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Chitosan derivatives as chiral selectors bonded on allyl silica gel: preparation, characterisation and study of the resulting high-performance liquid chromatography chiral stationary phases

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Abstract

Several mixed 10-undecenoyl/phenylaminocarbonyl or benzoyl derivatives of chitosan, differently substituted in the aromatic ring, were prepared, characterized and immobilized on allylsilica gel. The resulting high-performance liquid chromatography (HPLC) chiral supports were tested chromatographically. The influence of the starting polysaccharidic material, as well as that of the solvents used as mobile phase modifiers, on the preparation and performance of the resulting chiral stationary phases (CSPs) is discussed.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Chitosan

1. Introduction

Polysaccharide derivatives, especially those based on cellulose and amylose, have been extensively studied as chromatographic chiral selectors in recent decades. As a result, a number of chiral stationary phases (CSPs) have been developed and used in the resolution of enantiomers by HPLC.

Some other polysaccharides, such as xylan, dextran, chitin or chitosan, have occasionally been used as starting material for the preparation of CSPs. However, the trisphenylaminocarbonyl and the 3,5-dimethylphenylaminocarbonyl derivatives of chitosan [1,2], and the phenylcarbamate and 3,5-dimethylphenylcarbamate of the closely related chitin are the only derivatives described as chromatographic chiral selectors [3]. Recently, the preparation of a mixed 10-undecenoyl/3,5-dimethylphenylaminocarbonyl derivative of chitosan was reported [4]. This derivative was bonded on a silica gel matrix following the fixation method applied successfully to cellulose-based selectors [5,6]. The chromatographic results obtained with this derivative of chitosan encouraged us to prepare other carbamate and benzoate derivatives, in order to test their enantioselectivity as chiral selectors for HPLC.

The present study deals with the preparation and characterisation of bonded chitosan-based CSPs, having chloro or methyl substituted phenyl residues. Chitosan is a 2-deoxy-2-glucosamine polymer ob-

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tained from chitin, a 2-deoxy-2-acetamidoglucose polysaccharide, by hydrolysis. In order to assess the influence of the remaining acetyl groups on the stereoselectivity, analogous derivatives were prepared from chitosan with varying degrees of acetylation. The resulting CSPs were tested using either heptane/2-propanol or heptane/chloroform mixtures as mobile phase.

2. Experimental

^{1}H-NMR spectra were measured in a Varian GEMINI-300 spectrometer at 70°C. IR spectra were registered in a Perkin-Elmer FT-IR 1600 spectrometer. Elemental analyses were performed in a CE Instruments apparatus (Mod. EA 1108), using standard conditions, by the Serveis Científico-Tècnics de la Universitat de Barcelona (Spain). The CSPs were packed into stainless-steel tubes (150×4.6 mm ID) by the slurry method. The chromatographic experiments were performed on a HPLC system consisting of a Waters 600E pump, a Waters 717 auto sampler (Millipore, Milford, MA, USA), equipped with a Waters 996 photo-diode array detector and a Perkin-Elmer 241LC polarimetric detector (Perkin-Elmer, Uberlingen, Germany). The volume of sample injected was 3 μl. The void volume was determined using tri-*tert*-butylbenzene.

2.1. Preparation of the chitosan derivatives.

Low molecular weight chitosan (Mr 70 000, Fluka, Buchs, Switzerland) was used as starting material for the preparation of all derivatives (Fig. 1). The starting material used in the preparation of **1b**–**5b**, was subjected to an additional deacetylation treatment prior to derivatization. Thus, as already described [7], chitosan flakes were suspended in 50% sodium hydroxide at 100°C under argon for 2 h, then filtered off and thoroughly washed in water until neutrality.

Both deacetylated and non-deacetylated chitosan were purified by dissolution in acetic acid, filtration and reprecipitation by neutralisation. The resulting precipitates were exhaustively and successively washed in water, methanol and diethyl ether [8]. The materials obtained were characterized by their ^{1}H-NMR spectra, IR spectra and elemental analyses. The acetyl group content was estimated to be of the order of 20% for the non-deacetylated chitosan and lower than 5% for the deacetylated form.

