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Separation of pneumocandin and its analogues by HPLC for Scale-up by Varicol technology

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ABSTRACT

HPLC method has been developed for separation of pneumocandin isomers using silica gel; the effect of column loadings and flow rates on the separation profiles in terms of resolution and selectivity have been investigated for development of scalable continuous separation in Varicol process.

Keywords: Pneumocandin, HPLC, Selectivity, Resolution, Varicol process.

INTRODUCTION

Identification and characterization of impurities in Active Pharmaceutical Ingredients (APIs) is necessary for controlling them in API and drug product.

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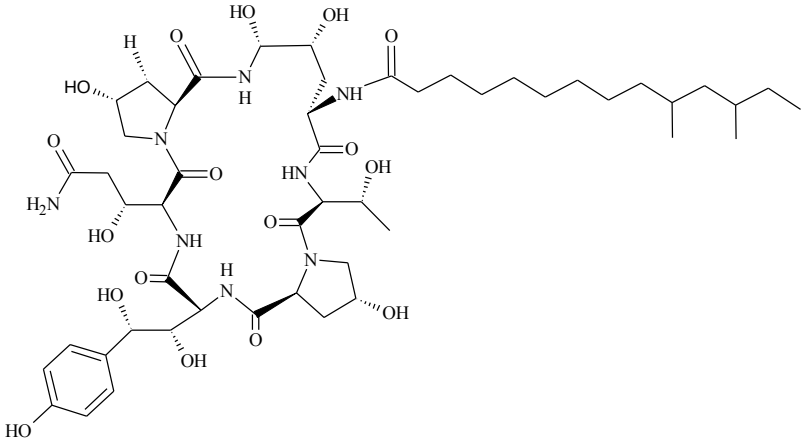

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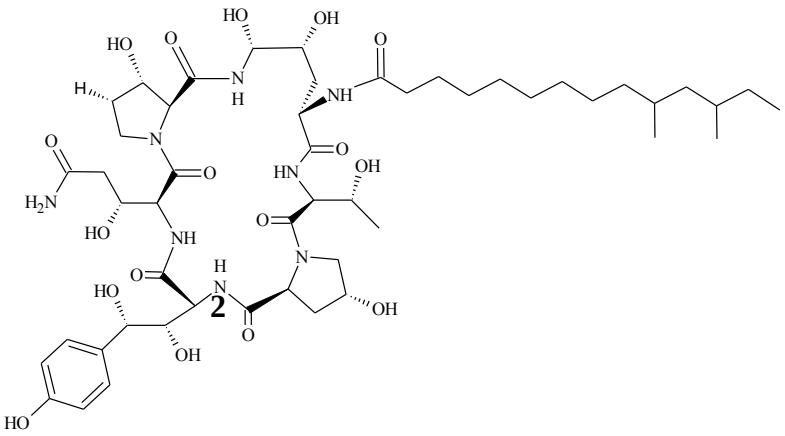

One of the principal requirements for any drug registration is to specify identified and unidentified impurities in drug substance/drug product as per ICH guidelines Q3A(R), Q3B(R) and Q3C[1]. These impurities need to be controlled when they are present at a level higher than the identification threshold in the final API. In our continued efforts to develop scalable separation of chiral compounds we have tackled the purification of crude pneumocandin B_0(**1**) from its major C_0 isomer(**2**)and other related analogues (Scheme 1). Pneumocandin**1** is a natural product produced as a secondary metabolite by fermentation of the fungus glarea lozoyensis[2,3]. It is a key intermediate in the production of caspofungin acetate[4-6]. Caspofungin is fungicidal against *Candida* spp. It acts by blocking the synthesis of a major fungal cell wall component, 1-3-beta-D-glucan[7-9]. It is manufactured by Merck and sold under the trade name *Cancidas*™. During the fermentation process many closely related analogues are also produced along with the desired isomer. Liquid chromatographic techniques are routinely used to purify such crude fermentation products. The active isomer **1** is reported to be separated by preparative chromatography using ethyl acetate-methanol-water mixture as an eluent on various silica based stationary media[10]. Purification of **1 by** traditional crystallization techniques is found to be challenging[11]. In the present study, we have developed HPLC method to scale up the purification of pneumocandin B_0 in continuous mode using Varicol technology[12], an advanced variant of the simulated moving bed (SMB) technology[13].

MATERIALS AND METHODS

API intermediates were provided by Ranbaxy Laboratories Ltd., India. Waters HPLC 2695 alliance separation module (customized with syringe and loop volume of 2.5 mL) with PDA 2998 and RI 2410 detectors were used for method development. HPLC grade solvents were used as obtained from Rankem and Qualigens. Customized columnKR60-SIL (250 × 4.6 mm) was procured from Akzo Nobel, USA. Varicol processes were carried out in *Novasep* VARICOL LAB equipment using commercially available solvents.

