Comparison of Separation Performances of Amylose- and Cellulose-Based Stationary Phases in the High-Performance Liquid Chromatographic Enantioseparation of Stereoisomers of β-Lactams

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ABSTRACT High-performance liquid chromatographic methods were developed for the separation of the enantiomers of 19 β-lactams. The direct separations were performed on chiral stationary phases containing either amylose-*tris*-3,5-dimethylphenyl carbamate, (Kromasil® AmyCoat™ column) or cellulose-*tris*-3,5-dimethylphenyl carbamate, (Kromasil® CelluCoat™ column) as chiral selector. The different methods were compared in systematic chromatographic examinations. The separations were carried out with good selectivity and resolution. The AmyCoat™ and CelluCoat™ columns appear to be highly complementary. The best separations of bi- and tricyclic β-lactam stereoisomers were obtained with the AmyCoat™ column, whereas the 4-aryl-substituted β-lactams were better separated on the CelluCoat™ column. The elution sequence was determined in all cases; no general rule could be established. *Chirality 22:120–128, 2010.*

KEY WORDS: column liquid chromatography; β-lactams; CelluCoat™ column; AmyCoat™ column

INTRODUCTION

β-Lactams or penams have been widely used in a variety of applications in a number of scientific areas, but especially in medicinal and synthetic chemistry. As the most commonly used group of antibiotics currently available, β-lactams function as lethal inhibitors of the growth of the cell walls of pathogenic bacteria.[1,2] Monocyclic β-lactams can be used as antibiotic agents (e.g., monobactams), and they are used as intermediates in the synthesis of β-amino acids,[3] short peptide segments,[4] taxoid antitumor agents,[5] alkaloids,[6] and different heterocycles of biological and medicinal interest.[7] *N*-Peptidyl-substituted 2-azetidinones obtained from 4-phenyl-2-azetidinone (4-aryl-substituted β-lactams) were earlier evaluated as inhibitors of serine protease elastase and cysteine protease papain.[8] A facile transformation of 3,4-disubstituted 2-azetidinones to chiral 5,6-dihydro-2-pyridones, which can serve as valuable chiral intermediates for different piperidine and indolizidine alkaloids and azasugars, was reported by Lee et al.[9] Various direct and indirect enzymatic[10,11] and enantioselective syntheses[12] of β-lactams have been described.

Since the biological activity of a β-lactam depends strongly on its stereochemistry, there is a clear need for analytical methods by which their enantiomeric purities can be determined. High-performance liquid chromatography (HPLC) on chiral stationary phases (CSPs) is an effective analytical tool for the resolution of chiral compounds on both analytical and preparative scales. β-Lactam stereoisomers were separated on different types of selectors as follows: (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine,[13–16] *tris*-carbamates of cellulose or amylose polysaccharide,[17–22] β-cyclodextrin,[23,24] and macrocyclic glycopeptide.[25,26] Jiang et al.[27] applied β-cyclodextrin-based capillary electrophoresis for the separation of the stereoisomers of β-lactam.

In this article, direct HPLC methods are described for the enantioseparation of racemic β-lactam stereoisomers (for the structures, see Fig. 1). The direct HPLC methods rely on the use of amylose-*tris*-3,5-dimethylphenyl carbamate or cellulose-*tris*-3,5-dimethylphenyl carbamate-based CSPs. For comparison purposes, most of the separations were carried out at constant mobile phase compositions and the separation performances of the amylose- and cellulose-based CSPs were compared. The experimental data allow a discussion of the influence of the mobile phase composition and specific structural features of the analytes on the retention and chiral recognition. The sequence of elution of the enantiomers was determined by the cochro-

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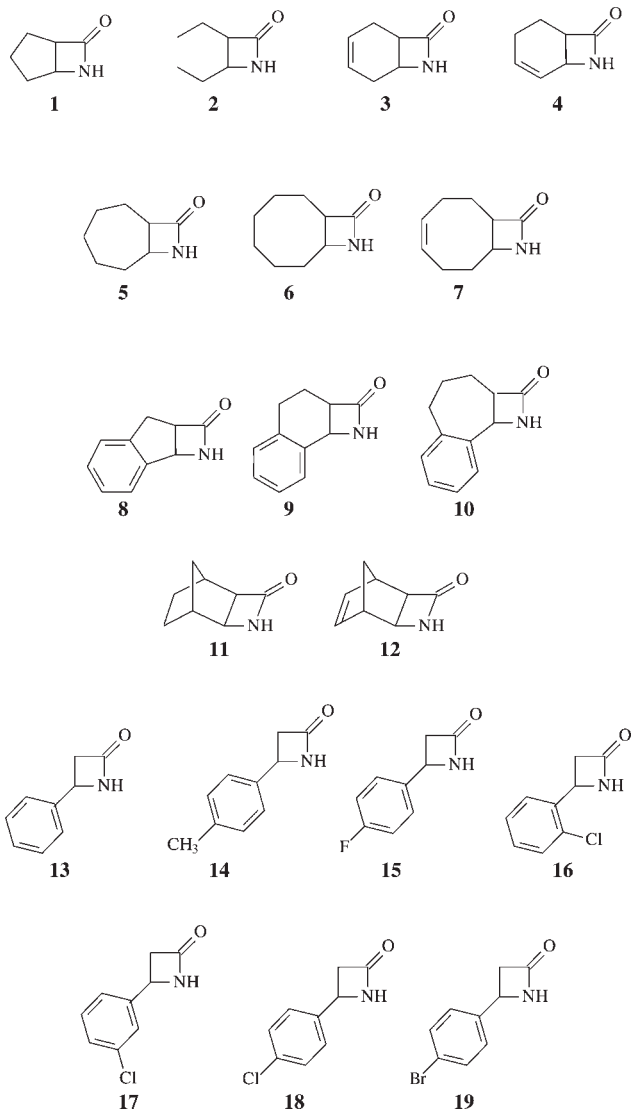

