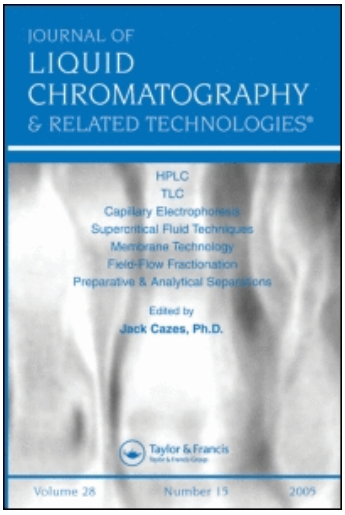

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Evaluation of a New Pepsin Enzyme Chiral Stationary Phase for the Optimized Separation of Seproxetine (S-Norfluoxetine) from R-Norfluoxetine

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EVALUATION OF A NEW PEPSIN ENZYME CHIRAL STATIONARY PHASE FOR THE OPTIMIZED SEPARATION OF SEPROXETINE (S-NORFLUOXETINE) FROM R-NORFLUOXETINE

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ABSTRACT

A thorough analysis of a new commercially available pepsin chiral stationary phase (CSP) has been completed using seproxetine (S-norfluoxetine) hydrochloride bulk drug substance and R-norfluoxetine hydrochloride as the test analytes. Chromatographic properties of this new Ultron ES-Pepsin column were investigated by varying key mobile phase parameters (pH, flow rate, buffer strength and organic concentration), column temperature and sample loading. After observing and plotting changes in retention, resolution and

theoretical plates based on corresponding variation in these parameters, it is possible to choose conditions for the separation that are optimum and robust. The subsequent method validation demonstrated acceptable precision, linearity, recovery, selectivity, limit of detection and ruggedness for the determination of R-norfluoxetine in seproxetine hydrochloride bulk drug substance.

INTRODUCTION

Pharmaceutical compounds are routinely evaluated for bulk drug substance purity, including quantitation of the unwanted enantiomer for chiral compounds. Typically, two analytical test methods are used. An achiral method is developed to determine impurities, including process related compounds and degradation products.

A second method, preferably a direct separation using a CSP, is used to determine the unwanted enantiomer. The technology of CSPs is an area of continual improvement and development, especially in light of increased regulatory requirements in the marketing of pharmaceutical products with stereogenic centers. In 1992, the FDA issued a formal guideline requiring compounds containing stereogenic centers be recognized and the activity of specific enantiomers be identified.[1] In response, the research community has introduced a wide selection of CSPs for high performance liquid chromatography (HPLC), including Pirkle type, cellulose-based, inclusion complexes, ligand exchange, macrocyclic antibiotics and protein bonded phases.[2-13] Protein-based columns have gained much attention due to their broad scope of chiral recognition and compatibility with aqueous mobile phases. Several protein CSPs are available as albumins, glycoproteins and enzymes, including bovine serum albumin (BSA), human serum albumin (HSA), α-acid glycoprotein (AGP), ovomucoid (OVM), avidin, fungal cellulase and α-chymotrypsin.[14-20]

A new CSP utilizing pepsin, a digestive enzyme, was investigated in this report. Pepsin is an acid protinase enzyme found in the gastric fluids of mammals.[21] The enzyme is bound to a 5 μm aminopropyl silica with a pore size of 120 Å using N,N-disuccinimidyl carbonate. Pepsin has an isoelectric point of less than 1, and is therefore targeted for the enantiomeric analysis of basic compounds. In this evaluation, pepsin as a CSP is analyzed for the separation of seproxetine from its unwanted enantiomer, R-norfluoxetine. The

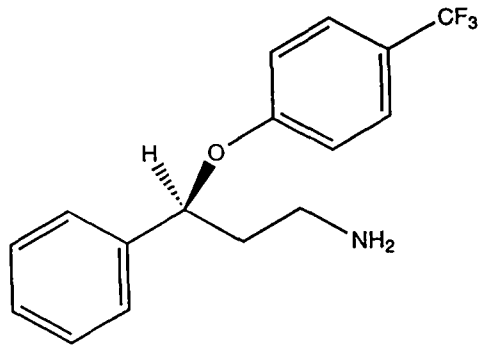

Figure 1. Structure of seproxetine.

