

Enantiomeric Analysis of Citalopram in Human Plasma by SPE and Chiral HPLC Method

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Citalopram is a serotonin transporter (SERT) inhibitor and is used as a potent anti-depressant drug. S-(+)-Citalopram is twice therapeutically active than its racemic mixture. The present article describes effective, selective and reproducible methods of chiral analysis of citalopram in human plasma. The sample preparation of human plasma was carried out by Solid Phase Extraction (SPE) C₁₈ cartridges with elution via methanol having 0.1% acetic acid. The chiral analysis was achieved on AmyCoat column (150 x 46 mm) of the amylase type CSP, by using n-hexane-2-propanol-DEA (95:05:0.2, v/v/v) as mobile phase. Flow rate of mobile phase was 0.5 mL/min with detection at 240 nm. The values of k [R-(-)-], k [S-(+)-], α and Rs were 3.56, 4.00, 1.12 and 1.22, respectively. Linearity was in the concentration range of 100-500 $\mu\text{g/L}$ with 10.0 ng as the detection limit. The percentage recovery by SPE was 98.00 and the validation parameters proved the precision of the method and its applicability for the determination of chiral citalopram in human plasma. S-(+)-Citalopram reacted more in human plasma than R-enantiomers.

Keywords: Citalopram, SPE-Chiral-HPLC, Enantiomeric Analysis, Human Plasma, Supra Molecular Chiral Recognition.

1. INTRODUCTION

Nowadays, a fast and complex lifestyle has compelled human beings world wide to suffer from depression and till now about 121 million people have so far been targeted [1]. The neural 5-hydroxytryptamine (5-HT) [serotonin] secreted from the presynaptic cleft of brain binds on the serotonin transporter (SERT), thus resulting into depression. Basically, SERT has a molecular structure of 12 trans-membrane α -helices (TMHs) and intracellular amino- and carboxy- terminals belonging to a family of sodium/chloride-dependent transporters, which is the major pharmacological target in the

treatment of several clinical disorders, including depression. Interaction with a low-affinity allosteric site on SERT modulates various drugs (fluoxetine, imipramine, citalopram, venlafaxine, and duloxetine) affinity at the binding site.

Among these drugs, citalopram is the best medication for depression due to its strong binding with 5-HT on SERT; by an allosteric mechanism [2]; resulting into inhibition of presynaptic reuptake of 5-HT and thereby its concentration level in the synaptic cleft is elevated. But it is a chiral molecule (Figure 1) with two enantiomers. S-(+)-Citalopram is twice therapeutically active than its racemic mixture. A search of literature indicates that enantiomers may differ in their pharmacological actions [3-5]. Therefore, the determination of enantiomeric purity is of great importance in pharmaceutical and pharmacological activities.

The US Food and Drug Administration (FDA) have issued guidelines to pharmaceutical and agrochemical industries to specify the enantiomeric purity of the optically active compounds prior to their marketing [6] and hence, the last decade has seen a rise of modern electrochemical techniques [7-53] for such purposes.

A thorough search of literature was carried out and only few papers are available on enantiomeric resolution of citalopram by HPLC on Chirobiotic V [54], acetylated 3-cyclobond [55], AGP [56], and Chiracel OD columns [57]. Our experience and literature dictate that amylose based chiral columns are very effective in enantiomeric separation of about 80 percent racemates [58]. In view of this, attempts have been made to resolve the enantiomers of citalopram on amylose chiral column under normal phase mode.

The pharmacokinetic and pharmaco-dynamic studies involving plasma profile of citalopram; with respect to time; require chiral ratio of citalopram in human plasma. For chiral analysis of citalopram in human plasma, sample preparation is an integral part and about 80 percent chromatographers are using Solid Phase Extraction (SPE) as the sample preparation method in various biological samples [59].

