Development and Validation of a Chiral HPLC Method for Quantitative Analysis of Enantiomeric Escitalopram

Asma Rahman[1,2], Mohammad Rashedul Haque[1], M. Muhibur Rahman[3] and Mohammad A. Rashid[1]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh
[2]Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka-1000, Bangladesh
[3]Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh

(Received: July 08, 2017; Accepted: September 03, 2017; Published (web): December 23, 2017))

ABSTRACT: In the present study a rapid, accurate and precise chiral HPLC method was developed and validated for enantiomeric separation of racemate citalopram and escitalopram according to the guidelines of United States of Pharmacopeia (USP) and International Conference on Harmonization (ICH). The chiral chromatographic separation was achieved with ammonium acetate/ ethanol/ 2-propanol/ methylene dichloride (100 : 150 : 70 : 30, v/v) at a flow rate of 0.5 ml/min using a chiral CD-PH column. The HPLC analyses were monitored at 254 nm. The method showed a good linearity with regression coefficient (r^2) of 0.998 in the range of 20.0-70.0 µg/ml for escitalopram. The detection limit (LOD), quantitation limit (LOQ) and average percentage of recovery for escitalopram were found to be 2.54, 7.68 µg/ml and 100.28% to 102.86%, respectively. The percentage of relative standard deviation (%RSD) for intra- and inter- day precision were found as 0.16% and 0.09%, respectively. The established method proved as reproducible with a %RSD value of less than 2 and having the robustness within specified limit. The present study also showed the enantiomeric purity or excess (%ee) of seven pharmaceutical preparations of escitalopram. Thus the proposed chiral method can be applied for the enantiomeric purity determination of escitalopram formulations.

Key words: Escitalopram, enantiomericpurity, method development, validation.

INTRODUCTION

Citalopram (1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbo-nitrile) is a selective serotonin reuptake inhibitor used as an antidepressant drug.[1] Citalopram possesses one asymmetric carbon (Figure 1) giving a pair of enantiomers, pharmacologically effective (S)-citalopram, and (R)-citalopram which is considered to be inactive.[2] Citalopram is also applied to treat panic, anxiety and obsessive compulsive disorders of pathological laughing and crying.[3]

The United States Food and Drug Administration (US FDA) and other regulatory agencies have made it mandatory for the manufacturers to investigate each enantiomer of the chiral drug individually.[4] According to the International Conference on Harmonization (ICH) guidelines, chiral identity, enantiomeric impurity and chiral assay tests are required for product specifications.[5] Chromatographic techniques, especially by HPLC have been given priority for the separation of enantiomers during the past several decades.[6-9] Numerous book chapters and review articles have dealt with the separation of chiral drugs by HPLC methods.[10-14] Chiral HPLC provides fast and accurate methods for chiral separation, and allows on-line detection and quantitation of both mass and optical rotation of enantiomers if appropriate detection devices are used.[15,16] Now a days, chiral HPLC methods are more widely used for direct chiral separations which are also more predictable than those using chiral additives in the mobile phase.[17]

Correspondence to: Mohammad A. Rashid
Tel.: +88-02-9661900-73, Extn. 8137;
Fax: +88-02-9667222, E-mail: rashidma@du.ac.bd

However, in order to quantitative analyses of the enantiomeric composition of citalopram, various analytical methods have been developed in recent years.[18-34] Most of them are involved in the employment of HPLC or supercritical fluid chromatography (SFC), equipped with chiral stationary phases.[5-30]

This study, presents a new, rapid, accurate, precise and the suitable analytical method for seperation of citalopram and the determination of enantiomeric purity of escitalopram with the chiral column composed of silica-based phenylcarbamated β-cyclodextrin (chiral CD-PH). To the best of our knowledge, this work is the first attempt of employing phenylcarbamated-β-cyclodextrin (CD-PH) as a chiral selector for enantiomeric separation and quantitation of citalopram. Performing the significant number of methods on trial and error techniques using a large number of polar and non-polar solvent mixtures as mobile phase, a new and suitable chiral HPLC method was developed for the enantiomeric separation of citalopram. This newly developed method demonstrated excellent resolution (>2) although the two enantiomers made the separation very difficult for their similar physical and chemical properties.[35] This method has been successfully applied on the determination of the content of escitalopram along with enantopurity in the seven marketed formulations available in Bangladesh.

