion as previously described [9]. Final mole fractions of CO_2 of 0.11 or 0.20 pressurized at 80 bar were studied.

2.3. Sample preparation

The samples were prepared using 99% uridine, 99% adenosine, 99% cytidine, and 98% guanosine (Sigma–Aldrich Corporation, St. Louis, MO, USA) in the organic solvent/buffer mixture at a concentration of 10 μg/mL. Toluene elution was used to mark the dead time. All samples were filtered through a 0.45 μm filter before use and kept at −15 °C while not in use.

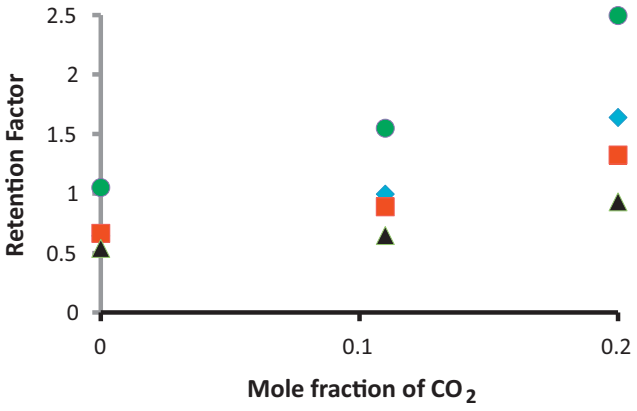

Fig. 1. Variation of retention factor as a function of added CO_2. Conditions: composed of 90/10 methanol/20 mM acetate buffer mobile phase, flow rate = 0.4 mL/min; n = 3. Adenosine (▲), uridine (■), cytidine (♦), and guanosine (●).

2.4. Data analysis

Chromatographic data were recorded using EZ Chrom Version 6.7 (Scientific Software Inc., Pleasanton, CA, USA). Data analysis was performed using PeakFit Version 4 (SPSS Inc., Chicago, IL, USA). All peak values were used as displayed from PeakFit. Efficiency calculations were performed using moment analysis in PeakFit.

2.5. Method

All parameters and equipment remained unchanged for the different mobile phase compositions except for the addition of a flow restrictor which was used at the end of the system when using the EFL mobile phases. All efficiency and retention data were taken in triplicate to ensure the reproducibility of the measurements.

3. Results and discussion

3.1. Retention

Fig. 1 shows that the retention of all the compounds increased as a function of increasing amounts of added CO_2 in a 90/10 methanol/aqueous buffer which includes 20 mM acetate mobile phase. Between 0 and 0.20 mole fraction of CO_2 adenosine showed a 72% increase in retention with a final k of 0.93; uridine showed a 66% increase in retention with a final k of 1.33; cytidine showed a 97% increase in retention with a final k of 1.64 and guanosine showed a 138% increase in retention with a final k of 2.5. The general trend was that analytes with larger retention factors had greater retention factor increases with the addition of carbon dioxide than the less retained analytes. This behavior is similar to that found for nucleosides and nucleotides with the addition of >85% acetonitrile to an acetonitrile/buffer mobile phase [20,21].

In HILIC, decreased mobile phase polarity is expected to increase the strength of the interaction of the analyte with the stationary phase as measured through an increased retention factor. Carbon dioxide is known to exhibit nonpolar solvent strength and predicted to be intermediate between that of hexane and carbon tetrachloride [22]. These common liquids of solvent strength similar to that of CO_2 are not miscible with H_2O or methanol. The mutual miscibility of the methanol/buffer solution with CO_2 provides the capability of changing the analyte retention substantially with proportions of added CO_2. However, it is interesting

