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Robustness of the chromatographic separation of alprenolol and related substances using a silica-based stationary phase and selective retention of metoprolol and related substances on a porous graphitic carbon stationary phase

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Abstract

In this study the robustness of two different LC methods for quantification of alprenolol and estimation of related substances was compared. In the first LC method a silica-based material, i.e., Hibar LiChrosorb RP-8, was used as the stationary phase and the mobile phase consisted of a counter-ion dissolved in acidic buffer and acetonitrile. The mobile phase in the other method consisted of alkaline methanol and as the stationary phase was porous graphitic carbon, Hypercarb, used. The robustness of the methods were investigated by experimental design and evaluated with multivariate calculations. The porous graphitic carbon system was far more robust than the silica system. The retention order of alprenolol and three related substances were the same, within the experimental design, when using the Hypercarb column. All the tested variables for the silica-based column were shown to have a significant effect on the retention behavior of the solutes. Most alarming is the retention shift of some of the solutes under the tested experimental intervals used. Separations of other closely related amino alcohols on Hypercarb are also presented. Metoprolol and related substances were baseline resolved with high column efficiencies, >60 000 plates/m, using the latest version of Hypercarb. © 1998 Elsevier Science B.V.

Keywords: Porous graphitized carbon; Stationary phases, LC; Experimental design; Mobile phase composition; Hypercarb; Hibar LiChrosorb RP-8; Chemometrics; Alprenolol; Metoprolol; Beta-blockers

1. Introduction

Chromatographic methods used for the quantitation of the active component and degradation products play an important role in the development of new drug products. It is of highest importance that these analytical methods are robust in order to obtain results of the highest possible quality. Experimental designs have previously been used to optimize chromatographic separations [1] as well as for robustness testing of analytical methods [2]. In both these articles the analytical results were evaluated with the partial least squares (PLS) regression method [3].

In this paper two different liquid chromatography (LC) methods for simultaneous quantification of alprenolol and estimation of related substances are presented. The two chromatographic methods were evaluated regarding their ruggedness against small changes in their mobile phase composition, respectively. One ion-pairing system with an acidic mobile phase and Hibar LiChrosorb RP-8 as the stationary

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phase was compared with an alkaline mobile phase and Hypercarb as the stationary phase. The porous graphitic carbon material used in Hypercarb was chosen as this support withstands all mobile phase pH values and therefore makes it possible to retain and separate the actual compounds as uncharged amines.

Due to its unique adsorption properties Hypercarb has been used to separate closely related substances as diastereoisomers [4,5] and positional isomers [6]. The possibility of using Hypercarb to separate metoprolol and closely related substances e.g., its positional isomers will also be presented.

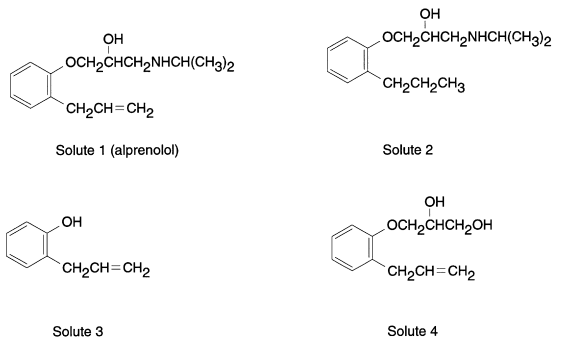

Fig. 1. Structures of alprenolol and related substances.

2. Experimental

2.1. Chemicals

Acetonitrile (HPLC grade) was obtained from Rathburn (Walkerburn, UK). Phosphoric acid (analytical-reagent grade), sodium dihydrogenphosphate and the silica column (Hibar LiChrosorb RP-8, 125× 4.0 mm I.D., 5 μm) were purchased from Merck (Darmstadt, Germany), sodium octylsulphonate (OSA) and methanol (HPLC grade) from Fisons (Loughborough, UK) and the sodium hydroxide pellets (analytical-reagent grade) from Eka Nobel (Surte, Sweden). The Hypercarb column (100×4.6 mm I.D., 5 μm) was supplied by Shandon (Astmoor, UK). The Brownlee Newguard guard column (RP-8, 15×3.2 mm, 7 μm) was from Applied Biosystems (San Jose, CA, USA). All the analytes (see structures in Fig. 1) were synthesized at Medicinal Chemistry Astra Hässle (Mölndal, Sweden).

