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Impact of methanol and acetonitrile on separations based on π–π interactions with a reversed-phase phenyl column

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Abstract

Studies were performed to investigate the roles of methanol and acetonitrile on the retention mechanism of an active pharmaceutical ingredient (API) and related compounds with a reversed phase phenyl column. Different retention orders were observed depending upon whether acetonitrile or methanol was used as the organic modifier. We propose that acetonitrile impedes the selective π–π interactions between the analyte molecules and the phenyl groups in the stationary phase. Further study with 1-naphthoic acid and 1-naphthol as test compounds in the HPLC separation provides additional support for the influence of acetonitrile on π–π interactions between analyte molecules and a phenyl stationary phase. This study suggests that methanol be used as the preferred organic modifier with phenyl columns to achieve selectivity based upon π–π interactions.
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Keywords: Phenyl columns; Secondary interactions; π–π interactions

1. Introduction

Ever since the introduction of the silica based reversed-phase HPLC columns in the 1970s, the stationary phases within these columns have primarily been some form of silica support modified with various aliphatic functional groups (e.g. $-CH_3$, $-C_4H_9$, $-C_8H_{17}$ and $-C_{18}H_{37}$). Other functional groups, such as phenyl ($-C_6H_5$), cyano ($-(CH_2)_3CN$), amino ($-NH_2$), and hydroxyl (–OH) have also been used to a lesser extent due to their significantly different selectivity mechanism and for their potential to improve peak shape with some classes of compounds [1]. HPLC columns with stationary phases modified with phenyl ($-C_6H_5$), cyano ($-(CH_2)_3CN$), and hydroxyl (–OH) groups are readily available from most major column suppliers. These columns introduce additional molecular interactions such as hydrogen bonding [2], π–π interaction [3–4] and ionic interaction [5–6] in addition to hydrophobic interaction, which offer additional retention mechanisms to obtain the desired chromatographic characteristics.

The π–π interaction is a type of electron donor-electron acceptor interaction, originating from π-electron systems in two unsaturated functional groups through either intermolecular or intramolecular interactions [7]. In a chromatographic system, these interactions can occur between π-electrons of the stationary phase and the analyte species [8–9]. An interaction between π-electron containing compounds such as the phenyl stationary phase, is favored when one compound is electron-rich (i.e. soft Lewis base) and one is electron-poor (i.e. soft Lewis acid) [10,11] such as the analyte. In light of the significance of π–π interactions as a separation force in HPLC, stationary phases containing various aromatic groups have been prepared, such as phenyl [12], pyrenyl [13], fluorenyl [8] and anthracenyl groups [14].

In this work, the authors investigated the unique selectivity power of π–π interactions occurring in a challenging chromatographic separation of an active pharmaceutical ingredient (API) and related impurities. A retention mechanism has been proposed, based upon π–π interactions occurring between the different analyte molecules and the phenyl-bonded phase. Experiments conducted with the test compounds, 1-naphthoic acid and 1-naphthol gave results consistent with the proposed retention mechanism. This proposed explanation may assist in

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organic modifier selection with phenyl columns and lead to further investigations into the retention mechanisms of different organic solvents and their impact on secondary interactions.

2. Experimental

2.1. Reagents

Organic solvents methanol and acetonitrile were HPLC grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA). The deionized water used in the preparation of standard solutions and eluents was obtained from a MILLI-Q water system (Millipore, Bedford, MA, USA). 1-Naphthoic acid, 1-naphthol and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich.

2.2. Apparatus

Chromatography was performed on a Waters (Milford, MA, USA) Alliance 2695 system with a 996 PDA detector. Chromatographic data were obtained and processed with Waters Millenium^{32} software. Xterra C18 columns (50 mm × 4.6 mm, particle size 3.5 μm) (150 mm × 3 mm, particle size 3.5 μm) were purchased from Waters. A Keystone Phenyl Hypersil^{R}-2 column (150 mm × 4.6 mm, particle size 5 μm) was obtained from Thermo Electron Corporation (Waltham, MA, USA).

