Published in final edited form as:

Electrophoresis. 2008 May ; 29(10): 2004–2015. doi:10.1002/elps.200700460.

Packed-column capillary electrochromatography and capillary electrochromatography-mass spectrometry using a lithocholic acid stationary phase

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Abstract

The preparation and characterization of a novel lithocholic acid (LCA)-based liquid crystalline (LC) stationary phase (SP) suitable for application in packed-column CEC and CEC coupled to MS is described. The extent of bonding reactions of LCA-SP was assessed using ¹H-NMR, ¹³C-NMR and elemental analysis. This characterization is followed by application of the LCA-SP for separation of β -blockers, phenylethylamines (PEAs), polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). Using the optimum mobile phase operating conditions (pH 3.0–4.5, 10 mM ammonium acetate, 85% v/v ACN), a comparison of the chromatographic ability of the aminopropyl silica phase vs. the LCA-bonded phase was conducted. The results showed improved selectivity for all test analytes using the latter phase. For example, the CEC-MS of β -blockers demonstrated that the LCA-bonded phase provides separation of six out of seven β -blockers, whereas the amino silica phase provides four peaks of several co-eluting β -blockers. For the CEC-MS analysis of PEAs, the LCA-bonded phase showed improved resolution and different selectivity as compared to the aminopropyl phase. An evaluation of the retention trends for PEAs on both phases suggested that the PEAs were retained based on varying degree of hydroxyl substitution on the aromatic ring. In addition, the MS characterization shows several PEAs fragment in the electrospray either by loss of an alkyl group and/or by loss of H₂O. Finally, the LCA-bonded phase displayed significantly higher separation selectivity for PAHs and PCBs as compared to the amino silica phase.

Keywords

Betablockers; Liquid crystal stationary phase; Phenylethylamines; Polyaromatic hydrocarbons; Polychlorinated biphenyls

1 Introduction

Materials that display structures, which are intermediate between the three-dimensional ordered crystalline state and the disordered or isotropic liquid state are known as liquid crystals. Liquid crystalline (LC) stationary phases (SPs) are widely used as a separation material in GC and have been around for over 40 years [1]. In addition, LC-SP has shown applications in

supercritical fluid chromatography [2,3] and HPLC [4-10]. However, in comparison to GC, the use of LC-SPs in HPLC and supercritical fluid chromatography is less prevalent. The LC-SPs in GC are often physically coated on the inner wall of the capillary, whereas in HPLC the LC-SPs are chemically bonded to withstand the high pressure and increased solubilizing capability of the mobile phase. In addition, the use of much lower temperature in HPLC compared to GC may provide different orientation and chemical property of the LC-SP, which in turn results in different chromatographic selectivity. An advantage of LC-SP over traditional reversed phase (*e.g.* C₁₈ bonded phase) in HPLC is that LC-SPs offer separations based upon unique selectivity. This selectivity is due in part to interesting morphological properties resulting from the rod-like shape and ordered arrangement of the LC-SP molecule [11]. Currently, some applications of chemically bonded LC-SP in HPLC include polyaromatic hydrocarbons (PAHs) [4,6,7,9], fullerenes [5], dipeptides [4], carotenoids [5] and steroids [9]. Therefore, the exploration of LC-SP for use in LC and similar chromatographic techniques is growing in popularity.

A complimentary technique to HPLC that combines the retention characteristics of LC and electrophoretic mobility of CZE is capillary CEC. Although in CZE, the neutral compounds cannot be separated due to zero electrophoretic mobility, the use of the SP in CEC provides additional selectivity based on hydrophobic and/or polar interactions, which enables separation of neutral compounds. In addition, one of the advantages of CEC is the use of small internal diameter fused-silica capillary (*i.e.* 75 μ m), which permits separations under a wide variety of operating modes. For example, the capillary can be either packed with SP, chemically modified *in situ*, or simply coated to provide several approaches to challenging analytical separations. Thus, as a microscale separation technique, CEC is becoming very popular. The state-of-the-art and additional applications of CEC and CEC-MS have been summarized in several recent reviews [12-15].

While the use of LC-SP for LC and GC have a long tradition, its use in CEC is an exciting new area that until recently has not received much attention. To date, the most popular approach for application of LC-SP in CEC entails etching the inner wall of the capillary followed by chemical modification of the open tubular capillary surface, open tubular CEC [16]. Moreover, the use of LC-SP using cholesterol as the SP in open tubular CEC has been applied for separations of peptides, mixtures of proteins, pyrimidine/purine bases, metabolic compounds, including serotonin, and other basic molecules [17-21]. Although cholesterol has been applied in open tubular CEC, currently there are no reports for the use of this biological ligand in packed column CEC. In contrast, several reports of cholesterol bonded to aminopropyl silica are described in HPLC [22-24].

