2006

Liquid crystal bonded stationary phases for high pressure liquid chromatography

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DOI: <https://doi.org/10.31979/etd.hby5-dcax>
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LIQUID CRYSTAL BONDED STATIONARY PHASES FOR
HIGH PRESSURE LIQUID CHROMATOGRAPHY

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Masters of Science

by

Susan M. Larrabee

May 2006

UMI Number: 1436928

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04/06/06

ABSTRACT
LIQUID CRYSTAL BONDED STATIONARY PHASES FOR
HIGH PRESSURE LIQUID CHROMATOGRAPHY

by Susan M. Larrabee

Two novel chromatography stationary phases for HPLC were evaluated for their properties and separation capabilities. The two stationary phases, cholesterol and 4-methoxy-4-allyloxy benzoate (MPAB), are liquid crystals, which give the columns unique and interesting properties. Some properties are molecular recognition capabilities, separation of chiral molecules, separation of steroids, the ability to use highly aqueous mobile phases, and non-linear retention with temperature change. Active pharmaceutical ingredients (APIs) were obtained from the pharmaceutical company Roche Palo Alto, LLC and used as analytes. Molecular classes tested include nucleoside analogues, purines, anthracenes, steroids and polar compounds. The cholesterol columns showed high selectivity for planar pro-drugs of nucleoside analogues as compared to a C8 stationary phase. Chiral separations could not be demonstrated with the chosen solutes and mobile phase conditions. In addition, the aqueous normal phase (ANP) mode of chromatography was demonstrated on some analytes using specific mobile phase conditions.

ACKNOWLEDGEMENT

Thank you to my advisors, Dr. J. J. Pesek and M. Matyska, for all their input and guidance, my research committee members, Dr. R. Terrill and Dr. S. Branz, for reviewing this work, and fellow students in the Chemistry Department at San Jose State University. I would like to acknowledge Roche Palo Alto, LLC for providing financial support. A special thank you to Dr. T. Alfredson, director of the Pharmaceuticals Department at Roche Palo Alto, LLC, for allowing this research to be conducted at the Roche Palo Alto site. Thank you to Dr. M. Brandl, manager of Discovery Research, Dr. D. Stefanidis, manager of Formulations, and all my colleagues in the Pharmaceuticals Department at Roche Palo Alto, LLC for their support and encouragement.

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1. Introduction

Liquid crystals, such as cholesterol, have been studied as bonded stationary phase for chromatography (1 - 4). These organic molecules are fluid, but have some crystalline properties that can provide chromatography columns unique and interesting separation capabilities (5). For over thirty years, liquid crystals coated onto capillary columns have been used to separate structural isomers by gas chromatography (5). New bonding technology of liquid crystals for silica stationary phase has allowed researchers to investigate their use in high pressure liquid chromatography (HPLC) (1, 5). The main type of liquid crystals researchers have bonded onto HPLC stationary phase have a rod-like, or calamatic structure, consisting of one or more ring systems, terminal groups and sometimes side groups (5). Because of their structure, liquid crystals can be induced by temperature and solvent concentration change to undergo phase transitions between the liquid and solid states (5, 6). In these phase transitions, called mesophases, the molecules are arranged in a structured fashion and are more ordered than liquids, but less ordered than solids (text). Some classifications of mesophases are shown in Fig. 1. For example, the smectic phase has molecules arranged in parallel and in sheets, whereas the nematic phase has molecules arranged in parallel, but not in sheets. Thirdly, the cholesteric phase is composed of chiral molecules arranged in sheets, with each sheet being turned slightly at a different angle (5, 6). Liquid crystals bonded to silica in an HPLC column will retain crystalline behavior and are therefore more ordered than C-18 molecules in a typical reverse phase column. This ordering can provide enhanced

capabilities such as separation of structural isomers (5). Separation seems to occur mainly due to the analytes moving into aligned “slots” of the liquid crystal (5). Since the mesophases are induced by temperature and solvent change, chromatography optimization can be achieved by changing column temperature and mobile phase composition.

Fig. 1. Liquid crystal transition phases (mesophases) including smectic, nematic and cholesteric (6).

The structures of two liquid crystals investigated, cholesterol and 4-methoxy-4-allyloxy benzoate (MPAB), are shown in Figs. 2 and 3. In the cholesteric bonded phase (cholesteryl-10-undecenoate), the cholesterol molecules are attached to the silica backbone with a flexible hydrocarbon linkage using a silanization/hydrosilation process (2, 7). Cholesterol is a steroid with four rings, a hydrocarbon tail, one planar side and eight chiral centers which allow it to be used for some chiral separations (2). It has been found that the cholesterol molecules arrange in different ordered fashions including the cholesteric phase. As stated previously, this alignment of adjacent cholesterol molecules can provide the column with some interesting properties (5). The second liquid crystal bonded stationary phase evaluated, MPAB, contains four oxygens and a

carboxyl group (8). The MPAB stationary phase has not been studied as much as the cholesteric bonded phase.

Fig. 2. Two-dimensional (A) and three-dimensional (B) structures of the cholesteryl bonded phase (cholesteryl-10-undecenoate). Black lines in 3-D view indicate the planar part of the phase (1).

Fig. 3. 4-Methoxyphenyl-4-allyloxybenzoate (MPAB) bonded phase (8).

To bond the desired liquid crystal moiety to silica particles, a two-step process called silanization/hydrosilation is performed (Fig. 4) (1, 9). This bonding method will produce a highly hydrolytically stable stationary phase with the silanols endcapped (1, 9). In the first step, called silanization, triethoxysilane (TES) is reacted with silica to form a monohydride intermediate (9). The silica surface will have a monolayer of hydride with most of the silanols removed and will have a high degree of cross-linking (9). Diffuse reflectance infrared Fourier transform (DRIFT) is used

SILANIZATION:

HYDROSILATION:

Fig. 4. Two-step bonding process, silanization/hydrosilation, to attach the liquid crystal moiety to the silica backbone (1, 9).

to confirm the formation of the hydride intermediate (9). The free Si-OH band at $\sim 3750\text{ cm}^{-1}$ will decrease and an Si-H stretching peak near 2250 cm^{-1} will appear (9). The second step, called hydrosilation, attaches the organic moiety to the new hydride surface using an unsaturated functional group creating a carbon-silicon bond (9). For the hydrosilation step, a catalyst (usually a metal complex such as hexachloroplatinic acid or a free radical initiator) is required (9). DRIFT spectra are taken to confirm the bonding of organic moiety to the silica. The Si-OH stretch at $\sim 3750\text{ cm}^{-1}$ will decrease and C-H stretching bands in the region of $3000\text{-}2800\text{ cm}^{-1}$ will appear (9). The bonded material is also characterized by elemental analysis to determine carbon content (9). Using the surface area value and the carbon content, the bonding density (also called surface coverage) can be calculated (9). The surface coverage of the bonded material is very important for liquid crystal HPLC because if it is too low, then adjacent bonded moieties would not have enough contact for the liquid crystal's physical properties to manifest themselves (5). Higher bonding densities would be expected to possess based on experimental evidence, more ordered structures (5).

Previous HPLC experiments with the cholesteric phase have demonstrated its properties and separation capabilities. Researchers found that the cholesteric bonded phase has intermediate hydrophobic/hydrophilic properties, which allows it to be used for both reversed phase and aqueous normal phase (ANP) separations (1). ANP is defined as normal phase chromatography using polar elution solvents such as acetonitrile and water instead of non-polar solvents such as hexane. Solute retention

times increase when the organic solvent in the mobile phase increases. When operating in the reverse phase chromatography mode, the cholesteric bonded column is less hydrophobic than C8 or C18 columns (1).

The relative hydrophobicity of the cholesterol phase was tested by injecting a hydrocarbon test mixture (benzene, toluene, ethylbenzene, isopropylbenzene, tert-butylbenzene and anthracene) on several columns (1). The columns included: cholesterol (with low and high surface area silica), C8 and C18 columns which were prepared with the silanization/hydrosilation process (1). The separations were performed with methanol-water mobile phases (1). Fig. 5 shows the chromatograms for the hydrocarbon test mixtures on the four different columns. With 80% methanol for the mobile phase, the cholesterol material with low surface area silica did not achieve separation for the six hydrocarbons. The cholesterol with the high surface area silica has slightly better separation than the lower surface area silica presumably because higher surface area silica can be bonded with more cholesterol molecules, and therefore there is more stationary phase for the solutes to interact with. The hydrocarbons on the cholesterol with high surface area silica eluted from about 4 to 10 minutes (anthracene eluting last). The C8 and C18 columns had complete separation of all six hydrocarbons using 80% methanol, with the hydrocarbons eluting from about 5.5 to 11 minutes (C8) and from about 6 to 20 minutes (C18). These results demonstrate that the cholesteric bonded phase is less hydrophobic than the C8 and C18 columns because the hydrocarbons elute earlier on the cholesterol column with the same mobile phase composition (1).

Separation of the six hydrocarbons can be achieved on the cholesterol column with high surface area when the methanol content of the mobile phase is lowered to 70% (1). With 70% methanol, anthracene elutes around 20 minutes, which is much later than the other five hydrocarbons. The relative retention of anthracene to *tert*-butylbenzene is higher on the cholesterol column than on the C8 or C18 columns. The selectivities (α , where: $\alpha = K_2'/K_1'$; 2 = anthracene, 1 = *tert*-butylbenzene) for the C8 and C18 columns are 1.1 and 1.4, respectively, whereas the selectivity for the cholesterol column is 1.8. The researchers attribute the higher selectivity of

Fig. 5. Separation of hydrocarbon test mixture on various chemically bonded stationary phases. (A) Cholesterol on low surface area silica; (B) cholesterol on high surface area silica; (C) C8 on high surface area silica; (D) C18 on high surface area silica; and (E) cholesterol on high surface area silica. Mobile phase: (A-D) MeOH-water (80:20) and (E) MeOH-water (70:30). Flow rate = 1 mL/min and detection at 254 nm. Solutes: 1-benzene, 2=toluene, 3=ethylbenzene, 4=isopropylbenzene, 5=*tert*-butylbenzene, 6=anthracene (1).

anthracene on the cholesterol column to a molecular shape recognition property (1). Since cholesterol is a liquid crystal, it retains a more ordered structure on the surface than the C8 and C18 columns do, allowing for an improved chromatic differentiation of anthracene from other (monocyclic) aromatic compounds (1).

Other studies have shown that the cholesteric bonded phase can be used for difficult to separate molecules including structural isomers of antibiotics and also steroids, due to its molecular shape recognition properties (1). Antibiotics can exist in a number of structural isomers that are difficult to resolve by reverse phase liquid chromatography. Fig. 6 shows chromatograms of ampicillin separations run on cholesterol and C18 columns with a methanol-water mobile phase. The cholesterol

Fig. 6. Separation of ampicillin on: (A) cholesterol on high surface area silica; and (B) commercial C18 column. Mobile phase: MeOH-water (70:30). Flow rate = 1 mL/min and detection at 254 nm (1).

column is able to separate two isomers, whereas the C18 column cannot. In addition to structural isomers, the cholesterol column can be used to separate steroids (1). Analysis of steroids is difficult and is usually performed using normal phase columns with gradient elution. A chromatogram of the separation of four steroids (androsterone, prednisone, corticosterone, and andrenosterone) on the cholesterol column with isocratic elution is shown in Fig. 7 (1). The improved resolution of antibiotics and the steroid separations may be due to shape selectivity from the more ordered structure of cholesterol (1).

Fig. 7. Separation of a steroid mixture on the high surface area cholesterol column. Mobile phase: MeOH-water (50:50). Flow rate = 1 mL/min and detection at 254 nm. Solutes: 1=4-androsterone; 2=prednisone; 3=corticosterone; and 4=andrenosterone (1).

In another study, the elution behavior of small polycyclic aromatic hydrocarbons (PAHs) was assessed on cholesteric bonded microcolumns (0.53 μm i.d. x 150 mm long) (2). PAHs, which are generally nonpolar, are categorized by their size (MW), shape, [length-to-breadth ratio (L/B) or the molecular size descriptor (F

number)], and their degree of planarity (2). The F number was proposed by Hurtubise *et al.* to describe molecular size with the following: $F = (\text{number of double bonds}) + (\text{number of primary and secondary carbons}) - 0.5 \times (\text{number of non-aromatic rings})$ (10). It was observed that PAH retention increases with increasing water concentration in the mobile phase, as in reverse phase chromatography (2). It was also observed that the cholesteric stationary phase exhibits a linear relationship between retention and a molecular size descriptor (F) for PAHs (2). As seen in the plot of $\log k'$ vs. F (Fig. 8), larger PAHs elute later than smaller PAHs for the size range shown. In addition, one of the interesting

Fig. 8. Relationship between $\log k'$ and F number for PAHs on the cholesteric bonded phase (2).

observations in the study was that the cholesteric bonded phase has high selectivity for planar molecules (2). Cholesterol is expected to preferentially retain planar PAHs longer than their non-planar analogues with the same or similar F number. On the plot in Fig. 8, the planar PAHs fall on the line ($y = x$), whereas the non-planar PAHs fall below the line (2). A widely used indicator of planarity recognition capability for

reversed phased stationary phases is the separation factor (α) of triphenylene and o-terphenyl (2). Typical values for a C18 column are between 1.1 and 1.7, but for the cholesterol column, values range between 2.0 and 2.5, which demonstrate the ability of the cholesterol phase to discriminate between planar and non-planar analogues for small-size PAHs (2). The authors hypothesize that this separation mechanism works because cholesterol moieties align together in an ordered fashion to form slots for the analytes to fit into and interact (2). As mentioned earlier, the cholesterol molecule has one side containing a planar site and another side that is not flat. The researchers conclude that the planar analytes are interacting with the planar site of cholesterol and are preferentially retained over the non-planar PAHs. They hypothesize that the planarity recognition diminishes when the PAH is larger than 4 rings because the planar side of cholesterol is relatively small (2).

A group of large PAHs, peropyrenes containing six to eleven rings, was tested on the cholesteric bonded stationary phase in another study using microcolumn liquid chromatography. Structures are shown in Fig. 9 (3). It was found that the retention of large PAHs depends on the planarity of the molecules (3). A plot of the retention factors vs. F numbers for these large PAHs is shown in Fig. 10. The elution order of these large PAHs does not follow the same trend as stated previously for small PAHs (where retention increases with increasing size) (2, 3). The large PAHs eluted before compounds with smaller molecular size (F number). For example, compound p (pentabenz[a,cd,j,lm]perylene), which has 10 rings, and compound q (hexabenz[a,cd,fi,lm,o]perylene), which has 11 rings, eluted earlier than compounds

i, j, m, n and o, which have 9 rings and lower F numbers than compounds p and q (3).

Compound k (tetrabenzo[a,cd,j,lm]perylene), with an F number of 17, eluted before compound f (benzo[pqr]naphtho[8,1,2-bcd]pyrene), which has a smaller F number

Fig. 9. Structures of the large PAH used as probes: **a**, benzo[ghi]perylene; **b**, dibenzo[b,def]chrysene; **c**, coronene; **d**, naphtho[bcd]perylene; **e**, dibenzo[cd,lm]perylene; **f**, benzo[pqr]naphtho[8,1,2-bcd]pyrene; **g**, benzo[a]coronene; **h**, tribenzo[a,cd,lm]perylene; **i**, benzo[lm]phenanthro[456,abcd]perylene; **j**, dinaphtho[2,1,8,7defg,2,1,8,7ijkl]pentaphene; **k**, tetrabenzo[a,cd,j,lm]perylene; **l**, tetrabenzo[a,cd,f,lm]perylene; **m**, benzo[rsf]phenanthro[cde]pentaphene; **n**, dibenzo[a,rsf]naphtho[cde]pentaphene; **o**, dibenzo[j,lm]naphtho[1,8-ab]perylene; **p**, pentabenzo[a,cd,fj,lm]perylene; **q**, hexabenzo[a,cd,f,j,lm,o]perylene (3).

Fig. 10. Retention factors of large PAH plotted against F number. The mobile phase was methanol-dichloromethane, 8:2. The letters correspond to those in Fig. 9 (3).

of 13 (3). Some of the larger PAHs elute earlier than the smaller ones in this study presumably because of their degree of planarity (3). For example, molecules p and q contain the 5-helicene substructure (Fig. 11) and are non-planar (3). These results demonstrate that the cholesteric bonded phase is more selective for planar compounds (planar molecules are retained longer than non-planar molecules) (3).

In addition to separating planar PAHs from non-planar analogues, structural isomers of antibiotics, and steroids, the cholesteric bonded phase can be used for separating some chiral molecules (1). Enantiomers cannot always be separated by reverse phase chromatography, and chiral separation must be employed, such as a cyclodextrin column (11). Fig. 12 shows a chromatogram of an enantiomer

Fig. 11. Substructures found to increase intramolecular strain in large PAH, and their manifestation in two PAHs (3).

separation of naphthylamine by the cholesterol column with a methanol-water mobile phase. The mechanism of separation is unknown, but probably based on the 3D structure of cholesterol, which has eight chiral centers (1).

In another published study, the cholesteric bonded phase was evaluated using the solutes 1,4-benzodiazepines and derivatives on a microcolumn (150 x 0.53-mm i.d., 1.4 $\mu\text{mol}/\text{m}^2$ bonding density) with acetonitrile-water mobile phases (4). The 1,4-benzodiazepines (which are weak bases) were classified into seven groups according to their structure (Figs.13 and 14). The chromatography results demonstrate that the cholesteric phase can discriminate between modifications in the 1,4-benzodiazepine structure. The elution order was different on the cholesterol

Fig. 12. Chromatograms of a mixture of two optical isomers of naphthethylamine. Mobile phase: MeOH-water (70:30), flow rate = 1 mL/min and detection at 254 nm (1).

Fig. 13. The benzodiazepine parent structure is characterized by the presence of a benzene ring fused to a saturated seven-membered ring with nitrogens at positions 1 and 4 (4).

column than on a C18 column (Fig. 15). For example, nitrazepam elutes first on the cholesterol column, but was retained after estazolam on the C18 column. On the cholesterol column, for groups A (keto containing) and B (triazolo ring containing), a methyl group at position N-1 increased retention (Tab. 1) (4). When a chlorine atom is present this methyl-group effect is enhanced, but when a nitrogen is present at position 8, the effect is lessened. For groups D (thienotriazolodiazepines) and E

Fig. 14. Structures of the benzodiazepines evaluated (4).

(thienodiazepines) the presence of a triazole ring causes faster elution on the cholesterol column. In group F (oxazolo derivatives), the orientation and/or attachment of a halogen affects retention on the cholesterol column greatly. For example, cloxazolam (having two chlorines) is retained much longer than oxazolam (having only one chlorine) and haloxazolam (having one bromine and one chlorine) (4).

Fig. 15. Chromatograms of mixture of benzodiazepines in (A) cholesteric and (B) ODS (C-18) phases at 35% acetonitrile concentration in water as the mobile phase. Peaks: 1=nitrazepam (Group A); 2=nimetazepam (group A); 3=estrazolam (group B); 4=brotizolam (group D); 5=clotiazepam (group E); 6=oxazolam (group F); 7-haloxazolam (group F); 8=cloxazolam (group F); and 9=medazepam (group G) (4).

Table 1. Molecular weight, log P, and retention factors of 20 benzodiazepines at different mobile phase compositions in cholesteric and ODS (C18) phases grouped according to structure (4).

Structure (4).

Group/no.	Benzodiazepines	MW	log P ^a	Retention factors						
				Acetonitrile concentration in water (%)						
				35	40	45	50	70	35	
				Cholesteryl						
				ODS						
Group A	1	Nitrazepam	281	2.54	0.74	0.61	0.32	0.23	0.04	4.06
	2	Clonazepam	316	2.84	0.84	0.67	0.33	0.25	0.03	4.72
	3	Oxazepam	287	2.10	0.92	0.67	0.35	0.26	0.07	3.76
	4	Flunitrazepam	313	2.35	0.95	0.78	0.39	0.27	0.04	6.54
	5	Nimetazepam	295	2.21	1.04	0.84	0.42	0.31	0.05	7.28
	6	Diazepam	285	3.18	1.38	1.3	0.62	0.41	0.08	13.6
	7	Fludiazepam	303	2.70	1.42	1.20	0.53	0.37	0.07	11.1
Group B	8	Estazolam	295	2.79	1.30	1.01	0.52	0.43	0.14	3.84
	9	Triazolam	342	3.20	1.63	1.27	0.63	0.50	0.16	4.68
	10	Alprazolam	309	3.20	1.66	1.30	0.65	0.54	0.19	4.8
Group C	11	Chlordiazepoxide	300	2.44**	1.71	1.27	0.72	0.63	0.20	4.84
Group D	12	Etizolam	343	3.10	1.75	1.35	0.65	0.51	0.15	3.84
	13	Brotizolam	394	2.83	1.76	1.38	0.66	0.52	0.15	6.34
Group E	14	Clotiazepam	319	3.20	2.26	1.75	0.76	0.53	0.10	23.7
Group F	15	Oxazolam	329	4.32	2.70	2.24	1.06	0.67	0.08	14.82
	16	Haloxazolam	377	3.50	3.67	2.60	1.52	1.12	0.24	29.14
	17	Cloxacolam	349	3.90	15.84	12.03	8.21	4.58	1.79	17.05
Group G	18	Medazepam	271	4.47	20.08	14.10	8.34	5.84	1.91	46.1

^a Calculated log P values taken from *Comprehensive Medicinal Chemistry*, Volume 6, Pergamon Press, UK, 1990.

** Experimentally determined, also taken from *Comprehensive Medicinal Chemistry*, Volume 6, Pergamon Press, UK, 1990.

The researchers conclude that the cholesteric bonded phase possesses three important structural features that might influence its molecular recognition capability: the carbonyl group on the hydrocarbon linker, the cholesterol moiety, and the long alkyl chain. Molecular modeling with the Chem3D Plus software illustrated possible sample and stationary phase interactions for nitrazepam (a keto compound) and medazepam (a deoxy derivative) (4). Nitrazepam's low retention on the cholesteric phase is due to its polar keto group and nitro group interacting more with the polar solvent than the cholesteric stationary phase. Medazepam has a larger surface area and contacts the cholesteric phase more than nitrazepam. The medazepam is retained

along the alkyl chain of the cholesteric phase, and medazepam's N-4 of the 7-membered ring interacts with the carbonyl linker. These results demonstrate cholesterol's molecular recognition property (4).

Another unique property of the cholesteric bonded phase is that it can be used with highly aqueous mobile phases, unlike C18 columns which collapse with high water content (1). Fig. 16 shows the $\log K'$ vs. water content for ampicillin on the cholesterol and C18 columns. The retention on the C18 column decreases above 80% water in the mobile phase. In contrast, the cholesterol column does not undergo phase collapse with the high water content, probably because of its rigid tetracyclic ring structure (1).

Fig. 16. Plot of $\log k$ vs. % water in the mobile phase for a sample of ampicillin. Columns: (A) cholesterol on high surface area silica; and (B) commercial C18. Mobile phase: MeOH-water (70:30). Flow rate = 1 mL/min and detection at 254 nm. (Data shown is the average of three measurements) (1).

This property can be utilized for poorly retained solutes, such as polar compounds, which need low organic composition in the mobile phase to elute later than the void volume.

As mentioned earlier, the transitional phases of liquid crystals can be induced by temperature (1, 5). In one experiment, researchers found that the cholesteric bonded phase can undergo re-ordering with temperature change due to its liquid crystal properties. Fig. 17 shows the retention of the antibiotic gentamycin vs. $1/\text{Temperature}$ (1). As the temperature decreases, the retention changes non-linearly, unlike a C18 column. This non-linear retention indicates that cholesterol is undergoing some reordering or phase transition. When trying to improve separations, this property can be exploited very easily by changing the column temperature over a small range.

Fig. 17. Plot of $\log k$ vs. $1/T \text{ (K}^{-1}\text{)}$ for two peaks in the chromatographic analysis of a gentamycin sample. Mobile phase: MeOH-water (50:50), flow rate = 1 mL/min and detection at 254 nm. (Data shown is the average of three measurements) (1).

To put this research into perspective to other research performed in this area, another research group, B. Buszewski and co-workers, have also used cholesterol bonded stationary phase for HPLC (12-17). These researchers prepared their cholesterol columns with a different bonding procedure than the silanization/hydrosilation method previously described (12). Aminopropyl was used for the linker attaching cholesterol to the silica and since it has only three hydrocarbons, this linker is shorter than the one used in Pesek's research. Additionally, the Buszewski's cholesteric stationary phase contained free amino and free silanol groups.