3. Results and discussion

A challenging chromatographic separation problem was encountered while developing analytical methods for the active pharmaceutical ingredient (A6) and two related impurities (all three are shown in Fig. 1). For accurate quantitation, an HPLC method was needed that could fully resolve all three compounds in one chromatographic run. The API molecule A6 has two chiral (R,R) centers. One of the related impurities is a diastereomer of A6 with a (R,S) configuration and the other (A5) is an intermediate of A6 which also has an (R,S) configuration. The only structural difference between A5 and A6(R,S) is an acetylthio (CH_3COS-) group and a bromide group (Fig. 1). CH_3COS- group contains a carbonyl group which may interact with other unsaturated functional groups through π–π interactions.

Separation of A5 and the A6 (R,S) was not achieved with an ODS column (see Fig. 2) when either methanol or acetonitrile was used as the mobile phase. However these two compounds were separated with a phenyl stationary phase when methanol was used as the organic modifier as shown in Fig. 3. Interestingly, when acetonitrile was used with the same phenyl column, no separation was achieved.

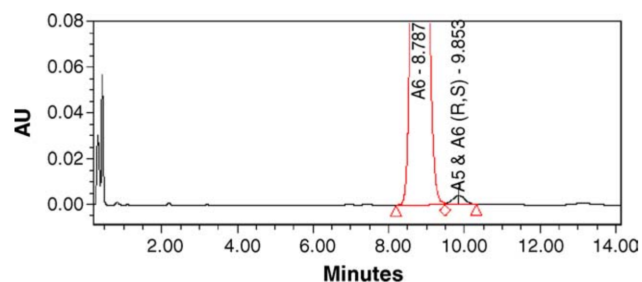

Fig. 2. Chromatogram obtained of a mixture solution containing 1 mg/ml A6, 0.02 mg/ml A5 and A6(R,S) each. Column: Waters Xterra C18, 50 mm × 4.6 mm, particle size 3.5 μm. Mobile phase: water/acetonitrile 68/32, flow rate 1.5 ml/min.

