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Enantioselective Separation and Determination of Formoterol in Bulk Drugs and Formulations by High Performance Liquid Chromatography

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A simple and rapid liquid chromatographic methods for enantioselective separation and determination of -(±)- enantiomers of formoterol (FMT) in bulk drugs and formulations using UV and polarimetric detectors connected in series was developed. The enantiomers were tested on numerous commercial HPLC columns. For formoterol the most convenient separation was determined on amylose chiralpak AD-H column. The mobile phase compositions were systematically studied to obtain the optimal chromatographic methods. Validation of methods in selected conditions shows that the chosen methods are selective and precise with linear response of detector for both pairs of enantiomers.

Key Words: β-Adrenoceptor agonists, Polysaccharide stationary phases, Chiral recognition, Polarimetric detector.

INTRODUCTION

There are two categories of antiasthma drugs, bronchodilators and antiinflammatory agents. Drugs used as bronchodilators include β2-adrenoceptor agonists, xanthines, cysteinyl-leukotriene receptor antagonists and muscarinic receptor antagonists. The β2-adrenoceptor agonists, their primary effect in asthma is to dilate the bronchi by a direct action on the β2-adrenoceptors on the smooth muscle. These drugs are usually given by inhalation of aerosol, powder or nebulized solution, but some may be given orally or by injection. Two categories of β2-adrenoceptor agonists are used in asthma.

Most of the chiral drugs available in the market are administered as racemate[1]. However, the great difference in pharmacological effects and pharmacokinetics between the two enantiomeric forms of many drugs is also well known[2,3]. Therefore, the pharmaceutical industry increasingly needs new analytical and preparative procedures capable of resolving and quantitation of drug enantiomers and the resolution of racemic mixtures is becoming a highly challenging area of separation technology.

Formoterol ((RR)-(9)-N-[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxypheny)-1-methylethyl]amino]ethyl]phenyl] formamide) (FMT). They are marketed as racemate of the enantiomers, which have the RR, SS configuration. The anti-bronchoconstrictor activity of formoterol lie with the (R,R) enantiomer and the (S,S) enantiomer does not exert any contractile effects when present in the racemate[4]. For formoterol, the (R,R)-enantiomer has shown to be more active than the other stereoisomers (R,S; S,R; S,S) of formoterol[5]. Maximum bronchodilation is achieved within 2 h, with effects persisting for approximately 12 h. This is significantly longer than the bronchodilatory effects of equivalent doses of salbutamol, fenoterol or terbuataline[6]. It has been reported to be more effective than a shorter-acting β2 agonist in the treatment of nocturnal and exercise-induced asthma[7,8].

Isotachophoresis (ITP)[9,10], capillary gel electrophoresis (CGE)[11,12], micellar electrokinetic chromatography (MEKC)[13,14] and capillary electrochromatography (CEC)[15,16] methods are available for analysis of formoterol, but chiral HPLC method based on polysaccharide based stationary phases are limited, so we have tried to develop a method on polysaccharide based stationary phases. Most experiments quoted in the literature were performed with β-cyclodextrin; γ-cyclodextrin was less frequently used, basically because of its higher price.

EXPERIMENTAL

All reagents were of analytical-reagent grade unless stated otherwise. HPLC-grade diethylamine, *n*-hexane, 1-propanol, 2-propanol and ethanol were purchased from S.D. Fine Chem., Mumbai, India. (±) enantiomers of formoterol were gifted from Neuland Laboratories Ltd., Hyderabad, India. All solutions were filtered through 0.45 µm membrane filters procured from Pall Pharmalab Filtration Pvt. Ltd., Mumbai, India.

The HPLC system composed of LC-10AT VP pump, SPD-10A VP UV detector and SIL-10AD VP auto injector and SCL-10A VP system controller attached with thermostat (all from Shimadzu, Kyoto, Japan). Polarimetric detector (IBZ Messtechnik GmbH, Hanover, Germany) was connected to UV detector in series for identification of the enantiomers. Chiralcel OD-H (250 × 4.6 mm; particle size 5μm), chiralcel OJ-H (250 mm × 4.6 mm; particle size 5 μm) and chiralpak AD-H (250 × 4.6 mm; particle size 5 μm) (Daicel Chemical Industries, Tokyo, Japan) were used for separation. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system.

