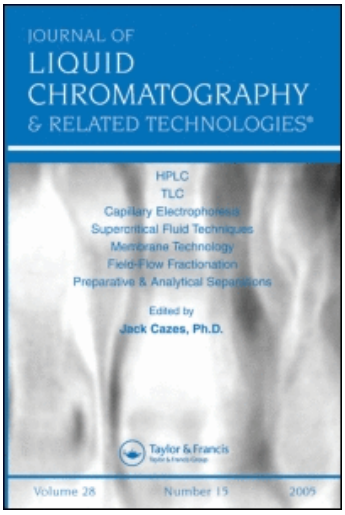

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THE CHIRAL SEPARATION OF OXYBUTYNIN ENANTIOMERS USING AN OVOMUCOID COLUMN

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ABSTRACT

The separation of oxybutynin enantiomers (racemic mixture of two enantiomers) was studied on an ovomucoid column. The optimized chiral separation baseline resolved the enantiomers in less than 10 minutes. Chromatographic variables that were found to have an effect on the chiral separation were studied and include: mobile phase pH, mobile phase ionic strength, type and concentration of organic modifier added to the mobile phase, column temperature, and the amount of analyte injected.

The enantiomeric separation was optimized on the ovomucoid column based on how each variable affected the separation. Calibration curves for a standard were linear over a range of 8.36 to 668.8 μg/g (ppm) with a correlation coefficient of 0.999 for both enantiomers. A detection limit of 4.5 μg/g and a quantitation limit of 9.0 μg/g were also found.

INTRODUCTION

The separation and quantitation of enantiomers present in pharmaceutical products is an important aspect in analytical chemistry and is performed to insure that the active ingredient is pure if it is a single enantiomer or has the correct ratio of enantiomers if the active is a racemic mixture. The ability to separate and quantitate enantiomers in a bulk drug or drug product is being addressed by regulatory agencies in the United States, Japan, and the European Union.[1-5] Areas where a chiral separation must be used for a single enantiomer or a racemic mixture are: bulk drug stability, drug product stability, pharmacology, toxicology, and pharmacokinetic studies. Without the development of a chiral separation, critical information about a drug would not be available.

Many chiral stationary phases have been developed over the past several years specifically for the separation of racemic mixtures. Protein-bonded stationary phases have become popular for chiral separations due to their direct optical resolution and the wide chiral recognition for enantiomers. Several of the more common protein phases include bovine serum albumin (BSA),[6-9] human serum albumin (HSA),[10,11] α_1-acid glycoprotein (AGP),[12-20] ovomucoid,[20-23] and α-chymotrypsin.[24] Recent publications discuss the retention mechanism of chiral stationary phases.[25,26] Analyte retention was attributed to a mixed mechanism: interactions due to nonselective sites and the second interaction due to the enantioselective sites. The protein stationary phases, however, are not very efficient and generally give broad sample peaks with less than 3500 theoretical plates.[27]

In this study, an ovomucoid (OVM) column was used for the separation of oxybutynin enantiomers. OVM has an isoelectric point of 4.5 and a molecular weight of 28,000. The molecule consists of a single 186 amino acid chain divided into three tandem homologous domains by 9 disulfide bonds, carbohydrate moieties (4 to 5 glycosylated asparagine residues), and sialic acid moieties which compose 0.5 to 1.0% of the total weight of the protein.[22,28] Research has shown that the retention mechanism for enantiomers on the OVM column consists of hydrophobic and ionic interactions between the enantiomers and the chiral stationary phase.[29] The OVM column has been shown to provide an effective separation of acidic and basic enantiomers without the need for derivatization. For many separations, small changes in mobile phase pH, column temperature and organic modifier concentration may dramatically influence retention and resolution.

The mobile phase parameters that influenced the chiral separation of oxybutynin on an OVM stationary phase were studied. The results that were found for each mobile phase parameter as well as the optimized enantiomeric separation are discussed.

EXPERIMENTAL

Reagents and Instrumentation

Oxybutynin hydrochloride was purchased from Aldrich Chemical Company (Milwaukee, WI). Citric acid, sodium hydroxide, methanol, isopropanol, ammonium acetate, acetic acid, and acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ, USA). Ethanol was purchased from Quantum Chemical Company (Newark, NJ, USA). HPLC-grade water was obtained by passing de-ionized water through a Nanopure II water purification system (Barnstead, Dubuque, IA, USA).