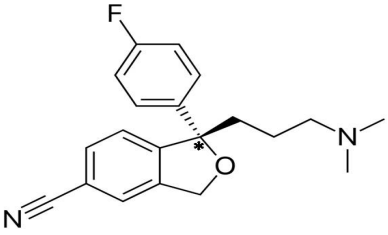

Figure 1. Structure of (R, S)-citalopram indicates chiral centre.

MATERIALS AND METHODS

Chemicals and equipment. The reference standard of citalopram and escitalopram were obtained from Incepta Pharmaceuticals Ltd., Savar, Bangladesh. Seven samples of escitalopram were purchased from local market. HPLC grade solvents like as ammonium acetate, ethanol, 2-propanol and methylene dicloride were purchased from Sigma-Aldrich, Germany.

Method development. For developing a new and easy chiral HPLC method, a large number of polar and non-polar solvent mixtures were used as mobile phase on trial and error technique using with two different chiral columns, such as Chiralcel OD-H and AGP. But good chromatographic condition was achieved on Chiral CD-PH.

Chromatographic conditions. Chromatographic condition was optimized with ammonium acetate/ ethanol/ 2-propanol/ methylene dichloride (100 : 150 : 70 : 30, v/v) at a flow rate of 0.5 ml/min on Chiral CD-PH column at 254 nm of detection. SIL 20 series Prominence HPLC (Shimadzu, Japan) equipped with an auto sampler (Model SIL-20 AC), dual pumps (Model 20 AD), column oven (Model CTO-20A), vacuum degasser (Model DGU-20A), UV-visible detector (Model SPD-20A), and LC solution software were used for the chiral analysis. The Chiral CD-PH (phenylcarbamated β-cyclodextrin) column, a silica-based chiral packing material coated by phenyl-carbamated β-cyclodextrin (CD) (250 × 4.6 mm) (Daicel Chemical Industries Ltd., Tokyo, Japan) was used. Before analysis with HPLC, all the solvents, mobile phases, standard and sample solutions were prepared by using sonication in ultrasonic bath (Ultrasons Medi-II, Spain) and filtered using 0.45 µm membrane filter tips..

Standard solutions. Five mg of escitalopram standard was weighed and transferred into a 50 ml volumetric flask and dissolved with diluent (mixture of ethanol and 2-propanol) applying sonication in an ultrasonic bath for 15 minutes. The solution was diluted with the solvent mixture up to the mark. The resulting solution had a concentration of 100 µg/ml. Dilution of this solution with appropriate volumes of the solvent mixture was carried out to obtain solutions of concentrations of 20, 35, 45, 55, and 70 µg/ml. Citalopram standard solution was also prepared to identify the enantiomers of citalopram.

Sample solutions of escitalopram. Take twenty capsules escitalopram of each of seven pharmaceutical manufacturers (code A, B, C, D, E, F, and G). Capsule powder equivalent to 5 mg of each brand was transferred to a 50 ml volumetric flask. The content was dissolved with suitable volume of diluent and sonicated for 15 minutes. Then the volume of the samples was made up with diluent and mixed well, and analyzed immediately.

Application of the method. This analytical method was applied to determine the % recovery of escitalopram commercial samples. Then the enantiomeric purity of samples were calculated by using the formula:[36-37]

enantiomeric purity = (% of major enantiomer - % of minor enantiomer).

RESULTS AND DISCUSSION

Specificity. Specificity of the test method was determined by analyzing standard substance against potential interferences. The method was found to be specific because of the absence of any interference to the test substance. Under the optimized chromatographic conditions, the chromatograms (Figure 2) confirmed the presence of (*S*)-and (*R*)-enantiomers in citalopram solution with the R_t at 11.74 ± 0.01 min and 28.32 ± 0.01 min, respectively without any interference.

System suitability.