In this work, we describe the synthesis of a charged LC-SP, which is a cholesterol-based lithocholic acid (LCA) attached to aminopropyl silica for its possible use as a separation media in packed column CEC and CEC-MS. A characterization of the reactions using several physiochemical techniques, including NMR spectroscopy and elemental analysis is presented. This characterization is followed by comparison of LCA phase to a non-LC-SP (*i.e.* amino silica phase) for separation of β -blockers, phenylethylamines (PEAs), polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). To our knowledge, this is the first report of LC-SP applied to packed-column CEC and CEC-MS.

2 Materials and methods

2.1 Reagents and chemicals

The 300 Å, 5 μ m particle size Kromasil silica was obtained from Akzo Nobel (Illinois, USA). 3-Aminopropyltriethoxysilane (99%), toluene (anhydrous, 99%), pyridine (99.8%), LCA (99%), morpholine (99%), and sodium sulfate (99%) were purchased from Sigma (St. Louis,

MO, USA). Triphosgene was obtained from Aldrich (Milwaukee, WI, USA). The HPLC-grade solvents such as dichloromethane, CH_2Cl_2 , methanol (MeOH), and ACN were purchased from Burdick and Jackson (Muskegon, MI, USA). The β -blockers were obtained as racemic mixtures from Sigma. The PEAs were also purchased from Sigma. The PAH mixture (PNA-550JM) and PCB mixture (PCB525-1JM) were obtained from ChemService (West Chester, PA, USA). For spiking studies, the individual PAHs were all obtained from Sigma. Similarly, the individual PCBs were purchased from ChemService.

2.2 Synthesis of the LCA-SP

2.2.1 Preparation of aminopropyl silica—A 1 g sample of previously dried 300 Å, 5 μm particle size Kromasil silica was weighed into a 250 mL round bottom (RB) flask containing 50 mL of toluene. While the content of the flask was stirred, ~ 3 mL of 3-aminopropyltriethoxysilane was added to the flask and this mixture was allowed to reflux overnight at 100°C. After 12 h of reaction, the mixture was cooled to room temperature and suction filtered. The product was washed with 300 mL MeOH, 300 mL deionized H_2O , and finally 300 mL MeOH. The ^{13}C -NMR resonance data showed three peaks in the range of 0-50 ppm, which correspond to the propyl alkyl chain of the aminopropyl silica.

2.2.2 Synthesis of LCA chloroformate (CF) (1)—LCA (20 g) was placed in a 500 mL RB flask. After adding 300 mL CH_2Cl_2 , the reaction mixture was stirred for 10 min followed by sonication for 2 min to disperse any large aggregates of LCA. Separately, 4 mL of pyridine was added to a small extraction funnel along with 100 mL of CH_2Cl_2 . Next, 5.3 g (0.018 mol) of triphosgene was added to the LCA solution contained in the RB flask. The pyridine in CH_2Cl_2 was then slowly added dropwise to the RB flask to neutralize any HCl produced. Finally, the contents of the RB flask was refluxed and stirred for 96 h. A resulting clear mixture was observed within 30 min of reaction time. After 96 h, ~ 100 mL of H_2O was added to RB flask and stirred for 1 h to dissolve all excess pyridinium chloride formed during the reaction. The mixture was extracted twice with H_2O , dried over anhydrous sodium sulfate and concentrated *in vacuo*. The resulting solid-product ((1), Scheme 1) appeared yellow to white. ^1H -NMR was used to characterize the conversion of LCA to LCA-CF. At 400 MHz using CDCl_3 as background solvent, the multiplet at, ~ 3.5 ppm shows downfield shift to ~ 4.8 ppm. Therefore, partial conversion of LCA to LCA-CF was achieved due to presence of LCA starting material (multiplet resonances at 3.5 ppm) along with an additional multiplet at 4.8 ppm due to LCA-CF product.

2.2.3 Attachment of LCA-CF to aminopropyl silica (2)—Aminopropyl silica (800 mg) was placed into a 250 mL RB flask along with excess (3 g) LCA-CF (1). Next, 300 mL of CH_2Cl_2 was added to disperse the contents. Morpholine (5 mL) was added as an activator and the mixture was then refluxed at 70°C overnight with slow stirring. The resulting white product ((2), Scheme 1) was suction filtered (Büchner) and washed with H_2O . Next, CH_2Cl_2 was used to rinse away any unreacted LCA-CF. Finally, the product was washed with HPLC-grade MeOH. The product was characterized using solid-state ^{13}C -NMR at 400 MHz and cross polarization-magic angle spinning. Chemical shifts corresponding to the cyclic ring network at 9.1, 20.6, 26.0, 33.7, 41.7, 56.0, 66.0, and 72.1 ppm were observed as well as 176.1 ppm for the terminal carboxylic acid. The LCA-CF carbonyl carbon attached to -NH group of the amino silica showed a chemical shift at 157.0 ppm.

