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Application of Silica-Based Hyper-Crosslinked Sulfonate-Modified Reversed Stationary Phases for Separating Highly Hydrophilic Basic Compounds

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Abstract

The separation and determination of hydrophilic basic compounds are of great importance in many fields including clinical and biological research, pharmaceutical development and forensic analysis. However, the most widely used analytical separation technique in these disciplines, reversed-phase liquid chromatography (RPLC), usually does not provide sufficient retention for several of the important classes of highly hydrophilic basic compounds including catecholamines, many drug metabolites and many drugs of abuse. Commonly eluents having little or no organic modifier and/or strong ion pairing agents must be used to achieve sufficient retention and separation. Use of highly aqueous eluents can lead to column failure by dewetting, resulting in poor retention, selectivity, reproducibility and slow recovery of performance. The use of a strong ion pairing agent to increase retention renders the separation incompatible with mass spectrometric detection and complicates preparative separations.

This paper describes the successful applications of a novel type of silica-based, hyper-crosslinked, sulfonate-modified reversed stationary phase, denoted as $\text{SO}_3\text{-HC-C}_8\text{-L}$, for the separation of highly hydrophilic cations and related compounds by a hydrophobically assisted cation-exchange mechanism. Compared to conventional reversed-phases, the $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase showed significantly improved retention and separation selectivity. Concurrently, due to the presence of both cation-exchange and reversed-phase retention mechanisms and the high acid stability of hyper-crosslinked phases, the separation can be optimized by changing the type or concentration of ionic additive or organic modifier, and by varying the column temperature. In addition, gradients generated by programming the concentration of either the ionic additive or the organic modifier can be applied to reduce the analysis time without compromising resolution. Furthermore, remarkably different chromatographic selectivities, especially toward cationic solutes, were observed upon comparison of the $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase with conventional reversed-phases. We believe that the combination of these two types of stationary phases will be very useful in two-dimensional liquid chromatography.

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Keywords

HPLC; Reversed-phase; Cation-exchange; Highly hydrophilic basic compounds; Selectivity; Stationary phases

1. Introduction

Highly hydrophilic basic compounds including catecholamines e.g. dopamine and epinephrine and hydrophilic metabolites of pharmaceuticals, e.g. glucuronides, are of great significance in the life sciences and medicine. For example, dopamine, norepinephrine and serotonin are neurotransmitters that communicate from one nerve cell to another. Imbalances in the amounts of various neurotransmitters are involved in depression, and serious neurologic disorders, such as Parkinson's disease. Clearly the analysis of such species is very important to understanding the pathways by which they are synthesized and degraded, and to diagnosis and treatment of the related diseases.

However, the separation of analytes as extremely hydrophilic as the above compounds by conventional RPLC which is after all based on hydrophobic, that is highly lipophilic interaction processes [1–6], is not easy. Such analytes are usually not sufficiently retained to be well separated due to their high hydrophilicity. In order to retain these polar analytes, long ODS columns together with a very high volume fractions of water even 100% aqueous eluents [7–11] is common. Nevertheless, use of a very low volume fraction of organic modifier on most hydrophobic stationary phases usually causes column failure by dewetting [12]. Even though this problem can be avoided by choosing from the relative limited variety of special reversed-phases designed for use in very water rich media [13], an additional and we believe much more serious problem is that use of such a small fraction of organic modifier leaves only a very narrow range in eluent conditions for optimizing the separation via manipulating the eluent composition. Strong ion pairing agents, such as surfactants with long alkyl chains, are also commonly used [7–9,14,15] to increase retention through either ion pairing in the mobile phase [14], or dynamic ion exchange in the stationary phase [5,16,17]. However, the addition of strong ion pairing reagents to the eluent is not desirable because (1) it makes the separation incompatible with mass spectrometric detection, (2) it is hard to recover the column after use since these additives tend to stick very strongly to the stationary phase, and (3) it is not practical in preparative separations. Another approach to increasing retention of highly hydrophilic basic compounds is to separate them as free bases at high pH [18–21]. However, most commercial silica-based stationary phases are not sufficiently stable at high pH to allow alkylamines to be deprotonated

Cation-exchange chromatography has also been used for the separation of highly hydrophilic basic compounds [22–25]. Nevertheless, high salt concentrations are needed to elute the cationic analytes; this has the additional drawback of being incompatible with mass spectrometric detection.

In this work, we used a previously described [26] sulfonate-modified reversed-phase, denoted $\text{SO}_3\text{-HC-C}_8\text{-L}$, which greatly improved the separation of a number of highly hydrophilic basic compounds in mixtures of related materials. The new sulfonated reversed-phase was prepared by introducing an exceedingly small number of sulfonyl groups (approximately $0.11\ \mu\text{moles/m}^2$) to a hyper-crosslinked reversed-phase [26–30]. After this minor degree of sulfonation, the hydrophobicity of the new phase was still quite comparable to that of ordinary C_8 phases. This was clearly demonstrated by the free energy of transfer per methylene unit and the column hydrophobicity selectivity parameter (H) obtained via the Snyder-Dolan phase characterization method [3,4,31–35]. The sulfonated phase functions very

nicely as an ordinary RPLC phase for polar and non-polar but non-electrolyte analytes. At the same time, the very small degree of sulfonation very greatly increased the retention of bases. The large C-term observed in the Snyder-Dolan characteristics of this phase [26,36] readily distinguished it from all other reversed-phases. Due to the coexistence of reversed-phase and strong Coulombic interactions, polar cationic compounds become much more retained and much better separated through a “*hydrophobically assisted cation-exchange mechanism*” [2, 37]. In addition, the separation can be optimized by varying the organic modifier composition, pH, displacer concentration, and temperature. Perhaps most importantly this new phase ($^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$) shows a remarkably different selectivity from conventional reversed-phase. Almost invariable protonated solutes are less retained in RPLC than are the deprotonated forms and even deaminated metabolites. The opposite is almost always true on this new phase. In addition, we believe this phase has considerable promise for use in two dimensional liquid chromatography when combined with a conventional reversed phase.

2. Experimental

2.1. Chemicals

Trifluoroacetic acid (TFA, ReagentPlus, 99%) was obtained from Aldrich (Milwaukee, WI, USA). Oxalic acid and EDTA (acidic form) were purchased from Mallinckrodt (Paris, KY, USA) and Acros Organic (Geel, Belgium) respectively. HPLC acetonitrile (MeCN) was obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC water was prepared by purifying house deionized water with a Barnstead Nanopure II deionizing system with an organic-free cartridge followed by a 0.45 μm Mini Capsule filter from PALL (East Hills, NY, USA). All chromatographic solutes were obtained from Aldrich or Sigma.

