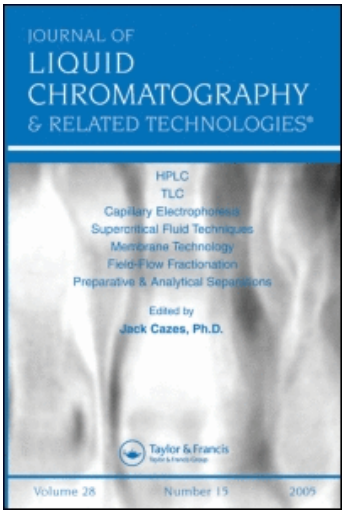

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SIMULTANEOUS RESOLUTION AND DETECTION OF A DRUG SUBSTANCE, IMPURITIES, AND COUNTER ION USING A MIXED-MODE HPLC COLUMN WITH EVAPORATIVE LIGHT SCATTERING DETECTION

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ABSTRACT

An alternative approach to developing individual potency, impurity, and counter ion methods is the simultaneous resolution and detection of the drug substance, impurities, and the counter ion in a single chromatogram. LY326315 hydrochloride was used as a model compound to demonstrate this concept. The separation was achieved using a conventional HPLC system with an Alltech mixed-mode column, a reversed phase eluant, and evaporative light scattering detection (ELSD). The mixed-mode column, which has both reversed phase and ion chromatography functionalities (e.g. phenyl/cation, C8/anion), coupled with ELSD offers a novel approach to simultaneously resolving and detecting pharmaceutical compounds and counter ions in a single chromatogram.

INTRODUCTION

Pharmaceutical compounds are routinely evaluated for drug substance purity, including quantitation of the counter ion, as well as, content of possible impurities and degradation products. Typically, three separate methods are often independently developed and used to analyze a drug substance for potency, non-volatile impurities, and the counter ion. The two methods for potency and impurities often employ reversed phase high performance liquid chromatography (HPLC) in conjunction with ultraviolet (UV) spectrophotometry detection. A third method for the counter ion has traditionally been performed by titration methods or ion chromatography (IC) with conductivity detection. An alternative approach using HPLC with a mixed-mode column and an evaporative light scattering detector (ELSD) would have the advantage of accomplishing these separations in a single chromatogram.

Mixed-mode (or mixed-interaction) stationary phases offer a rational approach to improving selectivity as compared to largely unimodal, conventional HPLC stationary phases.[1] In mixed-mode chromatography, multiple reaction mechanisms are employed to enhance selectivity. The Alltech mixed-mode column series combine reversed phase and ion-exchange phase capability in a single support. This multifunctional support consists of a high purity, 100 angstrom pore, spherical silica substrate which has been bonded with either anionic (amine) or cationic (carboxylate) functionalities in addition to conventional reversed phase (C_8, C_{18}, or phenyl) functionalities. Applications which previously required special conditions, such as ion-pair reagents or base deactivated supports, can be developed by alternately controlling the state of ionization via eluant pH adjustments along with buffer strength and organic modifier composition.[2] The current literature demonstrates the applicability of the mixed-mode columns for use with inorganic ions and carboxylic acids,[3] sulfonated azo dyes,[4] nucleic acid constituents,[5] and oligomers.[6]

The ELSD has gained acceptance as a sensitive universal detector.[7-8] The ELSD operates by nebulizing the volatile effluent from the HPLC column into a fine mist. The mist is then carried through a temperature controlled drift tube where the volatile components (mobile phase) are vaporized. A fine cloud of non-volatile solute particles is carried through a light beam causing light scattering where this scattered light is detected by a photomultiplier. Response is a function of the amount of light scattered and is proportional to the concentration.

