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# Evaluation of new mixed-mode UHPLC stationary phases and the importance of stationary phase choice when using low ionic-strength mobile phase additives

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ABSTRACT

In this study, the selectivity, retention properties, peak shape and loading capacity for bases were practically evaluated using two UHPLC mixed-mode hybrid CSH stationary phases modified by C18 or Phenyl group. The data were compared with the data obtained on other UHPLC hybrid stationary phases (BEH C18, BEH C8, BEH Phenyl and BEH Shield RP18) at both basic and acidic conditions using conventional HPLC buffers (50 mM ammonium formate/acetate) as well as low ionic-strength additives such as, e.g. 0.1–0.01% formic/acetic acid and 1 mM solution of ammonium formate/acetate, which are widely used in LC–MS applications.

Ten pharmaceutically important compounds encompassing acids, bases and neutral were included into the study. Due to properties of CSH sorbent (which possess positively charged surface besides RP group), much improved peak shapes and weaker retention was obtained for bases even at very low concentration of acidic additives. Such conditions are ideally suited for LC–MS analysis of bases, where typical RP chromatographic separation (retention and good selectivity at basic pH) and LS–MS conditions (efficient ionization at acidic pH) are not in agreement. On the other hand, acids were more strongly retained and for some compounds the peak shape was influenced negatively due to ion-exchange mechanism. Further, the behavior of acidic, basic and neutral solutes is discussed using various additives at both basic and acidic pH for all above stated columns. The robustness of retention times after pH change from basic to acidic was also evaluated. The new CSH stationary phases represent an interesting selectivity tool preferably for separation of basic compounds.



## 1. Introduction

In early beginning of ultra-high performance liquid chromatography (UHPLC) in 2004 there were only few stationary phases available. Initially they covered mostly reverse-phase separations including C18, C8 and Phenyl modified stationary phases. Recently, the range of applicability of UHPLC stationary phases has been widely extended including normal phase, ion-exchange and hydrophilic interaction chromatography (HILIC) applications as well [1]. The attention has been attracted to mixed-mode stationary phases, which allow for multiple retention process to occur simultaneously due to surface modification. Such modification enables to obtain further selectivity and to add dimensionality in 2D separations [2]. Typical mixed-mode stationary phases contain C18 reverse chain and simultaneously strong anion exchange (SAX) and/or weak anion exchange (WAX) group. Such sorbents are also widely used for SPE technique [3,4].

In reversed-phase (RP) chromatography the retention of ionizable analytes is influenced by the ionic properties of the packing caused by surface silanol groups, besides of hydrophobic interactions and hydrogen-bond interactions [5,6]. Positively charged analytes interact with negatively charged surface silanols via an ion-exchange mechanism, which results in an enhanced retention. Conversely, negatively charged analytes are subjected to ion-exclusion effect [5]. In order to suppress ion-exchange mechanism of silanol groups following approaches might be applied: (1) lowering pH of the mobile phase to suppress silanol ionization, (2) an addition of tertiary amines, that preferentially bind to charge silanol groups, (3) an increase in ionic strength of mobile phase, (4) an addition of more retentive buffer cations (e.g. potassium) or finally (5) use a column with low silanol activity [6].

When using the first approach, lowering pH of the mobile phase, the protonation of bases will be also increased. In fact, there is an important divergence in the development of appropriate conditions for LC–MS analysis of basic compounds. For a good retention of bases on RP stationary phase basic pH is required in order to obtain non-ionized base, which will be well retained on non-polar stationary phase. On the other hand, an efficient ionization of bases in an ion source of mass spectrometer is obtained at acidic conditions

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(protonization of bases). Several volatile additives, including formic acid, acetic acid, ammonium formate and ammonium acetate at low concentrations (<0.5% or <5 mM), are used for the purpose of ionization enhancement in mass spectrometry. Often a volatile additive is used rather as an ionization additive than a buffering one. Low concentration of volatile additives are required due to reduced signal suppression effects [7,8]. When using weakly acidic low ionic-strength additives, the ability to reduce the ion-exchange activity of residual silanol groups is substantially decreased, therefore wide or tailing peaks of basic compounds are often observed. D.V. McCalley described a serious loss in column efficiency for ionized basic drugs and peptides, when working with weakly acidic mobile phases of low ionic-strength suitable for mass spectrometry [9]. The loss in efficiency was attributed to overloading of C18 stationary phase.