The method described for the preparation of 10-undecenoyl/3,5-dimethylphenylaminocarbonyl de-

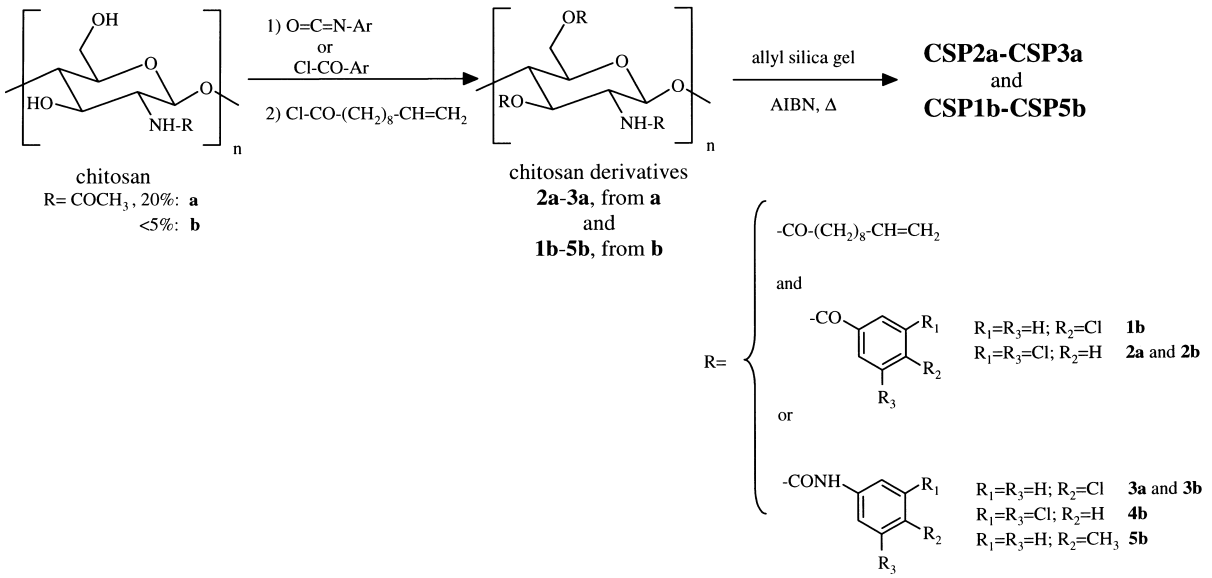

Fig. 1. Preparation of the chiral stationary phases.

rivative [4] was applied to obtain the chitosan derivatives in the present study. Thus, the starting chitosan, deacetylated or not, was suspended in pyridine and treated with an excess of the corresponding aryl isocyanate or benzoyl chloride (6 mol per mol of glucosamine units). The mixture was vigorously stirred at about 100°C until a viscous solution was obtained (from 30 min to 2 h, depending on the derivative). Then 0.5 mol of 10-undecenoyl chloride per mol of glucosamine units was added and the mixture was allowed to react for 24 h at 100°C.

All derivatized chitosans were isolated by precipitation in methanol or methanol/water mixtures, redissolved, reprecipitated and washed in methanol. When an arylaminocarbonyl derivative was prepared, the precipitate obtained was thoroughly washed in hot ethanol in order to remove the N,N′-bis(aryl)urea formed as a by-product. The polysaccharidic selectors thus obtained were characterized by their ^{1}H-NMR spectra and elemental analyses. The degree of substitution per glucosamine unit (DS) was calculated from elemental analyses (Table 1).

2.2. Preparation of chiral stationary phases

The mixed polysaccharide derivatives were immobilised on silica gel (5 μm, Nucleosil 100–5, Macherey-Nagel, Düren, Germany), previously modified by the introduction of allyl groups [5]. The fixation method described for other polysaccharide-based selectors was applied [4–6]. After the polymerisation reaction, the chiral supports obtained were suspended in tetrahydrofuran at reflux temperature for 2 h, filtered off and washed in hot tetrahydrofuran, chloroform and acetone. The resulting CSPs were characterised by elemental analysis (Table 1).

3. Results and discussion

As mentioned above, chitosan is a non-natural polysaccharide obtained from chitin, a 2-deoxy-2-acetamidoglucopyranosyl polymer, by hydrolytic treatment with concentrated alkali in order to remove the acetyl groups. However, this treatment does not yield fully deacetylated chitosan. A certain number of remaining acetyl groups are always present in the commercially available chitosan. This amount may differ from supplier to supplier or even from batch to batch. Thus, the acetyl content in the chitosan used in the preparation of the previously reported 10-undecenoyl/3,5-dimethylphenylaminocarbonyl derivative was estimated to be 13% [4]. However, the acetylation degree of the new batch of chitosan, obtained from the same supplier, was 20%.

