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High-performance liquid chromatographic enantioseparation of bicalutamide and its related compounds

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Abstract

Direct high-performance liquid chromatographic methods were developed for the enantioseparation of (*R*,*S*)-bicalutamide (**1**) and its analogs (±)-3-chloro-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide (**2**), (±)-*N*-(4-cyano-3-(trifluoro-methyl)phenyl)-2-methyloxirane-2-carboxamide (**3**), (±)-4-fluorophenylsulfonyl-2-hydroxy-2-methylpropionic acid (**4**) and (±)-3-hydroxy-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide (**5**). The methods involved the use of a cellulose-based Chiralcel OD-H, macrocyclic glycopeptide-based Chirobiotic T, TAG and R, β-cyclodextrin-based Cyclobond I 2000SN and *t*-butyl carbamate-derivatized quinine-based columns. The conditions affording the best resolution were found by selection and variation of the mobile-phase compositions, and the differences in separation capability of the methods were noted. The sequence of elution of the enantiomers was determined in all cases.

Keywords: High-performance liquid chromatography; Direct enantiomeric separation; Macrocyclic glycopeptide type stationary phases; Chirobiotic phases; Cellulose-*tris*-3,5-dimethylphenyl carbamate-based stationary phase; Chiralcel OD-H; Bicalutamide

1. Introduction

The nonsteroidal active pharmaceutical ingredient bicalutamide is the leading antiandrogen compound used for the treatment of prostate cancer [1]. This drug competes with testosterone and dihydrotestosterone for binding sites on the prostate and other androgen-sensitive tissues, it has little or no agonist activity [2,3] and it is well tolerated, with few side-effects [4]. Bicalutamide is a racemic mixture, but the pharmacological effect is attributed mostly to the *R*-(−) enantiomer. The (*S*) isomer can be metabolized and eliminated faster than the (*R*) isomer, which results in liver function stress. Accordingly, administration of the active enantiomer alone in order to reduce the dosage and the demand on the liver function may be highly advantageous. Among the possible ways to manufacture a single enantiomer of this compound include a suitable chiral resolution method for the racemic mixture or asymmetric synthesis.

A new synthetic route has been developed for the production of (*R*,*S*)-(±)- and (*R*)-(−)-bicalutamide (**1**) by the reaction of 3-chloro-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide (**2**) and sodium 4-fluorophenylsulfinate under phase-transfer conditions (Fig. 1) [5–7]. During the reaction, *N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-methyloxirane-2-carboxamide (**3**) can be detected as active species, and 4-fluorophenylsulfonyl-2-hydroxy-2-methylpropionic acid (**4**) and 3-hydroxy-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide (**5**) as side-products.

In the synthesis of such compounds, chirality is often of utmost importance and there is therefore great interest in devising methods for the separation and identification of enantiomers. The separation of optical isomers requires an asymmetric or chiral environment that allows diastereomeric interactions. For this purpose, high-performance liquid chromatography (HPLC) is widely applied.