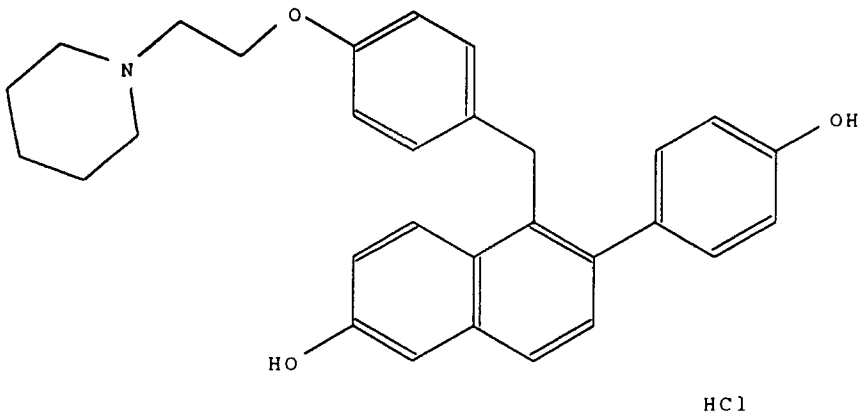


Figure 1. Structure of LY326315 hydrochloride.

The ELSD is not influenced by the UV spectral properties of solvents used for mobile phases, therefore the ELSD is not subject to baseline drift from gradient elution. The choice of acceptable solvents is expanded since the spectral background is not an issue with the ELSD. The ELSD is not affected with sample solvent interferences and sample response is independent of the chemical structure or optical characteristics of the solute and is therefore capable of detecting inorganic ions such as sodium[9] and chloride in addition to organic compounds.

A limitation of the ELSD requires the complete volatilization of all mobile phase components. Addition of nonvolatile components to the mobile phase would cause an elevated background by the continuous generation of solid particles into the light source. The elevated background decreases the sensitivity of the detector for the sample components.

The current literature demonstrates the applicability of the ELSD for use with phospholipids,[10-16] triglycerides, fats and fatty esters,[17-22] carbohydrates,[23-24] synthetic polymers,[25] steroids,[26] inorganic counter ions,[9,27,29] and pharmaceutical compounds.[9,28]

The purpose of this research was to demonstrate the concept of simultaneously resolving and detecting a pharmaceutical compound and its counter ion in a single chromatogram. The intent of this paper is to show the applicability of using mixed-mode HPLC columns in combination with ELSD

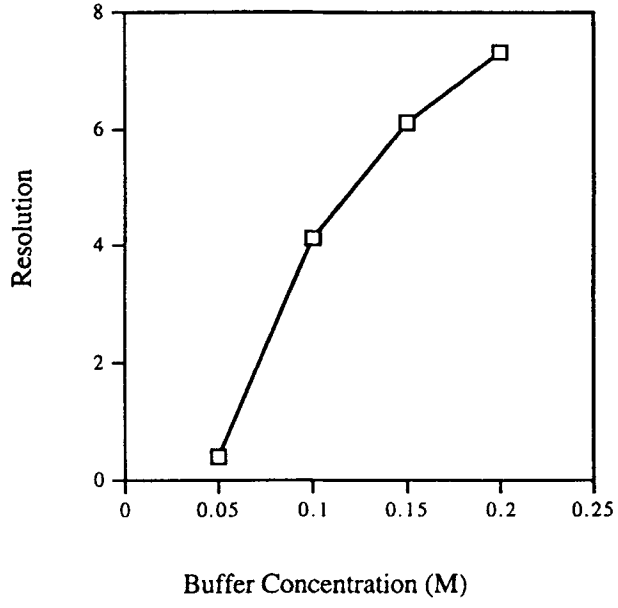


Figure 2. Effect of ammonium acetate buffer concentration on resolution between LY326315 and chloride using mixed-mode phenyl/cation column. Mobile phase: 50% methanol / 50% ammonium acetate buffer pH 4.0.

to resolve and detect a drug substance, impurities, and counter ion in a single chromatogram. LY326315 hydrochloride, a new selective estrogen receptor modulator (SERM), was selected as the model compound for this effort. The structure of LY326315 hydrochloride is shown in Figure 1.