Capacity factor (k)

Capacity factor (retention factor) is a measure of the retention time of a compound in the sample with a given combination of mobile phase and column. It is defined as k (A) = $(t_A - t_0)/ t_0$ in which t_A is the retention time of the compound and t_0 refers to retention time for an unretained compound. For an optimum separation, retention factor should be in the range of $0.5 < k < 10$. In the present work, k values for (*S*)-and (*R*)- enantiomers in citalopram were found as1.12 and4.04,respectively.

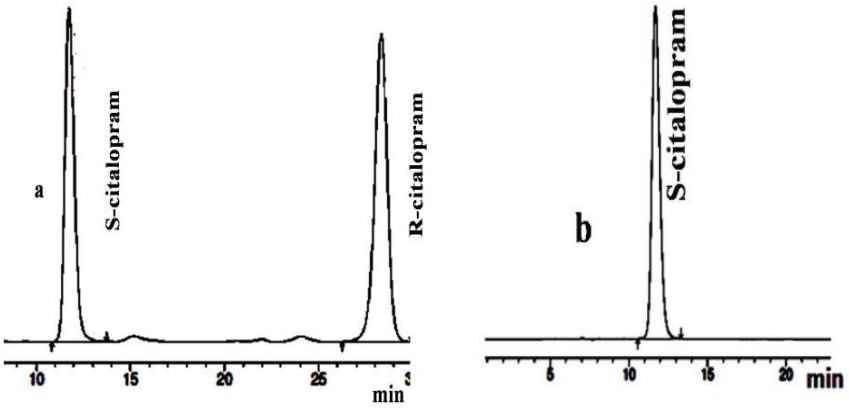

Figure 2. Chromatograms of (a) standard citalopram, and (b) standard *S*-citalopram

Selectivity factor (α)

Selectivity parameter is a measure of separation of two compounds in the sample under given conditions. For two components A and B it is defined as α = kA/kB (k is the respective capacity factor). Therefore, it is the ratio of the relative retentions of the two compounds. In the present study the selectivity parameter for separation of citalopram was found to be 3.60.

Resolution (R)

Resolution is a measure of the degree of separation between adjacent peaks. For two compounds A and B in a chromatographic run it is expressed as R = 2(tA − tB)/(wA + wB) in which tA and tB are the retention times and wA and wB refers to the width of the base of the component peaks. In this work the resolution value for separation of citalopram was 15.63.

This method also resulted in symmetric peak shape with tailing factors 1.16 and 0.91 as well as good number of theoretical plates with 23097 and 58185 for (*S*)- and (*R*)- citalopram, respectively. System suitability parameters are reported in Table 1.

Linearity and range. The calibration curve for escitalopram [(*S*)-citalopram] showed good linearity with coefficient of determination (r^2) value of 0.998 in the concentration range from 20-70 µg/ml. The linearity curve is shown in Figure 3 and the parameters are given in Table 2.

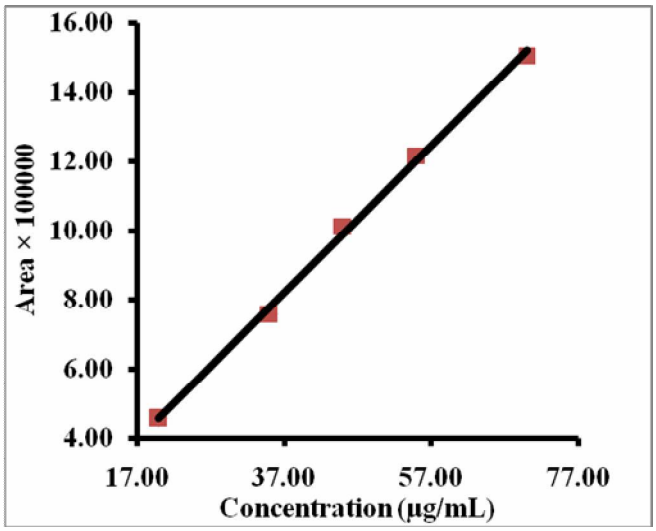

Figure **3**. Linearity curve for *S*-citalopram.