2.2. Analytical columns

The 5.0 $\times$ 0.46 cm Primesep 200 (5 μm , 100 $\text{\AA}$) column was purchased from SIELC Technologies (Prospect Heights, IL, USA). SB C₁₈ particles (5 μm , 80 $\text{\AA}$, 180 m^2/g) was donated by Agilent Technologies (Wilmington, DE, USA) and $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ particles (silica substrate: Zorbax, 5 μm , 80 $\text{\AA}$, 180 m^2/g) were synthesized and packed into 5.0 $\times$ 0.46 cm columns in our lab [26].

2.3. Chromatographic conditions

All chromatographic experiments were carried out on a Hewlett-Packard 1090 chromatography system, equipped with a binary pump, an autosampler, a temperature controller and a diode array UV detector (Hewlett-Packard, Wilmington, DE, USA). Data were collected and processed using Hewlett-Packard Chemstation software. The concentrations of mobile phase additives (in percentage or mM) were based on the total volume of the mobile phase including organic modifier and water. 0.2 mM oxalic acid or EDTA was added to the mobile phase to shield the adverse effect of residual tin contamination which could not be removed during phase synthesis. This is especially important in the separation of catecholamines since these analytes have metal chelating ability. The solutes were prepared at a concentration of about 1 mM in 50/50 MeCN/H₂O solutions and the injection volume was 0.5 μL .

3. Results and discussion

3.1. The separation of catecholamines

The structures of the catecholamines and related compounds used here are shown in Fig. 1. The compounds were first separated on a conventional ODS column, SB C₁₈, using a mobile phase which contained a strong ion pairing agent, hexanesulfonate, and only 10% acetonitrile (see Fig. 2.). Two different responses upon addition of hexanesulfonate are evident in the plot of k' versus the hexanesulfonate concentration: (1) a decrease in retention of the two acidic

solutes caused by the adsorption of hexanesulfonate on the stationary phase, and (2) an increase in retention of both amines and amino acids resulting from the ion-pairing/dynamic ion exchange retention mechanisms. However, the retentions of the amines and amino acids rapidly approach an upper limit upon addition of the hexanesulfonate. The rather limited enhancement in retention of cations by use of hydrophobic anionic additives in RPLC was also theoretically confirmed [5,6]. In addition, it is important to note that the more hydrophilic cations which really need to have an increase in retention actually *benefit least* from adding an ion pairing reagent. Due to their inherently low hydrophobicity, very hydrophilic cations have a lower ability to bind to the anionic additives and therefore show a smaller increase in retention and approach the limit more rapidly. For example, the retention factor of norepinephrine is still below 1 in the presence of 10 mM hexanesulfonate and this insufficient retention can not be significantly further increased by use of higher concentrations of hexanesulfonate based on the trend seen in Fig. 2. The poor separation due to the insufficient retention of these highly hydrophilic amines on the SB C₁₈ phase is clearly shown in Fig. 3A. Even though 10 mM hexanesulfonate and only 10% acetonitrile were used, five out of nine compounds eluted within the first 1.0–1.6 minutes (retention factors less than 2) and could not be baseline separated despite reasonable column efficiency.

We also tested the separation of these nine compounds on a Primesep 200 column designed especially for catecholamine separations. This is also a mixed-mode stationary phase showing both cation-exchange and reversed-phase interactions. The conditions recommended by the manufacture were used to achieve separations close to the optimum. As shown in Fig. 3B, with the same 10% acetonitrile, the amines and amino acids were more retained and better separated on Primesep 200 due to its cation-exchange retention mechanism. However, co-elution (compounds 2 and 3) was still observed and almost all the compounds (eight out of nine) were eluted within 0.5–4.0 minutes (retention factors less than 5.5); this is a quite narrow retention window that can hardly handle more complicated mixtures. Increasing retention on the Primesep 200 column by decreasing either the acetonitrile fraction or the TFA concentration could be difficult because both the acetonitrile and TFA concentrations (0.02% (v/v)) are already rather low. Clearly, the retentions of the highly hydrophilic cations were enhanced by use of the Primesep 200 column, but not sufficiently to achieve a wide retention range which would allow a flexible separation optimization.

Fig. 3C shows the much superior separation of the nine catecholamine compounds on the hyper-crosslinked sulfonate-modified reversed-phase ⁻SO₃-HC-C₈-L [26] using 24% acetonitrile and 0.02% (v/v) TFA at 65 °C. As can be seen from the chromatogram, all compounds are baseline separated in a much more extended retention window with a minimum critical pair resolution of 2.4 (compounds 4 and 5). Note the much increased concentration of acetonitrile. In the following sections, we will discuss the separation optimization on a ⁻SO₃-HC-C₈-L phase by varying the TFA concentration, the acetonitrile/water composition and column temperature.

3.1.1. The effect of TFA concentration—In principle, TFA can play three different roles in the cation-exchange separations studied here: (1) the proton from TFA, which at the concentrations employed here is almost fully dissociated, acts as the cationic displacer to exchange with cationic amines; (2) TFA in the mobile phase controls the pH and thus the charge status of the solutes; and (3) the trifluoroacetate resulting from the dissociation of TFA can act as an ion pairing agent in the mobile phase [5,6,38,39]. We believe that the third process has little effect on the retention of catecholamines because trifluoroacetate is not a sufficiently strong ion pairing agent to form ion pairs with the extremely hydrophilic catecholamines. This was confirmed in a subsidiary study on the effect of ion pairing agents in ion exchange processes [36]. The separation can be greatly improved by using the first two effects of TFA. As shown in Fig. 4, the different types of analytes (acids, bases and amino acids) exhibit quite

