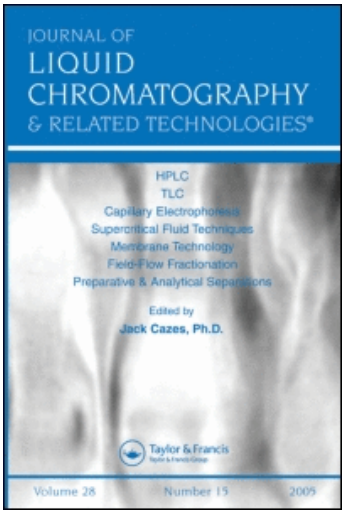

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Validated Chiral Liquid Chromatographic Method for the Enantiomeric Separation of Florfenicol

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Abstract: An isocratic normal phase liquid chromatographic (NP-LC) method was developed for the separation of florfenicol enantiomer in the bulk drug substance. Enantiomeric separation was achieved on a Chiralpack-AD column (250 × 4.6 mm) at a constant room temperature using mobile phase combination n-Hexane:Ethanol: Methanol (80 10 10, v/v/v) delivered at a flow rate of 1.0 mL/min. Analytes were monitored at 225 nm. The method was validated for precision, limit of detection (LOD), limit of quantification (LOQ), linearity, and accuracy.

Keywords: Florfenicol, Enantiomers, Chiral liquid chromatography, Validation

INTRODUCTION

Most of the commercial and investigational pharmaceutical compounds are enantiomers. The biological activities of chiral substances often depend upon their stereochemistry, thus, showing significant enantioselective differences in their pharmacokinetics and pharmacodynamics. There is a growing demand for the direct methods of enantiomeric separation of chiral drugs, as the US food and Drug Administration has issued an order to specify the enantiomeric purities of chiral drugs.[1]

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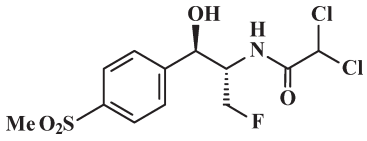

Figure 1. Structure of florfenicol.

Polysaccharide based chiral stationary phases for separation and quantification of enantiomeric impurity by liquid chromatography (LC) has gained popularity and are most widely used as these methods are direct.[2,3]

They can be used both in normal phase mode (NP-LC)[4] and in reversed phase mode (RP-LC).[5]

Florfenicol (Figure 1), a fluorinated derivative of thiamphenicol is a veterinary antibacterial. Florfenicol is a broad spectrum, primarily bacteriostatic, antibiotic with a range of activity similar to that of chloramphenicol, including many gram negative and gram positive organisms.[6] However, florfenicol does not carry the risk of inducing human aplastic anemia that is associated with chloramphenicol.[7] Florfenicol has a fluorine atom instead of the hydroxyl group located at C-3 in the structure of chloramphenicol and thiamphenicol. This may allow florfenicol to be less susceptible to deactivation by bacteria with plasmid transmissible resistance that involves acetylation of the C-3 hydroxyl group in the chloramphenicol and thiamphenicol, and prevents their interaction with bacterial ribosomes.[8,9] Only the D-threo enantiomer is active, and the inactive L-threo enantiomer needs to be monitored quantitatively for its control.

In terms of analytical methods for chiral separation of this drug, only one published paper is available,[10] which achieved enantiomeric separation of the two enantiomers using a pirkle type chiral HPLC column Whelk-O 1 (derived from 4-(3,5-dinitro benzamido)tetrahydrophenanthrene covalently bound to silica). The present work involves development and validation of a method for enantiomeric separation of the two enantiomers, with the limit of quantification (LOQ) 0.185 μg/mL, on a commonly available and widely used cellulose Polysaccharide based chiral stationary phase (CSPs) HPLC column in normal phase liquid chromatography (NP-LC).

EXPERIMENTAL

Chemicals and Reagents

Florfenicol reference standard, bulk substance, and enantiomer reference standard were provided by APL Research Centre (A Division of Aurobindo Pharma Ltd.) (Bachupally, Quthubullapur, Hyderabad-72, India). HPLC grade Methanol, n-Hexane was purchased from Merck (Worli, Mumbai,

India), and Ethanol AR grade 99.9% from Changshu Yangyuan Chemical, China. All the chemicals were used without further purification.

Apparatus

Chromatographic separation was performed on a high performance liquid Chromatography system with Waters alliance 2695 separations module and 2996 Photodiode array detector with *Empower pro* software for data handling, instrument control, and data acquisition. Chiralpak-AD (250 × 4.6 mm), packed with the tris 3,5 dimethyphenyl carbamate derivative of amylose, coated on 10 μm silica-gel support, was purchased from Daicel Chemical Industries (Tokyo, Japan).