2.2. Chromatography

Experiments were carried out on a chromatographic system consisting a Pharmacia LKB 2248 LC-pump (Sollentuna, Sweden), a Perkin-Elmer ISS-200 autosampler (Überlingen, Germany) and a Spectra-Physics Spectra 100 UV–Vis detector (San Jose, CA, USA). The temperature of the column (Hypercarb) and solvent reservoir was regulated in a Julabo 12B water bath by a Julabo P thermostat. The flow-rate was kept constant at 1.0 ml/min and 40 μl were injected in all the experiments. The pH of the phosphate buffer solution was measured with a PHM 83 autocal pH meter (Radiometer, Copenhagen, Denmark).

2.3. Statistical methods

All the designs were created in MODDE version 3.0 (Umetri, Sweden) and evaluated with the PLS regression method. Validation of the chemometric model was made with cross-validation. Modeling of the capacity factors and α values was separated in order to increase the fit of the statistical models.

As a measure of the model suitability, the fraction of the variation of the response explained by the model and the fraction of the variation of the response that can be predicted by the model was calculated [7,8].

The parameters used in this paper R^2 (the fraction of variation of the responses explained by the model) and Q^2 (the fraction of variation of the responses that can be predicted by the model) are given by

$$R^2 = \mathrm{SS}_{\mathrm{REG}}/\mathrm{SS} \quad (1)$$

$$Q^2 = 1 - \mathrm{PRESS}/\mathrm{SS} \quad (2)$$

where $\mathrm{SS}_{\mathrm{REG}}$ is the sum of squares of Y corrected for the mean, explained by the model, SS is the total sum of squares of Y corrected for the mean and PRESS is the prediction residuals sum of squares. R^2 and Q^2 are used as indicative criteria of the model fit.

The capacity factors were transformed to 10 log k'

throughout the investigation to increase the explanatory degree of the model [9].

2.4. Chromatographic responses

Chromatographic responses were the capacity factor (k') for the solutes and the α value for important separations. The capacity factor (k') was calculated using the equation

$$k' = (t_R/t_0) - 1 \quad (3)$$

where t_R is the retention time for the solute and t_0 is the retention time for an unretained solute (e.g., NO_3). The separation factor (α) was calculated using the equation

$$\alpha = k'_2/k'_1 \quad (4)$$

where k'_2 is the capacity factor for the last eluted analyte and k'_1 is the capacity factor for the first eluted analyte.

3. Results and discussion

3.1. Experimental designs for robustness testing of the chromatographic systems

In the silica method a reversed-phase liquid chromatographic system with UV detection at 275 nm is used both for the assay of alprenolol benzoate and for the estimation of three related substances, structures shown in Fig. 1. A Hibar LiChrosorb RP-8 (125×4 mm, 5 μm) column together with an Applied Biosystems RP-8 (7 μm) guard column was used. The mobile phase is made up of 290 ml acetonitrile and phosphate buffer pH 3.0 (ionic strength, $I=0.05$) to a total volume of 1000 ml. To the mobile phase sodium *n*-octylsulphonate is added to a final concentration of 2 m*M* in order to retain the two amines, structures shown in Fig. 1. To test this routine method a full factorial design with three centerpoints was used. Three mobile phase variables and three centerpoints lead to $2^3+3=11$ runs. This design resolved the linear and the cross-term behavior of the variables for the routine method. The mobile phase composition in the centerpoint is equal to the mobile phase stated above. In the design the pH of the phosphate buffer was varied from 2.5 to 3.5, the concentration of the ion-pairing agent was varied from 1.5 to 2.5 m*M* and the content of acetonitrile was varied from 27 to 31% (v/v).

