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Direct and indirect separations of five isomers of Brivanib Alaninate using chiral high-performance liquid chromatography☆

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ABSTRACT

Brivanib Alaninate is a novel chiral prodrug possessing two stereogenic centers. Simultaneous HPLC separation of five isomers of Brivanib Alaninate was systematically investigated on a wide variety of polysaccharide-based chiral stationary phases (CSPs) using underivatization and pre-column derivatization methods. The influence of derivatizing groups and mobile phase composition on the enantioseparation and retention behavior of Brivanib Alaninate compounds was studied. To better understand the chiral recognition mechanism, the temperature effect was also evaluated. The results of these studies led to the first complete HPLC resolution of all five isomers of Brivanib Alaninate as carbobenzyloxy (CBZ) derivatives on a cellulose benzoate CSP (OJ-H).

1. Introduction

Molecular chirality plays a central role in receptor–ligand interactions and functional biologic effects. Therefore the control and determination of the enantiomeric composition of chiral drug substances is a key issue for both the pharmaceutical industry and regulatory agencies [1]. Over the past three decades, high-performance liquid chromatography (HPLC) has been the most productive tool for resolving enantiomers for both analytical and preparative purposes, and its separation power has had a tremendous impact on the pharmaceutical industry [2]. Chiral separations can be achieved with HPLC through the following approaches: (a) direct separation of racemates to their corresponding enantiomers using chiral stationary phases (CSPs) [3]; or (b) indirect separation of diastereoisomers, formed by the reaction of the enantiomers with a chiral derivatizing agent, using achiral stationary phases [4]; or (c) separation of chiral derivatives, formed by the reaction with non-chiral derivatizating agents, using CSPs [5–10]. Direct methods based on CSPs are the preferred separation approaches, since they are simple and rapid to apply at both analytical and preparative scales. However, indirect methods often offer enhanced selectivity and detection sensitivity [8–10].

Enantioseparation of chiral compounds containing multiple stereogenic centers poses unique analytical challenges to the analyst [11]. A complete resolution of all potential stereoisomers for these compounds can only be achieved if the CSP used is highly discriminating for both enantiomers and diastereomers. This paper will highlight the challenges we faced in developing a chiral HPLC separation for Brivanib Alaninate, (S)-((R)-1-(4-(4-fluoro-2-methyl-1*H*-indol-5-yloxy)-5-methylpyrrolo[2,1-f][1,2,4]triazin-6-yloxy)propan-2-yl)2-aminopropanoate, a novel chiral prodrug possessing two stereogenic centers [12]. In addition to the four stereoisomers, Brivanib Alaninate also has the potential to form a positional isomer due to a moderately regioselective synthetic step (Fig. 1). A comprehensive HPLC method development approach was the proposed strategy for separating all five isomers of Brivanib Alaninate. This strategy was based on the consideration that a CSP is highly effective in resolving enantiomers with complementary intermolecular interaction sites [13]. When the method development strategy was executed, several different CSPs including CHIROBIOTIC V2, SUMICHIRAL 4800, ChiroSil SCA (+) and RCA (-), CYCLOBOND I 2000 RSP and polysaccharide-based CSPs were identified to possess enantioselectivity for both enantiomeric pairs (SR/RS and RR/SS) of Brivanib Alaninate. However, only polysaccharide-based CSPs showed the promise of simultaneously separating all five isomers using either direct or indirect methods [14].

Among the commercially available polysaccharide-based CSPs, polysaccharide phenylcarbamates and esters/benzoates represent an important class of CSPs, which have shown

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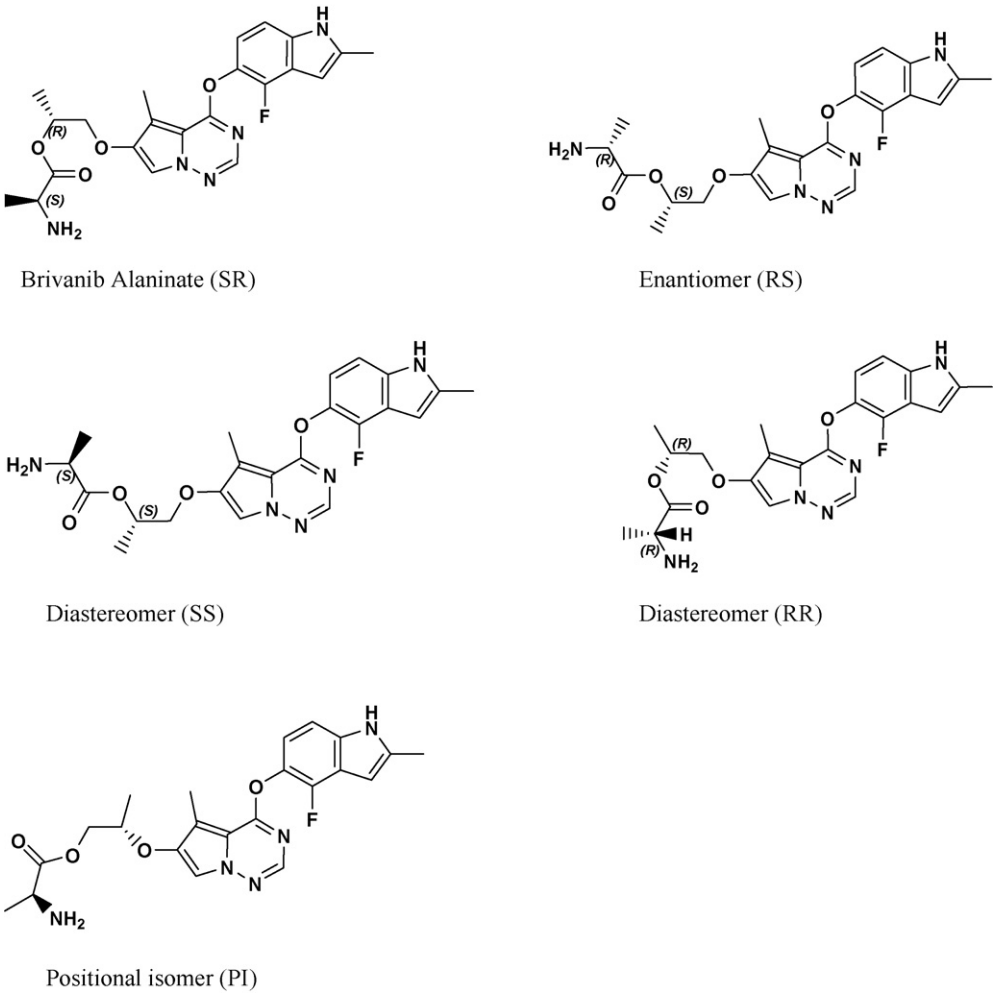

Fig. 1. Stereochemical structures of five isomers of Brivanib Alaninate.

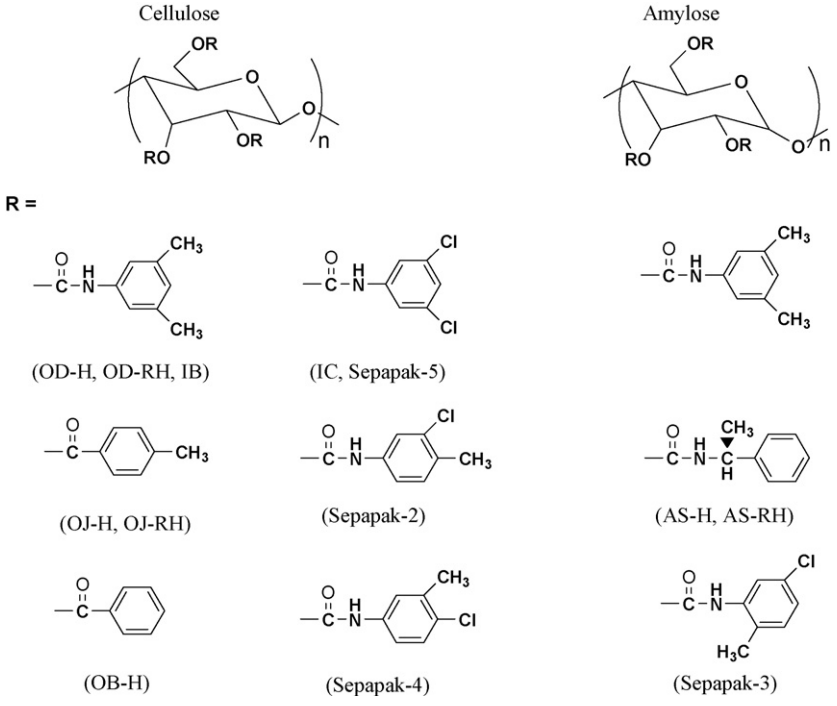

Fig. 2. Structures of derivatized cellulose and amylose chiral stationary phases used in the study.

Table 1
Chromatographic parameters for the direct enantioseparations of the two enantiomeric pairs of Brivanib Alaninate on polysaccharide stationary phases