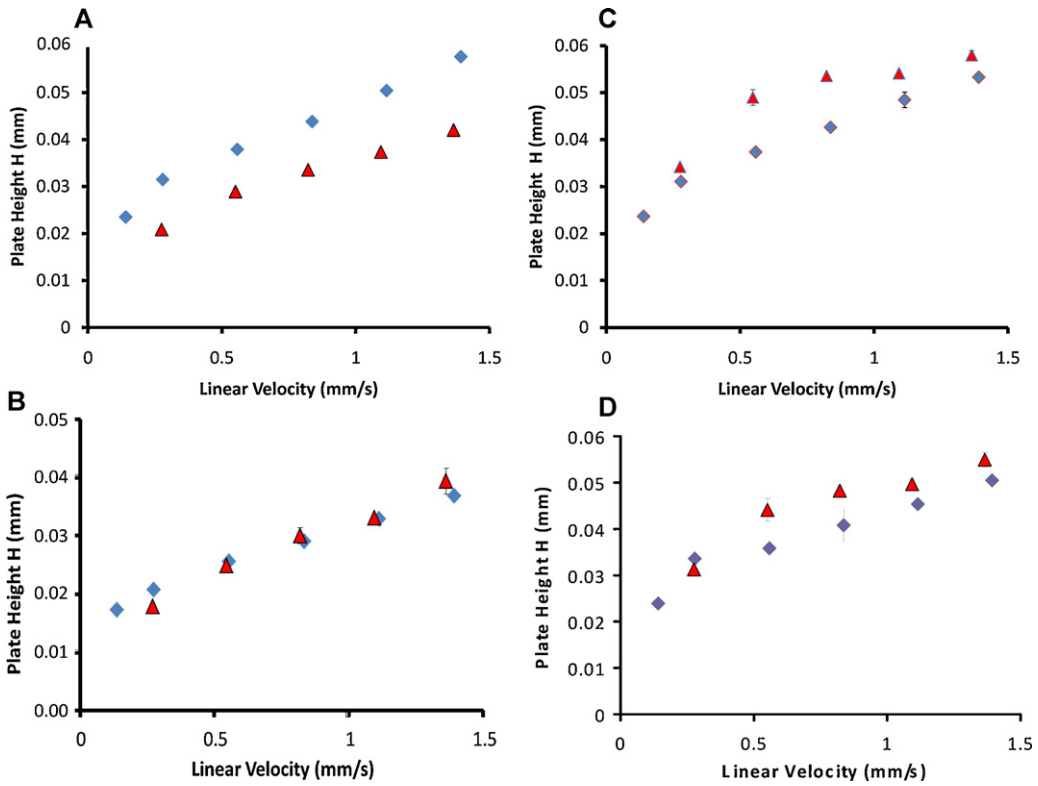

Fig. 2. (A) Effect of mobile phase composition on separation efficiency of adenosine (10 μg/mL) as a function of linear velocity. Conditions: 90/10 methanol/20 mM acetate buffer mobile phase and 0 mole fraction CO_2 (♦), 0.11 mole fraction CO_2 (▲). $n = 3$ for all points, error bars at 95% confidence intervals are within the symbol. (B) Effect of mobile phase composition on separation efficiency of uridine (10 μg/mL) as a function of linear velocity. Conditions: 90/10 methanol/20 mM acetate buffer mobile phase and 0 mole fraction CO_2 (♦), 0.11 mole fraction CO_2 (▲). $n = 3$ for all points, error bars at 95% confidence intervals are within the symbol. (C) Effect of mobile phase composition on separation efficiency of cytidine (10 μg/mL) as a function of linear velocity. Conditions: 90/10 methanol/20 mM acetate buffer mobile phase and 0 mole fraction CO_2 (♦), 0.11 mole fraction CO_2 (▲). $n = 3$ for all points, error bars at 95% confidence intervals are within the symbol. (D) Effect of mobile phase composition on separation efficiency of guanosine (10 μg/mL) as a function of linear velocity. Conditions: 90/10 methanol/20 mM acetate buffer mobile phase and 0 mole fraction CO_2 (♦), 0.11 mole fraction CO_2 (▲). $n = 3$ for all points, error bars at 95% confidence intervals are within the symbol.

that the addition of CO_2 in these proportions would result in a major change in solvent strength. The solvent strength change for a 2.3 mole ratio methanol/H_2O mixture was previously measured with the hydrogen-bond acidity and dipolarity/polarizability, as measured by Kamlet–Taft solvatochromic parameters, α and π, decreased by only 10% from the addition of up to 50 mol% CO_2 and the hydrogen-bond basicity of the mixture, as measured by the Kamlet–Taft β parameter, actually increased with the addition of 50 mol% CO_2 [23]. Further studies on the solvent strength of this methanol/H_2O/CO_2 mixture would be valuable to provide a deeper understanding to the change in observed retention of the nucleosides.