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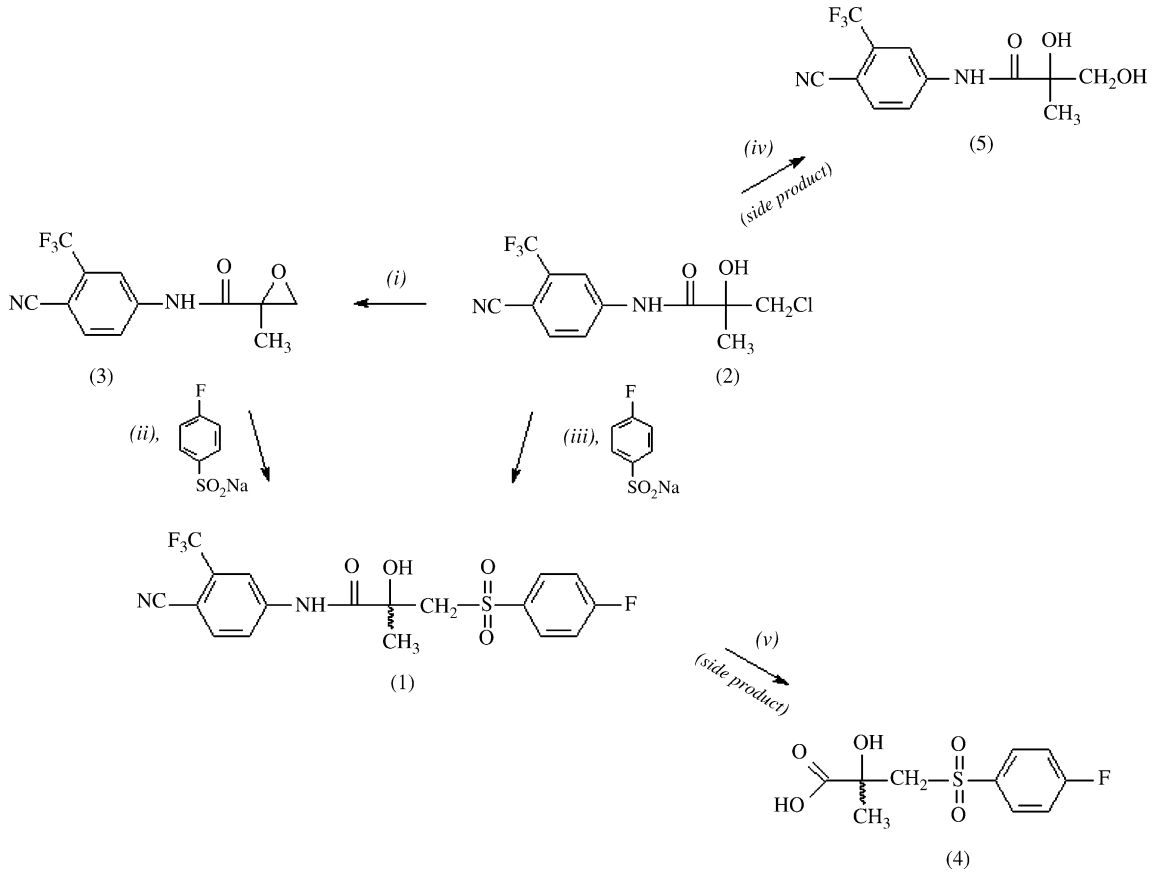

Fig. 1. Conditions: (i) NaOH in acetone, 68%; (ii) AcOH in MeOH, 43%; (iii) tetrabuthylammonium bromide, MeOH, 62%; (iv) and (v) isolation by chromatography.

Few examples are to be found in the literature on the enantioseparation of optically active **1**. Bargmann-Leyder et al. [8] presented a systematic comparison of liquid chromatography and supercritical fluid chromatography for cellulose-derived Chiralcel OD and amylose-derived Chiralpak AD chiral stationary phases (CSPs), using various chiral compounds, including **1** (ICI 176.334). James et al. [9] separated (*R*,*S*)-**1** on a Chiralcel OJ-H column, but no chromatographic conditions were reported. Tucker and Chesterson [10] resolved (*R*,*S*)-**1** by chromatographic separation as the diastereomeric (*R*)-camphanyl esters and determined the enantiopurity by HPLC with use of a Spherisorb-NH_2 column doped with (*R*)-(−)-*N*-benzoylphenylglycine. The detection limit for the (−) enantiomer in the presence of the (+) enantiomer was found to be only 5%.

The present paper describes the application of direct methods for enantioseparation of the racemic mixture of **1** and related compounds. The chromatographic separations are characterized in terms of the performance parameters retention, selectivity and resolution. The conditions affording the best resolution were determined and the differences in separation capability of the methods are discussed. The sequence of elution of the enantiomers was determined by spiking the racemic samples with enantiomers with known absolute configurations or optical rotations.

2. Experimental

2.1. Chemicals and reagents

Racemic (*R*,*S*)- and chirally pure (*R*)-**1** and its intermediate and side-products were synthetized in our laboratory according to the procedure depicted in Fig. 1 [5]. 3-Chloro-2-hydroxy-2-methylpropionic acid was obtained in good yield in the reaction of methacrylic acid and hypochlorous acid, and was resolved with classical resolution reagents, e.g. brucine or other chiral organic bases. The starting material (±)- or (+)-3-chloro-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide (**2**) was obtained by the acylation of 2-amino-3-trifluoromethyl benzonitrile with 3-chloro-2-hydroxy-2-methylpropionic acid in the presence of thionyl chloride and dimethyl acetamide. **2** can be transformed to the epoxy derivative **3**, or can be reacted directly with sodium 4-fluorobenzenesulfinate to yield **1**. The epoxy derivative **3** also reacted with sodium 4-fluorobenzenesulfinate to yield **1**. The synthesis of optically active derivatives was accomplished without any difficulty. No loss of optical activity was detected even during the transformation of **2** to **3** with sodium hydroxide. Both (+)-**2** and (+)-**3** gave (−)-**1** without loss of optical purity. Side-products **4** and **5** were isolated from the mother liquor.