Column	SR/RS				SS/RR				Mobile phase
	k'_1	α	R_s	First eluted[a]	k'_1	α	R_s	First eluted[a]	
Reversed phase									
AD-RH	4.16	1.01	0.4	SR	3.65	1.11	0.8	SS	A[b]
OD-RH	5.89	1.12	1.78	SR	5.64	1.02	0.6	RR	A[b]
OJ-RH	2.57	1.08	1.0	SR	2.57	–	–	–	A[b]
AS-RH	1.80	–[c]	–	–	1.72	–	–	–	A[b]
Sepapak-5	2.27	1.10	1.17	RS	2.37	1.18	2.11	SS	B
Polar organic phase									
AD-H	0.96	–	–	–	0.88	–	–	–	C
	0.64	–	–	–	0.63	1.10	0.6	SS	D
IA	0.85	–	–	–	0.87	–	–	–	C
	0.69	–	–	–	0.69	1.08	0.6	SS	D
OD-H	0.33	–	–	–	0.32	–	–	–	C
	0.49	1.21	1.70	SR	0.54	–	–	–	D
IB	0.33	–	–	–	0.33	–	–	–	C
	0.61	1.09	0.8	SR	0.67	–	–	–	D
AS-H	0.70	1.15	0.9	RS	0.69	1.13	0.8	SS	C
OJ-H	1.11	1.15	1.25	SR	1.13	1.11	1.0	RR	C
	0.95	1.23	1.10	SR	1.00	1.17	0.9	RR	F
	0.15	–	–	–	0.17	–	–	–	D
OB-H	0.22	1.54[d]	1.04	SR	0.27	1.25	0.8	RR	C
Sepapak-2	0.60	–	–	–	0.55	1.06	0.6	RR	C
	1.67	1.14	1.63	SR	1.79	–	–	–	D
Sepapak-3	0.34	–	–	–	0.33	1.10	0.6	SS	C
	0.57	–	–	–	0.61	1.10	0.5	SS	E
Sepapak-4	0.50	1.13	0.96	SR	0.46	1.20	1.35	RR	C
	0.42	1.12	0.7	SR	0.39	1.27	1.18	RR	F
	1.77	1.15	1.78	SR	1.86	1.11	1.36	RR	D
Sepapak-5	0.49	–	–	–	0.45	–	–	–	C
	1.54	–	–	–	1.69	1.12	0.8	SS	D
IC	1.15	1.05	0.7	SR	1.30	1.05	0.5	RR	C
	1.16	–	–	–	1.25	–	–	–	D
Normal phase									
AD-H	4.30	1.08	0.9	SR	4.54	1.14	2.00	SS	G[e]
IA	0.68	–	–	–	0.69	1.13	0.8	SS	H
OD-H	1.82	1.25	2.03	SR	1.83	1.08	0.8	RR	G[b]
IB	0.96	1.09	0.7	SR	0.99	–	–	–	H
AS-H	3.80	1.06	0.9	RS	3.80	1.17	2.07	SS	G[b]
OJ-H	6.46	1.09	1.25	SR	6.49	1.06	0.8	RR	H[b]
OB-H	1.96	–	–	–	2.05	–	–	–	H
IC	2.58	1.11	1.0	SR	2.46	1.19	1.46	RR	H[e]
	1.54	1.23	1.35	SR					I[e]
	0.75	1.27	1.68	SR	0.79	1.23	1.41	RR	J[e]
	3.17	1.24	3.17	SR	3.33	1.21	2.71	RR	K[e]
Sepapak-2	5.99	1.08	1.65	SR	5.89	1.13	2.79	RR	L[b]
Sepapak-3	1.82	–	–	–	1.87	–	–	–	H
Sepapak-4	1.72	1.20	1.43	SR	1.43	1.42	2.76	RR	H[e]

Mobile phase: (A) 20 mM NH_4OAc in water:ACN 60:40 (v/v); (B) 20 mM NH_4OAc in water:ACN 50:50 (v/v); (C) MeOH; (D) ACN with 0.1%DEA; (E) ACN; (F) EtOH; (G) IPA:Hexane:DEA 20:80:0.1 (v/v/v); (H) IPA:Hexane:DEA 50:50:0.1 (v/v/v); (I) MTBE:IPA:DEA 95:5:0.1 (v/v/v); (J) MTBE:MeOH:DEA 95:5:0.1 (v/v/v); (K) MTBE:Hexane:MeOH:DEA 50:45:5:0.1 (v/v/v/v); (L) EtOH:Hexane 15:85 (v/v).

[a] Configuration of the first eluted peak.
[b] 40 °C.
[c] No separation.
[d] Large α value may be a result of inaccurate measurement of k'.
[e] 25 °C.

excellent enantioselectivity for a wide range of racemates possessing a variety of chemical functionalities [15]. The chiral recognition ability of the polysaccharide stationary phases is influenced significantly by the substituents introduced on the phenyl group of the polymer [16–18]. The introduction of an electron-donating methyl group or an electron-withdrawing halogen at the meta- and/or para-position of the phenyl ring often improves the chiral recognition ability of the CSPs [19]. These modified polysaccharide CSPs have been successfully applied to enantioseparations utilizing normal [20,21], polar organic [21,22],

Table 2
Chromatographic parameters for the enantioseparations of the two derivatized enantiomeric pairs of Brivanib Alaninate on polysaccharide stationary phases

Column	Derivative	SR/RS				SS/RR				Mobile phase
		k'_1	α	R_s	First eluted[a]	k'_1	α	R_s	First eluted[a]	
Reversed phase										
AD-RH	AQC	3.54	1.29	1.0	RS	2.80	1.28	0.8	RR	A
	CBZ	6.78	–[b]	–	–	7.85	1.14	0.7	SS	B
	PTC	3.56	–	–	–	3.56	–	–	–	B
OD-RH	AQC	3.18	1.22	2.55	RS	3.18	1.13	1.53	RR	B
	CBZ	15.50	1.02	0.5	SR	16.43	–	–	–	B
	PTC	6.37	1.04	0.7	RS	6.59	1.08	1.24	RR	B
OJ-RH	AQC	1.01	–[b]	–	–	1.09	–	–	–	B
	CBZ	3.80	1.12	1.67	SR	4.44	1.15	2.14	RR	B
	PTC	3.34	1.10	1.21	SR	3.73	1.21	2.61	RR	B
AS-RH	AQC	1.62	1.17	1.14	SR	1.58	1.34	2.16	SS	B
	CBZ	6.22	–[b]	–	–	6.94	–[b]	–	–	B
	PTC	4.66	–[b]	–	–	5.21	1.22	2.37	RR	B
Sepapak-5	AQC	2.37	1.18	2.78	SR	2.44	1.05	0.8	RR	B
	CBZ	3.65	1.16	2.87	SR	4.46	–[b]	–	–	B
	PTC	2.16	1.10	1.65	SR	2.44	1.02	0.6	RR	B
Polar organic phase										
AD-H	CBZ	1.42	1.84	2.81	RS	1.69	1.60	2.28	RR	C
		0.42	–[b]	–	–	0.53	–	–	–	E
IA	CBZ	0.79	1.08	0.5	RS	0.90	–	–	–	C
		0.44	–[b]	–	–	0.50	–	–	–	E
OD-H	CBZ	0.86	1.13	1.17	SR	0.93	–	–	–	C
		0.45	–	–	–	0.45	–	–	–	E
IB	CBZ	0.56	1.06	0.6	SR	0.58	1.05	0.5	RR	C
		0.28	–	–	–	0.36	–	–	–	E
AS-H	CBZ	0.64	1.10	0.6	RS	0.75	1.21	1.35	SS	C
		0.72	–	–	–	0.73	1.19	1.0	SS	E
OJ-H	CBZ	3.74	1.48	4.80	SR	4.40	1.38	3.98	RR	C
		2.27	1.39	2.30	SR	2.65	1.54	3.07	RR	D
		0.03	–	–	–	0.03	–	–	–	E
OB-H	CBZ	0.25	–	–	–	0.29	1.67	0.8	SS	C
		0.15	1.34	0.7	RS	0.16	1.40	0.8	RR	E
IC	CBZ	0.48	1.21	1.76	SR	0.51	1.09	0.8	RR	C
		0.35	1.10	0.5	RS	0.46	–	–	–	E
Sepapak-2	CBZ	0.55	1.21	1.4	SR	0.66	–	–	–	C
		0.59	1.38	1.68	SR	0.70	1.24	1.23	SS	E
Sepapak-3	CBZ	0.62	–	–	–	0.63	1.07	0.6	SS	C
		0.25	–	–	–	0.33	1.18	0.7	RR	E
Sepapak-4	CBZ	0.59	1.27	2.44	SR	0.62	1.13	1.23	RR	C
		0.35	1.50	1.80	SR	0.43	1.07	0.6	RR	D
		0.67	1.54	3.20	SR	0.87	1.07	0.6	SS	E
Sepapak-5	CBZ	0.42	1.28	2.05	SR	0.45	1.10	0.9	RR	C
		0.17	–	–	–	0.15	–	–	–	F
Normal phase										
AD-H	CBZ	1.35	1.18	1.20	SR	2.04	1.13	1.37	RR	G
IA	CBZ	0.81	1.08	0.6	SR	1.08	1.10	0.8	SS	G
OD-H	CBZ	0.47	1.50	2.40	SR	0.56	–[b]	–	–	G
IB	CBZ	0.44	1.27	2.05	SR	0.51	–[b]	–	–	G
AS-H	CBZ	0.35	1.19	0.7	RS	0.43	1.10	0.5	SS	H
OJ-H	CBZ	2.46	1.45	2.80	SR	2.97	1.78	4.30	RR	H
OB-H	CBZ	0.36	1.15	0.6	SR	0.40	1.68	1.0	SS	H
IC	CBZ	0.45	1.34	2.12	SR	0.58	1.07	0.6	RR	G
Sepapak-2	CBZ	0.39	1.95	4.49	SR	0.55	–[b]	–	–	H
Sepapak-3	CBZ	1.02	1.12	0.5	SR	1.37	1.42	1.56	RR	H
Sepapak-4	CBZ	0.35	1.76	3.60	SR	0.44	1.09	0.7	RR	H

Mobile phase: (A) 20 mM NH_4OAc in water:ACN 30:70 (v/v); (B) 20 mM NH_4OAc in water:ACN 40:60 (v/v); (C) MeOH; (D) EtOH; (E) ACN; (F) ACN with 0.1% DEA; (G) EtOH:Hexane 50:50 (v/v); (H) EtOH:Hexane 70:30 (v/v).

[a] Configuration of the first eluted peak.

[b] No separation.