Buszewski's research studies focused on the separation capabilities and possible separation mechanisms for different analytes including aromatic hydrocarbons, isomers, and nucleosides on the cholesterol bonded phase. Specifically, the research studied the quantitative structure-retention relationships for aromatic hydrocarbons (12), the retention behavior and selectivity of polyaromatic hydrocarbons (13), and the lipophilicity determination of β -blockers (14). The researchers demonstrated that for a series of alkylbenzenes, retention increased linearly with increasing number of carbon atoms attached to the benzene ring, as in reverse phase chromatography (13). This result was consistent with the retention on the C18 column used as a comparison, except the retention was longer on the cholesterol column (13). In other studies, the cholesterol bonded phase was able to separate structural isomers of tocopherol (15) and estradiol diastereoisomers (16). And, when nucleosides were used as analytes, their retention increased on the cholesterol bonded phase with increasing pH in the mobile phase (17). Another finding from this study was that the retention of these nucleosides was longer on

the cholesterol column than a C18 column. Buszewski theorized that the stronger interaction with the cholesteric stationary phase was due to the similarity between the structures of nucleosides and the cholesterol molecule (aromatic rings). Buszewski hypothesized that the retention of some nucleosides, such as adenosine, on the cholesterol column was mostly based on Π - Π type and hydrophobic (van der Waals forces) interactions, but hydrogen bonding interactions could also be present (17).

In summary, the cholesterol bonded phase is a unique separation material and has properties and separation features that are not possessed by any commercially available stationary phase. It has reverse phase characteristics and is less hydrophobic than C8 or C18 columns. It also has molecular shape recognition abilities. Two other unique properties include non-linear retention with temperature change and no phase collapse with highly aqueous mobile phases. Finally, it has been used for some chiral separations.

The objective of this research is to evaluate the cholesterol and MPAB stationary phases with novel active pharmaceutical ingredients (APIs) obtained from Roche Palo Alto, LLC. This research investigated how the retention was affected by mobile phase compositions and temperatures, and also to compare the retention with the C8 stationary phase. Classes of molecules studied included nucleoside analogues and their pro-drugs, purines, steroids, anthracenes, and chiral compounds. In addition, polar solutes were investigated for aqueous normal phase (ANP) behavior.

2. Experimental

2.1 Materials

The solvents used for chromatographic experiments: acetonitrile (B& J Brand, Muskegon, MI), methanol (B & J Brand, Muskegon, MI), ethanol (Spectrum, Gardena, CA), formic acid (Fluka, Steinheim, GE), trifluoroacetic acid (J. T. Baker, Phillipsburg, NJ) and perchloric acid (VWR International, West Chester, PA) were obtained in reagent grade or HPLC grade. Purified water was prepared with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

The cholesterol and MPAB research columns were obtained from Dr. J. J. Pesek's laboratory at the Department of Chemistry, San Jose State University (San Jose, CA). The packing material for these research columns was synthesized at San Jose State. Triethoxysilane (TES), the silanization reagent, and cholesterol-10-undecenoate, the hydrosilation reagent for the cholesterol material, were purchased from Sigma-Aldrich (Milwaukee, WI). The MPAB was prepared using a synthetic procedure described by Apfel *et al.* (8, 18). Chloroplatinic acid hexahydrate (the Speier's catalyst) was purchased from Strem Chemicals (Newbury Port, MS). The catalyst was prepared in a nitrogen filled glove box by dissolving 0.4 g in 100 mL of isopropanol at a temperature of 93°C. The silicas used for the preparation of the stationary phases included Vydac HS (high surface area) and Vydac TP, purchased from The Separations Group (Hesperia, CA) and Astrosil, purchased from Stellar Phases (Langhorne, PA). The bonded stationary phases were packed into stainless steel columns by Microsolv Technology Corp. (Eatontown, NJ). The commercial

Zorbax SB-C8 phase was obtained from Agilent (Palo Alto, CA) and the commercial Cogent bidentate C18TM was obtained from Microsolv Technology Corp. (Eatontown, NJ). The details for the research columns and commercial columns (Cols. # 1 to 7) are as follows:

Column 1: Cholesterol phase, Vydac HS silica, 100 Å pore size, 330 m²/g surface area, 4.6 mm column diameter, 150 mm column length and 5 µm particle size;

Column 2: MPAB phase, Vydac TP silica, 300 Å pore size, 106 m²/g surface area, 4.6 mm column diameter, 150 mm column length and 6.5 µm particle size;

Column 3: Cholesterol phase, Astrosil silica, 100 Å pore size, 327 m²/g surface area, 4.6 mm column diameter, 150 mm column length and 4 µm particle size;

Column 4: Cholesterol phase, Astrosil silica, 100 Å pore size, 327 m²/g surface area, 4.6 mm column diameter, 75 mm column length and 4 µm particle size;

Column 5: Cholesterol phase, Astrosil silica, 100 Å pore size, 327 m²/g surface area, 4.6 mm column diameter, 75 mm column length and 5 µm particle size;

Column 6: Cogent bidentate C18TM phase, 100 Å pore size, 4.6 mm column diameter, 150 mm column length and 4 µm particle size;

Column 7: Zorbax SB-C8 phase, 80Å pore size, 180 m²/g surface area, 4.6 mm column diameter, 150 mm column length and 5 µm particle size.

Most solutes were obtained from Roche Palo Alto LLC (Palo Alto, CA, USA). Since the compounds obtained from Roche are proprietary, only partial structures will be shown. Ribavirin was obtained from MP Biomedicals, LLC (Aurora, OH, USA). Anthracene and cholesterol were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The solutes were dissolved in concentrations ranging from 0.01 to 0.1 mg/mL in either water, acetonitrile, water/acetonitrile (50:50) or DMSO depending on their solubility. The calculated log P's, acid pKa's (ApKa's) and basic pKa's (BpKa's) were obtained from the Roche database and are the theoretically calculated values. Log P is a measure of hydrophobicity, and is calculated by taking the log of the octanol-water partition coefficient ($K_{ow} = C_o/C_w$, where: C_o = concentration of compound in octanol layer, C_w = concentration of compound in water layer).

2.2 Instrumentation

A Waters 2690 pump and a Waters 996 photo-diode array detector with EmpowerTM software (Millford, MA) were employed. In addition, a Waters 2525 Binary Gradient Module and a Waters model ZMD Mass Spectrometry detector with MassLynxTM v. 4.0 software (Micromass Ltd., Manchester, UK) were used. Injection volumes were 10 μ L. Void volume times were estimated by calculation (11):

$$t_0 = V_m / F \quad V_m \sim 0.5 L d^2$$

where:

t_0	=	void volume time, min
V_m	=	void volume, mL
F	=	flow rate, mL/min
L	=	column length, cm
d	=	column diameter, cm

Therefore, the t_0 equals 0.79 minutes for the 4.6 x 75 mm columns at flow rate of 1.0 mL/min, 1.58 minutes at flow rate of 0.5 mL/min, and 1.13 minutes at flow rate of 0.7 mL/min. The t_0 equals 1.587 minutes for column size of 4.6 x 150 mm at flow rate of 1.0 mL/min.

3. Results and Discussion

3.1 Nucleoside Analogue Pro-Drugs

A nucleoside analogue parent drug and nine pro-drugs were tested with the cholesterol (Cols #1 and #3) and MPAB (Col #2) columns, and then compared to the C8 column (Col #7). The structure of the parent molecule resembles the nucleoside cytidine (ribose sugar attached to cytosine base) shown in Fig. 18. Pro-drugs have functional groups attached to the ribose at three positions for the purpose of increasing absorption of the drug through the gastrointestinal tract. The log P increased from -1.94 to the range of -0.275 to 4.77 (Table 2). Group 1 compounds have hydrocarbon chains attached to the ribose at two positions (compound 1) or to three positions (compounds 2 and 3) with ester linkages. This group has the highest log P's of the nine pro-drugs. Group 2 and 3 compounds have one functional group containing one or two phenyl groups attached to the ribose via a phosphate linkage. The mobile phase for the experiments consisted of acetonitrile, water, and trifluoroacetic acid (TFA).

Representative chromatograms for the cholesterol (Col #1) column and the C8 column are shown in Figs. 19 and 20. The peaks are sharper and narrower on the C8 column. The base peak widths for compound 7 are 3.2 minutes for the cholesterol (Col #1) column at 10% acetonitrile and 0.53 minutes for the C8 column at 40% acetonitrile. The peak tailing is about the same for both columns. For example, for compound 7 the USP tailing factor equals 1.13 for the cholesterol (Col #1) column at 10% acetonitrile, and 1.15 for the C8 column at 40% acetonitrile. The cholesterol column efficiency is

about seven times less than the C8 column. For the pro-drug compound 7, the USP plate count equaled 703.4 on the cholesterol column (10% acetonitrile in the mobile phase) and 4783.7 on the C8 (40% acetonitrile in the mobile phase).

Cytidine

Fig. 18. Backbone structure of the parent nucleoside analogue is similar to cytidine (ribose-cytosine). Pro-drugs contain side chains attached to the ribose at three possible positions.

Table 2. Molecular weights, pKa's and log Ps for nucleoside analogue parent and pro-drugs grouped according to structure

Group	No.	MW	ApKa	BpKa	Log P*
	parent	284.2	13.89	3.73	-1.94
Group 1	1	452.5		3.73	2.28
	2	494.5		3.73	3.18
	3	536.6		3.73	4.77
	4	540.4	9.3, 13.76		-0.275
Group 2	5	554.5	9.3, 13.76		0.254
	6	588.5	9.3, 13.76		0.568
	7	602.5	9.3, 13.73		0.687
Group 3	8	567.5	13.94	3.73	0.677
	9	601.5	13.94	3.73	0.711

* Calculated log P

The retention factors (K') for the parent nucleoside analogue and pro-drugs on the cholesterol (Col #1) column (0 to 25 % acetonitrile in the mobile phase) and on the C8 column (40% acetonitrile in the mobile phase) are shown in Table 3. K' is calculated using the following equation: $K' = (t_r - t_0)/t_0$, where: t_r = retention time in minutes and t_0 = void volume time in minutes). The elution order of these nucleoside analogues within each group is the same for both the cholesteric phase and the C8 phase. The parent drug elutes very early, before the void volume ($t_0 = 1.587$ minutes). The low retention time for the parent is expected since its log P equals only -1.94 and has low hydrophobicity. On the cholesterol (Col #1) column, the retention of the parent drug increases with decreasing acetonitrile in the mobile phase, but the parent is still unretained at 100% aqueous (retention time = 1.53 minutes). For the pro-drugs, the retention on the cholesterol column (Col #1) also decreases with increasing acetonitrile in the mobile phase as expected for reverse phase chromatography (Fig. 21). The Group 1 compound 3 elutes last on both the cholesterol (Col #1) column and the C8 column. On the cholesterol (Col #1) column, the other Group 1 compounds, 2 and 3, elute after group 3, but within the retention time range of Group 2 compounds. However, for the C8 column, the Group 1 pro-drugs 1 and 3 elute after all Group 2 and 3 compounds. Therefore, the cholesterol phase has different selectivity than the C8 phase.

Log K' vs. log P plots for the cholesterol (Col #1) and C8 columns are shown in Fig. 22 for the pro-drugs. For the C8 column, the Log K' vs. log P plot indicates that these two parameters are proportional and that the retention increases with increasing hydrophobicity (as expected for reverse phase chromatography). The Group 1

compounds have the highest log P and are the most hydrophobic. Therefore, the Group 1 compounds interact with the C8 stationary phase the most out of the nine pro-drugs and elute last (retention time = 5.8 to 34.7 minutes for Group 1 as compared to 2.8 to 4.8 minutes for Groups 2 and 3). For the cholesterol (Col #1) column, compound retention increases for increasing log P within each compound group. Although the acetonitrile content in the mobile phase (10%) is lower for the cholesterol column than for the C8 (40%), the retention of Group 1 molecules on the cholesterol column is lower because the cholesteric phase is less hydrophobic than the C8 phase. As previously stated, on the cholesterol (Col #1) column, the Group 1 pro-drugs 1 and 2 elute after Group 3, but within the retention time range of Group 2 pro-drugs even though their log Ps are higher. This phenomenon can be explained by the results in Fig. 22. The log K' for group 2 on the cholesterol (Col #1) column is higher than on the C8 column. In addition, the log K' for group 3 on the cholesterol (Col #1) is relatively higher than on the C8 column (compared to group 1). The Group 3 cholesterol (Col #1) data points are above the group 1 log K' vs. log P cholesterol (Col #1) line (if extended down to y = 0). But, for the C8 column, the Group 3 data points fall fairly close on the Group 1 log K' vs. log P line. If hydrophobicity was the only separation mechanism occurring on the cholesteric phase, the log K' vs. log P plots for Groups 2 and 3 would be lower on the graph. The theoretical log K's with just a hydrophobic mechanism present, would probably be about -0.7 to -0.9, much lower than the -0.1 to 0.7. The higher selectivity of the cholesterol phase to these Group 2 and 3 compounds could be due to their shape. Group 2 and 3 pro-drugs have a fairly long hydrocarbon chain containing one or two phenyl rings attached

to the ribose, whereas group 1 pro-drugs have short hydrocarbon attachments containing no phenyl or other aromatic groups. The planarity of the phenyl rings may cause Group 2 and group 3 molecules to fit into the slots created by the cholesterol's ordered structure. These results agree with previous research showing that the cholesteric phase preferentially retains planar PAHs over their non-planar analogues (2).

Fig. 19. Chromatograms of nucleoside analogue parent and Group 1 pro-drugs in (A) cholesteric (Col #1) and (B) C8 phases. Mobile phase: (A) ACN/water/TFA (10:90:0.05) and (B) ACN/water/TFA (40:60:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C. Detection = 280 nm. Solutes: Group 1 = 1, 2, 3.

A

B

Fig. 20. Chromatograms of nucleoside analogue parent, Group 2 and 3 pro-drugs in (A) cholesteric (Col #1) and (B) C8 phases. Mobile phase: (A) ACN/water/TFA (10:90:0.05) and (B) ACN/water/TFA (40:60:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C. Detection: Group 2 = 258 nm and Group 3 = 258 nm. Solutes: Group 2 = 4, 5, 6, 7; Group 3 = 8, 9.

Table 3. Retention factors of nucleoside analogue parent and pro-drugs at different mobile phase compositions run on the Cholesteric (Col #1) and C8 phases grouped according to structure

Group	No.	Log P*	Retention factor (K')							
			Acetonitrile (%)							
			25	20	15	10	8	5	0	40
			Cholesteric (Col #1)							C8
	parent	-1.94	-0.100	-0.078	-0.072	-0.065	-0.063	-0.059	-0.038	-0.030
Group 1	1	2.28	0.191	0.262	0.498	1.087				2.655
	2	3.18	0.251	0.490	1.085	2.603				5.394
	3	4.77	0.853	2.440	7.579					20.837
Group 2	4	-0.275	0.402	0.541	0.795	1.332	1.735	2.808		0.791
	5	0.254	0.457	0.653	1.036	1.939	2.650	4.655		1.209
	6	0.568	0.583	0.913	1.673	3.718	5.491	10.662		1.643
	7	0.687	0.629	1.032	1.992	4.738	7.118	14.405		2.039
Group 3	8	0.677	0.025	0.106	0.299	0.554	0.805	1.532		0.999
	9	0.711	0.062	0.265	0.376	0.907	1.325	2.545		1.151

* Calculated log P

Table 4. Log K' of nucleoside analogue parent and pro-drugs at different mobile phase compositions run on the Cholesteric (Col #1) and C8 phases grouped according to structure

Group	No.	Log P*	Log K'							
			Acetonitrile (%)							
			25	20	15	10	8	5	0	40
			Cholesteric (Col #1)							C8
	parent	-1.94								
Group 1	1	2.28		-0.58	-0.30	0.04				0.42
	2	3.18		-0.31	0.04	0.42				0.73
	3	4.77	-0.07	0.39	0.88					1.32
Group 2	4	-0.275	-0.40	-0.27	-0.10	0.12	0.24	0.45		-0.10
	5	0.254	-0.34	-0.19	0.02	0.29	0.42	0.67		0.08
	6	0.568	-0.23	-0.04	0.22	0.57	0.74	1.03		0.22
	7	0.687	-0.20	0.01	0.30	0.68	0.85	1.16		0.31
Group 3	8	0.677			-0.52	-0.26	-0.09	0.19		0.00
	9	0.711		-0.58	-0.43	-0.04	0.12	0.41		0.06

* Calculated log P

A

B

Fig. 21. Retention factor vs. % acetonitrile in the mobile phase for nucleoside analogue pro-drugs on the (A) cholesteric (Col #1) and (B) C8 phases. Solutes: Group 1 = 1, 2, 3; Group 2 = 4, 5, 6, 7; Group 3 = 8, 9.

Fig. 22. Log retention factor vs. log P for nucleoside analogue pro-drugs for cholesteric (Col #1) and C8 Phases. Mobile phase: cholesteric (Col #1) = ACN/water/TFA (10:90:0.05), C8 = ACN/water/TFA (40:60:0.05). Flow rate = 1.0 mL/min, Column temperature: 40°C, Detection: Group 1 and 3 = 280 nm, Group 2 = 258 nm. Solutes: Group 1 = 1, 2, 3; Group 2 = 4, 5, 6, 7; Group 3 = 8, 9.

Fig. 23. Chromatograms of nucleoside analogue parent and pro-drugs in cholesteric (Col #3) phase. Mobile phase: ACN/water/TFA (40:60:0.05). Flow rate = 1.0 mL/min, Column temperature = 40°C. Detection: Group 1 and 3 = 280 nm, Group 2 = 258 nm. Solutes: Group 1 = 1, 2, 3; Group 2 = 4, 5, 6, 7; Group 3 = 8, 9.

These nucleoside analogues were then analyzed using the cholesterol (Col #3) column with Astrosil silica as the backbone. Representative chromatograms are shown in Fig. 23 and resolution results are summarized in Table 5. The peaks are much sharper than the cholesterol (Col #1) column. The elution order within each group on the cholesterol (Col #3) column is the same as on the cholesterol (Col #1) column. However, the Group 1 compounds elute later in relation to Group 2 and 3 on the Col #3 column than on the Col #1. Compound 1 elutes after compound 6 on Col #3, but before compound 6 on Col #1. On the C8 column, compound 1 elutes after all Group 2 and 3 compounds.

The plots of $\log K'$ vs. $\log P$ for the cholesterol (Col #3) column are different from the cholesterol (Col #1) column (Fig. 24). The retention of Group 2 and 3 compounds is lower in relation to Group 1 compounds on Col #3 than on Col #1. As on Col #1, the elution order of compounds is different on Col #3 than the C8 column. Although the Group 2 and 3 compounds are not as preferentially retained on Col #3 as on Col #1, another separation mechanism other than hydrophobicity is evidently occurring on Col #3 as evidenced by compound 7 eluting after compound 1 at 40% ACN. On the C8 column, compound 7 elutes before compound 1 as expected, since compound 1 has a higher $\log P$. The results for Col #1 were then repeated and confirmed (Figs. 25 and 26). It is unclear why the two cholesteric phases have different retention trends for the group 2 compounds. The difference is likely due to the type of silica (Vydac HS or Astrosil) and the relative bonding density on the two columns.

Table 5. Log P's and retention factors of nucleoside analogue parent and pro-drugs at different mobile phase compositions ran on the Cholesteric (Col #3) and C8 phases grouped according to structure

Group	No.	Log P*	Retention factor (K')		
			Acetonitrile (%)		
			40	35	40
			Cholesteric (Col #3)		C8
	parent	-1.94			-0.030
Group 1	1	2.28	2.822	1.619	2.655
	2	3.18	4.594	2.595	5.394
	3	4.77	16.066	7.509	20.837
Group 2	4	-0.275	1.251	0.799	0.791
	5	0.254	1.695	1.052	1.209
	6	0.568	2.645	1.547	1.643
	7	0.687	3.086	1.773	2.039
Group 3	8	0.677	1.417	0.856	0.999
	9	0.711	1.918	1.111	1.151

* Calculated log P

Fig. 24. Log K' vs. log P for nucleoside analogue pro-drugs for cholesteric (Col #3) and C8 Phases. Mobile phase: cholesteric (Col #3) = ACN/water/TFA (40:60:0.05), C8 = ACN/water/TFA (40:60:0.05). Flow rate = 1.0 mL/min, Column temperature: 40°C, Detection: Group 1 and 3 = 280 nm, Group 2 = 258 nm. Solutes: Group 1= 1, 2, 3; Group 2 = 4, 5, 6, 7; Group 3 = 8, 9.

Fig. 25. Chromatograms of nucleoside analogue parent and pro-drugs in cholesteric (Col #1) phase, repeated. Mobile phase: ACN/water/TFA (10:90:0.05). Flow rate = 1.0 mL/min, column temperature = 40°C. Detection: Group 1 and 3 = 280 nm, Group 2 = 258 nm and. Solutes: Group 1 = 1-1, 2, 3; Group 2 = 4, 5, 6, 7; Group 3 = 8, 9.

Fig. 26. Log retention factor vs. log P for nucleoside analogue pro-drugs for cholesteric (Col #1), repeated, and C8 Phases. Mobile phase: cholesteric (Col #1) = ACN/water/TFA (10:90:0.05), C8 = ACN/water/TFA (40:60:0.05). Flow rate = 1.0 mL/min, column temperature: 40°C, Detection: Group 1 and 3 = 280 nm, Group 2 = 258 nm. Solutes: Group 1= 1, 2, 3; Group 2 = 4, 5, 6, 7; Group 3 = 8, 9.

Next, the MPAB column was tested with the nucleoside analogue parent and Group 1 pro-drugs. A chromatogram is shown in Fig. 27 and the retention data is shown in Table 6. The elution order of the Group 1 analytes is the same as observed for the two cholesterol columns and the C8 column. Retention increased with increasing log P (Fig. 28), as in reverse phase chromatography. At 40% acetonitrile in the mobile phase, the Group 1 pro-drugs eluted earlier than on the C8 column, so it is concluded that the MPAB phase is more hydrophobic than the C8 phase. Log K' was plotted against log P in Fig. 29. If Group 2 and 3 pro-drugs were tested on the MPAB phase, then perhaps more conclusions could be made as to its separation capabilities.

Fig. 27. Chromatograms of nucleoside analogue parent and pro-drugs in MPAB phase. Mobile phase: ACN/water/TFA (10:90:0.05). Flow rate = 1.0 mL/min, column temperature = 40°C. Detection: Group 1 and 3 = 280 nm, Group 2 = 258 nm and. Solutes: Group 1 = 1, 2, 3; Group 2 = 4, 5, 6, 7; Group 3 = 8, 9.

Table 6. Log P's and retention factors of nucleoside analogue parent and pro-drugs at different mobile phase compositions ran on the MPAB and C8 phases grouped according to structure

Group	No.	Cmpd	Log P*	Retention factor (K')							
				Acetonitrile (%)							
				40	30	25	20	15	10	0	40
				MPAB							C8
Group 1	parent	9187	-1.94	0.308	0.334	0.373	0.393	0.422	0.459	0.587	-0.030
	1	4199	2.28	0.895	1.712	2.752	4.364				2.655
	2	3740	3.18	1.186	2.651	4.682	8.209				5.394
	3	3741	4.77	2.267	7.506	16.326					20.837

* Calculated log P

Fig. 28. Retention factor vs. % acetonitrile in the mobile phase for nucleoside analogue parent and pro-drugs on the MPAB phase. Solutes: Group 1 = 1, 2, 3.

Fig. 29. Log retention factor vs. log P for nucleoside analogue pro-drugs for MPAB Phase. Mobile phase: ACN/water/TFA (40:60:0.05). Column temperature: 40°C, Detection: 280 nm. Solutes: Group 1 = 1, 2, 3.

To test the temperature effects, the Group 1 nucleoside analogue pro-drugs were run on the cholesterol (Col #1) column with temperatures ranging from 30 to 50°C using a mobile phase of acetonitrile/water/TFA (30:70:0.05). The chromatograms in Fig. 30 and data in Table 7 show that the retention decreases with increasing temperature. Plots

of $\log K'$ vs. $1/T$ for the cholesterol (Col #1) show that the retention increases linearly, as expected for a typical reverse phase column in the range of 30 to 50°C. The effects of temperature on the MPAB phase were also studied using the nucleoside analogue parent and group 1 pro-drugs at column temperatures 30 to 60°C. As seen in Fig. 31 and Table 8, the elution order for these compounds on the MPAB phase is the same as for the cholesteric (Col #1) phase. Retention decreased with increasing temperature for these nucleoside analogues from 30 to 60°C (Fig. 32). Perhaps retention at temperatures outside the range studied, or with group 2 and 3 pro-drugs would show some non-linearity.

Fig. 30. Chromatograms of nucleoside analogue parent and Group 1 pro-drugs in cholesteric (300Å) phase. Mobile phase: ACN/water/TFA (15:85:0.05). Flow rate = 1.0 mL/min, column temperature = 30, 40, 50°C. Detection: 280 nm. Solutes: Group 1= 1, 2, 3.

Table 7. Log P and retention factors of group 1 nucleoside analogue pro-drugs at different temperatures ran on the cholesteric (Col #1) phase, mobile phase: ACN/water/TFA (30:70:0.05)

RON water 177A (55.75:5.55)					
Group	No.	Log P*	Retention factor (K')		
			Temperature (°C)		
			30	40	50
Cholesteric (Col #1)					
Group 1	1	2.28	2.67	2.77	3.55
	2	3.18	3.45	3.76	4.67
	3	4.77	9.46	12.15	18.84

* Calculated log P

Fig. 31. Log retention factor vs. 1/Temperature for nucleoside analogue parent and pro-drugs on the cholesteric (Col #1) phase. Mobile phase: ACN/water/TFA (15:85:0.05). Flow rate = 1.0 mL/min, column temperature: 30, 40, 50°C, Detection = 280 nm. Solutes: parent, Group 1 = 1, 2, 3.

