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# Determination of fluoxetine hydrochloride enantiomeric excess using high-performance liquid chromatography with chiral stationary phases

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**Abstract**

Chromatographic methods using chiral stationary phases have been developed for the separation of fluoxetine hydrochloride enantiomers. Ovomucoid and tris(3,5-dimethylphenyl carbamate) cellulose stationary phases were used in the reversed- and normal-phase modes, respectively. Acceptable isomer separation was achieved at pH 3.5 with the ovomucoid phase, but peak shapes were broad and the separation was quite sensitive to the acetonitrile concentration in the mobile phase. Isopropyl alcohol and methyl-*tert*-butyl ether mobile phase modifiers each provided complete resolution using the derivatized cellulose column. Better separation robustness was obtained with a column temperature of 1°C using the isopropyl alcohol modifier. The methyl-*tert*-butyl ether system was robust at room temperature. Differences in relative enantiomer amounts of as little as 2% could be determined. The chromatographic conditions provided a much more discriminating test compared to an optical rotation method proposed for pharmacopeial use which had difficulty distinguishing individual enantiomers. The chiral chromatographic conditions were also applied to capsule formulations to demonstrate the presence of racemic fluoxetine hydrochloride. 

*Keywords:* Chiral separation; Chiral stationary phase; Derivatized cellulose; Fluoxetine hydrochloride; High-performance liquid chromatography; Ovomucoid

## 1. Introduction

Fluoxetine hydrochloride (Fig. 1) is a selective serotonin-reuptake inhibitor used for the treatment of depression and obsessive–compulsive disorders. The drug is used as the racemate, but the individual isomers do not have identical activity [1]. Since clinical and safety data have been established for the racemic drug, there is a quality control need to demonstrate that each batch of drug substance is indeed racemic and has not been enriched in either enantiomer. A control test might also be used to supplement identity tests to distinguish a racemate from samples of either pure enantiomer. Methods to determine enantiomeric enrichment would be useful in synthetic or preparative separation studies aimed at pro-

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ducing individual isomers. The European Pharmacopoeia has recently proposed an optical rotation test for enantiomeric enrichment in fluoxetine hydrochloride [2]; however, the very low rotations of the individual isomers in the solvent chosen for analysis render the test inadequate under most circumstances.

Several chromatographic methods have been published for the determination of fluoxetine enantiomers. Most methods have also included norfluoxetine, the desmethyl active metabolite of fluoxetine. Indirect methods include derivatization with *R*-napthylethyl isocyanate [3,4] or *R*-(−)-mandelic acid [5] followed by normal-phase liquid chromatographic separation of the resulting diastereomers. Gas chromatographic separation of diastereomers after derivatization with *S*-(−)-*N*-trifluoroacetylprolyl chloride has also been employed [6,7]. Piperaki et al. have studied a $\beta$-cyclodextrin chiral stationary phase (CSP) for direct separation of fluoxetine enantiomers [8,9]. Nearly complete resolution was achieved with retention times of 26–30 min. Investigations using capillary electrophoresis with cyclodextrins [10,11] or malto-oligosaccharides [12] as chiral selectors have also been reported.

Direct enantiomer separations using protein-based [13] or derivatized cellulose [14] CSPs have become quite common, but no methods for fluoxetine hydrochloride using these columns have been reported. Risley and Sharp achieved excellent separation of norfluoxetine enantiomers on a pepsin CSP, but fluoxetine enantiomers were not separated under any of the conditions investigated [15].

In this paper, conditions for the direct separation of fluoxetine enantiomers using an ovomucoid CSP or a tris(3,5-dimethylphenyl carbamate) cellulose CSP are reported. The effects of temperature and mobile phase modifiers on the separation using the cellulose-based column were also investigated. The conditions described have the advantage of not requiring sample derivatization and achieve better than baseline resolution in a shorter analysis time than high-performance liquid chromatographic (HPLC) conditions employing a $\beta$-cyclodextrin CSP [8,9]. The option of normal-phase or reversed-phase conditions provides a laboratory with flexibility in mode of separation as well as an alternative to cyclodextrin capillary electrophoresis. Chiral HPLC analysis is demonstrated as a much more discriminating method than optical rotation for assuring the racemic nature of fluoxetine hydrochloride drug substance or for investigations involving enantiomeric enrichment. Analysis of capsule formulations is also described.