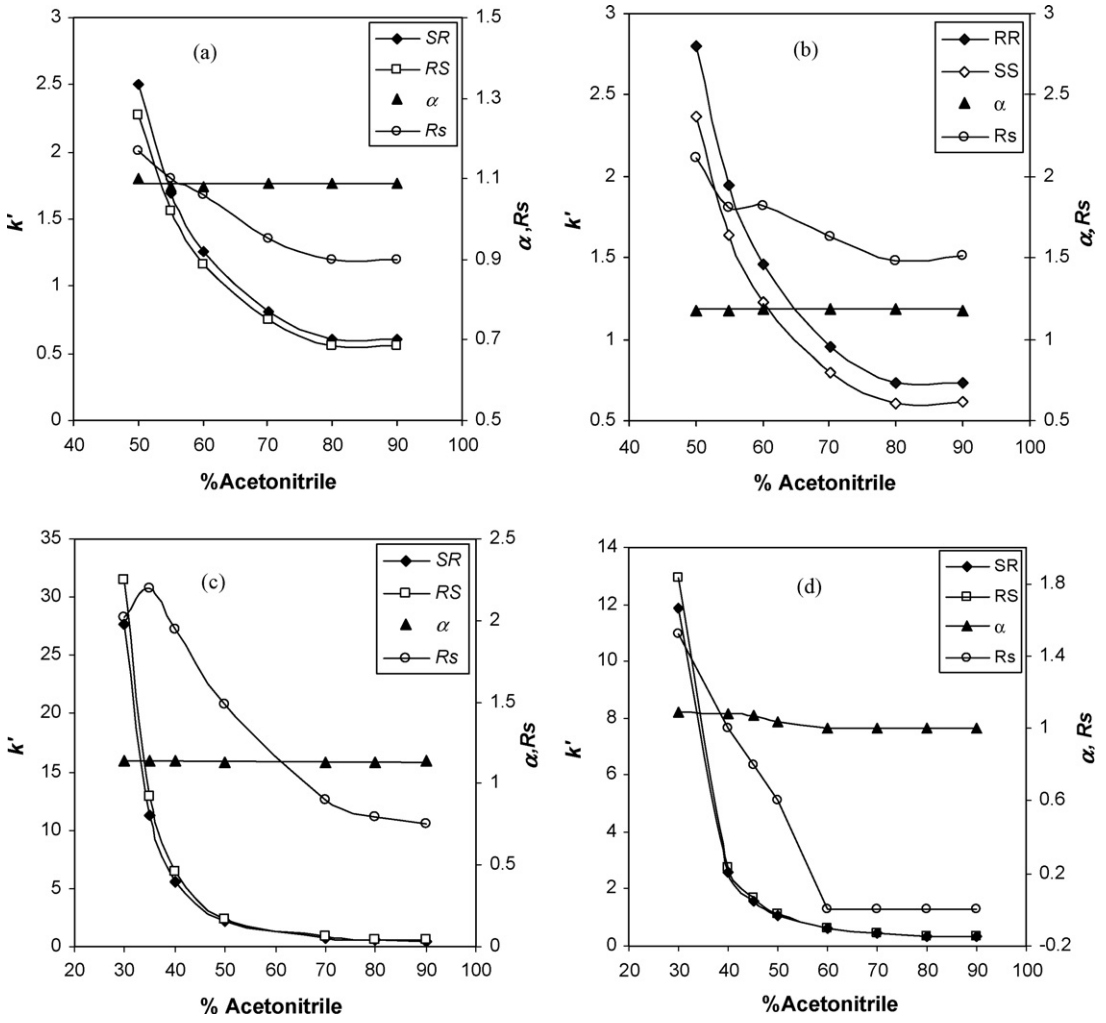

Fig. 3. Effect of the mobile phase composition on the retention (k'), enantioselectivity (α) and resolution (R_s) of Brivanib Alaninate (SR) and its enantiomer (RS) on Sepapak-5 (a and b), OD-RH (c) and OJ-RH column (d).

and reversed-phase modes [22,23]. Applicability in multiple elution modes is highly desirable for CSPs because it provides broader mobile phase selections for chiral separation to achieve desired goals.

In the present work, we report the systematic investigation of the direct and indirect chiral separations of Brivanib Alaninate and its isomers on various polysaccharide-based CSPs under reversed-phase, polar organic phase and normal phase conditions. Because of the number of chiral centers already on the analytes' molecules, derivatization with chiral derivatizing reagents was not applicable. The indirect separations investigated involved three non-chiral derivatizing approaches for the primary amine group of Brivanib Alaninate. A complete resolution of all five isomers following the formation of the carbobenzyloxy (CBZ) derivatives was achieved on a cellulose benzoate phase OJ-H under polar organic mobile phase conditions.

2. Experimental

2.1. Reagents and solvents

The individual stereoisomers and the positional isomer of Brivanib Alaninate and their CBZ derivatives were supplied by Bristol-Myers Squibb Process R&D. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamyl (AQC) and phenylthiocarbamyl (PTC) derivatives of Brivanib Alaninate were prepared according to the procedures in Sections 2.2 and 2.3, respectively. Both 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ·Tag™ reagent) and phenylisothiocyanate (PITC) kits were purchased from Waters (Milford, MA, USA). Ethanol (EtOH), 200-proof and ammonium acetate (NH_4OAc) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Heptane, hexane, diethylamine (DEA) and triethylamine (TEA) were obtained from EMD (San Diego, CA, USA). All other HPLC grade solvents, including methanol (MeOH), acetonitrile (ACN), methyl *tert*-butyl ether (MTBE), and isopropyl alcohol (IPA), were purchased from J.T. Baker (Phillpsburge, NJ, USA). Deionized water was purified through a Barnstead NANOpure® DIamond™ UV ultrapure water system (Dubuque, IA, USA).

2.2. Preparation of AQC derivatives

6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) is a fluorescent derivatizing agent for primary and secondary amines, and often used in amino acid and peptide analysis to enhance the detection sensitivity [5,24]. The AQC derivatives of Brivanib Alaninate were prepared using a modified procedure according to a previously reported method [24], i.e., equal molar amounts of AQC dissolved in ACN and freshly prepared solutions of each compound or mixture (~0.5 mg/mL) in either ACN or IPA were mixed in a vial. The vial was sealed and heated at 60–70 °C for 5 min on a

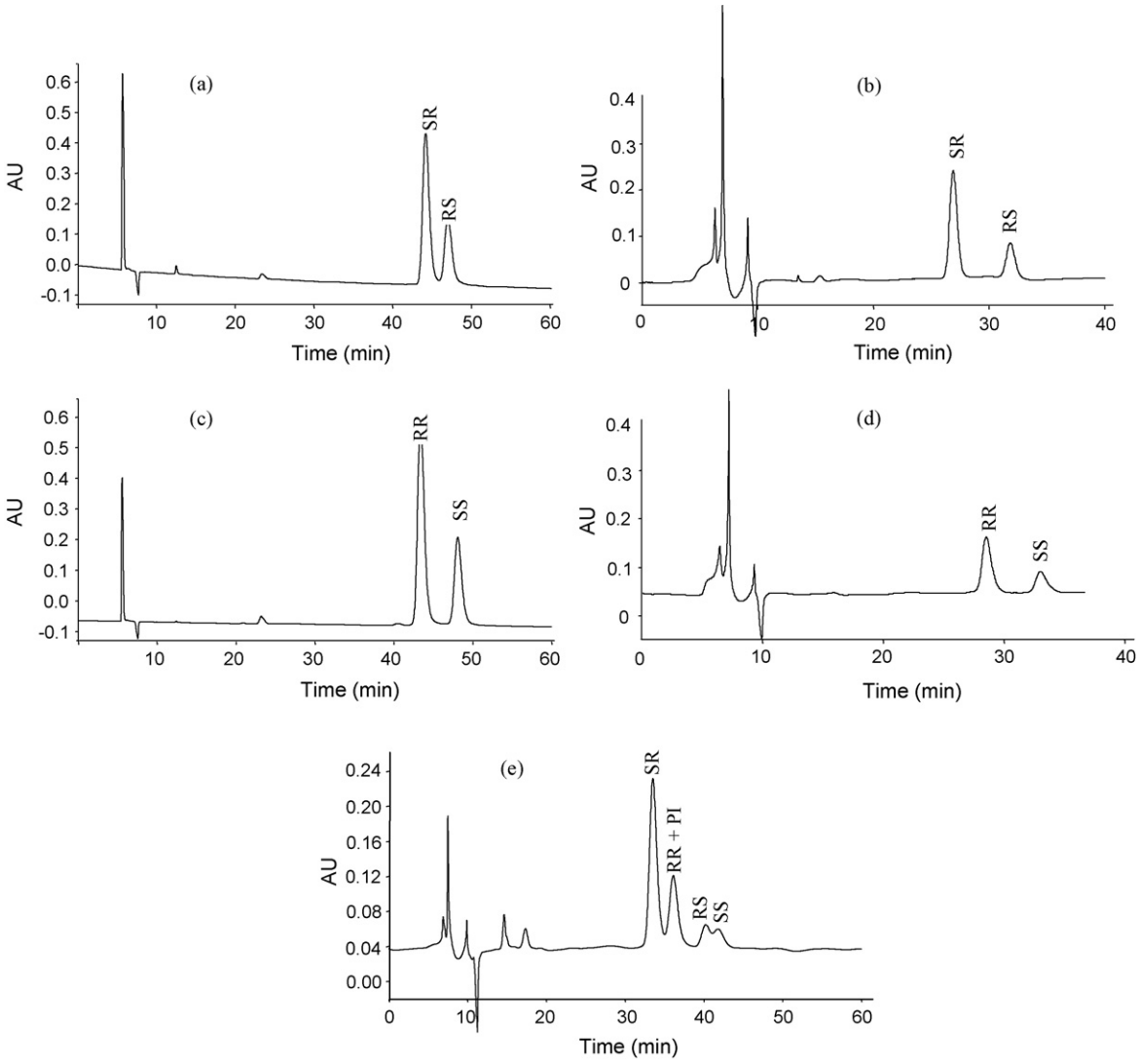

Fig. 4. Direct separation of the two enantiomeric pairs of Brivanib Alaninate on Sepapak-2 (a and c) and IC (b and d) columns, and five isomers of Brivanib Alaninate on IC (e). Mobile phases: (a and c): EtOH:Hexane 15:85 (v/v); (b, d and e): MTBE:Hexane:MeOH:DEA 50:45:5:0.1 (v/v/v/v).

temperature-controlled heating plate. The resulting solution was diluted with ACN and injected into a column without further purification.

2.3. Preparation of PTC derivatives

Phenylisothiocyanate (PITC, or Edman's reagent) was used for pre-column derivatization of amino acids and amino alcohols for reversed-phase HPLC separation [25,26]. The PITC solution was prepared by combining PITC, MeOH and TEA in a ratio of 70:10:5 by volume. The phenylthiocarbamyl (PTC) derivatives of Brivanib Alaninate were prepared by mixing a freshly prepared solution of each compound or mixture (~0.5 mg/mL) in ACN with an excess amount of PITC solution in a vial. After allowing the sample to react at room temperature for 20 min, the resulting solution was diluted with ACN and injected into a column without further purification.