2.3 Column packing procedure

The CEC-UV columns were packed according to our previously published procedure [25]. Briefly, slurry was prepared using ACN followed by sonication for 45 min. Next, a fused-silica capillary (75 μm id, 362 μm od) obtained from Polymicro Technologies (Phoenix, AZ, USA)

was packed to 30 cm using 300 bar as previously described [25]. The internally tapered CEC-MS columns were fabricated and packed according to our previously published method [26].

2.4 Buffer and sample preparation

The CEC running buffer was prepared using concentrated 100 mM NH_4OAc adjusted to the appropriate pH using HOAc . An appropriate aliquot of stock solution was then combined with ACN, triply deionized H_2O and TEA using volumetric pipettes, followed by 25 min sonication, filtering using 0.45 μm PTFE membrane syringe filter and finally degassing for 10 min. The mixture of seven β -blockers was prepared by taking 100 μL of each analyte dissolved in MeOH (1 mg/mL), followed by addition of 100 μL running buffer. A mixture of ten PEAs was prepared in the same manner. The 16-PAH mixture (200 $\mu\text{g/mL}$ in ACN) and the PCB mixture (500 $\mu\text{g/mL}$ in acetone) were diluted threefold using running buffer before analysis.

2.5 CEC instrumentation

All CEC-UV experiments were performed on an Agilent HP^{3D}CE system (Agilent Technologies, Palo Alto, CA) using 214 and 254 nm detection. For CEC-ESI-MS instrumentation, all experiments were performed on an Agilent HP^{3D}CE, which was interfaced to an Agilent 1100 series MSD quadrupole mass spectrometer equipped with a CE-MS adaptor kit and a sprayer kit. Sheath liquid was delivered by an Agilent 1100 series HPLC pump equipped with a 1/100 split flow. The ChemStation software (v. 10.02) was used for data processing.

3 Results and discussion

The characterization of the bonding reactions using ^1H -NMR, elemental analysis, and ^{13}C -NMR is first presented. The conversion of LCA to LCA-CF is described, followed next by attachment of propylamine to bare silica, then the attachment of LCA-CF to the modified aminopropyl silica. Finally, the LCA-bonded SP and the aminopropyl silica phases were packed in a capillary column and compared for the separation of several different classes of positively charged and neutral compounds.

3.1 Characterization of LCA-CF

LCA was first converted to CF. The ^1H -NMR of the LCA and the LCA after conversion to CF were compared (data not shown). The starting material LCA exhibited a multiplet for the ring protons at position 3 around 3.5 ppm, which was slightly downfield from the Chemdraw estimation of 3.1 ppm. After the conversion of LCA to CF at ring position 3 ((1), Scheme 1), a downfield shift to 4.8 ppm was observed for the multiplet, which is slightly downfield from the Chemdraw estimation of 3.9 ppm. This suggests that the reaction of LCA to LCA-CF was successful.

3.2 Characterization of aminopropyl silica and LCA-bonded phase

The elemental analysis of two prepared batches of aminopropyl silica and one batch of LCA-bonded phase were compared. After the bonding of LCA-CF to the aminopropyl silica, the elemental analysis in Table 1 showed significantly more C and H in mmol/g, although the nitrogen content remained the same between the two phases. Next, the solid-state ^{13}C -NMR spectra of the aminopropyl silica bonded to LCA-CF were compared (spectra not shown). For the aminopropyl silica, three peaks corresponding to the ^{13}C chemical shift of the propyl carbon chain were observed in the range of 8-43 ppm. In comparison, the ^{13}C -NMR spectra for the LCA-bonded phase showed multiple chemical shifts in the range of 0-80 ppm resulting from the extensive cyclic ring carbons, which were not present in the spectra of aminopropyl silica. In addition, the extent of bonding was determined by analysis of the chemical shift of the

carbonyl carbon attached to position 3 of the steroid. A small but distinct peak at 156.959 ppm indicated successful bonding. Finally, a comparison of the obtained bonded phase spectra to the literature cholesteric phase showed several similar resonances in the region of 0-80 ppm representing the chemical shifts of the cyclic ring carbons, as well as the resonance at 155 ppm representing the carbonyl carbon attached at position 3 of the steroid molecule [19].