## 2. Experimental

### 2.1. Reagents

HPLC-grade isopropyl alcohol, hexane, and acetonitrile were obtained from EM Science (Gibbstown, NJ). HPLC-grade methyl-*tert*-butyl ether was from Baxter (Muskegon, MI). Diethylamine from Aldrich (Milwaukee, WI) was distilled and then stored at 4°C to prevent oxidation. Water for mobile phases and sample solutions was purified with a Milli-Q system from Millipore (Milford, MA). HPLC-grade *o*-phosphoric acid (85%) was from Fisher (Fair Lawn, NJ). Samples of racemic fluoxetine hydrochloride and the individual (*R*)- and (*S*)-fluoxetine hydrochloride isomers were from Lilly Research Laboratories. Capsule formulations (20 mg strength) of fluoxetine hydrochloride from three different suppliers were analyzed.

### 2.2. Apparatus

The chromatographic system consisted of a Hitachi Model L-6200A pump (Naperville, IL), a Model 728 autoinjector (Alcott, Norcross, GA) with a fixed-loop injection valve (Valco, Houston, TX). A model 757 or 759 UV detector (Applied Biosystems, Ramsey, NJ) was used. Some chromatograms were obtained using a Model 600 pump (Waters, Bedford, MA). Chromatograms were recorded at 1 Hz and peak–area responses determined using an in-house data acquisition system based on a Hewlett-Packard HP1000 computer. Peaks were integrated manually using the graphical features of the software. A 15 cm × 4.6 mm i.d. Ultron ES-OVM, 5 μm particle size,

ovomucoid column with 1 cm guard column was from Mac-Mod (Chadds Ford, PA). The tris-(3,5-dimethylphenyl carbamate) cellulose CSP was a 25 cm × 4.6 mm i.d., 5 μm particle size, Chiracel OD-H column from Baker (Phillipsburg, NJ). Injection volumes of 10 and 20 μl were used for the ovomucoid and cellulosic columns, respectively. A flow rate of 1.0 ml $min^{-1}$ was used in all cases. The pH of mobile phase buffers was measured on the aqueous component alone. Other chromatographic conditions are described below. The polarimeter was a Model DIP-370 from Jasco (Easton, MD).

## 3. Results and discussion

### 3.1. Optical rotation

A pharmacopeial specification for an optical rotation of $-0.05°$ to $+0.05°$ from a solution of 2 g in 100 ml of 15/85 water/methanol (v/v) has been proposed [2]. Unfortunately, the choice of solvent for the test provides little discriminatory power. The (*S*) isomer of fluoxetine hydrochloride has a specific rotation of $+1.60°$ in methanol and $-10.85°$ in water [16]. The 15/85 water/methanol mixed solvent gave optical rotations of $+0.06°$ for the (*S*) isomer and $-0.04°$ for the (*R*) isomer at concentrations of 2 g/100 ml. Since the pure isomers meet or nearly meet the optical rotation limit for a racemate, the test under these conditions is meaningless. With the pharmacopeial requirement that the polarimeter must be capable of reading to the nearest 0.01°, measurement variability might also allow a pure enantiomer to meet the racemate rotation specification. Therefore, the use of optical rotation as an 'identity' test to distinguish a fluoxetine racemate from either of the individual isomers may not be possible. It would certainly not be possible to detect enrichment of either enantiomer in the presence of the other using the proposed conditions. While enantiomeric enrichment would not be expected in the synthesis of racemic fluoxetine hydrochloride, studies concerned with the individual isomers definitely require enantiomeric purity information.

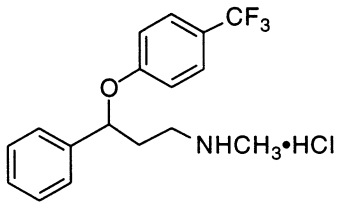


Fig. 1. Structure of fluoxetine hydrochloride.

Other solvents or a non-standard wavelength might improve the ability to determine an enantiomeric excess by optical rotation, but chiral chromatography was pursued as described below.