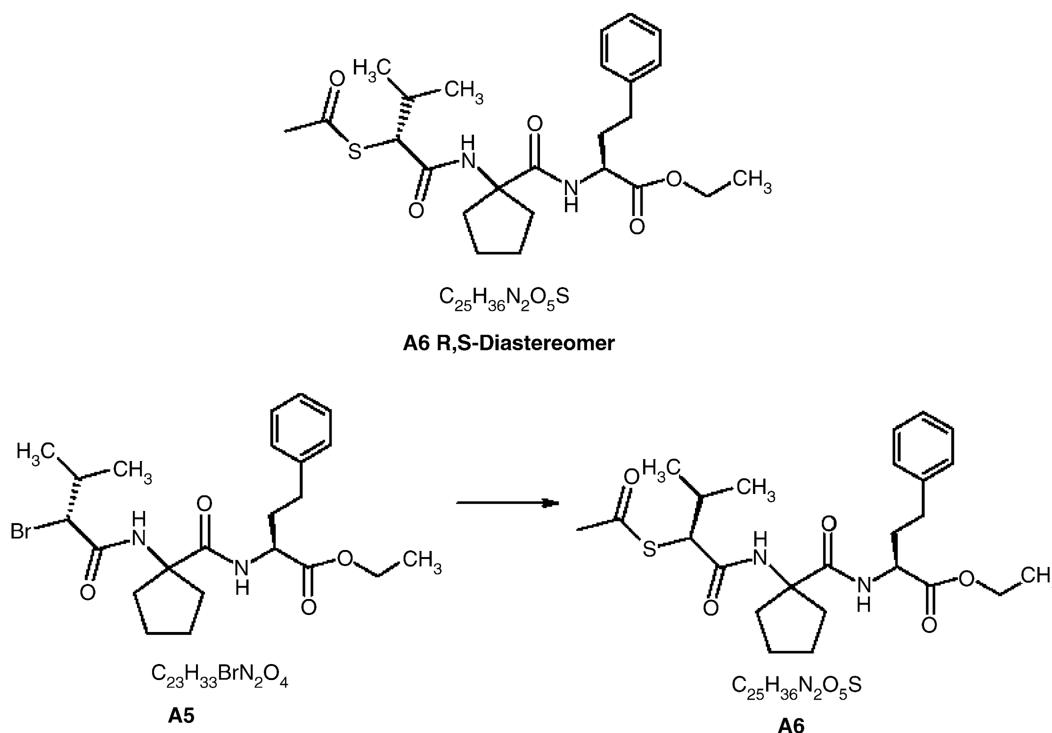

Fig. 1. Structures of A5, A6 and A6 R,S-diastereomer.

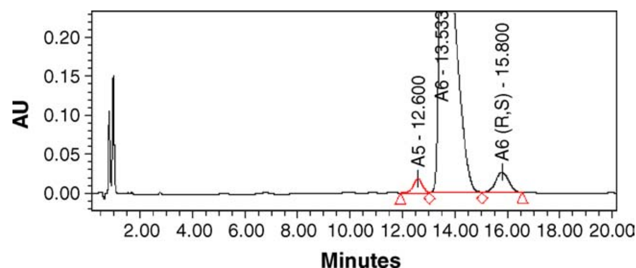

Fig. 3. Chromatogram obtained of a mixture solution containing 1 mg/ml A6, 0.02 mg/ml A5 and A6(R,S) each. Column: Keystone Hypersil-phenyl 150 mm × 4.6 mm, particle size 5 μm. Mobile phase: water/methanol 40/60, flow rate 1.5 ml/min.

To further investigate the separation of A5 and A6 (R,S) with a phenyl column, tertiary mobile phases of six different concentrations of acetonitrile, methanol and water were used (see Fig. 4). These chromatograms demonstrate a clear trend towards increasing resolution of A5 and A6 (R,S) as the ratio of methanol to acetonitrile increased. These results raised questions regarding the roles of both acetonitrile and methanol in the separation mechanism with the phenyl column. Earlier experiments with ODS columns indicated that A5 and A6 (R,S) have similar polarities and separation was not accomplished using either methanol or acetonitrile as organic modifier. The separation of A5 and A6 (R,S), achieved with the phenyl column, may be attributed to π–π interactions of the analytes with the phenyl stationary phase. Furthermore, as the relative acetonitrile concentration is increased these results suggest that an increasing suppression of these π–π interactions occurs. More specifically, this suppression when high concentrations of acetonitrile are present is likely due to interactions between acetonitrile, which has an unsaturated triple bond, C≡N, and the phenyl stationary phase.

Our results and proposed mechanisms are consistent with observations and preliminary explanations made by Martin, etc. in their studies into the separation of homogeneous triglycerides using aromatic stationary phases with both acetonitrile and methanol [15].

In a more dramatic example of this suppression of π–π interactions, the test compounds 1-naphthoic acid and 1-naphthol (Fig. 5) were studied. This compound pair was chosen because of their similar ODS column retention times and that 1-naphthoic acid has an additional carbonyl group in its molecular structure (similar to the previous example). When an ODS column was used with either acetonitrile or methanol as the organic modifier, a very similar separation was achieved with the 1-naphthoic acid eluting first, as shown in Fig. 6a and b. The elution order indicates that the 1-naphthoic acid is more polar (i.e. less hydrophobic) than the 1-naphthol.