Fig. 1. Structures of analytes. *cis*-6-azabicyclo[3.2.0]heptan-7-one (**1**), *cis*-7-azabicyclo[4.2.0]octan-8-one (**2**), *cis*-7-azabicyclo[4.2.0]oct-3-en-8-one (**3**), *cis*-7-azabicyclo[4.2.0]oct-4-en-8-one (**4**), *cis*-8-azabicyclo[5.2.0]nonan-9-one (**5**), *cis*-9-azabicyclo[6.2.0]decan-10-one (**6**), *cis*-9-azabicyclo[6.2.0]-dec-4-en-10-one (**7**), *cis*-3,4-benzo-6-azabicyclo[3.2.0]heptan-7-one (**8**), *cis*-4,5-benzo-7-azabicyclo[4.2.0]octan-8-one (**9**), *cis*-5,6-benzo-8-azabicyclo[5.2.0]nonan-9-one (**10**), *exo*-3-azatricyclo[4.2.1.$0^{2,5}$]nonan-4-one (**11**), *exo*-3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one (**12**), 4-phenyl-2-azetidinone (**13**), 4-(*p*-tolyl)-2-azetidinone (**14**), 4-(*o*-chlorophenyl)-2-azetidinone (**15**), 4-(*m*-chlorophenyl)-2-azetidinone (**16**), 4-(*p*-chlorophenyl)-2-azetidinone (**17**), 4-(*p*-fluorophenyl)-2-azetidinone (**18**) and 4-(*p*-bromophenyl)-2-azetidinone (**19**).

matography of racemic analytes with enantiomers with known absolute configurations.

EXPERIMENTAL

Chemicals and Reagents

The 12 racemic β-lactams, *cis*-6-azabicyclo[3.2.0]heptan-7-one (**1**), *cis*-7-azabicyclo[4.2.0]octan-8-one (**2**), *cis*-7-azabicyclo[4.2.0]oct-3-en-8-one (**3**), *cis*-7-azabicyclo[4.2.0]oct-4-en-8-one (**4**), *cis*-8-azabicyclo[5.2.0]nonan-9-one (**5**), *cis*-9-azabicyclo[6.2.0]decan-10-one (**6**), *cis*-9-azabicyclo[6.2.0]-dec-4-en-10-one (**7**), *cis*-3,4-benzo-6-azabicyclo[3.2.0]heptan-7-one (**8**), *cis*-4,5-benzo-7-azabicyclo[4.2.0]octan-8-one (**9**), *cis*-5,6-benzo-8-azabicyclo[5.2.0]nonan-9-one (**10**), *exo*-3-azatricyclo[4.2.1.$0^{2,5}$]nonan-4-one (**11**), and *exo*-3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one (**12**) were prepared in our laboratory by the cycloaddition of chlorosulfonyl isocyanate to the corresponding cycloalkanes or cycloalkadienes.[12,28] The racemic 4-aryl-substituted β-lactams, 4-phenyl- (**13**), 4-(*p*-tolyl)- (**14**), 4-(*p*-fluorophenyl)- (**15**), 4-(*p*-chlorophenyl)- (**16**), 4-(*p*-bromophenyl)- (**17**), 4-(*o*-chlorophenyl)- (**18**), and 4-(*m*-chlorophenyl)-2-azetidinone (**19**) were prepared from styrene or 2-, 3-, or 4-substituted styrene by chlorosulfonyl isocyanate addition. Enantiopure β-lactams were synthesized by a very efficient enzymatic method through catalysis of the enantioselective ring opening of β-lactams with H_2O in diisopropyl ether by Lipolase (lipase B from *Candida antarctica* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism and adsorbed on a macroporous resin).[29–33]

n-Hexane, *n*-heptane, methanol (MeOH), ethanol (EtOH), 1-propanol (PrOH), 2-propanol (IPA), 1-butanol (BuOH), *tert.*-butanol (*t*-BuOH) of HPLC grade were purchased from Sigma-Aldrich (St. Louis, MO), as were other reagents of analytical reagent grade.

The eluents were degassed in an ultrasonic bath, and helium gas was purged through them during the analyses.

Apparatus and Chromatography

The HPLC measurements were carried out on a Waters HPLC system consisting of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector, and a Millenium[32] Chromatography Manager data system (Waters Chromatography, Milford, MA). The chromatographic system was equipped with a Rheodyne Model 7125 injector (Cotati, CA) with a 20-μl loop.