3.2. Plate height

The variation in plate height, H, was studied for flow rates ranging from 0.2 to 1.0 mL/min at both 0 and 0.11 mole fraction CO_2 (Fig. 2A–D). In liquid chromatography, band dispersion caused by

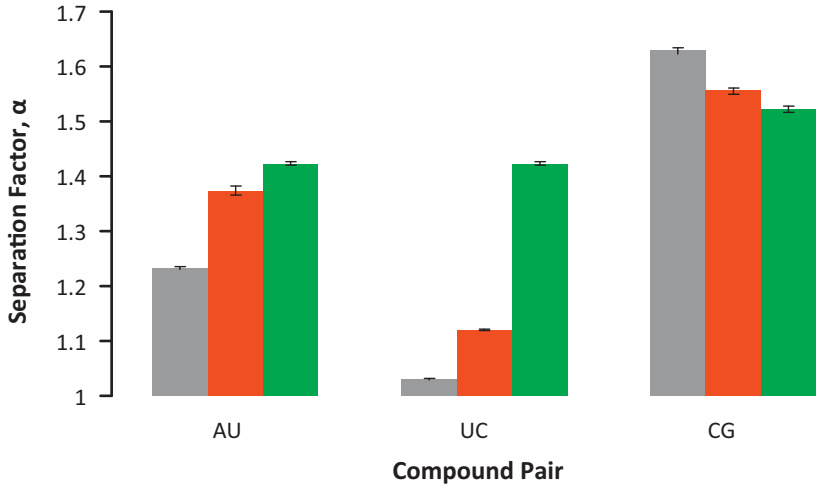

Fig. 3. Variation of separation factor (α) as a function of mobile phase composition. Conditions: flow rate = 0.4 mL/min, Mobile phase composed of 90/10 methanol/20 mM acetate buffer, 0 mole fraction CO_2 ( ), 0.11 mole fraction CO_2 ( ), 0.2 mole fraction CO_2 ( ) ($n = 3$).

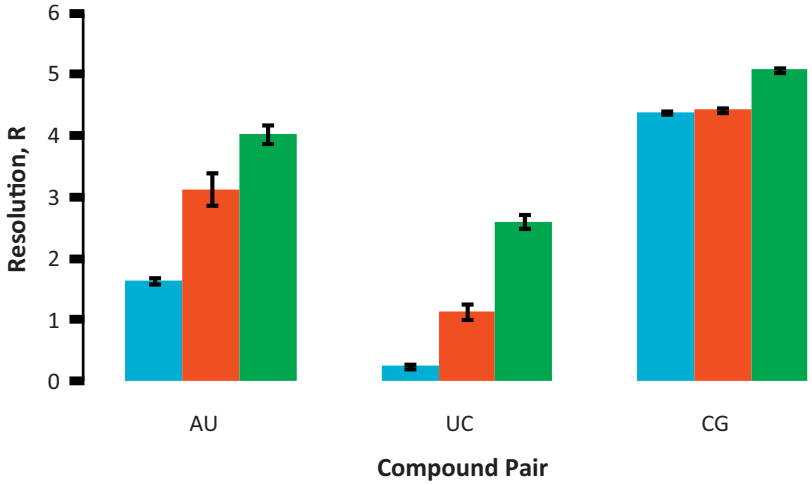

Fig. 4. Variation of resolution as a function of mobile phase composition. Conditions: flow rate = 0.4 mL/min, Mobile phase composed of 90/10 methanol/20 mM acetate buffer, 0 mole fraction CO_2 (■), 0.11 mole fraction CO_2 (■), 0.2 mole fraction CO_2 (■). ($n = 3$).