Chromatographic conditions: Chromatographic separation of enantiomers of formoterol achieved on chiralpak AD-H (250 × 4.6 mm; particle size 5 μm) column. For formoterol the mobile phase consisting of *n*-hexane:1-propanol:diethylamine (75:25:0.1 v/v/v) at 25 ºC were used. For formoterol the UV detector was kept at 245 nm. The flow rate was 1.0 mL/min and injection volume was 20 μl and total run time was 10 min.

Preparation of stock and standard solutions: Stock solutions of (±)-formoterol were prepared by dissolving 300.2 mg of (±) -formoterol weighed in respective 100 mL volumetric flasks, dissolved in 25 mL methanol and made up to the mark with the mobile phase. The stock solution were wrapped with aluminum foil and kept in the refrigerator at 5 ºC. The specified concentration of formoterol were taken as 300 μg/mL for the analysis.

RESULTS AND DISCUSSION

The column, mobile phase selectivity, effect of diethylamine and column temperature on resolution and retention were studied for optimizing the LC conditions for separation of enantiomers of formoterol.

Method optimization of formoterol enantiomers: The column, mobile phase selectivity, effect of diethylamine and column temperature on resolution and retention were studied for optimizing the LC conditions for separation of enantiomers of formoterol.

Column selectivity: Three different polysaccharide-based stationary phases were (i) chiralcel OJ-H (cellulose *tris*-(4-methylbenzoate)), (ii) chiralcel OD-H (cellulose *tris*-(3,5-dimethylphenylcarbamate) and (iii) chiralpak AD-H (amylose *tris*-(3,5-dimethylphenylcarbamate) columns were evaluated using 2-propanol, 1-propanol and ethanol as organic modifier in *n*-hexane. Chiralcel OD-H has not shown any selectivity for enantiomers of formoterol. While chiralcel OJ-H and chiralpak AD-H columns showed good separation for enantiomers of formoterol. Table-1 shows the selectivity and resolution of formoterol enantiomers on both the columns. It is clear from Table-1, that the enantiomers of formoterol were separated using 1-propanol, but enantiomers were retained long on chiralcel OJ-H (Fig. 1), whereas chiralpak AD-H column has shown excellent selectivity for the formoterol enantiomers with decreased retention (Fig. 2). So chiralpak AD-H column was chosen for further optimization.

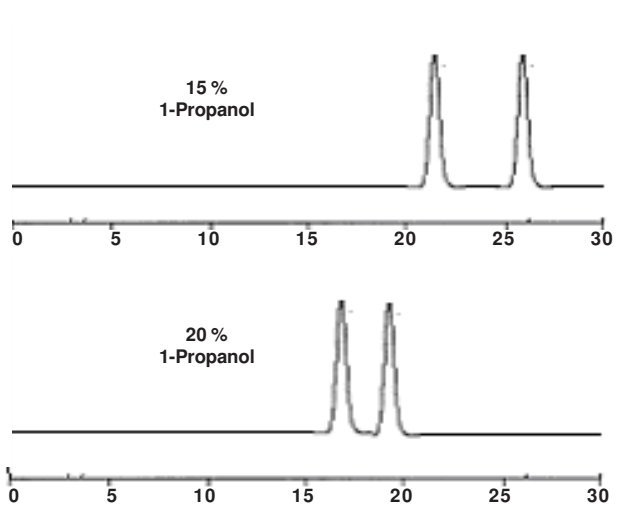

Fig. 1. Enantiomeric separation of formoterol on Chiralcel OJ-H column

Effect of organic modifier: The type and concentration of organic modifier was found to influence the retention and resolution of formoterol enantiomers on chiralpak AD-H. The effect of 2-propanol and 1-propanol as organic modifiers on resolution of formoterol enantiomers was investigated (Table-1). It is clear from the data that the selectivity and resolution of formoterol enantiomers were good with 1-propanol on chiralpak AD-H when compared to 2-propanol. So 1-propanol

TABLE-1
SELECTIVITY OF FORMOTEROL ENANTIOMERS ON CHIRALCEL OJ-H AND CHIRALPAK AD-H COLUMNS WITH DIFFERENT ORGANIC MODIFIERS AT 25º C AND 0.1 % DIETHYLAMINE

