Simultaneous determination of escitalopram impurities including the *R*-enantiomer on a cellulose tris(3,5-dimethylphenylcarbamate)-based chiral column in reversed-phase mode

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Abstract

A high-performance liquid chromatographic method was developed for the simultaneous determination of the related substances – three potential chemical impurities from escitalopram synthesis -, and the enantiomeric purity of escitalopram. The separation capacity of seven different polysaccharide-type chiral columns, including three amylose-based (Lux Amylose-1, Lux i-Amylose-1, Lux Amylose-2) and four cellulose-based (Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3 and Lux Cellulose-4) were screened in polar organic, and reversed-phase mode. Lux Cellulose-1, based on cellulose tris(3,5-dimethylphenylcarbamate) as chiral selector with acetonitrile-water mixture containing 0.1% diethylamine was identified as the most promising system. Using "one factor at a time" optimization technique the effect of column temperature, flow rate and mobile phase constituents on separation performance was checked and the critical resolution values were determined. U-shaped retention pattern was obtained when plotting the retention factors of the citalopram enantiomers versus the water content of the binary mobile

phases on Lux Cellulose-1 column. Thermodynamic analysis revealed an enthalpy-driven enantioseparation in polar organic and in reversed-phase mode as well. For further method optimizations a L9 orthogonal array table was employed. Using the optimized parameters (Lux Cellulose-1 column with 0.1% (v/v) diethylamine in water/acetonitrile 55/45 (v/v), 0.8 mL/min flow rate at 25 °C) baseline separation were achieved between all compounds. Our newly developed HPLC method was validated according to the ICH guidelines and its application was tested on a pharmaceutical formulation and proved to be suitable for routine quality control of related substances and the enantiomeric purity of escitalopram.

Keywords: Citalopram; Lux Cellulose-1; Enantioseparation; Chemoselectivity; Chiral separation

1. Introduction

Escitalopram (*S*-1-[3-(dimethylamino)propyl]f-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile), the *S*-enantiomer of citalopram, is a selective serotonin reuptake inhibitor for the treatment of major depressive disorder or generalized anxiety disorder [1]. Escitalopram presents a greater efficacy and faster onset of action compared to the racemic drug. The lower efficacy of citalopram is due to the inhibition of the effect of the *S*-enantiomer by the *R*-enantiomer, possibly via an allosteric interaction with the serotonin transporter [1–3]. Determination of *R*-enantiomer, as chiral impurity in escitalopram samples is a regulatory requirement. Moreover, all specified chemical impurities are of interest in the analysis of escitalopram. In pharmaceutical industry as well as in pharmacopoeia separate methods are used for the quantification of achiral (chemical) and chiral related substances. However, methods that enable simultaneous quantification of both chiral and chemical impurities can save valuable time and money. Using a single chiral column in HPLC or using an appropriate chiral selector in capillary electrophoresis can unify the analysis of enantiomeric purity and related substances [4–8].

Based on a literature search several capillary electrophoretic techniques were developed for enantioselective separation of citalopram, mainly using different cyclodextrins as chiral selector [9–16]. Sunghtong et al. developed a single capillary electrophoretic method for determination of citadiol enantiomers and *R*-citalopram in *S*-citalopram samples using a dual cyclodextrin system, containing β-cyclodextrin and sulfated β-cyclodextrin [16]. Citadiol is one of the synthetic intermediate of (es)citalopram. Despite the many advantages of capillary electrophoresis direct HPLC using chiral stationary phases (CSPs) is still the golden standard in this field.

Numerous chiral HPLC methods can also be found in the literature, focusing mainly on bioanalytical applications [17–19]. Interestingly, fewer works are focused on pharmaceutical analysis and the majority of this work deals only with quantifying the enantiomeric purity of the compound [13,20,21]. Two recent articles are available which determine not only *R*-citalopram, but other in-process impurities such as citadiol or escitalopram *N*-oxide [22,23]. As it can be observed from the previous studies the applied CSPs for the enantioseparation of citalopram show a high diversity. Polysaccharide-type stationary phases (Chiralcel OD, Lux Cellulose-2, Kromasil Amycoat) [17,24,25] macrocyclic glycopeptide-type CSPs (Chirobiotic V, V2) [26,27],

