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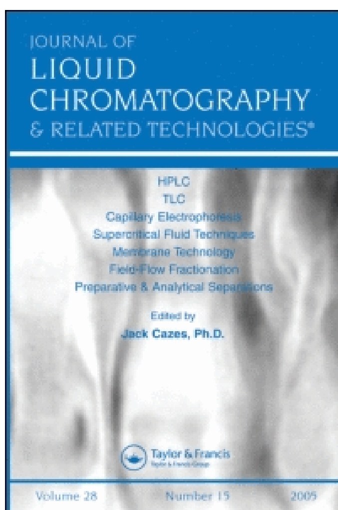

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A Retention Model for Polar Selectivity in Reversed Phase Chromatography as a Function of Mobile Phase Organic Modifier Type

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Abstract: Elucidation of solute retention as a function of the mobile phase modifier type under reversed phase conditions was investigated. The retention of steroid analogues were determined using methanol, acetonitrile, and tetrahydrofuran. Quantitative structure versus retention relationships (QSRRs) were then determined through the use of a de novo mathematical model. The results indicate that interactions between the solute and organic modifier that is extracted into the stationary phase play a significant role in the observed selectivity differences. Thermodynamic studies were conducted to further confirm the finding of the QSRRs determinations.

Keywords: QSRR, Steroid analogues, Polar selectivity

INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) is the most common, powerful, and reliable separation tool utilized for pharmaceutical analysis. Despite extensive investigations, retention mechanisms, including the factors that govern selectivity in reversed phase liquid chromatography, have not yet been clearly elucidated. Reversed phase systems are quite complex relative to those of normal phase systems due to the presence of a wider variety of potential interactions and the dynamic changes that occur in these systems as a function of the mobile phase

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composition and the type of stationary phase. The dominant retention mechanism in RP-HPLC has been attributed to partitioning, adsorption, or a combination thereof.[1–9] A number of mathematical models have been utilized to elucidate retention under reversed phase conditions. Linear free energy relationships (LFER) have provided some insight into retention mechanisms.[10–15] Quantitative structure versus retention relationships (QSRRs) have been utilized to predict retention and explain retention mechanisms based on the structure of the solute.[16,17]

An important parameter contributing to the retention of a solute in RP-HPLC is the mobile phase. Methanol-water and acetonitrile-water are the most commonly used mobile phases in RP-HPLC. The general model of reversed phase retention is based on molecular interactions occurring between the solute and components of the mobile and stationary phases.[18–23] In the mobile phase, it is believed that the dominant interaction between the solute and water is solvophobic expulsion of the solute from the mobile phase into the stationary phase. The organic modifier may also influence retention through its interactions with the solute, water molecules, and the stationary phase.

The apolar nature of the alkyl chains of the stationary phase relative to the bulk mobile phase usually results in an enrichment of the organic modifier in the stationary phase. Tetrahydrofuran and acetonitrile exhibit strong extraction into the stationary phase relative to methanol.[24–26] Methanol shows little difference in composition between the bulk mobile phase and the surface of the stationary phase in water-methanol-alkyl bonded phase systems. Conversely, acetonitrile exhibits preferential sorption onto the alkyl bonded phase in comparison to water for water-acetonitrile-alkyl bonded phase systems.[25] This enrichment of organic modifier in the stationary phase is manifested as an adsorbed phase on top of the bonded layer.[26–28] The methanol layer is determined to be monomolecular while the THF and acetonitrile layers have a thickness consistent with multiple layers. The influence of this adsorption layer on solute retention has not been the subject of much discussion. However, a retention model has been proposed whereby the solute partitions into this adsorbed organic layer, which is on top of the bonded phase, followed by adsorption onto the bonded layer.[26]

The interaction of the solute with the stationary phase is, thus, complex with at least four levels of interaction. The solute can interact with the alkyl chains, residual silanol sites, water in the stationary phase, and the extracted organic modifier in the stationary phase.[23] The first three types of interaction and their impact on solute retention have been the subject of intensive investigations. The fourth type of interaction has not been investigated to a similar extent.