Chromatographic Conditions

Enantioseparation was achieved on a Chiralpack-AD column (250 × 4.6 mm) at a constant room temperature using the mobile phase combination n-hexane: ethanol:methanol (80:10:10, v/v/v), delivered at a flow rate of 1.0 mL/min. Analytes were monitored at 225 nm. The injection volume was 20 μL.

Preparation of Test Solution and Control Solution

The test solution (0.5 mg/mL) was prepared by dissolving 50 mg of the bulk drug substance in 5 mL of methanol and diluting to 100 mL with mobile phase. The control solution was obtained with the florfenicol reference standard prepared the same as the test solution and further diluting to required concentration with mobile phase.

RESULTS AND DISCUSSION

Method Development

Polysaccharide based chiral stationary phases (CSPs) (cellulose and amylose derivatives) developed by Okamoto's group,[11] are mostly used in the normal-phase mode with n-hexane based mobile phases containing alcohols (% v/v) as the modifier. 2-Propanol and ethanol (EtOH) are the most commonly used modifiers for the separation of the enantiomers on these columns.[4] Three derivatives namely cellulose tris-(3,5-dimethyl phenyl carbamate), commercial name chiralcel OD; amylose tris-(3,5-dimethyl phenyl carbamate), commercial name chiralpak AD; and cellulose tris-(4-methyl benzoate), commercial name chiralcel OJ, are commercially available and the most

widely used polysaccharide-based chiral stationary phases. These CSPs have complementary properties and broad enantiorecognition capabilities for a wide range of pharmaceutical compounds.[12] As the compound contains an NH group, distant from the chiral center, separation selectivity can be modulated on tris-(3,5-dimethyl phenyl carbamate) columns. The aromatic functionality on the compound could provide an additional stabilizing effect to the solute-CSP complex by insertion of the aromatic portion of the solute into the chiral cavity.[13] Initial development trials were carried out on the chiralpak AD column with the mobile phase combination of n-hexane, and isopropyl alcohol in the ratio of 90:10 (v/v). The peaks were retained for a long time. Further increasing the ratio of isopropyl alcohol by a ten percent volume decreased the retention time, but the peak shape was broad with tailing. To improve the peak shape, a ten percent volume of ethanol was introduced in the first trial but the enantiomers merged. Trials with a mobile phase combination of n-hexane, and ethanol in the ratio of 70:30 (v/v) achieved good enantiomeric separation. Introduction of methanol in this trial improved the peak shapes further. The mobile phase ratio of n-hexane, ethanol, and methanol in the ratio of 80:10:10 (v/v/v) was optimized for good separation. Minor changes in the ratio 80:15:5 and 80:5:15 had no significant effect on the resolution between the enantiomers. In the optimized method, retention times of florfenicol and its enantiomer were about 9.7 and 15.8 min, respectively.

As per the ICH guidelines the method was validated in terms of the following parameters.[14]

System Suitability

The system suitability solution is prepared as mentioned in the preparation of the test solution and the control solution, to obtain a final concentration of florfenicol 500 μg/mL spiked with 2.5 μg/mL of florfenicol enantiomer. The resolution is not less than 3.0, as evident from Figure 2.

Solution Stability

Stability of the sample in the diluent was evaluated by injecting the freshly prepared test solution every hour, for 10 hours, at room temperature. Appearance, i.e., color of the solution did not change, no extra peaks appeared, and the cumulative relative standard deviation for the peak area of florfenicol enantiomer was less than 2.0%. Peak purity obtained from the photodiode array detector for the florfenicol enantiomer peak, shows that the peak is pure (purity angle is less than purity threshold). The results indicate that the solutions were stable up to 10 hours at room temperature.

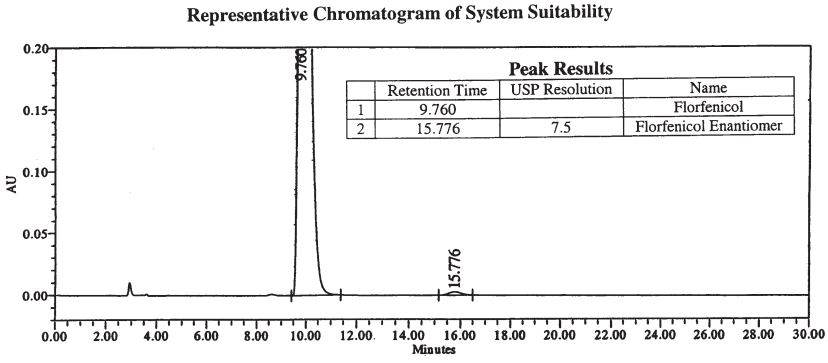

Figure 2. Representative chromatogram of system suitability.

Method Reproducibility

Method reproducibility was determined by measuring repeatability, precision, and intermediate precision (between-day precision). Repeatability of the method was determined by calculating %RSD for the enantiomer area from replicate injections (n = 6) of the system suitability solution. The %w/w of the enantiomer for six different preparations of the system suitability solution were calculated. Method precision was determined by calculating %RSD for these %w/w values.