2.4. High-performance liquid chromatography

HPLC separations were performed on a Waters Alliance system, Model 2695 (Milford, MA, USA) equipped with a Waters photodiode array (PDA, Model 2996) detector or a Waters dual wavelength UV–vis (Model 2487) detector. All analytes were dissolved in either ACN or IPA at concentrations ranging from 0.05 to 0.5 mg/mL. Injection volumes ranged from 5 to 10 μL. Chromatographic separations were carried out under isocratic conditions at a flow rate of 0.5 mL/min at ambient temperature, unless otherwise specified. Chromatograms were obtained at 237 nm or 245 nm. Column void times were determined from the first perturbation of baseline after injection of the sample solution. Temperature study was carried out using a HPLC column Chiller/Heater (Model TS-430) from Phenomenex (Torrance, CA, USA).

Sixteen different polysaccharide CSPs were evaluated in this study, including cellulose tris(3,5-dimethylphenylcarbamate) phases (Chiralcel OD-H, OD-RH and IB), amylose tris(3,5-dimethylphenylcarbamate) phases (Chiralpak AD-H, AD-RH, IA), amylose tris[(S)-1-phenylethylcarbamate] phases (AS-H and AS-RH), cellulose benzoate and methylbenzoate esters (Chiralcel OJ-H, OJ-RH and OB-H), cellulose chloromethyl phenylcarbamates (Sepapak-2 and 4), amylose chloromethyl phenylcarbamate (Sepapak-3), and cellulose tris(3,5-dichlorophenylcarbamate) phases (Chiralpak IC and Sepapak-5) (Fig. 2). The Chiralcel and Chiralpak columns are either coated or immobilized (IA, IB and IC columns only) on 5-micron silica support while the Sepapak

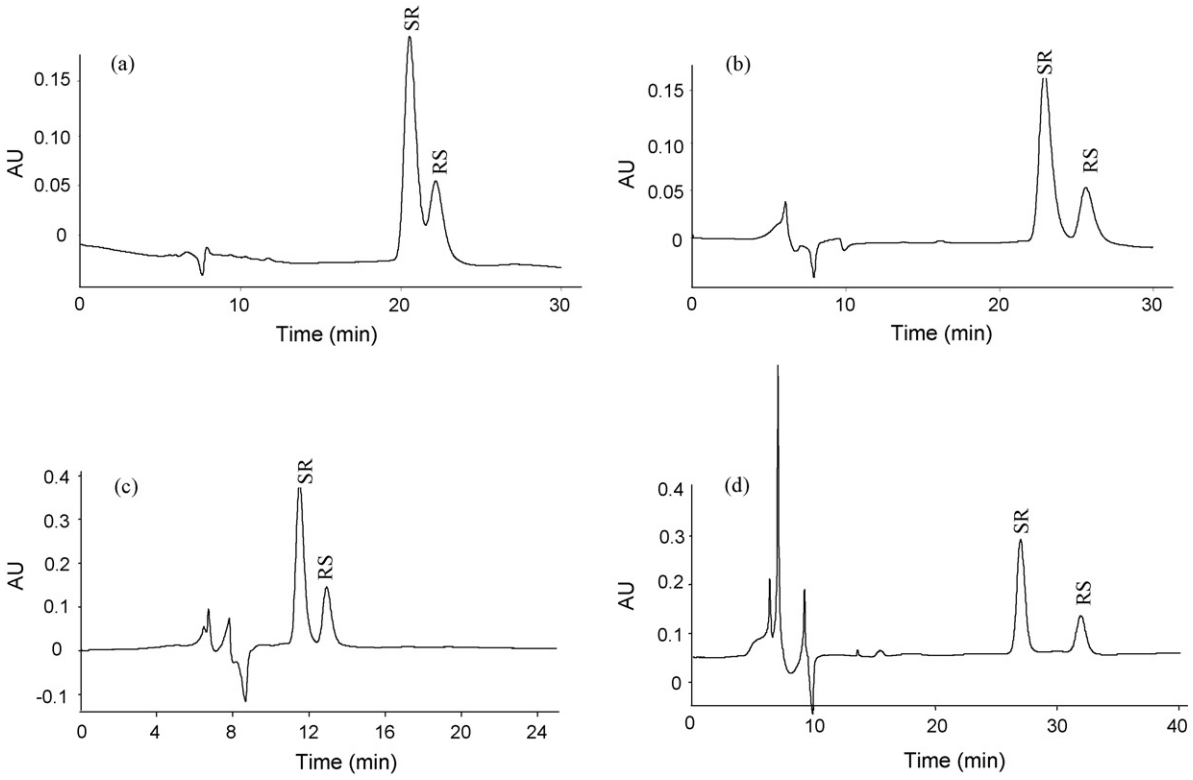

Fig. 5. Effect of the mobile phase composition on the enantioseparation of the SR/RS enantiomeric pair on an IC column: (a) IPA:Hexane:DEA 50:50:0.1 (v/v/v); (b) MTBE:IPA:DEA 95:5:0.1 (v/v/v); (c) MTBE:MeOH:DEA 95:5:0.1 (v/v/v); (d) MTBE:Hexane:MeOH:DEA 50:45:5:0.1 (v/v/v/v).

columns were packed with 3-micron silica particles. All columns have 4.6 mm i.d. × 250 mm configuration except for the AD-RH, OD-RH, AS-RH, OJ-RH and OB-H columns, which have 4.6 mm i.d. × 150 mm configuration. Chiralcel and Chiralpak columns were obtained from Chiral Technologies (West Chester, PA, USA), and Sepapak columns were purchased from Sepaserve GmbH (Münster, Germany).

3. Results and discussion

To separate all four stereoisomers and the positional isomer of Brivanib Alaninate, a chiral column must demonstrate high enantioselectivity and peak efficiency for both pairs of enantiomers (Fig. 1). The comprehensive chiral HPLC method development approach generated promising chiral separations of all five isomers of Brivanib Alaninate using polysaccharide-based CSPs [14]. Since the chiral recognition of the benzoate and phenylcarbamate derivatives of cellulose and amylose is greatly influenced by the type and position of the substituents on the phenyl group [16–18], the CSPs evaluated in this study carried a wide spectrum of substituted phenyl groups (Fig. 2). The potential of these CSPs to separate the two enantiomeric pairs of Brivanib Alaninate was fully explored using a variety of mobile phase conditions, such as normal phase (alcohol–hydrocarbon mixtures), reversed-phase (aqueous–organic mixtures) and polar organic solvents. The chromatographic results of capacity factor (k'), enantioselectivity (α) and resolution (R_s) for the direct enantioseparation of the four stereoisomers of Brivanib Alaninate are summarized in Table 1, and the results for indirect enantioseparation using non-chiral derivatization methods are presented in Table 2.

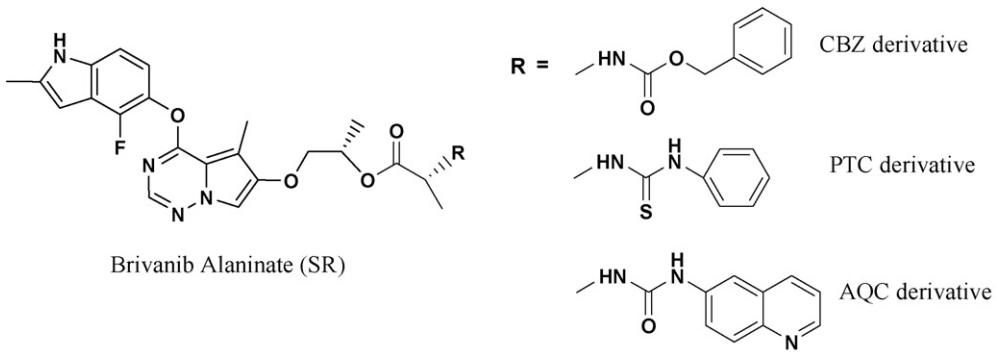

Fig. 6. A simplified scheme for the derivatization of Brivanib Alaninate with different approaches.

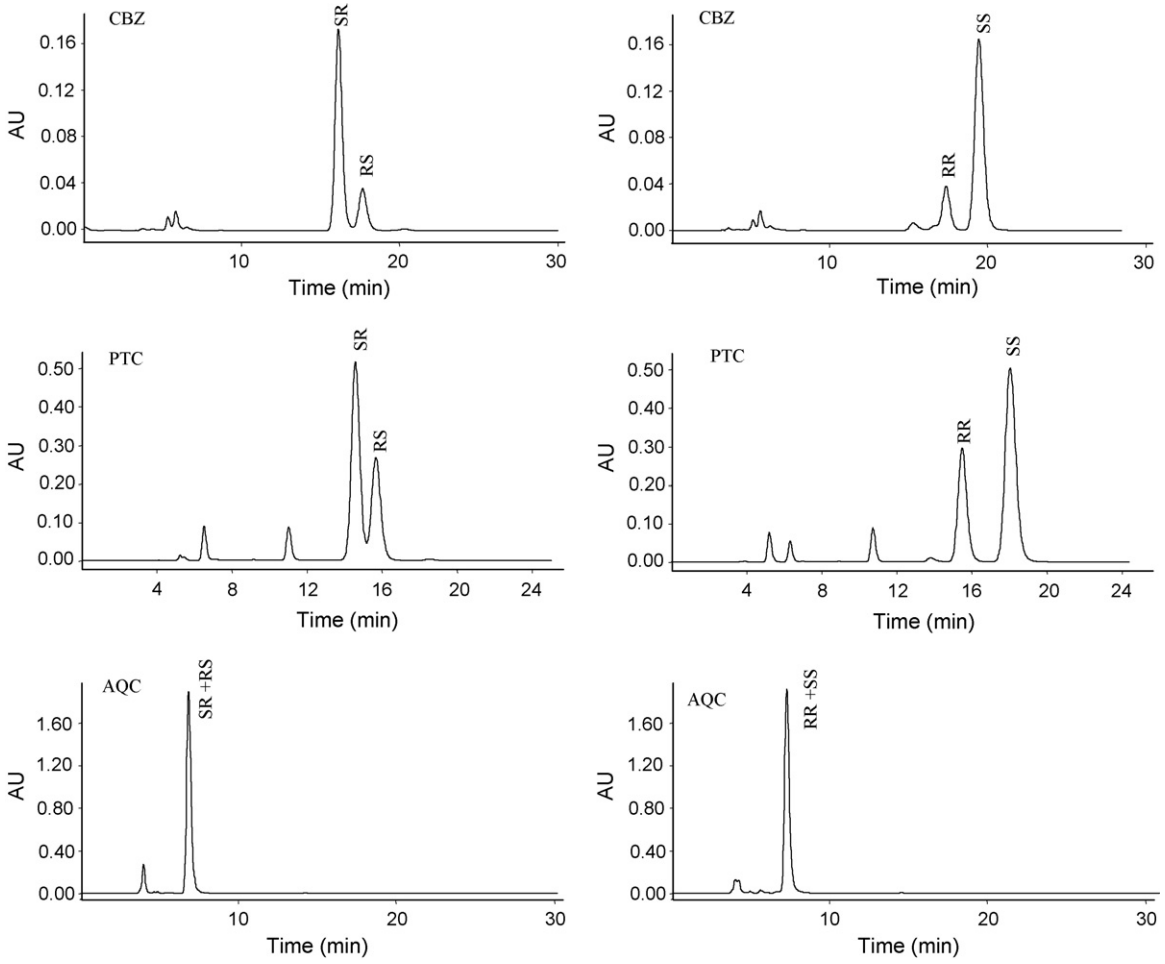

Fig. 7. Effect of different derivatization methods on the enantioseparation of the SR/RS and RR/SS enantiomeric pairs of Brivanib Alaninate on an OJ-RH column. Mobile phase: 20 mM NH_4OAc:ACN 40:60 (v/v).