Fig. 32. Chromatograms of nucleoside analogue parent and Group 1 pro-drugs in MPAB phase. Mobile phase: ACN/water/TFA (30:70:0.05). Flow rate = 1.0 mL/min, column temperature = 30, 40, 50, 60°C. Detection: 280 nm. Solutes: Parent, Group 1= 1, 2, 3.

Table 8. Log P's and retention factors of Group 1 nucleoside analogue pro-drugs at different temperatures ran on the MPAB phase, mobile phase: ACN/water/TFA (30:70:0.05)

Group	No.	Log P*	Retention factor (K')			
			Temperature (°C)			
			30	40	50	60
MPAB						
Group 1	parent	-1.94	0.35	0.33	0.32	0.31
	1	2.28	2.03	1.71	1.42	1.20
	2	3.18	3.22	2.65	2.13	1.74
	3	4.77	9.82	7.51	5.55	4.21

* Calculated log P

Fig. 33. Log retention factor vs. 1/Temperature for nucleoside analogue parent and prodrugs on the MPAB phase. Mobile phase: ACN/water/TFA (30:70:0.05). Flow rate = 1.0 mL/min, column temperature: 30, 40, 50°C, Detection = 280 nm. Solutes: parent, Group 1 = 1, 2, 3.

3.2 Purine Derivatives

Six derivatives of a purine backbone, similar to caffeine shown in Fig. 34, were analyzed using the cholesteric (Col #3) column and then compared to the C8 column (Col # 7) using acetonitrile/water/TFA, 0.05% for mobile phase. These purine derivatives have three attachments on the purine backbone. The Group A compound has one nitrogen containing ring, one phenyl ring and one hydrocarbon chain. Group B compounds contain two phenyl groups and one hydrocarbon chain attachment. Finally, the Group C compound has two phenyl rings and an oxygen containing ring. The molecular weights, pKa's and calculated log P's are shown in Table 9.

Fig. 34. Caffeine

Table 9. Molecular weight, pKas, and log P for purine derivatives grouped according to structure

Group	No.	MW	ApKa	BpKa	Log P*
Group A	1	428	-1.70	4.39	3.398
Group B	2	462	-1.22	4.76	4.88
	3	462	-1.26	4.51	4.606
	4	459	2.34	2.34	4.693
	5	446	-1.07	-1.07	5.556
Group C	6	440	-1.17	-1.17	5.061

* Calculated log P

Representative chromatograms for 35% acetonitrile in the mobile phase are shown in Fig. 35, and retention data is shown in Table 10. The peak shape is good for both columns, but the USP tailing factor is slightly better for the C8 column (1.09 to 1.64 for cholesterol, and 0.94 to 0.97 for C8). The efficiency for the cholesteric phase is on the same order of magnitude as for the C8 phase (USP Plate Count for compound 6 = 6,375 on cholesterol, and 12,227 on C8). The elution order on the cholesterol column is the same as for the C8 column. For all compounds, the retention decreases with increasing organic content in the mobile phase on the cholesterol column like reverse phase chromatography (Fig. 36). The log K' vs. % acetonitrile decreases linearly as shown in Fig. 37 and Table 11.

As the chromatograms of the test mixture show, peaks for compounds 1 and 2 are unresolved on the cholesterol column, but separated on the C8 column. However, when single injections are performed at 25 to 40% acetonitrile in the mobile phase, the retention of compound 1 is slightly longer than the retention of compound 2. For example, at 35% acetonitrile, the retention times are 7.954 and 7.847 minutes, for compounds 1 and 2, respectively. This elution order is opposite than for the C8 column

at 35% acetonitrile, where the retention times are 3.794 minutes for compound 1 and 4.592 minutes for compound 2. At 25% acetonitrile on the cholesterol column, the peaks still cannot be separated when the mixture is injected. Compounds 1 and 2 have different hydrophobicities ($\log P = 3.398$ and 4.88), molecular weights, and different functional groups at the three attachment positions. Perhaps a different separation mechanism other than hydrophobicity is occurring for at least one of these compounds on the cholesteric phase. In contrast, compounds 2 and 3, which are structural isomers with different $\log P$'s, are separated on both columns.

When $\log K'$ is plotted against the $\log P$ for the purine derivatives (Fig 38.), the trend for the cholesteric phase is similar to the C8, and the points are lower (shorter retention). For both columns, a correlation between the retention factor (K') and hydrophobicity ($\log P$) is not observed for the six derivatives or within each structural group. If a greater number of samples in each structural group were tested, a trend for each group might be observed.

To investigate the effects of temperature on retention, the purine derivatives were run on the cholesterol and C8 columns having column temperatures ranging from 27 to 60°C, using a mobile phase of acetonitrile/water/TFA (30:70:0.05). The chromatograms for the temperature study are shown in Fig. 39. As seen in the Van Hoff's plots (Fig. 40), the retention decreases linearly with increasing temperature for the cholesteric phase, with correlation coefficients of 0.995 to 0.997 for the regression lines. This linearity is the expected result for a typical reverse phase column and is observed for the C8 column. Perhaps at temperatures outside of this range (27 to 60°C), non-linearity would be

observed. For the temperatures tested, solutes 1 and 2 remain co-eluted using 30% acetonitrile. These results demonstrate that the cholesteric phase can separate some purine derivatives. The cholesteric phase might have a different separation mechanism than the C8 column for this class of molecules, as demonstrated by the co-elution of two very structurally and hydrophobicity different molecules (compounds 1 and 2).

Fig. 35. Chromatograms of purine derivatives on (A) cholesteric and (b) C8 phases. Mobile phase: ACN/water/TFA (35:65:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 272 nm. Solutes: Group A = 1; Group B = 2, 3, 4, 5; Group C = 6.

Table 10. Log P and retention factors for purine derivatives at different mobile phase compositions ran on the cholesteric and C8 phases grouped according to structure

Group	No.	Log P*	Retention factor (K')				
			Acetonitrile concentration in water (%)				
			40	35	30	25	35
			Cholesteric				C8
Group A	1	3.398	2.553	4.012	6.855	13.314	3.833
Group B	2	4.88	2.462	3.945	6.834	13.132	4.638
	3	4.606	3.278	5.423	9.652	18.996	7.036
	4	4.693	3.867	6.652	12.217	24.510	11.340
	5	5.556	5.429	9.632	18.329		15.315
Group C	6	5.061	6.147	10.894	21.345		18.072

* Calculated log P

Fig. 36. Retention Factor vs. % acetonitrile in the mobile phase for purine derivatives ran on the cholesteric phase. Solutes: Group A = 1; Group B = 2, 3, 4, 5; Group C = 6.

Fig. 37. Log K' vs. % acetonitrile in the mobile phase for purine derivatives run on the cholesteric phase. Solutes: Group A = 1; Group B = 2, 3, 4, 5; Group C = 6.

Table 11. Regression equations for log K' vs. % acetonitrile in the mobile phase for Purine Derivatives ran on the cholesteric phase

Compound	Regression Equation	R^2
1	$y = -0.0477x + 2.2927$	0.9926
2	$y = -0.0484x + 2.308$	0.9947
3	$y = -0.0508x + 2.529$	0.9956
4	$y = -0.0534x + 2.7071$	0.9969
5	$y = -0.0528x + 2.8433$	0.9989
6	$y = -0.0541x + 2.944$	0.9978

Fig. 38. Log retention factor vs. log P in the mobile phase for purine derivatives for cholesteric and C8 Phases. Mobile phase: ACN/water/TFA (35:65:0.05). Flow rate = 1.0 mL/min, column temperature: 40°C, Detection = 272nm. Solutes: Group A = 1; Group B = 2, 3, 4, 5; Group C = 6.

Fig. 39. Chromatograms of purine derivatives in (A) cholesteric and (b) C8 phases. Mobile phase: ACN/water/TFA (30:70:0.05). Flow rate = 1.0 mL/min, column temperature = 27, 30, 40, 50, 60°C, Detection = 272 nm. Solutes: Group A = 1; Group B = 2, 3, 4, 5; Group C = 6.

A **B**

Fig. 40. Log retention factor vs. 1/Temperature for purine derivatives on the (A) cholesteric and (B) C8 phases. Mobile phase: ACN/water/TFA (30:70:0.05). Flow rate = 1.0 mL/min, column temperature: 27 to 90°C, Detection = 272nm. Solutes: Group A = 1; Group B = 2, 3, 4, 5; Group C = 6.

3.3 Steroids

Six steroid-type drugs which are very similar to cholesterol, and also cholesterol (shown in Fig. 41) were tested on the cholesterol (Col #3) column and then compared to the C8 column (Col #7). Steroids were chosen to study because they are often not

separated by typical reverse phase conditions, but some researchers have been successful in separating them using the cholesteric phase (1). The six drugs tested in this research are similar to each other, except that some contain hydroxyl groups and fluorine atoms. In addition, the hydrocarbon tail on the five carbon steroid ring is different. The molecules are grouped together based on their elution order and not on their structure. The molecular weights and log P's are shown in Table 12. The mobile phase for the separations consisted of acetonitrile and water.

Fig. 41. Structure of cholesterol

Table 12. Molecular weight, log P and pKa's for steroids

Group	No.	MW	ApKa	Log P*
Group 1	1	348.48	13.39	3.209
Group 2	2	376.53		4.523
	3	388.47	4.93, 12.78	5.803
Group 3	4	386.66		9.520
	5	320.51		4.883
	6	320.51		4.883
	7	304.51		7.09

* Calculated log P

Representative chromatograms are shown in Figs. 42 and 43, and retention data is shown in Table 13 and 14. On the cholesteric phase, compound 1 is retained the least followed by Group 3 compounds. The Group 2 compounds are retained the longest, and

co-elute with each other at 25 to 50% acetonitrile. The Group 3 steroids also elute together for the % acetonitrile used (30 to 50%). The elution order of these steroids is the same on the C8 phase as on the cholesteric phase. In addition, the Group 2 and Group 3 molecules co-elute together within each group. Retention on both columns increases with decreasing % acetonitrile (Fig. 44), as expect for reverse phase chromatography. The log K vs. log P plot is shown in Fig. 45 for 30% acetonitrile in the mobile phase. There is no observable relationship between log K and log P. Retention does not seem to increase for increasing Log P on either column. The log K data for the cholesteric phase matches the data for the C8 phase, but is lower on the graph since the cholesteric phase is effectively less hydrophobic than the C8 phase. Some researchers have reported separating steroids with cholesterol columns using methanol in the mobile phase (1). Perhaps if methanol was used as the organic modifier, the Group 2 and Group 3 molecules could be separated.

3.4 Anthracenes

Two groups of anthracene drugs and also anthracene (Fig. 46) were tested on the cholesterol (Col #3) column and then compared to the C8 column (Col #7) to study how the molecular structure of planar molecules affects retention. The Group A compounds include anthracene plus drugs which have one attachment on the end ring. It also includes an impurity of one of the Group A molecules, with the structure being unknown. The group B anthracenes contain one attachment on the middle ring of the anthracene backbone. For both Group A and Group B drugs, the molecular moieties attached to the

anthracene backbone are hydrocarbon chains containing one phenyl group. Molecular

Fig. 42. Chromatograms of steroids on cholesteric phase. Mobile phase: (A) ACN/water (40:60) and (B) ACN/water (30:70), Flow rate = 1.0 mL/min, Column temperature = 30°C, Detection = 285 nm. Solutes: Group 1 = 1; Group 2 = 2, 3; Group 3 = 4-cholesterol; 5, 6, 7.

Fig. 43. Chromatograms of steroids on C8 phase using (A) gradient and (B) isocratic elution. Mobile phase: ACN/water, gradient = 2 to 95 %ACN in 25 minutes and isocratic = 25% ACN, Flow rate = 1.0 mL/min, Column temperature = 30°C, Detection = 285 nm. Solutes: Group 1 = 1; Group 2 = 2, 3; Group 3 = 4-cholesterol; 5, 6, 7.

Table 13. Log P and retention factors of steroids with different mobile phase compositions ran on the cholesteric phase

Compositions fall on the cholesteric phase						
Group	No.	Log P*	Retention factor (K')			
			Acetonitrile (%)			
			50	40	30	25
			Cholesteric			
Group 1	1	3.209	1.175	2.288	5.824	10.803
Group 2	2	4.523	2.161	4.832	12.459	22.057
	3	5.803	2.170	4.934	12.942	22.789
Group 3	4 **	9.520	1.696	3.405	7.250	10.728
	5	4.883	1.714	3.422	7.260	10.751
	6	4.883	1.711	3.417	7.253	
	7	7.09	1.712	3.417	7.242	

* Calculated log P, ** Cholesterol

Table 14. Log P and retention factors of steroids with different mobile phase compositions run on the C8 phase

Compositions run on the C8 phase							
Group	No.	Log P*	Retention factor (K')				
			Acetonitrile (%)				
			50	40	35	30	25
			C8				
Group 1	1	3.209	0.975	2.151	3.681	7.266	17.556
Group 2	2	4.523	2.855	6.819	11.306	19.868	37.339
	3	5.803	2.854	6.821	11.309	19.888	37.394
Group 3	4 **	9.520	3.214	7.081	11.072	17.750	27.682
	5	4.883	3.256	7.125	11.166	17.785	29.775
	6	4.883	3.253	7.129	11.161	17.799	29.683
	7	7.09	3.251	7.125	11.161	17.851	29.613

* Calculated log P, ** Cholesterol

A

B

Fig. 44. Retention factor vs. % acetonitrile in the mobile phase for steroids run on the (A) cholesteric and (B) C8 phases. Solutes: Group 1 = 1; Group 2 = 2, 3; Group 3 = 4-cholesterol; 5, 6, 7.

Fig. 45. Log Retention factor vs. log P for steroids ran on the cholesteric and C8 Phases. Mobile phase: ACN/water (30:70). Flow rate = 1.0 mL/min, Column temperature = 30°C, Detection = 285 nm. Solutes: Group 1 = 1; Group 2 = 2, 3; Group 3 = 4-cholesterol; 5, 6, 7.

weights and log Ps are shown in Table 15. Anthracene has the lowest log P of all ten solutes and so is effectively the least hydrophobic of the compounds tested. The mobile phase for the experiments consisted of acetonitrile, water and trifluoroacetic acid.

Representative chromatograms for Group A and Group B at different percent organic in the mobile phase are shown in Figs. 47 to 56. Retention data are shown in Table 16. For the group A anthracenes, the solutes are reasonably well separated on the C8 phase for all percentages of acetonitrile tested (70 – 40%). However, for the cholesteric phase, the Group A anthracenes are not separated at 70 or 65% acetonitrile because compounds 1 and 2 (an impurity of compound 4) co-elute together. In addition, compounds 3 and 4 co-elute. At 60 to 50% acetonitrile in the mobile phase, Group A compounds are fairly well separated on the cholesterol column. At 45% acetonitrile, complete separation of the five Group A molecules was achieved on the cholesteric

Fig. 46. Structure of anthracene; Group A anthracenes have an attachment on the end ring; Group B anthracenes have an attachment on the middle ring

Table 15. Molecular Weights and log Ps of anthracene compounds grouped according to structure

Group	No.	MW	Log P*
Group A	1	399.488	6.305
	2 **	N/A	N/A
	3	419.565	7.518
	4	435.608	7.251
	5 ***	178.233	4.49
Group B	1	385.48	5.431
	2	381.516	5.517
	3	381.516	5.787
	4	401.935	6.001
	5	446.386	6.151
	6	375.512	7.005

* Calculated log P

** Impurity of Compound A-4

*** Anthracene

phase (Fig. 52). For group B anthracenes, both phases separate the solutes about the same, and fairly well. A representative chromatogram at 50% acetonitrile is shown in Fig. 56.

In Figs. 57 and 58, retention is plotted against % acetonitrile in the mobile phase and in Figs. 59 and 60, log K' vs. % acetonitrile is shown. For both phases, retention increases with decreasing organic content in the mobile phase like reverse phase chromatography. The retention of anthracene is lower on the cholesteric phase than on

the C8 column as expected since the cholesteric phase is less hydrophobic. However, the other Group A compounds and the Group B compounds are retained longer on the cholesterol phase than on the C8 phase. Perhaps another mechanism other than hydrophobicity is operative on the cholesteric phase which makes the anthracene drugs interact to a greater extent with the stationary phase. The higher retention of these compounds on the cholesterol column could be due to their shape. As mentioned earlier, these compounds contain planar phenyl groups on the hydrocarbon tails. Possibly, the planar groups might fit into the ordered structure of the cholesterol causing them to interact longer with the stationary phase.

When looking at the chromatograms (Figs. 54 and 55), and retention data (Tables 16 – 19) for Group A, it is observed that the elution order on the cholesteric phase does not match the elution order on the C8 phase for all percentages of acetonitrile. Elution order is summarized in Table 20. On the C8 column for high % acetonitrile (70 and 65%) the elution order is compound 2 first, followed by 1, 3, 4, and then 5 eluting last. At all other percentages of acetonitrile tested for the C8 phase, the elution order is compound 1 first, followed by 2, 3, 4, and then 5 eluting last. On the cholesteric phase for 70% acetonitrile, the order of elution is compound 1 and 2 (co-eluting), then 4, followed by compounds 3 and 5 (co-eluting). At 65% acetonitrile, the elution order on the cholesterol column is slightly different: compounds 1 and 2 (co-eluting), 4, 5 and 3 (co-eluting). When the % acetonitrile in the mobile phase is 60% or 55%, the compounds are fairly well separated with the elution order of: compound 1 eluting first, then 2, 4, 5, with 3 being last. At 50% acetonitrile, the elution order on the cholesteric phase is 1, 2, 5,

4, with 3 still being last. Compounds 4 and 5 switch elution order when the % acetonitrile is decreased from 55 to 50%. At 45% acetonitrile, the elution order on the cholesteric phase is 1, 2, 5, 4, with 3 still being last. And finally, at 40% acetonitrile, the order on the cholesteric phase is 1, 5, 2, 4, with 3 still being last. Therefore, for 40 to 60% acetonitrile, compound 3 elutes last and compound 1 elutes first, but compounds 2, 4 and 5 will switch orders on the cholesteric phase as the % organic is changed. As the % organic decreases, the retention of anthracene compared to the other four anthracene compounds is decreasing. Or rather, the retention of the compounds 1 to 4 is increasing more relative to anthracene with decreasing organic composition in the mobile phase. Perhaps these anthracene drugs and impurity are interacting with the cholesteric stationary phase more than anthracene because of their planar structure, as previously mentioned.

The elution order for the Group B anthracenes on the cholesteric phase matches the order on the C8 phase (Fig. 56). Also, the elution order does not change when the % acetonitrile is changed. One interesting finding is that anthracene elutes later than the Group B solutes on both columns for the % acetonitrile tested even though its log P is the lowest. This result is different than what was observed for Group A compounds. For Group A compounds, anthracene eluted last on the C8 phase for all percentages of acetonitrile, but eluted within the Group A compounds from 40 to 60% acetonitrile on the cholesteric phase.

Log K' is plotted against log P for both phases at 55 and 50% acetonitrile in the mobile phase (Fig. 61). For Group B anthracene drugs, log K' increases with increasing

log P, indicating that for these solutes, retention increases with increasing hydrophobicity. This result is expected for reverse phase separations. On the other hand, for Group A anthracene drugs (which have the attachment on the end ring), a relationship between log K' and log P is not observed for either stationary phase. The trend for log K' vs. log P for the cholesteric phase does not match the C8 phase for Group A solutes. The differing plot indicates a different mechanism is occurring on the cholesteric phase. As previously mentioned, the retention of all compounds except anthracene, is lower on the C8 phase than on the cholesteric phase. If hydrophobicity was the only mechanism of separation on the cholesteric phase, it is expected that elution would be earlier on the cholesteric phase since it is less hydrophobic than the C8 phase. These experiments have shown that the cholesterol can separate anthracene drugs differently than a typical C8 column probably due to their shape. The molecular modeling program Chem3D Plus would be useful for investigating the possible interactions of molecular shape to the stationary phase (4).

3.5 Polar Compounds

Nine polar drugs were tested on several cholesteric columns to study the aqueous normal phase (ANP) mode of chromatography. As stated previously, ANP is defined as normal phase chromatography using polar elution solvents such as acetonitrile and water. The retention of solutes increases with decreasing aqueous %, in contrast to reverse phase chromatography, where the retention decreases with decreasing aqueous %. ANP could be utilized for polar solutes which are poorly retained on a typical reverse phase column

Fig. 47. Chromatograms of Group A anthracenes run on the (A) cholesteric and (B) C8 phases with 70% acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA (70:30:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 48. Chromatograms of Group A anthracenes run on the (A) cholesteric and (B) C8 phases with 65% acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA (65:35:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 49. Chromatograms of Group A anthracenes run on the (A) cholesteric and (B) C8 phases with 60% acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA (60:40:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 50. Chromatograms of Group A anthracenes run on the (A) cholesteric and (B) C8 phase with 55% acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA (55:45:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 51. Chromatograms of Group A anthracenes run on the (A) cholesteric and (B) C8 phase with 50% acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA (50:50:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 52. Chromatograms of Group A anthracenes run on the (A) cholesteric and (B) C8 phases with 45% acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA (45:55:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 53. Chromatograms of Group A anthracenes run on the (A) cholesteric and (B) C8 phases with 40% acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA (40:60:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 54. Chromatograms of Group A anthracenes run on the cholesteric phase with different % acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA, 0.05%; from 70 to 40% ACN. Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 55. Chromatograms of Group A anthracenes run on the C8 phase with different % acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA, 0.05%; from 70 to 40% ACN. Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 56. Chromatograms of Group B anthracenes run on the (A) cholesteric and (B) C8 phases with 50% acetonitrile in the mobile phase. Mobile phase: ACN/water/TFA (50:50:0.05), Flow rate = 1.0 mL/min, Column temperature = 40°C, Detection = 254 nm. Solutes: Group B = 1, 2, 3, 4, 5, 6.

Table 16. Log Ps and retention factors of anthracenes ran on the cholesteric phase at different mobile phase compositions grouped according to structure

Group	No.	Log P*	Retention factor (K')							
			Acetonitrile (%)							
			70	65	60	55	50	45	40	30
			Cholesteric							
Group A	1	6.305	0.846	1.088	1.439	1.986	2.845	4.202	6.740	
	2 **	N/A	0.756	1.065	1.569	2.425	4.021	7.219	14.167	
	3	7.518	1.252	1.766	2.613	4.089	6.854	12.399	25.504	
	4	7.251	0.972	1.394	2.088	3.294	5.579	10.212	20.766	
	5 ***	4.49	1.258	1.728	2.433	3.424	5.308	8.275	13.750	
Group B	1	5.431			0.641	0.943	1.422		3.660	12.548
	2	5.517			0.737	1.061	1.589		4.043	13.289
	3	5.787			0.772	1.132	1.721		4.537	15.616
	4	6.001			0.834	1.241	1.910		5.232	19.086
	5	6.151			0.914	1.363	2.112		5.958	22.478
	6	7.005			1.142	1.730	2.733		8.129	

* Calculated log P

** Impurity of compound A-4

*** Anthracene

Table 17. Log Ps and retention factors of anthracenes ran on the C8 phase grouped at different mobile phase compositions according to structure

Group	No.	Log P*	Retention factor (K')						
			Acetonitrile (%)						
			70	65	60	55	50	45	40
			C8						
Group A	1	6.305	0.587	0.793	1.084	1.558	2.317	3.563	6.046
	2 **	N/A	0.495	0.750	1.177	1.924	3.345	6.186	13.495
	3	7.518	0.567	0.866	1.364	2.243	3.928	7.209	16.086
	4	7.251	0.640	0.980	1.556	2.585	4.569	8.624	19.164
	5 ***	4.49	1.577	2.216	3.180	4.738	7.316	11.771	20.310
Group B	1	5.431			0.524	0.807	1.274		3.664
	2	5.517			0.572	0.868	1.358		3.881
	3	5.787			0.636	0.977	1.550		4.586
	4	6.001			0.675	1.049	1.687		5.197
	5	6.151			0.729	1.139	1.842		5.825
	6	7.005			1.099	1.735	2.877		9.686

* Calculated log P

** Impurity of compound A-4

*** Anthracene

Table 18. Log Ps and log K's of anthracenes ran on the cholesteric phase at different mobile phase compositions grouped according to structure

Group	No.	Log P*	Log K'							
			Acetonitrile (%)							
			70	65	60	55	50	45	40	30
			Cholesteric							
Group A	1	6.305	-0.073	0.036	0.158	0.298	0.454	0.623	0.829	
	2 **	N/A	-0.121	0.027	0.196	0.385	0.604	0.858	1.151	
	3	7.518	0.098	0.247	0.417	0.612	0.836	1.093	1.407	
	4	7.251	-0.012	0.144	0.320	0.518	0.747	1.009	1.317	
	5 ***	4.49	0.100	0.238	0.386	0.535	0.725	0.918	1.138	
Group B	1	5.431			-0.193	-0.025	0.153		0.563	1.099
	2	5.517			-0.132	0.026	0.201		0.607	1.123
	3	5.787			-0.112	0.054	0.236		0.657	1.194
	4	6.001			-0.079	0.094	0.281		0.719	1.281
	5	6.151			-0.039	0.134	0.325		0.775	1.352
	6	7.005			0.058	0.238	0.437			

* Calculated log P

** Impurity of compound A-4

*** Anthracene

Table 19. Log Ps and log K's of anthracenes ran on the C8 phase at different mobile phase compositions grouped according to structure

Group	No.	Log P*	Log K'						
			Acetonitrile (%)						
			70	65	60	55	50	45	40
			C8						
Group A	1	6.305	-0.232	-0.101	0.035	0.192	0.365	0.552	0.781
	2 **	N/A	-0.306	-0.125	0.071	0.284	0.524	0.791	1.130
	3	7.518	-0.246	-0.062	0.135	0.351	0.594	0.858	1.206
	4	7.251	-0.194	-0.009	0.192	0.413	0.660	0.936	1.282
	5 ***	4.49	0.198	0.346	0.502	0.676	0.864	1.071	1.308
Group B	1	5.431			-0.281	-0.093	0.105		0.564
	2	5.517			-0.243	-0.062	0.133		0.589
	3	5.787			-0.196	-0.010	0.190		0.661
	4	6.001			-0.171	0.021	0.227		0.716
	5	6.151			-0.137	0.056	0.265		0.765
	6	7.005			0.041	0.239	0.459		0.986

* Calculated log P

** Impurity of compound A-4

*** Anthracene

Table 20. Elution order of Group A anthracenes run on the cholesteric and C8 phases for different % acetonitrile in the mobile phase.

