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Short Communication

Direct resolution of racemic drugs using cellulase silica as a chiral stationary phase

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ABSTRACT

The enantiomers of propafenone, flecainide and its two analogues 4-hydroxyflecainide and 5-hydroxyflecainide, betaxolol, doxazosin, and terazosin and its analogue A-65297 were separated on a cellulase silica column (TrichSep-100). The effects of pH and the concentration of the organic modifier, 2-propanol, were studied.

INTRODUCTION

Immobilized proteins have been extensively used as chiral stationary phases for the direct resolution of racemates. α_1-Acid glycoprotein (AGP), bovine serum albumin and human serum albumin, immobilized on silica have been successfully used in the separation of racemic drugs and related substances [see refs. 1–3 for reviews]. Furthermore, the use of ovomucoid, a glycoprotein from chicken egg white, in enantioseparations has also been reported [4–6].

More recently, Erlandsson *et al.* [7] and Marle *et al.* [8] have reported on direct resolution of enantiomers using immobilized cellulase, a glycoprotein produced by the fungus *Trichoderma reesei*, as a chiral stationary phase. Also, two recent studies on this chiral phase were performed by Vandenbosch *et al.* [9,10]. The substances investigated on this new chiral column are mainly β-adrenergic blocking agents and local anaesthetics such as prilocaine and analogues.

In this communication, we report on the use of a cellulase silica column for the chiral separation of eight primary and secondary amines which have not previously been baseline resolved on cellulase.

EXPERIMENTAL

Apparatus

The liquid chromatographic system used was a Jasco Model PU-980 pump, a Jasco Model 851-AS autoinjector and a Jasco Model UV-975 variable-wavelength detector. A Barspec Data System was used to collect and process the chromatographic data.

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Chemicals

Terazosin hydrochloride and A-65297 were obtained from Abbott Labs. (North Chicago, IL, USA). Doxazosin mesylate was obtained from Pfizer (Karlsruhe, Germany). Flecainide acetate, 4-hydroxyflecainide acetate and 5-hydroxyflecainide were all obtained from 3M Health Care (Loughborough, UK). Betaxolol hydrochloride was obtained from Alcon-Couvreur (Puurs, Belgium), and propafenone hydrochloride was obtained from Knoll (Ludwigshafen, Germany). The structures of these substances are given in Fig. 1.

All other chemicals and solvents used were of analytical grade, available through normal commercial channels.

Chromatographic conditions

The column used was a TrichSep-100, particle size 10 μm, 10 cm × 4.6 mm I.D. (Skandinaviska GeneTec, Kungsbacka, Sweden).

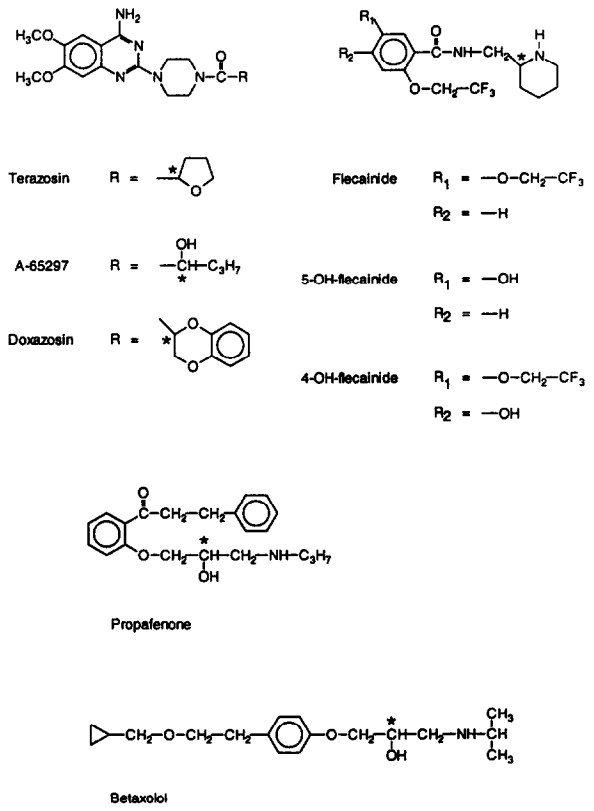

Fig. 1. Structures of the solutes.

The chromatographic experiments were performed at room temperature (20–25°C). Phosphate buffers were prepared from sodium dihydrogenphosphate and disodium hydrogenphosphate, and acetate buffers from sodium acetate and acetic acid. All mobile phases were filtered through a Millipore filter, type HV 0.45 μm, and degassed in an ultrasonic bath prior to use. The solutes were all dissolved in mobile phase at concentrations of 0.1–0.2 mM, and 20–50 μl of these solutions were injected. The flow-rate was 0.7 ml/min, and the detector was set at 240 nm.