When the chromatography column was switched to a phenyl column using methanol as the organic modifier, the elution order was reversed, as shown in Fig. 6c. This stronger retention of 1-naphthoic acid may be attributed to the additional π–π interaction provided by the carbonyl group. In all these separation experiments with 1-naphthoic acid and 1-naphthol, 0.1% of trifluoroacetic acid was added to the mobile phases to keep both

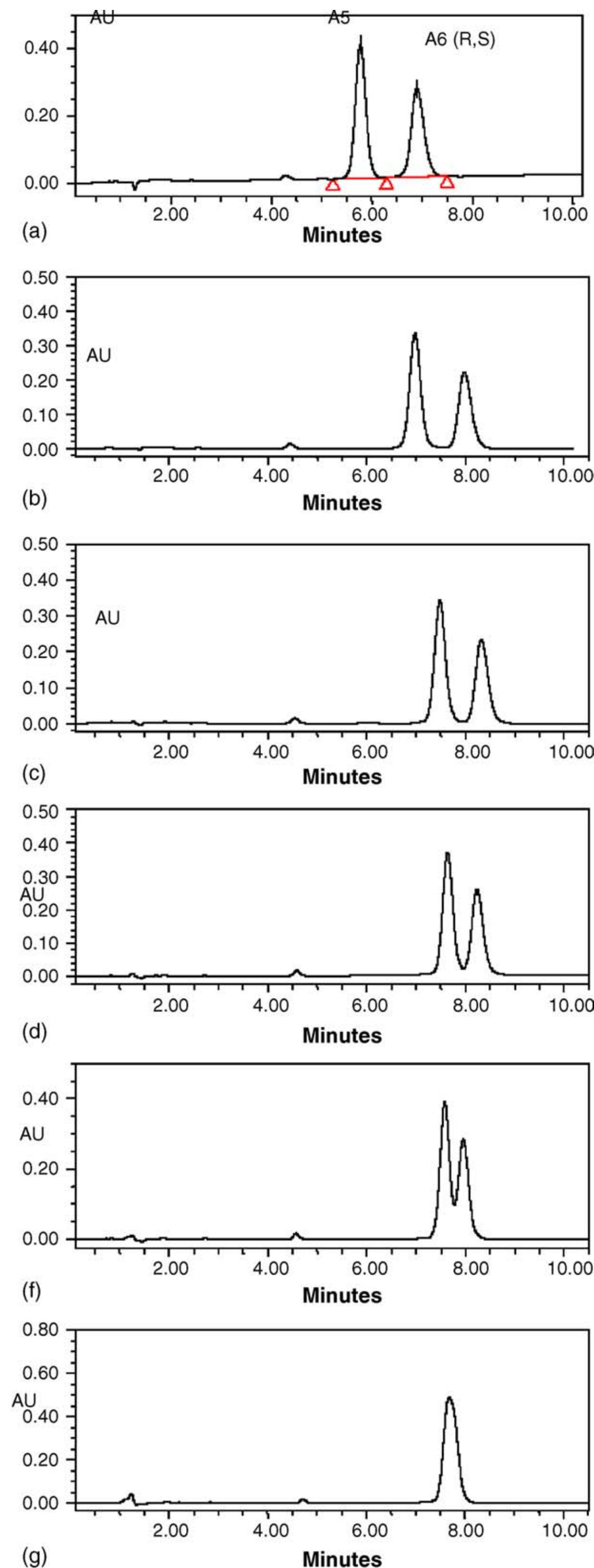

Fig. 4. Chromatograms of a mixture solution containing 0.5 mg/ml A6 (R,S) and, 0.5 mg/ml A5. Column: Keystone Hypersil-phenyl 150 mm × 4.6 mm, particle size 5 μm. Mobile phase A: water/methanol 42/58; mobile phase B: water/acetonitrile 58/42, flow rate 1.5 ml/min. Wavelength 210 nm. Chromatogram a, A/B: 100/0; chromatogram b, A/B: 70/30; chromatogram c, A/B: 50/50; chromatogram d, A/B: 30/70; chromatogram f, A/B: 15/85; chromatogram g, A/B: 0/100.