% Acetonitrile	Elution order for each phase	
	Cholesteric	C8
70	(1, 2), 4, (3, 5*)	2, 1, 3, 4, 5
65	(1, 2), 4, (5, 3)	2, 1, 3, 4, 5
60	1, 2, 4, 5, 3	1, 2, 3, 4, 5
55	1, 2, 4, 5, 3	1, 2, 3, 4, 5
50	1, 2, 5, 4, 3	1, 2, 3, 4, 5
45	1, 2, 5, 4, 3	1, 2, 3, 4, 5
40	1, 5, 2, 4, 3	1, 2, 3, 4, 5

* Compound 5 is anthracene

and as another mode of separation. The nine polar drugs tested were grouped according to their backbone structure. Group A drugs, which are tetramic acids, include a parent drug (compound 1) and two of its pro-drugs (compounds 2 and 3). Group B compounds contain the quinolinedione structure and are pro-drugs having the same parent molecule. Finally, Group C solutes are nucleoside analogues which have a backbone similar to the nucleoside cytidine. Compound 9 is a parent drug. The other two group C compounds, 7 and 8, are a mixture of two isomers and are pro-drugs (compound 9 not being the parent). The structures of saccharine, thiazine and cytidine are shown in Fig. 62. The molecular weight, pKa's and log Ps are shown in Table 21. Solute 9 has the lowest log P (-0.936). The log P of the Group C isomers is 1.827, while the rest of the polar compounds tested have log Ps ranging from 2.739 to 3.861.

Experiments were performed with methanol and water for the mobile phase. Representative chromatograms for the first cholesterol column (Col #4) studied are shown in Figs. 62 and 63, and retention data are shown in Table 22. For a mobile phase consisting of 45% methanol, all Group A and B solutes (tetramic acids and

A

B

Fig. 57. Retention factor vs. % acetonitrile for the Group A anthracenes run on the (A) cholesteric and (B) C8 phases. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

A

B

Fig. 58. Retention factor vs. % acetonitrile for the Group B anthracenes run on the (A) cholesteric and (B) C8 phases. Solutes: Group B = 1, 2, 3, 4, 5, 6.

Fig. 59. $\text{Log } K'$ vs. % acetonitrile for the Group A anthracenes run on the (A) cholesteric and (B) C8 phases. Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.

Fig. 60. $\text{Log } K'$ vs. % acetonitrile for the Group B anthracenes run on the (A) cholesteric and (B) C8 phases. Solutes: Group B = 1, 2, 3, 4, 5, 6.

A

B

Fig. 61. $\log K'$ vs. $\log P$ for the Group A and B anthracenes run on the cholesteric and C8 phases. Mobile phase consisted of (A) ACN/water/TFA (55:45:0.05) and (B) ACN/water/TFA (50:50:0.05). Solutes: Group A = 1, 2-impurity of compound 4, 3, 4, 5-anthracene.; Group B = 1, 2, 3, 4, 5, 6.

Fig. 62. Structures of (A) tetramic acid, (B) quinolinedione backbone and (C) the nucleoside cytidine

Table 21. Molecular weights, log Ps, pKa's of polar compounds grouped according to structure

Group	No.	MW	ApKa	BpKa	Log P*
A	1	549.6	7.91, 10.98	5.56	3.46
	2	605.7	7.91	5.28	3.315
	3	563.7	7.85	5.45	3.526
B	4	594.0	8.64	3.03	3.861
	5	519.6	8.64	-	2.739
	6	573.6	8.64	3.48	3.647
C	7	618.5	9.3, 13.76	-	1.827
	8	618.5	9.3, 13.76	-	1.827
	9	259.3	11.81	3.73	-0.936

* Calculated log P

quinolinediones) eluted by 4 minutes, whereas the Group C isomers eluted much later at about 21 and 24 minutes. When the methanol was decreased to 40%, the retention of Group A and B compounds increased. Retention factors ranged from 1.103 (compound 1) to 6.408 (compound 4). When the organic composition in the mobile phase was increased to 100% methanol, the retention for Group A and B compounds decreased.

Elution times ranged from 0.635 minutes (compound 2) to 0.642 minutes (compound 6), which are below the void volume of 0.79. These early eluting peaks (Group A and B compounds) had fairly wide peak widths. At 45% methanol, the base peak widths equaled: 1.550, 2.400, 1.767, 1.300, 2.350 and 1.833 for compounds 1, 4, 6, 5, 2 and 3, respectively. These peaks were not separated from each other at the % methanol tested.

Regarding the Group C solutes, the Group C isomers (compounds 7 and 8) elute last for all percentages of methanol tested (40 to 60, and 90 to 100%), even though the log P (1.827) is lower than the log P of all Group A and B compounds (2.739 to 3.861), which means the Group C isomers are less hydrophobic. Perhaps these isomers interact with the stationary phase longer because of their shape or size. In addition, the elution of the Group C isomers is significantly later than the other compounds. For example, at 45% methanol, the K' of Group C isomer peak 1 equaled 25.866, and the K' of the previously eluted peak (compound 6) equaled 2.009. The Group C isomers are fairly well separated from each other at 40 to 50% methanol in the mobile phase, even though the peaks are fairly broad. The USP resolution factor equaled 1.079, 1.306 and 1.530 at 50, 45 and 40% methanol, respectively. The peak widths at 45% methanol were 3.650 for peak 1 and 3.733 for peak 2. Chromatograms for Group C compound 9, which is the parent nucleoside analogue, are shown in Fig. 64. This polar molecule does not interact much with the stationary phase and elutes after only 3.6 minutes at 10% methanol.

The plots of retention factor vs. % methanol and log K' vs. percent methanol for the three polar groups are shown in Figs. 65 and 66. For all compounds, the retention

decreased for increasing % methanol indicating the reverse phase mode of chromatography is occurring.

Next, the compounds (except for compound 9) were tested on three more cholesteric columns (Col #1, Col #3, Col #5). The representative chromatograms using the cholesteric (Col #1) column are shown in Fig. 67, and the retention data is shown in Table 24. Peaks eluted very early and before the void volume ($t_0 = 1.587$) for 90 to 100% methanol. The retention factors ranged from -0.285 to 0.278 for 90% methanol and -0.277 to 0.391 for 100% methanol. The K' for this column was plotted against percent methanol in Fig. 68. For some compounds, the retention increased slightly when the methanol composition was increased from 90 to 100%. Although the retention values are very low at these % methanol compositions and the retention increase is slight, ANP mode may be occurring.

For the next cholesteric column studied (Col #3), the compounds also eluted before the void ($t_0=1.587$) for 90 to 100% methanol as the mobile phase (Fig. 69 and Table 25). The plot of K' vs. percent methanol shows that the Group B quinolinedione compounds (4, 5 and 6) decrease their retention when the percent methanol increases from 90 to 100% methanol as expected for reverse phase chromatography (Fig. 70). For the Group A tetramic acid compounds (1, 2 and 3), retention decreases from 90 to 95% methanol and then increases from 95 to 100% methanol. Finally, the Group C nucleoside analogue isomer (compound 7) increases retention with increasing methanol (90 to 100%). The increases observed are extremely small, but some aqueous normal phase mode might be occurring.

The last column tested with these polar solutes was the other short cholesterol column (Col #5). As all other columns, the compounds elute before the void volume ($t_0 = 0.79$) with 90 to 100% methanol as the mobile phase (Fig. 71). The retention factors equaled -0.200 to -0.181 for the Group A and B solutes, and 0.408 for the Group C compound 7 at 90% methanol. The K 's were -0.205 to -0.171 for the Group A and B solutes, and 0.349 for the Group C compound 7 at 100% methanol (Table 26). Compound 1 showed a slight increase in retention when methanol is increased from 90 to 100%.

Although it appears that some aqueous normal phase chromatography is occurring on the three columns, the data is derived from peaks eluting before the void volume. In addition, since the increases are so small, they might be insignificant and lie within the error range. Therefore, no conclusion can be made regarding whether aqueous normal phase mode is happening with these solutes. Perhaps if more data points were collected for the last three columns, the ANP mode would be more clearly observed.

3.6 Chiral Compounds

Eight chiral drugs were tested on the cholesteric column (Col# 4) to study the chiral separation capabilities of the cholesteric phase. The Group A drugs are nucleoside analogues with the backbone being similar to cytidine (Fig. 73). They include compound A-2 which is a parent drug with one chiral center (the D form), its isomer (the L form), and two of its pro-drugs. The D form was studied in the polar compound section (3.4)

Fig. 63. Representative chromatogram for polar solutes run on the cholesteric (Col #4) phase with 45% methanol in the mobile phase. (A) Individual Group A and B and C solutes (B) Test mix of Groups A, B, and C polar compounds. Mobile phase: MeOH/Water (45:55), Flow rate = 1.0 mL/min, Column temperature = ambient, Detection = 220 nm. Solutes: Group A = 1, 2, 3; Group B = 4, 5, 6; Group C = 7, 8, 9.

Fig. 64. Representative chromatograms for Group C polar compound 9 run on the cholesteric (Col #4) phase with different % methanol in the mobile phase. Mobile phase: MeOH/Water (10 to 80% MeOH), Flow rate = 1.0 mL/min, Column temperature = ambient, Detection = 220 nm. Solutes: Group C = 9.

Table 22. Retention times of polar compounds run on the cholesteric (Col #4) phase with different mobile phase compositions grouped according to structure

Retention time (minutes) - Cholesteric (Col # 4)										
Grp	No	Methanol (%)								
		40	45	50	60	70	80	90	95	100
A	1	1.661	1.069	0.882	0.645	0.622	0.609	0.641	0.638	0.641
	2	3.438	1.787	1.046	0.692	0.647	0.603	0.63	0.633	0.635
	3	2.523	1.489	1.034	0.689	0.64	0.613	0.637	0.638	0.64
B	4	5.852	2.752	1.277	0.716	0.689	0.62	0.64	0.636	0.64
	5	3.217	1.751	1.058	0.675	0.645	0.608	0.639	0.636	0.638
	6	4.872	2.377	1.223	0.713	0.686	0.619	0.642	0.638	0.642
C	7	47.07	21.224	10.704				1.112	1.036	1.074
	8	54.002	24.239	12.055						
		10	20	60	70	80				
	9	3.628	2.275	1.091	1.064	1.051				

t₀ = 0.79

$t_0 = 0.79$

Table 23. Retention factors of polar compounds run on the cholesteric (Col #4) phase with different mobile phase compositions grouped according to structure

Grp		No	Retention factor (K') - Cholesteric (Col # 4)								
			Methanol (%)								
			40	45	50	60	70	80	90	95	100
A	1	1.103	0.353	0.116	-0.184	-0.213	-0.229	-0.189	-0.192	-0.189	
	2	3.352	1.262	0.324	-0.124	-0.181	-0.237	-0.203	-0.199	-0.196	
	3	2.194	0.885	0.309	-0.128	-0.190	-0.224	-0.194	-0.192	-0.190	
B	4	6.408	2.484	0.616	-0.094	-0.128	-0.215	-0.190	-0.195	-0.190	
	5	3.072	1.216	0.339	-0.146	-0.184	-0.230	-0.191	-0.195	-0.192	
	6	5.167	2.009	0.548	-0.097	-0.132	-0.216	-0.187	-0.192	-0.187	
C	7	58.582	25.866	12.549				0.408	0.311	0.359	
	8	67.357	29.682	14.259							
		10	20	60	70	80					
	9	3.592	1.880	0.381	0.347	0.330					

t₀ = 0.79

$t_0 = 0.79$

A

B

Fig. 65. Retention factor vs. % methanol in the mobile phase for polar compounds run on the cholesteric (Col #4) phase. Solutes: (A) Group A = 1, 2, 3; Group B = 4, 5, 6 and (B) Group C = 7, 8, 9.

A

B

Fig. 66. Log K' vs. % methanol in the mobile phase for polar compounds run on the cholesteric (Col #4) phase. Solutes: (A) Group A = 1, 2, 3; Group B = 4, 5, 6 and (B) Group C = 7, 8, 9.

Fig. 67. Representative chromatogram for polar compounds run on the cholesteric (Col #1) phase with 10 % methanol in the mobile phase. (A) Individual Group A and B and C compounds (B) Test mix of Groups A, B, and C polar compounds. Mobile phase: MeOH/Water (90:10), Flow rate = 1.0 mL/min, Column temperature = ambient, Detection = 220 nm. Solutes: Group A = 1, 2, 3; Group B = 4, 5, 6; Group C = 7, 8, 9.

Table 24. Retention factors of polar compounds run on the cholesteric (Col #1) phase with different mobile phase compositions grouped according to structure

Grp	No	Retention factor (K')		
		Cholesteric (Col # 1)		
		Methanol (%)		
		90	95	100
A	1	-0.256	-0.272	-0.263
	2	-0.282	-0.278	-0.274
	3	-0.279	-0.275	-0.271
B	4	-0.277	-0.272	-0.270
	5	-0.285	-0.276	-0.277
	6	-0.282	-0.273	-0.271
C	7	0.146	0.156	0.149
	8	0.278	0.304	0.391

$t_0 = 1.587$

Table 25. Retention factors of polar compounds run on the cholesteric (Col #3) phase with different mobile phase compositions grouped according to structure

Group	No	Retention factor (K')		
		Cholesteric (Col #3)		
		Methanol (%)		
		90	95	100
A	1	-0.272	-0.331	-0.327
	2	-0.336	-0.347	-0.337
	3	-0.332	-0.340	-0.335
B	4	-0.257	-0.323	-0.328
	5	-0.297	-0.335	-0.335
	6	-0.285	-0.331	-0.331
C	7	0.255	0.265	0.313
	8		0.361	

$t_0 = 0.79$

Table 26. Retention factors of polar compounds run on the cholesteric (Col #5) phase with different mobile phase compositions grouped according to structure

Group	No	Retention factor (K')		
		Cholesteric (Col # 5)		
		Methanol (%)		
		90	95	100
A	1	-0.195	-0.180	-0.171
	2	-0.185	-0.187	-0.185
	3	-0.181	-0.182	-0.176
B	4	-0.200	-0.195	-0.201
	5	-0.199	-0.203	-0.205
	6	-0.194	-0.197	-0.197
C	7	0.408	0.315	0.349
	8		0.410	

$t_0 = 0.79$

A

B

Fig. 68. Retention factor vs. % methanol in the mobile phase for polar compounds run on the cholesteric (Col #1) phase. Solutes: (A) Group A = 1, 2, 3; Group B = 4, 5, 6 and (B) Group C = 7, 8, 9.

Fig. 69. Representative chromatograms for polar compounds run on the cholesteric (Col #3) phase with 10% methanol in the mobile phase. (A) Individual Group A and B and C compounds (B) Test mix of Group A, B, and C polar compounds. Mobile phase: MeOH/Water (90:10), Flow rate = 1.0 mL/min, Column temperature = ambient, Detection = 220 nm. Solutes: Group A = 1, 2, 3; Group B = 4, 5, 6; Group C = 7, 8, 9.

A

B

Fig. 70. Retention factor vs. % methanol in the mobile phase for polar compounds run on the cholesteric (Col #3) phase. Solutes: (A) Group A = 1, 2, 3; Group B = 4, 5, 6 and (B) Group C = 7, 8, 9.

Fig. 71. Representative chromatograms for polar compounds run on the cholesteric (Col #5) phase for 10% methanol in the mobile phase. (A) Individual Group A and B and C solutes (B) Test mix of Group A, B, and C polar compounds. Mobile phase: MeOH/Water (90:10), Flow rate = 1.0 mL/min, Column temperature = ambient, Detection = 220 nm. Solutes: Group A = 1, 2, 3; Group B = 4, 5, 6; Group C = 7, 8, 9.

A

B

Fig. 72. Retention factor vs. % methanol in the mobile phase for polar solutes run on the cholesteric (Col #5) phase. Solutes: (A) Group A = 1, 2, 3; Group B = 4, 5, 6 and (B) Group C = 7, 8, 9.

(labeled in the polar compound section as Group C compound 9). The two pro-drugs were included in these experiments to study the aqueous normal phase mode of chromatography in addition to chiral separations. The Group B solutes have a nitrogen heterocycle aromatic backbone and contain two chiral centers (Fig 72). These four enantiomers are compounds B-1 (the SS form), B-2 (the RS form), B-3 (the RR form) and B-4 (the SR form). The molecular weights, pK_as and LogP_s for the eight chiral compounds are shown in Table 21. Compounds A-1 and A2 (L and D isomers) have the lowest log P (-0.936).

Experiments were performed with methanol/water or acetonitrile/water for the mobile phase using several different flow rates and column temperatures (Table 28). For achieving a column temperature of 0° C, an ice bath was used. Decreased flow rates of 0.7 and 0.5 mL/min were used to avoid high column back pressure. For testing the chiral nucleoside analogues (Group A), the first condition was a mobile phase consisting of acetonitrile/water, a flow rate of 1.0 mL/min and a column temperature of 0° C. Representative chromatograms for Group A solutes using Condition 1 are shown in Fig. 74, and the corresponding retention data are shown in Table 29. At 50% acetonitrile, the L and D isomers (compounds A-1 and A-2) eluted early (1.2 minutes) and were not separated from each other. As the acetonitrile in the mobile phase was decreased, the L and D peaks eluted later, but were still not separated. At all the acetonitrile compositions used (20 to 50%) with condition 1, the L and D isomers eluted together. Retention factors ranged from 1.156 at 20% acetonitrile to 0.134 at 50% acetonitrile. The plots of retention factor vs. % acetonitrile and log K' vs. % acetonitrile show that the retention

decreases with increasing acetonitrile as expected for reverse phase chromatography (Figs. 75 and 76).

Fig. 73. Structures of (A) the nucleoside cytidine which has one chiral center and (B) the nitrogen heterocycle aromatic backbone which has two chiral centers

Table 27. Molecular weights, log Ps, pKa's of chiral compounds grouped according to structure

Group	No.	Description	MW	ApKa	BpKa	Log P*
A	1	L	259.24	11.81	3.73	-0.936
	2	D	259.24	11.81	3.73	-0.936
	3	Pro-drug of 2	371.36		3.73	1.27
	4	Pro-drug of 2	399.42		3.73	2.328
B	1	SS	406.39		3.93	1.592
	2	RS	406.39		3.93	1.592
	3	RR	406.39		3.93	1.592
	4	SR	406.39		3.93	1.592

* Calculated log P

Table 28. Experimental conditions

Group	Condition	Organic Modifier	Column Temperature	Flow Rate (mL/min)
A	1	Acetonitrile	Cooled with ice (0°C)	1.0
	2	Methanol	Cooled with ice (0°C)	0.7
	3	Methanol	Ambient room temperature	0.7
	4	Methanol	Ambient room temperature	1.0
B	1	Methanol	Cooled with ice (0°C)	0.7
	2	Methanol	Cooled with ice (0°C)	0.5
	3	Acetonitrile	Cooled with ice (0°C)	0.7

Next, the conditions were changed to methanol/water for the mobile phase, a flow rate of 0.7 mL/min and a column temperature of 0° C (Condition 2). Representative chromatograms of Group A compounds for this condition are shown in Fig. 77. With a mobile phase consisting of 10 to 50% methanol, the L and D isomer peaks eluted together. The two pro-drugs which have higher log Ps were separated from the L and D isomers. Retention of the L and D isomers is also low with these conditions (Table 30). The K' for the L and D isomers range from 0.598 at 50% methanol to 3.512 at 10% methanol (t_0 = 1.13 minutes). Plots of retention factor vs. % methanol (Fig. 78) and log K' vs. % methanol (Fig. 79) illustrate that retention decreases with increasing methanol for all four compounds, as in reverse phase chromatography. The aqueous normal phase mode of chromatography was not observed with these polar compounds at these conditions. In Fig. 80, log K' is plotted against log P for the four Group A solutes at three different percentages of methanol in the mobile phase (40, 45 and 50%). At these conditions, the log K' increases with increasing log P, and the compound with the highest log P (pro-drug A-4) elutes last.

The representative chromatograms for Group A chiral compounds using the third condition (methanol/water for the mobile phase, a flow of 0.7 mL/min and the column temperature at ambient) are shown in Fig. 81. The retention data is shown in Table 31. The L and D isomers still elute together at all percentages of methanol tested (0 to 80%). As with the first two conditions, at these compositions the L and D isomers elute early. However, at 100% water for the mobile phase, the retention time was 10.6 minutes. As

Figs. 82 and 83 illustrate, the retention decreased with increasing methanol in the mobile phase, like reverse phase chromatography.

For testing the Group A chiral compounds with the last condition, the mobile phase consisted of methanol/water, the flow equaled 1.0 mL/min and the column temperature was ambient. Chromatograms are shown in Fig. 84 and retention data are shown in Table 32. The L and D isomers were not separated at any of the percentages methanol in the mobile phase used (10 to 90%). The peaks eluted early, ranging from 3.2 minutes at 10% methanol down to 1.0 minute at 90% methanol. Separation was based on reverse phase chromatography since plots of retention factor vs. % methanol and $\log K'$ vs. % methanol show that retention decreases with increasing methanol (Figs. 85 and 86). For these nucleoside analogues, chiral separation could not be achieved with these experimental conditions.

The four chiral group B enantiomers were tested using Condition 1 (methanol/water mobile phase, flow rate of 0.7 mL/min and 0° C column temperature). Representative chromatograms for Condition 1 are shown in Fig. 87. At 90 to 100% methanol, the four enantiomers elute at about 1.5 to 1.9 minutes and are not separated from each other. When the methanol composition is lowered to 50%, the enantiomers elute at about 23 minutes and are still not separated from each other. The retention data for condition 1 is shown in Table 33. The retention factor is plotted against the % methanol in Fig. 88, and $\log K'$ is plotted against % methanol in Fig. 89. These graphs show that the retention decreases with increasing methanol composition in the mobile phase, as expected for reverse phase chromatography.

Fig. 74. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Condition 1. Mobile phase: ACN/Water (50:50), Column temperature = 0°C, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D, 3, 4.

Table 29. Retention factors of Group A chiral compounds run on the cholesteric phase (Col #4) using different mobile phase compositions at Condition 1: mobile phase = acetonitrile/water, column temp = 0°C, flow rate = 1.0 mL/min

Grp	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)				
			Acetonitrile (%)				
			20	25	30	40	50
A	1	L	2.084	1.480	1.252	0.865	0.622
	2	D	2.092	1.525	1.249	0.989	0.647
	3	Pro-drug					
	4	Pro-drug					

$t_0 = 0.79$

Fig. 75. Retention factor vs. % acetonitrile in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 1. Solutes: Group A=1-L, 2-D.

Fig. 76. Log retention factor vs. % acetonitrile in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 1. Solutes: Group A=1-L, 2-D.

Fig. 77. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Condition 2. (A) Individual solutes and test mix at 50% methanol and (B) test mixes at 45 and 40% methanol. Mobile phase: MeOH/Water, Column temperature = 0°C, flow rate = 0.7 mL/min, Detection = 276 nm. Solutes: Group A=1-L, 2-D, 3, 4.

Table 30. Retention factors of Group A chiral compounds run on the cholesteric phase (Col #4) using different mobile phase compositions in Cholesteric phase with Condition 2: mobile phase = methanol/water, column temp = 0° C, flow rate = 0.7 mL/min

Grp	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)			
			Methanol (%)			
			10	40	45	50
A	1	L	3.512	0.839	0.756	0.598
	2	D	3.580	0.871	0.766	0.624
	3	Pro-drug		9.982	6.274	4.301
	4	Pro-drug		27.906	15.991	9.442

$t_0 = 1.13$

Fig. 78. Retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 2. Solutes: Group A=1-L, 2-D, 3, 4.