cyclodextrin-type (Cyclobond I 200) [17] and protein-based chiral stationary phase (Chiral AGP) [23] were also used. Many of these earlier methods, especially those employing polysaccharide-type CSPs were performed in normal-phase chromatography, using toxic eluents, which, if possible, should be avoided in routine quality control. Therefore, an elaborated, economic, but also reliable and fast enantioselective method for the simultaneous analysis of enantiomeric impurity and chemical related compounds of escitalopram is therefore a crucial need for pharmaceutical analysis. The aim of the present study was to develop a method for simultaneous determination of *R*-citalopram, (3*RS*)-6-cyano-3-[3-(dimethylamino)propyl]-3-(4-fluorophenyl)isobenzofuran-1(3*H*)-one (IMP-1), (1*RS*)-1-(4-fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydroisobenzofuran-5-carbonitrile (desmethylcitalopram, IMP-2) and 3-[(1*RS*)-5-bromo-1-4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]-*N,N*-dimethylpropan-1-amine (IMP-3) (Figure 1) with an acceptance criteria of not more than 0.1% for each impurity. Detailed analysis of chromatographic parameters influencing the separation process, including thermodynamic analysis of enantioseparation was also studied.

2. Materials and Methods

Escitalopram oxalate and racemic citalopram HBr was ordered from Sigma-Aldrich Hungary (Budapest, Hungary). Chemical impurities such as (3*RS*)-6-cyano-3-[3-(dimethylamino)propyl]-3-(4-fluorophenyl)isobenzofuran-1(3*H*)-one, (1*RS*)-1-(4-Fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydroisobenzofuran-5-carbonitrile hydrochloride (Desmethylcitalopram Hydrochloride), as well as 3-[(1*RS*)-5-Chloro-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]-N,N-dimethylpropan-1-amine oxalate were the products of LGC GmbH (Luckenwalde, Germany). HPLC-grade MeOH, ACN, IPA and diethylamine were purchased from Merck (Darmstadt, Germany). The deionized water was prepared by a Milli-Q Direct 8 Millipore system. Lux Cellulose-1 (150 × 4.6 mm; particle size: 5 µm) [based on cellulose tris(3,5-dimethylphenylcarbamate)], Lux Cellulose-2 (150 × 4.6 mm; particle size: 5 µm) [based on cellulose tris(3-chloro-4-methylphenylcarbamate)], Lux Cellulose-3 (150 × 4.6 mm; particle size: 5 µm) [based on cellulose tris(4-methylbenzoate)], Lux Cellulose-4 (150 × 4.6 mm; particle size: 5 µm) [based on cellulose tris(4-chloro-3-methylphenylcarbamate)], Lux Amylose-1 (150 × 4.6 mm; particle size: 5 µm) [based on amylose tris(3,5-dimethylphenylcarbamate)], Lux i-

Amylose-1 (150 × 4.6 mm; particle size: 5 µm) [based on amylose tris(3,5-dimethylphenylcarbamate)], and Lux Amylose-2 (Am2) (150 × 4.6 mm; particle size: 5 µm) [based on amylose tris(5-chloro-2-methylphenylcarbamate) were all the products of Phenomenex (Torrance, CA, USA). Escigen 10 mg film-coated tablets were bought in a local pharmacy in Budapest, Hungary.

2.1 LC-UV analysis

Chromatographic experiments were performed on a Jasco HPLC system consisting of PU-2089 plus quaternary pump, AS-4050 autosampler, MD-2010 diode array detector, Jetstream 2 Plus thermostat. JASCO ChromNAV software was used for instrument control and data analysis. All separations were performed at 25 °C using 0.6 mL/min flow rate. UV detection was performed at 230 nm. In the screening phase, alcohols - MeOH and IPA – as well as ACN were used and the sample contains only enantio-enriched citalopram.

The developed method was validated for the simultaneous analysis of related substances and enantiomeric purity of escitalopram. MeOH was used as the solvent for the preparation of stock solutions throughout the study, and it was further diluted with the appropriate mobile phases if it was necessary. The final test solution of escitalopram used for simultaneous achiral and enantiomeric purity testing was about 4000 µg mL^{-1}. All impurity level percentages are reported to this concentration. An injection volume of 3 µL was used.