Table 1
Characterisation of the chitosan derivatives and the CSPs obtained

Chitosan derivative	Elemental analyses of chitosan derivatives				Substitution degree[a]		Chiral supports	Elemental analyses of the chiral stationary phases				g. polysac. derivative/ 100 g phase[b]	HEPT (cm)[c]
	%C	%H	%N	%Cl	Undec.	Ar		%C	%H	%N	%Cl		
1b	57.40	4.00	2.37	17.14	0.19±0.02	2.74±0.04	**CSP1b**	10.93	1.48	0.41	2.66	16.9	5.1×10^{-3}
2a	46.83	3.48	2.95	25.94	0.12±0.07	1.56±0.11	**CSP2a**	7.84	1.41	0.38	2.96	12.4	4.83×10^{-3}
2b	49.82	3.15	2.05	28.60	0.23±0.03	2.67±0.06	**CSP2b**	8.44	1.29	0.33	3.43	15.7	3.02×10^{-3}
3a	52.80	4.96	7.38	13.12	0.39±0.10	1.66±0.31	**CSP3a**	9.78	1.46	1.12	1.96	14.6	7.67×10^{-3}
3b	50.32	3.99	8.20	15.14	0.14±0.07	2.50±0.27	**CSP3b**	10.56	1.20	1.20	2.41	14.0	7.12×10^{-3}
4b	45.16	3.48	6.94	26.14	0.27±0.10	2.18±0.41	**CSP4b**	9.24	1.45	1.07	3.38	14.9	5.07×10^{-3}
5b	61.30	5.92	8.69		0.25±0.07	2.31±0.38	**CSP5b**	13.22	1.96	1.55		17.2	6.89×10^{-3}

[a] Calculations based on elemental analyses. The maximum substitution degree of a glucosamine unit is three. A 20% of remaining acetyl groups is taken into account in the calculations for derivatives **2a** and **3a**, and a 5% for **1b**–**5b**.
[b] Calculated from elemental analyses. Based on %N.
[c] Supports were packed in 15×0.46 cm stainless steel columns. Calculated using 1,3,5-tri-*tert*-butylbenzene.