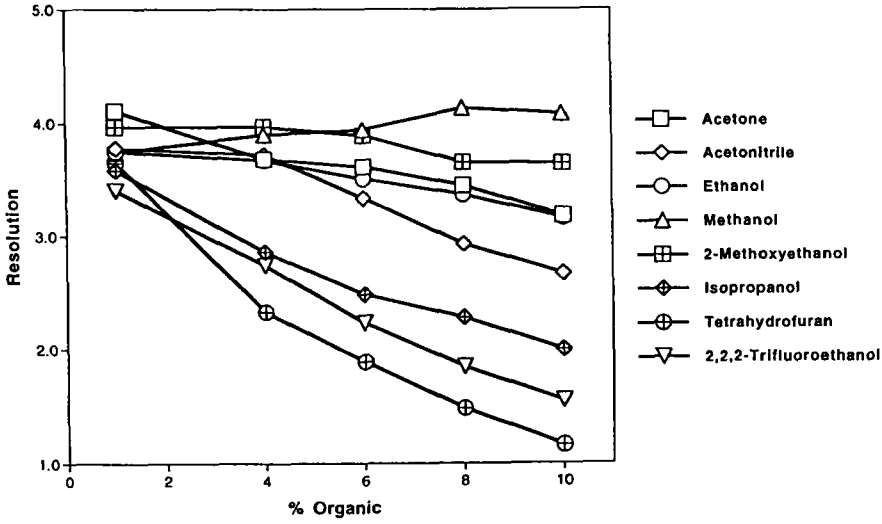

Figure 2. Th effect of organic modifier on the resolution of seproxetine and R-norfluoxetine. Aqueous mobile phase component: 20 mM KH_2PO_4 buffer.

structure of seproxetine is shown in Figure 1. Chromatographic conditions for the separation of seproxetine from R-norfluoxetine on the ES-Pepsin column were optimized, and final method conditions were validated.

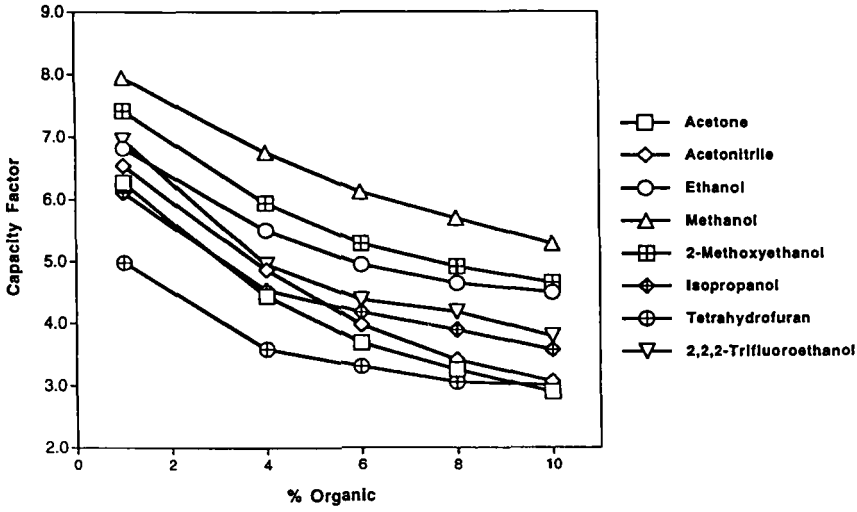

Figure 3. The effect of organic modifier on the capacity factor (K') for R-norfluoxetine. Aqueous mobile phase component: 20 mM KH_2PO_4 buffer.

EXPERIMENTAL

The Ultron ES-Pepsin column (15 cm x 4.6 mm) was obtained from Mac-Mod Analytical (Chadds Ford, PA) and manufactured by Shinwa Chemical Industries (Tokyo, Japan) for Rockland Technologies, Inc. (Newport, DE). A Hewlett-Packard (Wilmington, DE) 1050 series autoinjector and pump were used with an Applied Biosystems 1000S diode array detector (Foster City, CA). The column temperature was maintained with a Model 7950 Column Chiller from Jones Chromatography (Lakewood, CO). Chempure™ Brand organic modifiers distributed through Curtin Matheson Scientific (Houston, TX) were purchased. Ethanol (200 proof) was purchased from Quantum Chemical Corporation (Tuscola, IL) and the 2-methoxyethanol and 2,2,2-trifluoroethanol were obtained from Sigma-Aldrich Company (Milwaukee, WI). The potassium phosphate monobasic salt (KH_2PO_4) was obtained from Mallinckrodt® (Paris, KY) and the 85% o-phosphoric acid (H_3PO_4), 1.0 N hydrochloric acid and 0.1 N sodium hydroxide were acquired from Fisher Scientific (Fair Lawn, NJ). The water was deionized and filtered through a Millipore Milli-Q™ water purification system (New Bedford, MA). Seproxetine hydrochloride, R-norfluoxetine hydrochloride and fluoxetine hydrochloride were synthesized and degradation products were isolated at Eli Lilly and Company (Indianapolis,