For preparation of sample solutions, twenty film-coated tablets were weighted, then ground and mixed in a mortar. In a 10 mL volumetric flask, MeOH was added to an accurately weighted portion of the tablet powder corresponding to about 40 mg escitalopram. Then the suspension was sonicated for 30 min and centrifuged for 5 min applying 4000 rpm (Sartorius 2–16 P benchtop centrifuge, Goettingen, Germany). The clear supernatant was filtered through 0.22 µm pore size PVDF syringe filters (FilterBio membrane Co., LTD, Nantong City, China).

3. Results and Discussion

3.1 Method development

As a first step in method development, the chiral separation of citalopram enantiomers was attempted, as the most critical part of the given separation problem. Seven different polysaccharide-based CSPs, including amylose-based Lux Amylose-1, Lux i-Amylose-1 and Lux Amylose-2, as well as cellulose-based Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3 and Lux Cellulose-4 were tested in polar organic mode using 0.1% (v/v) DEA in MeOH, IPA or ACN as mobile phases, with 0.6 mL min^{-1} flow rate at 25 °C. The aim of this experiments was to choose the potential chiral selector(s) for further method development. All chromatograms from the scouting phase are depicted in Supplementary Figure S1-S3. Small peak splitting or deformation can be seen in few cases that could be the result of enantiorecognition. However, enantioseparation with R_s>0.5 was observed only in three cellulose-based stationary phases (Table 1).

Table 1 Chromatographic data obtained during the preliminary study, in terms of the retention time of the second-eluting enantiomer (t_2), resolution (R_s) and elution order for the chromatographic systems, where enantiorecognition was observed.

Column type	Mobile phase	t_2 (min)	R_s	Elution order
Lux Cellulose-3	IPA:DEA 100:0.1 (v/v)	4.41 min	0.6	S<R
Lux Cellulose-2	ACN:DEA 100:0.1 (v/v)	4.77 min	1.0	S<R
Lux Cellulose-1	ACN:DEA 100:0.1 (v/v)	5.50	1.2	R<S

It is interesting to see that using amylose-type CSPs or methanolic mobile phase no enantiorecognition was observed. Appropriate, distomer-first elution order was observed only in one case on Lux Cellulose-1 column with ACN:DEA 100:0.1 (v/v) mobile phase, and fortunately the highest resolution was also observed in this system. This separation system was chosen for further method development, despite the fact that some of the impurities elute with citalopram enantiomers (Figure 2A). Upon adding water to the polar organic mobile phase, both enantio- and chemoselectivity of the method improved (Figure 2A-D). The addition of more than 50% water to ACN resulted in the separation of all compounds (Figure 2D).

All three chemical impurities were available as racemates, however, our aim was not directed towards the chiral separation of these enantiomers, but the enantioseparation of citalopram and the separation of the chemical impurities in one single run. As it can be observed, in case of IMP-3, the individual enantiomers were also separated and they did not interfere with the determination of the analytes.

Figure 3 shows the effect of water content in ACN on the retention and resolution of citalopram enantiomers using Lux Cellulose-1 CSP. A U-shape retention and resolution profile was observed, that is typical for mixed-mode columns. In the first section until 20% of water content a HILIC-like behavior could be observed with a decrease in the retention factor with an increase of water content of the mobile phase. The transition from HILIC to reversed-phase mode was observed at a water content of 20%. Using more than 20% of water both the retention factor and resolution start to increase. Similar results were also described by other research groups on polysaccharide-based CSPs [28,29].

To find the optimal parameter ranges, first, a "one factor at a time" optimization technique was applied, tracking the obtained critical resolution values. The temperature between 10-40 °C, flow rate between 0.5 and 1 mL min^{-1} as well as DEA content between 0 and 0.15 %. Adding DEA, as a basic additive to the mobile phase was necessary for enantioseparation, however higher than 0.1% concentration did not have a significant influence on separation performance. Other parameters, such as temperature, flow rate, and water content in ACN – were further optimized using a L9 orthogonal array table. Both critical resolutions R_{s2} (resolution between IMP-2 and *R*-citalopram) and R_{s3} (resolution between *R*-citalopram and *S*-citalopram) were selected as response values. The chart of the experimental design, alongside with the critical resolution values obtained at each experimental run are in Table 2.