Table 2
Chromatographic results*

Racemic	CSP1b			CSP2a			CSP2b			CSP3a			CSP3b			CSP4b			CSP5b			Mobile
compounds	k'_1	α	*Rs*	k'_1	α	*Rs*	k'_1	α	*Rs*	k'_1	α	*Rs*	k'_1	α	*Rs*	k'_1	α	*Rs*	k'_1	α	*Rs*	phase[a]
1	0.41(+)	1.18	–	0.17	1.00	–	0.28	1.14	–	0.16	1.00	–	0.34	1.00	–	0.50(+)	1.36	0.57	0.23	1.00	–	**a** 98:2
2	0.43(+)	1.19	–	0.18	1.00	–	0.33	1.00	–	0.13	1.00	–	0.30	1.00	–	0.41(+)	1.34	0.65	0.26	1.00	–	**a** 98:2
3	7.85	1.32	0.83	1.71	1.00	–	2.80	1.00	–	13.23	1.00	–	11.84	1.00	–	3.10	1.66	1.55	11.55	1.00	–	**a** 98:2
4	5.30(−)	1.16	0.81	1.71(−)	1.10	0.82	3.17(−)	1.08	0.50	4.39(−)	1.15	–	7.36(−)	1.24	0.97	8.61	1.00	–	5.13	1.00	–	**a** 98:2
5	10.68	1.12	–	3.19	1.08	–	7.72(−)	1.12	0.61	10.17	1.00	–	16.46	1.00	–	7.17[c]	1.28	0.82	10.63	1.00	–	**a** 98:2
6	28.85	1.00	–	3.51(R)	1.22	–	7.84(R)	1.25	–	10.22	1.00	–	16.81	1.17	–	7.22[c]	1.00	–	14.16[b]	1.00	–	**a** 90:10
lorazepam	6.86(−)	1.34	0.54	1.98(−)	1.22	0.75	3.94(−)	1.19	0.52	10.62	1.00	–	16.35	1.00	–	6.17	1.26	–	12.40	1.00	–	**a** 80:20
lormetazepam	9.97(−)	1.19	–	3.42	1.09	–	7.61	1.00	–	17.66	1.00	–	28.02	1.13	–	21.94(−)	1.71	1.68	15.65	1.19	–	**a** 80:20
oxazepam	6.66(−)	1.29	–	1.58(−)	1.23	0.81	3.17(−)	1.27	0.82	10.75	1.00	–	17.32	1.00	–	8.63	1.00	–	12.74	1.00	–	**a** 80:20
temazepam	11.14(−)	1.19	–	4.09	1.07	–	9.04	1.00	–	20.50	1.00	–	25.97(−)	1.18	0.36	18.70(−)	1.76	1.49	13.51	1.32	0.74	**a** 80:20
warfarin	11.92	1.00	–	2.16	1.21	0.65	5.33(−)	1.22	0.58	14.61[b]	1.00	–	12.97[b]	1.00	–	9.42(−)	1.72	1.34	16.43	1.00	–	**a** 90:10
1	0.79(+)	1.19	0.69	0.39(+)	1.19	–	0.91(+)	1.20	1.30	0.95(+)	1.24	0.41	0.51(+)	1.25	0.49	1.45	1.00	–	0.79(+)	1.18	0.53	**b** 95:5
2	0.69(+)	1.18	0.55	0.34(+)	1.19	–	0.72(+)	1.23	1.23	0.71(+)	1.27	0.41	0.46(+)	1.17	–	1.21	1.00	–	0.82(+)	1.27	1.06	**b** 95:5
3	6.03(+)	1.36	2.33	4.46	1.00	–	5.84	1.03	–	13.09	1.00	–	10.95	1.00	–	3.89(+)	1.22	1.35	13.66(+)	1.11	–	**b** 75:25
4	7.06(−)	1.10	0.77	4.03	1.08	0.63	9.73	1.07	0.69	15.44(−)	1.16	–	8.41(−)	1.29	1.26	9.83	1.07	–	12.20	1.00	–	**b** 90:10
5	4.12	1.00	–	2.02	1.00	–	6.00	1.00	–	8.71	1.00	–	5.77	1.00	–	7.88(+)	1.70	2.30	5.25	1.00	–	**b** 75:25
6	4.46	1.00	–	2.07(R)	1.17	1.62	3.75(R)	1.22	1.83	10.26(R)	1.31	0.34	7.01(R)	1.28	0.79	4.00	1.00	–	13.63(R)	1.22	0.83	**b** 50:50
7	4.85	1.00	–	2.72	1.00	–	4.83(R)	1.06	–	11.45(R)	1.29	0.41	7.44(R)	1.31	1.00	4.73	1.00	–	15.11(R)	1.13	–	**b** 50:50
8	2.39	1.07	0.40	1.03(−)	1.15	0.94	4.43(−)	1.15	1.74	4.54	1.00	–	1.31	1.00	–	3.48(+)	1.13	0.47	3.13(−)	1.09	0.50	**b** 95:5
9	2.12	1.00	–	1.31	1.00	–	2.73	1.00	–	6.77	1.00	–	4.14(S)	1.15	–	2.71(S)	1.29	1.08	6.35(R)	1.13	–	**b** 50:50
lorazepam	8.55(−)	1.17	1.05	6.25	1.04	–	13.05	1.06	0.45	19.56[d]	1.00	–	14.74	1.00	–	5.20(−)	1.72	2.49	10.18	1.12	–	**b** 0:100
lormetazepam	4.95	1.00	–	4.72	1.00	–	7.79	1.00	–	17.50	1.00	–	13.19	ca 1	–	14.84(−)	1.49	1.79	6.96	1.00	–	**b** 50:50
oxazepam	7.24(−)	1.14	0.84	5.68	1.06	–	10.38	1.11	0.95	18.75[d]	1.00	–	13.74	1.00	–	4.88(−)	1.58	1.99	9.91	1.00	–	**b** 0:100
temazepam	4.66	1.06	0.32	4.19	1.00	–	8.04	1.00	–	18.62	1.00	–	11.93(−)	1.19	0.57	9.61(−)	2.45	4.19	6.25(−)	1.13	0.57	**b** 50:50
warfarin	2.45	1.00	–	8.80	ca. 1	–	2.55(−)	1.08	–	4.30	1.90	0.92	3.62	2.26	1.70	1.27(+)[e]	3.47	3.97	3.23	1.00	–	**b** 25:75

[*] k'_1, capacity factor of the first eluted enantiomer; α, selectivity factor; *Rs*, resolution factor. The configuration of optical rotation sign of the first eluted isomer is shown in parentheses. Column: 15×0.46 cm. Flow rate: 1 ml/min.