Although the elemental analysis data and the ^{13}C -NMR data suggested that the bonding of LCA-CF to the aminopropyl silica was successful, alternatively a comparison of the chromatographic separating ability of these two phases was conducted to further distinguish between the two materials. First, the capability of both phases for CEC-MS separation of cationic compounds (seven β -blockers and ten PEAs) was performed. Investigation of the most suitable operating conditions showed that a mobile phase containing 85/15 ACN/10 mM NH_4OAc , pH 3, 0.05% TEA could be utilized for the separation of β -blockers and PEAs. Next, the CEC-MS separation of ten PEAs was compared on the two phases using the aforementioned mobile phase. The PEAs separations was followed by the CEC-UV analysis of neutral compounds (PAHs and PCBs). Finally, data and comments on the EOF profile using thiourea as a dead time marker in CEC-MS and CEC-UV under various mobile phase conditions are presented.

3.3 CEC-MS of β -blockers

For CEC-MS separation of seven β blockers, Fig. 1 shows the comparison between the aminopropyl silica (Fig. 1A) and the LCA bonded phase (Fig. 1B). The results of the study demonstrate that the LCA-bonded phase provides six peaks for separation of six out of seven β -blockers, whereas the aminopropyl phase provides four peaks of several co-eluting β -blockers. From the extract ion chromatograms (EICs) shown as inset of Figs. 1A and B, the peak identification was assigned as shown in the caption. The LCA-bonded phase displayed better separation of several peak pairs. For example, atenolol/talinolol was baseline-resolved, whereas propranolol/metoprolol was partially resolved with LCA phases. On the other hand, these two peak pairs co-eluted with aminopropyl silica phase. With the exception of oxprenolol and atenolol, the β -blockers generally follow elution based upon decreasing mass-to-charge ratio (m/z). No significant correlation between $\log P$ and elution order was observed with the exception of talinolol ($\log P = 3.20$), which eluted last and had the highest $\log P$ value. Overall, it can be concluded that the selectivity of β -blockers on the LCA-bonded phase is higher than that of the aminopropyl phase.

The additional on-line CEC-MS characterization of the LCA-bonded phase and the aminopropyl silica using mobile phase conditions of Figs. 1A and B was conducted by analysis of a neutral marker and also comparison of the operating current on the two phases. Thiourea was chosen as the dead time or neutral marker as this compound was expected to be neutral at pH 3 (pK_a of thiourea is 1.44 ± 0.5 , Science Finder Scholar, v. 2002). The operating current in μA can provide insight of the flow of ions inside the packed CEC column. If the current profile is different for two phases run under the same operating conditions (*i.e.* packed bed length and mobile phase composition), it can be reasonable to state that the two materials are structurally different. First, an analysis of the current profile taken from the chromatograms showed that the LCA-bonded phase had a higher operating current of 11 μA as compared to the aminopropyl silica phase current of 9 μA . A comparison of the thiourea dead time marker showed that the retention on the LCA-bonded phase was 42 min as compared to 28 min on the aminopropyl phase (data not shown). As thiourea is effectively neutral at pH 3, the longer retention of thiourea can be attributed to slower EOF on the LCA-bonded phase. Therefore, it can be concluded that the surface property of the two phases are different and suggest that bonding of the LCA did in fact occur.

3.4 CEC-MS of PEAs

Next, the CEC-MS capability of the bonded phase for separation of a series of structurally similar PEAs was assessed. A comparison of the separation on the aminopropyl silica *versus* the LCA-bonded phase is shown in Figs. 2A and B, respectively. The results show that both SPs were able to separate nine out of ten PEAs within 18 min. However, several noticeable differences in separation selectivity were observed. For example, a reversal of elution order was seen for terbutaline and epinephrine on the two phases. To verify the elution order of the PEAs, the EIC for each m/z (shown as inset in Figs. 2A and B) was compared to the retention time of each PEA run individually on a much longer packed bed. For this experiment, the results of running individual PEA on the LCA-bonded phase are shown in Fig. S1 in the Supporting Information. From a comparison of the elution trend for each compound run individually (Fig. S1) to the EIC of the LCA-bonded phase (Fig. 2 inset B), it was obvious that the presence of multiple peak in several EIC channels resulted from the fragmentation of several PEA molecules. In general, the PEA fragment in the ESI source results either by loss of an alkyl group or by loss of hydroxyl group, or both. In this manner, the multiple peaks (a-o) in the EIC channel were assigned as shown in the Fig. 2 caption. Overall, at least three PEAs (isoproterenol, terbutaline, norepinephrine) were found to produce fragment ions with the same m/z ($m/z = 152$) as shown in Figs. 2A and B. This can be explained by the fragmentation pathway shown in Fig. 3. First, the PEA containing a primary amine such as norepinephrine (Fig. 3A) was found to fragment at the hydroxyl group to form a dicationic species. This fragmentation is followed by abstraction of the amino proton (acidic conditions, pH 3.0) by the negatively charged hydroxide group to form H_2O , leaving a neutral amine plus a positive charge at the site of hydroxyl abstraction.