Organic modifier	K_1	K_2	α	Rs
(A) Chiralcel OJ-H				
n-Hexane/1-Propanol = 90/10 + 0.1 % diethylamine	–	–	–	–
n-Hexane/1-Propanol = 87/13 + 0.1 % diethylamine	9.89	12.20	0.82	2.70
n-Hexane/1-Propanol = 85/15 + 0.1 % diethylamine	6.65	8.10	0.84	2.15
n-Hexane/1-Propanol = 80/20 + 0.1 % diethylamine	4.82	5.51	0.89	0.98
(B) Chiralpak AD-H				
n-Hexane/2-Propanol = 90/10 + 0.1 % diethylamine	1.41	1.58	0.93	1.23
n-Hexane/2-Propanol = 87/13 + 0.1 % diethylamine	6.24	7.27	0.87	2.07
n-Hexane/2-Propanol = 85/15 + 0.1 % diethylamine	4.13	4.72	0.89	1.70
n-Hexane/2-Propanol = 80/20 + 0.1 % diethylamine	2.79	3.13	0.91	1.50
n-Hexane/2-Propanol = 25/25 + 0.1 % diethylamine	1.24	1.37	0.94	1.10
n-Hexane/1-Propanol = 90/10 + 0.1 % diethylamine	8.41	11.27	0.76	4.77
n-Hexane/1-Propanol = 85/15 + 0.1 % diethylamine	4.00	5.65	0.76	4.84
n-Hexane/1-Propanol = 75/25 + 0.1 % diethylamine	1.34	1.79	0.83	2.85

was chosen as organic modifier for the separation. The effect of concentration of 1-propanol on chiralpak AD-H was studied. On decreasing the concentration of 1-propanol, retention factors as well as resolution (Table-1) were increased. At 25 % of 1-propanol in *n*-hexane resolution >2.5 was obtained. Further decrease of 1-propanol on chiralpak AD-H concentration led to peak broadening and higher retentions. As a compromise for higher resolution and lower retention, 25 % of 1-propanol in *n*-hexane was chosen for analysis.

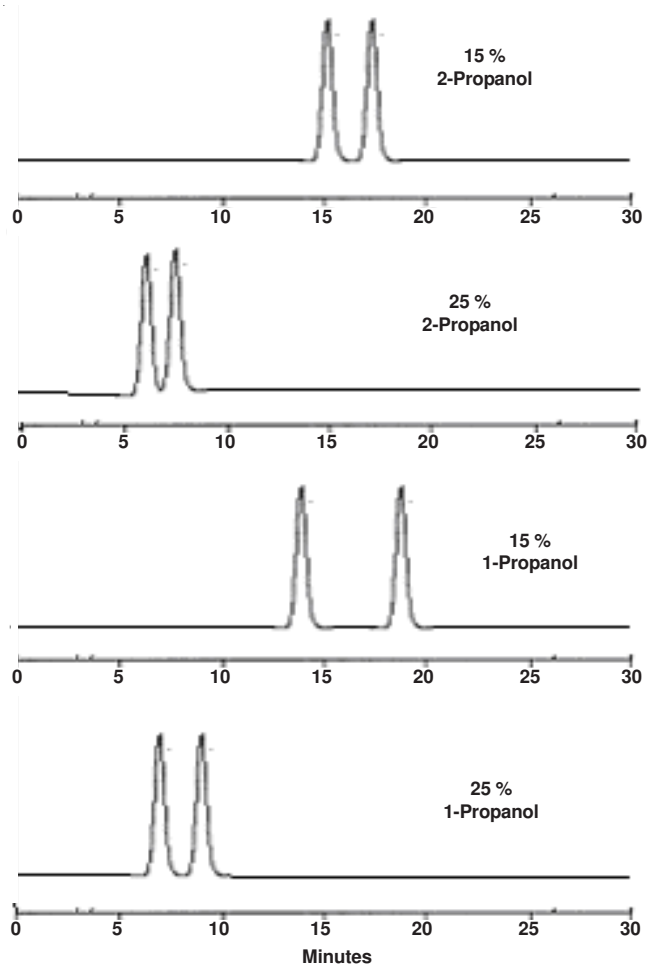

Fig. 2. Enantiomeric separation of formoterol on chiralpak AD-H column

Effect of concentration of diethylamine: To minimize peak tailing diethylamine was added to mobile phase. Diethylamine was not having much effect on retention factors. But on increasing the diethylamine concentration, peak shapes were sharpen and tailing was reduced. But increasing the diethylamine concentration from 0.1 to 0.3 %, increased the baseline noise and decrease the peak intensity. As a compromise 0.1 % of diethylamine was chosen as optimum for formoterol.