Table 1
Experimental design – Hibar LiChrosorb RP-18

No.	pH	MeCN	OSA	k'_1	k'_2	k'_3	k'_4
1	2.5	27	1.5	5.21	8.13	8.90	4.80
2	3.5	27	1.5	5.53	8.64	9.01	4.81
3	2.5	31	1.5	2.97	4.50	6.47	3.37
4	3.5	31	1.5	3.21	4.90	6.39	3.18
5	2.5	27	2.5	6.22	9.64	8.89	4.79
6	3.5	27	2.5	6.47	10.07	8.87	4.76
7	2.5	31	2.5	3.40	5.10	6.35	3.13
8	3.5	31	2.5	3.61	5.45	6.34	3.16
9	3.0	29	2.0	4.47	6.85	7.71	3.97
10	3.0	29	2.0	4.16	6.30	7.33	3.73
11	3.0	29	2.0	4.30	6.51	7.50	3.81

This method was compared with another reversed-phase system developed with a column based on porous graphitized carbon (Hypercarb). The mobile phase consists of 20 m*M* NaOH dissolved in methanol thermostatted to 22.5°C. The analytes were detected at 275 nm. To test the robustness of this chromatographic system a full factorial design varying two variables led to $2^2+3=7$ runs. The variables for the Hypercarb column were temperature, varied from 15 to 30°C and the concentration of NaOH varied from 10 to 30 m*M*.

All the mobile phase compositions and results for the routine method are given in Table 1. The mobile phase compositions and chromatographic responses for the Hypercarb method are given in Table 2.

3.2. Evaluation of the statistical models

When modeling the capacity factors for the solutes, structures shown in Fig. 1, using the Hibar

Table 2
Experimental design – Hypercarb

No.	Temperature	NaOH	k'_1	k'_2	k'_3	k'_4
1	15	10	7.18	10.14	0.69	2.57
2	30	10	5.45	7.40	0.60	2.08
3	15	30	7.12	10.11	0.57	2.63
4	30	30	5.37	7.29	0.50	2.07
5	22.5	20	6.28	8.70	0.57	2.36
6	22.5	20	6.25	8.68	0.53	2.34
7	22.5	20	6.13	8.50	0.57	2.33

Table 3
Evaluation of the statistical model – capacity factors

Chromatographic column	Solute	R^2	Q^2	Significant effects	
				Increase	Decrease
Hibar	1	0.994	0.972	pH, OSA	MeCN
LiChrosorb RP-8	2	0.992	0.963	pH, OSA	MeCN
	3	0.993	0.972	–	MeCN
	4	0.985	0.942	–	MeCN
Hypercarb	1	0.996	0.926	–	Te
	2	0.996	0.944	–	Te
	3	0.866	0.720	–	Te, Na
	4	0.992	0.926	–	Te

LiChrosorb RP-8 method about 99% of the experimental data (R^2) was explained by the statistical model, (see Table 3). The predictability (cross-validation, Q^2) of the statistical model was also high $>$96%. Analysis of variance (ANOVA) calculation showed no lack of fit and the residual normal probability plot illustrated no outliers. As no interaction terms were observed the three linear terms i.e., mobile phase pH, content of acetonitrile and concentration of OSA were enough to explain the variation in the experimental data. The high explanation degree indicates that no quadratic terms could be expected. Two PLS components were used for the modeling of the responses. The capacity factors were transformed to logarithmic values in order to increase the degree of explanation for the variation and prediction ability of the model. Modeling the α value showed similar results as the capacity factor with R^2 values $>$93% and Q^2 values $>$83% (see Table 4).

The evaluation of the modeling of capacity factors and α values for the Hypercarb system are shown in Tables 3 and 4, respectively. The explanation degree (R^2) of the capacity and separation factors was nearly as high as for the silica-based column. However, the predictability (Q^2) was somewhat lower but this can be explained. With cross-validation one measurement is removed from the worksheet and the statistical model tries to predict the removed value. For small designs all the measurements are important for the model and removing one reduces the prediction ability. Another plausible explanation is that the variation in the three centerpoints are of the same size as the total variation within the experimental design as is the desirable case in a robustness test (see Table 2).