different dependences of the retention factor on the concentration of TFA. For the two acids, the retention factor is almost independent of the TFA concentration. With pK_a s of 4.2–4.5, the two acids are almost completely protonated at all pHs studied and thus are uncharged. Therefore, their retentions are dominated by a simple reversed-phase mechanism and do not change with the amount of TFA as long as the acetonitrile concentration is fixed. For the four singly charged bases (open symbols), the retention factors vary linearly with $1/[TFA]$ and consequently we conclude that ion exchange between equally charged species (1:1) is the predominant retention mechanism [2,40]. For the three amino acids (solid symbols), a convex curve is obtained as shown in Fig. 4. This results because the carboxylate groups of amino acids have pK_a s lower than 2.6 and the carboxylate groups become partially deprotonated as the mobile phase pH approaches 2.6 at the lower TFA concentrations. The overall net positive charge of each amino acid is lower than 1 and decreases with the lower TFA concentrations. As a result, the amino acids are less retained by cation-exchange at low TFA concentration than they would be if their net positive charges were independent of pH. The separation can be improved greatly due to the very different relationships between retention and the TFA concentration. For example, 3-methoxytyramine and phenylalanine were much better separated at low TFA concentration: the selectivity increased from 1.03 at 0.1% (v/v) TFA to 1.56 at 0.02% (v/v) TFA. The elution order became inverted and the large separation space between dopamine and 3-methoxytyramine was much more efficiently used. The same significantly increased selectivity and inverted elution were also observed for the solute pairs of norepinephrine and 3,4-dihydroxy-L-phenylalanine, and epinephrine and tyrosine. If it were not for the decreased net charge caused by partial deprotonation of the carboxylate groups in the amino acids, we would not be able to see the dramatically improved separation because the selectivity between the two *singly* charged amines does not change under a pure ion exchange mechanism (this is discussed in more detail in section 3.2). Of the three TFA concentrations, 0.02% gave the best separation and we will use this concentration in the following studies.

3.1.2. The effect of acetonitrile composition—As in the separation of neutral species, varying the organic modifier concentration can also be used to change the selectivity in mixed-mode chromatography. The organic modifier exerts a variety of effects including: (1) changing the hydrophobic and hydrophilic interactions; (2) influencing the solvation of the ions; (3) altering the degree of ionization of weak acids or weak bases including both the analytes and buffers, and the ion exchange sites of the stationary phase [41,42] and (4) changing the Coulombic interaction between cations and the surface anions as the dielectric constant of the mobile phase is perturbed by changing the organic modifier. Due to this complex suite of effects, the various solutes respond differently to changes in the organic modifier (type or fraction) and the selectivity can be greatly altered. The nine solutes were separated at three different acetonitrile compositions all at 0.02% (v/v) TFA (see Fig. 5). As the amount of acetonitrile is increased, the followings are expected:

1. The retentions of the two weaker, thus uncharged, acids (pK_a : 4.2–4.5) that are dominated by the reversed-phase mechanism will decrease as observed in Fig. 5.
2. The retentions of bases and amino acids will decrease due to a decreased hydrophobic contribution to the hydrophobically assisted cation-exchange process responsible for their retention [26]. This is more obvious with the more retained hydrophobic solutes, such as 3-methoxytyramine. Similar phenomena were also observed in the other related ion exchange processes [43].
3. When the acetonitrile fraction is increased, the dielectric constant of the mobile phase decreases, resulting in stronger Coulombic interactions between the cations and surface anions. The degree and direction of the change in ion exchange retention is determined by the relative values of the charge to size ratios of the cationic analyte and the displacer. If the charge to size ratio of the cationic analyte is higher than that

of the displacer, an increase in ion exchange retention is expected. This presumably applies to our case, where the displacer (i.e. the proton) should have a lower charge to size ratio compared to the cationic analytes due to the large size of a solvated proton and the fact that on strong cation-exchangers protons are very weak displacers. This might not be the case on weak acid ion exchangers_[a1].

4. The retention, especially for the more hydrophilic solutes, will increase due to hydrophilic interaction chromatography (HILIC) at high acetonitrile composition [44]. This speculation is supported by the increased retention time of uracil (from 0.507 to 0.541 minutes) when the acetonitrile concentration was increased from 50% to 80% (v/v).
5. The retentions of amino acids will increase due to the reduced dissociation of carboxylate groups (pK_a : 1.8–2.4) and consequently increased net positive charge at higher levels of organic modifier [42,45].
6. TFA (pK_a = 0.23), the sulfonyl groups (pK_a = -2.5) on $^-SO_3-HC-C_8-L$ phase and amino groups (pK_a = 8.5–9.6) of the solutes are rather strong acids and bases. Therefore, the changes in the ionization of these groups are probably too small to be important.

With all the above effects operating simultaneously, we observed rather complex variations in retention with the acetonitrile composition (see Fig. 5). In addition, we also noticed that the bases and the amino acids tend to “bunch-up” and become poorly separated at the higher acetonitrile compositions. To minimize the complications due to variations in dissociation of the carboxylate groups and the occurrence of HILIC interactions, we limit our consideration of the selectivity changes to that of just the four amines as the acetonitrile composition increases from 24 to 50%; the “bunching” effect is still seen. This strongly suggests that the major cause of the decreased selectivity at high acetonitrile composition is reduced *hydrophobic assistance* to ion exchange [2]. This is also strong evidence that the hydrophobicity of the $^-SO_3-HC-C_8-L$ phase is essential to obtain the very good selectivity among these analytes. Further examples of improved selectivity related to increased hydrophobic assistance to the ion exchange process will be given in sections 3.2.

3.1.3. Temperature optimization—Largely due to the work of Snyder, temperature has gained more and more attention as a parameter for adjusting selectivity in both RPLC and ion exchange chromatography [43,46–48]. Using temperature to tune selectivity is convenient because no change in the stationary phase or the mobile phase is required. However, one of the challenges to using temperature to optimize separations is that conventional silica-based bonded phases, especially the more hydrophilic ion exchange phases, are not thermally stable above 50–60 °C [49,50]. Polymer-based, e.g. polystyrene and polymethacrylate, ion-exchange phases are chemically and thermally stable [50], but they have much lower separation efficiency, that is plate counts, than silica-based phases.

Because our $^-SO_3-HC-C_8-L$ phase is synthesized by sulfonating a highly acid stable $HC-C_8$ [26–30], it has very good acid stability even at high temperatures. This greatly increased acid stability has allowed us to explore temperature effects on this novel phase. The separation of the nine catecholamines and related compounds was conducted at 40, 50, 65, 80 and 100 °C, and $\ln k'$ versus $1/T$ is plotted in Fig. 6. As we can see, there is a fairly good linear relationship for each compound, which indicates the validity of a linear Van't Hoff type equation as shown below:

$$\ln k' = C - \frac{\Delta H^\circ}{R} \frac{1}{T} \quad (1)$$

which is derived from the fundamental equation:

$$\ln k' = -\frac{\Delta H^\circ(T)}{RT} + \frac{\Delta S^\circ(T)}{R} + \ln \beta \quad (2)$$

by assuming that ΔH° and ΔS° , the standard enthalpy and entropy change for solute transfer from mobile phase to stationary phase respectively, are independent of temperature. This is usually true over a narrow range of temperature. β is the phase ratio which is combined with the entropy term to become the intercept C in Eq. (1).