The retention time, t_R, was used to calculate the capacity factor, $k' = (t_R - t_0)/t_0$, where t_0 was determined by injection of water, that was assumed to be non-retarded. The enantioselectivity, α, was calculated as k_2'/k_1', where k_2' is the capacity factor of the more retained enantiomer. The peak symmetry was measured at baseline: the projection of the intersection point of the two peak tangents divided the baseline in two parts, a, the front side and b, the rear side. The asymmetry factor, *asf*, was then calculated as b/a. The resolution of incompletely resolved peaks was calculated as Kaiser's peak separation function, f/g [11,12]: one line was drawn joining the maxima of the two peaks; a second line was drawn from and perpendicular to the baseline through the valley between the peaks and up to meet the first line. This distance is defined as g. The distance between the valley and the intersection of the two lines is defined as f. The ratio $f/g = 1$ corresponds to complete separation. The resolution of completely resolved peaks was calculated using the equation

$$R_s = 2(t_{R2} - t_{R1})/w_1 + w_2$$

where w_1 and w_2 are the peak widths at baseline for the first and the second eluting band, respectively.

RESULTS AND DISCUSSION

The racemic drugs analyzed are shown in Fig. 1. The results of the analyses of these solutes are shown in Tables I and II. Generally speaking, good separations could be obtained when proper conditions were used and two factors, pH and

TABLE I

INFLUENCE OF VARIATION OF pH ON THE SEPARATION OF ENANTIOMERS

Mobile phase: 0.05 *M* acetate buffer with 0.065 *M* 2-propanol; flow-rate: 0.7 ml/min. asf_2 = Asymmetry factor for the second eluting peak; nc = not calculated.

Compound	Parameter	pH				
		3.5	4.5	5.0	5.5	6.5
Terazosin	k'_2	1.69	10.4	15.7	39.6	102.8
	α	[a]	1.21	1.29	1.36	1.46
	asf_2	nc	1.46	1.69	1.77	nc
	f/g	nc	0.49	0.95	0.94	1.63[b]
A-65297	k'_2	2.06	10.9	19.4	27.1	42.4
	α	1.43	1.59	1.68	1.75	2.15
	asf_2	1.67	1.76	1.87	1.82	1.44
	f/g	0.69	0.91	2.83[b]	2.76[b]	3.59[b]
Doxazosin	k'_2	5.96	34.0	72.6	103.1	152.7
	α	1.0	1.20	1.39	1.41	2.19
	asf_2	–	1.69	2.45	1.73	1.45
	f/g	0	0.41	0.89	0.89	2.93[b]
Flecainide	k'_2	0.19	0.71	1.40	2.89	12.9
	α	1.0	1.90	2.29	2.89	3.70
	asf_2	–	1.66	2.17	1.95	1.36
	f/g	0	0.51	0.93	0.94	3.00[b]
4-Hydroxyflecainide	k'_2	0.43	1.11	2.25	5.59	11.6
	α	1.0	2.46	3.20	4.20	4.89
	asf_2	–	1.96	2.16	2.54	2.98
	f/g	0	0.87	3.49[b]	3.99[b]	5.06[b]
5-Hydroxyflecainide	k'_2	0.29	0.42	0.93	1.69	5.67
	α	1.0	[a]	1.47	1.87	2.06
	asf_2	–	nc	2.00	1.88	1.73
	f/g	0	nc	0.89	1.97[b]	3.11[b]
Propafenone	k'_2	0.35	1.65	2.90	5.53	21.2
	α	1.0	1.2	1.38	1.73	2.42
	asf_2	–	1.27	3.11	2.94	1.47
	f/g	0	0.18	0.62	0.74	0.93
Betaxolol	k'_2	0	0.83	1.71	4.22	10.2
	α	1.0	1.76	2.02	2.59	3.00
	asf_2	–	2.23	2.69	2.73	2.63
	f/g	0	0.60	2.20[b]	2.62[b]	3.66[b]

[a] Separation tendency; *i.e.* $\alpha > 1.0$.
[b] Calculated as R_s.

2-propanol concentration in the mobile phase were found to be important for the regulation of enantioselectivity and retention.

Influence of pH on chiral selectivity and retention

An increase in pH of the mobile phase resulted in increased retention for all the amines studied in this report, as is shown in Table I. Similar findings have been reported for β-blockers [8,10] and local anaesthetics [8]. The isoelectric point of the cellulase protein is 3.9 and an increase in pH of the mobile phase would result in increased electronegativity of the protein. The

TABLE II

INFLUENCE OF CONCENTRATION OF 2-PROPANOL ON THE SEPARATION OF ENANTIOMERS

Mobile phase: 2-propanol in phosphate buffer pH 6.0 (ionic strength 0.01). Flow-rate: 0.7 ml/min. asf_2 and nc as in Table I.