The second fragmentation pattern observed for terbutaline (Fig. 3B) follows a somewhat similar pattern to norepinephrine. The molecule was found to first fragment at the isobutyl position, followed next by the loss of hydroxyl group to form H_2O as previously shown for norepinephrine. Isoproterenol, which is structurally similar to terbutaline was observed to fragment in a similar fashion except for the loss of the isopropyl group in the first step. After determining the elution order, the trend in elution was evaluated considering the molecular weight, MW, charge (z) at pH 3, m/z , log P , and finally the class as shown in Table 2.

A consideration of m/z and log P showed no correlation with the elution order on either phase. Alternatively, the class or degree of hydroxyl group substitution around the aromatic ring was found to compare well with retention of the PEAs. For the aminopropyl phase, the separation can be attributed to hydrophobic interactions between the propyl group of the SP and the short chain alkyl group of the PEAs. In addition, the amine group of this phase can hydrogen bond with the neutral alcohol groups of the PEAs. Therefore, the early eluting PEA follows elution relative to substitution of hydroxyl groups around the aromatic ring. Increasing the number of hydroxyl groups permits increased hydrogen bonding between the analyte and the protonated amine of the aminopropyl stationary phase. For example, pseudoephedrine, ephedrine and norephedrine (class III) have no hydroxyl substituent on the ring and eluted first (Table 2). The remaining PEAs containing one (*e.g.* class II, octopamine, synephrine, norphenylephrine) and two hydroxyl substituent (*e.g.* class I, isoproterenol, epinephrine, norepinephrine, terbutaline) on the ring eluted second and third, respectively with the exception of isoproterenol and norphenylephrine. This general trend in elution order based on class I-III PEAs was also found to hold true for the LCA-bonded phase. This suggests that some unbonded propylamine still exist on the LCA-bonded phase. Another possibility is that the hydroxyl group on the aromatic ring of the PEA hydrogen bonds with the carboxylic site of the LCA-SP. Moreover, the differences in elution order for later eluting PEAs (*e.g.* class I and class II) suggests that these PEA actually interact with the LCA-bonded phase in a somewhat different manner than the aminopropyl phase. For example, octopamine (peak 5, Fig. 2B) was able to be resolved from

isoproterenol (peak 6) on the LCA-bonded phase, whereas these compounds co-eluted on the aminopropyl phase. In contrast, norphenylephrine (peak 7, Fig. 2A) was able to be separated from isoproterenol on the aminopropyl phase, which co-eluted on the LCA-bonded phase. Most interestingly, the elution order of terbutaline and epinephrine was reversed between the two SPs (Fig. 2A vs. B). The different positioning of the hydroxyl groups on the ring most likely provides varying steric interactions that contribute to this separation. Overall, it can be concluded that for the separation of class II and class III PEAs, the LCA-bonded phase provides somewhat improved separation with different selectivity for several PEAs as compared to the aminopropyl phase.

The efficiency data of LCA phase was calculated from the chromatogram of Fig. 2(B) for all PEAs is summarized at the top of Table 3. In general, high efficiency was achieved for earlier eluting class III PEAs compared to the later eluting ones. For example, efficiencies of 152 000 and 140 000 plates/m are calculated from the peaks of class III PEAs (pseudoephedrine and ephedrine). On the other hand, for longer retained class II and class I compounds such as norphenylephrine and norepinephrine efficiencies were somewhat lower (*e.g.* 72 000 and 90 000 plates/m, respectively).

3.5 CEC-UV of PAHs

The capability of the LCA-bonded phase for CEC-UV separations of the 16 PAH test mixture was next evaluated. Initially, a study of mobile phase ACN content was conducted. The LCA-bonded phase was not well suited to run at lower ACN (*i.e.* 65% ACN) using 15 mM NH₄OAc at pH 5.0. However, the phase was well suited to be run under higher ACN content using conditions of 85% ACN, which were therefore utilized for optimizing the mobile phase pH. The results of the pH study are presented in Fig. 4 and are discussed below.

As shown in Fig. 4, a lower pH of 3 provided longer analysis time (*e.g.* 80 min) along with broader peak shapes. The longer retention times can be attributed to the increased ionic strength from addition of HOAc used to lower the pH of the NH₄OAc buffer. Furthermore, increased viscosity along with decrease in the electrical double-layer results in the longer retention shown above. At higher pH 4.0 and 4.5 little difference between the two separations is observed. The weaker ionic strength of the mobile phase at pH 4.5 results in faster analysis time although the resolution between the first two peaks is diminished. Interestingly, the same separation at pH 4.5 using the aminopropyl silica phase (shown as inset of Fig. 4) provided a single peak with no resolution of PAH. Overall, a pH 4.0 was chosen as the optimized mobile phase pH.