### 3.2. Ovomucoid CSP

An initial investigation of conditions for separation of fluoxetine isomers using an ovomucoid CSP followed the protocol suggested by Kirkland et al. [13]. Adjustment of pH and solvent strength was necessary to provide a compromise between separation of fluoxetine isomers and a reasonable run time. At pH 5.0 with 7% acetonitrile, the isomers were baseline separated but the run time was over 30 min. Increasing the solvent strength reduced the elution time, but also degraded the isomer separation, as shown in Fig. 2. The data in Fig. 2 also show that retention and separation

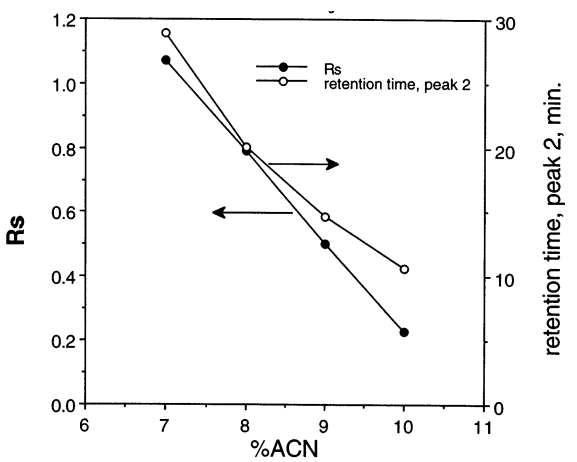


Fig. 2. Resolution and retention time of (*S*)-fluoxetine (peak 2) vs. % acetonitrile using an ovomucoid CSP. Mobile phase buffer = 10 mM phosphoric acid adjusted to pH 5.0 with 5 M KOH. Other conditions as given in Fig. 3.

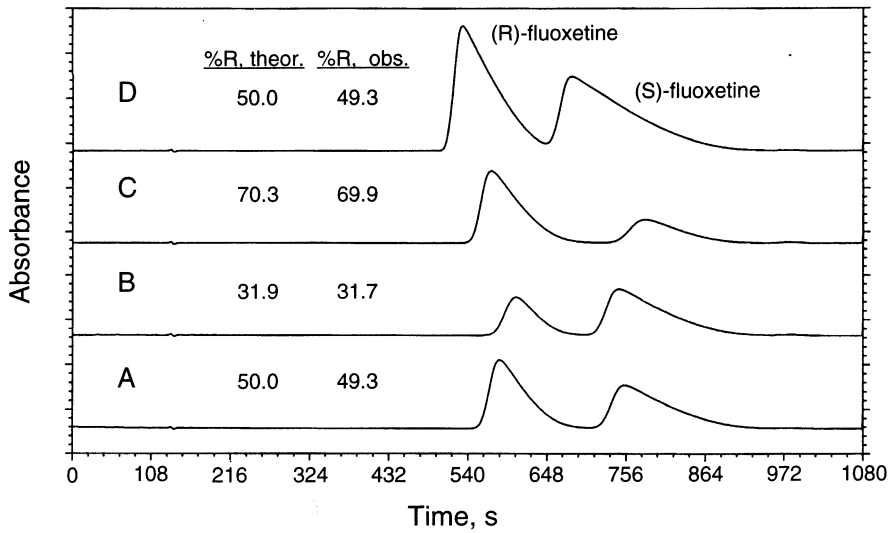


Fig. 3. Separation, recovery and effect of column loading of fluoxetine enantiomers using an ovomucoid CSP. Mobile phase and sample solvent: 98/2, 10 mM phosphoric acid, pH adjusted to 3.5 with 5 M KOH/acetonitrile (v/v). Injection volume, 10 μl; detection wavelength, 227 nm. (A) Racemate, 0.1 mg $ml^{-1}$; (B) solution enriched in (*S*)-fluoxetine; (C) solution enriched in (*R*)-fluoxetine; (D) racemate, 0.2 mg $ml^{-1}$.

with the ovomucoid CSP are very sensitive to solvent strength. Decreasing the pH to 3.5 and the solvent strength to 2% acetonitrile resulted in nearly baseline resolution with a run time of 18 min. A chromatogram under these conditions is shown in Fig. 3(A). The separation was still sensitive to solvent strength at pH 3.5 with incomplete resolution using 3% acetonitrile and coelution with 5% acetonitrile.

Although the peaks were somewhat broad and the separation was sensitive to solvent strength, these conditions could be used to determine fluoxetine isomer ratios. The relative standard deviation for determination of peak areas was 1.1% for a racemate and the linearity of area% response for solutions enriched in either isomer was demonstrated over a wide range, as shown in Fig. 4. Chromatograms showing recovery of spiked enantiomers are also given in Fig. 3(B) and (C). Retention time decreases with increasing isomer concentrations were observed, which is typical for this easily overloaded CSP. Chromatogram D in Fig. 3 shows a decrease in resolution compared to chromatogram A at half the concentration. Total isomer concentrations should be kept below 0.2 mg $ml^{-1}$ (2 μg injected) to maintain resolution. Overloading effects were not significant in solutions enriched in either enantiomer when the total isomer concentration was 0.1 mg/ml.

