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Direct chiral resolution of 15-deoxyspergualin using a cellobiohydrolase liquid chromatographic column

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Abstract

An LC method for the direct determination of the enantiomers of 15-deoxyspergualin was developed, optimized and validated. A commercially available LC column containing a cellulase enzyme (cellobiohydrolase I) stationary phase was used. The effect of various parameters on the separation and validation data are discussed.

Keywords: CBH-I; Cellobiohydrolase I; Resolution chiral; 15-Deoxyspergualin; Liquid chromatography

1. Introduction

15-Deoxyspergualin (DSG) is a synthetic analogue of spergualin, an antibiotic produced by *Bacillus laterosporus* [1]. DSG has been shown to possess antitumor and immunosuppressive activity [1–6]. The molecule (Fig. 1) contains a single asymmetric carbon and has two possible enantiomers. The total synthesis of racemic DSG [4] and the synthesis of optically active enantiomers [2] have been reported. The configuration about the C-11 asymmetric carbon is significant because the *S*-(−)-enantiomer has been shown to be the active form [2]. An indirect, chiral liquid chromatographic (LC) method utilizing 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as the chiral derivatization reagent has been developed [2]. In practice, the GITC derivatization method for DSG was shown to be cumbersome owing to the lengthy sample preparation procedure time of 60 min and chromatographic run time of 200 min [7]. In addition, only partial resolution of the enantiomers was obtained [7]. Therefore, a simpler and more efficient, direct method was sought to facilitate research and development on DSG.

In this investigation, the cellobiohydrolase I (CBH-1) LC column was the only commercially available LC column found to resolve the enantiomers of DSG. The CBH-1 column, invented by Erlandsson and co-workers [8,9] contains a cellulase enzyme as the chiral stationary phase, and has been shown to resolve the enantiomers of several β-blockers and other drug compounds.

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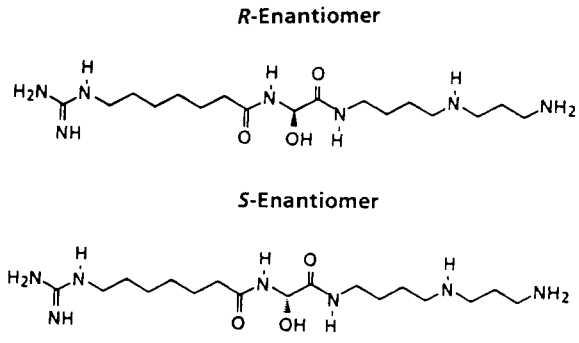

Fig. 1. Structures of the *R*- and *S*-enantiomers of 15-deoxyspergualin.

Additional chiral drugs were resolved when using CBH-1 as a chiral selector for capillary electrophoresis [10]. CBH-1 is a cellulolytic enzyme produced by the fungus *Trichoderma reesei* [11,12]. It is wedge-like in shape, possesses amphiphilic properties and catalyzes the hydrolysis of the non-reducing ends of cellulose chains to the disaccharide cellobiose [11]. Its structure consists of a catalytically active domain, a terminal domain and a glycosylated linker region connecting the two domains [11]. The role of the terminal domain and linker region is to bind cellulose and enhance the activity of the enzyme toward cellulose [11]. Because exogluconases such as CBH-1 have been reported to possess a tunnel-type opening to their active site and a region where the long, floppy polymer chain of cellulose can by attached for insertion [11,12], one can speculate that DSG, having an elongated, floppy structure, is a potential candidate for chiral recognition at a CBH-1 binding site. It is interesting that many of the analytes previously resolved by the CBH-1 column contain a hydroxyl group bound to an asymmetric carbon that is on a methylene chain containing adjacent amino groups [8–10].

The chiral assay developed for bulk DSG using the CBH-1 column was optimized by varying the parameters pH, organic solvent content, buffer concentration, sample concentration, column temperature and wavelength of UV absorption detection. Under the optimized conditions, the limit of detection (LD) for each enantiomer was estimated to be $<0.1\%$ (w/w), the resolution obtained was baseline (resolution ≥ 2.0) and the chromatographic runtime was reduced to 20 min. A description of the method, the effect of parameters and the validation data are reported and discussed.