Literature indicates few papers on solid phase extraction of citalopram in human plasma [60-63]. These methods have certain limitations such as time consuming, poor selective and less efficient. In view of all these facts, attempts have been made to develop fast, inexpensive, selective and reproducible SPE-Chiral-HPLC methods for enantiomeric resolution of citalopram (Figure 1) in human plasma. The results of these findings are discussed herein.

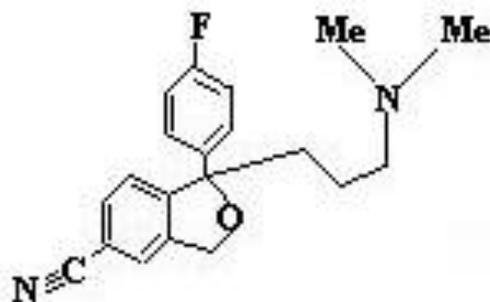


Figure 1. The chemical structure of citalopram

2. EXPERIMENTAL

2.1 Chemicals, Reagents and Instruments

The racemic and optically active samples of citalopram were obtained from Sigma Chemical Co., USA. The solutions of these samples (1.0 µg/mL) were prepared in LiChrosolve methanol. Methanol, n-hexane, 2-propanol, diethyl amine (DEA) and acetic acid of HPLC grade were purchased from Fisher Scientific (Fairlawn, New Jersey, USA).

Phosphoric acid and sodium phosphate (Na₂HPO₄) of A.R. grade were obtained from Merck India. Purified water was prepared using a Millipore Milli-Q (Bedford, M.A., U.S.A.) water purification system. C₁₈ Sep-Pak Classic (short body) cartridge was obtained from Waters, USA. pH was recorded with a pH meter (model 611, Orion Research Inc., USA). All HPLC experiments were carried out on an HPLC system of ECOM (Czech Republic) consisting of solvent delivery pump (model Alpha 10), manual injector, absorbance detector (Sapphire 600 UV-Vis.), Chromatography I/F module data integrator (Indtech. Instrument, India) and Winchrome software. Chiral column used was AmyCoat (150 x 46 mm, 3 µm silica particle size); a gift from Kromasil (Eka Chemicals Separation Products, Bohus, Sweden). The centrifuge machine used was of Remi, India (model C854/49/06).

2.2 Chiral-HPLC

The stock solutions (1.0 µg/mL) of the racemic, S-(+) and R-(-) citalopram used in this study were prepared in LiChrosolve methanol. An aliquot of 5.0 µL of each solution was injected on to a HPLC system described above.

The mobile phases used in this study was n-hexane-2-propanol-DEA (95:05:0.2, v/v/v) at 0.5 mL/min flow rate. The mobile phase was filtered and degassed before use daily. The separations were carried out at room temperature with detection at 240 nm.

The order of elution of the enantiomers was confirmed by using optically active pure enantiomers. The chromatographic parameters such as capacity (k), separation (α) and resolution (Rs) factors were calculated.

2.3 Solid Phase Extraction

5.0 µL solutions of racemic mixture and each enantiomers of citalopram (1.0 µg/mL) were mixed with 2.0 mL human plasma, separately and respectively. These samples were vortexed for 5.0 minutes and kept for 1 h followed by addition of 5.0 mL acetone, separately. These samples were centrifuged for 15.0 minutes at 10000 rpm.

The clear supernatants were separated and evaporated to dryness under nitrogen atmosphere. The residues were dissolved in 5.0 mL of phosphate buffer (25.0 mM, pH 3.0) and loaded on C₁₈ cartridges at 0.5 mL/min flow rate; pre-conditioned. Cartridges were washed with 2.0 mL of deionised water and dried with warm air.

The racemic and optically active citalopram were eluted from cartridges by methanol (5.0 mL) containing 0.10% acetic acid at 0.5 mL/min flow rate, separately and respectively. The eluents were dried under vacuum up to 0.10 mL and used for enantiomeric resolution by chiral-HPLC.