It has been demonstrated that changes in the type of organic modifier have little influence on the interactions of the solute in the mobile phase.[22] This effect is presumably due to the dominance of solute-water interactions over

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solute-organic modifier interactions. Consequently, changes in selectivity for a given stationary phase, as a function of different organic modifiers, can most likely be attributed to the interactions that occur between the solutes and the adsorbed organic layer on the stationary phase.[29–31] In this paper we investigate selectivity differences as a function of organic modifier type. It has been demonstrated that homologues are good choices for studying molecular interactions in chromatographic systems.[32–37] By comparing molecules with similar hydrophobic skeletons of similar size and shape, but differing in their polar functional groups, we may be able to determine the role of these polar groups in affecting selectivity. Polar selectivity is derived from polar interactions such as hydrogen bonding, dipole interactions, or electrostatic interactions. To this end, a series of steroids differing only in the number of double bonds, carbonyl groups, and hydroxyl groups were selected as probes for an investigation of polar selectivity with different organic modifiers. A de novo model was applied to generate QSRRs for the contribution of the polar substituents to retention as a function of organic modifier type.

In addition, the enthalpy and entropy of solute transfer from the mobile phase to the stationary phase were determined through the generation of van't Hoff plots. The enthalpy of interaction is related to the difference in chemical interactions of the solute with the stationary phase and the mobile phase. Studies have been previously performed to determine the thermodynamic properties and the influence of polar groups on solute retention.[38–42]

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EXPERIMENTAL

Materials

Cortisol (F; 4-pregnen-11β, 17, 21-triol-3, 20-dione), cortisone (E; 4-pregnen-17, 21-diol-3, 11, 20-trione), 6β-hydroxycortisol (6β-OHF; 4-pregnen-6β, 11β, 17, 21-tetrol-3, 20-dione), 6β-hydroxycortisone (6β-OHE; 4-pregnen-6β, 17, 21-triol-3, 11, 20-trione), 20β-dihydrocortisol (20β-DHF; 4-pregnen-11β, 17, 20β, 21-tetrol-3-one), 20β-dihydrocortisone (20β-DHE; 4-pregnen-17, 20β, 21-triol-3,11-dione), prednisone (1, 4-pregnadien-17, 21-diol-3, 11, 20-trione), and prednisolone (1, 4-pregnadien-11β, 17, 21-triol-3, 20-dione) were purchased from Steraloids Inc. (Newport, RI, USA). Acetonitrile, methanol, tetrahydrofuran (inhibitor-free), and water were of HPLC grade and purchased from Fisher (Pittsburgh, PA, USA).

Chromatographic Equipment

All experiments were performed on an Agilent HP1100 system with a column oven and a photodiode array detector (Santa Clarita, CA, USA).

The chromatographic data were acquired and analyzed by P.E. Nelson Turbochrom software (Cupertino, CA, USA). The columns used were Symmetry C18, 50 × 2.1 mm (Waters Corporation, MA, USA) with a particle size of 3.5 μm, and YMC ODS-AQ 50 × 4.6 mm (Waters Corporation, MA, USA) with a particle size of 3 μm.

Chromatographic Conditions

The mobile phases were 0.1% (v/v) formic acid (88%, J.T. Baker, NJ, USA) in water as the aqueous component, and acetonitrile, methanol, or tetrahydrofuran as the organic component. Samples were prepared by dissolution in 80/20 (v/v) deionized water/tetrahydrofuran diluent and were introduced into the chromatographic system through a 10 μL loop. Capacity factors, (k′), were determined as defined by

$$k' = (t_r - t_0)/t_0$$

where t_r is the retention time of the analyte peak and t_0 is the first perturbation in the baseline after injection of blank diluent.