Next, a spiking study for determination of the elution order of the 16-PAH mixture was conducted. From the results of the spiking study, the separation of three representative mixtures of PAHs clearly demonstrates the ability of the LCA-SP for baseline separation of PAHs differing in number of aromatic rings as shown in Fig. 5. From Fig. 5, the elution order was evaluated for insight into the molecular recognition properties of the LCA-bonded phase. A comparison of the log *P* with elution order showed that the PAHs follow a typical RP retention mechanism on the LCA-bonded phase based upon increasing carbon number or hydrophobicity as supported by increasing retention with increase in log *P*. In addition, the chromatographic efficiency and resolution data for these representative PAHs are provided in the bottom part of Table 3. Overall, acceptable plate numbers and resolution were achieved.

3.6 CEC-UV of PCBs

After analysis of PAHs, the capability of the LCA-bonded phase for separation of a series of PCBs was assessed. Similar to the study of PAHs, the PCBs were run at low (*e.g.* 65%) and high content (*e.g.* 85%) of ACN. The results of the study were to be the same as for PAHs, in that the LCA-bonded phase was not well suited for operating conditions as only one peak was

observed at 65% ACN. Similarly, a variation in the mobile phase pH in the range of pH 3-5 was evaluated. Overall, pH 4.0 was a good compromise between resolution and analysis time, as shown by the degree of separation of the early eluting PCBs. A comparison of the separation of eight PCBs on the LCA-bonded phase *versus* the aminopropyl silica showed that aminopropyl silica was not able to resolve any PCBs, as all eight PCBs co-eluted as one peak. In contrast, a representative chromatogram presenting a good separating capability of the LCA-bonded phase for PCBs is presented in Fig. 6. From analysis of Fig. 6, the elution order of the PCBs on the bonded phase generally follows increasing chlorine substituent group on the biphenyl ring. This trend follows an increase in hydrophobic nature of the analyte, which is a similar trend observed for PAHs (Fig. 5) and as shown in our previous work using MEKC [27,28].

3.7 EOF marker trends in CEC-MS and CEC-UV

Finally, the elution trend of the neutral marker (thiourea) at different mobile phase conditions for CEC-MS and CEC-UV was investigated. These data can lead to some useful insights regarding the EOF and overall allow conclusion for the most suitable operating conditions of the LCA-bonded phase. The CEC-MS mobile phase conditions varying the percentage of ACN were compared. A comparison between the aminopropyl phase and the LCA phase at 70% ACN content showed that the retention of thiourea is significantly longer on the LCA phase (~108 min, Fig. S2B in the Supporting Information) as compared to the amino phase (~54 min, Fig. S2A in the Supporting Information). Furthermore, this composition of 70% ACN provides poor peak efficiency of thiourea indicated by its wide peak base. Therefore, this mobile phase composition is concluded to be not suitable operating conditions, which is consistent with the analysis of PAHs and PCBs at 65% ACN, which also provided poor peak shapes. Next, a comparison of the two phases was conducted at higher organic composition of 85% ACN. A similar trend to 65% ACN was observed, whereas the aminopropyl phase showed shorter retention (~29 min, Fig. S2C in the Supporting Information) *vs.* the LCA phase (~42 min, Fig. S2D in the Supporting Information). The peak shapes and efficiencies were dramatically enhanced on both phases compared to lower ACN content. Furthermore, the decreased retention at 85% ACN as compared to 65% ACN along with higher efficiency suggests that 85% ACN is a much better operating condition. Lastly, the LCA-bonded phase was run at 90% ACN, which continued to decrease the retention of thiourea (~27 min, Fig. S2E in the Supporting Information). Therefore, two conclusions can be reached. First, a comparison of the thiourea retention time for different operating ACN conditions between the aminopropyl and LCA phases showed that slower EOF was always observed on the latter phase. Second, the retention time for thiourea on both phases decreased with increase in ACN, which is consistent with the change in dielectric constant/viscosity ratio and RP retention mechanism.

Next, the trend in thiourea retention time over the pH range of 3.0-4.5 was investigated under CEC-UV conditions, using 85% ACN for the LCA-bonded phase (data not shown). The trend of increasing retention time showed that the EOF marker slows down with decreasing pH. Two conclusions can be reached. First, decreased EOF at low pH is consistent with the theory. In addition, increasing ionic strength with added HOAc for pH adjustment also slows down the EOF and alternately affects the thickness of the electrical double-layer. Second, the useful operating range is below the pK_a of the carboxylic acid group of the LCA ($pK_a = 4.76 \pm 0.10$, Sci-Finder Scholar 2007). Hence, very low EOF is generated when the LCA phase is uncharged at low pH. A direct comparison at pH 4.5 between the LCA phase and the amino phase again showed that the thiourea marker is shorter retained on the amino phase (26 min) as compared to the LCA-bonded phase (34 min) (data not shown). This data further supports differences between the two materials.