2. Experimental

2.1. Chemicals

The $R(+)$- and $S(-)$-enantiomers and the racemate of DSG (15-deoxyspergualin, 1-amino-19-guanidino-11-hydroxy-4,9,12-triazanonadecane-10,13-dione) were obtained from the Bristol–Myers Squibb Pharmaceutical Research Institute. HPLC-grade potassium phosphate monobasic, 2-propanol (IPA) and acetonitrile (ACN) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ethylenediaminetetraacetic acid (EDTA; disodium salt dihydrate) was obtained from Aldrich (St. Louis, MO, USA). Distilled, deionized water was used throughout.

2.2. Chromatographic conditions, apparatus and instrumentation

The CBH-1 chiral analytical column, (150 × 4.0 mm i.d.), was purchased from Advanced Separations Technology (Whippany, NJ, USA). It is also available through the manufacturer (Chromtech, Sweden). Mobile phases, which were clear, were filtered through a 0.45 μm sintered-glass funnel and sonicated for about 10 min to remove air bubbles. The mobile phases were kept covered and were stored at 4°C when not in use to retard bacterial growth. The column temperature was controlled using a Model 7950 column thermostat from Jones Chromatography (Boulder, CO, USA). The LC system consisted of a Beckman Model 110B single-piston pump (Beckman, Somerset, NJ, USA), a Perkin-Elmer Model ISS-100 autoinjector with a 20 μl sample loop (Perkin Elmer–ABI, Elmwood Park, NJ, USA), and an ABI 783 variable-wavelength UV absorption detector. Data acquisition was accomplished using a VG Multichrom system (VG/Fisons, Danvers, MA, USA). Equilibration of the LC system took about 60 min at a flow rate of 0.5 ml min^{-1}. The column could be left in the chromatographic sys-

Table 1
Effect of pH on retention and resolution[a]

pH	S-Enantiomer		R-Enantiomer		R_s[d]
	t_r[b]	k'[c]	t_r[b]	k'[c]	
4.0	2.7	0	2.7	0	No resolution
5.0	4.1	0.52	4.1	0.52	No resolution
5.5	22.1	7.19	22.6	7.37	1.2
6.0	Retained	–	Retained	–	Retained > 60 min, not measured

[a]LC conditions: 0.06 M potassium phosphate, pH adjusted with orthophosphoric acid, 0.5 ml min^{-1}, ambient column temperature, sample concentration 0.05 mg ml^{-1} racemate in mobile phase, detection at 195 nm.
[b]t_r = Retention time in minutes.
[c]Capacity factor, $k' = (t_r - t_{r0})/t_{r0}$, where t_{r0} is the retention time (min) of the void volume or system peak.
[d]Resolution $R_s = 2(t_{r2} - t_{r1})/(W_1 + W_2)$, where t_{r1} and t_{r2} are the retention times of each enantiomer and W_1 and W_2 are the peak widths at the baseline. The procedure is described in Ref. [13].

tem overnight. When not in use, the column was stored in a solution of 8% IPA–water. The optimum sample concentration was 0.05 mg ml in the mobile phase. If a decrease in resolution occurred with time, the column could be regenerated by rinsing it with 2.5 mM EDTA for about 1 h at 0.5 ml min^{-1}, as recommended by the manufacturer.