Table 2 L9 orthogonal array table used for method optimization, with the obtained critical resolution values

Experimental No.	Temperature (°C)	Flow (mL/min)	Water content in ACN (%)	R_{s2}	R_{s3}
1	15	0.7	50	0.72	0.99
2	15	0.8	55	1.25	1.33
3	15	0.9	60	2.02	1.69
4	25	0.7	55	1.66	1.23
5	25	0.8	60	1.88	1.75
6	25	0.9	50	0.78	1.05
7	35	0.7	60	1.89	1.67
8	35	0.8	50	0.85	0.91
9	35	0.9	55	1.28	1.29
Results for R_{s2}					
K1	1.33	1.42	0.78		
K2	1.44	1.33	1.40		
K3	1.34	1.36	1.93		
R	0.10	0.09	1.15		
Results for R_{s3}					
K1	1.34	1.30	0.98		
K2	1.34	1.33	1.28		
K3	1.29	1.34	1.70		
R	0.05	0.04	0.72		

In order to analyze the impact of a particular factor on the enantioseparation, a range analysis was applied. The average R_s values were calculated for each of the three levels of a factor (K1–K3). The range values (R) mean the differences between the maximal and minimal K values, thus providing information about the impact of each factor upon R_{s2} and R_{s3}. As it can be observed, the most important factor to be considered is the water content, as it presents the highest range value.

The other two parameters have a smaller effect on the critical resolution values. Based on the results higher water content resulted in higher resolution values, however it was also accompanied by higher analysis time as well. Using 60 % water in the mobile phase, analysis time of less than 30 minutes was not possible, therefore a mobile phase consisting of water/acetonitrile 55/45 (v/v) with 0.1% DEA was chosen for further studies.

Regarding temperature, 25 °C was the best value for both investigated resolution values, as it offering the highest critical resolutions in the shortest analysis time. Analyzing the effect of flow rate, it can be observed that in the case of $R_{s2,}$ lower flow rate resulted higher resolution, but for Rs_3 higher flow rate was accompanied by higher resolution value. Considering the combined effect on both critical resolution values, 0.8 mL min^{-1} flow rate was chosen as optimum. Based on these values, Lux Cellulose-1 column thermostated at 25 °C, with a mobile phase consisting of 0.1% (v/v) diethylamine in water/acetonitrile 55/45 (v/v), delivered with a flow rate 0.8 mL/min was chosen as the final method. Using these circumstances, all analytes were baseline separated within 30 min; a representative chromatogram was depicted in Figure 4A.

3.2 Method validation and application

Validation of the optimized method was performed according to International Council for Harmonization guideline Q2 (R1) for all related substances and for *R*-citalopram as chiral impurity, with respect to sensitivity, linearity, accuracy, and precision. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on signal-to-noise ratios of 3:1 and 10:1 for the LOD and LOQ, respectively. The validation data are summarized in Table 3. Linearity of the method was evaluated at eight concentration levels for all impurities and calibration plots were represented by plotting peak areas against corresponding concentrations (expressed in µg/mL). The correlation coefficient was determined by linear least squares regression analysis and it is higher than 0.9987 in all cases. Moreover, for all impurities 95% confidence intervals of the y-intercepts included zero and random distribution of the residuals was observed. The accuracy and precision were analyzed by performing intra- (repeatability) and inter-day evaluation (two consecutive days) on three concentration levels for all impurities (low, medium, high) covering the linearity range, each solution being injected five times. For all impurities, accuracy (expressed in average recovery%) ranged from 98.32% to 101.59%, with less than 1% standard deviation. Intraday precision, (expressed as RSD%) was between 0.09% - 1.11%,

while RSD for intermediate precision was below 1.35%. Based on the results obtained during validation, the method proved to be sensitive, linear, accurate and precise for the determination of the selected impurities in escitalopram.