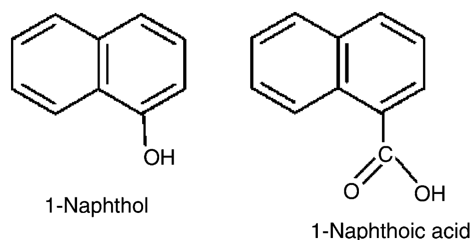

Fig. 5. Molecular structures of 1-naphthol and 1-naphthoic acid.

compounds fully protonated. (The pK_a's of 1-naphthoic acid, 1-naphthol, and trifluoroacetic acid are: 3.7, 9.34, −0.2, respectively [16].)

Interestingly, the elution order reversal seen on the phenyl column can be suppressed by switching to acetonitrile as the organic modifier. For example, if 12% acetonitrile is used 1-naphthol still elutes before 1-naphthoic acid but with somewhat reduced separation, as shown in Fig. 6d. When the acetonitrile concentration is further increased to 18%, the retention times of both compounds are reduced to varying degrees such that they coelute, as shown in Fig. 6e. Lastly, when 30% acetonitrile was added to the mobile phase, the retention times continued to shift earlier with full separation again achieved but this time with 1-naphthoic acid eluting before 1-naphthol, as shown in Fig. 6f. This elution pattern now mimics that seen on the ODS columns. Fig. 7 further demonstrates the relationship between capacity factors of the two test compounds and content of methanol or acetonitrile in mobile phase.

This retention behavior, as in the first example, can be explained by assuming that two separation mechanisms are affecting the chromatography: hydrophobic interactions and π–π interactions. When a phenyl column is used, at an acetonitrile concentration greater than 18%, π–π interactions between the compounds and the stationary phase are significantly weakened by the acetonitrile molecules, thus the retention order will be primarily determined by the hydrophobic interactions. When the acetonitrile concentration is reduced in the mobile phase, π–π interactions between the 1-naphthoic acid and the phenyl stationary phase start to play a more important role. In this specific case when the acetonitrile concentration is 18%, the hydrophobic interaction exactly balanced the π–π interactions and the two compounds coelute. As the acetonitrile concentration is further lowered, the π–π interactions between the 1-naphthoic acid and the phenyl stationary phase become relatively stronger.

Although more data with other aromatic group containing stationary phases and other test samples may be needed to