Accuracy. Accuracy of the method was examined by recovery experiments which were performed by spiking solutions of known amount of the drug with pre-analyzed sample. The data of the experiments were statistically analyzed using the formula [% Recovery = (Recovered conc. Injected conc.) x 100] to determine the recovery and the validity of the proposed method. To evaluate the accuracy of the method, successive analyses (n=3) of standard solutions of the drug were carried out. The average percentage of recovery was calculated and it was found to be 100.28% to 102.86% for (*S*)-citalopram against the concentration of 45, 60, and 65 µg/ml. All data are shown in Table 3.

Precision

Intra-dayprecision

For validation of the method repeatability was examined by determining the %RSD of a single solution of a particular concentration by injecting six times on the same day by using the formula [%RSD = (SD/Mean) × 100], where, SD = Standard deviation.

Inter-day precision

Intermediate precision was examined by determining the %RSD for a solution of single concentration by injecting three times on three different days. The results are presented in Table 4.

The intra-/inter- day precisions were found to be less than 2% for escitalopram indicated the developed method was precise.

Detection limit

The limit of detection (LOD) and the limit of quantitation (LOQ) were evaluated from the slope(s) of the calibration curve and the standard deviation (SD) of the peak areas using the following equations:

LOD = (SD/ Slope) × 3.3

LOQ = (SD / Slope) × 10. The results are shown in Table 1.

Table 1.Results of method validation parameters.

Parameters	(*S*)- citalopram
Linear equation	y = 21269x + 31568
Coefficient of determination ($r^2 > 0.995$)	0.998
Linear range	20-70 µg/ml
LOD (µg/ml)	2.54 µg/ml
LOQ (µg/ml)	7.68 µg/ml

Solution stability. The solution stability of standards and samples was established under normal bench top conditions, normal storage conditions, and sometimes in the instrument to determine where special storage conditions were necessary or not, for instance, refrigeration or protection from light. Escitalopram standard solution with concentration of 30 µg/ml was kept in a tightly capped volumetric flask at room temperature (25ºC) on the laboratory bench and at 4ºC in a refrigerator for 3days and its stability was tested. Solution stability study was carried out to calculate % RSD of area for three consecutive days at 25ºC and 4ºC. At 25ºC the value of %RSD was found to be 0.05%. At 4ºC the value of %RSD was 0.05% which demonstrated that the drug was fairly stable at normal and freezing temperatures.

Table 2. Results of system suitability parameters.

Parameters	(*S*)- citalopram	(R)- citalopram
Theoretical plates (≥2000) (n = 5)	23097	58185
Tailing factor (≤2) (n = 5)	1.16	0.91
Relative retention (k1 and k2) (n = 5)	1.12	4.04
Selectivity (α) (n = 5)	3.60	
Resolution (≥2) (n = 5)	15.63	

n = number of determinations

Table 3. Results of precision intra/inter-day validation of the method.

Precision (%RSD ≤ 2)	(*S*)- citalopram
Intra-day, n = 6	0.16%
Inter-day, n = 6	0.07%

n = number of determinations

Table 4. Accuracy of *S*-citalopram.

	Accuracy (n=3) (avg. % recovery) Standard + spike (µg/ml)	
Added concentration (µg/ml)	Recovered concentration (µg/ml)	% Recovery
45.00	46.29	102.86
60.00	60.53	100.89
65.00	65.18	100.28

Table 5. Robustness study of *S*-citalopram (n=3).

Change in flow rate (ml/min)	Average R_t of (S)- citalopram ± SD	% RSD
0.8	8.03 ± 0.01	0.12
0.6	11.74 ± 0.01	0.09
0.4	18.63 ± 0.01	0.03
Change in wavelength (nm	Average area of (S)- citalopram ± SD	% RSD
256	3107622 ± 6624.57	0.22
254	1123455 ± 1894.97	0.17
252	2658804 ± 5563.27	0.21

Table 6. Purity of escitalopram market samples.