Based on Eq. (1), the regression results and the ΔH° of the nine catecholamine compounds as well as some alkylphenones are given in Table 1. We see that all the ΔH° are negative, which means *the retention processes are exothermic* and the solutes become less retained as temperature is increased. In addition, by classifying the compounds according to their charge and then sorting the data based on increasing retention in each category, we found that:

1. Except for the two carboxylic acids, within each category of solute, the enthalpy changes is more exothermic for the more retained solutes. This agrees with the results of Rey [51] and Paull [52] in their study of metal ion separations on polymer-based cation-exchangers [51,52].
2. As the analyte's charge decreased from +1 towards zero and the cation-exchange contribution to retention decreased, the enthalpy of retention became more exothermic. That is, the less positively charged compounds tend to have a higher absolute enthalpy change and thus show greater dependence of retention on temperature. For example, with comparable retention, compound 4 (an amino acid with partial positive charge, $\Delta H^\circ = -3.61$ kJ/mol) has a much bigger enthalpy change than compound 5 (a base with one positive charge, $\Delta H^\circ = -0.84$ kJ/mol). Similar differences in enthalpy changes were also observed in studies on the ion exchange of monovalent and divalent metal cations, where divalent cations show much weaker dependence of retention on temperature than monovalent cations [51–54].
3. The intercept is the sum of the phase ratio term and the entropy term as shown in Eq. (2). Given the complexity of the retention process it is surely a gross assumption to take the phase ratio as being the same for all solutes. Nonetheless, we do so and take the differences in intercepts to be related to the differences in the entropy changes. Notwithstanding the approximation in Table 1, we see a clear trend in intercepts by solute type. Within each category, the intercepts are fairly similar. However, between different types, the intercepts, and thus the entropy changes, are very different. The cation-exchange dominated retention of the fully charged bases show a much more positive entropy change (favorable) than the reversed-phase dominated retention of acids and alkylphenones. The partially charged amino acids exhibit entropy changes that fall between the neutral and completely charged basic compounds. This means that at a very high temperature where hydrophobic interactions are greatly reduced, neutral compounds will have very little retention, whereas the ion exchange dominated cations will likely still be retained.

Based on the above observations of the different enthalpy and entropy changes for various types of solutes, it is clear that as the temperature increases the retention factors decrease and tend to converge to a common limiting value for each solute class. The limiting retention factors are in the order: bases > amino acids > acids ~ neutral compounds. The retention convergence of the solutes in each class at high temperatures further confirms the importance of hydrophobic assistance to retention to improving the selectivity of the ion exchange processes.

Due to differences in retention enthalpies among the various solutes, temperature can be a powerful tool for manipulating selectivity. By substituting the slopes and intercepts of Table 1 into Eq. (1), a window diagram [55–57] of the minimum resolution versus temperature can be obtained. Based on the window diagram, the optimum temperature at which we should separate the nine catecholamine compounds is 64 °C, where the minimum resolution reaches the highest value, 2.4. Fig. 3C shows a chromatogram of the separation performed at 65 °C, which is very close to the optimum. We see good agreement with the theoretical prediction: all the compounds are very well separated with a minimum resolution of 2.4. While 64 °C is not what one would call “high temperature”, it is certainly above the typical values reported for optimum stability of most bonded phases especially at low pH [49].

3.1.4. Gradient elution—Based on sections 3.1.1 and 3.1.2, we know that retention, especially for the more retained species, can be significantly altered by changing either the TFA concentration or the acetonitrile volume fraction. Separations with much shorter total analysis time can be achieved by use of either TFA (see Fig. 7A) or acetonitrile gradients (see Fig. 7B). As shown in these chromatograms, with proper gradient design, all analytes can be baseline separated within *eight minutes* using a TFA gradient and within *twelve minutes* with an acetonitrile gradient. Both separation times are much less than the fifteen minutes required in an isocratic baseline separation obtained at even 65 °C (see Fig. 3C). In addition, compared to isocratic elution in the previous sections, the peaks are much sharper in both gradients. The peak widths at the half height in the TFA gradient (0.03–0.12 min) are quite narrow and comparable to the values achieved in typical reversed-phase gradients. The peak widths in the acetonitrile gradient are wider (0.03–0.25 min). However, the acetonitrile gradient might be superior to the TFA gradient in LC-MS because there should be less signal suppression from the lower TFA and higher acetonitrile concentrations [58].

3.2 The separation of aminophenols

Aminophenols are another class of highly hydrophilic basic compounds of great interest to the pharmaceutical industry. For example, 4-aminophenol is the major toxic impurity of p-acetamidophenol (Tylenol) generated during metabolism and drug degradation as a result of the hydrolysis of acetamidophenol. Additionally, aminophenols are commonly used in cosmetics, such as sun screen creams and dyes for hair coloring. However, according to the safety data for 4-aminophenol, it is a potential mutagen and teratogen and its presence in the drug substance must be well controlled. Therefore, the separation and quantitation of 4-aminophenol in the presence of a large amount of p-acetamidophenol is of great importance and RPLC is one of the most frequently used techniques for their analysis. Because of its extreme hydrophilicity, 4-aminophenol is almost always separated at a pH greater than the pK_a (5.50) of the anilinium group to achieve higher retention [18–21]. Nevertheless, even the retention of the uncharged form is usually still insufficient. In the remainder of this section, we will show the very successful separation of p-acetamidophenol, 4-aminophenol and its two isomers: 2-, and 3-aminophenol on the $^{-}SO_3-HC-C_8-L$ phase under acidic conditions.

For comparison, the separation of the four compounds was first carried out on a SB C_{18} column as shown in Fig. 8A. Although only 10% acetonitrile was used, 2-, 3- and 4-aminophenols shown little retention and the peaks were seriously overlapped with the baseline disturbance around the column dead time. However, under the same mobile phase conditions, a much better separation, as shown in Fig. 8B, was achieved on the $^{-}SO_3-HC-C_8-L$ phase. Interestingly, the elution order between the neutral acetamidophenol and the three aminophenols was reversed due to the strong cation-exchange retention of the cationic amines on the $^{-}SO_3-HC-C_8-L$ phase. Such *gross changes in elution order for neutral and cationic solutes between conventional RPLC phases and the $^{-}SO_3-HC-C_8-L$ phase are common.*