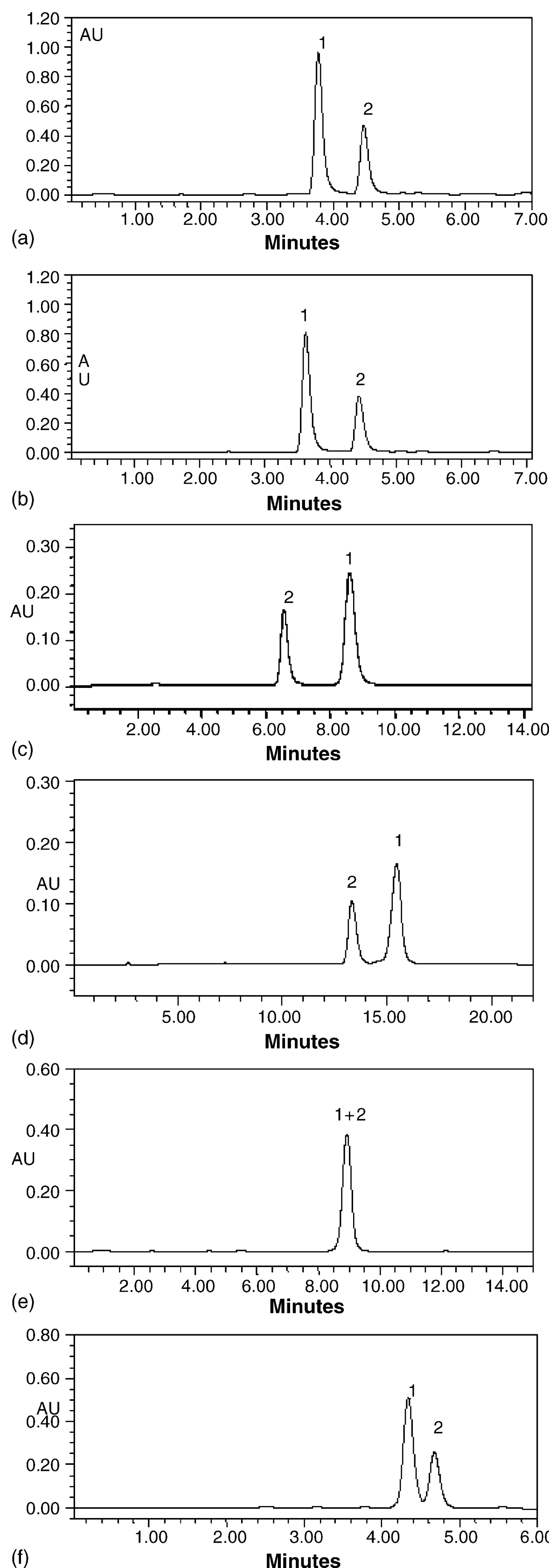

Fig. 6. chromatograms of a mixture of 0.5 mg/ml each of l-naphthoic acid (peak 1) and l-naphthol (peak 2), chromatograms a and b, Xterra C18 column (150 mm × 3 mm, 3.5 μm); chromatograms c–f, Keystone Hypersil-phenyl column (150 mm × 4.6 mm, 5 μm). chromatographic conditions: (a) water/acetonitrile: 50/50, 0.1% TFA, 0.5 ml/min; (b) water/MeOH: 50/50, 0.1% TFA, 0.5 ml/min; (c) water/MeOH: 70/30, 0.1% TFA, 0.8 ml/min; (d) Water/Acetonitrile: 88/12, 0.1% TFA, 0.8 ml/min; (e) Water/Acetonitrile: 82/18, 0.1% TFA, 0.8 ml/min; (f) Water/Acetonitrile: 70/30, 0.1% TFA, 0.8 ml/min. Detection wevlength: 250 nm.

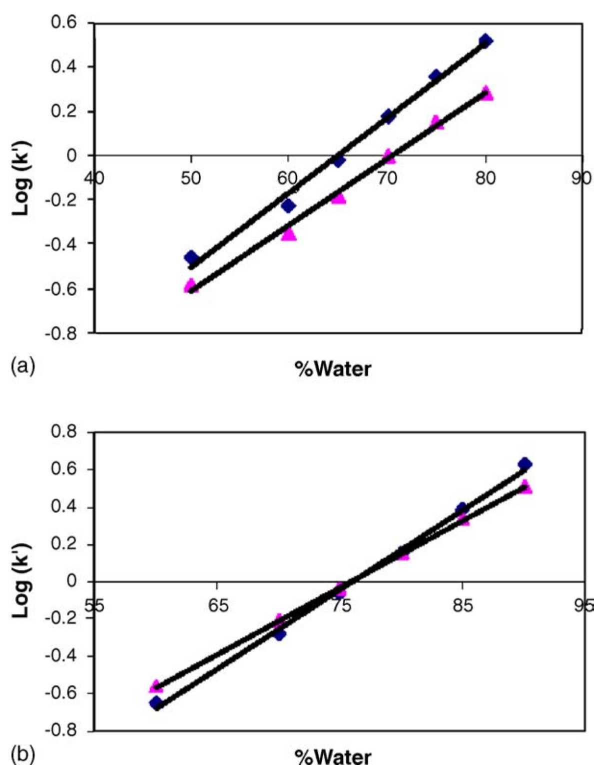

Fig. 7. Log (k') vs. %water for test mixtures of 1-naphthoic acid (♦) and 1-naphthol (▲) with Keystone Hypersil-phenyl column (150 mm × 4.6 mm, 5 μm). Organic modifier: (a) methanol; (b) acetonitrile.