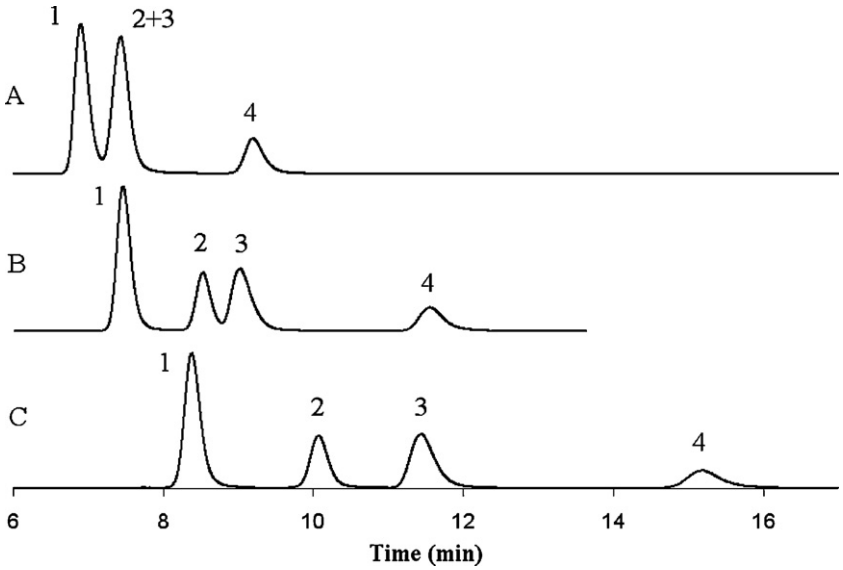

Fig. 5. Separation of selected nucleotides with different mobile phase compositions. Conditions: flow rate = 0.4 mL/min, Mobile phase composed of 90/10 methanol/20 mM acetate buffer: (A) LC, (B) 0.11 mole fraction, (C) 0.20 mole fraction. Analytes: adenosine (1), uridine (2), cytidine (3), and guanosine (4).

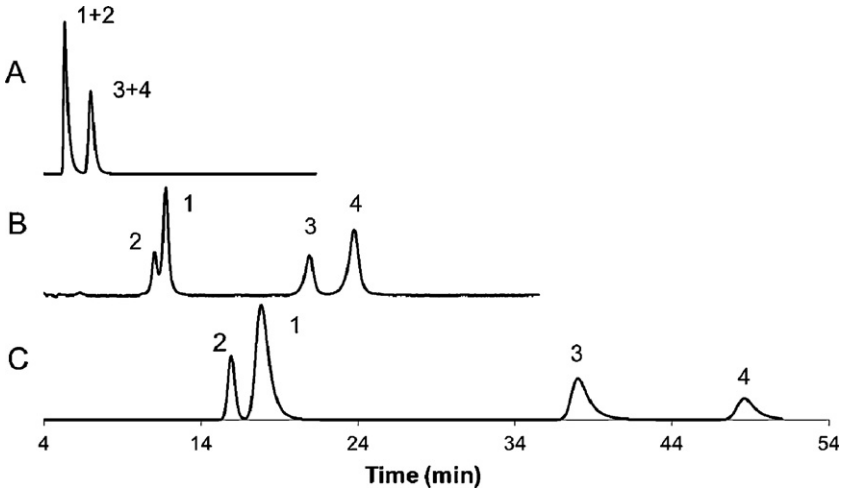

Fig. 6. Separation of selected nucleotides with different mobile phase compositions. Conditions: flow rate is 0.6 mL/min, aqueous phase composed of 20 mM acetate buffer, the % of acetonitrile is varied: (A) 70% acetonitrile, (B) 85% acetonitrile, (C) 90% acetonitrile. Analytes: adenosine (1), uridine (2), cytidine (3), and guanosine (4).

the laminar flow in the column is a significant contribution to the overall measured dispersion. This contribution to band dispersion is inversely proportional to the diffusion coefficient of the mobile phase and directly proportional to a function involving the retention factor of the analyte. The addition of CO_2 greatly increases the diffusivity of the mixed mobile phase, which should lower band dispersion. However, data highlighted in the previous section show that the retention of the nucleosides also increased. The plate height of the different compounds was affected differently by the addition of CO_2 depending on the change in retention factor. Indeed, the plate height decreased with the addition of CO_2 for the least retained nucleoside: adenosine. The next least retained molecule, uridine, had negligible change in plate height under EFL conditions as compared with traditional LC conditions. For the more retained analytes, cytidine and guanosine at low mobile phase velocities, the plate height increased for the EFL conditions compared to that from the methanol/H_2O mixed solvent. However for cytidine and guanosine at mobile phase velocities above 1 mm/s, the plate heights are approximately the same using either the EFL mixture or methanol/H_2O mixture. Therefore, when working at higher flow rates, the EFLC mobile phases have similar or higher efficiencies than the corresponding LC mobile phases. No improvement in efficiency would be gained at the higher flow rates when comparing EFLC to LC with commonly used solvents.

3.3. Separation factor

The selectivity factor α was calculated for each pair of adjacent peaks. Fig. 3 shows the changes in α as a function of increasing CO_2 proportions and highlights the fact that the retention of each compound increases at differing rate. When comparing α for 0 and 0.20 mole fraction CO_2, the adenosine–uridine peak pair and the uridine–cytidine peak pair showed an increase from 1.23 to 1.42 and 1.03 to 1.24, respectively. However, the cytidine–guanosine peak pair showed a decrease in α from 1.73 to 1.52. This decrease in α was not detrimental to the separation since there was still ample selectivity between cytidine and guanosine under both EFL conditions.