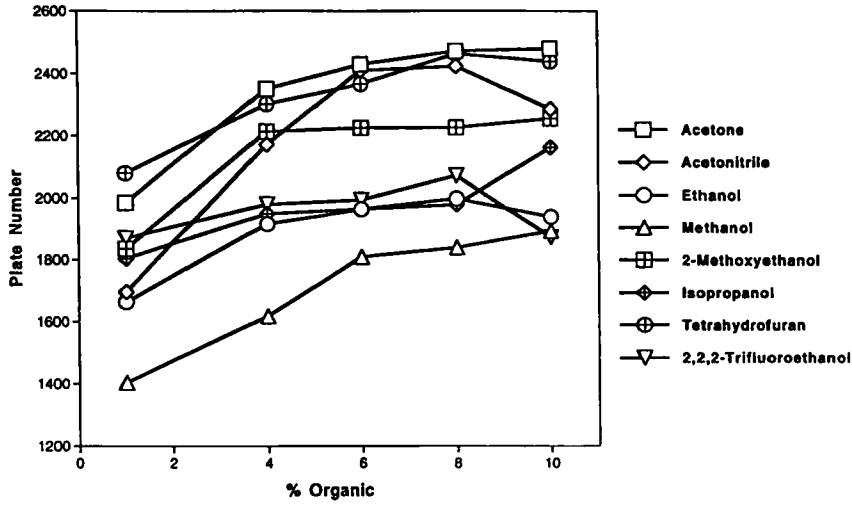

Figure 4. The effect of organic modifier on the plate numbers for R-norfluoxetine. Aqueous mobile phase component: 20 mM KH_2PO_4 buffer.

IN).[22-24] Unless otherwise noted, the sample for injection consisted of a mixture of 0.03 mg/mL of seproxetine plus 0.02 mg/mL of R-norfluoxetine prepared in 20 mM KH_2PO_4 buffer, the flow rate was 1.0 mL/min. and the column temperature was ambient. An injection volume of 10 μL and UV detection set at 225 nm were used throughout this study.

METHOD OPTIMIZATION

Effect of Organic Modifier

The Ultron ES-Pepsin column was evaluated for the separation of seproxetine and its unwanted enantiomer, R-norfluoxetine, using the eight organic solvents: acetone, acetonitrile, ethanol, methanol, 2-methoxyethanol, isopropanol, tetrahydrofuran and 2,2,2-trifluoroethanol. The organic composition was increased from 1 to 10% with a buffer comprised 20 mM KH_2PO_4 with no pH adjustment. The resolution of the enantiomers was greater than 1.5 (baseline) for all the organic modifiers tested as shown in Figure 2.

Generally, resolution decreased with increasing organic composition in the mobile phase. In the case of methanol, a resolution of approximately 4.0 was maintained as the methanol composition was increased. The effect of organic mobile phase composition on the capacity factor (K') for the first eluting peak, R-norfluoxetine, is shown in Figure 3. The use of methanol resulted in the greatest overall K' values whereas tetrahydrofuran yielded the lowest. The effect of changing organic mobile phase composition on the number of theoretical plates is shown in Figure 4. Acetone resulted in the highest theoretical plate numbers and methanol the lowest. 2-Methoxyethanol was an excellent organic modifier when considering the small resolution change from 1 to 10% organic modifier, the number of theoretical plates and overall peak shape. However, this solvent is not commonly used for chromatographic work due to safety concerns, expense and questionable long-term effects on the column longevity. The spectral properties of acetone and 2,2,2-trifluoroethanol make them inappropriate choices when considering the need for trace analysis of the unwanted enantiomer (R-norfluoxetine) with an UV maximum at 225 nm. Although methanol resulted in the lowest theoretical plate numbers of all the organic modifiers tested, it exhibited comparable resolution and K' values to 2-methoxyethanol and the advantage of its consistent resolving capability is important, especially when developing a rugged and universal method. A mobile phase comprised 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer was used for further optimization of other parameters. This composition produced substantial resolution, along with reasonable theoretical plates and a desirable K' value.