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RESULTS AND DISCUSSION

Mathematical Modeling

QSRRs are mathematical models that relate chemical structure and chromatographic retention. The retention factor expresses the mass distribution of a solute between the stationary and mobile phases. The substituent contribution factor, τ, as calculated from RP-HPLC data is defined as:[43]

$$\tau_x = \log k'_{RX} - \log k'_{RH} \quad (1)$$

where RH represents the parent molecule and RX represents a molecule where a hydrogen has been replaced by a substituent X. For congeners having multiple substituents, a more complex mathematical treatment, such as a de novo model, is required.[43–48] This model assumes that retention can be expressed as the sum of the contributions of the substituents and the retention of the parent congener. In this model, the retention of different congeners is described by the summation of the $\log k'$ of the parent congener and terms representing the products of the substituent contribution factor and the indicator variable for each substituent. The substituent contribution factor is unique for a given functional group under specific chromatographic conditions. The indicator variable (I) represents the occurrence

of each possible substituent. The retention of each congener can then be represented by the equation:

$$\log_{k'i} = \log k'_{RH} + I_i X_a \tau_a + I_i X_b \tau_b + \cdots \quad (2)$$

The linear equations generated for each congener are then simultaneously solved by multiple regression analysis. The regression coefficients obtained express the contribution of that substituent under the given chromatographic conditions. The intercept, log k'_{RH}, represents the retention time of the parent congener.

In our study, cortisone was used as the parent congener (Figure 1). Congeners were used where the carboxyl groups at C_{11} and C_{20} were substituted with hydroxyl groups, a double bond was inserted between C_1 and C_2, or the proton at C_6 was substituted with a hydroxyl group. These substitutions resulted in a total of 8 congeners (Figure 2). Data were collected with a YMC column using 0.1% formic acid as the aqueous modifier and either 23% acetonitrile, 45% methanol, or 17% THF as the organic modifier. The percent organic modifier was selected to generate similar capacity factors for the parent congener. The variables are listed in Table 1, while Table 2 shows the results of the regressions.

The effect of the substituent on solute retention is twofold. First, if the substituent is more polar it may reduce the hydrophobic interaction between the solute and the alkyl chains of the stationary phase. Secondly, the substituent may affect the interaction between the solute and the extracted organic modifier, resulting in an increase or decrease of retention depending on the nature of the interaction. The contribution of the substituents to solute retention can be determined from evaluation of the regression coefficients (Table 2). The introduction of the double bond between C_1 and C_2 led to a similar decrease in retention (negative sign of the coefficient) with all mobile phases. The introduction of the hydroxyl group for a proton at C_6 results in a decrease of retention for all organic modifiers. However, the magnitude of the effect differs. With THF, the decrease in retention of the hydroxyl substituted congener is smaller relative to using methanol or

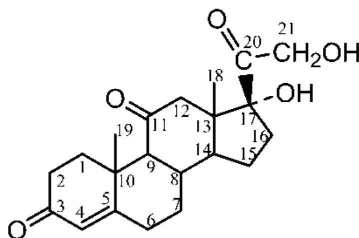

Figure 1. Structure of parent congener, cortisone.

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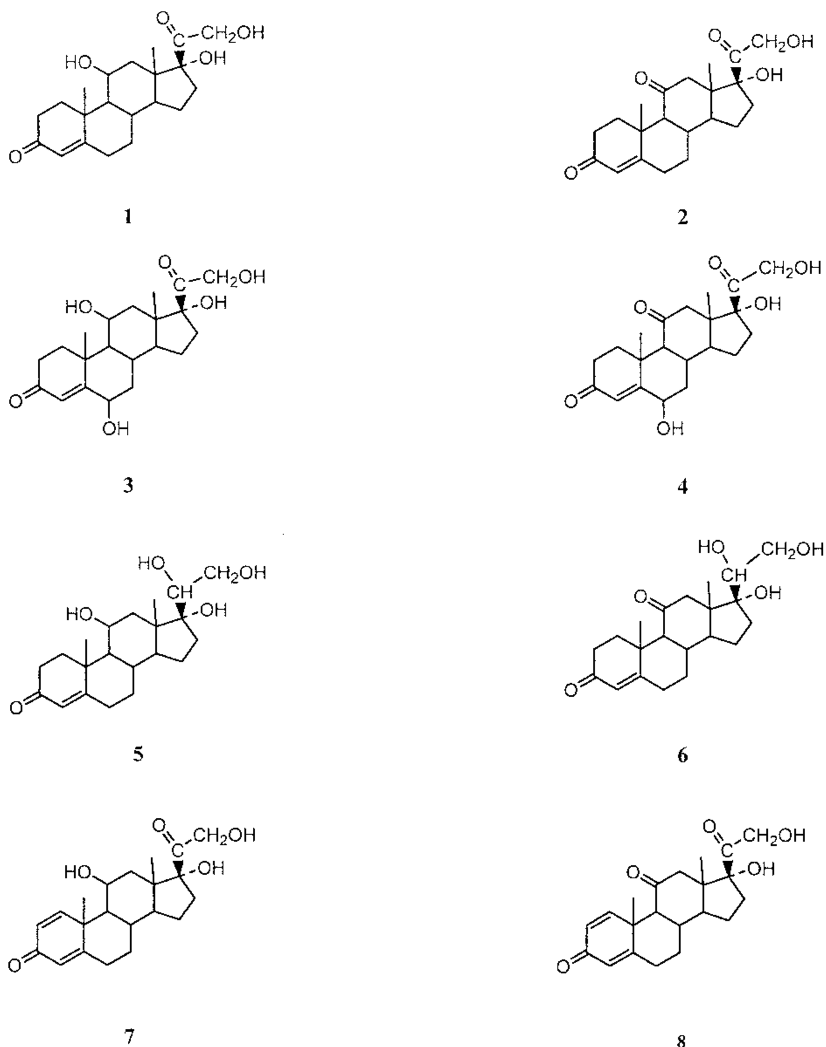