The columns used for direct separations were amylose-*tris*-3,5-dimethylphenyl carbamate (Kromasil® AmyCoat™) or cellulose-*tris*-3,5-dimethylphenyl carbamate-based (Kromasil® CelluCoat™) CSPs, measuring 150 × 4.6 mm I.D. with a particle size of 5 μm, both from Eka Chemicals AB (Bohus, Sweden). The dead-times (t_0) of the columns were determined by injecting 1.0 mM potassium bromide dissolved in IPA. The columns were thermostated in a Spark Mistral column thermostat (Spark Holland, Emmen, The Netherlands). The precision of temperature adjustment was ±0.1°C.

Stock solutions of the analytes (1 mg ml^{-1}) were prepared by dissolution in the starting mobile phase.

RESULTS AND DISCUSSION

Two CSPs were utilized for the direct enantioseparation of the 19 β-lactam stereoisomers in this study: the amylose-*tris*-3,5-dimethylphenyl carbamate-based AmyCoat™ and cellulose-*tris*-3,5-dimethylphenyl carbamate-based CelluCoat™. Both columns were used in the normal-phase mode. Relevant separation data on compounds **1-19**, including retention factors (k'), separation factors (α), and

TABLE 1. Chromatographic data, retention factors (k'), separation factor (α), resolution (R_S), and elution sequence for the direct separation of the stereoisomers of β-lactams on CelluCoat™ CSP

Analyte	Mobile phase	k'_1	α	R_S	Elution sequence[a]
1	**a**	1.28	1.00	0.00	–
	b	1.71	1.00	0.00	–
	c	4.96	1.00	0.00	–
2	**a**	1.54	1.05	[b]	1*R*,6*S*
	b	2.10	1.05	[b]	1*R*,6*S*
	c	4.82	1.06	[b]	1*R*,6*S*
3	**a**	2.11	1.09	1.15	1*S*,6*R*
	b	3.26	1.09	0.95	1*S*,6*R*
4	**a**	1.71	1.00	0.00	–
	b	2.63	1.00	0.00	–
	c	5.92	1.02	[b]	1*S*,6*R*
5	**a**	1.63	1.00	0.00	–
	c	4.79	1.03	[b]	1*R*,7*S*
6	**a**	1.54	1.05	[b]	1*R*,5*S*
	b	2.58	1.00	0.00	–
7	**a**	1.72	1.04	0.30	n.d.
	b	2.79	1.00	0.00	–
	c	6.40	1.00	0.00	–
8	**a**	2.50	1.31	4.00	1*S*,5*S*
9	**a**	2.23	1.11	1.45	1*R*,6*R*
10	**a**	2.50	1.52	7.40	1*R*,7*R*
11	**a**	1.24	1.15	1.55	1*S*,2*R*,5*S*,6*R*
12	**a**	1.47	1.13	1.50	1*R*,2*R*,5*S*,6*S*
13	**a**	3.30	1.00	0.00	–
	b	5.72	1.05	1.30	*R*
14	**a**	2.55	1.20	2.80	*R*
15	**a**	3.03	1.10	1.40	*R*
	b	6.13	1.11	1.85	*R*
16	**a**	3.33	1.12	1.85	*S*
17	**a**	3.64	1.00	0.00	–
	b	7.40	1.04	0.65	*R*
	c	11.94	1.07	1.45	*R*
18	**a**	3.67	1.06	1.15	*R*
	b	6.74	1.09	1.60	*R*
19	**a**	3.96	1.06	0.90	*R*
	b	7.75	1.09	1.45	*R*

Column, CelluCoat™; mobile phase, **a.** *n*-heptane/IPA = 90/10 (v/v), **b.** *n*-heptane/IPA = 95/5 (v/v), **c.** *n*-heptane/IPA = 97/3 (v/v); flow rate, 0.5 ml min^{-1}; detection, 205 nm; temperature, ambient; t_0, 3.88 min.
[a]Configuration of the first-eluted enantiomer.
[b]Sign of separation.

resolutions (R_S) for each analyte with several mobile phases are given in Tables 1 and 2. On the CelluCoat™ column with a mobile phase of *n*-heptane/IPA (90/10 (v/v), the stereoisomers of all the analytes were retained, but only slight or no enantioseparation was observed for the analytes of **1**, **2**, **4**, **5-7**, **13**, and **17** (Table 1). On AmyCoat™ with the same mobile phase, the stereoisomers of all the analytes were retained again and most of the stereoisomers were baseline separated. (The only exceptions were **2**, **11**, **13**, **14**, and **16**, Table 2.) However, by changing the mobile phase composition most of the analytes were baseline separated on both CSPs.

The alcohol content of the mobile phase strongly influenced the chromatographic behavior. As can be seen in Tables 1 and 2 on the CelluCoat ™ and AmyCoat™ CSPs, and in Figure 2 for compounds **2**, **9**, **11**, and **13** on the AmyCoat™ CSP with the *n*-heptane/IPA mobile phase system, k' strongly decreased with increasing alcohol content, whereas the changes in α and R_S differed. For the β-lactam analogs, slight changes in α were registered with increasing IPA content, whereas R_S changed in parallel with k', i.e., R_S in most cases decreased with decreasing k' (special behavior was observed for analyte **2**, where R_S exhibited a maximum curve as a function of the IPA content). The concentration of the alcohol exerted a slight effect on the enantioselectivity, i.e., the ratio of the nonchiral and chiral interactions between the CSP and the analytes depend only slightly on the concentration of the alcohol (Fig. 2).