The sensitivity of the separation to solvent strength could present ruggedness problems for day-to-day use. The separation was reproduced, however, on three different ovomucoid columns.

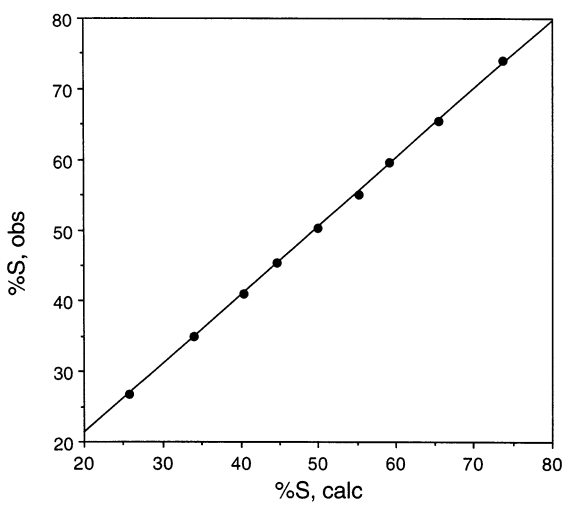


Fig. 4. Observed vs. calculated percentage of (*S*)-fluoxetine using conditions given in Fig. 3.

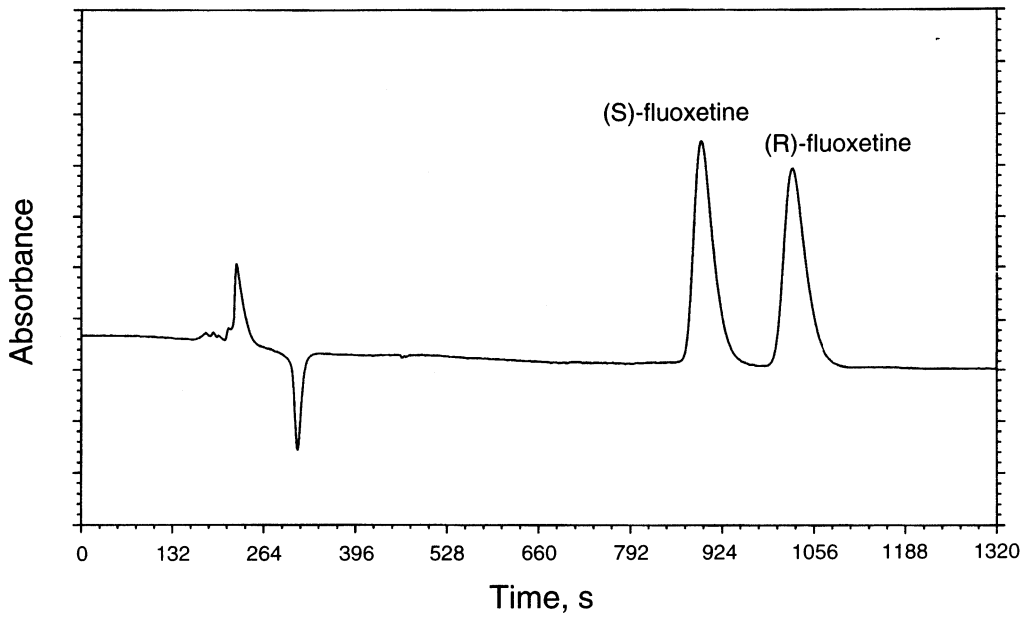


Fig. 5. Separation of fluoxetine enantiomers using a tris-(3,5-dimethylphenyl carbamate) cellulose CSP with IPA mobile phase modifier. Mobile phase: 98.5/1.5, hexane + 0.2% diethylamine/isopropyl alcohol (v/v). Sample preparation: 5 mg fluoxetine HCl dissolved in 2 ml IPA and diluted to 25 ml with hexane + 0.2% DEA. Injection volume, 20 μl; detection wavelength, 260 nm.

Ease of sample preparation is an advantage using these conditions, since fluoxetine hydrochloride is soluble in acidic aqueous solutions.