Fig. 79. Log Retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 2. Solutes: Group A=1-L, 2-D, 3, 4.

Fig. 80. Log K' vs. log P for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 2. Solutes: Group A=1-L, 2-D, 3, 4.

Fig. 81. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Condition 3. Mobile phase: MeOH/Water (A) (10:90) and (B) (0:100). Column temperature = ambient room temperature, flow rate = 0.7 mL/min, Detection = 276 nm. Solutes: Group A=1-L, 2-D.

Table 31. Retention factors of Group A chiral compounds run on the cholesteric phase (Col #4) using different mobile phase compositions with Condition 3: mobile phase = methanol/water, column temp = ambient room temperature, flow rate = 0.7 mL/min

Grp	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)					
			Methanol (%)					
			0	10	60	70	80	90
A	1	L	8.421	3.283	0.377	0.327	0.317	0.373
	2	D	8.676	3.388	0.381	0.330	0.314	0.373
	3	Pro-drug						
	4	Pro-drug						

$t_0 = 0.79$

Fig. 82. Retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) with Condition 3. Solutes: Group A=1-L, 2-D.

Fig. 83. Log retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) with Condition 3. Solutes: Group A=1-L, 2-D.

Fig. 84. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with condition 4. (A) Individual solutes at MeOH/Water (10:90) and (B) Test Mix at Different % MeOH in the Mobile phase. Mobile phase = MeOH/water, Column temperature = ambient room temperature, flow rate = 1.0 mL/min, Detection = 220 nm. Solutes: Group A=1-L, 2-D.

Table 32. Retention factors of Group A chiral compounds run on the cholesteric phase (Col #4) using different mobile phase compositions with Condition 4: mobile phase = methanol/water, column temp = ambient room temperature, flow rate = 1.0 mL/min

Grp	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)				
			Methanol (%)				
			10	60	70	80	90
A	1	L	3.022	0.420	0.357	0.335	0.382
	2	D	3.010	0.418	0.358	0.334	0.386
	3	Pro-drug					
	4	Pro-drug					

$t_0 = 0.79$

Fig. 85. Retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 4. Solutes: Group A=1-L, 2-D.

Fig. 86. Log retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 4. Solutes: Group A=1-L, 2-D.

Next, the group B enantiomers (compounds B-1, B-2, B-3 and B-4) were tested with a mobile phase consisting of methanol/water, a lower flow rate of 0.5 mL/min and 0° C column temperature (Condition 2). Representative chromatograms for Condition 2 are shown in Fig. 90, and the retention data are shown in Table 34. With a mobile phase consisting of 45 and 50% methanol, the four enantiomers elute at about 29 minutes and 11 minutes, respectively. The four enantiomers are not separated from each other with these conditions. When the methanol composition is reduced to 40%, the solutes elute at about 31.5 minutes and remain co-eluted. As the plots of K' vs. % methanol and $\log K'$ vs. % methanol show, retention decreases with increasing % methanol (Figs. 91 and 92). Therefore, the reverse phase mode of chromatography is occurring.

Finally, the conditions were changed to an acetonitrile/water mobile phase, a flow rate of 0.7 mL/min, and a column temperature of 0° C (Condition 3). Representative chromatograms for Condition 3 are shown in Fig. 93. Using a mobile phase of 50% acetonitrile, the four group B enantiomers elute at about 2.0 minutes and are not separated from each other. At 40% acetonitrile, the solutes elute at 3.0 minutes and remain unresolved. And, at 30% acetonitrile, the four solutes elute together at about 6.7 minutes. The retention factors for Condition 3 are shown in Table 35. They ranged from about 4.9 at 30% acetonitrile down to about 0.73 at 50% acetonitrile. K' is plotted against % acetonitrile in Fig. 94. As with the other two conditions, the retention factor of the four enantiomers decreases with increasing percentage of acetonitrile in the mobile phase indicating that reverse phase chromatography is occurring. A plot of $\log K'$ vs. % acetonitrile shows that $\log K'$ decreases linearly with increasing % acetonitrile (Fig. 94).

Using these three chromatography conditions, the four enantiomers could not be separated. The conditions to separate chiral molecules on the cholesteric phase are still not understood. Perhaps an acidic modifier needs to be added to the mobile phase.

Six of the chiral drugs including the Group A nucleoside analogue isomers (L and D forms), and the four Group B enantiomers were then tested with an acidified mobile phase to determine if chiral separation could be achieved on the cholesterol phase (Col # 4). The four chromatography conditions used are listed in Table 36. Acetonitrile and methanol were used as organic modifiers and 0.5% formic acid and 0.05% perchloric acid were used to lower the mobile phase pH.

Representative chromatograms for Group A molecules using Acid Condition 1 (methanol, 0.5% formic acid) are shown in Figs. 96 and 97. At 60% methanol, the nucleoside analogue L and D isomers elute together at 1.0 minute. In addition, at all percentages of methanol tested (60 to 90%), the isomers were not separated. The retention factors for the L and D isomers are low, 0.1 to 0.2 (Table 37). The plot of K' vs. % methanol shows that the retention of the two isomers decreases slightly with increasing methanol in the mobile phase as in reverse phase chromatography (Fig. 98).

For Acid Condition 2, the organic modifier was changed to acetonitrile. Representative chromatograms for Group A solutes at 60% acetonitrile and 20% acetonitrile are shown in Fig. 99. The L and D isomers elute at about 1 minute for both conditions and are not separated from each other. The test mix chromatograms in Fig. 100 show that the L and D isomers co-elute at all % acetonitrile in the mobile phase tested (90 to 20%). At 90% acetonitrile, the retention time of the isomers is about

Fig. 87. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with condition 1. (A) Individual solutes at 50% MeOH and (B) Test Mix at Different % MeOH in the Mobile phase. Mobile phase = MeOH/water, Column temperature = 0°C, flow rate = 0.7 mL/min, Detection = 220 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Table 33. Retention factors of Group B chiral compounds run on the cholesteric phase (Col #4) using different mobile phase compositions with Condition 1: mobile phase = methanol/water, column temperature = cooled with 0° C, flow rate = 0.7 mL/min,

Grp	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)			
			Methanol (%)			
			50	90	95	100
B	1	SS	19.700	0.617	0.400	0.333
	2	RS	19.370	0.624	0.403	0.371
	3	RR	19.902	0.630	0.396	0.339
	4	SR	19.286	0.661	0.406	0.341

$t_0 = 1.13$

Fig. 88. Retention factor vs. % methanol in the mobile phase for Group B chiral compounds run on the cholesteric phase (Col# 4) using Condition 1. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 89. Log Retention factor vs. % methanol in the mobile phase for Group B chiral compounds run on the cholesteric phase (Col# 4) using Condition 1. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 90. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Condition 2. (A) Individual solutes at 40% MeOH and (B) Test Mix at Different % MeOH in the Mobile phase. Mobile phase = MeOH/water, Column temperature = 0° C, flow rate = 0.5 mL/min, Detection = 220 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Table 34. Retention factors of Group B chiral compounds run on the cholesteric phase (Col #4) using different mobile phase compositions with Condition 2: Column temperature = 0° C, mobile phase = methanol/water, flow rate = 0.5 mL/min

Group	No.	Descr.	Retention factor (K')	
			Cholesteric (Col # 4)	
			Methanol (%)	
			40	45
B	1	SS	19.396	17.185
	2	RS	18.942	17.105
	3	RR	19.185	17.508
	4	SR	19.822	18.197

$t_0 = 1.58$

Fig. 91. Retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 2. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 92. Log retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Condition 2. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 93. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Condition 3. Mobile phase = ACN/water (A) (50:50), (B) (40:60) and (C) (30:70), Column temperature = 0°C, flow rate = 0.7 mL/min, Detection = 220 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Table 35. Retention factors of Group B chiral compounds run on the cholesteric phase (Col #4) using different mobile phase compositions in with Condition 3: mobile phase = acetonitrile/water, column temperature = 0° C, flow rate = 0.7 mL/min

Group	No.	Descr.	Retention factor (K')		
			Cholesteric (Col # 4)		
			Acetonitrile (%)		
			30	40	50
B	1	SS	4.839	1.679	0.724
	2	RS	4.935	1.692	0.748
	3	RR	4.835	1.646	0.731
	4	SR	4.918	1.687	0.753

$t_0 = 1.13$

Fig. 94. Retention factor vs. % acetonitrile in the mobile phase for Group B chiral compounds run on the cholesteric phase (Col# 4) using Condition 3. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 95. Retention factor vs. % acetonitrile in the mobile phase for Group B chiral compounds run on the cholesteric phase (Col# 4) using Condition 3. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Table 36. Experimental conditions with an acidic mobile phase

Group	Condition	Organic Modifier	Acid	Column Temperature	Flow Rate (mL/min)
A, B, C and D	1	Methanol	0.5% Formic Acid	Ambient room temperature	1.0
	2	Acetonitrile	0.5% Formic Acid	Ambient room temperature	1.0
	3	Methanol	0.05% Perchloric Acid	Ambient room temperature	1.0
	4	Acetonitrile	0.05% Perchloric Acid	Ambient room temperature	1.0

2.5 minutes, which is higher than their retention time at 60% and 20% acetonitrile (1.0 minute). The retention of these Group A solutes decreases with decreasing % acetonitrile in the mobile phase (Table 38 and Fig. 101). At 90% acetonitrile, the retention factor equals 1.3, and equals -0.15 at 60% acetonitrile. The increasing retention with increasing acetonitrile in the mobile phase indicates that the aqueous normal phase mode of chromatography is occurring. Only the reverse phase chromatography mode was observed in previous experiments with compound 2 (the D isomer). Therefore, the formic acid which lowers the mobile phase pH must be required for the ANP mode to

occur. In addition, the ANP mode was only observed with acetonitrile as the organic modifier, not methanol.

For Acid Condition 3, the mobile phase consisted of 0.05% perchloric acid and methanol. Representative chromatograms for Group A solutes at 60% methanol are shown in Fig. 102. The L and D isomers were not separated from each other with these conditions, or at any of the percentages of methanol tested (90 to 60%) (Fig. 103). Although the chromatograms for 80 and 90% methanol show two peaks, one peak is the solvent front. The isomers are retained poorly. The retention factor equals 0.32 at 60% methanol and decreases to 0.09 at 90% methanol (Table 39). The decreasing retention with increasing methanol indicates that reverse phase chromatography is occurring (Fig. 104).

Representative chromatograms for Group A solutes at the last acid condition (0.05% perchloric acid and acetonitrile) are shown in Figs. 105 and 106. When using 60% and 20% acetonitrile, the L and D isomers co-elute at 1.0 minute (60% acetonitrile) and 1.5 minutes (20% acetonitrile). Separation of the isomers could not be achieved at any of the percentages of acetonitrile tested, 90 to 20% (Fig. 107). The retention of Group A molecules increased with decreasing % acetonitrile (Table 40 and Fig. 108), like reverse phase chromatography. Perhaps the ANP mode was not observed because the perchloric acid was at a lower concentration, 0.05% instead of 0.5%.

Representative chromatograms for the Group B enantiomers (SS, RS, RR, SR) tested with Acid Condition 1 (methanol, 0.5% formic acid) are shown in Fig. 109. At 60% methanol, the four enantiomers co-eluted at 2.5 minutes. In addition, the solutes

Fig. 96. Representative chromatograms for Group A chiral compounds run on the uholesteric phase (Col# 4) with Acid Condition 1. Mobile phase: MeOH/Water/Formic acid (60:40:0.5), Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Fig. 97. Representative chromatograms for Group A chiral compounds on the cholesteric phase (Col# 4) with Acid Condition 1 at different % methanol concentrations in the mobile phase. Mobile phase: MeOH/Water/Formic acid, Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Table 37. Retention factors of Group A chiral compounds run on the cholesteric phase (Col #4) with different mobile phase compositions at Acid Condition 1: mobile phase = methanol/water/formic acid, column temp = ambient room temp, flow rate = 1.0 mL/min

Group	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)			
			Methanol (%)			
			60	70	80	90
A	1	L	0.229	0.204	0.185	0.122
	2	D	0.220	0.197	0.186	0.123

$t_0 = 0.79$

Fig. 98. Retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Acid Condition 1. Solutes: Group A=1-L, 2-D.

Fig. 99. Representative Chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 2. Mobile phase: ACN/Water/Formic acid (A) (60:40:0.5) and (B) (20:80:0.5), Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Fig. 100. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 2 at different % acetonitrile concentrations in the mobile phase. Mobile phase: ACN/Water/Formic acid, Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Table 38. Retention factors of Group A chiral compounds run on the cholesteric phase (Col #4) with different mobile phase compositions at Acid condition 2: mobile phase = acetonitrile/water/formic acid, column temp = ambient room temp, flow rate = 1.0 mL/min

Grp	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)							
			Acetonitrile (%)							
			90	80	70	60	50	40	30	20
A	1	L	1.281	0.260	-0.049	-0.152	-0.195	-0.188	-0.160	-0.094
	2	D	1.315	0.263	-0.049	-0.152	-0.196	-0.189	-0.163	-0.085

$t_0 = 0.79$

Fig. 101. Retention factor vs. % acetonitrile in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group A=1-L, 2-D.

Fig. 102. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 3. Mobile phase: MeOH/Water/Perchloric acid (60:40:0.05), Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Fig. 103. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 3 at different % methanol concentrations in the mobile phase. Mobile phase: MeOH/Water/Perchloric acid, 0.05%. Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Table 39. Retention factors of Group A chiral compounds run on the cholesteric phase (Col #4) with different mobile phase compositions at Acid Condition 3. mobile phase = methanol/water/perchloric acid, column temp = ambient room temp, flow rate = 1.0 mL/min

Group	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)			
			Methanol (%)			
			60	70	80	90
A	1	L	0.315	0.272	0.266	0.090
	2	D	0.316	0.272	0.268	0.087
$t_0 = 0.79$

Fig. 104. Retention factor vs. % methanol in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Acid Condition 3. Solutes: Group A=1-L, 2-D.

Fig. 105. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 4. Mobile phase: ACN/Water/Perchloric acid (60:40:0.05), Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Fig. 106. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 4. Mobile phase: ACN/Water/Perchloric acid (20:80:0.05), Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Fig. 107. Representative chromatograms for Group A chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 4 at different % acetonitrile concentrations in the mobile phase. Mobile phase: ACN/Water/Perchloric acid, 0.05%. Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A=1-L, 2-D.

Table 40. Retention factors of Group A chiral compounds run on the cholesteric phase (Col #4) with different mobile phase compositions at Acid Condition 4: mobile phase = acetonitrile/water/perchloric acid, column temp = ambient room temp, flow rate = 1.0 mL/min

Grp	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)							
			Acetonitrile (%)							
			90	80	70	60	50	40	30	20
A	1	L	-0.242	-0.144	-0.135	-0.119	-0.069	-0.004	0.096	0.259
	2	D	-0.241	-0.142	-0.133	-0.117	-0.066	0.002	0.098	0.258

$t_0 = 0.79$

Fig. 108. Retention factor vs. % acetonitrile in the mobile phase for Group A chiral compounds run on the cholesteric phase (Col# 4) using Acid Condition 4. Solutes: Group A=1-L, 2-D.

co-elute when the % methanol in the mobile phase equaled 70, 80 and 90% (Fig. 109).

Table 41 and Fig. 111 show the retention factors for these four enantiomers at different percentages of methanol. Retention decreases with increasing % methanol, as expected for reverse phase chromatography.

For Acid Condition 2, the methanol was replaced with acetonitrile.

Representative chromatograms for Group B chiral compounds are shown in Fig. 112. At 60% and 20% acetonitrile, the enantiomers are not separated from one another. The chromatograms for Group B test mixes show that the four enantiomers co-elute at all percentages of acetonitrile (90 to 20%) (Fig. 113). For these solutes, the retention

increased slightly with increasing % acetonitrile, from 80 to 90% acetonitrile, indicating the ANP mode of chromatography is occurring (Table 42). The amount of increase is not as large as with the Group A solutes. The retention factors increased from 0.56 at 80% acetonitrile to 0.75 at 90% acetonitrile. The plot of K' vs. % acetonitrile shows that K decreases from 20 to 80% acetonitrile (as reverse phase chromatography), and slightly increases from 80 to 90% acetonitrile (Fig. 114).

Representative chromatograms for the third acid condition (methanol, 0.05% perchloric acid) are shown in Figs. 115 and 116. The four enantiomers were not separated from one another at all percentages of methanol tested (60 to 90%). The retention times decreased with increasing % methanol in the mobile phase (Table 43 and Fig. 117).

Finally, the Group B solutes were tested with Acid Condition 4 (acetonitrile, 0.05% perchloric acid). The four enantiomers co-eluted at about 1.4 minutes with 60% acetonitrile in the mobile phase (Fig. 118). When the acetonitrile amount was reduced to 30%, the four Group B solutes were still not separated from each other (Fig. 119). The peaks co-eluted at all percentages of acetonitrile tried (30 to 90%) (Fig. 120). The retention time of the solutes decreased with increasing acetonitrile in the mobile phase (Table 44 and Fig. 121).

In summary, chiral separation could not be achieved for these two groups of molecules using these acid conditions. Perhaps a different type of acid or organic modifier is required. Or maybe a combination of acetonitrile/methanol together is needed for chiral separation to occur.

Fig. 109. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 1. Mobile phase = MeOH/water/formic acid (60:40:0.5). Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 110. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 1. Test mix at different % MeOH in the mobile Phase. Mobile phase = MeOH/water/formic acid, 0.5%. Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Table 41. Retention factors of Group B chiral compounds run on the cholesteric phase (Col #4) with different mobile phase compositions using Acid Condition 1: mobile phase = methanol/water/formic acid, column temperature = ambient room temp, flow rate = 1.0 mL/min

Group	No.	Descr.	Retention factor (K')			
			Cholesteric (Col # 4)			
			Methanol (%)			
			60	70	80	90
B	1	SS	1.997	1.103	0.663	0.459
	2	RS	2.101	1.097	0.663	0.463
	3	RR	2.125	1.104	0.667	0.459
	4	SR	2.091	1.090	0.662	0.462
$t_0 = 0.79$

Fig. 111. Retention factor vs. % methanol in the mobile phase for Group B Chiral Compounds run on the cholesteric phase (Col# 4) using Acid Condition 1. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 112. Representative chromatograms for Group B chiral compounds ran on the cholesteric phase (Col# 4) with Acid Condition 2. Mobile phase = ACN/water/formic acid (A) (60:40:0.5) and (B) (20:80:0.05). Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 113. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 2. Test mix at different % acetonitrile in the mobile phase. Mobile phase = ACN/water/formic acid, 0.5%. Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Table 42. Retention factors of Group B chiral compounds run on the cholesteric phase (Col #4) with different mobile phase compositions using Acid Condition 2: mobile phase = acetonitrile/water/formic, column temperature = ambient room temp, flow rate = 1.0 mL/min

Group	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)							
			Acetonitrile (%)							
			20	30	40	50	60	70	80	90
B	1	SS	16.676	4.705	2.001	1.118	0.753	0.597	0.563	0.744
	2	RS	16.590	4.752	2.025	1.139	0.763	0.599	0.568	0.749
	3	RR	16.667	4.709	2.001	1.116	0.756	0.595	0.565	0.744
	4	SR	16.520	4.738	2.024	1.132	0.763	0.601	0.567	0.751

$t_0 = 0.79$

Fig. 114. Retention factor vs. % acetonitrile in the mobile phase for Group B chiral compounds run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 115. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 3. Mobile phase = MeOH/water/perchloric acid (60:40:0.05). Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 116. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 3. Test mix at different % MeOH in the mobile phase. Mobile phase = MeOH/water/perchloric acid, 0.05%. Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Table 43. Retention factors of Group B chiral compounds run on the cholesteric phase (col #4) with different mobile phase compositions using Acid Condition 3: mobile phase = methanol/water/perchloric acid, 0.05%. Column temperature = ambient room temp, flow rate = 1.0 mL/min

Group	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)			
			Methanol (%)			
			60	70	80	90
B	1	SS	2.496	1.199	0.700	0.473
	2	RS	2.485	1.192	0.697	0.473
	3	RR	2.503	1.201	0.699	0.472
	4	SR	2.457	1.189	0.694	0.470

$t_0 = 0.79$

Fig. 117. Retention factor vs. % methanol in the mobile phase for Group B chiral compounds run on the cholesteric phase (Col# 4) using Acid Condition 3. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 118. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 4. Mobile phase = ACN/water/perchloric acid (60:40:0.05). Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 119. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 4. Mobile phase = ACN/water/perchloric acid (30:60:0.05). Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Fig. 120. Representative chromatograms for Group B chiral compounds run on the cholesteric phase (Col# 4) with Acid Condition 4. Test mix at different % ACN in the mobile phase. Mobile phase = ACN/water/perchloric acid, 0.05%. Column temperature = ambient room temp, flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

Table 44. Retention factors of Group B chiral compounds run on the cholesteric phase (Col #4) with different mobile phase compositions using Acid Condition 4: mobile phase = acetonitrile/water/formic, column temperature = ambient room temp, flow rate = 1.0 mL/min

Group	No.	Descr.	Retention factor (K') - Cholesteric (Col # 4)						
			Acetonitrile (%)						
			30	40	50	60	70	80	90
B	1	SS	6.470	2.424	1.246	0.490	0.487	0.568	0.778
	2	RS	6.562	2.463	1.270	0.491	0.485	0.572	0.790
	3	RR	6.484	2.422	1.248	0.492	0.480	0.567	0.778
	4	SR	6.547	2.459	1.265	0.487	0.481	0.570	0.786

$t_0 = 0.79$

Fig. 121. Retention factor vs. % acetonitrile in the mobile phase for Group B chiral compounds run on the cholesteric phase (Col# 4) using Acid Condition 4. Solutes: Group B = 1-SS, 2-RS, 3-RR, 4-SR.

3.7. Aqueous Normal Phase (ANP) Chromatography

To investigate the ANP chromatography mode further, forty-seven compounds (eleven groups) were studied on the cholesteric phase (Col #4) using an acidified mobile phase. The first four groups of compounds (Groups A, B, C and D) tested together were grouped according to their backbone structure. Group A compounds are saccharine derivatives and group B compounds are thiazines. The Group C drugs are nucleoside

analogues based on a backbone similar to cytosine. Compound 5 is a parent drug and the one used previously in the polar compound section 3.5 (labeled compound 9) and in the chiral compound section 3.6 (labeled compound 2, the D isomer). It has the lowest log P (-0.936) of the Group A, B, C and D compounds. The Group C solutes also include three pro-drugs of a different parent nucleoside analogue. Finally, the Group D compound is a benzopyran containing an amide bond. The structures of tetramic acid, quinolinedione, cytosine and benzopyran backbones are shown in Fig. 122. The molecular weights, pKas and log Ps are shown in Table 45. The Group A, B and C compounds are fairly polar. Experiments with the Group A, B, C and D compounds were performed with four acid conditions shown in Table 2. The mobile phase pH was lowered using 0.5% formic acid or 0.5% perchloric acid. The amount of perchloric acid used was increased from what was used in the chiral compound section (0.05%) to 0.5%, to determine if the ANP mode occurs with a higher percentage of acid.

Fig. 122. Structures of (A) saccharin, (B) quinolinedione backbone, (C) the nucleoside cytidine and (D) benzopyran

Table 45. Molecular weights, log Ps and pKa's of for Group A, B, C and D

Group	No.	MW	ApKa	BpKa	Log P*
A	1	535.614	7.91, 10.98	5.56	2.961
	2	428.482	7.91	5.8	4.293
B	3	519.572	8.64	-	2.739
	4	535.614	8.65, 8.86	-	3.457
C	5	259.236	11.81	3.73	-0.936
	6	397.425	12.12	3.73	1.539
	7	425.479	12.09	3.73	2.597
	8	453.532	12.07	3.73	3.655
D	9	396.416	-	-	2.795

* Calculated log P

Table 46. Experimental conditions with acidic mobile phases

Group	Condition	Organic Modifier	Acid	Column Temperature	Flow Rate (mL/min)
A, B, C and D	1	Methanol	0.50% Formic Acid	Ambient room temperature	1.0
	2	Acetonitrile	0.50% Formic Acid	Ambient room temperature	1.0
	3	Methanol	0.50% Perchloric Acid	Ambient room temperature	1.0
	4	Acetonitrile	0.50% Perchloric Acid	Ambient room temperature	1.0

Representative chromatograms for the Group A, B, C and D solutes using Acid Condition 1 (methanol and 0.50% formic acid) are shown in Figs. 123 and 124. Retention data are shown in Tables 46 and 47. At 60% methanol, compound 5, which has the lowest log P, eluted the earliest, at 0.964 minutes. All the compounds are separated from each other, except for compounds 2 and 3, which elute together. These two compounds belong to different classes (compound 2 is a saccharin and compound 3 is a quinolinedione). In addition, compounds 2 and 3 have different log Ps (4.293 and 2.739) and compound 3 has a BpKa whereas compound 2 doesn't. The last compound to elute is the Group D compound 9 with a retention time of 12.83 minutes. This peak is fairly broad. The retention factors and log K's were plotted against the percent methanol

in the mobile phase for Acid Condition 1 in Figs. 125 and 126. For all Group A, B, C and D compounds, the retention factor decreased with increasing methanol as in reverse phase chromatography. The $\log K'$ vs. $\log P$ for the Groups A, B, C and D for 60 and 90% methanol in the mobile phase are shown in Fig. 127. There is no correlation between $\log K'$ and $\log P$, except it appears that for Group C, retention increases with increasing $\log P$.