The nature of the alcohol greatly influenced the retention and resolution. Figure 3 reveals that, on the AmyCoat™ and CelluCoat™ CSPs for analytes **2**, **9**, and **13** at constant alcohol concentration (0.39 mol l^{-1} in the *n*-hexane/alcohol mobile phase system), the change in k' did not appear to correlate with the carbon number of the alcohols. From the six examples (Fig. 3), it can be concluded that MeOH gives smaller k' on the AmyCoat™

TABLE 2. Chromatographic data, retention factors (k'), separation factor (α), resolution (R_S) and elution sequence for the direct separation of the stereoisomers of β-lactams on AmyCoat™ CSP

Analyte	Mobile phase	k'_1	α	R_S	Elution sequence[a]
1	**a**	1.65	1.24	2.40	1*R*,5*S*
	b	2.81	1.34	3.50	1*R*,5*S*
	c	6.74	1.21	2.60	1*R*,5*S*
2	**a**	1.74	1.00	0.00	–
	d	9.82	1.06	0.85	1*R*,6*S*
3	**a**	1.97	1.09	0.90	1*S*,6*R*
4	**a**	1.74	1.13	1.50	1*S*,6*R*
5	**a**	1.81	1.14	1.45	1*S*,7*R*
6	**a**	1.49	1.40	3.75	1*S*,8*R*
7	**a**	1.66	1.25	2.70	1*S*,6*R*
8	**a**	2.46	1.98	11.3	1*S*,5*S*
9	**a**	2.39	1.30	3.80	1*S*,6*S*
10	**a**	3.79	1.56	8.65	1*S*,7*S*
11	**a**	2.00	1.02	[b]	–
	c	7.58	1.09	1.65	1*S*,2*R*,5*S*,6*R*
12	**a**	1.95	1.07	0.80	1*S*,2*S*,5*R*,6*R*
13	**a**	2.40	1.02	[b]	–
	c	7.55	1.08	1.25	*R*
14	**a**	2.21	1.05	0.45	*R*
	c	7.75	1.04	0.75	*R*
15	**a**	2.01	1.21	2.75	*S*
16	**a**	2.33	1.00	0.00	–
	b	4.97	1.04	0.65	*R*
17	**a**	2.55	1.13	1.80	*R*
18	**a**	2.43	1.09	1.25	*R*
19	**a**	2.60	1.15	2.15	*R*

Column, AmyCoat™; mobile phase, **a.** *n*-heptane/IPA = 90/10 (v/v), **b.** *n*-heptane/IPA = 95/5 (v/v), **c.** *n*-heptane/IPA = 97/3 (v/v), **d**. *n*-heptane/IPA = 98/2 (v/v); flow rate, 0.5 ml min^{-1}; detection, 205 nm; temperature, ambient; t_0, 3.90 min.
[a]Configuration of the first-eluting enantiomer.
[b]Sign of separation.