HPLC-grade methanol (MeOH), acetonitrile (MeCN), hexane, ethanol (EtOH) and 2-propanol (IPA) were obtained from Merck (Darmstadt, Germany). Triethylamine (TEA), diethylamine (DEA), glacial acetic acid (HOAc), trifluoroacetic acid (TFA) and other reagents were of analytical-reagent grade, and were also obtained from Merck. The inorganic component used in the reversed-phase method was prepared from Milli-Q water, which was further purified by filtering on a 0.45-μm filter, type HV, Millipore (Molsheim, France).

0.1% Triethylammonium acetate (TEAA) buffers were prepared by titration of 0.1% (by volume) aqueous solutions of TEA with AcOH to a suitable pH. Mobile phases for normal-phase, reversed-phase and polar-organic chromatography were prepared by mixing the indicated volumes of buffers and/or solvents and were further purified by filtration through a 0.45-μm Millipore filter, type HV. The eluents were degassed in an ultrasonic bath, and helium gas was purged through them during the analyses. When new mobile phase or column was chosen, the chromatographic system was conditioned by passing the eluent (ca. 50 ml) through the column until a stable baseline signal and reproducible retention factors were obtained for the subsequent injections. The dead-times (t_0) of the columns were determined by injecting 20-μl of 0.001 M potassium bromide (Chirobiotic and Cyclobond columns), 0.01 M thiourea (Prontosil column) and hexane/ethanol in different compositions (Chiralcel OD-H).

Stock solutions of analytes (1 mg ml^{-1}) were prepared by dissolution in water or in the starting mobile phase and a further 5–10-fold dilution was applied.

2.2. Apparatus

The HPLC measurements were carried out on a Waters Breeze HPLC system consisting of a 1525 binary pump, a 487 dual-channel absorbance detector, a 717 plus autosampler, a Model 5CH column thermostat and Breeze data manager software (Waters Chromatography, Milford, MA, USA). Chromatographic system was equipped with a Rheodyne Model 7125 injector (Cotati, CA, USA) with 20-μl loops.

The macrocyclic glycopeptide-based stationary phases used for analytical separation were a teicoplanin-containing Chirobiotic T, a teicoplanin aglycone-containing Chirobiotic TAG, a vancomycin-containing Chirobiotic V, a vancomycin aglycone-containing Chirobiotic VAG and a ristocetin A-containing Chirobiotic R column, 250 mm × 4.6 mm I.D., 5-μm particle size (for each column) (Astec, Whippany, NJ, USA). The cellulose-*tris*-3,5-dimethylphenyl carbamate-based CSPs were Chiralcel OD-H and Chiralcel OD-RH columns, (150 mm × 4.0 mm I.D., 5-μm particle size for each) (Daicel, Tokyo, Japan). The weak ion-exchanger column packed with a *t*-butyl carbamate-derivatized quinine analog was Prontosil 120-5 *t*BuCQN, 5-μm particle size, 150 mm × 3.0 mm I.D. (Bischoff, Leonberg, Germany). The β-cyclodextrin-based columns Cyclobond I 2000, Cyclobond I 2000SN and Cyclobond I 2000RSP, α-cyclodextrin-based Cyclobond III, 250 mm × 4.6 mm I.D., 5-μm particle size (for each column) were also from Astec. The columns were thermostated. The precision of temperature adjustment was ±0.1 °C.

3. Results and discussion

The analytes were chromatographed and detected without pre- or postcolumn derivatization. All compounds in Tables 1–3 were evaluated by using several stationary phases with a minimum of three different normal-phase, reversed-phase or polar-organic mobile phases. To simplify the presentation, only the chromatographic results obtained the optimal mobile phase composition and/or the conditions that gave the best resolution on different columns are presented in the tables.