The separation of alprenolol and the three related substances using Hibar LiChrosorb RP-8 as the stationary phase are shown in Fig. 2. The corresponding separation using Hypercarb is given in Fig. 3. The chromatographic separations were done using the centerpoint settings from the two different experimental designs as the respective mobile phase compositions.

3.3. Comparison of robustness

The influence of descriptor variables on the responses for the two chromatographic systems are given in Tables 3 and 4, respectively.

Despite the minor variation in the variables (Table 1), important effects were observed for the Hibar LiChrosorb RP-8 system (Tables 3 and 4). The change in acetonitrile concentration (27–31%, v/v) significantly effects the retention for all the four

Table 4
Evaluation of the statistical model – separation factors

Chromatographic column	α	R^2	Q^2	Significant effects	
				Increase	Decrease
Hibar	$\alpha_1 = k'_1/k'_4$	0.981	0.912	pH, OSA	MeCN
LiChrosorb RP-18	$\alpha_2 = k'_2/k'_1$	0.933	0.832	–	MeCN
	$\alpha_3 = k'_3/k'_2$	0.984	0.910	AcCN	pH, OSA
Hypercarb	$\alpha_1 = k'_4/k'_3$	0.848	0.759	Na	–
	$\alpha_2 = k'_1/k'_4$	0.885	0.584	–	Te
	$\alpha_3 = k'_2/k'_1$	0.992	0.460	–	Te

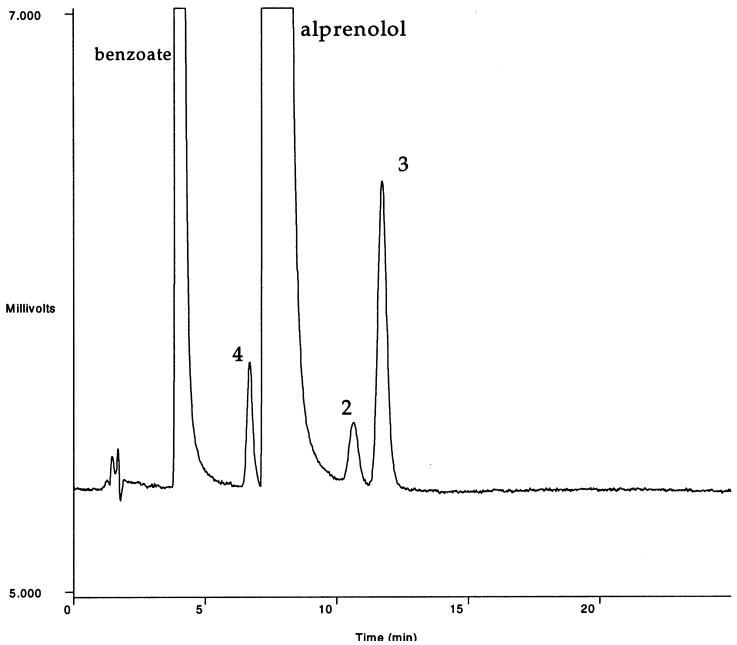

Fig. 2. Typical chromatogram using Hibar LiChrosorb RP-8 as the solid-phase. Mobile phase: 2 m*M* sodium *n*-octylsulphonate in phosphate buffer (pH = 3.0, $I = 0.05$)–acetonitrile (71:29). Concentration: alprenolol (400 μg/ml) other three 0.2–0.4% (w/w).