Compound	Parameter	Concentration of 2-propanol (M)			
		0.13	0.39	0.65	1.3
Terazosin	k'_2	∞	68.1	52.5	23.9
	α		1.43	1.39	1.36
	asf_2		1.95	1.88	2.20
	f/g		0.97	0.99	0.96
Flecainide	k'_2	13.8	12.2	12.2	12.5
	α	3.56	3.73	3.79	3.45
	asf_2	2.67	1.92	2.12	2.26
	f/g	3.70[a]	4.21[a]	4.71[a]	4.61[a]
4-Hydroxyflecainide	k'_2	22.9	19.1	17.9	14.4
	α	5.28	4.82	4.17	2.55
	asf_2	3.81	2.16	2.14	2.55
	f/g	5.13[a]	5.56[a]	5.31[a]	3.69[a]
5-Hydroxyflecainide	k'_2	9.06	9.42	10.2	11.9
	α	1.92	1.61	1.34	1.1
	asf_2	3.63	1.86	2.10	nc
	f/g	2.21[a]	2.14[a]	0.97	0.12
Betaxolol	k'_2	13.8	15.4	15.7	15.6
	α	2.96	3.42	3.84	4.53
	asf_2	3.24	2.55	2.54	2.98
	f/g	2.88[a]	3.80[a]	4.56[a]	4.80[a]
Propafenone	k'_2	21.6	17.1	16.1	12.5
	α	2.14	2.38	2.38	2.12
	asf_2	3.23	2.17	2.62	3.15
	f/g	0.86	0.93	0.97	0.96

[a] Calculated as R_s.

amines studied would be predominantly protonated in the pH range 3.5–6.5 and the increased retention observed at higher pH might therefore at least in part be explained by increased electrostatic attraction between the solute and the protein. Table I also demonstrates that the enantioselectivity, α, increased with increasing pH. Most of the enantiomeric pairs eluted as single peaks at the lower pH values studied. In some cases, *e.g.* terazosin at pH 3.5, a peak shoulder was clearly seen, an indication for a minor separation of the enantiomers. Since pure enantiomers were not available it was not possible to calculate these α values with sufficient accuracy, and in Table I they are given as a "separation tendency".

In order to obtain baseline separation of the enantiomeric pairs studied, the pH had to be around 6. For some of the solutes, an increase in 2-propanol content was also required. Examples of the separations obtained are shown in Figs. 2 and 3.

Influence of organic modifier, 2-propanol, on retention and chiral selectivity

The retention and enantioselectivity can also be controlled by the addition of an uncharged organic modifier to the mobile phase. On the AGP column, the modifier most extensively studied is 2-propanol [13]. The influence of 2-propanol has also been studied on the cellulase column, where an increase in the concentration

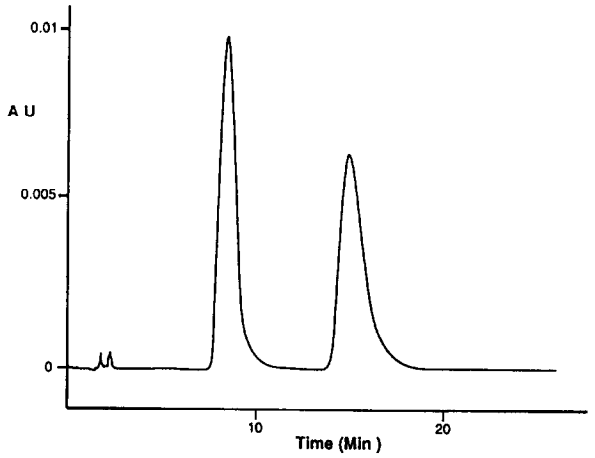

Fig. 2. Resolution of the enantiomers of 5-hydroxyflecainide. Column: TrichSep-100 (100 × 4.6 mm I.D.); mobile phase: 0.05 *M* acetate buffer pH 6.5 containing 0.065 *M* 2-propanol. Amount injected: 2 nmol.

of the alcohol improved the enantioselectivity and peak symmetry for the amines metoprolol, propranolol and prilocaine, while the enantioselectivity was almost unaffected for warfarin and omeprazole (acid and ampholyte, respectively) [8].

For this part of the study we chose phosphate buffer instead of acetate buffer, as phosphate buffer has been reported to give slightly higher stereoselectivity, peak symmetry and resolution for propranolol as compared to acetate buffer [8].