Effect of pH

Hydrochloric acid and sodium hydroxide were used to attain the various pH adjustments of the 20 mM KH_2PO_4 buffer solutions. The resolution of the enantiomers was very sensitive to changes in pH. The resolution increased with increasing pH as did K' as illustrated in Figure 5. In fact, pH appears to be the most selective parameter to enhance the chiral separation for this column. The large resolution values between seproxetine and R-norfluoxetine over a wide pH range allowed for some flexibility in choosing the pH for this method but the tradeoff is an increasing K' value with increasing resolution. Generally accepted guidelines for determining adequate retention is to establish a K' range from 2 to 20; thus from the data in Figure 5, the pH range from 3.9 to 5.3 would be acceptable. Other considerations, such as, sharp peaks for adequate detection, large resolutions for robustness and shorter run time for multiple sample analyses, are also important. To keep the analysis time under 30 minutes and resolution above two, any pH from above 3.8 to below 4.9

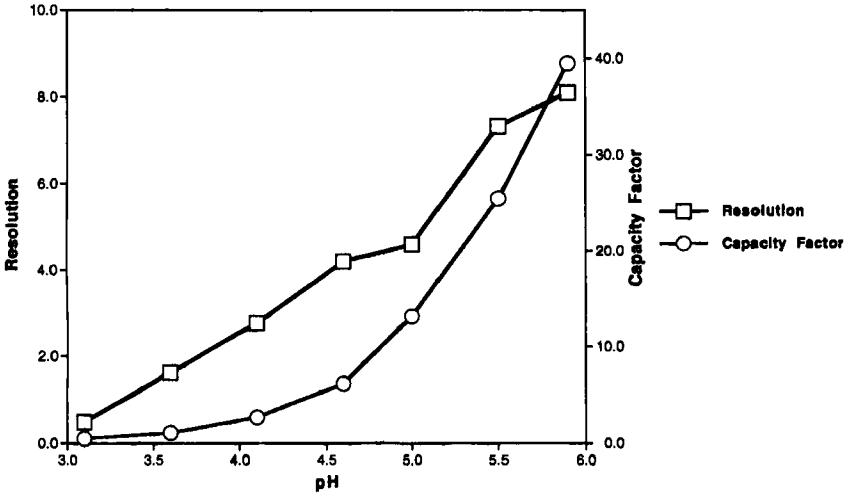

Figure 5. The effect of pH on the resolution of seproxetine and R-norfluoxetine and on the capacity factor (K') for R-norfluoxetine. Mobile phase: 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer (pH adjusted with either 1.0 N HCl or 1.0 N NaOH).

would be sufficient. Using 20 mM KH_2PO_4 buffer resulted in a pH of approximately 4.6 and therefore eliminated a procedural step for further pH adjustment; thus a simpler and more rugged method could be obtained. Further evaluation of other parameters used 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer without pH adjustment.

Effect of Buffer Concentration

Potassium phosphate monobasic was the only buffer tested in concentrations ranging from 1 to 40 mM for this evaluation of the ES-Pepsin column. Although varying the buffer concentration did not have the striking effect that pH variations produced, there were trends in the resulting chromatography. The concentration resulting in the maximum resolution was from 5 to 10 mM. The best peak shape, however, was obtained using 10 to 20 mM KH_2PO_4 concentrations. The total resolution change over the buffer concentration range tested was approximately 0.7 with resolution decreasing below 5 mM or above 10 mM (Figure 6). The effect of buffer concentration on column plate number peaked at an approximate concentration range from 10 to 20 mM. The number of theoretical plates quickly decreased when the buffer