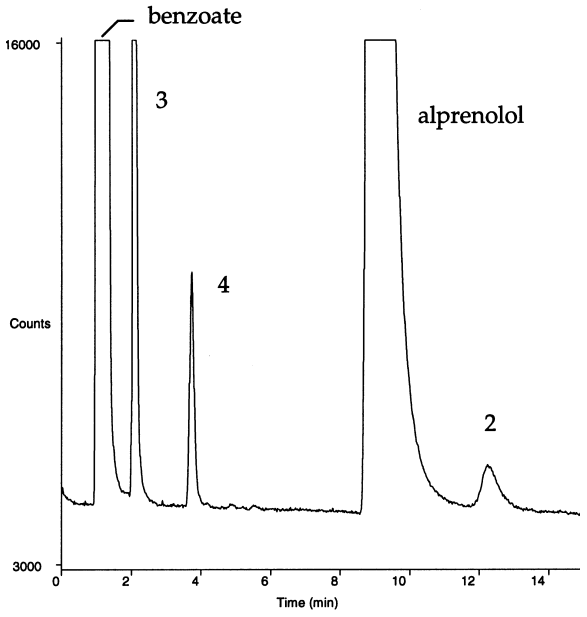

Fig. 3. Typical chromatogram using Hypercarb as the solid-phase. Mobile phase: 20 m*M* sodium hydroxide in methanol. Concentration: alprenolol (50 μg/ml) other three 1% (w/w).

solutes. However, the variation in the counter-ion concentration (1.5–2.5 m*M*) effects only the two amines, solutes 1 and 2 while only minor effects were observed by the change in the mobile phase pH (2.5–3.5). The effect on the capacity factors can be negligible if the separation is sufficient and no problem with peak identification or peak shape arise. However, most alarming is if some of the solutes change retention order. In the examined Hibar LiChrosorb RP-8 method solutes 4 and 1 change retention order as also do solutes 2 and 3 as can be seen in the contour plots in Fig. 4. The reason for the change in retention order is the selective effect of the counter-ion concentration on the retention for the two amines, solutes 1 and 2, combined with the effect of the change in content of acetonitrile, Figs. 4 and 5.

For the Hypercarb method a significant effect on retention was observed when changing the column temperature (15–30°C) (Table 3). However, all the solutes were effected in the same way and no change in retention order was noticed. A significant effect on retention was detected by varying the sodium hydroxide concentration (10–30 m*M*) for solute 3

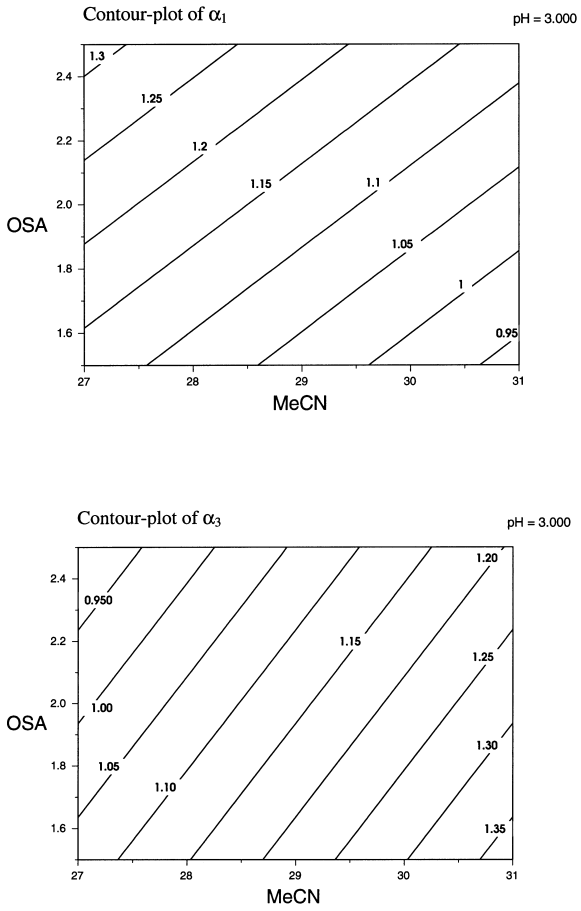

Fig. 4. Effect of counter-ion concentration and content of acetonitrile on selectivity using Hibar LiChrosorb RP-8 as stationary phase. α_1 and α_3 as in Table 4.