[a] **a**: Heptane/2-propanol; **b**: Heptane/chloroform.

[b] Heptane/2-PrOH 80:20.

[c] Heptane/2-PrOH 90:10.

[d] Heptane/chloroform 25:75.

[e] Heptane/chloroform 0:100.

In order to assess the influence of the variability in the starting material on the chromatographic behaviour of the resulting CSPs, two analogous derivatives were prepared from the new batch of chitosan (**2a** and **3a**) and from an additionally deacetylated chitosan (**2b** and **3b**). The degree of substitution (DS) of the 3,5-dichlorobenzoyl (**2a**) and the 4-chlorophenylaminocarbonyl (**3a**) derivatives was lower than expected. Hence, problems were encountered during the coating step in the preparation of the corresponding stationary phases (**CSP2a** and **CSP3a**). In spite of the reduced solubility of these derivatives, especially **3a**, the corresponding CSPs were succesfully prepared and packed after several attempts.

In principle, the use of completely deacetylated chitosan would avoid the variability observed in the acetyl content of the commercial material, and would facilitate the characterisation of the derivatives. In order to obtain chiral selectors with a higher DS and, therefore, to increase their solubility, chitosan was further deacetylated prior to derivatization. The deacetylation treatment yielded a practically non-acetylated product, as assessed by ^{1}H-NMR and IR spectra. The final acetyl content, calculated by elemental analysis, was 0–5%. The maximum value, 5%, was taken into account for further calculations.

As expected, the use of deacetylated chitosan in the preparation of **2b** and **3b** resulted in highly substituted chiral selectors, with increased solubility. Both derivatives were satisfactorily bonded on allylsilica gel. The corresponding stationary phases **CSP2b** and **CSP3b** were tested and compared to **CSP2a** and **CSP3a**, respectively. The chromatographic results show that the enantioselectivities of the deacetylated and the non-deacetylated chitosan derivatives are similar (Table 2). However, the increased solubility and the easier manipulation of the deacetylated selectors during the preparation of the CSPs justify the additional hydrolytic step. All other derivatives were prepared from the deacetylated material.

In general, derivatives **1b**–**5b** presented high DS and good solubility in many solvents, especially the benzoates **1b** and **2b**, which were partially soluble even in alcohols. The successful bonding of all the derivatives onto the silica matrix prevents the loss of chiral selector when any of those solvents is used as