Samples	content of *S*-citalopram	R-citalopram as impurity	purity of *S*-citalopram
A	100.00%	0.00%	100.00%
B	100.00%	0.00%	100.00%
C	100.00%	0.00%	100.00%
D	99.70%	0.30%	99.40%
E	99.98%	0.02%	99.96%
F	99.55%	0.45%	99.10%
G	99.72%	0.28%	99.44%

n=3; where, n = number of determinations,

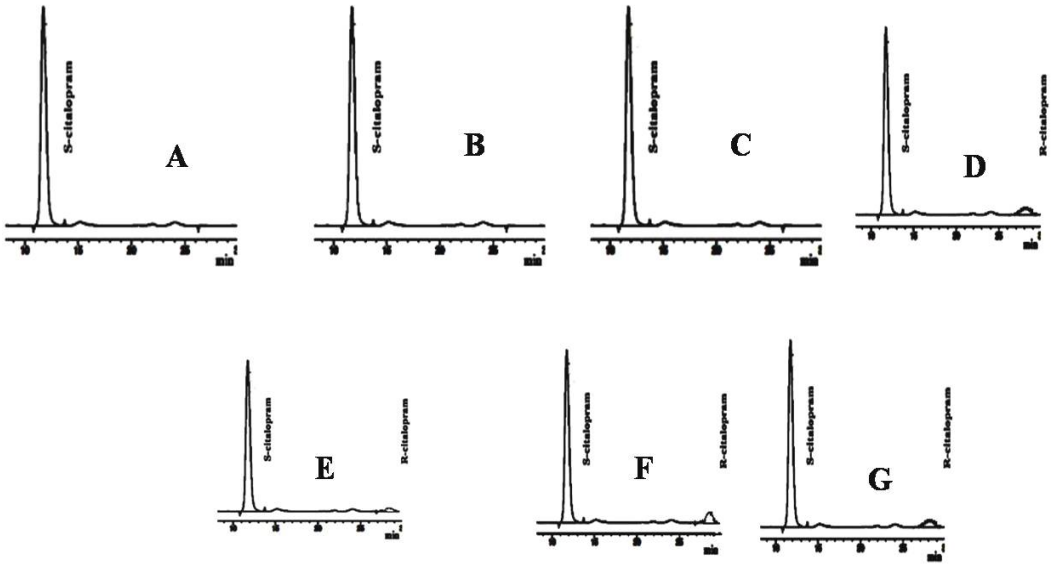

Figure 4. Chromatograms of escitalopram market preparations (A-G) used for testing.

Robustness. The robustness of the test method was carried out changing the flow rate ± 10%, *i.e.* 0.6 ml to 0.8 ml/min) and wavelength ± 2 nm, *i.e.* 254 nm to 256 nm), and n = 3. The method was found to be robust. %RSD was calculated for each variation. Values obtained are given in Table 5.

Method application. This analytical method was applied to quantitate the content of (*S*)-citalopram in samples and as well as to calculate the %purity. The average content of (*S*)-citalopram was found to vary from 99.10% to 100.00% in the formulations while the average content of (*R*)-citalopram found as impurity varied from 0.02% to 0.45%. The percent enantiomeric purity (%ee) of samples were determined. The results are recorded in table6 and all the chromatogram of samples are shown in figure 4 and table 6.

CONCLUSION

This study presented a newly developed and validated method for the enantioseparation of citalopram and determination of escitalopram in pharmaceutical preparation. The new method was found to be simple, accurate, and precise with improved resolution compared to reported methods. So the established method can be successfully applied for the routine analysis of escitalopram in pharmaceutical formulations.

ACKNOWLEDGEMENTS

The authors express their gratitude to Incepta Pharmaceuticals Ltd., Savar, Bangladesh for providing reference samples.

Conflict of Interests

The authors declared that there is no conflict of interests regarding the publication of this paper.