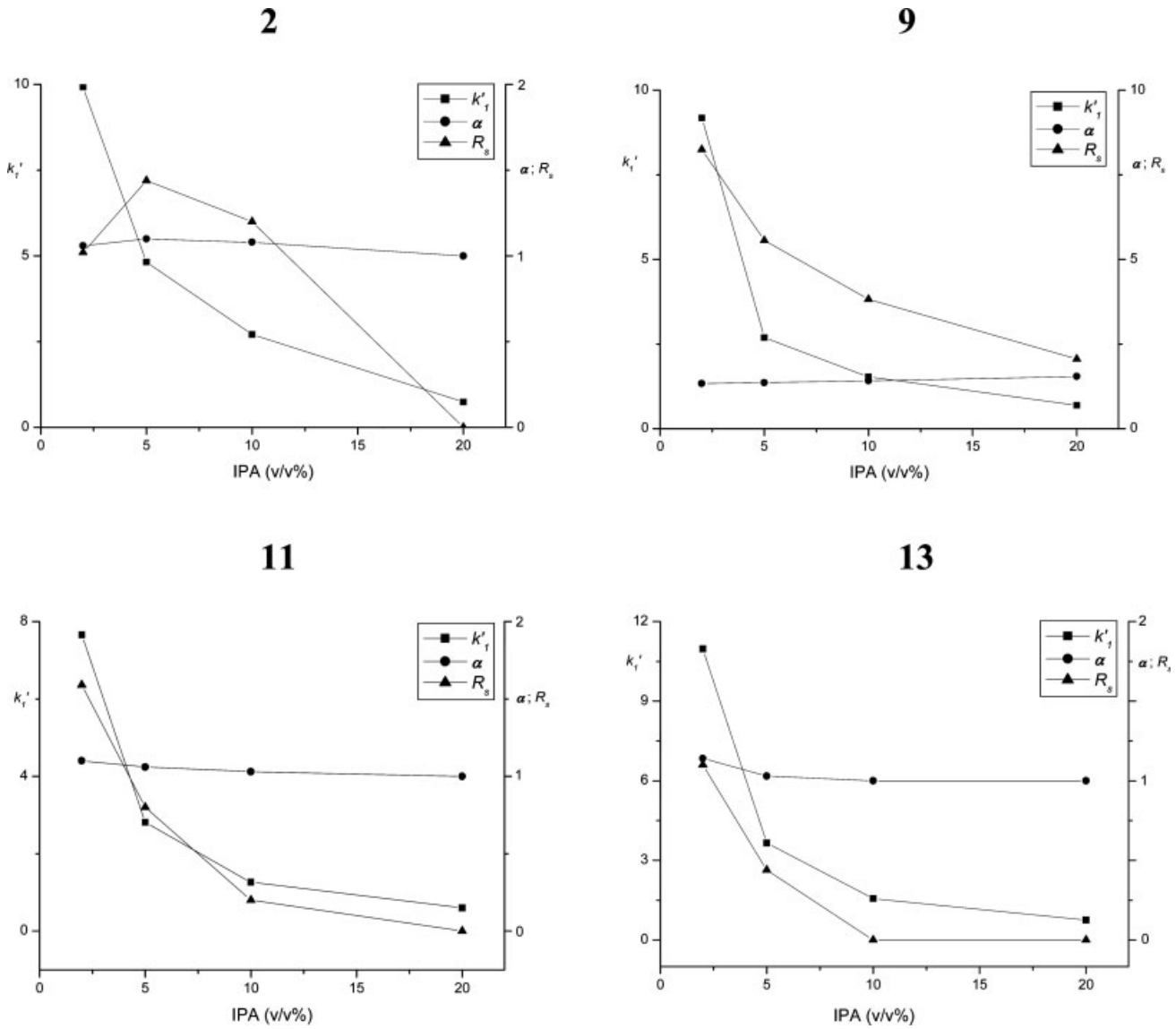

Fig. 2. Effects of IPA content on retention factor of first-eluting enantiomer (k'_1), separation factor (α) and resolution (R_S) for analogs **2**, **9**, **11**, and **13** on AmyCoat™ CSP. Chromatographic conditions: mobile phase, *n*-hexane/IPA = 98/2–80/20 (v/v); flow rate, 0.5 ml min^{-1}; detection, 205 nm, (■) k'; (●) α; (▲) R_S.

CSP and higher k' on the CelluCoat™ CSP. The enantioselectivity did not change dramatically when the different alcohols were applied in the same molar concentration. Wang and Winslow[34] found that the changes caused in the CSP structure by the different alcohols may affect the chiral selectivity of the CSP, depending on the size and structure of the analyte. The influence of the nature of alcohol on resolution exhibited a large effect but no general rule could be established. When separation was observed sometimes, the application of alcohols with bulky and branched side-chains, such as PrOH, IPA, BuOH, and *t*-BuOH resulted higher R_S but the selectivity seemed to be the same or sometimes even lower. Probably the selector swells more in these solvents resulting better mass-transfer kinetics and higher R_S values.

For most of the analytes investigated, a structure-retention relationship was observed on both stationary phases. With increasing number of carbon atoms attached to the β-lactam ring (analytes **1**-**7**), k' usually increased (Tables 1 and 2). This increase in the normal-phase mode can be attributed to the increased steric effect (bulkiness) of the analytes. For analytes **11** and **12**, the k' values on the CelluCoat™ CSP were somewhat lower, whereas and on the AmyCoat™ CSP, they were somewhat higher than that of analyte **5**, which also contained a seven-membered condensed ring. It is generally recognized that, at the supramolecular level, the lamellar arrangement of the polysaccharide chains provides a multitude of chiral cavities, and therefore multiple interaction sites.[35] In the case of the *tris*-3,5-dimethylphenyl carbamate-based CSPs, the polar