3. Results and discussion

3.1. Initial separation

DSG's lack of structural rigidity increases the challenge of developing a chiral assay. The molecule lacks an aromatic moiety or a cyclic ring structure which would provide the possibility of the typical intermolecular interactions required of many of the chiral selectors such as π to π interactions or inclusion complexation. In fact, several chiral LC column types and capillary electrophoresis (CE) techniques failed to achieve chiral resolution. Resolution of the enantiomers of DSG was not attained using α_1-acid glycoprotein, bovine serum albumin, human serum albumin, ovomucoid, β-cyclodextrin, valine (Sumichiral OA 3100) or cellulose LC columns (Chiralcel OB, OC, OD, OD-R and OJ), or by using CE with various cyclodextrins, proteins, enzymes and chiral surfactants as chiral selectors. Because CBH-1 was found to be commercially unavailable at the time of the investigation, it could not be tried as a chiral selector using CE. The chiral LC columns were investigated under a variety of mobile phase conditions. Partial resolution was initially observed using the CBH-1 column in combination with a mobile phase of 0.02 M potassium phosphate (pH 5.5) at ambient temperature. Variation of the parameters ensued to optimize the separation.

3.2. Variation of parameters

The initial separation was obtained by varying the pH of a phosphate buffer mobile phase. As shown in Table 1, pH is a critical parameter and the range for optimum resolution is narrow (pH 5.5 ± 0.2). The acidity constants for the amino groups of DSG have not been determined. However, based on comparison with similar molecules (amino acids such as lysine), it is most likely that the guanidino, 1-amino and 4-amino functional groups remain protonated under the conditions of pH that were studied [14,15]. In particular, the pK_a of the primary amine has been estimated to be between 9 and 10 [14,15]. Therefore, the changes in the chromatographic properties of DSG with pH shown in Table 1 are not likely to be due to a change in the state of protonation of the analyte but are probably due to a change in the surface charge and the conformation of the enzyme. The isoelectric point of CBH-1 is pH 3.9 [9]. Assuming the bound enzyme has similar prop-

erties to those when in solution, the enzyme is nearly neutral at pH 4.0. Therefore, the polar, tri-protonated DSG has no apparent affinity for the stationary phase at pH 4.0 and elutes with the system peak at 2.7 min (Table 1). As the pH is increased and the net negative charge of the enzyme becomes larger, there is a greater electrostatic attraction of the cationic analyte for the stationary phase, resulting in increased retention times. The conformation of CBH-1 has been studied using circular dichroism and appears to be pH dependent [9]. It was found that reversible conformational changes occur between pH 2.2 and 8.1 [9]. A change in conformation could also affect the properties of the binding site, which would also alter enantioselectivity and elution times.

It is well known that the solution properties of certain proteins (conformation, binding behaviour, etc.) can be affected by temperature and by organic and inorganic additives. Indeed, it is the variable properties of proteins that can be manipulated by solution additives that make them broadly applicable in terms of attaining enantioselectivity for a large number of drug compounds of various structural types. In support of this supposition with respect to CBH-1, solution parameters such as ionic strength, organic solvent additives and temperature were shown to have a significant effect upon the chromatographic properties of DSG.

The gradual addition of ACN (0–5%, v/v) to the mobile phase reduced the retention times but significantly increased the resolution (Table 2). Maximum resolution ($R_s = 2.4$) was obtained at 5% (v/v) ACN. At 0% ACN, R_s was 1.8. The gradual addition of IPA (0–5%) reduced the resolution slightly and reduced the retention times to a lesser extent than ACN in spite of IPA being the stronger solvent (Table 3). Marle et al. [9] have reported that increasing the concentration of IPA reduced the retention and increased the enantioselectivity for several amines. In the case of DSG, the addition of ACN appears to parallel the reported trends. However, IPA has a deleterious effect on resolution. Thus, the role of organic modifier is more complex than previously reported. A discussion on the types of possible interaction of solution additives with α_1-acid glycoprotein can be found in Ref. [16] and may be comparable.