REFERENCES

1. Petersen, H. 1998. Method for the preparation of citalopram. WO 9819513A2.
2. Hyttel, J. and Larsen, J.J. 1985. Selective serotonin antidepressants. *Acta Pharmacol. Toxicol*. **56**,146-153.
3. Mork, A., Kreilgaard, M. and Sanchez, C. 2003. The R-enantiomer of citalopram counteracts escitalopram-induced increase in extracellular 5-HT in the frontal cortex of freely moving rats. *Neuropharmacology* **45**, 167-173.
4. Rauws, A.G. and Groen, K. 1994.Current Regulatory (draft) Guidance on chiral medicinal products: Canada, EEC, Japan, United States. *Chirality* **6**, 72-75.
5. Branch, S.K. 2005. Guidelines from the International Conference on Harmonisation (ICH). *J. Pharm. Biomed. Ana.* **38**, 798-805.
6. Bortocan, R., Lanchote, V.L., Cesarino, E.J. and Bonato, P.S. 2000. Enantioselective analysis of disopyramide and mono-N-dealkyldisopyramide in plasma and urine by high-performance liquid chromatography on an amylose-derived chiral stationary phase. *J. Chromatogr. B* **744**, 299-306.
7. Hiep, B.T., Khanh, V., Hung, N.K., Thuillier, A. and Gimenez, F. 1998. Determination of the enantiomers of chlorpheniramine and its main monodesmethyl metabolite in urine using achiral–chiral liquid chromatography. *J. Chromatogr. B* **707**, 235-240.
8. Brandsteterova, E. and Wainer, I.W. 1999. Achiral and chiral high-performance liquid chromatography of verapamil and its metabolites in serum samples. *J. Chromatogr. B* **732**, 395-404.
9. Campanero, M.A., Calahorra, B., Valle, M., Troconiz, I.F. and Honorato, J. 1999. Enantiomeric separation of tramadol and its active metabolite in human plasma by chiral high-performance liquid chromatography: application to pharmacokinetic studies. *Chirality* **11**, 272-279.
10. Vermeulen, N.P.E. and Koppele, J.M. te. 1993. Stereoselective biotransformation: toxicological consequences and implications. In Drug Stereochemistry: *Analytical Methods and Pharmacology*, 2nd Ed.; Wainer, W.I., Ed.; Marcel Dekker, Inc.: New York, 245-280.
11. Gubitz, G. and Schmid, M.G. 2001. Chiral separation by chromatographic and electromigration techniques. *Biopharm. Drug Dispos.* **22**, 291-336.
12. Allenmark, S.G. Chromatographic Enantioseparation: Methods and Applications, 2nd Ed.; Ellis Horwood Ltd: New York, 1991.
13. Taylor, D.R. and Maher, K. 1992. Chiral separations by high-performance liquid-chromatography. *J. Chromatogr. Sci.* **30**, 67-85.
14. Haginaka, J. 2002. Review: pharmaceutical and biomedical applications of enantio separations using liquid chromatographic techniques. *J. Pharm. Biomed. Anal.***27**, 357-372.
15. Boehme, W., Wagner, G. and Oehme, U. 1982. Spectrophotometric and polarimetric detectors in liquid chromatography for the determination of enantiomer ratios in. complex mixtures. *Anal. Chem*. **54**, 709-711.
16. Purdie, N. and Swallows, K.A.1989. Analytical applications of polarimetry, optical rotatory dispersion, and circular dichroism. *Anal. Chem*. **61**, 77A-89A.
17. Koppenhoefer, B., Nothdurft, A., Pierrot-Sanders, J., Piras, P., Popescu, C., Roussel, C., Stiebler, M. and Trettin, U. 1993. Chirbase, a graphical molecular database on the separation of enantiomers by liquid-, supercritical fluid-, and gas chromatography. *Chirality* **5**, 213-219.
18. Unceta, N., Aranzazu Goicolea, M. and Barrio, R. J. 2011. Analytical procedures for the determination of the selective serotonin reuptake inhibitor antidepressant citalopram and its metabolites. *Biomed. Chromatogr*. **25**, 238-257.
19. Rochat, B., Amey, M. and Baumann, P. 1995. Analysis of enantiomers of citalopram and its demethylated metabolites in plasma of depressive patients using chiral reverse-phase liquid chromatography. *Ther. Drug.Monit*. **17**, 273-279.
20. Rochat, B., Amey, M., Van Gelderen, H., Testa, B. and Baumann, P.1995. Determination of the enantiomers of citalopram, its demethylated and propionic acid metabolites in human plasma by chiral HPLC. *Chirality* **7**, 389-395.
21. Rocha, A., Marques, M. P., Coelho, E. B. and Lanchote, V. L. 2007. Enantioselective analysis of citalopram and demethylcitalopram in human and rat plasma by chiral LC-MS/MS: application to pharmacokinetics. *Chirality* **19**, 793-801.
22. Kosel, M., Eap, C. B., Amey, M. and Baumann, P. J. 1998. Analysis of enantiomers of citalopram and its demethylated metabolites using chiral liquid chromatography. *J.Chromatogr. B* **719**, 234-38.
23. MacLeod, S. L., Sudhir, P. and Wong, C. S.2007. Stereoisomer analysis of waste water-derived beta-blockers, selective serotonin reuptake inhibitors, and salbutamol by high-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **1170**, 23-33.
24. Zheng, Z. C., Jamour, M. and Klotz, U. 2000. Stereoselective HPL Cassay for citalopram and its metabolites. *Ther Drug Monit.* **22**, 219-224.
25. Geryk, R., Vozka, J., Kalıkova, K. and Tesarova, E. 2013. HPLC method for chiral separation and quantification of antidepressant citalopram and its precursor citadiol. *Chromatographia.* **76**, 483-489.
26. Haupt, D. 1996. Determination of citalopram enantiomers in human plasma by liquid chromatographic separation on a Chiral-AGP column. *J. Chromatogr*. B **685**, 299-305.
27. Bartolincic, A., Sporec, A., Druskovic, V. and Vinkovic, V. 2006. Development of practical and accurate HPLC methods for enantioselective analysis of fluoxetine and citalopram. *Chem. Anal*. **51**, 509-526.
28. Li, M., Bao, Z. B., Su, B. G., Xing, H. B., Yang, Y. W. and Ren, Q. 2013. Enantiomeric separation of citalopram base by supercritical fluid chromatography. *J. Sep. Sci.* **36**, 3093-3100.