3.1. Separation of enantiomers on cellulose-based columns

Table 1 reports results on the separation of the enantiomers of the **1** and its related analogs **3** and **5** on a Chiralcel OD-H column. The stereoisomers of **1** and **5** were separated at different hexane/IPA and hexane/EtOH mobile phase compositions. The chromatographic system behaved as a real normal-phase system, i.e. with decreasing alcoholic modifier concentration, the retention factor increased, and an alcohol content of less than 5% resulted in extremely large retention times. The addition of DEA to the mobile-phase system, which in most cases led to

Table 1
Chromatographic data, retention factor (k'), separation factor (α) and resolution (R_S) of bicalutamide and its synthetic side products on cellulose based stationary phase

Compound	CSP	Eluent (v/v)	k'_1	k'_2	α	R_S	Elution sequence
1	OD-H	90/10, a	16.84	17.92	1.06	0.86	(−) < (+)
1	OD-H	95/5/0.1, b	34.99	36.56	1.04	0.85	(−) < (+)
1	OD-H	90/10, c	6.64	7.36	1.11	1.20	(−) < (+)
3	OD-H	60/40, a	0.39	0.63	1.61	2.11	(−) < (+)
5	OD-H	90/10, a	2.73	2.92	1.07	0.86	(−) < (+)
5	OD-H	95/5, a	10.48	11.20	1.07	0.92	(−) < (+)
5	OD-H	96/4/0.2, b	12.25	23.26	1.08	0.90	(−) < (+)
5	OD-H	98/2/0.1, b	36.44	40.10	1.10	1.12	(−) < (+)

Chromatographic conditions: column, OD-H, Chiralcel OD-H; a, hexane/IPA (v/v); b, hexane/IPA/DEA (v/v/v), c, hexane/ethanol (v/v); flow rate, 0.5 ml min^{-1}; detection, 254 nm; temperature, 25 °C; dead-time, t_0 = 3.90 min.

Table 2
Chromatographic data, retention factor (k'), separation factor (α) and resolution (R_S) of bicalutamide and its synthetic side products on macrocyclic glycopeptide-based stationary phases

Compound	CSP	Eluent (v/v/v)	k'_1	k'_2	α	R_S	Elution sequence
1	T	10/90, a	2.12	2.97	1.40	2.25	(+) < (−)
4	T	100/0.1/0.1, b	0.90	1.06	1.18	1.05	(−) < (+)
5	T	10/90, a	1.67	2.29	1.37	2.44	(+) < (−)
1	TAG	10/90, a	3.96	5.07	1.28	0.80	(+) < (−)
1	TAG	30/70, c	0.22	0.37	1.68	1.20	(+) < (−)
1	TAG	40/60, c	0.67	0.93	1.38	1.33	(+) < (−)
1	TAG	50/50, c	2.23	2.92	1.31	1.62	(+) < (−)
4	TAG	100/0.1/0.1, b	0.68	1.02	1.50	1.52	(−) < (+)
1	V	10/90, a	2.69	3.11	1.16	0.90	(−) < (+)
1	V	40/60, a	5.73	6.71	1.17	1.01	(−) < (+)
1	VAG	10/90, a	2.13	2.40	1.15	1.00	(−) < (+)
4	R	100/0.1/0.1, b	0.61	1.01	1.66	2.76	(−) < (+)

Chromatographic conditions: column, T, Chirobiotic T; TAG, Chirobiotic TAG; V, Chirobiotc V; VAG, Chirobiotic VAG; R, Chirobiotic R; mobile phase: a, hexane/IPA (v/v); b, MeOH/AcOH/TEA (v/v/v); c, 0.1% aqueous TEAA (pH 4.1)/MeOH (v/v); flow rate, 0.8 ml min^{-1}; detection, 254 nm; temperature, 25 °C; dead-time, Chirobiotic T, t_0 = 2.50 min, Chirobiotic TAG, t_0 = 2.77 min, Chirobiotc V, t_0 = 3.28 min, Chirobiotic VAG, t_0 = 3.25 min and Chirobiotic R, t_0 = 4.21 min.

improved peak shapes and resolution, exerted no influence on the separation selectivity. The hexane/EtOH system was found to be more advantageous for the enantioseparation of **1**, while the hexane/IPA system proved more useful for the separation of the stereoisomers of **5**. For the stereoisomers of **5**, no enantioseparation was achieved in the hexane/EtOH system (data not shown). A decrease in column temperature or in flow rate had little effect on the resolution (data not shown). The stereoisomers of **3**, the active species involved in the synthesis of **1**, showed excellent resolution on the Chiralcel OD-H column. Since chiral stationary phases with amino functions are very sensitive to the reactive epoxide group of **3**, the normal-phase method was suitable for this chiral impurity determination (Fig. 1). With the Chiralcel OD-RH column, which has the same selector as Chiralcel OD-H, but used in reversed-phase mode, application of the H_2O/MeCN, 0.1 M aqueous phosphate buffer/MeCN or 0.05 M aqueous potassium hexafluorophosphate/MeCN mobile-phase systems resulted in unresolved peaks of the enantiomers of all investigated analytes. Unfortunately, the cellulose-based stationary phases were inefficient in the separation of **2** and **4**, in either the normal-phase or reversed-phase mode. However, significant improvements in these separations were achieved on the application of the macrocyclic glycopeptide-based CSPs.