The optimized and validated method was applied to the analysis of real samples, in the form of film-coated tablets with a nominal content of 10 mg escitalopram in the form of escitalopram oxalate. Representative chromatogram are shown in Figure 4B. From the impurities only *R*-citalopram can be identified in the sample, the quantity of other impurities is under 0.05%. The content of *R*-citalopram was 0.71 ± 0.01%, which meets the requirements of the limit stated in the United States Pharmacopoeia (not higher than 3%).

Table 3. Summary of data obtained during method validation for the simultaneous determination of related substances and enantiomeric purity.

Parameter	Level	IMP-1	IMP-2	*R*-cit	IMP-3a	IMP-3b
Range (µg/mL)		2-40	2-40	2-40	4-40	4-40
Range (%)		0.05-1	0.05-1	0.05-2	0.1-1	0.1-1
r^2		0.9990	0.9989	0.9995	0.9987	0.9985
LOD (µg/mL)		0.51	0.60	0.60	1.20	1.20
LOQ (µg/mL)		1.7	2.0	2.0	4.0	4.0
Accuracy	I. (4 µg/mL)	100.42	100.25	99.12	98.32	98.36
	II. (16 µg/mL)	98.99	99.61	100.49	100.45	101.0
	III. (32 µg/mL)	99.58	101.59	99.58	101.12	99.45
Intraday precision	I. (4 µg/mL)	0.61%	0.55%	0.75%	1.11%	0.89%
	II. (16 µg/mL)	0.65%	0.09%	0.43%	0.42%	0.75%
	III. (32 µg/mL)	0.54%	0.13%	0.80%	0.51%	0.33%
Intermediate precision	I. (4 µg/mL)	0.27%	0.74%	0.30%	1.01%	1.35%
	II. (16 µg/mL)	0.08%	0.12%	0.45%	0.42%	0.35%
	III. (32 µg/mL)	0.05%	0.33%	0.22%	0.23%	0.32%

3.3 Thermodynamic study

Thermodynamic study is a useful and widely applied method to investigate chiral recognition mechanism [30–33]. Chromatographic runs performed at different temperatures provided an opportunity to compare the thermodynamic parameters in reversed phase and polar organic mode for the enantioseparation of citalopram. To reveal the effect of temperature upon retention and selectivity, the classical van't Hoff analysis was applied. Although this approach is often used, due

to its simplicity, it does not distinguish between enantioselective and non-enantioselective interactions, thus, the thermodynamic values obtained herein are only apparent [34].

The differences in the change of standard enthalpy $\Delta(\Delta H°)$ and standard entropy $\Delta(\Delta S°)$ for the enantiomeric pair, for reversed-phased and polar organic mode on the Lux Cellulose-1 column was calculated by plotting $\ln\alpha$ vs $1/T$, based on the following equation:

$$\ln \alpha = -\frac{\Delta\Delta \mathrm{H}^{\circ}}{\mathrm{RT}} + \frac{\Delta\Delta \mathrm{S}^{\circ}}{\mathrm{R}} \quad (1)$$

where R stands for universal gas constant, T is the temperature, expressed in Kelvin and α is the selectivity factor.

The iso-enantioselective temperatures (T_{iso}) were also calculated, as:

$$T_{iso} = \Delta(\Delta H°)/\ \Delta(\Delta S°) \quad (2)$$

T_{iso} is where the enthalpy and entropy compensate each other, the two enantiomers coelute and no separation would be achieved. For temperature above T_{iso} the separation is entropy-controlled, whereas below T_{iso} it is enthalpy-controlled. By changing the temperature from entropy-controlled area to enthalpy-controlled enantiomer order reversal is expected. Q values were used for visualizing the relative contribution of enthalpy and entropy terms to the free energy of adsorption:

$$Q = \Delta(\Delta H°)/\mathrm{T}\mathrm{x}\Delta(\Delta S°)_{298\mathrm{K}} \quad (3)$$

Thermodynamic data are summarized in Table 4.