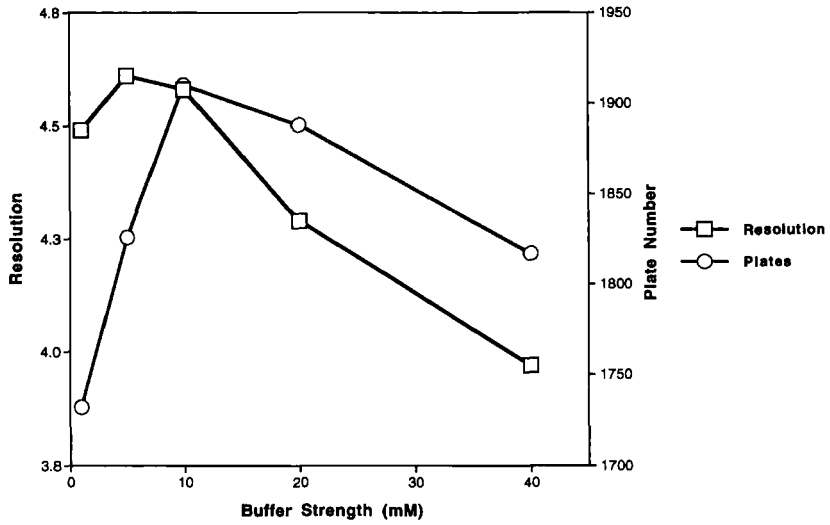

Figure 6. The effect of KH_2PO_4 buffer strength on the resolution of seproxetine and R-norfluoxetine and on the plate numbers for R-norfluoxetine. Organic mobile phase component: 6% methanol.

strength drops below 10 mM (Figure 6). Likewise, peak shape deteriorated and tailing increased at buffer strengths below 5 mM. Capacity factor values decreased with increasing buffer concentration, and therefore buffer strength can be used for retention control. Reasonable K' values were seen throughout the range tested. The large resolution value for the separation of seproxetine from R-norfluoxetine minimized the importance of varying buffer concentration. Such high resolutions may not always be obtained, therefore this parameter could be used to optimize separations at or near baseline resolution. A 20 mM KH_2PO_4 buffer concentration for this analysis was used due to a reasonable K', good peak shape and a more rugged working range.

Temperature

Holding all other parameters constant, a mixture of seproxetine and R-norfluoxetine was injected onto the ES-Pepsin column conditioned at temperatures ranging from 10°C to 35°C in increments of 5°C. The resolution and theoretical plates increased with increasing temperature as illustrated in Figure 7. The resolution only changed about 0.4 units over the range tested.

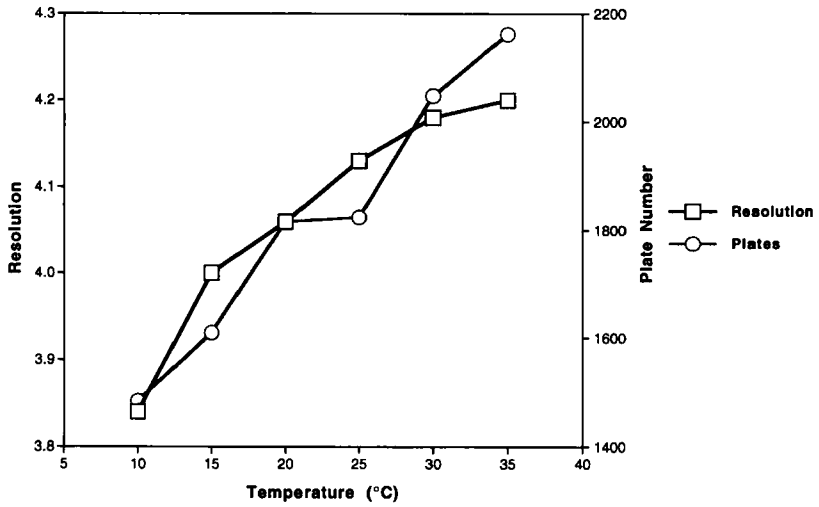

Figure 7. The effect of temperature on the resolution of seproxetine and R-norfluoxetine and on the plate numbers for R-norfluoxetine. Mobile phase: 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer.

Column temperature was shown to have a greater impact on theoretical plate values than either mobile phase composition or buffer strength. Both peak tailing and K' values decreased linearly with increasing column temperature. In general, higher temperature provided better chromatography, however, the results did not account for the potential of decreased column life at prolonged elevated temperatures. Although there was an advantage in using an elevated column temperature, this was outweighed by the need to obtain a simple and rugged analytical method. If resolution or other factors were not adequate, altering the column temperature was another parameter that could be used for affecting method optimization. For the purposes of optimizing a method for the enantiomeric analysis of seproxetine and R-norfluoxetine, ambient conditions were selected.