3.1. Direct separation of the two enantiomeric pairs of Brivanib

3.1.1. Enantioseparation under reversed-phase mode

Among the polysaccharide-based CSPs tested, the benzoate and phenylcarbamate derivatives of cellulose and amylose CSPs demonstrated promising separations under reversed-phase conditions. In general, the cellulose phases performed better than the amylose phases in separating the isomers of interest. The cellulose 3,5-dimethylphenylcarbamate phase (OD-RH) provided the best separation for the SR/RS enantiomeric pair, while cellulose 3,5-dichlorophenylcarbamate (Sepapak-5) was noted as providing the best separation for the RR/SS enantiomeric pair. It is interesting to observe that the OD-RH and Sepapak-5 columns demonstrated not only a difference in magnitude of enantioselectivity, but also a reversal in elution order for the two enantiomeric pairs. Previous studies revealed that the polar carbamate moieties on the phenylcarbamate derivatives of polysaccharides are the major interaction sites for chiral recognition, and the secondary structure of polysaccharide backbone is maintained by the H-bonding between the N—H group and the neighboring glucose unit [17,18]. Both N—H and C=O groups on carbamate moieties can interact with a chiral compound through H-bonding or dipole–dipole interaction or both. The introduction of either electron-withdrawing dichloro- (as in Sepapak-5) or electron-donating dimethyl- (as in OD-RH) substituents on the phenyl group of these CSPs (Fig. 2) would change the acidity of the N—H group on the carbamate linkage and the polarity of the C=O group. Consequently, the interaction sites for chiral recognition and the secondary structure of polysaccharide backbone would be changed. These effects may be responsible for the reversed elution order of Brivanib Alaninate enantiomers on the OD-RH and Sepapak-5 columns under similar conditions.

The effect of the reversed-phase mobile phase composition on the enantioseparation of the Brivanib Alaninate isomers was further investigated on three derivatized cellulose phases: Sepapak-5, OD-RH and OJ-RH (Fig. 3). As expected, the retention factor (k') of the Brivanib Alaninate isomers demonstrated typical reversed-phase behavior; i.e., k' increased gradually with decreased amount of ACN in the mobile phase. Dramatic changes in retention of the analytes were observed on all of the columns studied when the ACN content was lowered to less than 50% in the mobile phase. Similar retention behavior was previously reported on derivatized cellulose phases [7,27]. Given the presence of both indole and triazine moieties in the structure of Brivanib Alaninate (Fig. 1), the observed retention dependence can be a consequence of the increasing importance of hydrophobic interactions between CSPs and analytes when the aqueous component of the mobile phase becomes dominant. Unlike other CSPs such as cyclodextrin [28] and macrocyclic glycopeptide [29] phases which can also be used in reversed-phase, polar organic and normal phase modes, no polysaccharide CSPs have been reported to give U-shape retention behavior for any chiral analytes reported to date. This is also true for Brivanib Alaninate isomers, suggesting that H-bonding interactions play a minor role

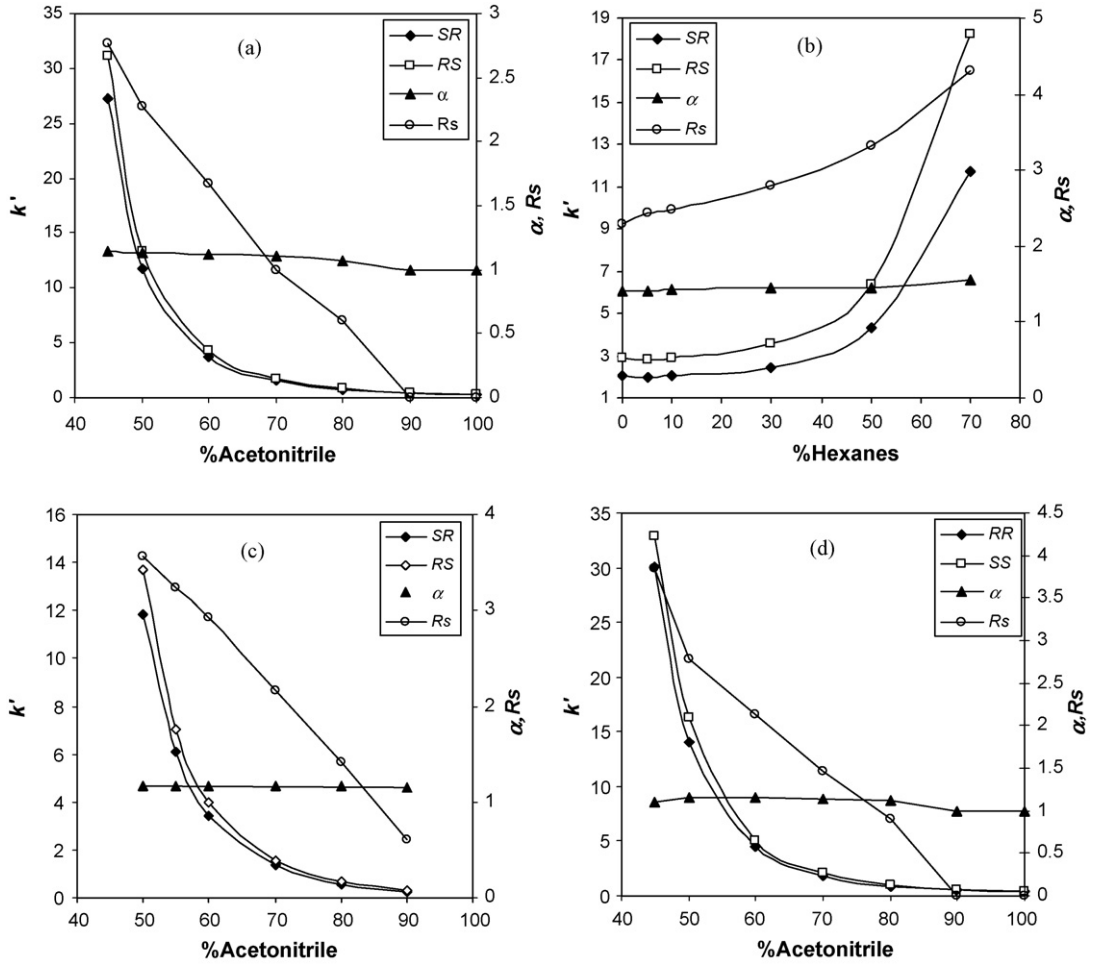

Fig. 8. Effect of the mobile phase composition on the retention (k'), enantioselectivity (α) and resolution (R_s) of the CBZ derivatives of the SR/RS enantiomers on (a) OJ-RH, (b) OJ-H and (c) Sepapak-5 columns, and the RR/SS enantiomers on (d) OJ-RH column.

in the retention of these analytes on the polysaccharide CSPs under reversed-phase conditions. Within the entire range of mobile phase composition investigated (Fig. 3), the resolution (R_S) for both enantiomeric pairs followed the same trend on all the columns studied, i.e., R_S increased with the decrease of organic content in the mobile phase. The effect of mobile phase composition on enantioselectivity (α), however, showed a dependency on the CSPs. The enantioselectivity of the separation of both enantiomeric pairs remained invariant on the Sepapak-5 and OD-RH columns (Fig. 3a–c), while α increased progressively from 1.0 to 1.09 on the OJ-RH column when the ACN content in the mobile phase decreased from 90% to 30% (Fig. 3d). This finding implies that while the hydrophobic interaction between the CSP and the Brivanib Alaninate isomers made insignificant contribution to the chiral recognition with the Sepapak-5 and OD-RH columns, it played a role in the chiral separation of the analytes on the OJ-RH column under reversed-phase conditions.

3.1.2. Enantioseparation under polar organic mode

The use of polar organic separation mode, which utilizes polar organic solvents such as alcohols, acetonitrile or their mixtures as mobile phase, offers a few advantages over reversed-phase and normal phase modes, such as higher separation efficiency and increased solute solubility, and has drawn increasing attention in recent years [21,30]. Under polar organic mode, several columns, including OJ-H, OB-H and Sepapak-4, showed reasonable high enantioselectivity for both enantiomeric pairs (Table 1). The best separation was observed on the Sepapak-4 column using acetonitrile as the mobile phase. Due to short retention time (most k' were lower than 1.0), it was difficult to achieve reasonable resolution (R_S) for underivatized analytes in polar organic mode. The two cellulose chloromethyl phenylcarbamate phases investigated (Sepapak-2 and 4) differed only in the placement of the substituted groups on the phenyl moiety (Fig. 2). Sepapak-4 had good selectivity for both enantiomeric pairs under the same mobile phase conditions. Sepapak-2 showed enantioselectivity for both enantiomer pairs but not under the same mobile phase conditions. As discussed in Section 3.1.1, the performance difference between these two CSPs was expected since the placements of chloro and methyl substituents on the phenyl moiety would have strong influence on the acidity of the N—H group on the phenylcarbamate residue, which contributes significantly to the chiral recognition with polysaccharide CSPs [17,18].