RESULTS AND DISCUSSION

Pneumocandins are produced by *Glarea lozoyensis*[2-3]. Modifications of the cyclic peptide nucleus led to the identification of caspofungin acetate, the first β-1,3-glucan synthesis inhibitor indicated for the treatment of serious fungal infections[7-9]. Solids obtained from fermentation broth generally contain pneumocandin B_0 and C_0 isomers, and other closely related impurities (Scheme 1). The crude pneumocandin B_0 is 80-85% pure with 10.6% C_0 isomer and an assay of about 49%. Purification of pneumocandin B_0 by conventional crystallization methods is found to be challenging[14]. WO 2005/026323 reports preparative chromatographic methods for the separation of **1** and related analogues on silica gel and modified silica gel based stationary media. Herein, the separation of the closely related pneumocandin isomers was carried out using Varicol technology, an SMB variant. Varicol simulation

software was used for designing and optimizing Varicol process under isocratic conditions by using HPLC data.

The HPLC data was generated with 16 µm silica (KR60-SIL), keeping in mind its suitability for the scale-up, but in the screening for a suitable stationary phase, 5 µm size was considered. KR60-SIL offered the best selectivity between **1** and **2** compared to that with diol, cyano and amino-derivatized silica (Table 1). In addition, the selectivity between **1** and **2** was found to more or less the same with both 5 µm and 16 µm silica with the values of 1.25 (at a loading of 0.050 mg) and 1.37(at a loading of upto 2.5 mg), respectively. Furthermore, the selection of mobile phase (ethyl acetate–methanol–water mixture) was based on a good compromise between dissolution of the crude product and separation of **1** and **2**. Void volume was calculated using 1, 3, 5-Tri-t-butylbenzene as void volume marker[15].

Table 1.Stationary phase screening for pneumocandin B_0 and C_0 isomers

Stationary phase	Mobile phase	K'(B_0)	K' (C_0)	Selectivity
KR60-5-SIL	EtOAc[a]/MeOH[b]/water 84/9/7 (v/v/v)	7.72	9.71	1.25
KR60-5-diol	EtOAc/MeOH/water 84/9/7 (v/v/v)	17.06	20.25	1.18
KR60-5-CN	EtOAc/MeOH 80/20 (v/v)	0.23	----	Co-elution
KR100-5-NH_2	EtOAc/MeOH/water 84/9/7 (v/v/v)	20.16	23.36	1.15

The crude pneumocandin B_o is 80-85% pure with 10.6% C_0 isomer and an assay of about 49%.The closely related chiral isomers were separated on KR60-SIL column using ethyl acetate–methanol–water mixture (84:9:7, v/v/v) as the mobile phase with good selectivity and resolution even at a high loading of 2.5 mg (Table 2). The flow rate and loading plots are shown in figure 1. It may be noticed that even at high flow rates and sample loadings, the B_0 and C_0 peaks are well resolved. At 0.5 mg sample loading, with the increase in the flow rates, the theoretical plates and resolution decreased as anticipated, while the column back pressure increased with it. At higher sample loadings, ranging between 1 mg to 2.5 mg, all the analytical parameters were more or less similar that observed for the 0.5 mg sample loading. However, at 3.5 mg loading, the theoretical plates and the resolution drastically decreased. In addition, it is evident from the chromatograms that at higher flow rates, the resolution of the peaks decrease, and both peaks follow linear adsorption at all column loadings. The product was also purified in a Varicol process to about 93.38 % (with 0.8% C_0 content) with an assay of about 80% with a feed concentration of 10 g/L, using five columns with a distribution of 0.9 (zone 1), 1.5 (zone 2), 1.5 (zone 3) and 1.1 (zone 4), and with feed, eluent, extract, raffinate and recycling flow rates of 21.05 mL min^{-1}, 109.98 mL min^{-1}, 76.5 mL min^{-1}, 54.46 mL min^{-1} and 303.8 mL min^{-1}, respectively. This material could be further purified to the desired level by conventional crystallization.

Table 2.HPLC analytical parameters for pneumocandin B_0 and C_0 isomers at various flow rates and sample loadings.

Flow Rate (mL/min)	Wave length (nm)	Concentration (µg/µL)	Quantity injected (µg)	N1	N2	K_1'	K_2'	α	Resolution	Pressure drop (bar)
LiCrosphere Si-60, 16 µm, 4.0×250 mm; Mobile phase: EtOAc-MeOH-water (84:9:7, v/v/v); Solubility in mobile phase: 35 g/L										
1.5	278	25	500	1158	474	8.09	11	1.26	1.8	1.65
3.0	278	25	500	689	314	12.79	16.96	1.31	1.30	6.75
7.5	278	25	500	335	---	12.85	16.71	1.30	---	15.5
1.5	278	25	500	1199	452	8.59	11.74	1.37	1.80	---
1.5	278	25	1000	1172	467	8.68	11.79	1.36	1.80	---
1.5	278	25	1500	1147	458	8.76	11.85	1.35	1.70	---
1.5	278	25	2000	1116	454	8.71	11.72	1.34	1.70	---

1.5 1.5	278 278	25 25	2500 3500	1262 1027	465 16	8.70 8.89	11.67 10.77	1.34 1.21	1.70 1.0	--- ---

[a]EtOAC: ethyl acetate; [b]MeOH: methanol

[a]Number of theoretical plates that a real column possesses, $N = 5.55 \times t_R^2/w_{1/2}^2$, where $w_{1/2}$ is the peak width at half-height.