Flow Rate

A sample mixture of seproxetine and R-norfluoxetine was injected at different flow rates ranging from 0.2 mL/min. to 1.0 mL/min. All other parameters described earlier were held constant during this analysis. As expected, the resolution and theoretical plate numbers decreased with

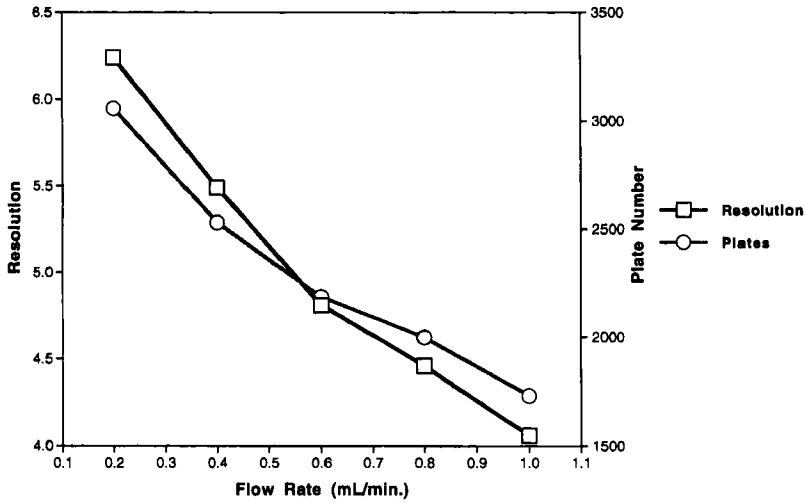

Figure 8. The effect of flow rate on the resolution of seproxetine and R-norfluoxetine and on the plate numbers for R-norfluoxetine. Mobile phase: 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer.

increasing flow rate as illustrated in Figure 8. Although a lower flow rate would yield better resolution, the tradeoff was sample analysis time; at a flow rate of 0.2 mL/min. the seproxetine peak eluted at 95 minutes. In the case of these enantiomers, the excellent resolution obtained under various conditions allowed for the higher flow rate of 1.0 mL/min. in light of a quicker analysis time.

Sample Loading

Consistent with other protein-based columns, the ES-Pepsin column has a relatively low sample loading capacity. The recommended analyte injection is 1 μg on column. Sample loading was tested by injecting five samples of the enantiomers from 1 μg to 6 μg onto the column. As the analyte concentration was increased over this range, resolution decreased by 70%, tailing approximately doubled and the number of theoretical plates decreased by 30%. Based on experimental data for seproxetine, a maximum sample loading of 1.5 μg on the ES-Pepsin column was the limit before resolution, tailing and plate number began deteriorating.