Next, the Group A, B, C and D compounds were tested on the cholesterol column using Acid Condition 2 (acetonitrile and 0.50% formic acid). Representative chromatograms are shown in Figs. 128 to 131 and retention times are shown in Tables 49 and 50. At 60% acetonitrile in the mobile phase, the retention times range from 0.958 minutes (compound 5) to 3.275 minutes (compound 9). These retention times are shorter than with Acid Condition 1. Then, at 90% acetonitrile in the mobile phase, the retention times range from 2.028 minutes (compound 4) to 2.616 minutes (compound 5). For example, compound 1 elutes first at 90%, fourth at 80%, third at 70% and fifth at 60% acetonitrile. Compound 5 elutes last at 90%, fifth at 80% and first at 70% and 60% acetonitrile.

The plots of retention and $\log K'$ vs. % acetonitrile in the mobile phase show that retention of Group A compounds 1 and 2, Group B compounds 3 and 4, and Group D compound 9 decreases with increasing acetonitrile as in reverse phase chromatography (Figs. 132 and 133). However, the retention of Group C compound 5 decreases from 20 to 50% acetonitrile, and then increases from 50 to 90% acetonitrile. Therefore, the increase in retention with increasing organic content in the mobile phase indicates that

Fig. 123. Representative chromatograms of Group A, B, C and D compounds run on the cholesteric phase (Col #4) for 60% methanol in the mobile phase using Acid Condition 1. Mobile phase: MeOH/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: (A) Group A – 1, 2; Group B – 3, 4; Group D – 9 and (B) Group C – 5, 6, 7, 8.

Fig. 124. Representative chromatograms of Group A, B, C and D compounds run on the cholesteric phase (Col #4) for different % methanol in the mobile phase using Acid Condition 1. Mobile phase: MeOH/Water/Formic acid. Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: (A) Test mix of Group A – 1, 2; B – 3, 4; C – 6, 7, 8; and D – 9; (B) Group C – 5.

Table 47. Retention times of Group A, B, C and D compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 1

Group	No	Retention time (minutes) - Cholesteric (Col # 4)			
		Methanol (%)			
		60	70	80	90
A	1	4.416	2.163	1.484	1.276
	2	8.912	3.669	2.068	1.519
B	3	8.909	3.569	1.991	1.45
	4	6.129	2.594	1.57	1.22
C	5	0.964	0.946	0.937	0.887
	6	1.348	1.147	1.041	1.002
	7	3.335	1.789	1.262	1.032
	8	1.973	1.38	1.13	1.031
D	9	12.83	4.603	2.325	1.588

$t_0 = 0.79$

Table 48. Retention factors of Group A, B, C and D compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 1

Group	No	Retention factor (K') - Cholesteric (Col # 4)			
		Methanol (%)			
		60	70	80	90
A	1	4.590	1.738	0.878	0.615
	2	10.281	3.644	1.618	0.923
B	3	10.277	3.518	1.520	0.835
	4	6.758	2.284	0.987	0.544
C	5	0.220	0.197	0.186	0.123
	6	0.706	0.452	0.318	0.268
	7	3.222	1.265	0.597	0.306
	8	1.497	0.747	0.430	0.305
D	9	15.241	4.827	1.943	1.010

$t_0 = 0.79$

A

B

Fig. 125. Retention factor vs. % methanol in the mobile phase for Groups A, B, C and D run on the cholesteric phase (Col# 4) using Acid Condition 1. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9 and (B) Group C = 5, 6, 7, 8.

A

B

Fig. 126. Log K vs. % methanol in the mobile phase for Groups A, B, C and D run on the cholesteric phase (Col# 4) using Acid Condition 1. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9 and (B) Group C = 5, 6, 7, 8.

A

B

Fig. 127. Log K' vs. log P for Groups A, B, C and D compounds run on the cholesteric phase (Col# 4) using Acid Condition 1 at (A) 60% and (B) 90% Methanol in the mobile phase. Solutes: Group A = 1, 2; Group B = 3, 4; Group C = 5, 6, 7, 8; Group D = 9.

the ANP chromatography mode is occurring. In addition, for the other Group C compounds (6, 7 and 8) retention increases when the acetonitrile increases from 70 to 90% acetonitrile in the mobile phase. Therefore, the ANP mode of chromatography is occurring on the cholesteric phase with Acid Condition 2 for the Group C compounds, but not the Group A, B and D compounds. The Group C compounds are nucleoside analogues each containing a primary amine with a BpKa of 3.73. The Group A, B and D compounds, which do not exhibit ANP, only contain secondary amines (except for compound 2 which only has a tertiary amine). The Group A tetramic acid compounds 1 and 2 have BpKa's of 5.56 and 5.8, respectively. The Group B quinolinediones (compounds 3 and 4) and the Group D compound do not have BpKa's for their amine groups.

Within the four Group C nucleoside analogues, compound 5 elutes the earliest at 60%, and the latest at 90% acetonitrile. The difference could be due to the log P since the Bpka's are all equal to 3.73 and the Apka's are similar (11.8, 12.1, 12.1 and 12.1). Compound 5 has the lowest log P of the Group C compounds, so elutes earliest at 60% acetonitrile because the separation is based on hydrophobicity. The log K' vs. log P is plotted in Fig. 134. At 60% acetonitrile in the mobile phase, the log K' for the Group C compounds seems to increase with increasing log P. The log K' of these compounds are lower than the Log Ks for the A, B and D compounds. But at 90% acetonitrile, there is no relationship between the log K' and log P for the Group C compounds. And, the log K' vs. log P line for the Group C compounds is above the Group A, B and D data points. The difference between the 60% and 90% graphs is due to the Group C

compounds being retained by the ANP mode, while the other groups are retained based on the reverse phase mode.

The group A, B, C and D solutes were analyzed with the third condition being methanol and 0.50% perchloric acid. Representative chromatograms are shown in Figs. 135 and 136, and the retention data are shown in Tables 51 and 52. At 60% methanol, compound 5 elutes near the void volume ($t_0 = 0.79$ minutes) at 0.959 minutes. At 90% methanol, compound 5 elutes even closer to the void volume, 0.878 minutes. The plots of retention factor vs. % methanol and $\log K'$ vs. % methanol show that retention of all compounds decreases with increasing % methanol as in reverse phase chromatography (Figs. 137 and 138). Therefore, the ANP chromatography mode is not occurring with these mobile phase conditions.

Finally, the group A, B, C and D solutes were tested using Acid Condition 4 (acetonitrile and 0.50% perchloric acid). Chromatograms for individual compounds at 60% and 90% acetonitrile in the mobile phase are shown in Figs. 140 and 141, respectively. The chromatograms for mixtures using different percentages of acetonitrile in the mobile phase are shown in Fig. 142. The corresponding retention data are shown in Tables 53 and 54. At 60% acetonitrile in the mobile phase, the compounds elute between 0.849 minutes (compound 5) and 3.408 minutes (compound 9). At 90% the mobile phase, the peaks elute earlier from 0.852 minutes (compound 5) to 1.231 minutes (compound 9). The retention factor was plotted against the % acetonitrile in Fig. 143, and the $\log K'$ was plotted against the % acetonitrile in Fig. 144.

Fig. 128. Representative chromatograms for Group A, B, C and D compounds on the cholesteric phase (Col #4) with 60% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9 and (B) Group C = 5, 6, 7, 8.

Fig. 129. Representative chromatograms of Group A, B, C and D compounds run on the cholesteric phase (Col #4) for 90% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (90:10:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9 and (B) Group C = 5, 6, 7, 8.

Fig. 130. Representative chromatograms of Group C compound 5 run on the cholesteric phase (Col #4) for different % acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (90:10:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group C – 5.

Fig. 131. Representative chromatograms for test mixes of Group A, B, C and D compounds run on the cholesteric phase (Col #4) for different % acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/0.50% Formic acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A = 1, 2; Group B = 3, 4; Group C = 6, 7, 8; and Group D = 9.

Table 49. Retention times of Group A, B, C and D compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Grp	No	Retention time (minutes) - Cholesteric (Col # 4)							
		Acetonitrile (%)							
		90	80	70	60	50	40	30	20
A	1	1.038	1.125	1.375	1.966				
	2	1.162	1.351	1.831	2.983				
B	3	1.183	1.325	1.704	2.597				
	4	1.028	1.124	1.386	2.026				
C	5	2.616	1.427	1.075	0.958	0.909	0.916	0.946	1.034
	6	2.35	1.555	1.325	1.283				
	7	2.609	1.851	1.717	1.914				
	8	2.449	1.68	1.49	1.542				
D	9	1.228	1.444	1.979	3.275				

$t_0 = 0.79$

Table 50. Retention factors of Group A, B, C and D compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Grp	No	Retention factor (K') - Cholesteric (Col # 4)							
		Acetonitrile (%)							
		90	80	70	60	50	40	30	20
A	1	0.314	0.424	0.741	1.489				
	2	0.471	0.710	1.318	2.776				
B	3	0.497	0.677	1.157	2.287				
	4	0.301	0.423	0.754	1.565				
C	5	2.311	0.806	0.361	0.213	0.151	0.159	0.197	0.309
	6	1.975	0.968	0.677	0.624				
	7	2.303	1.343	1.173	1.423				
	8	2.100	1.127	0.886	0.952				
D	9	0.554	0.828	1.505	3.146				

$t_0 = 0.79$

A

B

Fig. 132. Retention factor vs. % acetonitrile in the mobile phase for Groups A, B, C and D run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9. and (B) Group C = 5, 6, 7, 8.

A

B

Fig. 133. Log K' vs. % acetonitrile in the mobile phase for Groups A, B, C and D run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9. and (B) Group C = 5, 6, 7, 8.

A

B

Fig. 134. Log K' vs. log P for Group A, B, C and D compounds run on the cholesteric phase (Col# 4) using Acid Condition 2 at (A) 60% and (B) 90% Acetonitrile in the mobile phase. Solutes: Group A = 1, 2; Group B = 3, 4; Group C = 5, 6, 7, 8; Group D = 9.

Fig. 135. Representative chromatograms for Group A, B, C and D compounds run on the cholesteric phase (Col #4) with 60% methanol using Acid Condition 3. Mobile phase: MeOH/Water/Perchloric acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A = 1, 2; Group B = 3, 4; Group C = 5, 6, 7, 8; Group D – 9.

Fig. 136. Representative chromatograms for test mixes of Group A, B, C and D compounds run on the cholesteric phase (Col #4) with different % methanol using Acid Condition 3. Mobile phase: MeOH/Water/0.50% Perchloric acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A = 1, 2; Group B = 3, 4; Group C = 5, 6, 7, 8; Group D – 9.

Table 51. Retention times of Group A, B, C and D compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 3

Group	No	Retention time (minutes) - Cholesteric (Col # 4)			
		Methanol (%)			
		60	70	80	90
A	1	6.168	2.838	1.761	1.344
	2		4.724	2.432	1.604
B	3	9.634	3.772	2.052	1.469
	4	6.688	2.727	1.61	1.232
C	5	0.959	0.927	0.893	0.878
	6	1.286	1.09	1.005	0.996
	7	3.148	1.661	1.186	1.02
	8	1.869	1.298	1.077	1.019
D	9	13.952	4.927	2.418	1.613

$t_0 = 0.79$

Table 52. Retention factors of Group A, B, C and D compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 3

Group	No	Retention factor (K') - Cholesteric (Col # 4)			
		Methanol (%)			
		60	70	80	90
A	1	6.808	2.592	1.229	0.701
	2		4.980	2.078	1.030
B	3	11.195	3.775	1.597	0.859
	4	7.466	2.452	1.038	0.559
C	5	0.214	0.173	0.130	0.111
	6	0.628	0.380	0.272	0.261
	7	2.985	1.103	0.501	0.291
	8	1.366	0.643	0.363	0.290
D	9	16.661	5.237	2.061	1.042

$t_0 = 0.79$

A

B

Fig. 137. Retention factor vs. % methanol in the mobile phase for Groups A, B, C and D run on the cholesteric phase (Col# 4) using Acid Condition 3. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9; and (B) Group C = 5, 6, 7, 8.

A

B

Fig. 138. Log K' vs. % methanol in the mobile phase for Groups A, B, C and D run on the cholesteric phase (Col# 4) using Acid Condition 3. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D – 9; and (B) Group C = 5, 6, 7, 8.

Fig. 139. Log K' vs. log P for Groups A, B, C and D run on the cholesteric phase (Col# 4) using Acid Condition 3 at 60% Methanol in the mobile phase. Solutes: Group A = 1, 2; Group B = 3, 4; Group C = 5, 6, 7, 8; Group D = 9.

For all compounds except compound 5, the retention decreased as the % acetonitrile increased in the mobile phase, indicating that the ANP mode is not occurring. However, for compound 5, the retention increased slightly from 80 to 95% acetonitrile from 0.834 minutes to 0.898 minutes. The increase can be observed more clearly in the Fig. 144 graph. Therefore, the ANP mode may be occurring for the nucleoside analogue compound 5, but the effect is not as dramatic as with the acetonitrile/formic acid mobile phase.

These results demonstrate that the aqueous normal phase mode of chromatography occurs when a mobile phase of acetonitrile/water/0.50% formic acid is employed. Only the reverse phase mode was observed with a mobile phase of methanol/water/0.50% formic acid and methanol/water/0.50% perchloric acid. One compound (compound 5) exhibited a small amount of ANP behavior with the acetonitrile/water/0.50% perchloric acid. The compounds that exhibited the ANP

chromatography behavior were polar nucleoside analogues containing a primary amine group with a BpKa of 3.73.

Fig. 140. Representative chromatograms of Groups A, B, C and D compounds run on the cholesteric phase (Col #4) for 60% acetonitrile in the mobile phase using Acid Condition 4. Mobile phase: ACN/Water/Perchloric acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D – 9 and (B) Group C = 5, 6, 7, 8.

Fig. 141. Representative chromatograms for Groups A, B, C and D run on the cholesteric phase (Col #4) with 90% acetonitrile in the mobile phase using Acid Condition 4. Mobile phase: ACN/Water/Perchloric acid (90:10:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D – 9 and (B) Group C = 5, 6, 7, 8.

Fig. 142. Representative chromatograms for test mixture of Groups A, B, C and D run on the cholesteric phase with different % acetonitrile in the mobile phase using Acid Condition 4. Mobile phase: ACN/Water/Perchloric acid, 0.50%, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group A = 1, 2; Group B = 3, 4; Group C = 5, 6, 7, 8; Group D – 9.

Table 53. Retention times of Group A, B, C and D compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 4

Retention time (minutes) - Cholesteric (Col # 4)										
Grp	No	Acetonitrile (%)								
		95	90	80	70	60	50	40	30	20
A	1		1.069	1.199	1.531	2.311				
	2		1.165	1.398	1.949					
B	3		1.18	1.336	1.736	2.677				
	4		1.027	1.128	1.405	2.08				
C	5	0.898	0.852	0.834	0.837	0.849	0.905	0.944	1.011	1.174
	6	0.891	0.883	0.903	0.949	1.057				
	7	0.922	0.929	0.974	1.114	1.44				
	8	0.901	0.904	0.934	1.02	1.215				
D	9		1.231	1.462	2.031	3.408				

$t_0 = 0.79$

Table 54. Retention factors of Group A, B, C and D compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 4

Grp	No	Retention factor (K') - Cholesteric (Col # 4)								
		Acetonitrile (%)								
		95	90	80	70	60	50	40	30	20
A	1		0.353	0.518	0.938	1.925				
	2		0.475	0.770	1.467					
B	3		0.494	0.691	1.197	2.389				
	4		0.300	0.428	0.778	1.633				
C	5	0.137	0.078	0.056	0.059	0.075	0.146	0.195	0.280	0.486
	6	0.128	0.118	0.143	0.201	0.338				
	7	0.167	0.176	0.233	0.410	0.823				
	8	0.141	0.144	0.182	0.291	0.538				
D	9		0.558	0.851	1.571	3.314				

$t_0 = 0.79$

A

B

Fig. 143. Retention factor vs. % acetonitrile in the mobile phase for Groups A, B, C and D compounds run on the cholesteric phase (Col# 4) using Acid Condition 4. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9 and (B) Group C = 5, 6, 7, 8.

A

B

Fig. 144. $\text{Log } K'$ vs. % acetonitrile in the mobile phase for Groups A, B, C and D compounds run on the cholesteric phase (Col# 4) using Acid Condition 4. Solutes: (A) Group A = 1, 2; Group B = 3, 4; Group D = 9 and (B) Group C = 5, 6, 7, 8.

Fig. 145. Log K' vs. log P for Group A, B, C and D compounds run on the cholesteric phase (Col# 4) using Acid Condition 4 at 60% Acetonitrile in the mobile phase. Solutes: Group A = 1, 2; Group B = 3, 4; Group C = 5, 6, 7, 8; Group D – 9.

More compounds including nucleoside analogues were investigated using the acetonitrile/0.50% formic acid mobile phase (Acid Condition 2). Group E compounds are nucleoside analogue pro-drugs of the group C compound 5 parent. The molecular weights, pKas and log Ps are shown in Table 55. The compounds are all primary amines, except for compound 8, which is a secondary amine. Representative chromatograms for the Group E nucleoside analogue pro-drugs are shown in Figs. 146 to 148. The retention

Table 55. Molecular weights, log P's and pKa's of Group E compounds

Group	No.	MW	ApKa	BpKa	Log P*
E	1	358.368	11.81	8.28, 1.89	-0.451
	2	371.363	-	3.73	1.27
	3	399.416	-	3.73	2.328
	4	459.468	-	3.73	3.003
	5	431.414	-	3.73	2.205
	6	487.522	-	3.73	4.321
	7	455.524	-	3.73	4.444
	8	601.665	13.95	2.4	6.863

* Calculated log P

data are shown in Tables 56 and 57. At 90% acetonitrile in the mobile phase, compound 8 elutes first at 1.218 minutes and compound 7 elutes last at 2.546 minutes. When the mobile phase contains 60% acetonitrile, compound 1 elutes first at 1.411 minutes and compound 8 elutes last at 1.218 minutes. The chromatograms for the mixtures at different % acetonitrile in the mobile phase show that the compound order is different depending on the % acetonitrile in the mobile phase (Fig. 148). Compound 8 elutes last at 60% acetonitrile, but first at 95% acetonitrile. If one wanted to isolate and collect compound 8, the 95% acetonitrile condition would be sufficient.

The retention of all Group E solutes except for compound 8 increases with increasing acetonitrile in the mobile phase from about 70 or 80% acetonitrile to 95% acetonitrile (Figs. 149 and 150). Therefore, all compounds except compound 8 are exhibiting the ANP mode of chromatography. Only reverse phase chromatography is occurring for the Group E nucleoside pro-drug compound 8. Its retention factor decreased from 6.111 at 60% acetonitrile to 0.520 at 95% acetonitrile. This compound is the only one out of the eight Group E compounds that does not contain a primary amine. Its amine group is substituted so the BpKa decreased from 3.73 to 2.4 making the amine group less basic. For the solutes that exhibit ANP behavior, compounds 6 and 7 initiate the ANP mode at 80% acetonitrile, and compounds 2, 3, 4 and 5 initiate at 70% acetonitrile. Compound 1 starts even earlier, at approximately 60% acetonitrile. The earlier ANP behavior for compound 1 could be due to its lower log P of -0.451. All other Group E compounds have LogPs greater than 1.0. Or the difference could be due to the basicity of the amine group. Compound 1 has a BpKa of 8.28, whereas the other ANP

Group E compounds have BpKa's of 3.73. However, the parent compound (Group C compound 5) started its ANP behavior at 50% acetonitrile and it has a LogP of -0.936 and an amine group with a BpKa of 3.73. The plot of log P vs % acetonitrile in the mobile phase where the ANP behavior starts shows there is a correlation ($R^2 = 0.827$) between hydrophobicity and ANP initiation (Fig. 151). As the hydrophobicity (log P) increases, the ANP behavior starts at a higher % acetonitrile. The most polar compounds (Group C compound 5 and Group E compound 1) start the ANP behavior the earliest.

Next, eleven more nucleoside analogue pro-drugs called Group F were tested with Acid Condition 2 on the cholesteric phase. The Group F compounds are also pro-drugs of the nucleoside analogue parent in Group C (compound 5). They all have amine groups, but only six have primary amine groups. The molecular weights, pKa's and log Ps are shown in Table 58. The log Ps range from -0.163 (compound 1) to 5.379 (compound 11).

Representative chromatograms for the Group F nucleoside analogues run on the cholesteric phase with 90% acetonitrile in the mobile phase are shown in Fig. 153. Compound 2 elutes last at 16.477 minutes, and compound 3 elutes first at 1.073 minutes. The elution of Group F compounds is not in order of hydrophobicity. For example, compound 2 has a very low log P (-0.952), but elutes last. Therefore, another separation mechanism besides hydrophobicity must be occurring. At 60% acetonitrile in the mobile phase, the elution order is different than at 90% acetonitrile (Fig. 154). Compound 2 elutes first at 0.982 minutes and compound 9 elutes last at 3.886 minutes. At these

Fig. 146. Representative chromatograms for Group E compounds run on the cholesteric phase (Col #4) at 90% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (90:10:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group E – 1, 2, 3, 4, 5, 6, 7, 8.

Fig. 147. Representative chromatograms for Group E compounds run on the cholesteric phase (Col #4) at 60% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group E – 1, 2, 3, 4, 5, 6, 7, 8.

Fig. 148. Representative chromatograms of Test Mixture for Group E Compounds run on the cholesteric phase (Col #4) at different % acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/0.50% Formic acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group E – 1, 2, 3, 4, 5, 6, 7, 8.

Table 56. Retention times of Group E Compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Group	No	Retention time (minutes) - Cholesteric (Col # 4)				
		Acetonitrile (%)				
		95	90	80	70	60
E	1	2.83	1.411	1.042	0.938	0.904
	2	3.215	2.274	1.514	1.317	1.303
	3	2.847	2.299	1.599	1.443	1.506
	4	2.807	2.233	1.662	1.062	1.848
	5	2.937	2.209	1.562	1.437	1.539
	6	2.609	2.404	1.878	1.937	2.486
	7	2.965	2.546	1.947	1.977	2.471
	8	1.201	1.218	1.54	2.53	5.618
$t_0 = 0.79$						

Table 57. Retention factors of Group E compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Group	No	Retention factor (K') - Cholesteric (Col # 4)				
		Acetonitrile (%)				
		95	90	80	70	60
E	1	2.582	0.786	0.319	0.187	0.144
	2	3.070	1.878	0.916	0.667	0.649
	3	2.604	1.910	1.024	0.827	0.906
	4	2.553	1.827	1.104	0.344	1.339
	5	2.718	1.796	0.977	0.819	0.948
	6	2.303	2.043	1.377	1.452	2.147
	7	2.753	2.223	1.465	1.503	2.128
	8	0.520	0.542	0.949	2.203	6.111
$t_0 = 0.79$

Fig. 149. Retention factor vs. % acetonitrile in the mobile phase for Group E compounds on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group E – 1, 2, 3, 4, 5, 6, 7, 8.

Fig. 150. Log K' vs. % acetonitrile in the mobile phase for Group E compounds on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group E – 1, 2, 3, 4, 5, 6, 7, 8.

Fig. 151. % Acetonitrile in the mobile phase where ANP initiates vs. log P for Group C and E compounds run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group C = 5, 6, 7, 8; Group E=1, 2, 3, 4, 5, 6, 7.

Fig. 152. Log K' vs. log P for Group E compounds run on the cholesteric phase (Col# 4) using Acid Condition 2 at 60% Acetonitrile in the mobile phase. Solutes: Group E – 1, 2, 3, 4, 5, 6, 7, 8.

Table 58. Molecular weights, log Ps, pKa's of Group F compounds

Group	No.	MW	ApKa	BpKa	Log P*
F	1	358.4	11.78	7.95, 3.73	-0.163
	2	356.4	12.3	7.95, 3.73	-0.952
	3	373.4	11.81, 13.95	2.4	1.606
	4	485.50	13.95	2.4	3.812
	5	457.5		8.25, 7.65	1.266
	6	455.5		2.17	2.667
	7	476.5		2.98, 1.32	1.597
	8	427.5		3.73	3.386
	9	541.6	3.95	2.4	5.928
	10	455.5		3.73	4.444
	11	515.6		3.73	5.379

* Calculated log P

conditions, the separation seems to be based on hydrophobicity since compound 2 has the lowest log P (-0.952) and compound 9 has the highest log P (5.928). Retention data are shown in Tables 59 and 60.