3. RESULTS AND DISCUSSION

3.1 Chiral HPLC

The capacity (k), separation (α) and resolution (R_s) factors for the resolved enantiomers of citalopram were calculated and given in Table 1, which shows the base line and successful resolution of citalopram on AmyCoat column (150 x 46 mm) column of Kromasil, Sweden. The typical chromatograms of the resolved citalopram enantiomers are shown in Figure 2 and 3 for standard solution and extracted from human plasma, respectively. It has been observed that the R-(-)-enantiomer eluted first followed by S-(+)-enantiomer. The values of k [R-(-)-], k [S-(+)-], α and R_s were 3.56, 4.00, 1.12 and 1.22, respectively. Linearity was in the concentration range of 100-500 $\mu\text{g/L}$ with 10 ng as the limit of detection. A comparison of Figure 2 and 3 indicates that the peaks area of Figure 3 is less than Figure 2. It may be due to some interactions of both enantiomers with plasma proteins. Furthermore, it is very interesting to observe that the peak of S-(+)-enantiomer is smaller than R-(-)-enantiomers, which is due to higher reactivity of S-(+)-enantiomer in comparison to R-(-)-antipode. A variation in the chromatographic parameters was carried out to obtain the best resolution. To optimize the chromatographic conditions, various ratios of 2-propanol and diethyl amine were tested. As a result of extensive experiments the optimized chromatographic conditions were developed and reported herein. The effect of percent variation of 2-propanol on R_s and α of citalopram enantiomers is shown in Figure 4. A perusal of this Figure indicates that the best percentage of 2-propanol as 5%. Low and high percentages of 2-propanol were resulted into lower values of R_s and α than 1.0 indicating poor resolution. The variation of diethylamine was also carried out but no remarkable change in the values of R_s and α was observed.

Table 1. The retention times (t_r) capacity (k), separation (α) and resolution (R_s) factors for the enantiomeric resolution of citalopram on AmyCoat column (150 x 46 mm).

Citalopram Enantiomers	t_r	k	α	R_s
Standard Sample				
R-(-)-Enantiomer	19.21	3.56		
S-(+)-Enantiomer	21.09	4.00	1.12	1.22
Plasma Sample				
R-(-)-Enantiomer	19.20	3.55		
S-(+)-Enantiomer	21.08	4.00	1.12	1.22

For details see experimental section.

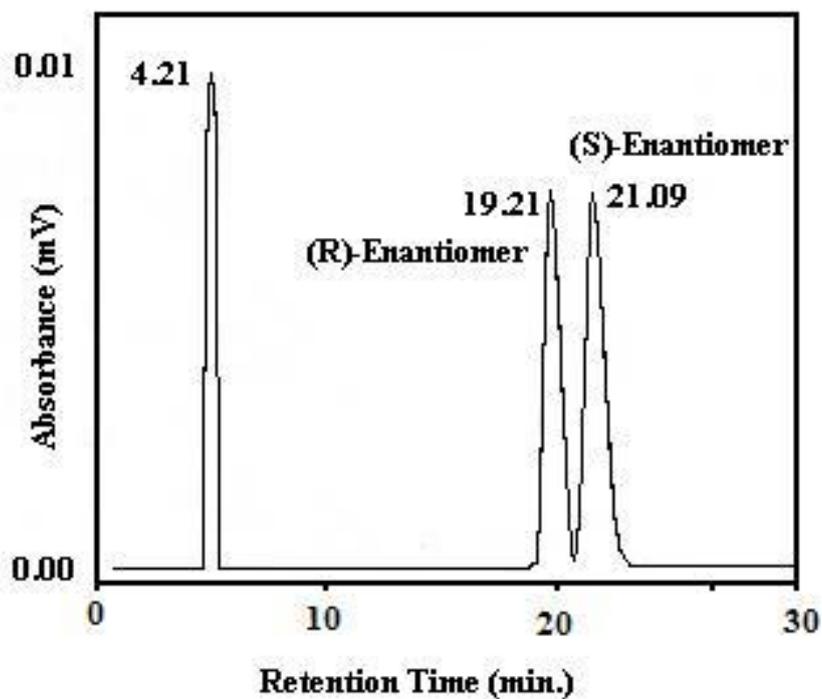


Figure 2. Chromatogram showing the enantiomeric resolution of citalopram (standard solution) Experimental conditions as given in text