3.2. Separation of enantiomers on macrocyclic glycopeptide-based CSPs

Table 2 lists the chromatographic data obtained on different macrocyclic glycopeptide-based CSPs under different chromatographic conditions. The data reveal that the enantiomers of **1**, **4** and **5** could be separated in one or other of the chromatographic modes. In the normal-phase mode, with a hexane/IPA mobile-phase system the enantiomers of **1** and **5** were separated with high efficiency on the Chirobiotic T column. This column was less effective in the reversed-phase mode (data not shown), but partial separation with $R_S > 1$ was obtained for **4** in the polar-organic mode in the MeOH/AcOH/TEA mobile-phase system.

Excellent enantiomeric separations of **1** and **4** were achieved on the aglycone-type teicoplanin-based Chirobiotic TAG column in either reversed-phase or polar-organic mode. The resolution of analyte **1** (which increased with decreasing MeOH content and at optimized conditions $R_S > 1.5$) could be achieved with a 0.1% aqueous TEAA (pH 4.1 or 6.5)/MeOH mobile phase system.

The vancomycin-containing Chirobiotic V and Chirobiotic VAG columns resulted in some selectivity only in the normal-

Table 3
Chromatographic data, retention factor (k'), separation factor (α) and resolution (R_S) of bicalutamide and its synthetic side products on β-cyclodextrin and quinine-carbamate-based stationary phases

Compound	CSP	Eluent (v/v)	k'_1	k'_2	α	R_S	Elution sequence
2	2000 SN	80/20, a	4.62	4.96	1.07	0.70	(+) < (−)
2	2000 SN	90/10, b	5.67	6.10	1.08	1.25	(+) < (−)
2	2000 SN	92.5/7.5, b	9.44	10.19	1.08	1.30	(+) < (−)
3	2000 SN	90/10, a	11.58	11.95	1.03	0.80	(−) < (+)
3	2000 SN	90/10, b	4.45	4.60	1.03	0.70	(−) < (+)
4	QN	10/90, c	6.99	8.16	1.17	1.63	(+) < (−)

Chromatographic conditions: column, 2000 SN, Cyclobond I 2000 SN; QN, Prontosil 120-5 *t*BuCQN; mobile phase: a, hexane/IPA (v/v); b, hexane/ethanol (v/v); c, 0.2 M aqueous NH_4OAc/MeOH (pH 6.0) (v/v); flow rate, 2000 SN, 0.8 ml min^{-1}, QN, 0.5 ml min^{-1}; detection, 254 nm; temperature, 25 °C; dead-time, Cyclobond I 2000 SN, t_0 = 4.40 min and Prontosil, t_0 = 1.80 min.

phase mode for the enantiomers of **1** (Table 2). The resolutions were lower as compared with the data obtained on Chirobiotic T and were similar to the R_S values obtained on the Chiralcel OD-H column, but on Chiralcel OD-H column these separations occurred at very high retention times.

Of the macrocyclic glycopeptide-based columns, the Chirobiotic R column, containing ristocetin A as the chiral selector, provided the highest selectivity for **4**, especially in the polar-organic mode. **4** could in most cases be resolved on macrocyclic glycopeptide-based Chirobiotic columns, with a primary or secondary amino group as a bonding site. This reveals the importance of the interaction between carboxy group of the analyte and the protonated amino group of the chiral selector in the chiral recognition. The enhanced interaction between the selector and the analyte resulted in baseline separations.