The instrumentation consisted of a Thermo Separations SCM1000 degasser, P4000 quaternary pump, AS3000 variable loop autosampler with built-in column oven, UV6000 photodiode array detector, and ChromQuest Data System (Thermo Separation Products, San Jose, CA, USA). The Ultron ES-OVM chiral column was purchased from Mac Mod Analytical Inc. (Chadds Ford, PA, USA).

Procedures

Several standards were prepared at a concentration of 1 mg/g in Nanopure™-grade water. The working standards were prepared by diluting the 1 mg/g standards with Nanopure™-grade water. A sample size of about 100 μg/g (ppm) was typically used for all studies. A flowrate of 1.0 mL/min was used for all separations with UV detection at 238 nm. A column temperature of 30°C was used with an injection volume of 25 μL.

RESULTS AND DISCUSSION

The mobile phase parameters that had a significant effect on the retention and resolution of oxybutynin (Figure 1) enantiomers on the ovomucoid (OVM) column were: type and concentration of organic modifier, mobile phase pH, ionic strength, and column temperature. The amount of analyte injected into the chromatographic system was also evaluated to determine what effect concentration would have on resolution. Each of these parameters was studied to evaluate the influence on the separation of oxybutynin enantiomers.

Organic Modifier

The concentration of organic modifier was found to have a major effect on retention and resolution. Figure 2 shows the influence that the mobile phase concentration of ethanol had on oxybutynin retention. As the concentra-

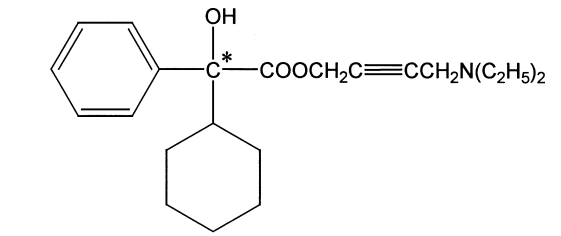

Figure 1. The structure of oxybutynin.

tion of ethanol in the mobile phase was increased, a corresponding decrease in enantiomer retention was observed. This is consistent with previous reports for other enantiomers on the OVM column.[28]

Two other organic modifiers were also studied: acetonitrile and methanol. Acetonitrile was found to be unacceptable under all chromatographic conditions studied since almost no resolution between the two oxybutynin enantiomers could be attained. Methanol provided results that were similar to ethanol, however the two enantiomers could not be baseline resolved under the best mobile phase conditions.

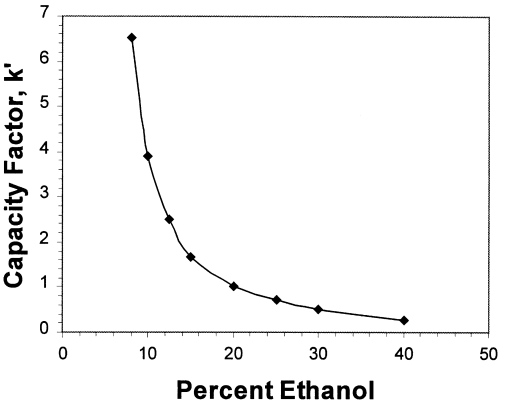

Figure 2. The effect of ethanol concentration on oxybutynin retention. Mobile phase: 40 mM citric Acid, pH 5.0, ethanol.