The separation of the four compounds on the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phase was optimized by varying the TFA concentration and the acetonitrile fraction (see Fig. 9). The most challenging separation is that of the three isomeric aminophenols. With 50% acetonitrile and 0.1% (v/v) TFA, the three aminophenols were not baseline separated. 2-, and 3-aminophenols almost coeluted. The TFA concentration was first varied to improve the separation. As discussed above for the separation of catecholamines (section 3.1), TFA not only buffered the mobile phase but also provided protons to act as the displacer for the cationic solutes. Based on the pK_a values (pK_a : 4.2–5.5), all the aminophenols are essentially completely protonated at all TFA concentrations (pH 1.9–2.5). The linear relationship between k' and $1/[\text{TFA}]$ for the three aminophenols (see Fig. 9A) clearly shows the dominance of ion exchange between protons (from the TFA) and the positive amines. Although we see a significant increase in retention as the TFA concentration is decreased, the separation between the critical pair 2-, and 3-aminophenols shows only a slight improvement resulting from the increased retention. This is because in an ion exchange dominated mechanism, the most important contributor to resolution, selectivity ($\alpha = k'_2/k'_1$, $k'_2 > k'_1$), does not change as the displacer concentration is varied as is shown by Eq. (3).

$$\alpha = \frac{k'_2}{k'_1} = \frac{k'_{\text{RP},2} + B_{\text{IEX},2}/[\text{C}^+]_m}{k'_{\text{RP},1} + B_{\text{IEX},1}/[\text{C}^+]_m} \approx \frac{B_{\text{IEX},2}/[\text{C}^+]_m}{B_{\text{IEX},1}/[\text{C}^+]_m} = \frac{B_{\text{IEX},2}}{B_{\text{IEX},1}} = \frac{\varphi_{\text{IEX}} \cdot K_{\text{A/C},2} \cdot \Lambda}{\varphi_{\text{IEX}} \cdot K_{\text{A/C},1} \cdot \Lambda} = \frac{K_{\text{A/C},2}}{K_{\text{A/C},1}} \quad (3)$$

Where k'_{RP} and B_{IEX} are the intercept and slope of k' versus $1/[\text{C}^+]_m$ respectively [2,26]. The pure reversed-phase contribution k'_{RP} is of lesser importance in an ion exchange dominated separation. φ_{IEX} and Λ denote the phase ratio and ion exchange capacity as described in [26]. After all the simplifications are made we see that selectivity is determined primarily by the ratio of the two equilibrium constants $K_{\text{A/C},1}$ and $K_{\text{A/C},2}$. Therefore, to increase selectivity of two ionic solutes with the same type and amount of charge we need to be able to vary the relative equilibrium constants.

The relative equilibrium constants can be effectively changed in two ways. One is to use different displacers and the other is to vary the organic modifier in terms of type or composition. Because of the convenience of varying the amount of organic modifier, we further optimized the separation of the three aminophenols by changing the acetonitrile fraction at constant 0.1% (v/v) TFA concentration. As shown in Fig. 9B, upon decreasing the acetonitrile concentration, all retentions increased due to the increase in hydrophobically assisted cation-exchange retention for the three aminophenols [26] and the increase in reversed-phase retention for the acetamidophenol. The most important result is that the three aminophenols are better separated at the lower acetonitrile fraction. For example, when the amount of acetonitrile was decreased from 50% to 10%, the selectivity between 2-aminophenol and 3-aminophenol increased from 1.06 to 1.20; this will clearly result in a very significant increase in resolution and the two compounds became baseline separated with a resolution of 2. The chromatogram of the optimized separation under 10% acetonitrile is shown in Fig. 8B. It is clear that the organic modifier composition is a very important variable to effectively tune the selectivity of ionic compounds in hydrophobically assistant ion-exchange processes.

3.3 Comparison of the selectivity using $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ and conventional reversed-phase

3.3.1. Selectivity comparison under isocratic conditions—From the applications discussed in the previous sections, it is clear that the selectivities of $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ and a conventional reversed ODS phase, SB C₁₈, are very different especially when one compares neutral to cationic solutes. Nineteen solutes with widely varying functionalities including acids, bases, zwitterions, and neutral compounds were used to further compare the differences in

selectivity. A full list of analytes is given in Table 2 with the number of positive charges of each solute under the test chromatographic conditions. Because of the difference in functionality and pK_a , the solutes possess positive charge number varying from zero to one when 0.1% TFA was in the mobile phase. Due to the fundamentally different retention mechanisms of the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ and the SB C_{18} phases, it is impossible to achieve comparable retention ranges using the same eluent composition on both phases. Therefore, different acetonitrile compositions were used on the two phases to obtain similar retention windows. 10% and 25% acetonitrile was used on the SB C_{18} and $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phases respectively. As shown in Table 2, with the different acetonitrile fractions, the minimum and maximum k' values are very similar on the two phases. Although the retention windows are similar, the mean and median of the retentions are much higher on the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ than on the SB C_{18} phase. Furthermore, the standard deviation, which represents the spread of retentions, is also higher on the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phase. This means that the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phase provides an overall higher retention and better separation for the nineteen solutes studied despite the use of a larger amount of acetonitrile on this phase than on the SB C_{18} phase. Clearly if compounds with even more hydrophilicity were encountered, one could more readily accommodate them on the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phase by reducing the volume fraction of acetonitrile which would be much more difficult on the C_{18} phase as the organic modifier used with it is already only 10%. A κ - κ plot is shown in Fig. 10 to further compare the selectivity between the two phases. Such plots were devised by Horvath [59] to test the similarity in the energetics of retention. A slope close to 1.0 with a good correlation coefficient indicates homoenergetic retention and thus very similar mechanisms. Low correlation coefficients and a slope other than 1.0 indicate significant differences in retention mechanism and thus selectivity. As can be seen from the plot, the data points are extraordinarily highly scattered and the R^2 is only 0.109 and the slope is 0.22. This clearly demonstrates that the selectivities are very different on the two phases. We doubt very much that any of the 350 reversed phases in the Snyder-Dolan compendium [4] would show anything like this degree of orthogonal selectivity. Despite the fact that retention is hydrophobically assisted ion-exchange, there is very little relationship between the retentions on these two clearly different types of phases. This result strongly suggests that the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phase could be very useful when combined with a conventional ODS column for two dimensional liquid chromatography (2D-LC) [60].