29. Zhang, J. Y., Chen, J., Yin, C. Q., Li, S. R. and Bai, Z. W. 2009. Preparation and enantio separation of copolymer chiral stationary phases with (1R, 2R)-(+)-1, 2-dipheny-lethylenediamine as the chiral building block. *J. Liq. Chromatogr. Relat. Technol*. **33**, 46-60.
30. Chen, J., Li, M.Z., Xiao, Y.H., Chen, W., Li, S.R. and Bai, Z.W. 2011. The comparison in enantio separation ability of the chiral stationary phases with single and mixed selector-the selectors derived from two D-tartrates.*Chirality*. **23**, 228-236.
31. El-Gindy, A., Emara, S., Mesbah, M. K. and Hadad, G. M. 2006. Liquid chromatography determination of citalopram enantiomers using beta-cyclodextrin as a chiral mobile phase additive. *J. AOAC Inter.* **89**, 65-70.
32. Dixit, S. and Park, J. H. 2014. Penicillin G as a novel chiral selector in capillary electrophoresis. *J. Chromatogr. A* **1325**, 134-138,
33. Mandrioli, R., Fanali, S., Pucci,V. and. Raggi, M. A. 2003. Enantiomeric separation of citalopram and its metabolites by capillary electrophoresis. *Electrophoresis* **24**, 2608-2616.
34. Sungthong, B., Jac, P. and Scriba, G. K. E. 2008. Development and validation of a capillary electrophoresis method for the simultaneous determination of impurities of escitalopram including the *R*-enantiomer. *J. Pharm. Biomed. Anal*. **46**, 959-965.
35. Maier, N. M., Franco, P. and Linder, W. 2001. Separation of enantiomers: needs challenges, perspectives. *J. Chromatogr. A* **906**, 3-33.
36. Francis, A., Carey and Richard, J. and Sundberg, K. 2007. Advanced organic chemistry Part A: Structure and Mechanism, Plenum Publishers. 5th edition, Chapter 2,
37. http://web.chem.ucla.edu/~harding/IGOC/E/enantiomeric_excess.html (date of access May 22, 2017).
38. International Conference on Harmonisation (ICH), validation of analytical procedures: Text and Methodology Q2 (R1) in November 2005.