Figure 2. Structures of the 8 congeners. 1—Cortisol; 2—Cortisone; 3—6β-OHF; 4—6β-OHE; 5—20β-DHF; 6—20β-DHE; 7—Prednisolone; 8—Prednisone.

acetonitrile. The substitution of a hydroxyl group for a carbonyl group at C_{20} led to a smaller decrease in retention, with methanol as the organic modifier relative to acetonitrile and THF. For the C_{11} position, substitution of a hydroxy group for a carbonyl group led to a decrease in retention for the congener with acetonitrile, but this decrease was significantly less than those observed for substitution at the C_{20} position. Even more remarkable, this substitution leads to an increase in retention for the substituted congener in

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Table 1. Congener variables for the regression analysis

Compound	C=C	C11	C20	C6
1	0	1	0	0
2	0	0	0	0
3	0	1	0	1
4	0	0	0	1
5	0	1	1	0
6	0	0	1	0
7	1	1	0	0
8	1	0	0	0

methanol and THF. Overall, these effects lead to the differences in selectivity seen for THF, methanol, and acetonitrile (Figure 3).

Since the stationary phase is constant, the observed difference in selectivity cannot be attributed to differences in interactions between the solutes and the stationary phase. The bulk mobile phase is also a less likely cause of these differences, since water is, in all cases, the predominant component of this phase participating in solute interactions. The most likely cause for these differences is the interactions occurring between the solute and the extracted organic modifier in the stationary phase. The differences in selectivity may then be traced to the properties of these organic modifiers.

Acetonitrile exhibits a greater potential for dipole interaction (0.90), than methanol (0.44) and tetrahydrofuran (0.48). Methanol exhibits greater hydrogen bond acidity (0.43) relative to acetonitrile (0.07) and tetrahydrofuran (0.00). Methanol and tetrahydrofuran exhibit similar hydrogen bond basicity (0.53 and 0.47, respectively) relative to acetonitrile (0.32).[10] The data in Table 2 indicates that a change from a carbonyl group to a hydroxyl group results in a small decrease or even a slight increase in retention, when methanol is the organic modifier. There is a significantly greater decrease in retention when acetonitrile is the organic modifier, and the effect on retention in tetrahydrofuran is intermediate between that of methanol and acetonitrile. This effect may be due to the limited ability of the

Table 2. Regression analysis of results from the YMC column

	Acetonitrile	Methanol	Tetrahydrofuran
K0	2.62	2.65	2.74
C=C	−0.09	−0.07	−0.07
C11	−0.18	0.13	0.11
C20	−0.62	−0.12	−0.62
C6	−2.20	−2.35	−1.83
Multiple R	0.996	0.990	0.965