Effect of column temperature: The effect of column temperature on resolution and retention of formoterol enantiomers was studied in the range 298-313 K (25-40 ºC) on chiralpak AD-H column. On increasing the temperature, retentions (Fig. 3) as well as resolutions were decrease (Table-2). Under thermodynamically equilibrium conditions, free energy accompanying the separation of two enantiomers related to retention factors by the following equation.

$$\Delta G^{o} = - RT \ln k \quad (1)$$

where, k is the retention factor, R the gas constant and T is the temperature in kelvin. An expansion of eqn. (1) to involve the enthalpy (H) and entropy (S) terms yield.

$$\ln k = - \Delta H^{o}/RT + \Delta S^{o}/R \quad (2)$$

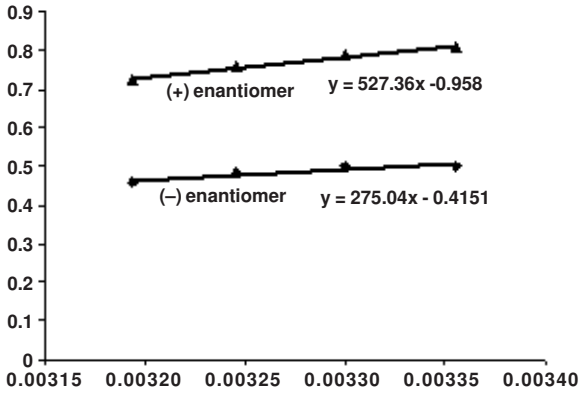

Fig. 3. Van't Hoff plots of the formoterol enantiomers at 25 % of 1-propanol on chiralpak AD-H column

TABLE-2
EFFECT OF TEMPERATURE ON SELECTIVITY AND RESOLUTION OF FORMOTEROL ENANTIOMERS ON CHIRALPAK AD-H COLUMN

Temperature	k_1	k_2	α	Rs
25	1.37	1.89	0.82	2.45
30	1.34	1.79	0.83	2.50
35	1.31	1.72	0.84	2.26
40	1.27	1.58	0.88	1.93

Van't Hoff plots were drawn for logarithm of retention factor (ln k) *versus* inverted temperature (1/T) in K for the two isomers, which yielded straight lines 465.03 × +1.243 and 1073.2x - 2.958 for (+)-formoterol and (-)-formoterol enantiomers, respectively. ΔH^{o} and ΔS^{o} for the two enantiomers were obtained from slope and intercept of the straight lines, respectively. The change in free energy accompanying the separation of two enantiomers was given by:

$$\Delta\Delta G^{o} = \Delta\Delta H^{o} - T\Delta\Delta S^{o} \quad (3)$$

The enthalpy change ($\Delta\Delta H^{o}$), entropy change ($\Delta\Delta S^{o}$) and Gibb's free energy change ($\Delta\Delta G^{o}$) accompanying the separation were recorded in Table-3. The data indicated that the

TABLE-3
THERMODYNAMIC DATA CALCULATED FROM THE VAN'T HOFF PLOTS OF FORMOTEROL ENANTIOMERS

Enantiomer	ΔH^{o} (KJ mol^{-1})	$\Delta\Delta H^{o}$ (KJ mol^{-1})	ΔS^{o} (JK^{-1} mol^{-1})	$\Delta\Delta S^{o}$ (JK^{-1} mol^{-1})	$\Delta\Delta G^{o}$ (KJ mol^{-1}) at 298K
-(+)-Formoterol	-3.866	-10.334	-5.056	-14.258	-0807
-(-)-Formoterol	-8.922		-24.592		

ΔH^{o} = Slope × R; ΔS^{o} = Intercept × R; $\Delta\Delta G^{o}=\Delta\Delta H^{o} - T\Delta\Delta S^{o}$

separation of formoterol enantiomers was an enthalpy driven process.