### *3.3. Tris-(3,5-dimethylphenyl carbamate) cellulose CSP*

Initial investigations using this CSP focused on a hexane mobile phase with isopropyl alcohol (IPA) as the polar modifier. Diethylamine (0.2%) was added to improve the peak shape of fluoxetine, which is a secondary amine. Mobile phase IPA concentrations less than 2% were necessary to provide adequate retention and separation of fluoxetine isomers. A chromatogram using 1.5% IPA is shown in Fig. 5. Small enantiomeric excesses of either isomer were detectable using these conditions, as shown in Fig. 6. Based on an integration variability of about 1% for the racemic mixture, an estimate of the smallest detectable difference in enantiomer content would be 2%. Larger differences would be easily detectable. These conditions were not robust, however, since the resolution of fluoxetine isomers was very sensitive to the amount of IPA in the mobile phase. In fact, reproducible separation from day to day was difficult to obtain without careful and sometimes time-consuming adjustments in the IPA content of the mobile phase.

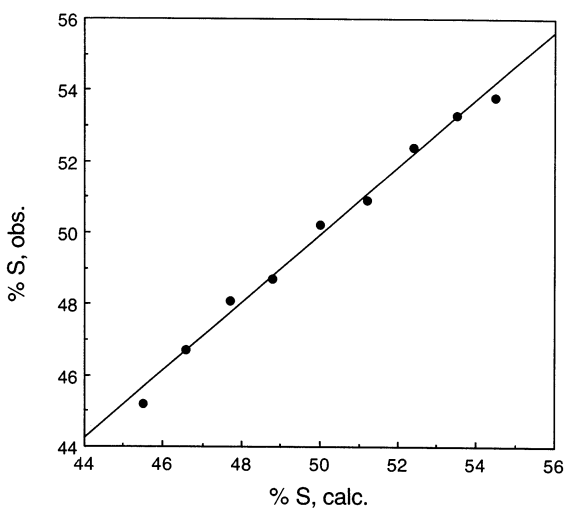


Fig. 6. Observed vs. calculated percentage of (*S*)-fluoxetine using conditions given in Fig. 5.

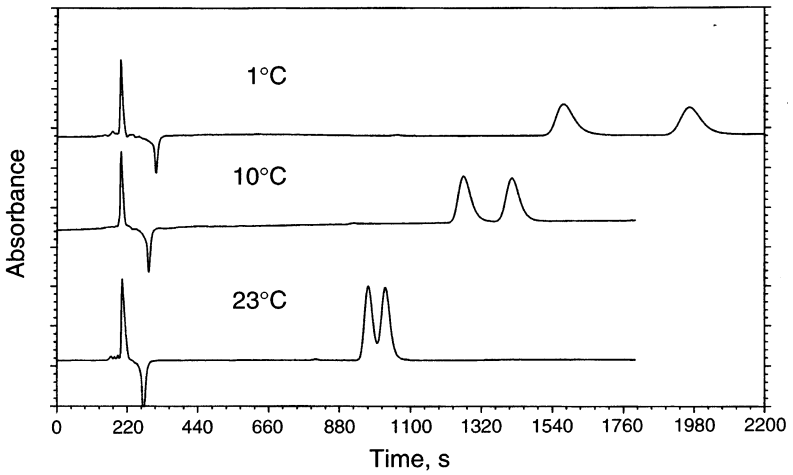


Fig. 7. Effect of temperature on fluoxetine isomer separation using a tris-(3,5-dimethylphenyl carbamate) cellulose CSP. All conditions except for column temperature are the same as given in Fig. 5.

The separation was also affected significantly by column temperature (Fig. 7) and this parameter was investigated as a way to improve robustness. The peak separation was much greater at low temperature, which allowed an increase in IPA concentration range to be used. Operating at 1°C provided a range of IPA concentrations of 2–6% over which resolution was obtained. At 23°C, changes in IPA concentration of as little as 0.5% could make a difference between complete resolution or coelution. Low-temperature operation is therefore recommended for improved robustness with a hexane/IPA mobile phase.