The selection of organic solvent as the mobile phase in polar organic mode is as important to the enantioseparation as the type of CSPs selected. Different polysaccharide phases have different organic solvent preferences for the same pair of enantiomers. For the SR/RS enantiomeric pair, the best separations on the OD-H, Sepapak-2, and 4 columns were observed using acetonitrile rather than alcohols as the mobile phase, while alcohols showed better enantioseparations on the cellulose benzoate phases (OJ-H and OB-H) when compared to acetonitrile. The dependence of enan-

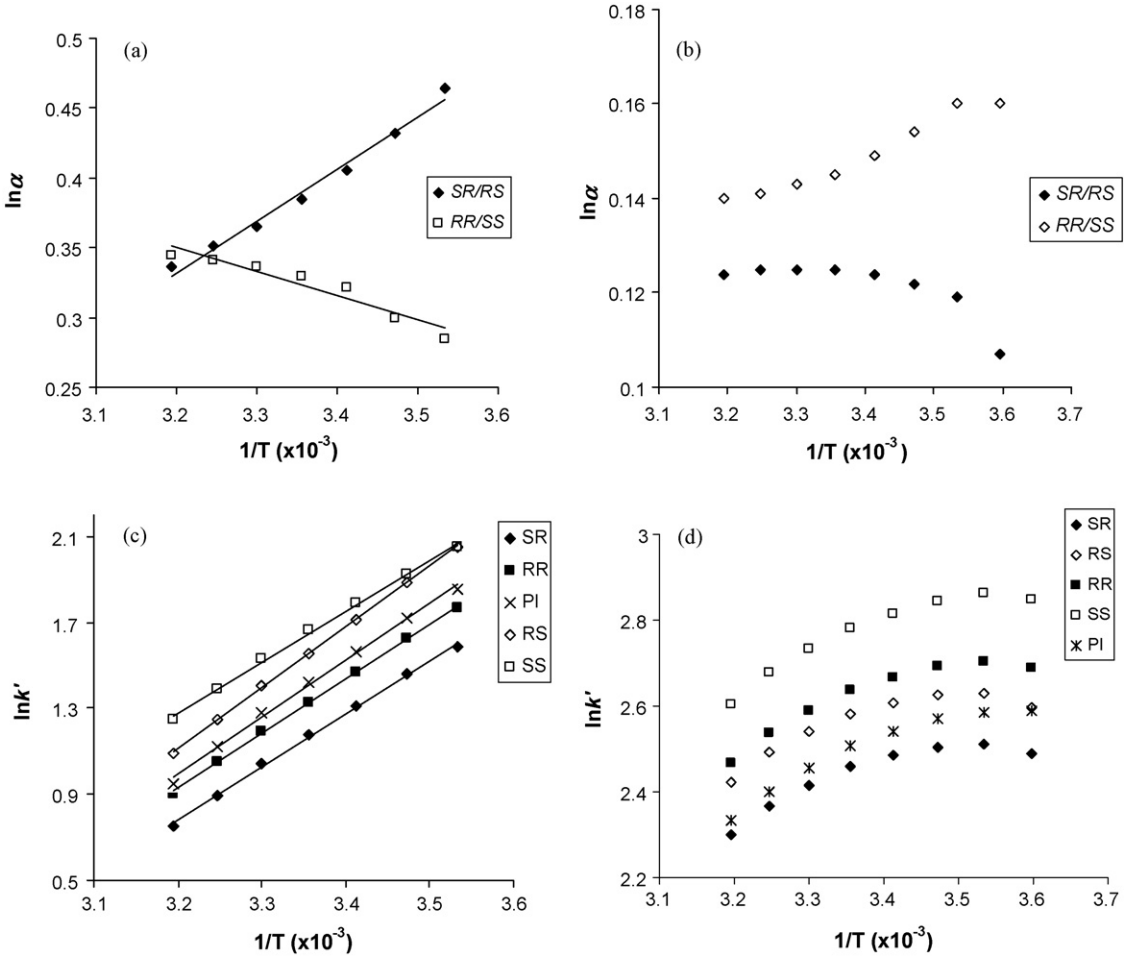

Fig. 9. The effect of column temperature on the enantioselectivity (α) and retention (k') of the CBZ derivatized Brivanib Alaninate compounds on an OJ-H column in polar organic mode (a and c) and OJ-RH in reversed-phase mode (b and d). Mobile phase: (a and c) MeOH; (b and d) 20 mM NH_4OAc/ACN 50/50 (v/v).

tioseparation on the type of organic solvent as mobile phase for different CSPs implies that both hydrophobic and H-bonding interactions were involved in the chiral recognition process under polar organic mobile phase conditions. In addition, since Brivanib Alaninate contains a primary amine group, it is important to use basic additives in the mobile phase in order to maintain peak efficiency in many cases.

3.1.3. Enantioseparation under normal phase mode

Better enantioselectivity of polysaccharide CSPs is often observed under normal phase conditions since H-bonding, which plays an extremely important role in chiral recognition with polysaccharide CSPs, is greatly enhanced under these conditions [15]. This is consistent with the enantioseparations observed for the Brivanib Alaninate isomers. Compared the enantioseparations of Brivanib Alaninate isomers under three different mobile phase conditions, normal phase mode typically showed equivalent or better enantioselectivity than either reversed-phase or polar organic mode (Table 1). Much better enantioseparations were observed on the IC, Sepapak-2 and Sepapak-4 CSPs under normal phase conditions. Some representative enantioseparations are shown in Fig. 4. The elution order of enantiomers was observed to remain the same on the same column during the transition of mobile phase modes. However, it is noteworthy that the elution order of both SR/RS and RR/SS enantiomeric pairs on the amylose phase AS-H and that of the RR/SS pair on AD-H was opposite to that on the cellulose phases OJ-H and OD-H (Table 1).

As shown in Table 1, the coated polysaccharide CSPs outperformed those immobilized phases for the enantioseparation of Brivanib Alaninate enantiomers. Using the OD-H and IB phases as examples, the coated OD-H phase baseline resolved the SR/RS enantiomeric pair, and partially resolved the RR/SS pair. The IB immobilized phase, however, could only partially resolve the SR/RS enantiomeric pair and was unable to separate the RR/SS pair. A similar trend was also observed on the AD-H (coated phase) and IA (immobilized phase) columns. The comparison between the Sepapak-5 and IC CSPs could not be made since the coated cellulose 3,5-dichloromethylphenyl carbamate stationary phase in the Sepapak-5 column is highly soluble in normal phase alcohol–alkane mobile phases. Despite the observation that the immobilization process may reduce the enantioselectivity of polysaccharide CSPs for Brivanib Alaninate isomers, immobilized CSPs are still attractive since they afford much broader range of mobile phase solvent choices. This advantage is clearly demonstrated in Fig. 5 and Table 1. Superior enantioselectivity for the SR/RS pair was obtained when the hexane–alcohol containing mobile phase was replaced with MTBE–hexane–alcohol mixture. When other organic solvents, such as methylene chloride, ethyl acetate, tetrahydrofuran and di-*n*-butyl ether, were combined with IPA as mobile phase, lower α and k' values were obtained for the same pair of enantiomers (data not shown). These results suggest that dipole–dipole and steric interactions may participate in the chiral recognition with the polysaccharide CSPs used in this study. Mobile phases containing MTBE, hexane and methanol generated the best enantioseparations

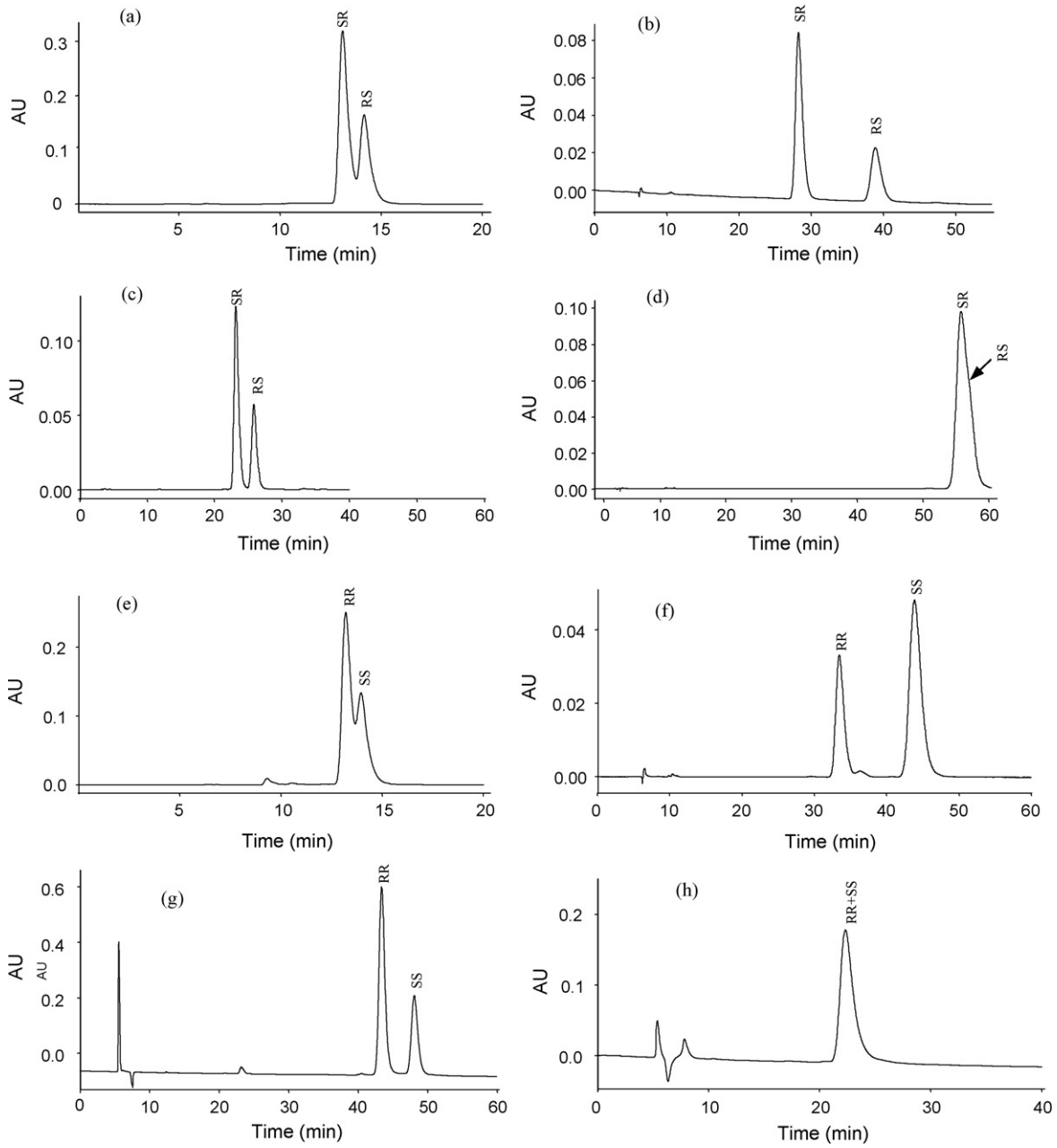

Fig. 10. Comparison of the enantioseparations using direct (a, c, e and g) and CBZ derivatization (b, d, f and h) methods on the OJ-H (a, b, e and f), OD-RH (c and d) and Sepapak-2 (g and h) columns. Mobile phase conditions: (a, b, e and f) MeOH; (c) 20 mM NH_4OAc:ACN 60:40 (v/v); (d) 20 mM NH_4OAc:ACN 45:55 (v/v); (g) EtOH:Heptane 15:85 (v/v); (f) EtOH:Hexane 30:70 (v/v).

for both Brivanib Alaninate enantiomeric pairs on the IC column (Fig. 4b and d). An optimized separation of all five Brivanib Alaninate isomers on the IC column is shown in Fig. 4e. Even under the optimized mobile phase conditions, only four peaks were observed for a mixture of five isomers with RR isomer co-eluting with the positional isomer and the RS isomer partially overlapping with the SS isomer.