4 Concluding remarks

This work has described the preparation and characterization of a LCA-SP suitable for application in packed-column CEC-MS and CEC-UV. The LCA ligand was first converted to CF followed by attachment to aminopropyl silica. Reaction of the LCA to CF was confirmed *via* ^1H -NMR while the attachment of the LCA-CF to the modified silica was assessed using elemental analysis and finally ^{13}C -NMR. The elemental analysis comparing aminopropyl silica to the LCA-bonded phase showed significantly more carbon and hydrogen on the LCA-bonded phase although the nitrogen content remained constant. Next, the ^{13}C -NMR (using cross polarization-magic angle spinning) comparison of the two phases showed significant differences in peak resonances. This comparison suggested that the surface property of the two phases were different.

The capability of the LCA phase for applications in CEC-MS and CEC-UV was assessed using several mixtures of cationic and neutral compounds important for pharmaceuticals and environmental industry. First, the analysis for small charged molecules, including β -blockers and PEAs by CEC-MS showed that the LCA-bonded phase exhibited higher selectivity compared to the aminopropyl silica phase. For the analysis of neutral, more hydrophobic molecules such as PAHs and PCBs, the LCA-bonded phase demonstrated superior separation power compared to aminopropyl silica phase with a typical RP retention mechanism. A comparison of the EOF trend between the two phases under the same conditions showed that the thiourea marker was always shorter retained on the amino phase as compared to the bonded phase. In summary, this work is the first report of LCA-bonded LC-SP suitable for packed-column CEC-MS and CEC-UV applications.

Acknowledgments

This project was supported by National Institute of Health (Grant No. GM 62314) and Solvay Pharmaceuticals (Marietta, GA). Dean Norton would like to thank Dr. Syed Asad Ali Rizvi and Dr. Brian Crow for their participation in this project. In addition, thanks to Dr. Johannes Leisen at Georgia Tech for ^{13}C -NMR characterization of the stationary phases.

Abbreviations

CF, chloroformate; EIC, extract ion chromatogram; LCA, lithocholic acid; LC, liquid crystal; MeOH, methanol; PCB, polychlorinated biphenyl; PEA, phenylethylamine; RB, round bottom; SP, stationary phase.

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Figure 1.

CEC-MS comparison of (A) aminopropyl silica phase (B) LCA-bonded phase for separation of seven component β -blockers. Conditions: packed bed 25 cm, total length 58 cm, buffer, 85/15 ACN/10 mM NH_4OAc , pH 3, 0.05% TEA. Sheath liquid: 70/30 MeOH/10 mM NH_4OAc delivered at 500 $\mu\text{L}/\text{min}$. ESI spray chamber: drying gas flow rate 6 L/min, nebulizer pressure 5 psi, drying gas temp. 200°C, Vcap 2700 V, fragment V 80, CEC 12 kV runs, 4 kV 4 s injection. Analytes 1 mg/mL in MeOH, injection sample 80/20 analyte/running buffer. Peak identification: 1 = oxprenolol, 2 = alprenolol, 3 = pindolol, 4 = propranolol, 5 = metoprolol, 6 = atenolol, 7 = talinolol. The inset provides the EIC for each m/z monitored.

Figure 2.

Separation of a mixture of ten PEAs compounds on the (A) aminopropyl silica phase and (B) LCA-bonded phase. Conditions are same as Fig. 1. The protonated molecular ion $[M+H]^+$ was monitored for all analytes. Peak identification; 1 = pseudoephedrine; 2 = ephedrine; 3 = norephedrine; 4 = synephrine; 5 = octopamine; 6 = isoproterenol; 7 = norphenylephrine; 8 = terbutaline; 9 = epinephrine, 10 = norepinephrine. The inset provides the EIC for each m/z monitored, for both (A) and (B) a: [norephedrine $M+H$] $^+$, b: [isoproterenol $M+H-C_3H_8O$] $^+$, c: [terbutaline $M+H-C_4H_{10}O$] $^+$, d: [norepinephrine $M+H-H_2O$] $^+$, e: [octopamine $M+H$] $^+$, f: [norphenylephrine $M+H$] $^+$, g: [pseudoephedrine $M+H$] $^+$, h: [ephedrine $M+H$] $^+$, i: [epinephrine $M+H-H_2O$] $^+$, j: [synephrine $M+H$] $^+$, k: [terbutaline $M+H-C_4H_8$] $^+$, l: [norepinephrine $M+H$] $^+$, m: [epinephrine $M+H$] $^+$, n: [isoproterenol $M+H$] $^+$, o: [terbutaline $M+H$] $^+$. Packed bed lengths 25 cm, total length 60 cm.