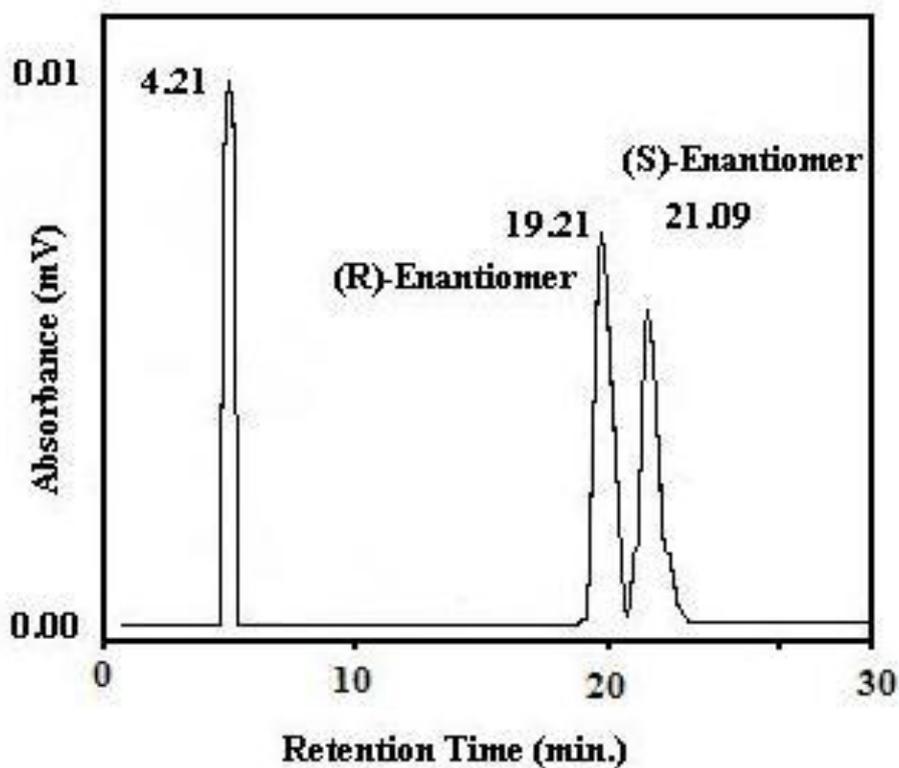


Figure 3. Chromatogram showing the enantiomeric resolution of citalopram (in human plasma) Experimental conditions as given in text.