3.2. Separation of derivatized stereoisomers

It is clear from the direct enantioseparations obtained on the polysaccharide CSPs that all of these CSPs show relatively low enantioselectivity (α value ranged from 1.01–1.27) for both enantiomeric pairs and poor selectivity for the diastereomers (Table 1). None of the polysaccharide CSPs investigated was able to simultaneously separate all five isomers of Brivanib Alaninate. These results are not unexpected since the chiral center on the propanoyl moiety of the Brivanib Alaninate molecule lacks a suitable functional group for chiral recognition (Fig. 1). On the other hand, the bulky indolyloxy pyrrolotriazinyl group, which attached to the same chiral center indirectly via an oxyethyl linkage, contributed significantly to analyte's retention, but apparently it had limited contribution to the chiral recognition of Brivanib Alaninate stereoisomers, as illustrated by the marginal enantioselectivity (α) values shown in Table 1 and Fig. 3.

It has been previously reported that the enantioselectivity can be effectively improved through derivatization, since the chiral recognition process is highly sensitive not only to the CSPs and mobile phase, but also to any changes of the analyte's structure [5,6,8]. Due to their ease and effectiveness of derivatizing amino

group, and structural diversity of the resulted products, three different derivatization approaches were chosen in order to evaluate how the introduction of different functionality to the alanine side of the molecule would affect the enantioseparation. It is evident from the structures shown in Fig. 6 that at least two critical changes were introduced to the analyte structure through derivatization: (a) the N—H group, a H-bond donor, was converted to a carbamate, a thiourea and an urea, respectively, all of which could act as both H-bond donor and acceptor; (b) the benzyl, phenyl and quinolyl groups introduced by the three derivatization approaches would afford additional π-π and steric interaction sites on the alanine side of the molecule to counter the dominant fluoromethylindolyloxy methylpyrrolotrazinyl group. Consequently, the separation of Brivanib Alaninate enantiomers and diastereomers was greatly impacted by derivatization. The results of the enantioseparations for the two derivatized enantiomeric pairs of Brivanib were summarized in Table 2.

3.2.1. Effect of different derivatizating groups on enantioseparation

The influence of different amino derivatizating groups on the enantioseparation of Brivanib Alaninate isomers was studied in reversed-phase mode. The data in Table 2 clearly indicates that the enantioseparation of the derivatized Brivanib isomers was highly dependent on both the type of the N-derivatizing group and the CSPs. For the SR/RS enantiomeric pair, the AQC derivatives were baseline resolved on the OD-RH and Sepapak-5 columns, while the best enantioseparations for the CBZ derivatives were obtained on the OJ-RH and Sepapak-5 columns. Baseline separations of all three different derivatives of the SR/RS enantiomeric pair were only obtained on the Sepapak-5 column. For the RR/SS enantiomeric pair, the OJ-RH column demonstrated excellent enantioselectivity (α) and resolution factor (R_s) for the CBZ and PTC derivatives. The OD-RH and AS-RH columns performed better when separating the AQC and PTC derivatives, however, Sepapak-5 and AD-RH column were much less effective in separating the RR/SS enantiomeric pair. Typical chromatograms which demonstrate the effect of different N-derivatizing group on the enantioseparation of both Brivanib enantiomeric pairs on the OJ-RH column are given in Fig. 7. Overall, cellulose-based CSPs outperformed amylose-based CSPs in resolving the derivatized isomers of Brivanib Alaninate, and the OJ-RH column outperformed all other studied columns for the separation of both enantiomers and diastereomers (Table 2).

Under reversed-phase conditions, the retention of the three different derivatives generally followed the same trend for both enantiomeric pairs on all tested columns: CBZ derivative > PTC derivative ≥ AQC derivative. This result is likely related to the ability of three different derivatives to form intramolecular H-bonds. Compared to the CBZ derivatives, it is much easier for both the AQC and PTC derivatives to form intramolecular H-bond. Since the electron-withdrawing ability of the C=S bond is less than that of the C=O bond, the tendency for the three derivatives to form intramolecular H-bond follows the order: AQC derivative > PTC derivative > CBZ derivative. The formation of an intramolecular H-bond resulted in a change in the shape of the derivative and its potential to interact with the chiral recognition sites on the CSPs. Consequently, the CBZ derivative among three derivatives was found to have the strongest interaction with all of the CSPs investigated. These results indicate that the best fit with the polysaccharide cavity follows the order: CBZ derivative > PTC derivative > AQC derivative. Another interesting finding in this study is that a reversal in the elution order of enantiomers was observed when different functional groups were introduced to the N-terminal. For example, the CBZ derivatives and the AQC/PTC derivatives had opposite elution orders for the SR/RS enantiomeric pair on the OD-RH column, and the AQC derivatives and PTC derivatives of the RR/SS enantiomeric pair had opposite elution orders on the AS-RH column. This phenomenon was previously reported on other CSPs [7,13]. In addition, opposite elution order was observed on different polysaccharide CSPs for the same enantiomeric pair. For instance, the SR enantiomer of the PTC derivatives eluted first on the Sepapk-5 column, but the RS enantiomer of the PTC derivatives eluted first on the OD-RH column. In the following sections of study, the CBZ derivatives of Brivanib Alaninate isomers were chosen for further studies under all three mobile phase modes (Table 2).

3.2.2. Retention behavior of CBZ derivatized isomers of Brivanib

As mentioned earlier, the CBZ derivatization introduced an additional aromatic group to the alanine moiety of the Brivanib Alaninate molecule. This structural change would enhance the hydrophobic interactions between analyte and CSPs resulting in longer retention times for the derivatized compounds under the same mobile phase conditions. This postulation was confirmed for both enantiomeric pairs on all polysaccharide CSPs studied (Figs. 3 and 8). Using the results on the OJ-RH column under reversed-phase conditions as an example, at 45% of ACN in the mobile phase, the k' values of the CBZ derivatives of the SR/RS enantiomers were above 27 (Fig. 8a) while the k' values of the underivatized SR/RS enantiomers were less than 2 (Fig. 3d). It was observed that the retention time of the CBZ-derivatives of the SR/RS enantiomers increased substantially when the ACN content of the mobile phase was decreased below 70%, while the retention time of the underivatized SR/RS enantiomers did not increase significantly until the ACN content in the mobile phase decreased below 50%. This observation indicates that hydrophobic interactions play a more important role in the retention of the derivatized analytes than that of the underivatized analogs under reversed-phase conditions. The CBZ derivatized analytes showed typical reversed-phase retention behavior under reversed-phase conditions and typical normal phase behavior under normal phase conditions (Fig. 8). Like their underivatized counterparts, the CBZ derivatized Brivanib Alaninate isomers showed similar dependence of enantioselectivity (α) and resolution (R_s) on the mobile phase composition for all CSPs tested.

3.2.3. Temperature effect on the enantioseparation of CBZ derivatives

It is interesting to note that the two enantiomeric pairs of Brivanib Alaninate expressed different preferences for CSPs as shown in Table 2. Under reversed-phase conditions, the Sepapak-5 column afforded baseline separation for the SR/RS enantiomeric pair regardless of the derivatization modification of the molecule, and it showed significantly less enantioselectivity for the RR/SS enantiomeric pair. In contrast, the OD-RH and AS-RH columns showed higher enantioselectivity for the RR/SS enantiomeric pair than that for the SR/RS enantiomeric pair. Under polar organic and normal phase conditions, the OD-H, Sepapak-2 and 4 columns were more effective in resolving the SR/RS enantiomeric pair, while the Sepapak-3 and OJ-H were more effective in resolving the RR/SS enantiomeric pair.

To better understand the mechanism behind this observation, the effect of the column temperature on the enantioseparation of the two enantiomeric pairs of Brivanib Alaninate was investigated under polar organic mode and reversed-phase mode, and the results are shown in Fig. 9. In polar organic mode, the plots of the variation of $\ln(\alpha)$ vs. $1/T$ (Fig. 9a) and $\ln(k')$ vs. $1/T$ (Fig. 9c) gave acceptable linear relationship for both enantiomeric pairs indicating that the enantioselective retention mechanism remained invariant and the chiral stationary phase did not have conformational changes within the studied temperature range. The

Table 3
Thermodynamic parameters for the enantioseparations of SR/RS and RR/SS enantiomers of Brivanib Alaninate (experimental conditions were the same as in Fig. 9)

Compound	OJ-H			OJ-RH		
	$\Delta\Delta H^\circ$ (kJ mol^{-1})	$\Delta\Delta S^\circ$ (J mol^{-1})	R^2	$\Delta\Delta H^\circ$ (kJ mol^{-1})	$\Delta\Delta S^\circ$ (J mol^{-1})	R^2
SR/RS	−3.1	−7.1	0.986	–	–	0.596
RR/SS	1.44	7.5	0.921	−0.47	−0.36	0.957

thermodynamic parameters $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ were calculated from the slope and intercept, respectively, of the ln(α) vs. 1/T plot, according to the van't Hoff model, and the results are given in Table 3. The values of $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ are negative for the enantioseparation of SR/RS enantiomers on the OJ-H column, indicating that the enantioseparation of SR/RS enantiomers in polar organic mode is an enthalpy driven process and the enantioselectivity factor (α) decreased with increasing temperature. The values of $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ are positive for RR/SS enantiomers, meaning that the enantioseparation of RR/SS enantiomers in polar organic mode is an entropy driven process and the enantioselectivity factor (α) increased with increasing temperature. This result is a good indication of that both enantiomer pairs may undergo different chiral recognition mechanism [30]. For the separations in reversed-phase mode on the OJ-RH column, however, the van't Hoff plot (Fig. 9d) for all five isomers of Brivanib Alaninate and ln(α) vs. 1/T plot for both SR/RS and RR/SS enantiomeric pairs (Fig. 9b) gave poor linear correlation, implying that conformational changes of the OJ-RH CSP may occur when the column temperature was varied, the values of $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ had opposite sign for the two enantiomeric pairs, suggesting that the two enantiomeric pairs responded differently to the conformational changes of the CSP (Table 3). More interestingly, the values of $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ had opposite sign for the same pair of enantiomers under polar organic mode and reversed phased mode. A possible explanation is that a change of mobile phase induced the change of chiral recognition mechanism through changing the conformation of polysaccharide CSP. The critical effect of mobile phase composition on the chiral recognition mechanism has also been observed on other chiral stationary phases [31,32].