EXPERIMENTAL

The LY326315 hydrochloride drug substance and related impurities were synthesized at Eli Lilly and Company (Indianapolis, IN). Sodium chloride was purchased from Mallinckrodt Chemicals, Inc. (Paris, KY). Ammonium acetate and glacial acetic acid were purchased from EM Science (Gibbstown, NJ). Chempure™ brand methanol was purchased from Curtin Matheson Scientific, Inc. (Houston, TX). The water was deionized and filtered through a Millipore Milli-Q™ water purification system (New Bedford, MA).

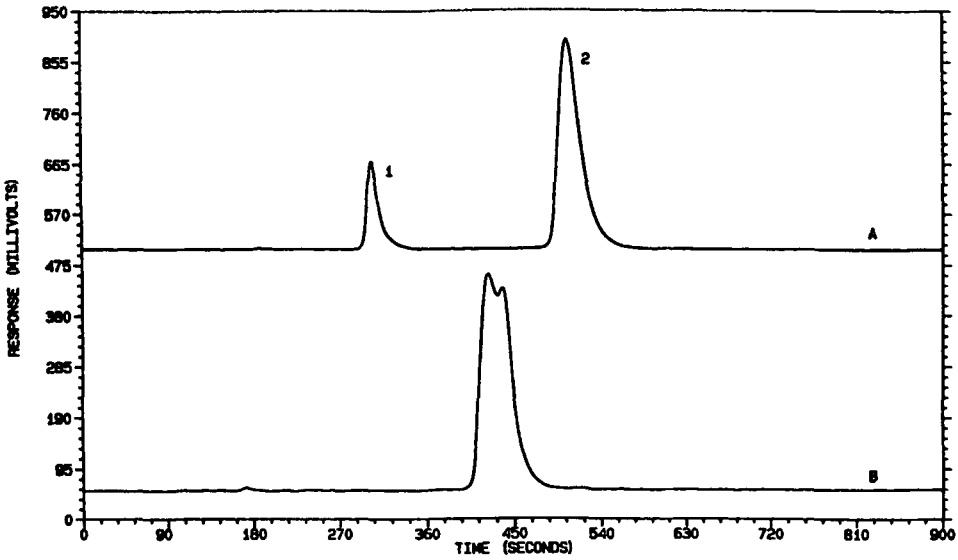


Figure 3. Chromatograms showing the effect of buffer concentrations at 0.05 M and 0.15 M on resolution between the LY326315 and chloride peaks using mixed-mode phenyl/cation column. Mobile phase: 50% methanol / 50% ammonium acetate buffer pH 4.0. (A) = 0.15 M buffer, (B) = 0.05 M buffer. Peak 1 = chloride, peak 2 = LY326315.

The mixed-mode HPLC columns - phenyl/cation, phenyl/anion, C_8/cation, and C_{18}/anion (250 mm x 4.6 mm, 7 µm particle size) - were obtained from Alltech Associates, Inc. (Deerfield, IL). A Shimadzu (Kyoto, Japan) series 10A autoinjector and pump were used with a Sedex 55 evaporative light scattering detector (Richard Scientific, Novato, CA). NF grade nitrogen (Air Products and Chemicals, Inc., Allentown, PA) was used as carrier gas for the ELSD.

The chromatography parameters examined were: ammonium acetate buffer strength (0.05 M - 0.20 M), buffer pH (4.0 - 5.5), organic modifier composition (0% - 55%), and detector temperature (28°C - 90°C). A mobile phase flow rate of 1.0 mL/minute was used.

Samples were prepared in 50% methanol / 50% water. Injection volume was 100 µL. The column temperature was ambient. The ELSD detector was set at a nitrogen pressure of 1 bar.