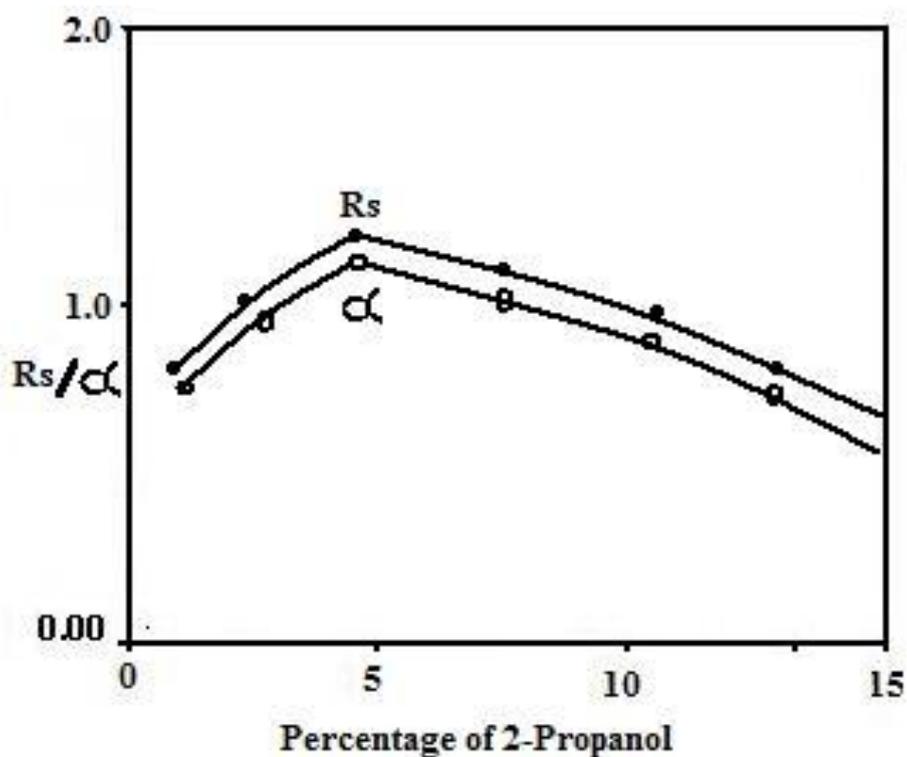


Figure 4. The effect of 2-propanol on R_s and α value

3.2 Chiral Recognition Mechanism

Better resolution capacity of amylose may be attributed to the fact that the amylose CSP is more helical in nature and possesses well defined grooves, making it different from the corresponding cellulose analogues, which appeared to be more linear and rigid in nature [64]. Therefore, amylose provides better chiral environment to the citalopram.

The chiral recognition mechanism at a molecular level on amylose based CSP is still unclear although it has been reported that the chiral resolution by this CSP is achieved through the different bondings such as π - π , hydrogen and dipole-dipole induced interactions, van der Waal forces and steric effects between the chiral stationary phase and the enantiomers [65-67]. The structure of this drug (Figure 1) contains aromatic rings, electronegative atoms *viz.* fluorine and oxygen. Therefore, the resolution of the enantiomers of citalopram was achieved through π - π interactions, hydrogen bonding and dipole-dipole induced interactions of the enantiomers with CSP. Besides, the contribution of steric effect in chiral resolution has also been reported [68, 69]. Accordingly, the enantiomers of the reported drug fit stereogenically in the different fashion into the chiral grooves of the stationary phase, which are stabilized by these interactions of different magnitudes and, hence, the resolution of enantiomers occurred [70].

Attempts have been made to explain the chiral recognition mechanisms of citalopram at supra-molecular level by developing models as shown in Figure 5. Such models have already been observed in literature for this purpose [71, 72-91].