The retention factor vs. % acetonitrile is plotted in Fig. 155 and the log K vs. % acetonitrile is plotted in Fig. 156. For pro-drugs 4, 6 and 9, their retention decreases with increasing % acetonitrile. As stated previously, this elution profile follows the reverse phase chromatography mode where separation is based on hydrophobicity and the most hydrophobic solutes elute last. The other pro-drugs (compounds 1, 2, 3, 7, 8, 10 and 11) exhibit the ANP mode of chromatography. Some of these compounds contain primary amine groups with BpKa's of 3.73 (compounds 1, 2, 8, 10 and 11), or 8.25 and 7.65 (compound 5). Two compounds containing substituted amine groups exhibited ANP: compound 3 having a BpKa of 2.4 and compound 7 having BpKa's of 2.98 and 1.32. The solutes which don't exhibit ANP mode have secondary amines with a BpKa of 2.4

(compound 4), 2.17 (compound 6), or no BpKa (compound 9). It appears that a BpKa of at least 2.4 is necessary for a compound to exhibit the ANP mode. It is unclear why compound 3 exhibits ANP, whereas compound 4 does not, since both compounds contain secondary amines with calculated BpKa's of 2.4. Perhaps the actual BpKa of compound 3 is actually greater than the calculated BpKa, or the compound is more hydrophobic.

Regarding the solutes where ANP occurs, compounds 1, 2 and 5 display increased retention as early as 60% acetonitrile in the mobile phase. Compound 10 starts to exhibit ANP behavior at 70% acetonitrile and compounds 7, 10 and 11 exhibit the ANP mode at 80% acetonitrile. Fig. 157 shows the plot of % acetonitrile for ANP initiation vs. log P. The correlation is poor ($R^2 = 0.5066$). Perhaps compound 5 starts the ANP mode earlier than the theorized trend because its BpKa (7.65) is higher than 3.73. Also, compounds 3 and 7 could sit higher than the line because their BpKa's of 2.4 and 2.98 are lower than 3.73.

Log K' is plotted against the log P at 90% and 60% acetonitrile in the mobile phase for the ANP and reverse phase compounds (Fig 158). At 60% acetonitrile, log K' increases with increasing log P for both sets of compounds as expected for reverse phase chromatography. The log K' vs. log P line for the reverse phase compounds (3, 4, 6 and 9) is above the line for ANP compounds (1, 2, 5, 7, 8, 10, 11). But, at 90% acetonitrile in the mobile phase, the ANP line does not increase with increasing log P as in reverse phase chromatography because the separation mechanism is different. In addition, the ANP line is above the line for the reverse phase compounds.

Fig. 153. Representative chromatograms of Group F compounds run on the cholesteric phase (Col #4) at 90% acetonitrile in the mobile using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (90:10:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group F = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11.

Fig. 154. Representative chromatograms of Group F compounds run on the cholesteric phase (Col #4) at 60% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group F = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11.

Table 59. Retention times of Group F compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Group	No	Retention time (minutes) - Cholesteric (Col # 4)				
		Acetonitrile (%)				
		95	90	80	70	60
F	1		10.454	4.024	1.624	1.072
	2		16.477	2.571	1.297	0.982
	3	1.1	1.073	1.032	1.063	1.156
	4	1.102	1.104	1.195	1.472	2.153
	5			3.983	1.603	1.066
	6	1.102	1.094	1.144	1.337	1.761
	7	2.074	1.531	1.365	1.454	1.722
	8	3.233	2.467	1.745	1.66	1.882
	9	1.203	1.203	1.423	2.072	3.886
	10	3.022	2.597	1.971	1.997	2.493
	11	4.572	2.981	2.319	2.601	3.745
<hr/>						
$t_0 = 0.79$						

Table 60. Retention factors of Group F compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Group	No	Retention factor (K') - Cholesteric (Col # 4)				
		Acetonitrile (%)				
		95	90	80	70	60
F	1		12.233	4.094	1.056	0.357
	2		19.857	2.254	0.642	0.243
	3	0.392	0.358	0.306	0.346	0.463
	4	0.395	0.397	0.513	0.863	1.725
	5			4.042	1.029	0.349
	6	0.395	0.385	0.448	0.692	1.229
	7	1.625	0.938	0.728	0.841	1.180
	8	3.092	2.123	1.209	1.101	1.382
	9	0.523	0.523	0.801	1.623	3.919
	10	2.825	2.287	1.495	1.528	2.156
	11	4.787	2.773	1.935	2.292	3.741
<hr/>						
$t_0 = 0.79$

Fig. 155. Retention factor vs. % acetonitrile in the mobile phase for Group F compounds run on the cholesteric phase (Col# 4) with Acid Condition 2. Solutes: Group F = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11.

Fig. 156. Log K' vs. % acetonitrile in the mobile phase for Group F compounds run on the cholesteric phase (Col# 4) with Acid Condition 2. Solutes: Group F = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11.

Fig. 157. % Acetonitrile in the mobile phase where ANP initiates vs. log P for Group F compounds on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group F = 1, 2, 3, 5, 7, 8, 10, 11.

Next, five more nucleoside analogues (Group G1) and two other compounds (Group G2) were tested on the cholesteric phase using the same chromatography conditions. Group G1 nucleoside analogues include two parent drugs which are isomers (compounds 1 and 2) and three pro-drugs. Compound 3 is a pro-drug of parent compound 1 and compounds 4 and 5 are pro-drugs of parent compound 2. These nucleoside analogues have primary amines with BpKa's of 3.73. The Group G2 compound 6 is a tetramic acid molecule with two secondary amines present in the ring structure. The other Group G2 solute, compound 7, is a synthetic peptide containing three amide bonds (Fig 159). The molecular weights, pKa's and LogP's are shown in Table 61.

A

B

Fig. 158. $\log K'$ vs. $\log P$ for Group F compounds run on the cholesteric phase (Col# 4) using Acid Condition 2 at (A) 90% and (B) 60% Acetonitrile in the mobile phase. Solutes: Group F = 1, 2, 3, 5, 7, 8, 10, 11.

Fig. 159. Structure of amide bond

Table 61. Molecular weight, log P, pKa's of Group G1 and G2 compounds

Group	No.	MW	ApKa	BpKa	Log P*
G1	1	284.2	13.89	3.73	-1.937
	2	284.2	13.89	3.73	-1.937
	3	494.5	-	3.73	3.184
	4	494.5	-	3.73	3.184
	5	536.6	-	3.73	4.771
G2	6	375.9	-	8.82	2.854
	7	713.9	-	7.37	3.737

* Calculated log P

At 90% acetonitrile in the mobile phase, compound 4 elutes first at 2.5 minutes and compound 7 elutes last at 11.2 minutes (Fig. 160). With 60% acetonitrile in the mobile phase, compound 2 elutes first (1.0 minute) and compound 5 elutes last (3.0 minutes (Fig 161). Retention data are shown in Tables 62 and 63. The plots of retention factor vs. % acetonitrile and log K' vs. % acetonitrile show that all seven Group G 1 and G2 compounds exhibit the aqueous normal phase mode of chromatography (Figs. 162 and 163). It is interesting that ANP occurs for the tetramic acid compound 6 because it has only a secondary amine group. However, the amine is very basic with a BpKa of 8.82 so the nitrogen can be protonated. The parent Group G1 nucleoside analogues 1 and 2 initiate their retention increase with increasing acetonitrile at 60% acetonitrile in the mobile phase. The nucleoside analogue pro-drugs (compounds 3, 4 and 5) initiate the ANP at 80% acetonitrile. The earlier start of ANP with the parent compounds is probably due to their low hydrophobicity. The Group G2 compounds start their ANP increase at 60% acetonitrile. There is no correlation between log P and the point where

retention increases with increasing acetonitrile when all Group G compounds are plotted together (Fig 164).

Log K' is plotted against log P for the Group G1 and G2 compounds using 90% and 60% acetonitrile in the mobile phase (Fig. 165). At 60% acetonitrile, log K' increases linearly with increasing log P as expected for reverse phase chromatography. However, at 90% acetonitrile, there is no relationship between log K' and log P for the Group G1 compounds because their separation is not based on reverse phase chromatography.

Next, ten more compounds (Groups H1 and H2) were tested on the cholesteric phase (Col #4) with the acetonitrile and 0.05% formic acid mobile phase (Acid Condition 2). The H1 compounds are purines based on a backbone somewhat similar to adenine and the H2 compound has a backbone similar to the nucleotide cytosine (Fig. 166). The molecular weights, pK_a 's and log P s for the H1 and H2 compounds are shown in Table 64. Group H1 compounds contain some secondary amine groups. The H2 compound 10 contains a primary amine group and a substituted amine group. Fig. 167 shows the representative chromatograms for Group H1 and H2 compounds using 95% acetonitrile in the mobile phase. Compounds 1 to 9 elute with similar retention times (1.1 to 1.2 minutes). The H2 compound 10 is much more retained, having a retention time of 6.6 minutes. The log P s of all H compounds are similar and range from 3.9 to 5.1, with compound 10 having a Log P of 4.5. At 60% acetonitrile in the mobile phase, solute retention times range from 1.4 to 2.9 minutes, with compound 7 eluting first and compound 10 eluting last (Fig. 168). The retention data is shown in Tables 65 and 66.

Fig. 160. Representative chromatograms for of Group G compounds run on the cholesteric phase (Col #4) at 90% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (90:10:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group G1= 1, 2, 3, 4, 5; G2 = 6, 7.

Fig. 161. Representative chromatograms for of Group G compounds run on the cholesteric phase (Col #4) at 60% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group G1= 1, 2, 3, 4, 5; G2 = 6, 7.

Table 62. Retention times of Group G compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Group	No	Retention time (minutes) - Cholesteric, (Col # 4)				
		Acetonitrile (%)				
		95	90	80	70	60
G1	1	6.324	3.36	1.593	1.152	0.995
	2	6.291	3.433	1.566	1.129	0.979
	3	4.146	2.65	1.88	1.808	2.057
	4	3.669	2.477	1.799	1.748	2.009
	5	3.954	2.709	2.095	2.252	3.019
G2	6	7.188	4.723	2.261	1.701	1.588
	7		11.166	4.201	2.783	2.402

$t_0 = 0.79$

Table 63. Retention factors of Group G compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Group	No	Retention factor (K') - Cholesteric, (Col # 4)				
		Acetonitrile (%)				
		95	90	80	70	60
G1	1	7.005	3.253	1.016	0.458	0.259
	2	6.963	3.346	0.982	0.429	0.239
	3	4.248	2.354	1.380	1.289	1.604
	4	3.644	2.135	1.277	1.213	1.543
	5	4.005	2.429	1.652	1.851	2.822
G2	6	8.099	4.978	1.862	1.153	1.010
	7		13.134	4.318	2.523	2.041

$t_0 = 0.79$

A

B

Fig. 162. Retention factor vs. % acetonitrile in the mobile phase for Group G compounds run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: (A) Group G1=1, 2, 3, 4, 5; (B) G2 = 6, 7.

A

B

Fig. 163. Log K' vs. % acetonitrile in the mobile phase for Group G compounds run on the Cholesteric phase (Col# 4) using Acid Condition 2. Solutes: (A) Group G1=1, 2, 3, 4, 5; (B) G2 = 6, 7.

Fig. 164. % Acetonitrile in the mobile phase where ANP initiates vs. log P for Group G compounds run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group G1 = 1, 2, 3, 4, 5; Group G2 = 6, 7.

Figs. 169 and 170 show the plots of retention vs. % acetonitrile and log K' vs. % acetonitrile in the mobile phase for group H compounds. For all the Group H1 purine compounds, the retention decreases with increasing % acetonitrile as in reverse phase chromatography. The ANP does not occur because the Group H1 compounds do not contain primary amine groups which can readily protonate. The Group H2 compound 10 increases its retention with increasing % acetonitrile in the mobile phase from 70% to 95%. Therefore, the ANP mode of chromatography is occurring with the Group H2 compound because it has a primary amine group with a BpK_a of 4.07.

The last set of compounds tested on the cholesteric phase (Col #4), are nucleoside analogues (Group I). Ribavirin (compound 1) is a parent drug containing a D-ribose molecule and Levovirin-5'-Valine Ester (LVV-Val, compound 2) is the pro-drug of ribavirin's isomer levovirin (Fig. 171). The molecular weights, pK_a 's and log P s are

A

B

Fig. 165. Log K' vs. log P for Group G compounds run on the cholesteric phase (Col# 4) using Acid Condition 2 at (A) 90% and (B) 60% acetonitrile in the mobile phase. Solutes: Group G1=1, 2, 3, 4, 5; G2 = 6, 7.

Fig. 166. Structures of (A) the purine adenine and (F) the nucleotide cytosine

Table 64. Molecular weights, log Ps, pKa's of Group H compounds

Group	No.	MW	ApKa	BpKa	Log P*
H1	1	447.8	-1.42, 12.59	1.58, 0.67	3.949
	2	457.9	-1.42	1.62, 0.67	5.279
	3	431.8	-1.38	1.73, 0.67	5.397
	4	430.8	13.29	6.81, 2.68	5.743
	5	463.4	9.59	4.43, 2.65	4.704
	6	446.8	0.54	8.94, 2.91	5.106
	7	448.4	11.35	6.27, 2.91	4.935
	8	389.3	12.15	-	4.751
	9	330.3	-1.16	3.78, 1.32	4.561
H2	10	470.3	-	4.07	4.478

* Calculated log P

Fig. 167. Representative chromatograms of Group H compounds run on the cholesteric phase (Col #4) for 95% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (95:5:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group H1 = 1, 2, 3, 4, 5, 6, 7, 8, 9; Group H2 = 10.

Fig. 168. Representative chromatograms of Group H compounds run on the cholesteric phase (Col #4) for 60% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 270 nm. Solutes: Group H1 = 1, 2, 3, 4, 5, 6, 7, 8, 9; Group H2 = 10.

Table 65. Retention times of Group H compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Group	No	Retention time (minutes) - Cholesteric, Col # 4				
		Acetonitrile (%)				
		95	90	80	70	60
H1	1	1.099	1.086	1.098	1.216	1.473
	2	1.234	1.233	1.391	1.815	2.788
	3	1.131	1.135	1.276	1.628	2.46
	4	1.22	1.179	1.244	1.486	2.029
	5	1.106	1.085	1.096	1.21	1.474
	6	1.113	1.089	1.1	1.201	1.436
	7	1.056	1.051	1.076	1.199	1.425
	8	1.08	1.082	1.122	1.21	1.637
	9	1.235	1.224	1.358	1.722	2.506
H2	10	6.557	4.619	2.758	2.53	2.933
$t_0 = 0.79$						

Table 66. Retention factors of Group H compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition 2

Group	No	Retention factor (K') - Cholesteric, Col # 4				
		Acetonitrile (%)				
		95	90	80	70	60
H1	1	0.391	0.375	0.390	0.539	0.865
	2	0.562	0.561	0.761	1.297	2.529
	3	0.432	0.437	0.615	1.061	2.114
	4	0.544	0.492	0.575	0.881	1.568
	5	0.400	0.373	0.387	0.532	0.866
	6	0.409	0.378	0.392	0.520	0.818
	7	0.337	0.330	0.362	0.518	0.804
	8	0.367	0.370	0.420	0.532	1.072
	9	0.563	0.549	0.719	1.180	2.172
H2	10	7.300	4.847	2.491	2.203	2.713
$t_0 = 0.79$

Fig. 169. Retention factor vs. % acetonitrile in the mobile phase for Group H compounds run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group H1 = 1, 2, 3, 4, 5, 6, 7, 8, 9; Group H2 = 10.

Fig. 170. Log K' vs. % acetonitrile in the mobile phase for Group H compounds run on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group H1 = 1, 2, 3, 4, 5, 6, 7, 8, 9; Group H2 = 10.

A

B

Fig. 171. Log K' vs. log P for Group H compounds run on the cholesteric phase (Col# 4) using Acid Condition 2 at (A) 95% and (B) 60% Acetonitrile in the mobile phase. Solutes: Group H1 = 1, 2, 3, 4, 5, 6, 7, 8, 9; Group H2 = 10.

shown in Table 67. The ribavirin molecule has a very low log P of -2.348 and LVV-Val has a slightly higher log P of -1.574. These two molecules only absorb at 215 nm, which is also where the formic acid absorbs. Therefore, mass spectroscopy was used for the detector. The formic acid had to be reduced to 0.1% to keep the salt from building up onto the detector cell. The chromatograms were extracted using the M+Na⁺ (267) data for ribavirin and the M+H⁺ (344) data for LVV-Val.

Fig. 172. Structures of (A) Ribavirin and (B) Levovirin-5'-Valine Ester

Table 67. Molecular weights, log P's and pKa's of Group I compounds

Group	No.	Cmpd	MW	ApKa	BpKa	Log P*
I	1	Ribavirin	244.260	13.22	2.73	-2.348
	2	Levovirin-5'-Valine Ester	343.338	13.26, 13.95	7.95, 2.73	-1.574

* Calculated log P

Representative chromatograms for Group I compounds 1 and 2 run on the cholesteric phase are shown in Figs. 173 to 176. The chromatogram of ribavirin run at 95% acetonitrile in the mobile phase has a saddle with two peaks eluting from 0.98 to 2.19 minutes (Fig. 173). At 90% acetonitrile, there is a broad peak eluting from 0.75 to 1.5 minutes. At 80%, the ribavirin peak elutes at 0.96 minutes and has better peak shape.

At the other percentages of acetonitrile tested (70 to 30%), the ribavirin peak elutes between 0.81 to 0.87 minutes. The LVV-Val compound also shows strange retention behavior at 95% acetonitrile in the mobile phase (Fig. 175). A very broad peak elutes from 4.75 to 8.0 minutes. Then, at 90% the peak is a little less broad, eluting from 4.0 to 5.5 minutes. When the acetonitrile % is reduced to 80%, the LVV-Val peak has a decent peak shape and elutes at 2.0 minutes. At all other percentages of acetonitrile amounts tested, the LVV-Val peak shape is good and retention times ranged from 1.2 to 0.94 minutes. The retention data are shown in Tables 68 and 69. For the retention time of ribavirin at 95% acetonitrile, the second peak of the saddle (2.19 minutes) was used. For LVV-Val at 95 and 90% acetonitrile, the average retention times of the broad peaks were used.

The plots of retention factor vs. % acetonitrile and $\log K'$ vs. % acetonitrile show that for both group I compounds, the retention increased with increasing acetonitrile at high % acetonitrile in the mobile phase (Figs. 177 and 178). Therefore, these nucleoside analogues exhibit aqueous normal phase mode of chromatography. Ribavirin contains one primary amine group with a BpKa of 2.73 and LVV-Val contains two primary amine groups having BpKa's of 7.95 and 2.73, which causes the ANP behavior. The saddle peak shape of ribavirin and the broad peak shape of LVV-Val could be due to the combination of the low BpKa of 2.73 and the lower amount of formic acid in the mobile phase, 0.1%. Perhaps only half of the ribavirin is being protonated with the formic acid. If a higher amount of formic acid, such as 0.5%, were used maybe ribavirin would elute

as one peak at 95% acetonitrile. These peak shapes might also be due to solubility problems.

The retention increase due to ANP initiates at 50% acetonitrile for ribavirin and 60% for LVV-Val. The lower ANP starting point for ribavirin must be due to its lower log P of -2.348.

Fig. 173. Representative chromatograms of Group I compound 1 (Ribavirin) run on the cholesteric phase (Col #4) with 95 to 70% acetonitrile in the mobile phase using Acid Condition. Mobile phase: ACN/Water/0.1% Formic acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = Mass Spec: 267 (M+Na⁺), Solutes: Group I = 1-Ribavirin.

Fig. 174. Representative chromatograms of Group I compound 1 (Ribavirin) run on the cholesteric phase (Col #4) with 60 to 30% acetonitrile in the mobile phase using Acid Condition. Mobile phase: ACN/Water/0.1% Formic acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = Mass Spec: 267 ($M+Na^+$), Solutes: Group I = 1-Ribavirin.

Fig. 175. Representative chromatograms of Group I compound 2 (LVV-Val) run on the cholesteric phase (Col #4) with 95 to 70 % acetonitrile in the mobile phase using Acid Condition. Mobile phase: ACN/Water/0.1% Formic acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = Mass Spec: 344 (M+H⁺), Solutes: Group I = 2-Levovirin 5'-Valine Ester (LVV-Val).

Fig. 176. Representative chromatograms of Group I compound 2 (LVV-Val) run on the cholesteric phase (Col #4) with 60 to 30 % acetonitrile in the mobile phase using Acid Condition. Mobile phase: ACN/Water/0.1% Formic acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = Mass Spec: 344 (M+H⁺), Solutes: Group I = 2-Levovirin 5'-Valine Ester (LVV-Val).

Table 68. Retention times of Group I compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition

Grp	No	Cmpd	Retention Time (minutes) - Cholesteric (Col # 4)							
			Acetonitrile (%)							
			30	40	50	60	70	80	90	95
I	1	Ribavirin	0.87	0.85	0.87	0.81	0.85	0.96	1.03	2.19
	2	Levovirin 5'-Valine Ester	0.98	0.96	0.94	1.02	1.24	1.95	4.83	6.13

$t_0 = 0.79$

Table 69. Retention factors of Group I compounds run on the cholesteric phase with different mobile phase compositions using Acid Condition

Grp	No	Cmpd	Retention factor (K') - Cholesteric (Col # 4)							
			Acetonitrile (%)							
			30	40	50	60	70	80	90	95
I	1	Ribavirin	0.101	0.076	0.101	0.025	0.076	0.215	0.304	1.772
	2	Levovirin 5'-Valine Ester	0.241	0.215	0.190	0.291	0.570	1.468	5.114	6.759

$t_0 = 0.79$

Fig. 177. Retention factor vs. % acetonitrile in the mobile phase for Group I compounds run on the cholesteric phase (Col# 4) using Acid Condition. Solutes: Group I = 1-Ribavirin, 2-Levovirin-5'-Valine Ester (LVV-Val).

Fig. 178. Log K' vs. % acetonitrile in the mobile phase for Group I compounds run on the cholesteric phase (Col# 4) using Acid Condition. Solutes: Group I = 1-Ribavirin, 2-Levovirin-5'-Valine Ester (LVV-Val).

The molecular properties for all compounds which exhibited aqueous normal phase mode of chromatography are shown in Table 70. These compounds include some nucleoside analogues, a tetramic acid, a synthetic peptide (compound G2-7) and the nucleotide derivative (compound H2-10). They contain basic amine groups. Most have at least one primary amine with BpKa's of at least 3.73. A few molecules contain only secondary amines, with BpKa's of 13.95 (compound F-3), 2.98 (compound F-7) and 8.82 (compound G2-6). Compound G2-7 contains three secondary amines with a BpKa of 7.37. The properties for compounds which did not exhibit ANP are shown in Table 71. These compounds include two tetramic acids, the quinolinediones, the benzopyran, some nucleoside analogues, and the purine compounds. The compounds either only contain secondary or tertiary amines (the tetramic acids, quinolinediones, benzopyran and nucleoside analogues) or aromatic tertiary amines (the purines). The nucleoside

Table 70. Molecular properties for compounds which exhibited aqueous normal phase mode of chromatography on the cholesteric phase

Group	No.	Molecular Class and Description	MW	Amine Type Present	BpKa	Log P*
C	5	Nucleoside analogue parent	259.2	primary	3.73	-0.936
	6	Nucleoside analogue pro-drug	397.4	primary	3.73	1.539
	7	Nucleoside analogue pro-drug	425.5	primary	3.73	2.597
	8	Nucleoside analogue pro-drug	453.5	primary	3.73	3.655
E	1	Nucleoside analogue pro-drug (parent is C-5)	358.4	primary	8.28, 1.89	-0.451
	2	Nucleoside analogue pro-drug (parent is C-5)	371.4	primary	3.73	1.27
	3	Nucleoside analogue pro-drug (parent is C-5)	399.4	primary	3.73	2.328
	4	Nucleoside analogue pro-drug (parent is C-5)	459.5	primary	3.73	3.003
	5	Nucleoside analogue pro-drug (parent is C-5)	431.4	primary	3.73	2.205
	6	Nucleoside analogue pro-drug (parent is C-5)	487.5	primary	3.73	4.321
	7	Nucleoside analogue pro-drug (parent is C-5)	455.5	primary	3.73	4.444
F	1	Nucleoside analogue pro-drug (parent is C-5)	358.4	two primary	7.95, 3.73	7.95
	2	Nucleoside analogue pro-drug (parent is C-5)	356.4	two primary	7.95, 3.73	7.95
	3	Nucleoside analogue pro-drug (parent is C-5)	373.4	secondary	13.95	2.4
	5	Nucleoside analogue pro-drug (parent is C-5)	457.5	primary	8.25, 7.65	8.25
	7	Nucleoside analogue pro-drug (parent is C-5)	476.5	secondary	2.98, 1.32	2.98
	8	Nucleoside analogue pro-drug (parent is C-5)	427.5	primary	3.73	3.73
	10	Nucleoside analogue pro-drug (parent is C-5)	455.5	primary	3.73	3.73
	11	Nucleoside analogue pro-drug (parent is C-5)	515.6	primary	3.73	3.73
G1	1	Nucleoside analogue parent, isomer of G1-2	284.2	primary	3.73	-1.937
	2	Nucleoside analogue parent, isomer of G1-1	284.2	primary	3.73	-1.937
	3	Nucleoside analogue, pro-drug (parent is G1-1)	494.5	primary	3.73	3.184
	4	Nucleoside analogue, pro-drug (parent is G1-1)	494.5	primary	3.73	3.184
	5	Nucleoside analogue, pro-drug (parent is G1-2)	536.6	primary	3.73	4.771
G2	6	Tetramic acid	375.9	secondary	8.82	2.854
	7	Synthetic peptide	713.9	three secondary	7.37	3.737
H2	10	Nucleotide derivative	470.3	primary and secondary	4.07	4.478
I	1	Nucleoside analogue = Ribavirin	244.3	primary	2.73	-2.348
	2	Nucleoside analogue pro-drug = Levovirin-5'-Valine Ester	343.3	two primary	7.95, 2.73	-1.574

* Calculated log P

analogues which did not exhibit ANP are compounds E-8, F-4, F-6 and F-9. Compound F-9 does not have a BpKa. The BpKa's for compounds E-8, F-4 and F-9 are: 2.4, 2.4 and 2.17, respectively. The two nucleoside analogues having only a secondary amine present which did exhibit the ANP mode (F-3 and F-7) have BpKa's of 2.4 and 2.98. Therefore, it seems that a basic amine is required with a BpKa of greater than 2.4. It is interesting to note that two of the tetramic acids did not show ANP and their BpKa's are above 2.4. Compound A1 has BpKa of 5.56 and A2 has BpKa of 5.8. The tetramic acid that did exhibit the ANP mode (G2-6) has a BpKa of 8.82. In summary, primary amines are likely to exhibit the ANP mode of chromatography. In some cases, secondary amines will also exhibit the ANP mode.