Optimized conditions: Thus, a mobile phase containing *n*-hexane:1-propanol:diethylamine (75:25:0.1 v/v/v) was chosen for the separation of formoterol enantiomers on chiralpak AD-H column maintained at 25 ºC. The flow rate was kept at 1.0 mL/min throughout the analysis. The chromatographic separation of (+) -formoterol, (-) -formoterol in the optimized conditions using UV detector and polarimetric detector. Table-4 Assay of Formoteral in bulk drugs and formulations. The method was validated in terms of accuracy, precision and linearity as per ICH guidelines.

TABLE-4
ASSAY OF FORMOTERAL IN BULK DRUGS AND FORMULATIONS

S. No.	No. Inj.	Taken	Recovered	% Recovered	R.S.D. (%)*
I	1	0.0990	0.0982		
	2	0.0990	0.0987	99.75	0.55
	3	0.0990	0.0993		
II	1	0.0488	0.0490		
	2	0.0488	0.0485	100.22	1.02
	3	0.0488	0.0495		
III	1	0.0252	0.0250		
	2	0.0252	0.0247	99.87	1.82
	3	0.0252	0.0256		
I	1	0.0981	0.0987		
	2	0.0981	0.0989	100.20	0.48
	3	0.0981	0.0980		
II	1	0.0492	0.0484		
	2	0.0492	0.0494	99.84	1.04
	3	0.0492	0.0491		
III	1	0.0247	0.0251		
	2	0.0247	0.0245	99.75	2.44
	3	0.0247	0.0239		

*n=3 injections

Validation of Formoterol enantiomers:

System suitability: The solution of formoterol-(±) (100 μg mL^{-1}) prepared in the mobile phase was used for system suitability studies. The chiralpak AD-H column was stabilized for 0.5 min in the optimized conditions and three replicate injections were made. The system was deemed to be suitable if resolution between the two formoterol enantiomers is not less than 2.5 and tailing factor is not more than 1.3 (at 10 % base).

Precision: Precision of the method was tested by preparing six individual solutions of formoterol- (±) and making triplicate injections for each solution. The % RSD of the assay was less than 0.68 %. Inter and intraday assay precision was performed by analyzing the solutions for five times in a day for three consecutive days. The % RSD of the assay was less than 0.89 % for the both isomers.

Linearity: Calibration graphs are drawn in the range of 50-600 μg mL^{-1} of formoterol enantiomers by preparing fresh solutions every day for 3 days. Curves were linear with $r^2 > 0.9999$ and the regression equations for (+) -formoterol and (-) -formoterol were Y = 11883 × 6902 and 11910 × 7724, respectively.

Accuracy: Accuracy was determined by spiking formoterol solution at five levels in the range 50-150 % with respective to specified level (300 μg mL^{-1}) and analyzing the each solution in triplicate for 3 days. Percentage recoveries were between 99.24 and 100.79 %.

Robustness: Robustness of the method was checked by making small deliberate changes in the operating parameters. Variation of 0.5 % of 1-propanol did not affect the resolution except that retentions were changed. The effect of temperature has been studied by analyzing sample at 25 ± 1 ºC. The resolution remained still above 2.5. The effect of flow rate was studied by analyzing the samples with 0.9 and 1.1 mL/min flow rates. In both the cases resolution was above 2.5. The effect of diethylamine was studied by adding 0.09 % and 0.11 % diethylamine to the mobile phase and it has not any effect on resolution and retentions.

Limit of detection and limit of quantification: Limit of detection and limit of quantification were calculated using signal/noise (S/N) ratio method. Limit of detection is taken as a concentration of analyte where S/N was 3 and it was found to be 0.20 μg mL^{-1} for both the enantiomers. Limit of quantification is taken as concentration of analyte where S/N is 10 and it was found to be 0.7 μg mL^{-1} for both the enantiomers.

Conclusion

Separation and determination of formoterol enantiomers was studied on polysaccharide stationary phases. Chiralpak AD-H column has shown excellent selectivity for formoterol enantiomers. The effect of organic modifiers and temperature on resolution and retention of enantiomers have been evaluated to optimize the mobile phase composition. The enantiomeric separation was found to be an enthalpy driven processes for formoterol. The methods were validated with respect to accuracy, precision, linearity, limit of detection, limit of quantification and robustness. The developed methods are quite simple, rapid, sensitive and enantioselective and could be of use for determination of enantiomeric purity of formoterol in bulk drugs and formulations.

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