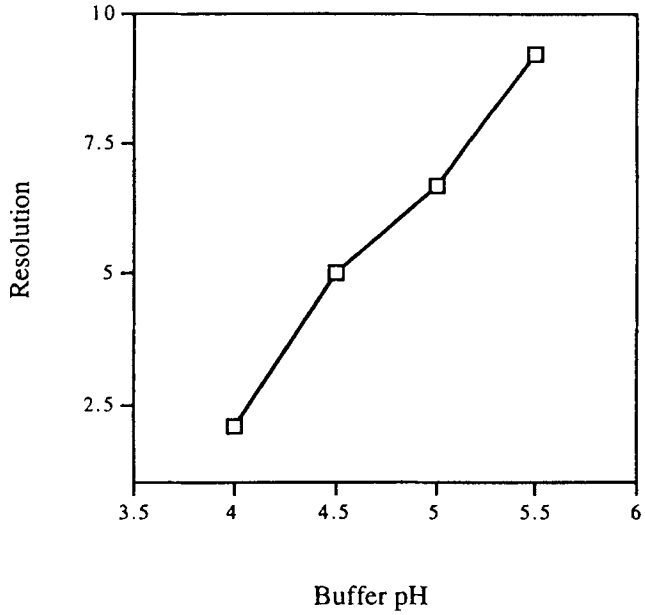


Figure 4. Effect of buffer pH on resolution of LY326315 and chloride using mixed-mode phenyl/cation column. Mobile phase: 50% methanol / 50% 0.1 M ammonium acetate buffer.

RESULTS AND DISCUSSION

Effect of Buffer Strength

Ammonium acetate was the only buffer tested, in concentrations ranging from 0.05 M to 0.20 M, for the evaluation of the phenyl/cation mixed-mode column. Ammonium acetate buffer was selected because it is volatile and therefore compatible with the ELSD. Ammonium acetate is also necessary to enhance the response of chloride. LC/MS studies have been previously conducted indicating the formation of ammonium chloride clusters as the moiety being detected by the ELSD.[29] Increasing the buffer concentration had a significant effect by increasing the resolution between the LY326315 and chloride peaks (Figure 2). At the low concentration of 0.05 M, the two peaks overlapped (Figure 3). Adequate resolution was obtained with a 0.10 M buffer concentration. The retention of chloride decreased while the retention of LY326315 increased as buffer concentration increased. Buffer strength had

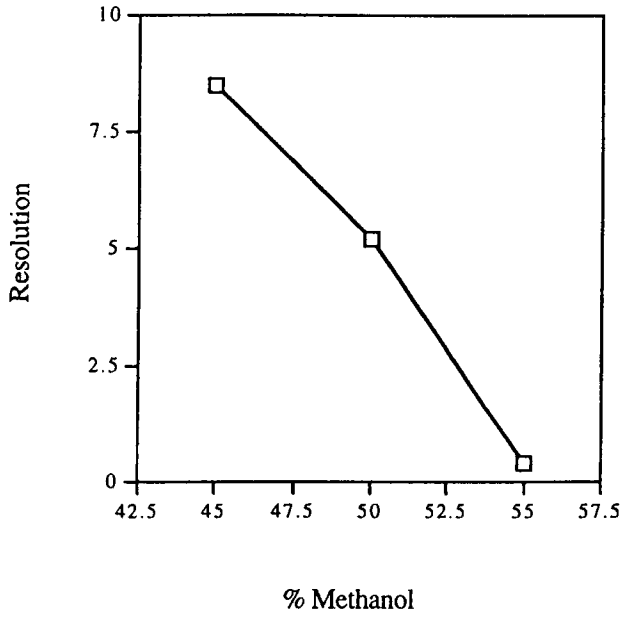


Figure 5. Effect of percent methanol in mobile phase on resolution between LY326315 and chloride using mixed-mode phenyl/cation column. Mobile phase: methanol / 0.1 M ammonium acetate buffer pH 4.5.

little effect on the peak shape in the chromatography. The amount of additives should be kept to a minimum when using an ELSD to maintain maximum sensitivity, less baseline noise, and less preventative maintenance; therefore a mobile phase containing ammonium acetate buffer at a strength of 0.10 M was used for further optimization of other parameters.