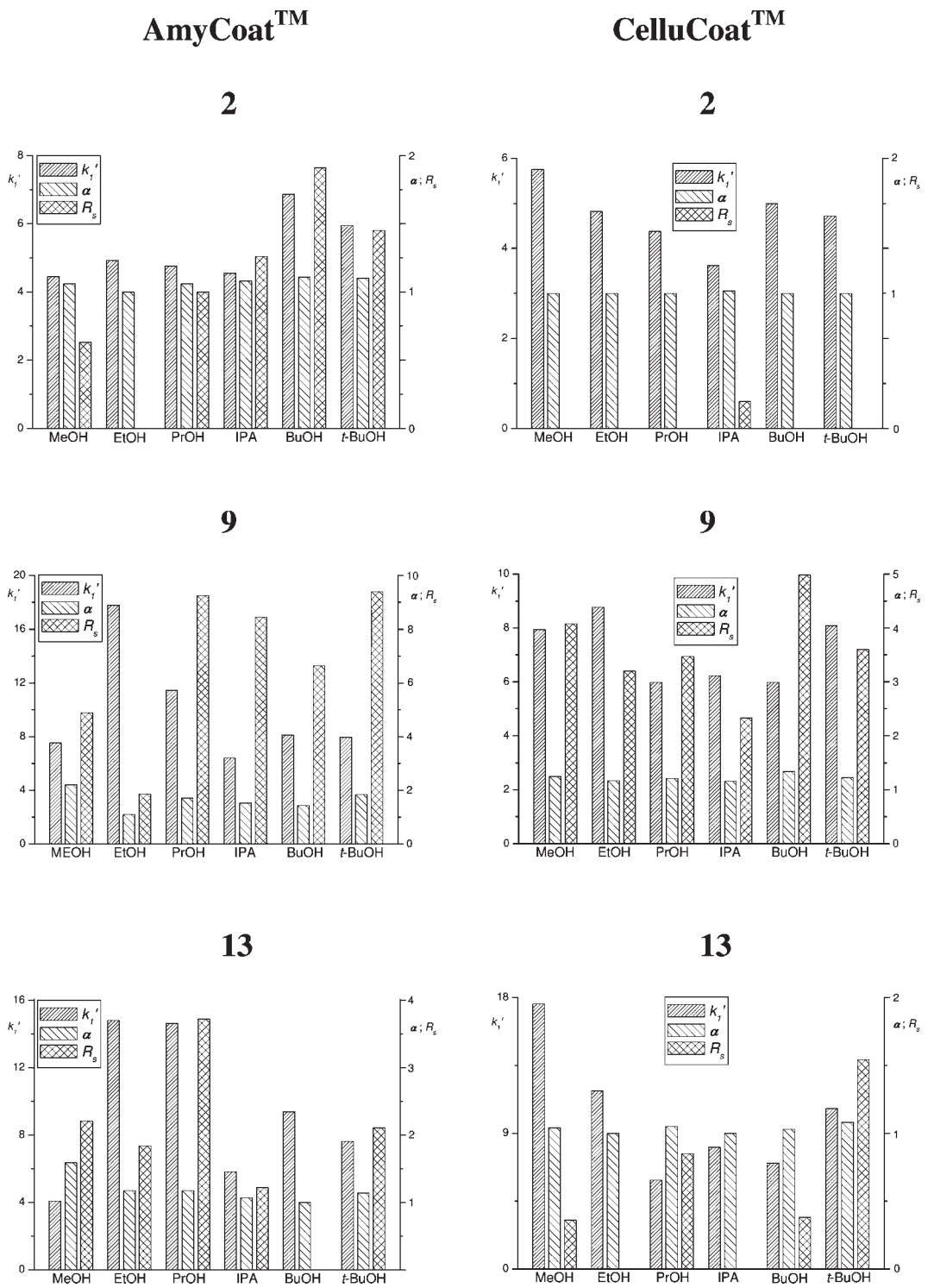

Fig. 3. Effects of the nature of the alcoholic modifier on the retention factor of the first-eluting enantiomer (k_1'), the separation factor (α) and the resolution (R_S) for analytes **2**, **9**, and **13** on AmyCoat™ and CelluCoat™ columns. Chromatographic conditions: mobile phase, *n*-hexane/alcoholic modifier (see text); alcoholic modifiers, MeOH, EtOH, PrOH, IPA, BuOH and *t*-BuOH; flow rate, 0.5 ml min^{-1}; detection, 205 nm.

carbamate residues are located inside, whereas the hydrophobic aromatic groups are outside the polymer chain. The enantiomers can therefore interact with the carbamate groups via H-bonding with the —NH— and —CO= groups and dipole–dipole interactions involving the —CO= moiety. Besides these polar interactions, π–π interactions between the phenyl groups of the CSP and an aromatic group of the solute may play some role in chiral