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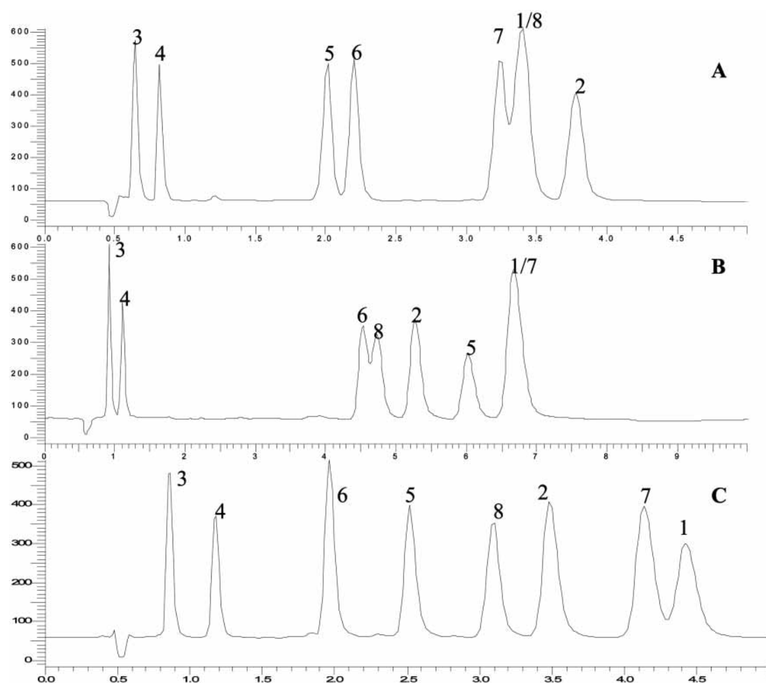

Figure 3. Chromatograms showing the elution order of all 8 congeners as a function of organic modifier with 0.1% formic acid as the buffer phase on YMC ODS-AQ column at ambient temperature. A) 25% acetonitrile, 1.5 mL/min flow rate; B) 45% methanol, 1.2 mL/min flow rate; C) 20% tetrahydrofuran, 1.5 mL/min flow rate.

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solutes to undergo hydrogen bonding with acetonitrile. In contrast, both methanol and THF would undergo stronger hydrogen bonding interactions with the hydroxyl substituted congeners relative to the carbonyl substituted congeners. To affirm that these findings were not specific to the YMC column, similar studies were performed with a Waters Symmetry column with identical results (Table 3).

Table 3. Regression analysis of results from the waters symmetry column

	Acetonitrile	Methanol	Tetrahydrofuran
K0	2.57	2.63	2.63
C=C	−0.07	−0.03	−0.06
C11	−0.19	0.13	0.13
C20	−0.52	−0.11	−0.57
C6	−2.88	−2.90	−2.03
Multiple R	0.994	0.988	0.970

Thermodynamic Studies

The thermodynamic properties for the solute transfer of the steroids from the mobile phase to the stationary phase were determined through generation of van't Hoff plots.

Studies were performed with formic acid as the aqueous modifier, with 45% methanol, 25% acetonitrile, or 20% tetrahydrofuran as the organic modifier, and with a YMC AQ column. The retention time of each solute was determined at 5, 10, 15, 20, 25, 30, 35, 40, and 45°C. The enthalpy and entropy terms for solute transfer (ΔH° and $\Delta S^\circ/R + \ln\varphi$, respectively) were determined from the slope and the intercept of the generated plots for each solute (Table 4). Solutes 3 and 4 were excluded from this study because their small retention times would introduce large errors in the determination of their thermodynamic properties.

A comparison of the thermodynamic properties as a function of mobile phase exhibited some significant differences (Figure 4). The enthalpic values obtained with methanol and THF were significantly more negative in comparison to those obtained with acetonitrile. In addition, the entropic terms were significantly more positive with acetonitrile as compared to methanol and THF. These results confirm differences in the retention mechanism with acetonitrile versus methanol and THF. The stationary phase is the same so the enthalpy for the interaction of a given solute with the stationary phase should be similar regardless of the mobile phase. The same argument can be applied for interactions of the solute with silanol sites and with water extracted into the stationary phase. Using these water rich systems, the interactions of the solute with the mobile phase should be dominated by the interactions between the solute and water. Differences, however, may occur in the interaction between the solute and the extracted organic modifier in the stationary phase. These interactions will vary in

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Table 4. The enthalpic and entropic contributions to solute transfer from the three mobile phases to the stationary phase for the 6 congeners