Recent work has shown the utility of aprotic mobile phase modifiers for optimization of separations using cellulosic CSPs [14]. Results using methyl-*t*-butyl ether (MTBE) instead of IPA are shown in Fig. 8. With MTBE, a concentration of 25% was necessary to elute fluoxetine, but good resolution over a range of 23–27% was obtained. Interestingly, the retention order using MTBE was reversed from that obtained with IPA as modifier. Fluoxetine hydrochloride was not soluble in this mobile phase and a major baseline disturbance was observed when a small amount of IPA was used to solubilize the sample. Therefore, the sample was dissolved in water and extracted into mobile phase after the addition of base. This provided an excellent baseline at the expense of increased sample preparation time. Complete recovery of spiked enantiomeric excesses was also achieved with this system (Fig. 8). Separations using the IPA or MTBE systems were each reproduced on two different tris-(3,5-dimethylphenyl carbamate) cellulose columns to demonstrate column-to-column ruggedness.

The above conditions were applied to the analysis of fluoxetine hydrochloride capsule formulations by dissolving the active ingredient from the contents of the capsule in water, filtering off insoluble excipients and then extracting the solution as before. Analysis of capsules from three different sources confirmed the presence of racemic fluoxetine hydrochloride in each case.

## 4. Conclusions

The performance of the chiral HPLC systems investigated is summarized in Table 1. The separation afforded by the tris-(3,5-dimethylphenyl carbamate) cellulose column using hexane/MTBE/DEA mobile phase was least sensitive to small changes in mobile phase composition, but required an extraction for sample preparation. All three methods are suitable for the determination

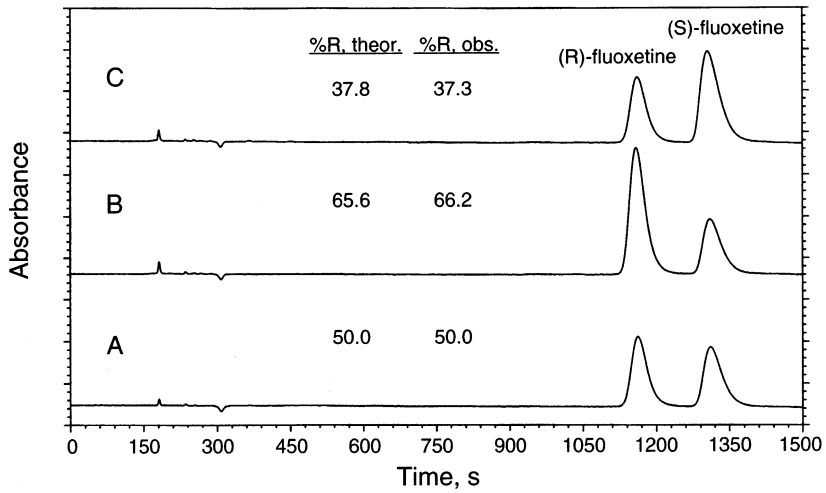


Fig. 8. Separation of fluoxetine enantiomers using a tris-(3,5-dimethylphenyl carbamate) cellulose CSP with MTBE mobile phase modifier. Mobile phase: 75/25/0.2, hexane/methyl-*t*-butyl ether/diethylamine (v/v/v). Sample preparation: 1 ml fluoxetine HCl (1 mg $ml^{-1}$ in water) extracted as free base into 5 ml of mobile phase after addition of 1 drop of 50% NaOH. Injection volume, 20 μl; detection wavelength, 260 nm. (A) racemate; (B) solution enriched in (*R*)-fluoxetine; (C) solution enriched in (*S*)-fluoxetine.

Table 1
Summary of fluoxetine hydrochloride enantiomer separations

| Column | Mobile phase | Col. temp. (°) | Resolution | Tailing factor[a] | Plates[a] | Ret. time (min)[a] |
|---|---|---|---|---|---|---|
| Ultron ES-OVM | 10 mM, pH 3.5, potassium phosphate/ACN: 98/2 | 23 | 1.4 | 2.1 | 600 | 9.7 |
| Chiracel OD-H | Hexane/IPA/DEA: 98.5/1.5/0.2 | 20 | 2.5 | 1.5 | 5040 | 14.9 |
| Chiracel OD-H | Hexane/IPA/DEA: 98.5/1.5/0.2 | 1 | 3.4 | 1.4 | 3560 | 26.3 |
| Chiracel OD-H | Hexane/MTBE/DEA: 75/25/0.2 | 23 | 2.8 | 1.5 | 4430 | 18.2 |

[a] First peak.

of enantiomeric excess in fluoxetine hydrochloride drug substance or capsule samples and are far superior to optical rotation measurements in 15/85 water/methanol. Development of appropriate sample preparation/concentration procedures and investigation of the separation of norfluoxetine enantiomers might extend the applicability of these methods to biological fluid analysis.

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