A sub-ambient temperature was found to be advantagious since the resolution could be im-

Table 2
Effect of acetonitrile on retention and resolution[a]

Acetonitrile (%, v/v)	*S*-Enantiomer		*R*-Enantiomer		R_s
	t_r (min)	k'	t_r (min)	k'	
0.0	26.85	9.35	33.18	11.77	1.79
0.6	20.84	7.02	26.14	9.05	1.90
1.3	18.47	6.12	23.40	8.00	2.02
2.5	14.80	4.69	18.95	6.29	2.16
5.0	10.28	2.95	13.45	4.17	2.41
8.0	9.34	2.59	12.08	3.65	2.32

[a]LC conditions: 0.06 M potassium phosphate (pH 5.5), 0.5 ml min^{-1}, column temperature 5°C, sample concentration 0.05 mg ml^{-1} racemate in mobile phase, UV detection at 195 nm.

Table 3
Effect of 2-propanol on retention and resolution[a]

IPA (%, v/v)	*S*-Enantiomer		*R*-Enantiomer		R_s
	t_r (min)	k'	t_r (min)	k'	
0.0	26.85	9.33	33.18	11.76	1.79
1.0	25.34	8.75	31.10	10.96	1.78
3.0	25.14	8.67	30.63	10.78	1.71
5.0	23.42	8.01	30.16	10.60	1.56

[a]LC conditions: 0.06 M potassium phosphate (pH 5.5), 0.5 ml min^{-1}, column temperature 5°C, sample concentration 0.05 mg ml^{-1} racemate in mobile phase, UV detection at 195 nm.

Table 4
Effect of column temperature on retention and resolution[a]

Temperature (°C)	*S*-Enantiomer		*R*-Enantiomer		R_s
	t_r (min)	k'	t_r (min)	k'	
Ambient (about 22°C)	10.05	2.88	12.05	3.63	1.77
15	10.79	3.15	13.09	4.03	1.91
10	10.62	3.08	13.36	4.14	2.09
5	10.96	3.21	14.15	4.44	2.30

[a]LC conditions: 5% (v/v) ACN–0.06 M potassium phosphate (pH 5.5), 0.5 ml min^{-1}, sample concentration 0.05 mg ml^{-1} racemate in mobile phase, UV detection at 195 nm.

Table 5
Effect of potassium phosphate concentration on retention and resolution[a]

Potassium phosphate concentration (M)	S-Enantiomer		R-Enantiomer		R_s
	t_r (min)	k'	t_r (min)	k'	
0.015	56.88	20.88	74.19	27.53	1.74
0.03	33.39	11.84	43.24	15.63	2.07
0.06	12.14	3.67	15.61	5.00	2.24

[a]LC conditions: 5% (v/v) ACN (pH 5.5), 0.5 ml min^{-1}, column temperature 5°C, sample concentration 0.05 mg ml^{-1} racemate in mobile phase, UV detection at 195 nm.

proved by lowering the column temperature to 5°C (Table 4). The enhancement of resolution by using temperature as a variable when employing the various protein columns has been reported [17]. The potassium phosphate concentration was found to be a significant parameter (Table 5). Retention times decreased and the resolution increased on raising the phosphate concentration from 0.015 to 0.06 M. Using the studied variables, the separation was optimized and is shown in Fig. 2.

3.3. Assay validation and application

The enantiomeric assay was partially validated under the optimized conditions given in Fig. 2. The linearity of the response of each enantiomer was excellent in the 0.01–0.1 mg ml^{-1} concentration range (correlation coefficient >0.99). Owing to the poor sample-loading capacity of the column, the analytical concentration of sample required is 0.05 mg ml^{-1}. Above this concentration, there is some loss in resolution that is likely due to overloading of the active sites of the stationary phase [9]. The linearity of each enantiomer's response was excellent in the lower concentration range of 0.4–5.0% (w/w relative to the main sample component of the opposite enantiomer at 0.05 mg ml^{-1}) (correlation coefficient >0.99 for each enantiomer). The reproducibility of response was satisfactory for each enantiomer (concentration of 0.05 mg ml^{-1} in mobile phase) with RSDs of 0.8% and 1.3% for six determinations of the S- and R-enantiomers, respectively. Sensitivity was shown to be maximized using 195 nm as the wavelength of detection.