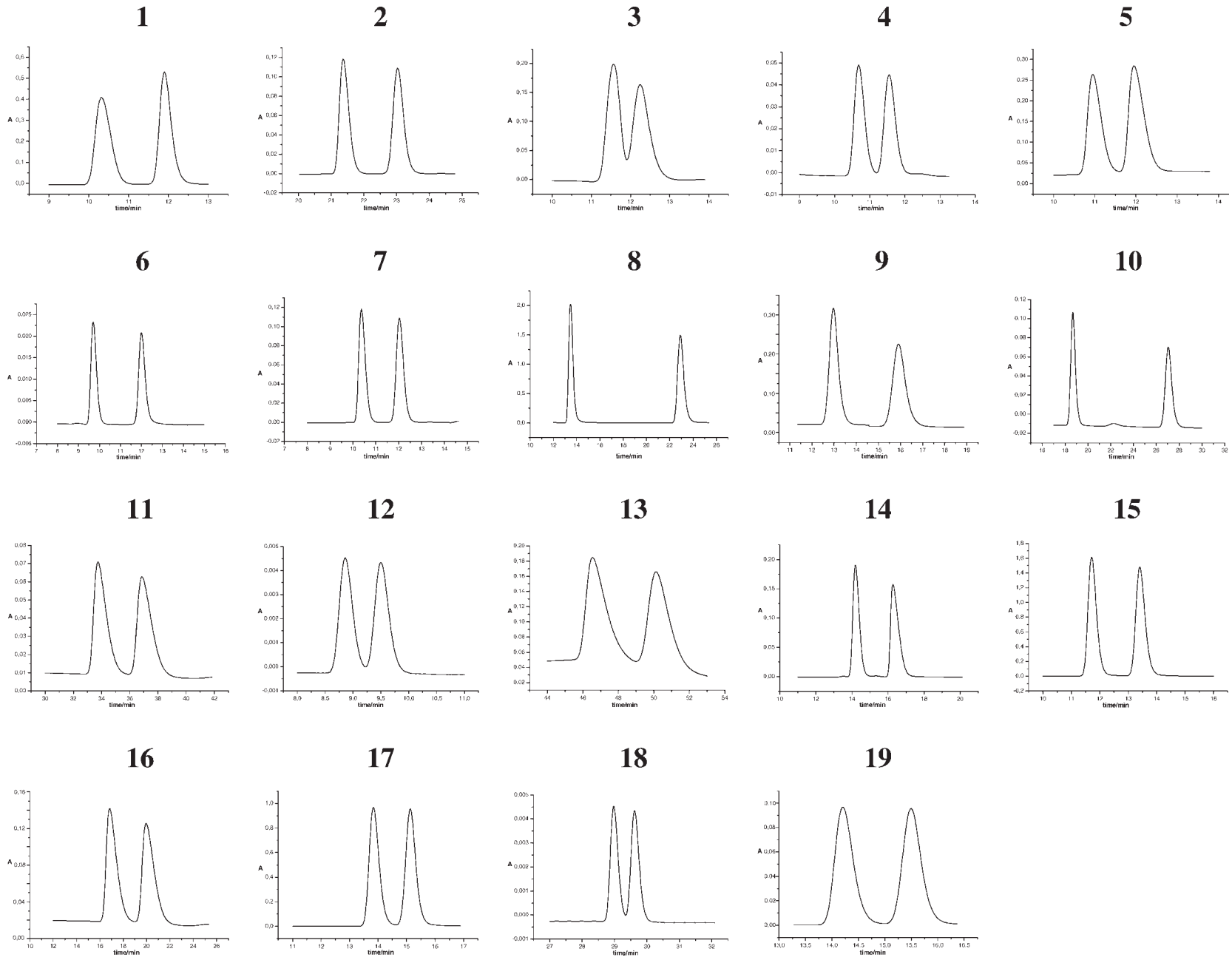

Fig. 4. Selected chromatograms of enantioseparations on either the AmyCoat™ or the CelluCoat™ column. Chromatographic conditions: column, AmyCoat™ for **1**, **2**, **4-11**, **15**, **17**, and **19**, CelluCoat™ for **3**, **12-14**, **16**, and **18**; mobile phase, *n*-heptane/IPA = 90/10 (v/v) for **1**, **3-10**, **12**, **14-17**, and **19**, *n*-heptane/IPA = 98/2 (v/v) for **11**, *n*-heptane/*n*-BuOH = 97/3 (v/v) for **2**, *n*-heptane/*t*-BuOH = 97/3 (v/v) for **13**, and *n*-heptane/IPA = 95/5 (v/v) for **18**; detection, 205 nm; flow rate, 0.50 ml min^{-1}; temperature 25°C; for the elution sequences, see Tables 1 and 2.