Table 4 Thermodynamic parameters, *Δ(ΔH°)*, *Δ(ΔS°)*, *TxΔ(ΔS°)*, *Δ(ΔG°),* van't Hoff equation, correlation coefficients and *Q* values

System/Mode	van't Hoff equations	r^2	-Δ(ΔH°) (kJ mol^{-1})	-Δ(ΔS°) (J mol^{-1}K^{-1})	-TxΔ(ΔS°)$_{298K}$ (kJ mol^{-1})	-Δ(ΔG°)$_{298K}$ (kJ mol^{-1})	$T_{(iso)}$ (°C)	Q
Reversed phase*	lnα=148.66x-0.3642	0.9988	1.2	3.0	0.9	0.3	135	1.4
Polar organic mode**	lnα=586.8x-1.707	0.9989	4.9	14.2	4.2	0.6	70.8	1.2

* Lux Cellulose-1 column with 0.1% (v/v) diethylamine in water/acetonitrile 55/45 (v/v), 0.8 mL/min flow rate

** Lux Cellulose-1 column with 0.1% (v/v) diethylamine in 100% acetonitrile, 0.8 mL/min flow rate

As it can be observed, retention factors and selectivities decrease with increasing temperature, and positive *Q* values were obtained in both cases, which means that the enantioseparation was mainly driven by enthalpic contributions. It can also be seen that thermodynamic parameters such us *(Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°)* are lower in polar organic mode than in reversed phase mode in our case, moreover the T_{iso} value is relative low in both mode, however lower value was observed for polar organic mode.

4. Conclusion

A single-run chemo- and enantioselective method was developed for the determination of related substances of escitalopram, including its enantiomeric pair on a polysaccharide column. The initial screening phase was based on the determination of chiral discrimination capabilities of polysaccharide-type chiral stationary phases in polar organic and reverse-phase mode, as the most critical part of the method. Lux Cellulose-1, based on cellulose tris(3,5-dimethylphenylcarbamate) was identified as the most promising column, using a mobile phase consisting of water-acetonitrile mixtures, containing 0.1% diethylamine as basic modifier Upon tracking the retention times of the enantiomers as a function of the water content of the mobile phase, U-shaped retention curves were obtained, revealing a gradual transition from HILIC-like to typical reverse-phase behavior. The thermodynamic characterization using classical van't Hoff analysis revealed an enthalpy-driven

separation. Chemo- and enantioselectivity of the method was further fine-tuned using an L9 orthogonal array table and provided baseline separation of all analytes under optimized conditions (Lux Cellulose-1 column with a mobile phase consisting of water/acetonitrile 55/45 (v/v), containing 0.1% (v/v) diethylamine. The method was subsequently validated according to ICH guidelines and applied on real, commercial samples, containing escitalopram. The single-run, chemo- and enantioselective method could offer a valuable cost and time-saving alternative to the presently often applied approach using a chiral- and a separate achiral chromatographic system.

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Figure legends

Figure 1 Chemical structure and abbreviations of the compounds used in this study

Figure 2 Representative chromatograms on Lux cellulose-1 column with different acetonitrile water ratio. **Figure 2A** 100% acetonitrile, **Figure 2B** water/acetonitrile 80/20 (v/v), **Figure 2C** water/acetonitrile 60/40 (v/v), **Figure 2D** water/acetonitrile (40/60 v/v). All mobile phases contain 0.1% diethylamine as basic additive. Other chromatographic parameters: 0.6 mL/min flow rate and 25 °C column temperature. 1: IMP-1, 2: IMP-2, 3: R-citalopram, 4: escitalopram 5: IMP-3

Figure 3 Plots of the retention and resolution factors as a function of the water content in acetonitrile on Lux Cellulose-1 column. (Chromatographic conditions: mobile phase 0.1% DEA in the indicated eluent composition, flow rate: 0.6 mL min^{-1}; column temperature: 25 °C)

Figure 4 Representative chromatograms obtained during method optimization and application. **Figure 4A** Solution of escitalopram sample spiked with 0.1% impurities; **Figure 4B** Solution of escitalopram 10 mg tablet. Experimental conditions: Lux Cellulose-1 column with 0.1% (v/v) diethylamine in water/acetonitrile 55/45 (v/v), 0.8 mL/min flow rate at 25 °C.