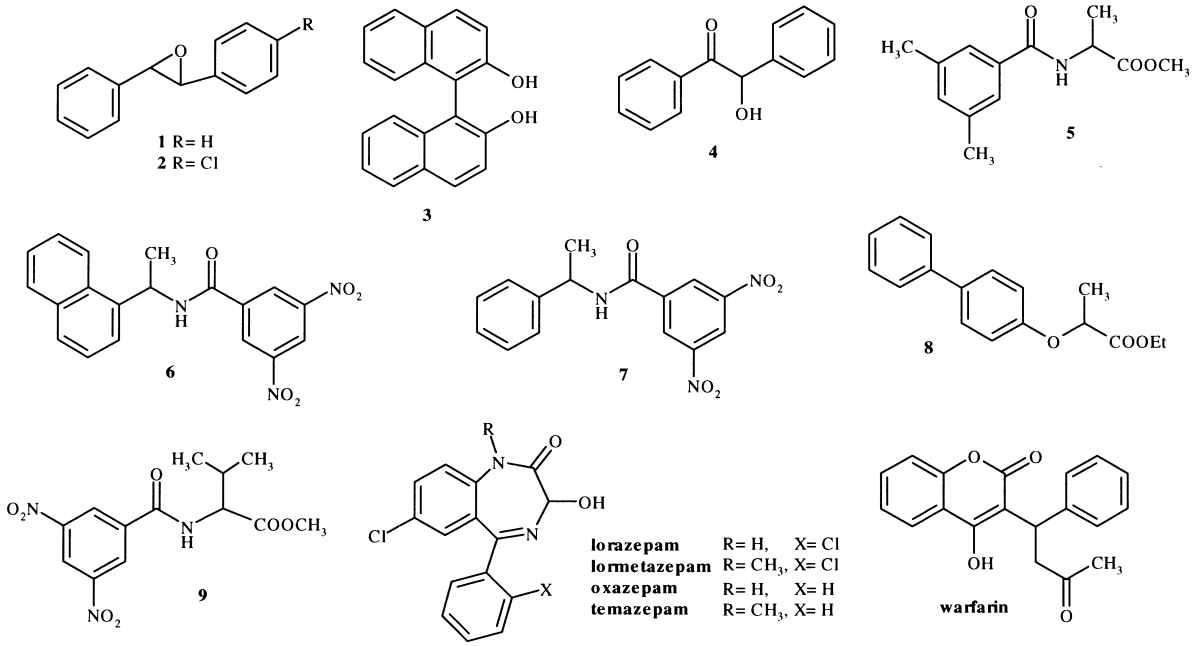

Fig. 2. Chemical structures of some of the racemic compounds tested.

mobile phase. Thus, all the CSPs were tested both in heptane/2-propanol and heptane/chloroform mixtures. Results are presented in Table 2. In Fig. 2 the chemical structures of some of the racemic test compounds are shown.

If structural considerations are taken into account, the expected chromatographic enantioselectivity for chitosan derivatives must be closely related to that of cellulose derivatives. As for cellulose, β(1–4) bonds are established between glucosidic units in chitosan. Thus, in contrast to the low enantioselectivity of benzoyl derivatives of amylose [9,10], benzoyl derivatives of chitosan (**CSP1b** and **CSP2a/b**) are more enantioselective for many racemic compounds.

However, noticeable differences can be observed when chitosan and cellulose derivatives are compared. For example, **CSP3b** and **CSP5b** show poor discrimination ability when using heptane/2-propanol mixtures as mobile phase, in contrast to the analogous cellulose derivatives (**CSP4** and **CSP2** in [6], respectively). Nevertheless, this is not a general behaviour for chitosan derivatives. Thus, **CSP4b**