3.3. Comparison between direct and indirect enantioseparation

Recently it has been reported that CBZ derivatized chiral amines consistently demonstrated enhanced chiral resolution on polysaccharide and Pirkle-type CSPs under HPLC and SFC conditions [8]. Such an unambiguous conclusion cannot be drawn from the enantioseparation of Brivanib Alaninate isomers whose molecules possess two chiral centers (Tables 1 and 2). For the SR/RS enantiomeric pairs, the CBZ derivatives showed better enantioseparation in terms of α and R_s than the underivatized analytes on almost all columns except AD-RH and OD-RH columns. For the RR/SS enantiomers, CBZ derivatized isomers were consistently less resolved on all CSPs except cellulose benzoate CSPs (OJ-RH, OJ-H and OB-H). AQC and PTC derivatizations seemed to work better for the RR/SS enantiomeric pair than CBZ derivatization depending on the CSPs (Table 2). Some representative chromatograms are presented in Fig. 10. Derivatized and underivatized Brivanib Alaninate isomers also showed different solvent preference in several cases. A reversal in elution order was observed before and after derivatization (in the case of enantioseparations on the OD-RH and Sepapak-5 columns) and when changing the mobile phase from alcohol to acetonitrile for derivatized analytes in polar organic mode (in the cases of the SR/RS enantiomers on IC column and RR/SS enantiomers on Sepapak-3 and Sepapak-4 column).

Polar organic mode was proven to be extremely successful for the enantioseparation of CBZ derivatized Brivanib Alaninate isomers. Among the sixteen polysaccharide CSPs studied, AD-H, OJ-H, Sepapak-2 and Sepapak-4 columns showed excellent resolution (R_s) for both CBZ derivatized enantiomeric pairs under polar organic mode (Table 2). While the cellulose benzoate ester OJ phase showed similar retention for the first eluted peak (k'_1) of both CBZ derivatized enantiomeric pairs in reversed-phase and polar organic phase, the enatioselectivity (α) and resolution (R_s) were greatly enhanced under polar organic conditions.

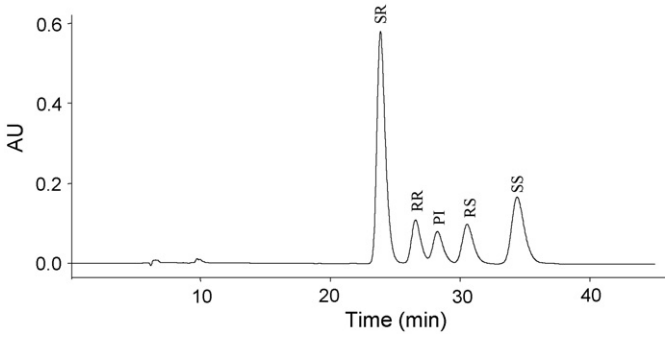

Fig. 11. Simultaneous separation of five Brivanib Alaninate isomers using CBZ derivatization method on an OJ-H column. Mobile phase: MeOH; column temperature: 30 °C. See Section 2 for other conditions.

3.4. Simultaneous separation of five isomers of Brivanib

The present systematic investigation of derivatization effect on the enantioseparation of Brivanib Alaninate isomers reveals that derivatization is an effective way to enhance enantioselectivity. Cellulose 4-methylphenyl benzoates (OJ-H and OJ-RH) were identified to be the best CSPs for the separation of five isomers of CBZ derivatized Brivanib Alaninate. These CSPs demonstrated excellent selectivity not only for the two enantiomeric pairs but more importantly for the diasteroismers and positional isomer. The temperature study in Section 3.2.3 also showed that column temperature was an important experimental parameter used to control the separation of Brivanib Alaninate isomers (data not shown). The best separation of all five CBZ derivatized Brivanib Alaninate isomers was achieved on the OJ-H column in polar organic mode with methanol as mobile phase at elevated temperature (Fig. 11), while reversed-phase and normal phase were less effective in separating these isomers.

4. Conclusions

The systematic study of 16 chiral columns under three different mobile phase modes clearly showed that the enantioseparation of Brivanib Alaninate isomers was sensitive to the structure changes of the analytes and CSPs. Depending on the derivatization approaches and CSPs, indirect analysis could produce superior enantioseparation for the two enantiomeric pairs and higher selectivity for the diastereomers of Brivanib Alaninate than direct analysis. No U-shape retention behavior was observed on any polysaccharide CSPs tested for Brivanib Alaninate isomers. A reversal in the enantiomer elution order could be realized by several ways, including changing CSPs, changing organic solvents in polar organic mode, and using

different derivatization methods. The temperature study revealed that different chiral recognition mechanisms were involved in the enantioseparation of the two enantiomeric pairs of Brivanib Alaninate. Successful separation of five isomers of Brivanib Alaninate was developed on the OJ-H column by using CBZ derivatization method and methanol as mobile phase.

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References

[1] Food and Drug Administration, FDA's policy statement for the development of new stereoisomeric drugs, 57 Fed. Reg. 22 (1992) 249.
[2] A.M. Krstulovic, Chiral Separations by HPLC: Applications to Pharmaceutical Compounds, Ellis Hornwood Limited, Chichester, 1989.
[3] G. Subramanian, Chiral Separation Techniques—A Practical Approach, 2nd ed., Wiley-VCH, Weinheim, 2001.
[4] T. Toyóoka, J. Biochem. Biophys. Methods 54 (2002) 25.
[5] S. Chen, M. Pawlowska, D.W. Armstrong, J. Liq. Chromatogr. 17 (1994) 483.
[6] C. Czerwenka, M. Lämmerhofer, W. Lindner, J. Sep. Sci. 26 (2003) 1499.
[7] K.-A. Lee, S. Yeo, K.H. Kim, W. Lee, J.S. Kang, J. Pharm. Biomed. Anal. 46 (2008) 914.
[8] C.M. Kraml, D. Zhou, N. Byrne, O. McConnell, J. Chromatogr. A 1100 (2005) 108.
[9] A. Patti, S. Pedotti, C. Sanfilippo, Chirality 20 (2008) 63.
[10] C. Hsieh, S. Wang, A.-L. Kwan, H.-L. Wu, J. Chromatogr. A 1178 (2008) 166.
[11] H.Y. Aboul-Enein, J. Chromatogr. A 906 (2001) 185.
[12] Z.-W. Cai, Y. Zhang, R.M. Borzilleri, L. Qian, S. Barbosa, D. Wei, X. Zheng, L. Wu, J. Fan, Z. Shi, B.S. Wautlet, S. Mortillo Sr., R. Jeyaseelan, D.W. Kukral, A. Kamath, Marathe, C. Arienzo, G. Derbin, J.C. Barrish, A. Robl, J.T. Hunt, J. Lombardo, J. Fargnoli, R.S. Bhide, J. Med. Chem. 51 (2008) 1976.
[13] M. Lammerhofer, W. Lindner, J. Chromatogr. A 741 (1996) 33.
[14] B.L. He, Y. Shi, Am. Pharm. Rev. 11 (2008) 47.
[15] Y. Okamoto, E. Yashima, Angew. Chem. Int. Ed. 37 (1998) 1020.
[16] Y. Okamoto, M. Kawashima, K. Hatada, J. Chromatogr. 363 (1986) 173.
[17] B. Chankvetadze, E. Yashima, Y. Okamoto, J. Chromatogr. A 670 (1994) 39.
[18] B. Chankvetadze, L. Chankvetadze, S. Sidamonide, E. Kasashima, E. Yashima, Y. Okamoto, J. Chromatogr. A 787 (1997) 67.
[19] Y. Okamoto, R. Aburatani, T. Fukumoto, K. Hatada, Chem. Lett. (1987) 1857.
[20] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403.
[21] B. Chankvetadze, C. Yamamoto, Y. Okamoto, J. Chromatogr. A 922 (2001) 127.
[22] B. Chankvetadze, I. Kartozia, C. Yamamoto, Y. Okamoto, J. Pharm. Biomed. Anal. 27 (2002) 467.
[23] K. Tachibana, A. Ohnishi, J. Chromatogr. A 906 (2001) 127.
[24] S.A. Cohen, D.M. Michaud, Anal. Biochem. 211 (1993) 279.
[25] J.A. White, R.J. Hart, J.C. Fry, J. Automatic Chem. 8 (1986) 170.
[26] K.R. Anumula, P.B. Taylor, Anal. Biochem. 197 (1991) 113.
[27] A. Patti, S. Pedotti, C. Sanfilippo, Chirality 19 (2007) 344.
[28] Q. Zhong, L. He, T.E. Beesley, W.S. Trahanovsky, P. Sun, C. Wang, D.W. Armstrong, J. Chromatogr. A 1115 (2006) 19.
[29] B. Zhang, R. Soukup, D.W. Armstrong, J. Chromatogr. A 1053 (2004) 89.
[30] K.G. Lynam, R.W. Stringham, Chirality 18 (2006) 1.
[31] A. Peter, G. Torok, D.W. Armstrong, G. Toth, D. Tourwe, J. Chromatogr. A 828 (1998) 177.
[32] A. Berthod, B.L. He, T.E. Beesley, J. Chromatogr. A 1060 (2004) 205.