Intermediate precision was determined by performing method precision by a different analyst on a different day. The results in Table 1 show that method reproducibility was good.

Limits of Detection and Quantification

The limit of detection (LOD) and limit of quantification (LOQ) was established from the linearity curve by the following equation. The results listed in Table 1 indicate that the LOD and LOQ values were 0.061 μg/mL and 0.185 μg/mL, respectively, with good precision at LOQ. Precision at LOQ was established by calculating %RSD for replicate injections (n = 6) of reference standard prepared at LOQ level.

$$\text{LOD} = \frac{3.3 \times \text{Residual sum of squares}}{\text{slope}}$$

$$\text{LOQ} = \frac{10 \times \text{Residual sum of squares}}{\text{slope}}$$

Table 1. Validation Results of the developed chiral LC method

Validation parameter	Results	
Repeatability (n = 6, %RSD)		
a) System precision (area of enantiomer)	1.0	
b) Method precision (%w/w of enantiomer)	2.5	
Intermediate precision (n = 12, %RSD)		
Method precision (%w/w of enantiomer)	3.0	
LOD-LOQ		
Limit of detection (μg/mL)	0.061 μg/mL	
Limit of quantification (μg/mL)	0.185 μg/mL	
Precision at LOQ (%RSD)	6.2	
Linearity	For drug	For enantiomer
Calibration range (μg/mL)	0.10 to 3.75 μg/mL	0.10 to 3.75 μg/mL
Calibration points	7	7
Slope	42842	40855
Intercept	1945	1
Residual Sum of Squares	2087	1898
Correlation coefficient	0.9994	0.9995

Linearity

Standard solutions were prepared at concentration levels from LOQ to 3.0 μg/mL (0.1, 0.50, 1.25, 2.0, 2.5, 3.0, and 3.75 μg/mL) as described in Preparation of Test solution and Control Solution for florfenicol and its enantiomer. The regression curve was obtained by plotting the peak areas versus the concentration. The results listed in Table 1 indicated that the correlation coefficient was good and more than 0.99.

Accuracy (Recovery)

Standard addition and recovery experiments were conducted by spiking the florfenicol sample solutions with its enantiomer at levels 0.25%, 0.50%, and 0.75% w/w of the florfenicol target analyte concentration, each in triplicate. The mean recovery of florfenicol enantiomer was between 95.9 and 102.3, respectively, with %RSD between 0.5 and 1.0, respectively, indicating good accuracy of the method. The results are listed in Table 2.

Robustness

The robustness of a method is the ability of the method to remain unaffected by small but deliberate changes in parameters such as flow rate, mobile phase

Table 2. Recovery data of enantiomer

Concentration/ sample ID	Amount added (%w/w)	Amount found (%w/w)	Recovery (%)	Statistical analysis
0.25% – Sample 1	0.251	0.242	96.4	Mean: 95.9
0.25% – Sample 2	0.252	0.239	94.8	SD: 0.92
0.25% – Sample 3	0.248	0.239	96.4	%RSD: 1.0
0.50% – Sample 1	0.508	0.522	102.8	Mean: 102.3
0.50% – Sample 2	0.498	0.507	101.8	SD: 0.50
0.50% – Sample 3	0.502	0.514	102.4	%RSD: 0.5
0.75% – Sample 1	0.756	0.760	100.5	Mean: 99.9
0.75% – Sample 2	0.747	0.741	99.2	SD: 0.67
0.75% – Sample 3	0.756	0.757	1001	%RSD: 0.7

composition, and column oven temperature. The effect of resolution for the system suitability solution was monitored in this study. The flow rate of the mobile phase is 1.0 mL/min and was varied to 0.9 mL/min and 1.1 mL/min. The effect of change in percent organic, i.e., ethanol:methanol, (1 1, v/v) ratio was studied by varying its concentration to −2 and +2% absolute, i.e., 78% n-hexane: 22% ethanol:methanol (10 10, v/v) and 82% n-hexane: 18% ethanol:methanol (10 10, v/v). The column temperature is ambient (laboratory temperature maintained at 25 ± 2°C) and was varied to 35°C. The effects of each of these parameters were studied while the rest of the parameters were kept constant, as mentioned in an above section. The resolution remained unaffected by these small changes as shown in Table 3, thus proving the method to be rugged.

Table 3. Robustness data of the method

Parameter	USP resolution between the enantiomers
Flow rate (mL/min)	
0.9	3.7
1.0	3.7
1.1	3.5
[Ethanol: methanol, 10:10 (v/v)] percentage in mobile phase	
18	4.2
20	3.7
22	5.3
Column temperature (°C)	
Ambient (25 ± 2°C)	3.7
35	3.7

CONCLUSION

The developed and validated normal phase liquid chromatographic method offers simplicity and sufficient sensitivity for the quantitative determination of the florfenicol enantiomer in the bulk drug substance.

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