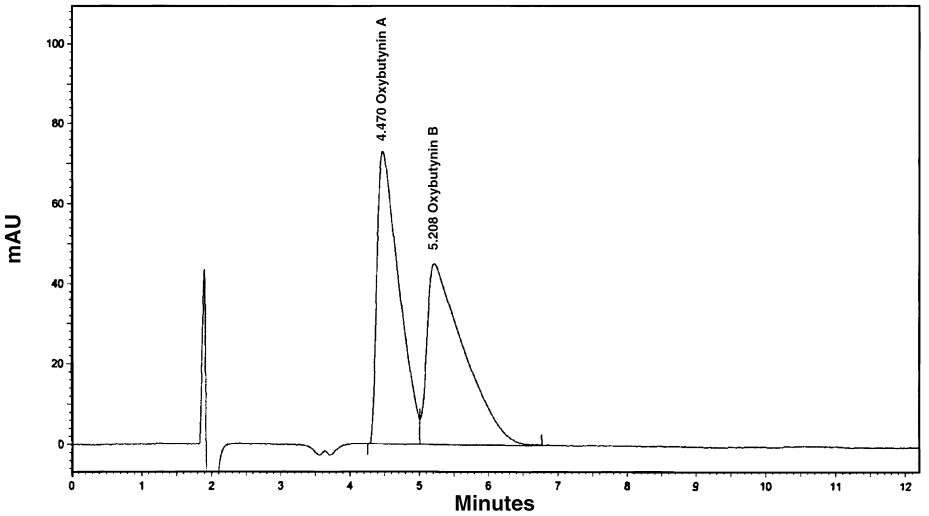

Figure 3. The separation of oxybutynin using methanol as the organic modifier in the mobile phase. Mobile phase: 10 mM citric acid, pH 5.0, 30% methanol.

Figure 3 shows the best separation that was obtained when methanol was used. Ethanol was the only organic modifier that provided baseline resolution for both enantiomers on the OVM column and confirms the results that were reported by Iredale et al.[28] Therefore, ethanol was the organic modifier used for all additional studies.

The optimum mobile phase concentration of ethanol that provided good peak shape and baseline resolution between the two enantiomers was determined to be 12%. Lower concentrations showed improved resolution, however, the peaks became quite broad as well as excessive peak tailing. At higher concentrations of ethanol the two enantiomers were not baseline resolved.

Mobile Phase pH

The effect that mobile phase pH had on oxybutynin retention was also studied. The goal for this study was three-fold: to determine how the mobile phase pH would affect enantiomer retention, resolution, and peak tailing. Protein columns are known to show much greater degrees of peak tailing than achiral silica-based stationary phases; therefore a compromise usually must be made between baseline-resolution and peaks that have significant tailing. In

many cases, changing one or more mobile phase parameters (i.e., pH, ionic strength) may improve enantiomeric resolution, however, unacceptable peak shape may be the result. The results that were observed during this study were similar to those found by Iredale et al.[28] They reported that as the pH of the mobile phase was decreased from 6.0 a corresponding decrease in the net negative charge of the protein took place, as well as, a corresponding change in the Coulombic interactions between the ovomucoid and charged analytes.

A transition in the net charge of the protein takes place over the pH range of 3 to 6 since the isoelectric point of ovomucoid is about 4.5. At a mobile phase pH below 4.5 the stationary phase would be cationic, whereas above pH 4.5 the stationary phase would be anionic in nature. Cationic analytes were found to have lower retention at lower pH mobile phases due to cation-cation repulsion whereas at mobile phases above pH 4.5 higher retention was observed due to favorable ionic interactions. This study indicated that retention and enantioselectivity on the immobilized ovomucoid stationary phase are a function of hydrophobic interactions and Coulombic interactions between the analyte and the immobilized protein.

Figure 4 shows the effect that the mobile phase pH had on analyte retention. Retention was found to increase as the pH was raised from 3 to 7 using a sodium hydroxide solution (mobile phase: 40 mM citric acid, 12% ethanol). The only conditions where the oxybutynin enantiomers were found to be resolved were at mobile phase pH 4 and 5. At pH 4, the two enantiomers were baseline resolved, however, one enantiomer eluted near the solvent front which

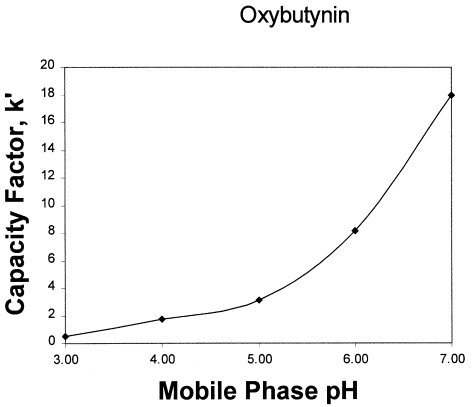

Figure 4. The effect of mobile phase pH on oxybutynin retention. Mobile phase: 40 mM citric acid, 12% ethanol.

was found to be unacceptable. Mobile phase pH 5.0 provided baseline resolution as well as acceptable retention and peak tailing.