Table 71. Molecular properties for compounds which did not exhibit aqueous normal phase mode of chromatography on the cholesteric phase

Group	No.	Molecular Class and Description	MW	Amine Type Present	BpKa	Log P*
A	1	Tetramic acid	535.6	secondary	5.56	2.961
	2	Tetramic acid	428.5	tertiary	5.8	4.293
B	3	Quinolinedione	519.6	secondary	-	2.739
	4	Quinolinedione	535.6	secondary	-	3.457
D	9	Benzopyran	396.4	secondary	-	2.795
E	8	Nucleoside analogue pro-drug (parent is C-5)	601.7	secondary	2.4	6.863
F	4	Nucleoside analogue pro-drug (parent is C-5)	485.5	secondary	2.4	2.4
	6	Nucleoside analogue pro-drug (parent is C-5)	455.5	secondary	2.17	2.17
	9	Nucleoside analogue pro-drug (parent is C-5)	541.6	secondary	-	2.4
H1	1	Purines	447.8	aromatic, tertiary	1.58, 0.67	3.949
	2	Purines	457.9	aromatic, tertiary	1.62, 0.67	5.279
	3	Purines	431.8	aromatic, tertiary	1.73, 0.67	5.397
	4	Purines	430.8	aromatic, tertiary	6.81, 2.68	5.743
	5	Purines	463.4	aromatic, tertiary	4.43, 2.65	4.704
	6	Purines	446.8	aromatic, tertiary	8.94, 2.91	5.106
	7	Purines	448.4	aromatic, tertiary	6.27, 2.91	4.935
	8	Purines	389.3	aromatic, tertiary	-	4.751
	9	Purines	330.3	aromatic, tertiary	3.78, 1.32	4.561

* Calculated log P

For all the compounds which exhibited ANP behavior, the % acetonitrile of ANP initiation was plotted against the LogP (Fig. 179). The correlation between hydrophobicity and ANP initiation when all compounds are plotted is poor ($R^2 = 0.5642$). Perhaps there is only a relationship between molecules containing the same type of basic amine. When compounds containing BpKa's either much greater or lower than 3.73 are removed, the graph shows a better relationship between hydrophobicity and ANP initiation, $R^2 = 0.811$ (Fig. 180). Compounds removed from the plot and their corresponding BpKa's include: E-1 (8.28), F-3 (2.4), F-5 (8.25 and 7.65), F-7 (2.98), G2-6 (8.82) and G2-7 (7.37). Although this correlation coefficient is not 1.00, there is possibly a relationship. If actual log Ps were used instead of calculated log P's, and if the ANP initiation were measured more precisely, the graph might have a better correlation.

Fig. 179. % Acetonitrile in the mobile phase where ANP initiates vs. log P on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group C – 5, 6, 7 and 8; Group E – 1, 2, 3, 4, 5, 6 and 7; Group F – 1, 2, 3, 5, 7, 8, 10 and 11; Group G1 – 1, 2, 3, 4, and 5; Group G2 – 6 and 7; Group H2 – 10, Group I – 1 (ribavirin) and 2 (LVV-Val).

Fig. 180. % Acetonitrile in the mobile phase where ANP initiates vs. log P on the cholesteric phase (Col# 4) using Acid Condition 2. Solutes: Group C – 5, 6, 7 and 8; Group E – 2, 3, 4, 5, 6 and 7; Group F – 1, 2, 8, 10 and 11; Group G1 – 1, 2, 3, 4, and 5; Group H2 – 10, Group I – 1 (ribavirin) and 2 (LVV-Val).

Next, some of the compounds which exhibited the aqueous normal phase mode of chromatography on the cholesteric phase, were then tested on the bidentate-C18TM phase (Col #6) using the Acid Condition 2 (acetonitrile and 0.5% formic acid). The bidentate-C18TM column was prepared using the same silanation/hydrosilation procedure as the cholesteric phase. Representative chromatograms for the Group A, B, and C solutes ran on the bidentate-C18TM phase are shown in Figs. 181 and 182. The corresponding retention data are shown in Tables 72 and 73. At 60% acetonitrile in the mobile phase, Group C compound 5 eluted first (1.6 minutes) and Group B compound 4 eluted last (4.8 minutes). The compound elution order on the bidentate-C18 phase is the same as on the cholesteric phase at this mobile phase condition. At 90% acetonitrile, the Group A and B solutes (1, 3 and 4) elute first, 1.9 to 2.0 minutes. The Group C solutes (5, 6, 7 and 8)

elute later, from 4.9 to 6.8 minutes, with compound 7 eluting last. In Figs. 183 and 184, the retention factors are plotted against the % acetonitrile for the Group A, B and C compounds run on the cholesteric phase and the bidentate-C18TM phase. The log K' vs. % acetonitrile for the bidentate-C18TM phase is plotted in Fig. 185. The Group A and B solutes have decreasing retention with increasing % acetonitrile in the mobile phase as in reverse phase chromatography. For the Group C compounds (5, 6, 7 and 8), the retention increased with increasing % acetonitrile on the bidentate-C18TM phase, so the ANP mode of chromatography is occurring. The start of the increase in retention for the Group C solutes on the bidentate-C18TM phase matches the cholesteric phase results. The log K' vs. log P plot shows that at 60% acetonitrile, the retention of Group C compounds increases with increasing hydrophobicity as in reverse phase chromatography (Fig. 186). However, at 90% acetonitrile in the mobile phase, a relationship between log K' and log P is not observed for the Group C solutes, indicating that another separation mechanism besides reverse phase chromatography is occurring. Therefore, the observed results for the Group A, B and C solutes ran on the bidentate-C18TM phase are similar to what was seen on the cholesteric phase.

Seven of the Group E nucleoside analogues (compounds 1, 2, 3, 5, 6, 7 and 8) were tested on the bidentate-C18TM phase with the same acidic mobile phase conditions. Representative chromatograms are shown in Figs. 187 to 189. The corresponding retention data are shown in Tables 74 and 75. The retention factor vs. % acetonitrile was plotted in Figs. 190 and 191, and the LogK vs. % acetonitrile was plotted in Fig. 191. For the Group E compounds 1, 2 and 3, the retention on the bidentate-C18TM phase

Fig. 181. Representative chromatograms of Group A, B and C compounds run on the bidentate-C18TM phase (Col #6) for 60% acetonitrile using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: (A) Group A=1; Group B = 3, 4; and (B) Group C = 5, 6, 7, 8.

Fig. 182. Representative chromatograms of Group A, B and C compounds run on the bidentate-C18TM phase (Col #6) for 90% acetonitrile using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (90:10:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: (A) Group A=1; Group B = 3, 4; and (B) Group C = 5, 6, 7, 8.

Table 72. Retention times of Group A, B and C compounds with different mobile phase compositions run on the bidentate-C18TM phase using Acid Condition 2

Group	No	Retention time (minutes) - bidentate-C18 TM (Col # 6)				
		Acetonitrile (%)				
		95	90	80	70	60
A	1	1.958	1.996	2.231	2.957	4.747
B	3	1.997	1.975	3.357	3.117	5.04
	4	1.877	1.904	2.188	2.913	4.819
C	5	7.058	4.931	2.442	1.839	1.619
	6	7.646	5.196	3.135	2.515	2.42
	7	7.815	6.814	4.448	4.155	4.766
	8	8.311	5.67	3.666	3.119	3.254
<hr/>						
$t_0 = 1.587$						

Table 73. Retention factors of Group A, B and C compounds with different mobile phase compositions run on the bidentate-C18TM phase using Acid Condition 2

Group	No	Retention factor (K') - bidentate-C18 TM (Col # 6)				
		Acetonitrile (%)				
		95	90	80	70	60
A	1	0.234	0.258	0.406	0.863	1.991
B	3	0.258	0.244	1.115	0.964	2.176
	4	0.183	0.200	0.379	0.836	2.037
C	5	3.447	2.107	0.539	0.159	0.020
	6	3.818	2.274	0.975	0.585	0.525
	7	3.924	3.294	1.803	1.618	2.003
	8	4.237	2.573	1.310	0.965	1.050
<hr/>						
$t_0 = 1.587$						

A

B

Fig. 183. Retention factor vs. % acetonitrile in the mobile phase for Group A and B solutes run on the (A) Cholesterol (Col #4) and (B) bidentate-C18TM phases (Col# 6) using Acid Condition 2. Solutes: Group A = 1; Group B = 3 and 4.

A

B

Fig. 184. Retention factor vs. % acetonitrile in the mobile phase for Group C solutes run on the (A) cholesterol (Col #4) and (B) bidentate-C18TM phases (Col# 6) using Acid Condition 2. Solutes: Group C = 5, 6, 7, and 8.

A

B

Fig. 185. Log K' vs. % acetonitrile in the mobile phase for Group A, B and C solutes run on the bidentate-C18TM phase (Col #6) using Acid Condition 2. Solutes: (A) Group A = 1; Group B = 3, 4; and (B) Group C = 5, 6, 7, 8.

A

B

Fig. 186. Log K' vs. log P in the mobile phase for Group A, B and C compounds run on the bidentate-C18TM phase (Col #6) using Acid Condition 2 at (A) 60% and (B) 90% acetonitrile in the mobile phase. Solutes: Group A = 1; Group B = 3, 4; and Group C = 5, 6, 7, 8.

increases with increasing % acetonitrile in the mobile phase. In addition, retention for compounds 5, 6 and 7 increase with increasing % acetonitrile, but compound 8 does not. These retention results match the results on the cholesteric phase. Also, the initiation of the ANP on the bidentate-C18TM phase matches that on the cholesteric phase.

Next, the Group F compound 11, Group G1 compounds (1, 2, 3, 4 and 5) and Group G2 compounds (6 and 7) were tested on the bidentate-C18TM phase.

Representative chromatograms for the Group F compound 11 and Group G1 and G2 compounds run on the bidentate-C18 phase are shown in Figs. 194, 196, 197 and 198. The retention time data are shown in Tables 76, 77, 78 and 79. The plots of retention factor vs. % acetonitrile and log K' vs. % acetonitrile show that ANP occurs for these compounds on the bidentate-C18TM phase as on the cholesteric phase (Figs. 195, 199, 200 and 201). In addition, the initiation of ANP on the bidentate-C18TM phase occurs at the same % acetonitrile as on the cholesteric phase.

The elution order and retention range for compounds run on the cholesteric and the bidentate-C18TM phases are shown in Table 80. The capacity factors for the compounds run on the cholesteric phase are shorter than on the bidentate-C18TM phase. For example, Compound F-11 eluted at 4.6 minutes on the cholesteric phase and 7.6 minutes on the bidentate-C18TM phase with 95% acetonitrile in the mobile phase. The earlier elution could be due to fewer theoretical plates since the cholesteric column was 75 mm long compared to the 150 mm long bidentate-C18TM column. Or the shorter retention could be attributed to the cholesteric phase being less hydrophobic than the bidentate-C18TM phase. In addition, for Groups A, B, C, E and G, the order of elution on

Fig. 187. Representative chromatograms of Group E compounds run on the bidentate-C18TM phase (Col #6) for 90% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (90:10:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: Group E = 1, 2, 3, 5, 6, 7 and 8.

Fig. 188. Representative chromatograms of Group E compounds run on the bidentate-C18TM phase (Col #6) for 60% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: Group E = 1, 2, 3, 5, 6, 7 and 8.

Fig. 189. Representative chromatograms of two Group E compounds run on the bidentate-C18TM phase (Col #6) for different % acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/0.50% Formic acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: Group E = (A) 1 and (B) 8.

Table 74. Retention times of Group E compounds run on bidentate-C18TM phase (Col #6) with different mobile phase compositions using Acid Condition 2

Group	No	Retention time (minutes) - bidentate-C18 TM (Col # 6)				
		Acetonitrile (%)				
		95	90	80	70	60
E	1	2.35	1.754	1.512	1.438	1.412
	2	3.496	2.914	2.239	2.066	2.104
	3	3.742	3.162	2.51	2.418	2.63
	5	3.582	3.026	2.461	2.43	2.738
	6	4.213	3.81	3.591	4.203	6.133
	7	4.533	4.091	3.745	4.273	5.996
	8	2.584	3.021	5.326	11.854	
		$t_0 = 1.587$				

Table 75. Retention factors of Group E compounds run on bidentate-C18TM phase (Col #6) with different mobile phase compositions using Acid Condition 2

Group	No	Retention factor (K') - bidentate-C18 TM (Col # 6)				
		Acetonitrile (%)				
		95	90	80	70	60
E	1	0.481	0.105	-0.047	-0.094	-0.110
	2	1.203	0.836	0.411	0.302	0.326
	3	1.358	0.992	0.582	0.524	0.657
	5	1.257	0.907	0.551	0.531	0.725
	6	1.655	1.401	1.263	1.648	2.865
	7	1.856	1.578	1.360	1.693	2.778
	8	0.628	0.904	2.356	6.469	
		$t_0 = 1.587$				

A

B

Fig. 190. Retention factor vs. % acetonitrile in the mobile phase for Group E compounds run on the (A) Cholesteric (Col #4) and (B) bidentate-C18TM phases using Acid Condition 2. Solutes: Group E = 1, 2, 3.

A

B

Fig. 191. Retention factor vs. % acetonitrile in the mobile phase for Group E compounds run on the (A) Cholesteric(Col #4) and (B) bidentate-C18™ phases using Acid Condition 2. Solutes: Group E = 5, 6, 7, 8.

Fig. 192. Log K' vs. % acetonitrile in the mobile phase for Group E compounds run on the bidentate-C18TM using Acid Condition 2. Solutes: Group E = 1, 2, 3, 5, 6, 7, and 8.

Fig. 193. Log K' vs. log P in the mobile phase for Group E compounds ran on the bidentate-C18TM using Acid Condition 2 at 60% acetonitrile in the mobile phase. Solutes: Group E = 1, 2, 3, 5, 6, 7, and 8.

Fig. 194. Representative chromatograms of Group F compound 11 run on the bidentate-C18TM for different % acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/0.5% Formic acid, Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: Group F=11.

Table 76. Retention times of Group F compound 11 run on the bidentate-C18TM phase for different % acetonitrile in the mobile phase using Acid Condition 2.

Group	Cmpd	Retention time (minutes) - bidentate-C18 TM (Col # 6)				
		Acetonitrile (%)				
		95	90	80	70	60
F	11	7.586	6.633	6.241	7.774	12.365
<hr/>						
t ₀ = 1.587						

Table 77. Retention factors of Group F Compound 11 run on the bidentate-C18TM phase for different % acetonitrile in the mobile phase using Acid Condition 2.

Group	Cmpd	Retention factor (K') - bidentate-C18 TM (Col # 6)				
		Acetonitrile (%)				
		95	90	80	70	60
F	11	3.780	3.180	2.933	3.899	6.791
<hr/>						
t ₀ = 1.587						

the columns is slightly different. For example, at 70% acetonitrile, the elution order of Groups A, B and C is 5, 6, 1, 4, 8, 3, 7 on the cholesteric phase and 5, 4, 6, 1, 3, 8, 7 on the bidentate-C18TM phase. And for 90% acetonitrile, the elution order of Group G is 4, 3, 5, 1, 2, 6, 7 on the cholesteric phase and 2, 4, 1, 3, 5, 6, 7 on the bidentate-C18TM phase. Therefore, the cholesteric and bidentate-C18TM phases may offer different selectivity.

In summary, for ANP chromatography mode to be observed, it seems that the solute needs to have a basic primary or secondary amine with a pK_a greater than 2.74. The mechanism of ANP is not understood, but is likely due to the presence of the hydride surface since both the cholesteric and bidentate-C18TM phases exhibited ANP. The acidic mobile phase probably protonates the basic solutes' amine groups thus allowing the solutes to interact with the silica surface of the column. For some reason, at the

higher % acetonitrile content in the mobile phase, the protonated solute prefers to interact with the stationary phase longer.

A

B

Fig. 195. Retention factor vs. % acetonitrile in the mobile phase for Group F compound 11 run on the (A) cholesterol (Col #4) and (B) bidentate-C18TM (Col# 6) phases using Acid Condition 2. Solutes: Group F = 11.

Fig. 196. Representative chromatograms of Group G compounds run on the bidentate-C18TM phase (Col# 6) for 95% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (95:5:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: Group G1 = 1, 2, 3, 4, 5; Group G2 = 6 and 7.

Fig. 197. Representative chromatograms of Group G compounds run on the bidentate-C18TM phase (Col# 6) for 90% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (90:10:0.0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: Group G1 = 1, 2, 3, 4, 5; Group G2 = 6 and 7.

Fig. 198. Representative chromatograms of Group G compounds run on the bidentate-C18TM phase (Col# 6) for 60% acetonitrile in the mobile phase using Acid Condition 2. Mobile phase: ACN/Water/Formic acid (60:40:0.50), Column temperature = ambient, Flow rate = 1.0 mL/min, Detection = 276 nm. Solutes: Group G1 = 1, 2, 3, 4, 5; Group G2 = 6 and 7.

Table 78. Retention times of Group G1 and G2 compounds run on the bidentate-C18TM phase (Col# 6) with different mobile phase compositions using Acid Condition 2

Group	No	Retention time (minutes) - bidentate-C18 TM (Col # 6)				
		Acetonitrile (%)				
		95	90	80	70	60
G1	1	6.638	4.767	2.377	1.761	1.562
	2	6.783	4.515	2.379	1.738	1.535
	3	6.277	4.983	3.9	3.83	4.569
	4	5.66	4.711	3.704	3.621	4.378
	5	6.117	5.813	5.199	5.949	8.817
G2	6	7.806	6.518	3.448	2.526	2.344
	7	23.35	15.077	6.449	4.091	3.517

$t_0 = 1.587$

Table 79. Retention factors of Group G1 and G2 compounds run on the bidentate-C18TM phase (Col# 6) with different mobile phase compositions using Acid Condition 2

Group	No	Retention factor (K') - bidentate-C18 TM (Col # 6)				
		Acetonitrile (%)				
		95	90	80	70	60
G1	1	3.183	2.004	0.498	0.110	-0.016
	2	3.274	1.845	0.499	0.095	-0.033
	3	2.955	2.140	1.457	1.413	1.879
	4	2.566	1.968	1.334	1.282	1.759
	5	2.854	2.663	2.276	2.749	4.556
G2	6	3.919	3.107	1.173	0.592	0.477
	7	13.713	8.500	3.064	1.578	1.216

$t_0 = 1.587$

A

B

Fig. 199. Retention factor vs. % acetonitrile in the mobile phase for Group G1 compounds run on the (A) cholesterol (Col # 4) and (B) bidentate-C18TM (Col# 6) phases using Acid Condition 2. Solutes: Group G1 = 1, 2, 3, 4, 5.

A

B

Fig. 200. Retention factor vs. % acetonitrile in the mobile phase for Group G2 compounds run on the (A) cholesterol (Col # 4) and (B) bidentate-C18TM (Col# 6) phases using Acid Condition 2. Solutes: Group G2 = 6 and 7.

A

B

Fig. 201. Log K' vs. % acetonitrile in the mobile phase for Group G compounds run on the bidentate-C18TM phase (Col# 6) using Acid Condition 2. Solutes: (A) Group G1 = 1, 2, 3, 4, 5; and (B) Group G2 = 6 and 7.

A

B

Fig. 202. Log K' vs. log P in the mobile phase for Group G compounds run on the bidentate-C18TM phase (Col# 6) using Acid Condition 2 at (A) 90% and (B) 60% acetonitrile in the mobile phase. Solutes: Group G1 = 1, 2, 3, 4, 5; Group G2 = 6 and 7.

Table 80. Elution order and retention range of compounds run on the cholesteric and bidentate-C18TM phases using acetonitrile/water/0.50% formic acid mobile phase

Group	% ACN	Order on cholesteric phase	Order on bidentate-C18 TM phase
A, B, C	95	N/A	4, 1, 3, 5, 6, 7, 8 (1.9 to 8.3 minutes)
	90	4, 1, 3, 6, 7, 5, 8 (1.0 to 2.4 minutes)	4, 3, 1, 5, 6, 8, 7 (1.9 to 6.8 minutes)
	80	4, 1, 3, 5, 6, 8, 7 (1.1 to 1.9 minutes)	4, 1, 5, 6, 3, 8, 7 (2.2 to 4.4 minutes)
	70	5, 6, 1, 4, 8, 3, 7 (1.1 to 1.7 minutes)	5, 4, 6, 1, 3, 8, 7 (1.8 to 4.1 minutes)
	60	5, 6, 8, 7, 1, 4, 3 (1.0 to 2.6 minutes)	5, 6, 8, 1, 7, 4, 3 (1.6 to 5.0 minutes)
E	95	8, 6, 1, 3, 5, 7, 2 (1.2 to 3.2 minutes)	1, 8, 2, 5, 3, 6, 7 (2.4 to 4.5 minutes)
	90	8, 1, 5, 2, 3, 6, 7 (1.2 to 2.5 minutes)	1, 2, 8, 5, 3, 6, 7 (1.8 to 4.1 minutes)
	80	1, 2, 8, 5, 3, 6, 7 (1.0 to 1.9 minutes)	1, 2, 5, 3, 6, 7, 8 (1.5 to 5.3 minutes)
	70	1, 2, 5, 3, 6, 7, 8 (0.9 to 2.5 minutes)	1, 2, 3, 5, 6, 7, 8 (1.4 to 11.8 minutes)
	60	1, 2, 3, 5, 7, 6, 8 (0.9 to 5.6 minutes)	1, 2, 3, 5, 6, 7, 8 (1.4 to 6.1 minutes)
F	95	11 (4.6 minutes)	11 (7.6 minutes)
	90	11 (3.0 minutes)	11 (6.6 minutes)
	80	11 (2.3 minutes)	11 (6.2 minutes)
	70	11 (2.6 minutes)	11 (7.8 minutes)
	60	11 (3.8 minutes)	11 (12.4 minutes)
G1 and G2	95	4, 5, 3, 2, 1, 6 (3.7 to 7.2 minutes)	4, 5, 3, 1, 2, 6 (5.7 to 7.8 minutes)
	90	4, 3, 5, 1, 2, 6, 7 (2.5 to 11.2 minutes)	2, 4, 1, 3, 5, 6, 7 (4.5 to 15.1 minutes)
	80	1, 2, 4, 3, 5, 6, 7 (1.6 to 4.2 minutes)	1, 2, 6, 4, 3, 5, 7 (2.4 to 6.5 minutes)
	70	2, 1, 6, 4, 3, 5, 7 (1.6 to 4.2 minutes)	2, 1, 6, 4, 3, 7, 5 (1.7 to 5.9 minutes)
	60	2, 1, 6, 4, 3, 7, 5 (1.0 to 3.0 minutes)	2, 1, 6, 7, 4, 3, 5 (1.5 to 8.8 minutes)

4. Conclusion

The molecular recognition properties of the cholesteric phase were demonstrated using pro-drugs with planar phenyl attachments and using anthracene molecules. The experiments using the MPAB phase did not demonstrate any different separation mechanisms from the C8 phase; however, the MPAB phase can be used to separate nucleoside analogues. The separation mechanism of purines was the same on the cholesteric phase as on the C8 phase. Chiral separations could not be demonstrated on the cholesteric phase with the two groups of chiral molecules using the chromatography conditions tested. Steroid separation was similar on the cholesteric phase as on the C8 phase with the mobile phase conditions tested. But, one interesting finding was that on the cholesteric phase, the cholesterol solute showed different retention characteristics than the other steroids tested. Finally, the cholesteric and bidentate-C18TM phases exhibit the aqueous normal phase mode of chromatography with polar solutes containing basic amines using acetonitrile/water/formic acid as the mobile phase.

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