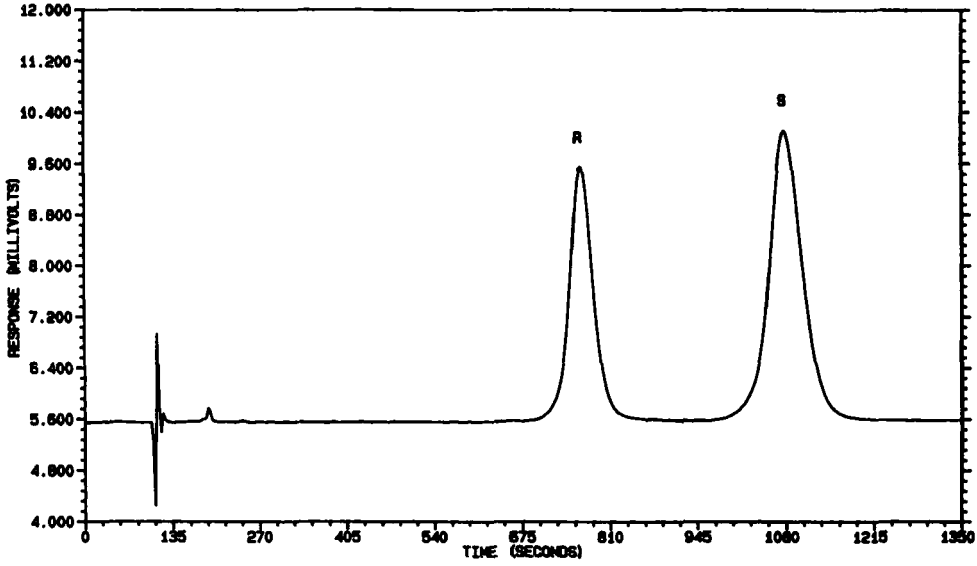

Figure 9. A sample chromatogram of the separation of seproxetine and R-norfluoxetine. Mobile phase: 6:94 (v/v) methanol /20 mM KH_2PO_4 buffer; detection: UV absorbance, 225 nm; flow rate: 1.0 mL/min.; injection volume: 10 µL; sample: 0.05 mg/mL of a 3:2 mixture of seproxetine:R-norfluoxetine; temperature: ambient. Peak identification: (R) = R-norfluoxetine, (S) = seproxetine.

METHOD VALIDATION

HPLC Conditions

The mobile phase was comprised 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer. A flow rate of 1.0 mL/min., UV detection at 225 nm and sample injections of 10 µL were used with the Ultron ES-Pepsin column. A sample chromatogram of a 0.05 mg/mL solution (3:2; seproxetine:R-norfluoxetine) is shown in Figure 9.

Linearity

It is usual practice to perform linearity determinations over a wide range of sample concentrations to fully assess the linear dynamic range of the

detection system. The linearity of the method was determined by injecting 20 samples prepared from seproxetine hydrochloride and R-norfluoxetine hydrochloride which were serial dilutions from stock solutions of these compounds. The samples encompassed a range of 0.01 - 406 μg/mL for each of seproxetine and R-norfluoxetine. The linear working range for the method was determined to be 0.1 - 150 μg/mL. This range included 16 samples and resulted in a correlation coefficient of 0.9999 for both seproxetine and R-norfluoxetine.

Precision

The precision of the method was evaluated in two ways. First, ten replicate injections of the same sample were injected to determine the reproducibility of the method apart from analyst error. Second, ten separate sample preparations were injected singly to determine the overall precision of the method. Approximately 11.5 mg from a bulk drug substance lot of seproxetine hydrochloride was transferred into respective 100 mL volumetric flasks and diluted to volume with 20 mM KH_2PO_4 buffer resulting in the target nominal concentration of 0.1 mg/mL of seproxetine. The samples were quantitated using a peak versus total peak area approach. The samples averaged 0.9% R-norfluoxetine in the validation lot with a 2.3% relative standard deviation (RSD) for the ten replicate injections and a 2.7% RSD for the ten separate sample preparations.

Selectivity

As part of the USP guidelines for validation, a method must be proven to be selective for the analyte of interest. For this method selectivity was assessed by separating seproxetine from R-norfluoxetine and three degradation products: p-trifluoromethylphenol, 3-phenyl-3-hydroxypropylamine and 3-amino-1-phenyl-1-propene. The degradation pathways have previously been reported.[20] The relative retention times for 3-phenyl-3-hydroxypropylamine, p-trifluoromethylphenol, 3-amino-1-phenyl-1-propene and R-norfluoxetine as compared to seproxetine were 0.15, 0.21, 0.22 and 0.71, respectively.

Surprisingly, the enantiomers from a racemic mixture of fluoxetine hydrochloride could not be separated using any of the conditions tested in this report. The main metabolite of fluoxetine is norfluoxetine which is identical in

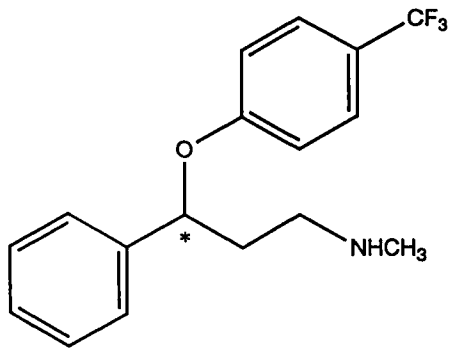

Figure 10. Structure of fluoxetine.

structure to fluoxetine except it is demethylated. The structure of fluoxetine is shown in Figure 10. Fluoxetine had a relative retention of 0.85 as compared to seproxetine.

Recovery

The recovery was determined by a standard addition technique whereby three separate preparations of two seproxetine hydrochloride bulk drug substance lots containing 0.1% and 0.9% of R-norfluoxetine initially were spiked with an additional 1% of R-norfluoxetine from a stock solution of this enantiomer. The average percent recovery for these two sample lots were 91.5% and 101.1%, respectively.