At pH 3, the enantiomers eluted in the solvent front, whereas at pH 6 and 7 no resolution was observed. Peak tailing was also severe at the higher pH mobile phases. The data from this study indicated that a mobile phase of pH 5 would provide the best enantiomeric separation.

Effect of Citric Acid Concentration

Chiral columns may be affected by the concentration of buffers as well as the ionic strength of the mobile phase depending on the type of interaction that takes place between an enantiomer and the stationary phase. Oda and co-workers[30] found that the buffer concentration over a range of 10 to 300 mM phosphate did not affect analyte retention. This suggests that electrostatic interactions were not the driving force in the chiral separation for the compounds studied. The retention behavior was determined to be influenced by Coulombic interactions.

A similar result was observed in this study for the oxybutynin enantiomers. At very low levels of citrate buffer (1 mM) analyte retention was high, but once the mobile phase concentration reached 3 mM, analyte retention did not significantly change, even for a mobile phase that contained 100 mM citrate buffer. Figure 5 shows the effect that the citrate buffer had on the first oxybutynin enantiomer that eluted.

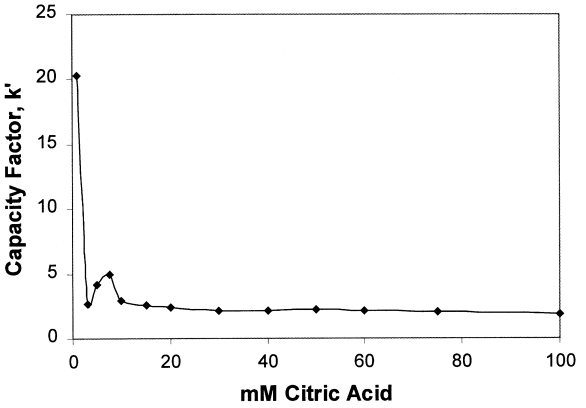

Figure 5. The effect of citric acid concentration on oxybutynin retention. Mobile phase: citric acid, pH 5.0, 12% ethanol.

The resolution of the oxybutynin enantiomers was found to increase with increasing citrate buffer concentration. Table 1 shows how the resolution of the two enantiomers were affected as the concentration of citrate buffer was increased. The retention of the first enantiomer did not change but resolution improved over the entire buffer concentration range that was studied. This indicates that the improved resolution may be influenced by Coulombic interactions, such as ion exchange and or salting out effects.[30] Although resolution improved with increasing citrate buffer concentration, the retention of the more retained enantiomer increased as did peak tailing. A good compromise for resolution, retention, and peak tailing was determined to be at a citrate buffer concentration of 40 mM.

Effect of Column Temperature

Kirkland and McCombs[26] studied the effect that column temperature had on enantiomer retention and resolution. This study indicated that selectivity and resolution might be improved by temperature changes. Chiral resolution of

Table 1

The Effect of Citrate Buffer Concentration on Oxybutynin Enantiomer Resolution

Citrate Buffer mM	Resolution
1.0	0.0
3.0	0.9
5.0	0.9
7.5	0.7
10	0.7
15	1.1
20	1.1
20	1.1
40	1.5
50	1.8
60	2.0
75	2.4
100	2.4

acidic drugs was found to decrease with increasing temperature while the retention of basic drugs was found to increase as the temperature was increased until a maxima was reached.

This data showed that the column temperature should be carefully controlled for optimum reproducibility of analyte retention as well as for quantitative data.

The effect that column temperature had on oxybutynin retention and enantiomeric resolution was studied over the range of 25 to 50°C where a mobile phase of 40 mM citric acid, pH 5.0 and 12% ethanol was used. The results that were found over this temperature range are shown in Figure 6. As the column temperature was increased a corresponding decrease in retention was observed. This would be expected since the mass transfer kinetics are faster at higher temperatures, which results in lower enantiomeric retention.

The effect that the column temperature had on the resolution of the enantiomers is shown in Table 2. Resolution was found to decrease when the column temperature was increased. Oxybutynin, which is a basic compound, did not follow what would be expected according to research reported by Kirkland and McCombs.[26]

This may indicate that oxybutynin is retained on a different portion of the protein than the drugs studied by Kirkland[26] or the maxima is reached at a lower column temperature. From this data, it was determined that a column temperature of 30°C should provide a good separation for the oxybutynin enantiomers.