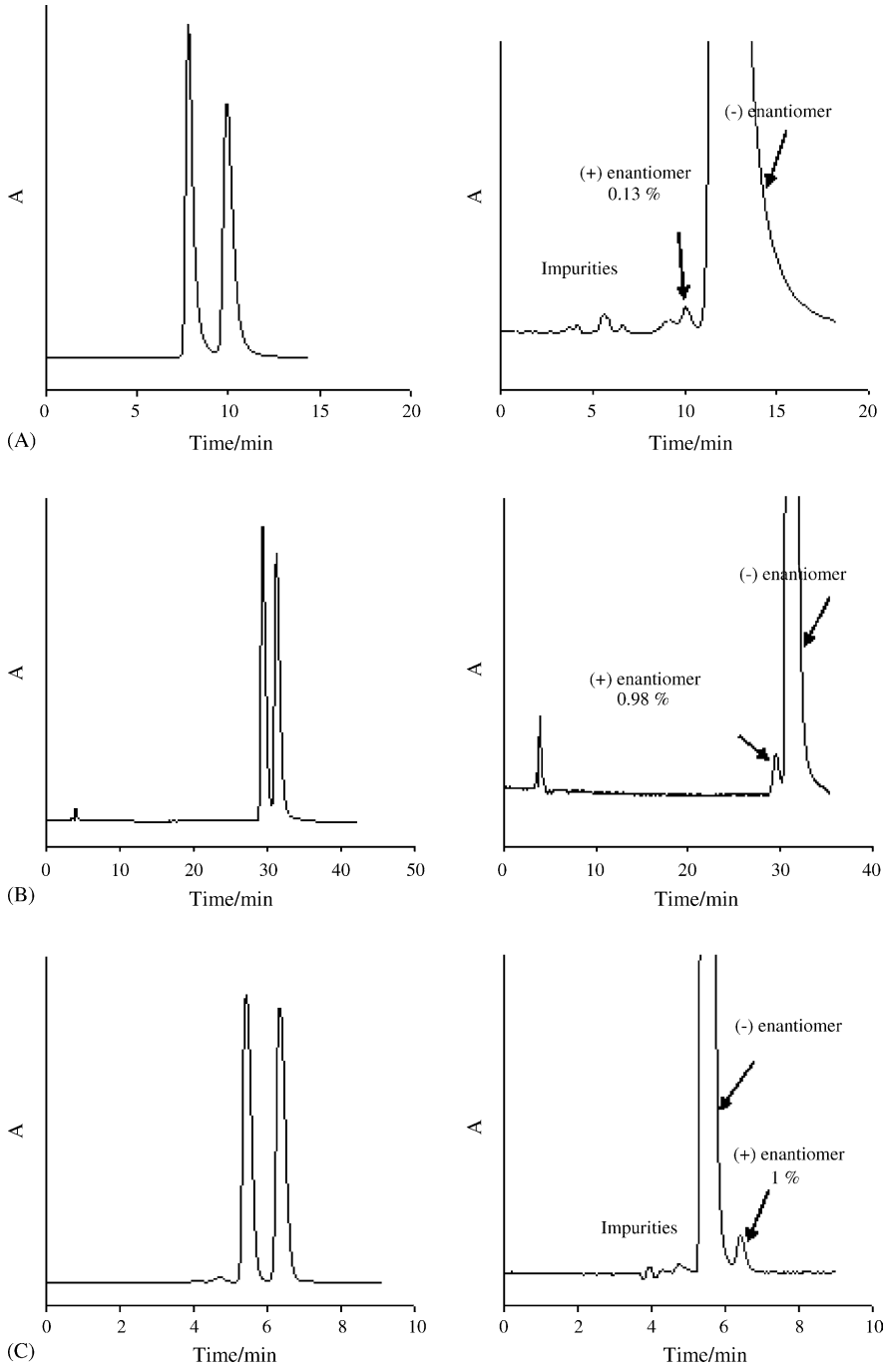

Fig. 2. Selected chromatograms of enantioseparation of bicalutamide and its starting material and intermediates of synthesis: (A) (*R*,*S*)-bicalutamide (**1**); (B) (±)-3-chloro-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropaneamide (**2**), (C) (±)-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-methyloxirane-2-carboxamide (**3**); chromatographic conditions: column, (A) Chirobiotic T, (B) Cyclobond I 2000 SN, (C) Chiralcel OD-H; mobile phase composition, (A) hexane/IPA = 10/90 (v/v), (B) hexane/ethanol = 92.5/7.5 (v/v), (C) hexane/IPA = 60/40 (v/v); flow rate, 0.8 ml min^{-1}; detection, 254 nm.