draw a general conclusion about the influence of acetonitrile on phenyl stationary phase, this work provides a preliminary rationale on how acetonitrile alters the separation and selectivity compared to methanol with a phenyl based column. It must be recognized that other factors that influence separation, such as hydrogen bonding, should not be ignored especially when there are substantial hydrogen bonding interactions between analytes and mobile phases. Despite that interpretations based on other separation factors are possible, interpretation with π–π interaction seems to be consistent with the experimental results in this study. Literature search indicates that little attempt has been made to explain the different separation patterns obtained with acetonitrile and methanol using phenyl columns. Although it was found that acetonitrile may affect π–π interaction with a phenyl column in various ways [15,17], no correlation was made to explain the retention time changes as a function of the organic modifier used. In a recent study [18], Marchand tested a series of aromatic compounds and aliphatic compounds using a phenyl column with both acetonitrile and methanol as the mobile phase organic modifiers. They concluded that π–π interactions were enhanced by methanol versus acetonitrile, which is consistent with our interpretation that acetonitrile weakens π–π interactions. In practice, the primary reason for one to use a phenyl column is to provide a complementary selectivity pattern as compared to an ODS column. Therefore, it is important to note that when developing HPLC methods on phenyl columns, the use of acetonitrile may in fact be suppressing the most important separation mechanism of phenyl columns.

To obtain a separation which is difficult with an ODS column, methanol may be a preferred choice since it will not interfere with the π–π interaction mechanism. π–π interactions will still play a role in the separation with analytes of significantly different aromaticity even when acetonitrile is used in the mobile phase, since acetonitrile may not completely block strong π–π interactions [19]. However, it was noticed that in [18], when acetonitrile concentration increased in the mobile phase, π–π interaction played a less significant role on separation, which is consistent with the data obtained in our work. Furthermore, a literature review of additional studies of phenyl columns supports the rationale proposed in our work. For example, Goss [12] tested the separation of salicylic acid and related compounds with both a phenyl column and an ODS column with methanol as the organic modifier. A review of all the chromatographic tests performed in that paper consistently demonstrate that retention order changes between phenyl columns and ODS columns are consistent with the mechanisms proposed in our work (i.e. π–π interactions leading to longer retention for compounds with more unsaturated bonds with phenyl columns).

Another example shows acetonitrile suppresses π–π interactions the secondary phenyl column separation mechanism. In that study, examining separation conditions with both a phenyl column and a C8 column when using acetonitrile, resulted in the same retention order for a complicated set of compounds [20]. In contrast, when using methanol as the organic modifier, the phenyl column gave an altered retention order, whereas the C8 continued to give the same elution order. Literature review of other studies revealed that invariably, methanol, instead of acetonitrile, was always the organic modifier used to alter the retention order with a phenyl column compared to columns of ODS stationary phases [21–23].

4. Conclusion

In this study, the impact of different organic modifiers (i.e. methanol and acetonitrile) on the HPLC separation of several select compounds, with a phenyl column, was investigated. It was noticed that while with no separation of A5 and A6(R,S) was observed when acetonitrile was used as the organic modifier with a phenyl column, good separation was achieved with methanol. A rationale was proposed to explain the difference based upon acetonitrile blockage of π–π interactions between analyte molecules and the phenyl groups in the stationary phase. Additional HPLC studies with 1-naphthoic acid and 1-naphthol provided further support to this rationale. As a result of this work, when using phenyl columns, methanol is recommended as an organic modifier of first choice, since it is most likely to give the maximum selectivity difference when compared to that of ODS columns.

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