In this study, the effect of 2-propanol concentration was investigated for six of the solutes, as presented in Table II. Note that two of the solutes are being structurally related to meto prolol and propranolol. Compared to metoprolol and propranolol, these two solutes, betaxolol and propafenone, showed a slightly different retention pattern. The highest enantioselectivity for betaxolol was obtained at high 2-propanol content, while the enantioselectivity of propafenone was almost not affected. Interestingly, the increasing enantioselectivity of betaxolol was caused by a decrease in retention of the first eluting enantiomer, while the retention of the second eluting enantiomer initially increased and eventually reached a plateau. The partial resolution ($f/g = 0.85$) of propafenone enantiomers on cellulase at pH 5 was recently reported by Vandenbosch *et al.* [9].

The enantioselectivity of the two analogues of flecainide was also strongly dependent on the concentration of 2-propanol. As can be seen in Fig. 4, the first eluting enantiomer of 5-hydroxyflecainide was strongly retained by an increased

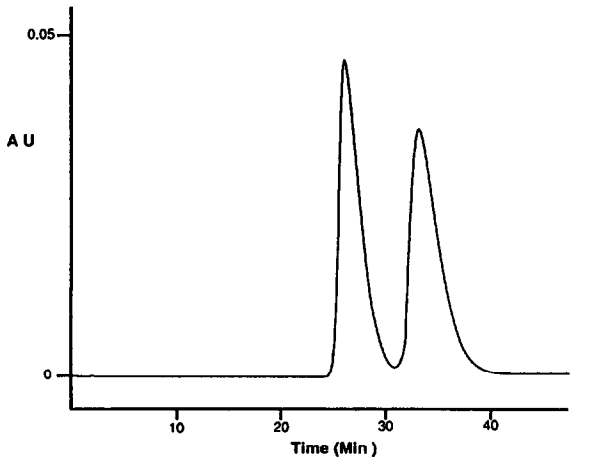

Fig. 3. Resolution of the enantiomers of terazosin. Column: TrichSep-100 (100 × 4.6 mm I.D.); mobile phase: 0.05 *M* acetate buffer pH 5.0 containing 0.065 *M* 2-propanol. Amount injected: 3 nmol.

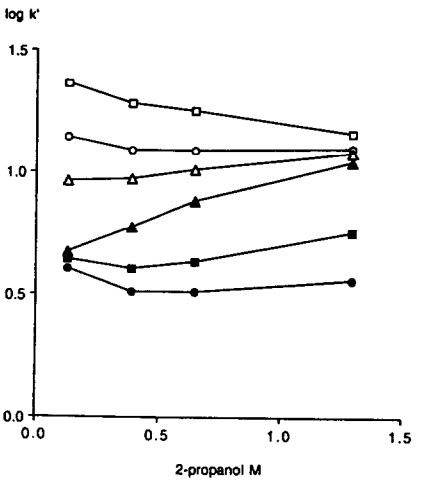

Fig. 4. Influence of mobile phase content of 2-propanol on the capacity factors of the flecainides. Column: TrichSep-100 (100 × 4.6 mm I.D.); mobile phase: phosphate buffer (ionic strength 0.01) pH 6.0 containing different amounts of 2-propanol. ●, ○ = flecainide; ■, □ = 4-hydroxyflecainide; ▲, △ = 5-hydroxyflecainide.

concentration of 2-propanol. The retention of the second enantiomer increased also, but not as dramatically, and thus the largest enantioselectivity was obtained at a low content of 2-propanol. A low concentration of 2-propanol was also favourable for the enantioselectivity of 4-hydroxyflecainide. This was however due to a decrease in retention of the second eluting enantiomer, while the first eluting one showed a complicated dependence on 2-propanol content. Interestingly, the first eluting enantiomers of all three flecainides were eluted close together at low 2-propanol concentration, while an increase in 2-propanol separated these three peaks. The opposite was true for the second eluting enantiomers and thus an intermediate concentration should be chosen to resolve all six peaks. This might be an interesting pharmaceutical application since 4-hydroxyflecainide and 5-hydroxyflecainide are regarded as impurity and degradation product, respectively, of flecainide. The observed differences in the effect of 2-propanol on the flecainides demonstrate that the enantioselectivity is sensitive to the substitution pattern on the aromatic ring, as has also been reported for β-blockers [8].

CONCLUSIONS

The enantiomers of eight chiral compounds were resolved on the cellulase silica phase, TrichSep-100. The enantioselectivity obtained with this column was high, *e.g.* $\alpha = 5.28$ for 4-hydroxyflecainide.

The retention and enantioselectivity can easily be regulated by changing the pH of the mobile phase and the concentration of the uncharged organic modifier 2-propanol. The effects of these changes, however, are strongly dependent on solute structure. Further studies are needed to elucidate the complex nature of the chiral recognition properties of this new chiral stationary phase.

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