With the aim to reduce the consequences of the ion-exchange effect of silanol groups many companies have introduced new stationary phases with decreased silanol activity. The greatest contribution was made by hybrid particle technologies. These materials contain organic moieties such as methyl or ethyl groups in their structures, which provides higher chemical and mechanical resistance as well as a significant reduction (by nearly one third) of number of silanol sites [10,11]. Despite all these improvements in column technology, serious peak deformation can still occur under certain experimental conditions. More favorable behavior was noted by D. V. McCalley et al on a mixed-mode RP/embedded cation-exchange stationary phase [12]. Even though the silanol groups also occur on such stationary phase, they may be shielded by embedded ionic groups. Therefore, an increase in loadability of bases is a substantial advantage.

New UHPLC mixed-mode stationary phases based on hybrid support were introduced in 2010 as a new family of CSH (charged surface hybrid) analytical columns [13]. So far, the properties of this so called "charged surface hybrid" sorbent have not been described yet in practical applications. The aim of this study was to evaluate the selectivity, retention properties, peak shape and loading capacity of basic compounds using a mixture of pharmaceutical compounds of different structures. Two UHPLC mixed-mode CSH stationary phases modified by C18 and Phenyl groups were evaluated. The obtained data were compared with the data from other UHPLC hybrid stationary phases belonging to the bridged ethyl hybrid (BEH) family (BEH C18, BEH C8, BEH Phenyl and BEH Shield RP18).

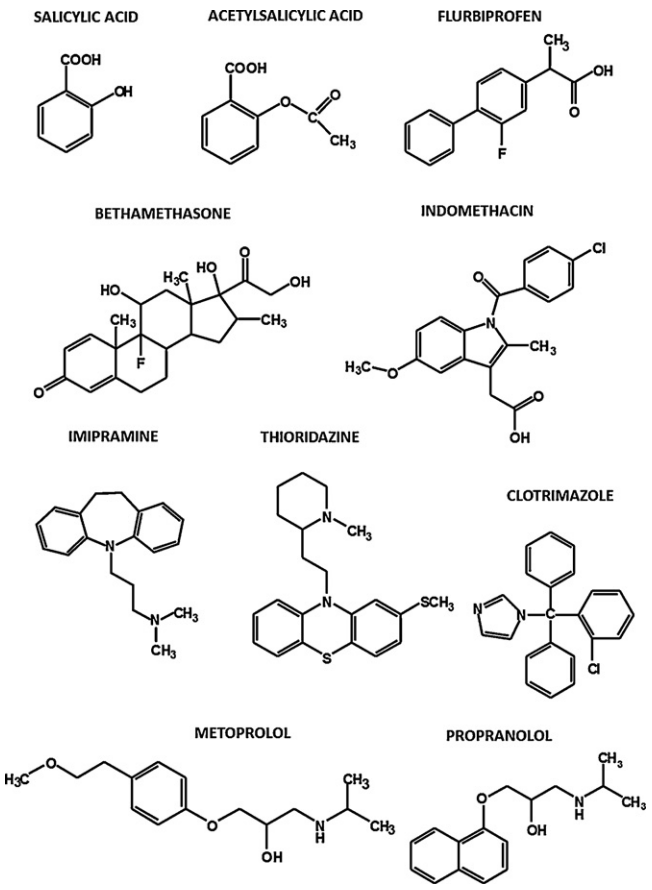


**Fig. 1.** Structures of compounds selected for this study.

## 2. Experimental

### 2.1. Chemicals and reagents

Working standards of metoprolol, salicylic acid, acetylsalicylic acid, propranolol, betamethasone, imipramine, clotrimazole, thioridazine, indomethacin and flurbiprofen were used for the purpose of this study. The structures are shown in Fig. 1. All compounds were