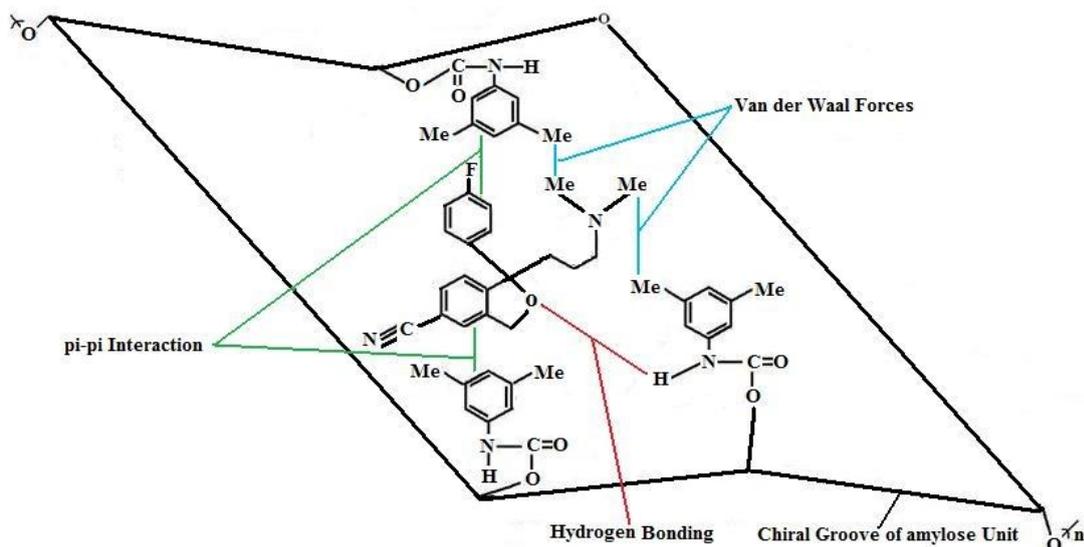


Figure 5. Supra molecular chiral recognition model of citalopram showing various interactions

3.3 Solid Phase Extraction

To achieve the maximum percentage recoveries of citalopram enantiomers various SPE parameters were optimized. The varied variables were flow rates of buffer containing citalopram enantiomers and eluting solvents, pH and ion strength of buffer, selection of eluting solvents and addition of organic acids in eluting solvents. Besides, various types of SPE cartridges were also used for this purpose. The best SPE conditions were phosphate buffer (25 mM, pH 3.0) (containing drug enantiomers) with 0.5 mL/min as the flow rate. The drug enantiomers eluted with 5.0 mL methanol containing 0.10% acetic acid at 0.5 mL/min flow rate. The flow rates of buffer and methanol (0.1 mL/min.) were also optimized who gave 98.5% recoveries but they consumed more time. The other solvents tried were ethanol, acetone, ethyl acetate and dichloromethane but the best results were obtained with methanol only. The percentage recoveries by SPE for these enantiomers were 98.00 percent.

3.4. Validation of the methods

The validation of the reported method was carried out by running five sets ($n = 5$) of SPE and Chiral-HPLC procedures under identical conditions. The regression analysis was carried out using Microsoft Excel program and the results are given in Table 2 which indicates that the standard deviation (SD) was ± 0.10 for enantiomeric resolution and peak areas respectively. The correlation coefficient (R^2) and confidence levels for chiral-HPLC method were 0.9999 and 99.0% respectively. Similarly, the standard deviation for SPE experiment was ± 0.11 while the values of correlation coefficient and confidence levels were 0.9999 and 99% respectively. The linearity range was from 10 to 500 $\mu\text{g/L}$ and the correlation coefficients for calibration curves were higher than 0.999 as

determined by least square analysis. The detection limit for the developed method was 10 ng for both enantiomers. The inter and intra days (7 days) assays analyses were also carried out, which indicated no deviation from the reported results showing the stability of this drug under the reported chromatographic conditions.

Table 2. Regression analyses data for enantiomeric analysis of citalopram enantiomers in human plasma.

Rs			PA			SPE (% Recovery)		
SD	CC	CL(%)	SD	CC	CL(%)	SD	CC	CL(%)
R-(+)-Enantiomer								
±0.10	0.9999	99.00	±0.10	0.9999	99.00	±0.11	0.9999	99.00
S-(+)-Enantiomer								
±0.10	0.9999	99.00	±0.11	0.9999	99.00	±0.11	0.9999	99.00

CC: Correlation coefficient, CL: Confidence level, PA: Peak area, SD: standard deviation and SPE: Solid phase extraction. n = 5

4. CONCLUSION

A successful chiral resolution of citalopram on amylase based CSP was achieved under normal phase mode. Taking into the consideration the results obtained, it is concluded that the enantiomeric resolution of citalopram on amylase column is governed by π - π interactions, hydrogen bondings, and dipole induced dipole interactions and steric. The percentage recoveries of (+)- and (-)-enantiomers in SPE using C₁₈ cartridge were 98.00 percent, respectively. The reported SPE-Chiral-HPLC system is simple, efficient and reproducible and can be used for the enantiomeric resolution of citalopram in any biological sample. The developed methodologies are also useful for studying chiral pharmacokinetic and dynamic studies.

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