Limit of Detection/Quantitation

The Limit of Detection (LOD) can be defined as the lowest concentration of sample that can be clearly detected above baseline noise. Typically this value is three times the level of baseline noise. The LOD for this method was determined to be 0.05 μg/mL. The limit of quantitation (LOQ) might be estimated as three times the LOD or can be determined from the linearity validation experiments. For this method the LOQ was determined experimentally to be 0.1 μg/mL. When using a nominal sample concentration of 0.1 mg/mL of seproxetine, quantitation of R-norfluoxetine can be achieved at a level of 0.1%.

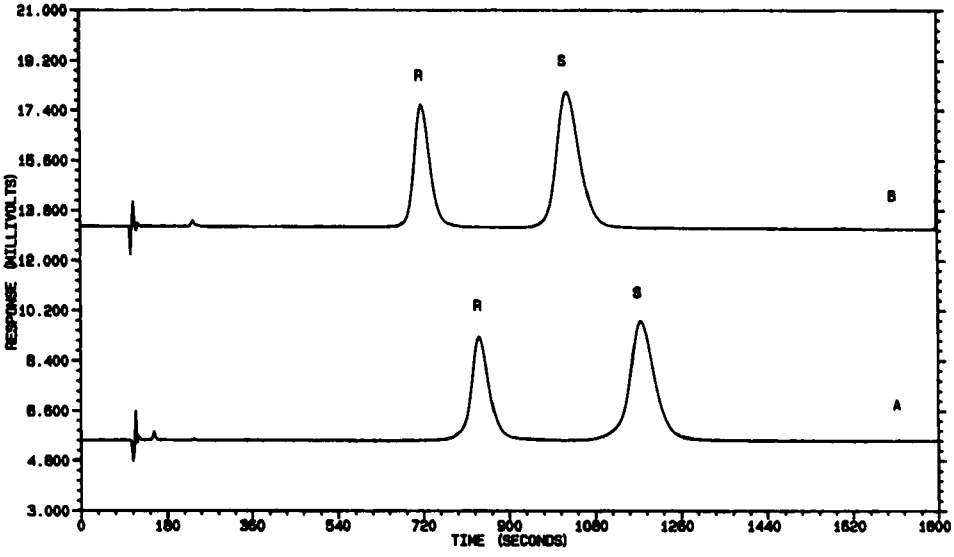

Figure 11. Sample chromatograms for the separation of seproxetine and R-norfluoxetine using two different batch numbers of the Ultron ES-Pepsin column with identical instrument and chromatographic conditions: (A) Ultron ES-Pepsin column with > 250 injections, lot # 2061023 and (B) Ultron ES-Pepsin column with < 10 injections, lot # 2061030. Mobile phase: 6:94 (v/v) methanol/20 mM KH_2PO_4 buffer; detection: UV absorbance, 225 nm; flow rate: 1.0 mL/min.; injection volume: 10 µL; sample: 0.05 mg/mL of a 3:2 mixture of seproxetine:R-norfluoxetine; temperature: ambient. Peak identification: (R) = R-norfluoxetine, (S) = seproxetine.

Ruggedness

The effects of the key mobile phase parameters (pH, flow rate, buffer strength and organic concentration), column temperature and sample loading have been discussed in the method optimization section (Figures 2-8). Although the excellent separation of seproxetine and R-norfluoxetine was easily achieved, these parameters were also important for maintaining consistency of the separation over long-term use of the method.

In addition to studying these effects, another ES-Pepsin column, from a different batch number, was compared to the column used for the optimization and validation. Figure 11 shows a comparison of the chromatograms generated by the two columns using identical conditions. The favorable comparison of

the two chromatograms in Figure 11 is a good indication of the ruggedness of the method for long-term use.

CONCLUSION

The Ultron ES-Pepsin column has been demonstrated to be an excellent CSP for the separation of seproxetine and R-norfluoxetine. The column was shown to be compatible with several different organic modifiers and a relatively wide range of other key mobile phase parameters (pH, buffer strength and organic concentration).

Although this report focused on the separation of seproxetine and R-norfluoxetine, we have separated other basic chiral drug substances using this column, thus the ES-Pepsin column is likely to be applicable to the separation of many other basic chiral compounds.

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