[b]Retention factor, $k_1' = (t_R - t_M) / t_M$, where t_R is the retention time and t_M is the time taken for the mobile phase to pass through the column.

[c]Selectivity factor (α) is the ratio of relative retention factors (k'); $\alpha = k_2' / k_1'$

[d]Resolution factor, $R_s = (t_{R2} - t_{R1}) / 0.5 \times (t_{w1} + t_{w2})$, where t_R is the retention time and t_w is obtained from the intersection of the tangents with the baseline

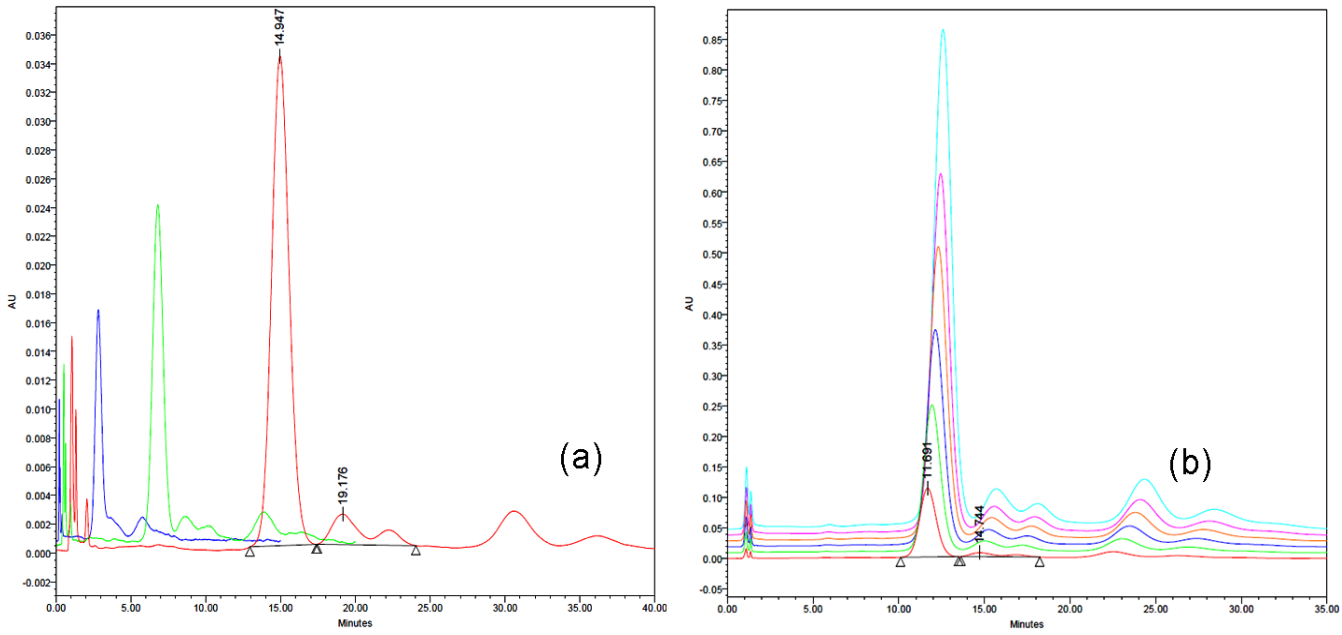

Figure 1. Separation of pneumocandin B_0 and C_0 isomers using EtOAc-MeOH-water (84:9:7, v/v/v)on KR60-16-SIL (16µm, 4.6×250 mm) at different (a) flow rates and (b) column loadings.

APPLICATIONS

The prepared compounds are important API as described above. Pneumocandin**1** is a natural product produced as a secondary metabolite by fermentation of the fungus glarea lozoyensis[2-3]. It is a key intermediate in the production of caspofungin acetate[4-6]. It acts by blocking the synthesis of a major fungal cell wall component, 1-3-beta-D-glucan[7-9].

CONCLUSIONS

HPLC method for separation of closely related B_0 and C_0 isomers of pneumocandin was developed using silica gel. Purification of pneumocandin B_0 were scaled up in a continuous process using Varicol technology, an SMB variant. The study is quite unique - while separation of enantiomers or chiral compounds on chiral CSPs in SMB or its advanced versions like Varicol process is quite obvious,

separation of chiral isomers, particularly when the isomer ratio is far from 50:50 composition, is uncommon.

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