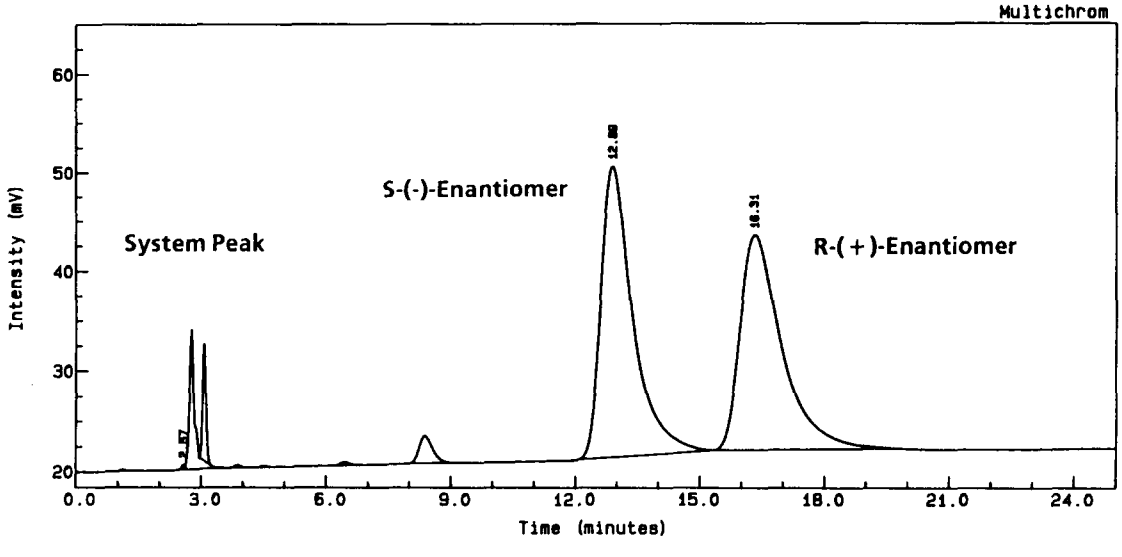

Fig. 2. Chromatogram of batch 61BR of the racemic mixture of 15-deoxyspergualin under the optimized conditions: 5% (v/v) ACN–0.05 M potassium phosphate (pH 5.5), 0.5 ml min^{-1}, sample concentration 0.05 mg ml^{-1} in mobile phase, column temperature 5°C, UV detection at 195 nm.