3.4. Resolution

Resolution was greatly improved under enhanced-fluidity conditions as illustrated in Fig. 4. The resolution between each pair of adjacent peaks increased as a function of increasing fraction of carbon dioxide in the mobile phase. For the adenosine–uridine pair, resolution increased by over 180% between 0 and 0.20 mole fraction CO_2, giving a final resolution of over 3. For the uridine–cytidine pair, originally co-eluted under LC conditions, a resolution of nearly 2 was achieved using 0.20 mole fraction CO_2. For the cytidine–guanosine pair, resolution increased by over 22% between 0 and 0.20 mole fraction CO_2, giving a final resolution of over 3.75. The increase in resolution is clearly noted by comparing the chromatograms (Fig. 5) which clearly shows baseline resolution for both 0.11 and 0.20 mole fraction CO_2. The significant change in selectivity for uridine and cytidine impacts the improved resolution significantly with the optimal addition of CO_2 clearly between 0.11 and 0.20 mole fraction CO_2 because unnecessary space between the resolved chromatographic bands is present in the chromatogram using 0.20 mole fraction CO_2. Fig. 5 also clearly illustrates that the increase in resolution with the addition of CO_2 did come at the cost of increased analysis time. While doubt could exist on the possibility of separating effectively compounds such as these highly polar nucleosides with a CO_2 modified mobile phase, the chromatograms clearly illustrate effective separations with no problems with lack of analytes' solubility. At first look, this does seem contrary to the known miscibility of the nucleosides in water and alcohols. For example, adenosine and cytidine and uridine are all soluble in water and guanosine is slightly soluble in water [24]. Also, adenosine is insoluble in alcohols while cytidine and uridine are only slightly soluble in alcohols, [24,25] and no data on the solubility of guanosine in alcohols were readily obtained. However, as early as 1954, Francis noted that the addition of CO_2 in moderate concentrations had a strong homogenizing action upon liquids [26]. We have also previously noted similar increased solubility of high molecular weight polymers and polar analytes through the addition of CO_2 to liquid mixtures [27].

3.5. Comparison with acetonitrile/aqueous buffer mobile phases

The final question to address in this study was: Is this separation truly better than that obtained with those possible using acetonitrile (ACN)/aqueous buffer mixed mobiles phases which as mentioned before are the commonly used standard mobile phases for HILIC? Fig. 6 shows the chromatograms of the nucleosides over a range of solvent strengths for ACN/aqueous buffer mixtures (70/30–90/10 acetonitrile/aqueous buffer) under isocratic conditions with the same aqueous buffer. The 90/10 acetonitrile/buffer phase is the only acetonitrile/aqueous buffer mobile phase that provided baseline resolution of adenosine and uridine. However, the time required for this separation was approximate 49 min at the given flow rate which is much slower than that found less than 16 min using the EFLC mobile phase. Also, the order of elution in the acetonitrile/aqueous buffer is the same (U, A, C, G) as observed in other HILIC separations using a diverse array of stationary phases, such as diol, amine and amide functionalized supports [5,18–20]. However, the methanol/H_2O/CO_2 mobile phase reverses the order of elution of U and A, with A eluting before U. The change of elution order with the addition of CO_2 without changing the stationary phase may be valuable for other nucleobase separations.

Comparing the methanol based EFL results to the acetonitrile based LC results, it can be seen that the EFL gave superior selectivity characteristics in isocratic mode. The large differences in final selectivity between the various peak pairs indicate that using an acetonitrile-based LC phase would require the use of a gradient to allow the separation in a reasonable amount of time. Thus an EFL is a superior choice for isocratic HILIC separation work in this case. However, it should be noted that others have published nucleoside separations with comparable total separation time and selectivity using other stationary phases [28].

4. Conclusions

Baseline separation of the RNA nucleosides was achieved using methanol/aqueous buffer/CO_2 EFLC. This isocratic separation was markedly faster than that obtained using comparable isocratic conditions using acetonitrile/aqueous buffer mobile phases. The combination of these two attributes shows this method to be a fast, straightforward method of doing biological separations of nucleosides in the HILIC mode. Addition of CO_2 is a unique means of decreasing the polarity of the mobile phase and hence increasing retention of polar solute. Lower mobile polarity is possible with the addition of CO_2 than through addition of a nonpolar conventional solvent because of poor miscibility of the conventional a nonpolar solvent (hexane) with polar solvents (alcohols/water).

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