Effect of Buffer pH

Glacial acetic acid was used to attain the various pH adjustments between 4.0 and 5.5 in increments of 0.5 for the 0.10 M ammonium acetate buffer solutions. The resolution between the LY326315 and the chloride peaks was sensitive to changes in pH. The resolution increased with increasing pH as illustrated in Figure 4. Similar to the effect with buffer concentration, the retention of chloride decreased while the retention of LY326315 increased as the buffer pH increased. However, unlike buffer concentration changes,

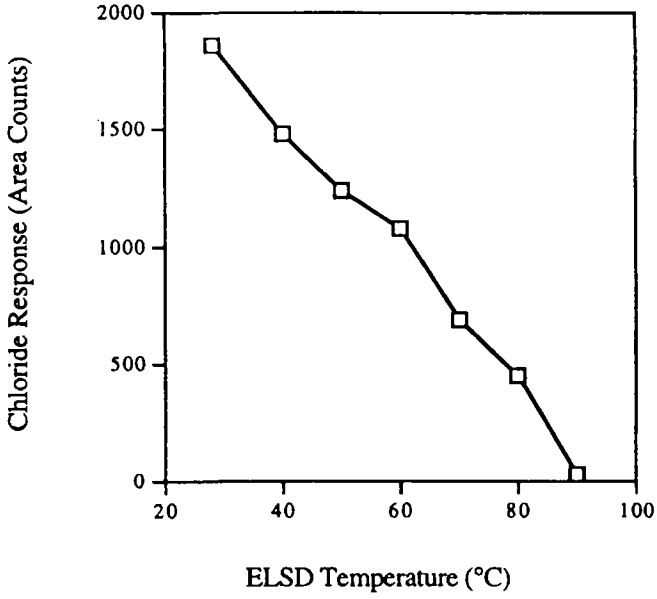


Figure 6. Effect of ELSD temperature on chloride response.

chromatographic peak shape was compromised as pH increased. More peak tailing was observed for LY326315 as the buffer pH was increased. Thus, a mobile phase containing 0.1 M ammonium acetate buffer at pH 4.5 was used for further optimization of other parameters.

Effect of Organic Modifier Composition

Methanol was used as the organic modifier for these experiments, in mobile phase concentrations ranging from 45% to 55% in increments of 5%. The resolution between the LY326315 and chloride peaks was sensitive to changes in methanol concentration. The resolution decreased with increasing methanol concentration as illustrated in Figure 5.

The percent methanol had little effect on ion retention but significant effect on LY326315 retention. A mobile phase consisting of 50% methanol / 50% 0.1 M ammonium acetate buffer at pH 4.5 was used for further work.

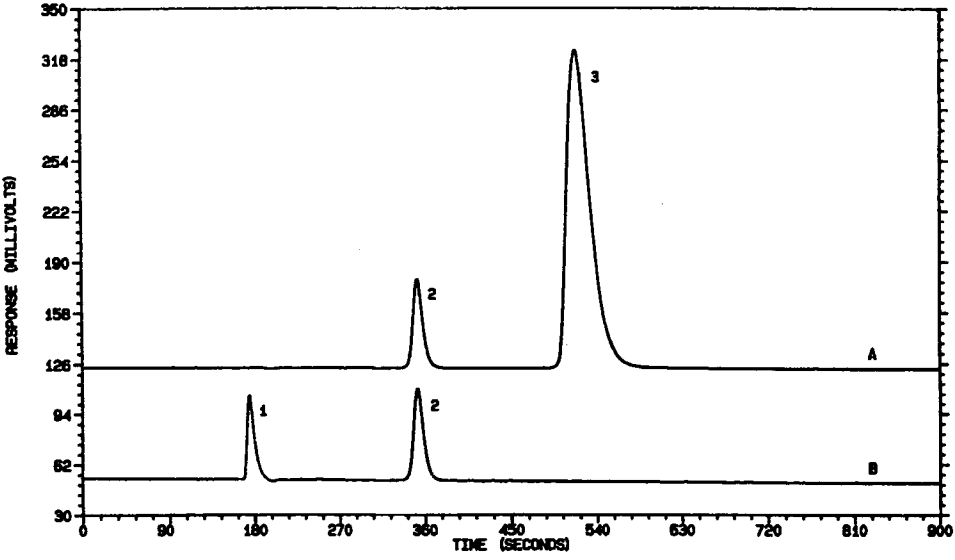


Figure 7. Chromatogram of LY326315 hydrochloride (A) as resolved with the mixed-mode phenyl/cation column and detected by ELSD and a chromatogram of a sodium chloride standard (B). Peak 1 = sodium, peak 2 = chloride, peak 3 = LY326315.