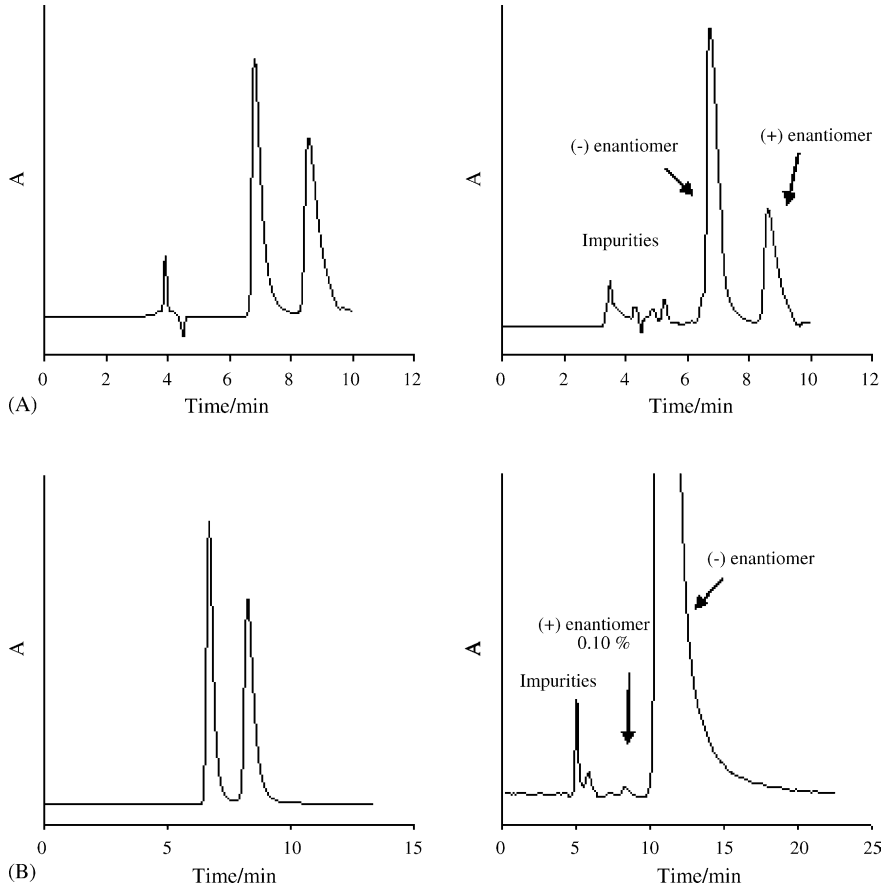

Fig. 3. Selected chromatograms of enantioseparation of side products of bicalutamide synthesis: (A) (±)-4-fluorophenylsulfonyl-2-hydroxy-2-methylpropionic acid (**4**); (B) (±)-3-hydroxy-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide (**5**) Chromatographic conditions: column, (A) Chirobiotic R, (B) Chirobiotic T; mobile phase composition, (A), MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v), (B) hexane/IPA = 10/90 (v/v); flow rate, 0.8 ml min^{-1}; detection, 254 nm; analyte **4** was enantiomerically enriched for (−).

3.3. Separation of enantiomers on β-cyclodextrin and quinine carbamate-based CSPs

In the course of this project, several cyclodextrin-based chiral stationary phases were tested. The α-cyclodextrin-based Cyclobond III, and the β-cyclodextrin-based Cyclobond I, Cyclobond I 2000 SN and Cyclobond I 2000 RSP columns contained native or derivatized cyclodextrins as selectors and offered the possibility for inclusion complex formation inside the cyclodextrin cavity. Despite the different possibilities of interactions, only the naphthylethyl carbamate-derived β-cyclodextrin-based Cyclobond I 2000 SN column was active in the separation of the stereoisomers of **1**-related compounds (Table 3). For the stereoisomers of **2**, which is the starting material in the chiral synthesis (Fig. 1), an almost baseline separation was achieved in a hexane/EtOH mobile-phase system. The hexane/IPA mobile phase system proved somewhat less efficient. The same held true for the separation of the very reactive epoxide-containing **3**, for which the resolution was below 1.0 in both the hexane/EtOH and hexane/IPA eluent systems.

Excellent separation was achieved for the enantiomers of **4** on a Prontosil 120-5 *t*BuCQN column, where the chiral selector was a *t*-butyl carbamate-derivatized quinine analog. This selector was successfully applied earlier for different *N*-acyl-derivatized amino acids [11,12]. On this column, baseline separation could be achieved in reversed-phase mode.

Selected chromatograms for the separation of stereoisomers of individual analytes and the artificial mixtures of analytes **1**–**5** are depicted in Figs. 2–4.

3.4. Elution sequence

The sequence of elution of the enantiomers was determined. On the Chiralcel OD-H column, the (−) enantiomer eluted before the (+) enantiomer. A similar elution sequnce was observed for **1** on the Chiralcel OJ-H column [7]. On the Chirobiotic T and TAG columns for **4** similar elution sequence, while for **1** and **5** a reverse elution sequence, i.e. (+) before (−) was observed. On the Chirobiotic R, V and VAG columns the elution sequence (−) before (+) was obtained (Table 2). On the β-cyclodextrin based Cyclobond I 2000 SN and *t*-butyl carbamate-derivatized quinine analog columns, different elution sequences were observed for **2–4**. The opposite elution sequences observed on different columns revealed the importance of the identification of the elution sequence and the choice of column selection in order to determine the minor component as the first-eluting peak in the chromatogram (Figs. 2 and 3).