Figure 3. Proposed fragmentation pathway of PEAs, (A) norepinephrine by loss of H₂O, and (B) terbutaline by loss of isobutyl alkyl group followed by loss of H₂O.

Figure 4.

Variation of the mobile phase pH for separation of 16-PAH test mixture on the LCA phase. The inset shows the same separation achieved at pH 4.5 using the aminopropyl silica phase. Conditions: 85/15 ACN/15 mM NH₄OAc, packed length 30 cm; run voltage = 10 kV; injection: 10 kV for 18 s; injection sample 85/15 analyte/running buffer. Peak identification: peak 1 = acenaphthylene; peak 2 = naphthalene, acenaphthene, peak 3 = phenanthrene; peak 4 = fluoranthene; anthracene, fluorene; peak 5 = chrysene, pyrene, 1,2-benzanthracene; peak 6 = benzo[*k*]fluoranthene, benzo[*b*]fluoranthene; peak 7 = 1,2:5,6-ibenzanthracene; benzo[*a*]pyrene; peak 8 = indeno(1.2.3-*cd*)pyrene; peak 9 = benzo[*ghi*]perylene.

Figure 5. Separation of different PAH mixtures on the LCA-bonded phase. Conditions are the same as Fig. 4 except for the mobile phase pH 4.0.

Figure 6. Separation of a four-PCB mixture on the LCA-bonded phase. Conditions are the same as Fig. 4 except for the mobile pH 4.0.

Scheme 1.

Synthetic scheme for the attachment of lithocholic acid (LCA) to aminopropyl silica. First step involves the conversion of LCA to LCA-chloroformate (1), followed by attachment to aminopropyl silica to yield LCA-bonded phase (2).

Table 1Elemental analysis of the aminopropyl silica intermediate and the LCA-bonded phase^{a)}

Kromasil Si-C₃-NH₂	Batch 1 (more NH₂ silane added)
C	1.72
H	5.00
N	4.90
Kromasil Si-C₃-NH₂	Batch 2 (less NH₂ silane added)
C	1.65
H	4.80
N	4.60
Kromasil Si-C₃-NH-LCA	
C	4.79
H	9.80
N	4.30

^{a)} C,H,N elemental analyses are mmol/g.

Table 2
Elution order, mass (*m*), charge (*z*), *m/z* ratio and log *P* of the PEAs

Compound	Elution order (Si-C ₃ -NH ₂) (Si-C ₃ -NH-LCA)		MW	(<i>z</i>)	<i>m/z</i>	log <i>P</i>	Class
Pseudoephedrine	1	1	165.23	+1	0.0061	0.93	III
Ephedrine	2	2	165.23	+1	0.0061	0.93	III
Norephedrine	3	3	151.21	+1	0.0066	0.86	III
Synephrine	4	4	167.21	+1	0.0060	0.67	II
Octopamine	5	6	153.18	+1	0.0065	0.15	II
Isoproterenol	6	5	211.26	+1	0.0047	0.94	I
Norphenylephrine	7	9	153.18	+1	0.0065	0.15	II
Epinephrine	8,9	7,8	183.20	+1	0.0054	2.04	I
Terbutaline	8,9	7,8	225.28	+1	0.0044	-	I
Norepinephrine	10	10	169.18	+1	0.0059	-0.24	I

Table 3

Chromatographic performance of the LCA-bonded phase for analysis of positively charged (top) and neutral (bottom) compounds^{a)}

Peak Identification:	Efficiency (plates/m)	Resolution
Positively charged compounds		
1=pseudoephedrine	152 000	
2=ephedrine	140 000	2.3
3=norephedrine	136 000	4.1
4=synephrine	96 000	1.6
5=octopamine	136 000	3.9
6=isoproterenol	72 000	1.5
7=norphenylephrine	72 000	0.0
8=terbutaline	72 000	1.1
9=epinephrine	84 000	1.5
10=norepinephrine	90 000	2.4
Neutral compounds		
11=naphthalene	29 900	-
12=1,2-benzanthracene	36 300	3.2
13=benzo[<i>a</i>]pyrene	36 300	3.4
14=acenaphthene	56 000	-
15=benzo[<i>b</i>]fluoranthene	66 000	6.3
16=indeno(1.2.3- <i>cd</i>)pyrene	59 500	4.9
17=anthracene	52 800	-
18=chrysene	56 100	2.9
19=benzo[<i>k</i>]fluoranthene	39 600	1.9
20=benzo[<i>b</i>]fluoranthene	39 600	0.0
21=1,2:5,6-dibenzanthracene	56 100	1.8

^{a)}Chromatographic data of PEAs and PAHs were calculated for the separation obtained in Fig. 2B and Fig. 5, respectively.