3.3.2. Selectivity comparison under gradient conditions in the separations of regulated intoxicants—The selectivity of the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ and SB C_{18} phases were further compared in the separation of regulated intoxicants under gradient conditions. In this comparison, we used a novel instrument configuration developed in our lab [61] for a high-throughput screening method for drugs of abuse. The 47 basic drugs used to develop the fast gradient elution HPLC system [61] were chosen as the solutes (see table 3). As discussed in section 3.3.1, because of the fundamental difference in retention mechanism, different chromatographic conditions must be applied on the two phases to achieve comparable retention ranges. An acetonitrile gradient from 10% to 56% and a salt (TEAH^+) gradient from 0 to 50mM in 2.50 min were used on SB C_{18} and $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phases respectively. Perchloric acid was selected as the buffer for both gradients to increase the retention time and loading capacity of some of the weakly retained cationic drugs on SB C_{18} through ion pairing [61]. The retention times obtained on the two separation systems are plotted in Fig. 11 in a manner similar to κ - κ plot. The scattered pattern of the data and the low correlation between the two sets of retention times ($R^2 = 0.49$, S.E. = 0.31) indicate the large difference in the selectivity of the two systems, and strongly suggest that $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ is a good candidate to construct a successful 2D-LC separation system when used in conjunction with a conventional reversed-phase.

4. Conclusions

Based on the results and the observations in the above applications of our silica-based hyper-crosslinked sulfonate-modified reversed-phase $\text{SO}_3\text{-HC-C}_8\text{-L}$, we conclude that:

1. Compared to conventional reversed-phases, the $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase significantly improved the separation of highly hydrophilic cations, such as catecholamines and aminophenols, by greatly increasing their retentions through a hydrophobically assisted cation-exchange mechanism.
2. The hydrophobicity of the $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase is essential to give good selectivity between two cations of equal charges and similar sizes and therefore similar Coulombic interaction with the surface anionic sites. This is supported by the following observations:
 - a. The more hydrophobic mixed-mode stationary phase $\text{SO}_3\text{-HC-C}_8\text{-L}$ provides better separation for hydrophilic catecholamines than the relatively more hydrophilic mixed-mode stationary phase Primesep 200 even when a higher acetonitrile fraction and a higher column temperature are employed.
 - b. Cations are better separated at lower column temperature and lower organic modifier composition when the hydrophobic interaction is more pronounced.
3. The low cation-exchange capacity of the $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase [26] is very important because:
 - a. It avoids the use of strong displacers, such as alkylamines, at high concentrations for eluting hydrophilic cationic analytes. The weakest cation exchange displacer, the proton, is strong enough to elute all the highly hydrophilic amines studied here at a concentration less than only 13 mM (corresponding to 0.1% (v/v) TFA). This is a great advantage as it decreases baseline noise in UV detection, increases the signals in MS detection and reduces corrosion of instrument components as well as the induced loss of bonded phase [62].
 - b. The low degree of substitution with sulfonyl groups helps to maintain a relatively high degree of hydrophobicity of the stationary phase which is important for selectivity of both cationic and neutral organics.
4. The separation of highly hydrophilic cations on the $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase can be optimized by following the rules summarized below:
 - a. For extremely weakly retained solutes, low displacer concentrations should be used to significantly increase retention.
 - b. For analytes that are sufficiently retained but not well separated, such as the aminophenols, decreasing the fraction of organic modifier is recommended to enhance the selectivity by increasing the ability of mobile phases to differentiate the small difference in solutes' hydrophobicity.
 - c. For a mixture of analytes with pK_a 's close to the mobile phase pH, such as the mixture of catecholamine and related compounds, varying mobile phase pH can effectively tune the selectivity.
 - d. Gradient elution in terms of either displacer (e.g. TFA) concentration or the fraction of organic modifier is recommended for complicated samples to reduce analysis time and maintain good resolution.

- e. Due to the high stability of the hyper-crosslinked networks, the $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase can be used at high temperatures. This advantage allows the selectivity to be tuned by varying temperature as discussed in section 3.1.3.
5. In addition to cationic compounds, neutral compounds are also quite well retained on the hydrophobic $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase. *This is an absolutely essential characteristic of this new phase as it permits the simultaneous analysis of these two types of analytes which are frequently found in the same sample.*
6. The $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase shows very different selectivity from conventional reversed-phase, such as SB C₁₈. This is clearly demonstrated by the very low correlation in $\kappa\text{-}\kappa$ plots based on the retentions of a set of solutes with highly variegated properties and by the comparison of the retention times of 47 drugs of abuse obtained on the two phases using different gradient conditions. This implies that a successful 2D-LC could be constructed by coupling the $\text{SO}_3\text{-HC-C}_8\text{-L}$ phase and a conventional reversed-phase.

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ACIDS:

**1. 3,4-dihydroxyphenylacetic acid
(4.25)**

**2. homovanillic acid
(4.41)**

BASES:

**5. norepinephrine
(8.58)**

**6. epinephrine
(8.59)**

**7. dopamine
(8.93)**

**9. 3-methoxytyramine
(9.54)**

AMINO ACIDS:

**3. 3,4-dihydroxy-L-phenylalanine
(2.32, 8.68)**

**4. tyrosine
(2.20, 9.11)**

**8. phenylalanine
(1.83, 9.13)**

Fig. 1.

The structures and pK_a s of the catecholamine and related compounds (compounds are numbered based on the elution order in Fig. 3C)

Fig. 2.

The effect of ion pairing agent concentration on the retention of catecholamines. ■: phenylalanine; □: 3-methoxytyramine; ■: homovanillic acid; ◆: tyrosine; ◇: dopamine; ▲: 3,4-dihydroxy-L-phenylalanine; *: 3,4-hydroxyphenylacetic acid; ○: epinephrine; △: norepinephrine. Chromatographic conditions: SB C₁₈ column, 10/90 MeCN/H₂O containing 0.1% (v/v) TFA, 0.2 mM EDTA and sodium hexanesulfonate, T = 40 °C, F = 1.0 mL/min.

Fig. 3. The chromatograms of the separation of catecholamines on different phases

(A) SB C₁₈. Chromatographic conditions: 10/90 MeCN/H₂O containing 0.1% (v/v) TFA, 0.2 mM EDTA and 10 mM sodium hexanesulfonate, T = 40 °C.

(B) Primesep 200. Chromatographic conditions: 10/90 MeCN/H₂O containing 0.02% (v/v) TFA, T = 40 °C.

(C) ⁻SO₃-HC-C₈-L. Chromatographic conditions: 24/76 MeCN/H₂O containing 0.02% (v/v) TFA and 0.2 mM EDTA, T = 65 °C.

Other chromatographic conditions: 5.0×0.46 cm column, F = 1.0 mL/min.

Solute designation is the same as in Fig. 1.

Fig. 4.

The relationship between k' and $1/[TFA]$ in the separation of catecholamines. □: 3-methoxytyramine; ■: phenylalanine; ◇: dopamine; ○: epinephrine; △: norepinephrine; ◆: tyrosine; ▲: 3,4-dihydroxy-L-phenylalanine; ■: homovanillic acid; *: 3,4-hydroxyphenylacetic acid. Chromatographic conditions: 24/76 MeCN/H₂O containing 0.02, 0.06 or 0.1% (v/v) TFA and 0.2 mM oxalic acid, T = 40 °C, F = 1.0 mL/min.