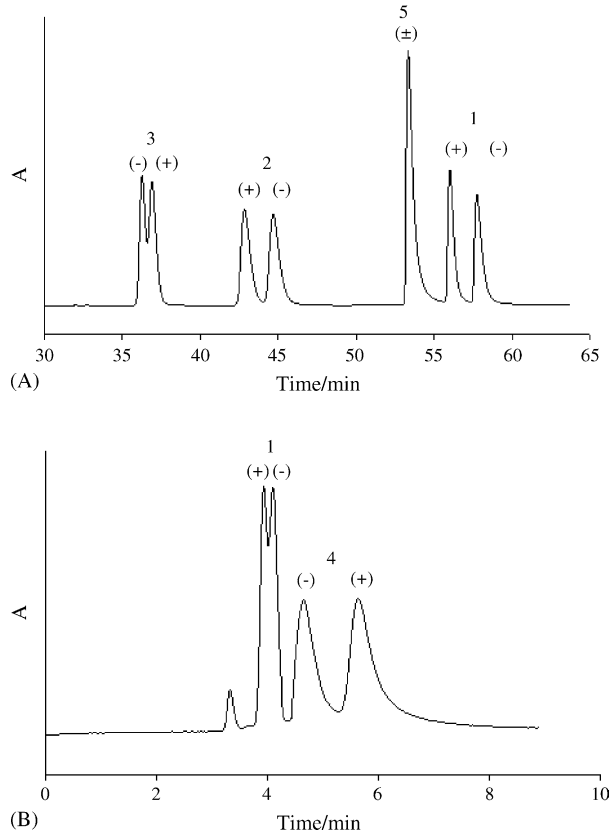

Fig. 4. Chromatograms of artificial mixtures of stereoisomers of analytes **1**–**5**: **1**, (*R*,*S*)-bicalutamide; **2**, (±)-3-chloro-*N*-(4-cyano-3-(trifluoromethyl)-phenyl)-2-hydroxy-2-methylpropaneamide; **3**, (±)-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-methyloxirane-2-carboxamide; **4**, (±)-4-fluorophenylsulfonyl-2-hydroxy-2-methylpropionic acid; **5**, (±)-3-hydroxy-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide Chromatographic conditions: column, (A) Cylobond I 2000 SN, (B) Chirobiotic TAG; mobile phase, (a) hexane, (b) EtOH, (c) MeOH/AcOH/TEA; mobile phase compositions, (A) gradient elution, 0–20 min 95% a + 5% b, 21–45 min, 90% a + 10% b, 46–65 min, 70% a + 30% b, 66 min, 95% a + 5% b, (B) MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v); flow rate, 0.8 ml min^{-1}; detection, 254 nm.

4. Conclusions

The enantioseparation of (*R*,*S*)-bicalutamide (**1**), its starting material (±)-3-chloro-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-hydroxy-2-methylpropanamide (**2**), its active species involved in the reaction, (±)-*N*-(4-cyano-3-(trifluoromethyl)phenyl)-2-methyloxirane-2-carboxamide (**3**) and its synthetic side-products, (±)-4-fluorophenylsulfonyl-2-hydroxy-2-methylpropionic acid (**4**) and (±)-3-hydroxy-*N*-(4-cyano-3-(trifluoromethyl)-phenyl)-2-hydroxy-2-methylpropanamide (**5**) was carried out by the use of a cellulose-based Chiralcel OD-H column, macrocyclic-glycopeptide based Chirobiotic T, TAG and R columns and a *t*-butyl carbamate-derivatized quinine analog based Prontosil 120-5 *t*BuCQN column. Among the columns investigated, the teicoplanin and teicoplanin aglycone-containing Chirobiotic T and TAG columns provided the best resolutions for the stereoisomers of analytes **1**, **4** and **5**, while stereoisomers of **2** and **3** were well separable on a Cyclobond I 2000 SN and a Chiralcel OD-H column, respectively. By application of these CSPs, excellent resolutions were achieved for most of the investigated compounds in the reversed-phase, normal-phase or polar-organic mobile phase systems. The differences between the separation capabilities of the methods were noted. The elution sequences on the different columns were determined and are reported.

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