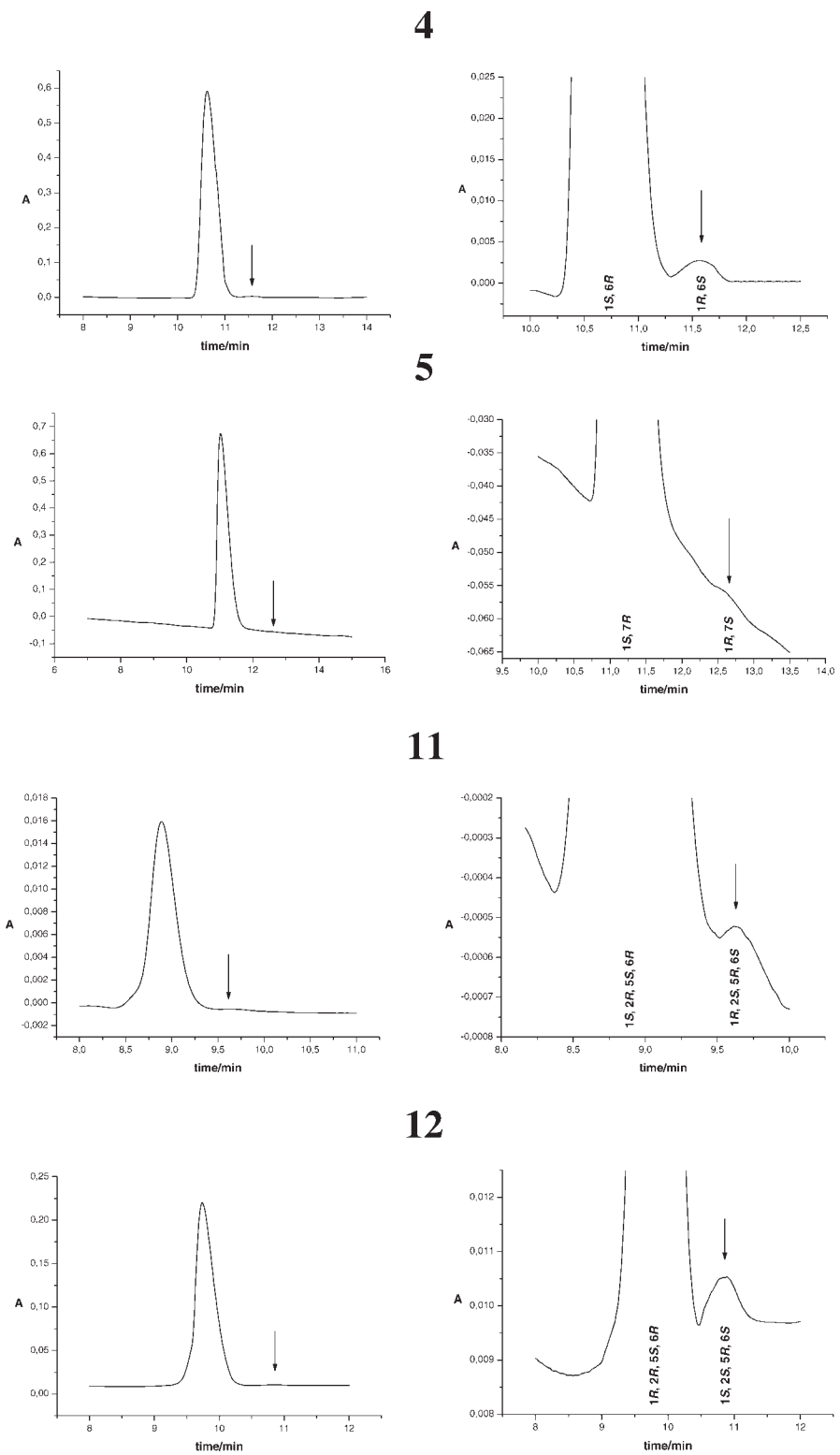

Fig. 5. Separation of minor enantiomers of **4**, **5**, **11**, and **12** when it is present in an excess of the major isomer. Chromatographic conditions: column, AmyCoat™ for **4** and **5**, CelluCoat™ for **11** and **12**; mobile phase, *n*-heptane/IPA = 90/10 (v/v); detection, 205 nm; flow rate, 0.50 ml min^{-1}; temperature 25°C.

recognition. The three-ring systems of **11** and **12** probably fit better sterically into the amylose cavity than into the cellulose cavity, and thus the interactions with the amylose-based CSP are the more favored; probably **11** and **12** have stronger achiral interactions with AmyCoat™ CSP. However, the chiral recognition and chiral interac-

tion seems to be more favored on CelluCoat™ CSP (Tables 1 and 2). The comparison of the retention behavior of analytes with the same ring number, but not containing or containing a double bond (i.e., **2** vs. **3** and **4**; **6** vs. **7**; and **11** vs. **12**), or the comparison of analytes not condensed with or condensed with an aromatic ring (**1** vs. **8**; **2** vs. **9**; and **5** vs. **10**) drew attention to the importance of π–π interactions in the retention. Introduction of a double bond or an aromatic ring into the molecules increased the k', and this increase in most cases (especially for the condensed aromatic ring) being accompanied by increases in selectivity and resolution (Tables 1 and 2).

The largest k' values of **8-10** and especially **13-19** in the mobile phase *n*-heptane/IPA = 90/10 (v/v) are possibly due to the enhanced π–π interactions between the phenyl ring of the analytes and the 3,5-dimethylphenyl ring of the selector (Tables 1 and 2). A further tendency could be observed for analytes **15**, **18**, and **19**. At constant mobile phase composition, k' increased in the sequence fluorine-chlorine-bromine. The size and polarizability of the halogen substituted molecules increase in the sequence fluorine-chlorine-bromine. Probably the polar interaction between the CSP and the molecule increases when fluorine was substituted by chlorine or bromine resulting in larger k'. The larger size of the analyte may contribute to the retention by the increased steric effect (bulkiness) as was observed for analytes **1-7** with increasing number of carbon atoms attached to the β-lactam ring. The position of the chloro substituent in analytes **16**, **17**, and **18** exerted marked effects on the chromatographic behavior. At constant mobile phase composition, the k' values for these analytes were similar on the CelluCoat™ and AmyCoat™ CSPs, but as concerns chiral recognition the *para*-chloro substituted analog was separated on both CSPs with similar enantioselectivity, whereas for the *orto*-chloro substituted analog CelluCoat™ CSP and for *meta*-chloro substituted analog AmyCoat™ CSP proved to be much more efficient (Tables 1 and 2).

In all cases when separation occurred, the sequence of elution was determined, but no general rule could be established for the stereoisomers of the β-lactams on either the amylose- or the cellulose-based phase; interestingly, a difference in elution sequence between AmyCoat™ and CelluCoat™ CSPs was observed for analytes **5**, **6**, **9**, **10**, and **12**. This highlights the importance of identification of the stereoisomers in each chromatographic run.

Selected chromatograms for the enantioseparation of analytes **1-19**, evaluated on the CelluCoat™ or AmyCoat™ CSPs are depicted in Figure 4. Depicted chromatograms with R_S values $R_S > 2.0$ allow the determination of enantiomeric impurity even impurity eluted second. If the separation presented insufficient for enantiomeric purity assessment resolution can be increased by decreasing of the eluent strength of the mobile phase or by changing the type of alcohol and the column (CelluCoat™ or AmyCoat™). Figure 5 depicts separation of minor enantiomers eluting second of **4**, **5**, **11**, and **12** when it is present in an excess of the major isomer (LOD < 0.5%).

CONCLUSIONS

HPLC methods were developed for the separation of the enantiomers of 19 β-lactams. The direct separations were performed on CSPs containing either amylose-*tris*-3,5-dimethylphenyl carbamate (AmyCoat™ column) or cellulose-*tris*-3,5-dimethylphenyl carbamate (CelluCoat™ column) as chiral selector. By variation of the chromatographic parameters, the separation of the stereoisomers was optimized; as a result, baseline resolution was achieved for the β-lactams in at least one chromatographic system. The AmyCoat™ and CelluCoat™ columns appear to be highly complementary. The elution sequence was determined in all cases.

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LITERATURE CITED

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