Effect of Detector Temperature

The Sedex ELSD detector permits the drift tube to be temperature controlled. With the pressure set at 1.0 bar and detector gain at 7, the temperature was increased to determine if it had an effect on peak sensitivity. Figure 6 shows the effect of ELSD temperature on chloride response. Significant chloride peak area loss was observed as the detector temperature was increased. This decrease in response is attributed to a breakdown in the ammonium chloride cluster moiety with increasing temperatures. The response of LY326315 was not significantly affected by detector temperature. Detector temperature was set at 28°C to maximize peak area response for chloride.

Optimized Conditions

Figure 7 was obtained using the following optimized conditions on the mixed-mode phenyl/cation column: the isocratic mobile phase consisted of 50% methanol/50% 0.1 M ammonium acetate buffer at pH 4.5 (adjusted with acetic acid). The ELSD settings were: temperature at 28°C, 1.0 bar, and gain of 7.

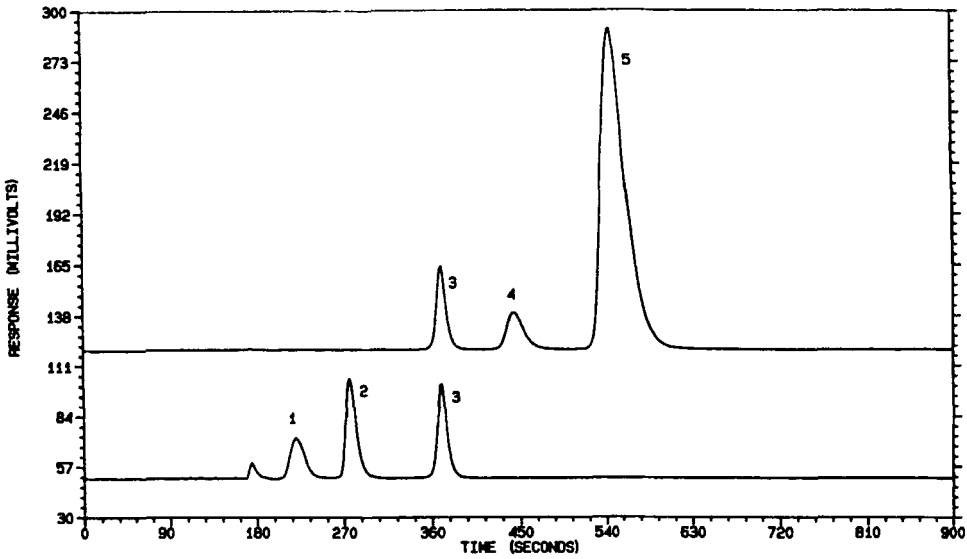


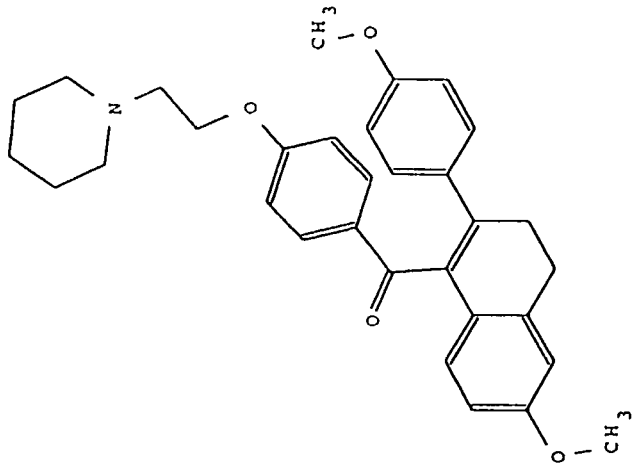
Figure 8. Chromatograms of LY326315 hydrochloride and spiked impurities (process intermediates) as resolved from the mixed-mode phenyl/cation column and detected by ELSD. Peak 1 = compound 174266, peak 2 = compound 151630, peak 3 = chloride, peak 4 = compound 317695, peak 5 = LY326315. Peak at approximately 180 seconds is undetermined.