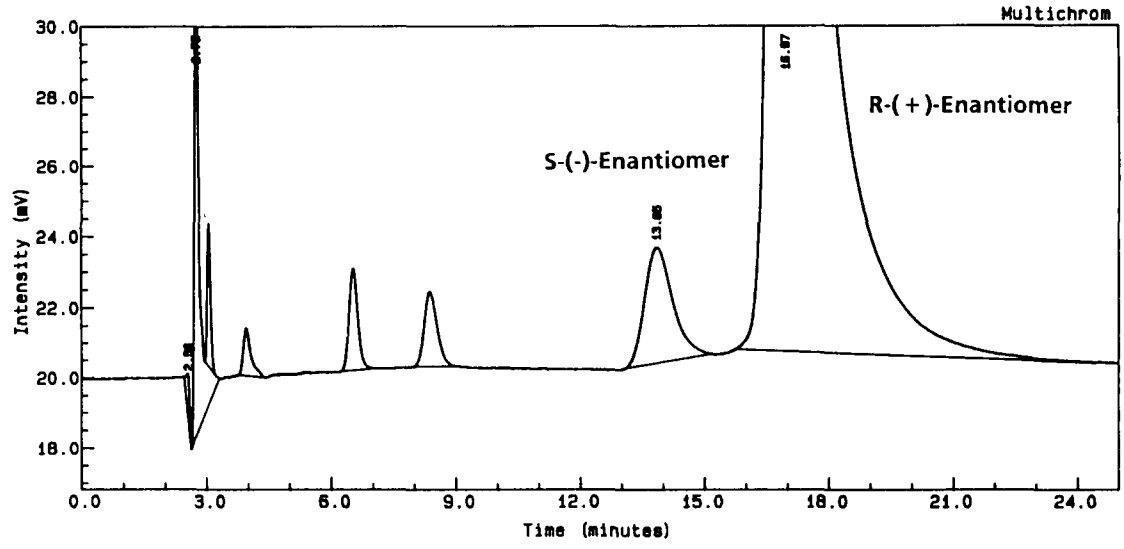

Fig. 3. Chromatogram of batch 003 of the *R*-enantiomer containing about 4.5% of the *S*-enantiomer. Chromatographic conditions as in Fig. 2.

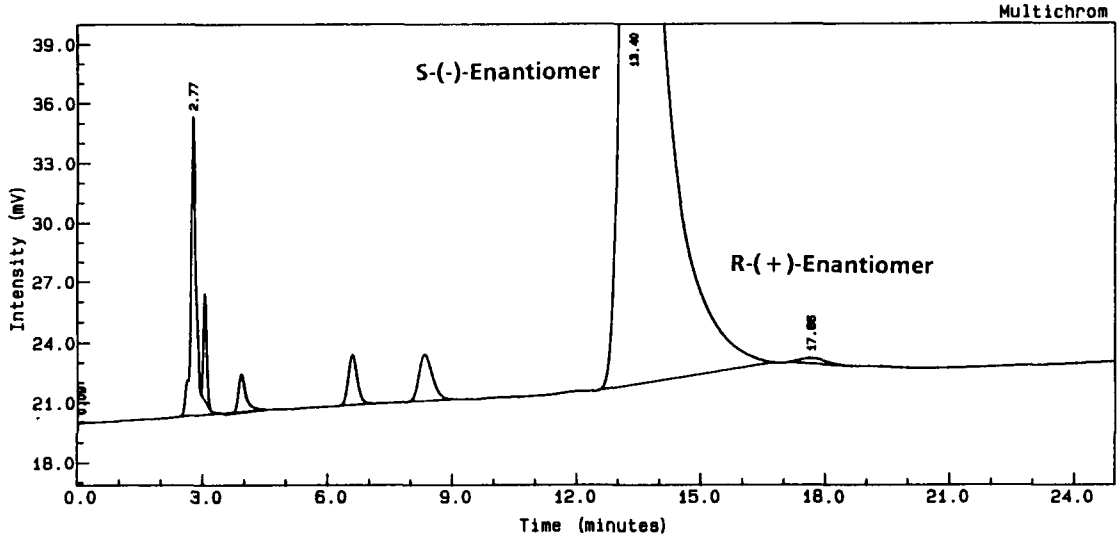

Fig. 4. Chromatogram of batch 003 of the *S*-enantiomer containing about 0.7% of the *R*-enantiomer. Chromatographic conditions as in Fig. 2.

Chromatograms of a typical batch of each enantiomer are shown in Figs. 3 and 4. A typical batch of a racemic mixture is shown in Fig. 2. The limit of detection of each enantiomer was estimated to be about 0.1% (w/w) (0.050 μg ml^{-1}) by a statistical linearity method [18,19].

Interconversion was not detected for enantiomers and racemic samples kept for 48 h at room temperature and in the mobile phase. The ruggedness of the method appeared acceptable since there was no significant column-to-column variation. Care must be taken to rinse the column

with IPA–water prior to storage. Leaving the column in mobile phase for longer than 1 day may result in a loss of column performance in terms of resolution and peak shape.

4. Conclusion

A direct, chiral LC method for 15-deoxyspergualin was developed using a commercially available cellobiohydrolase I column. The parameters organic solvent, pH, ionic strength and column temperature were found to affect significantly the enantioselectivity and retention times. Using a wavelength of detection of 195 nm, the detection limits for each enantiomer were estimated to be $<0.1\%$ (w/w). The method affords baseline resolution with an efficient run time of 20 min and qualifies for use as a stereospecific identity test, an assay required by the US Food and Drug Administration [20].

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