Fig. 5.

The relationship between k' and acetonitrile fraction in the separation of catecholamines. □: 3-methoxytyramine; ■: phenylalanine; ◇: dopamine; ○: epinephrine; ▲: norepinephrine; ◆: tyrosine; ▲: 3,4-dihydroxy-L-phenylalanine; ■: homovanillic acid; *: 3,4-hydroxyphenylacetic acid. Chromatographic conditions: MeCN/H₂O containing 0.02% (v/v) TFA and 0.2mM oxalic acid, T = 40 °C, F = 1.0 mL/min.

Fig. 6.

The effect of temperature on the retentions and separation of catecholamine and related compounds on $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phase. Solute designation is the same as in Fig. 1.

Chromatographic conditions: 24/76 MeCN/ H_2O containing 0.02% (v/v) TFA and 0.2 mM EDTA, $T = 40, 50, 65, 80, \text{ or } 100^\circ\text{C}$, $F = 1.0 \text{ mL/min}$.

Fig. 7.

Chromatograms of the gradient elutions of catecholamine and related compounds on SO_3^- -HC-C₈-L phase. (A) TFA gradient. Eluent A: 24/76 MeCN/H₂O containing 0.02% (v/v) TFA and 0.2 mM oxalic acid; eluent B: 24/76 MeCN/H₂O containing 0.1% (v/v) TFA and 0.2 mM oxalic acid; gradient profile: 0–100% B in 0.00–6.00 min, 100–0% B in 6.00–6.01 min, and 0% B in 6.01–8.00 min. (B) MeCN gradient. Eluent A: 24/76 MeCN/H₂O containing 0.02% (v/v) TFA and 0.2 mM oxalic acid; eluent B: 80/20 MeCN/H₂O containing 0.02% (v/v) TFA and 0.2 mM oxalic acid; gradient profile: 0% B in 0.00–4.00 min, 0–100% B in 4.00–12.00 min, 100–0% B in 12.00–12.01 min, and 0% B in 12.01–14.00 min.

Other chromatographic conditions: T = 40 °C, F = 1.0 mL/min, wavelength = 280 nm. Soutes designation is the same as in Fig. 1.

Compound 8 can not be observed at 280 nm. Based on the chromatograms obtained at 210nm, its retention time is 6.34 min in (A) and 9.06 min in (B)

Fig. 8. The separation of acetamidophenol, 2-, 3-, and 4-aminophenol on different phases. (A) SB C₁₈, 10/90 MeCN/H₂O with 0.1% (v/v) TFA and 0.2 mM oxalic acid; (B) $\text{SO}_3\text{-HC-C}_8\text{-L}$, 10/90 MeCN/H₂O with 0.1% (v/v) TFA and 0.2 mM oxalic acid. Other chromatographic conditions: 5.0×0.46 cm column, T = 40 °C, F = 1.0 mL/min, wavelength = 210 nm.

Fig. 9.

The separation optimization of acetamidophenol, 2-, 3-, and 4-aminophenol on the $^{-}\text{SO}_3\text{-HC-C}_8\text{-L}$ phase. (A) k' versus $1/[\text{TFA}]$. 50/50 MeCN/ H_2O with 0.025%, 0.05, or 0.1% (v/v) TFA and 0.2 mM Oxalic acid; (B) k' versus MeCN composition. 10/90, 25/75 or 50/50 MeCN/ H_2O with 0.1% (v/v) TFA and 0.2 mM oxalic acid. Solutes: Δ : 2-aminophenol; $\square$: 3-aminophenol; $\diamond$: 4-aminophenol; $*$: acetamidophenol.

Other chromatographic conditions: 5.0×0.46 cm column, $T = 40^\circ\text{C}$, $F = 1.0$ mL/min, wavelength = 210 nm.

Fig. 10. Selectivity comparison between $\text{SO}_3\text{-HC-C}_8\text{-L}$ and SB C_{18} phases via $\kappa\text{-}\kappa$ plot based on the retention data in Table 2

Slope = $0.22_{\pm 0.15}$; Intercept = $0.62_{\pm 0.10}$; $R^2 = 0.109$; S.E. = 0.41

Chromatographic conditions and solutes are the same as in Table 2.

Fig. 11.

Selectivity comparison between the TEAH⁺ gradient on ⁻SO₃-HC-C₈-L and acetonitrile gradient on SB C₁₈ in the separation of regulated intoxicants.

Slope = 0.59 ± 0.09 ; Intercept = 0.20 ± 0.10 ; $R^2 = 0.49$; S.E. = 0.31

Chromatographic conditions and solutes are the same as in Table 3.

Table 1
Analysis of Temperature Dependence of Retention of the Catecholamines.

Solute Type	Charge Number ^a	Solute Designation ^b	Linear Regression Results			ΔH° ^g
			Slope ^c	Intercept ^d	r ² ^e	
Bases	1	5 ^h	101 _{±5.1}	1.87 _{±0.02}	0.992	-0.84 _{±0.04}
		6 ^h	211 _{±11}	1.80 _{±0.03}	0.993	-1.8 _{±0.09}
		7 ^h	370 _{±10}	1.54 _{±0.03}	0.998	-3.1 _{±0.08}
		9 ^h	524 _{±28}	1.70 _{±0.08}	0.992	-4.4 _{±0.2}
Amino Acids	0-1 ⁱ	3 ^h	265 _{±19}	0.91 _{±0.06}	0.985	-2.2 _{±0.2}
		4 ^h	434 _{±13}	0.72 _{±0.04}	0.997	-3.6 _{±0.1}
		8 ^h	620 _{±9.6}	0.95 _{±0.03}	0.999	-5.2 _{±0.08}
		1 ^h	1470 _{±71}	-6.0 _{±0.2}	0.993	-12 _{±0.6}
Acids	~0 ^j	2 ^h	1130 _{±47}	-4.0 _{±0.1}	0.995	-9.4 _{±0.4}
		acetophenone	1430 _{±63}	-2.8 _{±0.2}	0.994	-11.9 _{±0.5}
		propiphenone	1700 _{±75}	-2.8 _{±0.2}	0.994	-14.1 _{±0.6}
		butyrophenone	1930 _{±85}	-2.8 _{±0.3}	0.994	-16.1 _{±0.7}
Neutral Compounds	0	valerophenone	2340 _{±110}	-3.3 _{±0.3}	0.996	-19.4 _{±0.9}

^aThe number of positive charges on each compound under the test chromatographic conditions

^bSolute designation is the same as in Fig. 1.