	Acetonitrile		Methanol		Tetrahydrofuran	
Compound	ΔH° (kJ/mol)	$\Delta S^\circ/R + \ln\varphi$ (J/mol·K)	ΔH (kJ/mol)	$\Delta S^\circ/R + \ln\varphi$ (J/mol·K)	ΔH° (kJ/mol)	ΔS° (J/mol·K)
1	−1.41	1.25	−17.8	−4.57	−20.3	−5.91
2	−4.73	0.03	−17.1	−4.54	−18.0	−5.22
5	4.25	2.92	−15.8	−3.87	−19.2	−6.04
6	1.20	1.80	−15.5	−4.04	−16.5	−5.22
7	−2.09	0.92	−19.1	−5.08	−21.4	−6.40
8	−4.68	−0.07	−17.3	−4.74	−17.8	−5.24

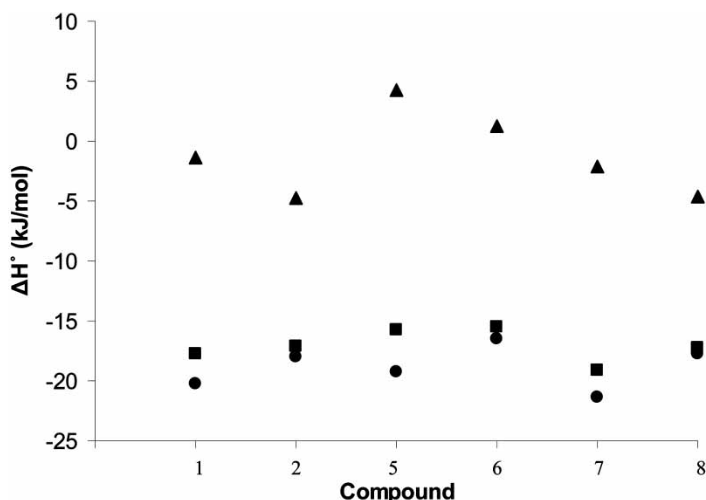

Figure 4. Comparison of the solute transfer enthalpies for the 6 congeners as a function of the organic modifier in the mobile phases—acetonitrile (▲), methanol (■), and tetrahydrofuran (●).

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accordance with the hydrogen bonding and dipolar interaction capability of the organic modifier. In this particular case, the steroids can undergo hydrogen bonding through their hydroxyl groups with both methanol and THF and, to a lesser extent, with acetonitrile. This concept is, thus, consistent with the higher negative enthalpies observed with methanol and THF.

Polar selectivity can also be investigated through thermodynamic studies. For example, the polar selectivity at C_{11} can be determined by evaluation of the ΔΔH and ΔΔS values for the following pairs 1 and 2, 5 and 6, and 7 and 8 (Table 5). In methanol and THF the ΔΔH values are negative while in acetonitrile they are positive, indicating again, that the hydroxyl groups are undergoing a stronger interaction with the methanol and THF relative to acetonitrile.

Table 5. Calculated ΔΔH and ΔΔS values for congener pairs

	Acetonitrile		Methanol		Tetrahydrofuran	
Compound	ΔΔH° (kJ/mol)	ΔΔS° (J/mol · K)	ΔΔH° (kJ/mol)	ΔΔS° (J/mol · K)	ΔΔH° (kJ/mol)	ΔΔS° (J/mol · K)
1, 2	3.32	1.22	−0.69	−0.03	−2.34	−0.69
5, 6	3.04	1.12	−0.32	0.17	−2.71	−0.82
7, 8	2.59	0.99	−1.77	−0.34	−3.64	−1.16

CONCLUSIONS

Selectivity differences were observed for steroid analogues as a function of the organic modifier in reversed phase chromatography. These differences can be attributed to interactions that occur between the solutes and the organic modifier that is extracted into the stationary phase. Hydrogen bonding interactions of the solute with methanol and THF lead to stronger retention for solutes when hydroxyl groups are substituted for carbonyl groups. These solutes appear to undergo weaker interactions when acetonitrile is in the mobile phase, as reflected in the enthalpic contributions for the transfer of the solute from the mobile phase to the stationary phase.

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