The chromatograms in Figure 8 show where process related impurities, compounds 174266, 151630, and 317695 elute in relation to the LY326315 and chloride peaks. These impurities are resolved from each other, the drug substance, and chloride. The structures of these impurities are shown in Figure 9.

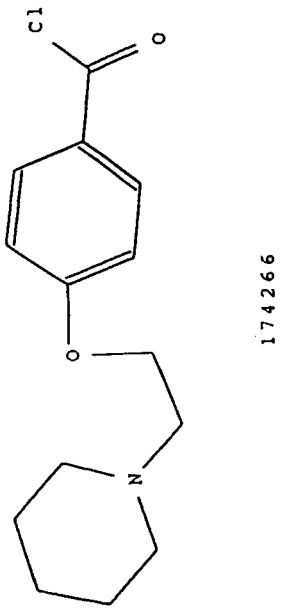
Other Mixed-Mode Columns

The mixed-mode cation columns were designed to retain cations and the mixed-mode anion columns likewise retain anions.[2] Therefore in these experiments with the mixed-mode phenyl/cation column, the greater retention of chloride compared to sodium was an unexpected result.

Figure 9. (right) Structures of process-related impurities, compounds 174266, 151630, and 317695.

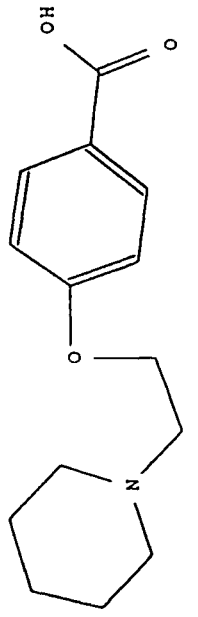


317695



HCl

174266



HCl

151630

Three other Alltech mixed-mode columns - phenyl/anion, C_8/cation, and C_{18}/anion - were tested using the same mobile phase established for the mixed-mode phenyl/cation column. The buffer pH at 4.5 was ideal since the recommended pH is <5 for the anion columns and >4 for the cation columns. The mixed-mode anion columns retained chloride and LY326315 considerably longer than the cation columns. However, on all the mixed-mode anion and cation columns tested, chloride was retained more than sodium.

Literature and manufacturer reports indicate no previous use of mixed-mode columns to analyze small ions such as chloride and sodium. A possible explanation for the chloride retention on both the anion and cation mixed-mode columns involves the mobile phase buffer component which is ammonium acetate. On the mixed-mode phenyl/cation column, which has the carboxylic acid functionality, sodium and chloride are reacting with ammonium acetate forming a sodium acetate complex and an ammonium chloride complex. The ammonium chloride is adsorbing like an ion-pair reagent on the phenyl portion of the column. Sodium acetate has a lower affinity for the stationary phase and therefore has little retention. On the mixed-mode phenyl/anion column, which has the amine functionality, both mechanisms are occurring - ion exchange and ion-pairing for ammonium chloride - therefore creating longer retention when compared to the phenyl/cation column. Other possible explanations include ion interactions with exposed silanols on the column's silica support, size exclusion effects, or a combination of some of the above. Further experiments would be needed to determine the primary contributors to chloride or sodium ion retention.

CONCLUSION

The applicability of a mixed-mode HPLC column in combination with an evaporative light scattering detector for the simultaneous resolution and detection of a drug substance, impurities, and the counter ion has been demonstrated. This novel approach demonstrates a means to simultaneously resolve and detect pharmaceutical compounds, impurities, and counter ions in a single chromatogram, in contrast to developing three separate methods. The mixed-mode column can be used with typical reversed phase mobile phases and with conventional HPLC systems. Since the ELSD is capable of detecting many types of solutes, regardless of functional groups, its versatility was demonstrated here for both organic compounds and inorganic ions. In combination, the mixed-mode column with ELSD provides a new alternative for the analytical chemist performing method development.

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