^cThe slope of ln k' vs. 1/T based on the data in Fig. 6.

^dThe intercept of ln k' vs. 1/T based on the data in Fig. 6.

^eThe squared correlation coefficient of ln k' vs. 1/T based on the data in Fig. 6.

^fThe standard error of ln k' vs. 1/T based on the data in Fig. 6.

^gThe standard enthalpy change for solute transfer calculated from the corresponding slope

^hCompounds are ordered from top to bottom based on ascending retention in each category

ⁱThe one positive charge from the amino group is partially cancelled out by the deprotonation of the carboxylate group as discussed in section 3.1.1

^jThe carboxylate groups in the acids are almost totally protonated and shows no charge as discussed in section 3.1.1

Chromatographic conditions: the same as in Fig. 6

Table 2Retention Factors of Cationic and Related Solutes on SB C₁₈ and ⁻SO₃-HC-C₈-L Phases.

	Stationary Phase		SB C ₁₈	⁻ SO ₃ -HC-C ₈ -L
	TFA		0.1%	0.1%
	%MeCN		10	25
Solute Symbol	Solute Name ^a	Charge Number ^b		k'
a	4-Aminophenol	1	0.12	2.72
b	3-Aminophenol	1	0.27	3.44
c	3-Aminobenzoic acid	0-1	0.37	2.99
d	2-Aminophenol	1	0.40	3.90
e	Phenylephrine	1	0.55	4.26
f	Aniline	1	0.61	6.24
g	4-Aminobenzoic acid	0-1	0.67	1.82
h	5-Hydroxy-L-tryptophan	0-1	0.84	3.57
i	5-Hydroxy-tryptamine	1	0.89	6.06
j	Acetamidophenol	0	1.05	0.38
k	Salbutamol	1	1.41	5.61
l	3-Aminobenzoic acid	0-1	3.47	2.85
m	Ephedrine	1	3.71	15.79
n	Methylephedrine	1	4.57	22.48
o	Tryptophan	0-1	4.59	9.98
p	Tryptamine	1	5.64	21.78
q	Indole-3-acetyl-L-lysine	0-1	8.21	11.22
r	Benzoic acid	~ 0	13.65	2.10
s	Acetophenone	0	23.39	3.17
			Statistics of k' Values	
	Minimum		0.12	0.38
	Maximum		23.39	22.48
	Mean		3.92	6.86
	Median		1.05	3.90
	Standard Deviation		5.85	6.53

^a Solutes are listed in order of k' on SB C₁₈^b The number of positive charges on each compound under the test chromatographic conditions

Chromatographic conditions:

SB C₁₈: 10/90 MeCN/H₂O with 0.1% (v/v) TFA and 0.2 mM oxalic acid⁻SO₃-HC-C₈-L: 25/75 MeCN/H₂O with 0.1% (v/v) TFA and 0.2 mM oxalic acid

Other chromatographic conditions:

5.0×0.46 cm column, T = 40 °C, F= 1.0 mL/min

Table 3
Retention Times of 47 Regulated Intoxicants on SB C₁₈ and ⁻SO₃-HC-C₈-L Phases.

Solute Symbol	Solute Name	Retention Time (min)	
		SB C ₁₈	⁻ SO ₃ -HC-C ₈ -L
a	Morphine	0.213	0.409
b	Oxymorphone	0.290	0.372
c	Hydromorphone	0.338	0.46
d	Phenylpropanolamine	0.403	0.419
e	7-Aminoclonazepam	0.405	0.59
f	Cathinone	0.441	0.471
g	Pseudoephedrine	0.488	0.473
h	Codeine	0.488	0.529
i	Ephedrine	0.492	0.486
j	Methcathinone	0.500	0.532
k	7-Aminoflunitrazepam	0.533	0.644
l	Oxycodone	0.580	0.59
m	6-Acetylmorphine	0.594	0.558
n	Amphetamine	0.597	0.546
o	MDA	0.620	0.578
p	PMA	0.666	0.607
q	Methamphetamine	0.684	0.639
r	MDMA	0.694	0.678
s	Phentemine	0.732	0.626
t	PMMA	0.741	0.712
u	MDEA	0.820	0.778
v	Bromazepam	0.783	0.569
w	Benzoyllecgonine	0.862	0.596
x	MBDB	0.886	0.831
y	Estazolam	0.973	0.73
z	Nordiazepam	1.026	0.748
a*	Oxazepam	1.078	0.536
b*	Chlordiazepoxide	1.160	0.904
c*	Hydrocodone	1.188	0.704
d*	Zolpidem	1.222	1.189
e*	Clobazam	1.228	0.804
f*	Nitrazepam	1.243	0.655
g*	Diazepam	1.360	0.973
h*	Desalkylflurazepam	1.390	0.709
i*	2-Hydroxyethylflurazepam	1.417	0.609
j*	Alprazolam	1.436	1.011
k*	Midazolam	1.451	1.294

Solute Symbol	Solute Name	Retention Time (min)	
		SB C ₁₈	⁻ SO ₃ -HC-C ₈ -L
l*	Flurazepam	1.550	1.594
m*	Clonazepam	1.568	0.706
n*	Lorazepam	1.584	0.546
o*	Triazolam	1.688	1.19
p*	Temazepam	1.755	0.774
q*	Cyclobenzaprine HCl	1.812	2.268
r*	Flunitrazepam	1.834	0.822
s*	Lometazepam	1.903	0.848
t*	Prazepam	1.950	1.668
u*	Amitriptyline	1.971	2.458

Chromatographic conditions:

SB C₁₈: Eluent A: 20mM perchloric acid in H₂O; eluent B: 20mM perchloric acid in 80/20 (v/v) MeCN/H₂O; gradient profile: 12.5–70% B in 0.00–2.50 min, 70–12.5% B in

2.50–2.51 min, and 12.5% B in 2.51–2.80 min.

⁻SO₃-HC-C₈-L: Eluent A: 20mM perchloric acid in 63/37 (v/v) MeCN/H₂O; eluent B: 20mM perchloric acid and 50mM TEA.HCl in 63/37 (v/v) MeCN/H₂O; gradient profile: 0–100% B in 0.00–2.50 min, 100–0% B in 2.50–2.51 min, and 0% B in 2.51–2.80 min

Other chromatographic conditions:

5.0×0.21 cm column, T = 40 °C, F = 1.0 mL/min, wavelength = 210 nm