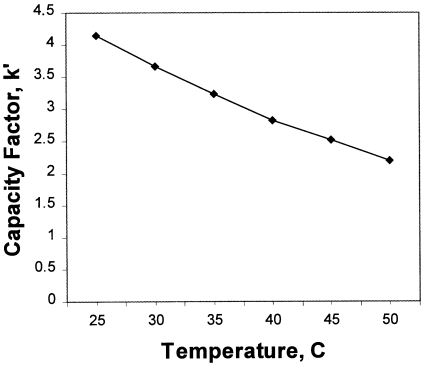

Figure 6. The effect of column temperature on oxybutynin retention. Mobile phase: 40 mM citric acid, pH 5.0, 12% ethanol.

Table 2

The Effect of Temperature on Oxybutynin Enantiomer Resolution

Temperature °C	Resolution
25	2.19
30	2.17
35	1.72
40	1.31
45	1.19
50	0.92

Optimized Separation

The optimized separation for the oxybutynin enantiomers is shown in Figure 7. The mobile phase was composed of 40 mM citric acid, pH 5.0 (adjusted with NaOH) that contained 12% ethanol. A flow-rate of 1.0 mL/min and a column temperature of 30°C were used. The two enantiomers were baseline resolved with a runtime of ten minutes.

Calibration Curves and Sample Loading

Calibration curves were established over the range of 8.36 to 668.8 μg/g (ppm) of oxybutynin. A correlation coefficient of greater than 0.999 was observed for each enantiomer indicating that the system is linear over this range. The limit of detection (3:1 signal:noise) was found to be 4.5 μg/g while the limit of quantitation was determined to be 9.0 μg/g.

Enantiomeric resolution was found to decrease as the amount of oxybutynin injected was increased. Table 3 shows the effect that the amount of oxy-

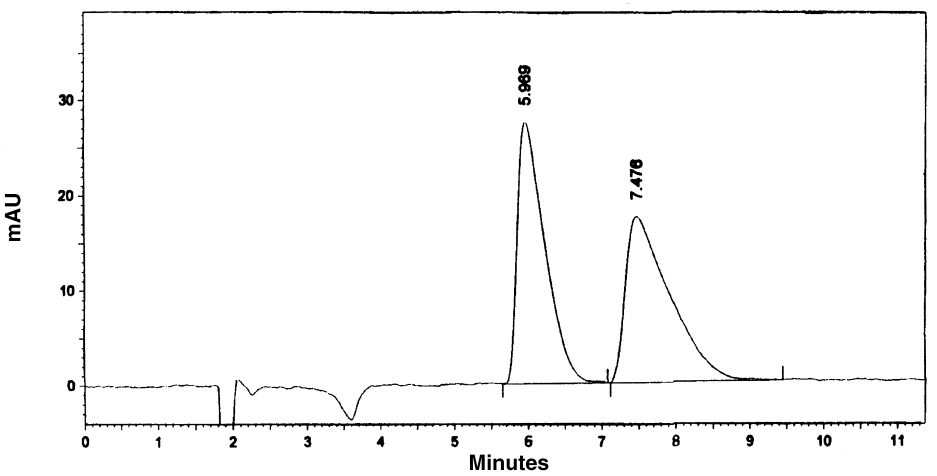

Figure 7. The optimized enantiomeric separation for oxybutynin. Mobile phase: 40 mM citric acid, pH 5.0, 12% ethanol.

Table 3

The Effect of the Amount of Oxybutynin Injected on Enantiomer Resolution

Oxubutynin Injected μg/g (ppm)	Resolution
80	3.03
164	1.97
244	1.84
328	1.71
408	1.56
488	1.17
652	1.21
816	0.83
980	0.86
1144	0.65
1304	0.90
1468	0.88
1632	0.86

butynin injected had on enantiomeric resolution. When the amount of oxybutynin injected was greater than about 652 μg/g (ppm), resolution was found to decrease quickly. Resolution, as well as peak tailing, was found to be the best at lower levels of oxybutynin. This indicates that the OVM column is sensitive to the amount of sample injected and this should be taken into account when determining how much analyte may be chromatographed.

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