**Table 1**
The values of peak symmetry factor at selected chromatographic conditions. The numbers correspond as follows: (1) metoprolol, (2) acetylsalicylic acid, (3) salicylic acid, (4) propranolol, (5) betamethasone, (6) imipramine, (7) clotrimazole, (8) thioridazine, (9) flurbiprofen, (10) indomethacin.

| Compound | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| **0.1% formic acid in mobile phase** | | | | | | | | | | |
| BEH C18 | 1.30 | 1.15 | 1.39 | 1.75 | 1.42 | 1.79 | 1.97 | 1.87 | 1.34 | 1.34 |
| BEH C8 | 1.48 | 1.71 | 2.30 | 1.50 | 1.47 | 1.51 | 1.48 | 1.51 | 1.72 | 1.71 |
| CSH C18 | 1.17 | 1.13 | 1.85 | 1.20 | 1.16 | 1.15 | 1.12 | 1.16 | 1.07 | 1.08 |
| CSH Phenyl | NA | 1.19 | 2.23 | 1.29 | 1.14 | 1.26 | 1.26 | 1.21 | 1.12 | 1.12 |
| BEH Phenyl | 1.53 | 1.51 | 1.34 | 1.32 | 1.59 | 1.66 | 1.79 | NA | NA | 1.25 |
| BEH Shield RP 18 | 1.87 | 1.10 | NA | 1.42 | 1.07 | 1.37 | NA | 1.25 | 1.04 | 1.03 |
| **10 mM ammonium acetate pH 3.0 in mobile phase** | | | | | | | | | | |
| BEH C18 | 1.19 | 1.09 | 1.22 | 1.36 | 1.22 | 1.36 | 1.38 | 1.45 | 1.30 | 1.30 |
| BEH C8 | 1.41 | 1.62 | 1.52 | 1.35 | 1.36 | 1.41 | NA | 1.38 | 1.45 | 1.49 |
| CSH C18 | 1.26 | 1.12 | 1.40 | 1.40 | 1.08 | 1.08 | NA | 1.18 | 1.07 | 1.08 |
| CSH Phenyl | 1.36 | NA | 1.58 | NA | 1.14 | 1.21 | 1.25 | 1.14 | 1.12 | 1.13 |
| BEH Phenyl | NA | 1.45 | NA | 1.48 | 1.35 | 1.50 | 1.50 | 1.13 | 1.13 | 1.29 |
| BEH Shield RP 18 | 1.35 | 1.12 | 1.22 | 1.29 | 1.07 | 1.24 | 1.07 | 1.21 | 1.05 | 1.06 |
| **1 mM ammonium acetate pH 3.0 in mobile phase** | | | | | | | | | | |
| BEH C18 | NA | 1.14 | NA | 1.95 | 1.40 | 1.84 | 1.81 | 1.83 | 1.37 | 1.38 |
| BEH C8 | 1.62 | 2.06 | 2.28 | 1.35 | 1.32 | 1.35 | NA | NA | 1.58 | 1.63 |
| CSH C18 | 1.28 | 1.25 | 2.15 | 1.28 | 1.11 | 1.25 | 1.10 | 1.26 | 1.18 | 1.13 |
| CSH Phenyl | 1.30 | 1.22 | 2.86 | 1.22 | 1.29 | 1.29 | 1.02 | 1.16 | 1.16 | 1.15 |
| BEH Shield RP 18 | 0.99 | 1.14 | NA | 1.41 | 1.18 | NA | 0.99 | 1.29 | 1.04 | 1.03 |