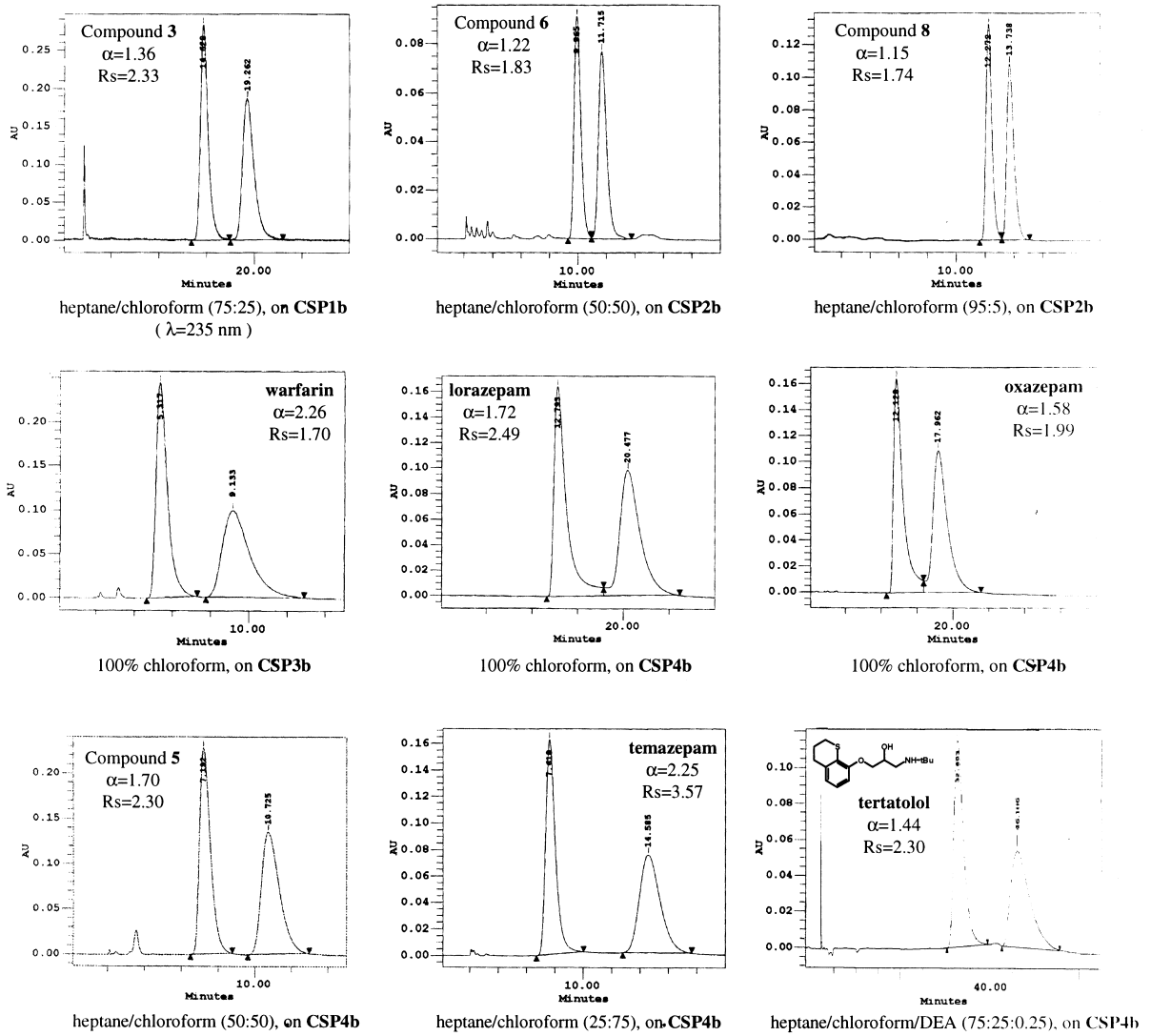

Fig. 3. Resolution of some racemic test compounds on different chitosan-based CSPs (flow rate: 1 ml/min; UV detection: 254 nm, except otherwise indicated).

shows enantioselectivity values of the same order as the corresponding cellulose derivative (**CSP5** in Ref. [6]).

In earlier studies on cellulose derivatives, changes in enantioselectivity were observed when the organic modifier in the mobile phase was changed from 2-propanol to chloroform. Usually, a reduction in the number of successfully resolved compounds was simultaneously observed. However, a marked increase in the application domain of the CSPs based on chitosan derivatives was observed when chloroform was used in the mobile phase. Not only higher enantioselectivity values, but also higher resolution factor values are obtained in these conditions. This is more noticeable for **CSP3b**, **CSP4b** and **CSP5b**, based on phenylaminocarbonyl derivatives, than for benzoyl derivatives (**CSP1b** and **CSP2b**).

This effect may be related to the behaviour of the chiral selectors in the solvents used as mobile phases. Phenylaminocarbonyl derivatives are more swollen by chloroform than by alcohols. Thus, in the former those chiral selectors may become more accessible to analytes. Benzoyl derivatives are swollen by both chloroform and 2-propanol. Therefore, they may maintain a similar accessiblility for analytes when these solvents are used. This is more noticeable for the 4-substituted aryl carbamates **3b** and **5b**, which show poor enantioselectivity in heptane/2-propanol mixtures, that improves in heptane/chloroform eluents.

Among the chitosan derivatives tested, the 3,5-dichlorophenylaminocarbonyl derivative (**4b**) has the most remarkable chiral discrimination ability. The corresponding **CSP4b** shows the best chromatographic results, and it is able to completely resolve drugs such as benzodiazepines, warfarin or tertatolol (Fig. 3). In contrast, it is the only CSP that is not able to resolve compounds **1** and **2** in heptane/chloroform mobile phases, though in heptane/2-propanol the separation is achieved.

4. Conclusions

Small variations in the amount of acetyl group contained by the starting chitosan do not significantly affect the chromatographic discrimination ability of the chiral selectors prepared from it. However, increased DS can be obtained if deacetylation process is performed prior to derivatization. Highly substituted derivatives show improved solubility and easier manipulation, which facilitates the development of better chiral supports.

The chromatographic behaviour of the chiral selectors seems to be related to their solubility. Thus, when chloroform is used as mobile phase modifier, the selectors swell and become more accessible to analytes. As a result, improved chromatographic results are achieved. Benzoate derivatives show similar behaviour in both 2-propanol and chloroform mixtures due to the same effect.

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