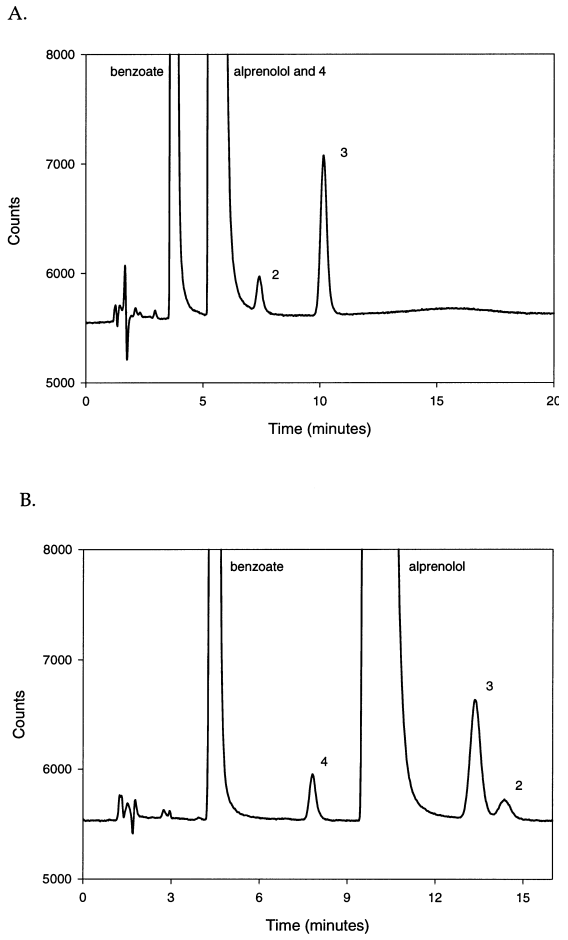

Fig. 5. Reversal of elution order on Hibar LiChrosorb RP-8. Mobile phase: (A) 1.5 m*M* sodium *n*-octylsulphonate in phosphate buffer (pH=2.5, I=0.05)–acetonitrile (69:31); (B) 2.5 m*M* sodium *n*-octylsulphonate in phosphate buffer (pH=2.5, I=0.05)–acetonitrile (73:27).

(Table 3). The selectivity factor that includes the capacity factor of solute 3 ($\alpha_1 = k'_4/k'_3$) was also effected by the sodium hydroxide concentration (Table 4).

Despite the fact that the ranges of the variables were suitable for a robustness test and not for an optimisation, significant and important effects were found in the Hibar LiChrosorb RP-8 system. As long as the α value is kept high or is increased the method is considered stable and robust. If the α value decreases i.e., approaches 1.0 in the tested region, the analysis method is unstable and separation must be verified, for example by system suitability solutions. A comparison of variation in the separation factors for the two systems within the experimental designs is given in Table 5. As problems occur with reversed retention order i.e., the α value passes 1.0 in at least two cases for the silica-based method within the experimental design but not for the porous graphitic carbon method making the

Table 5
Variation in selectivity factors within the experimental design

Chromatographic method	α_1	α_2	α_3
Hibar LiChrosorb RP-18	0.88–1.36	1.50–1.56	0.88–1.44
Hypercarb	3.46–4.64	2.60–2.80	1.36–1.42

α_1 to α_3 as in Table 4.

latter method a seemingly attractive alternative to the former one.

3.4. Selectivity of closely related amino alcohols using Hypercarb as solid-phase

Selective adsorption to the flat and homogenous surface of the porous graphitic carbon material has previously been used to separate closely related substances e.g., positional isomers and diastereoisomers [4–6]. Therefore, we tested Hypercarb as an adsorption phase for metoprolol and some closely related substances. Influence on retention of the position of a substituent in the aromatic ring, type of alkyl group attached to the nitrogen and the number of methylene groups between the asymmetrical carbon atom and the nitrogen atom were studied. The same group of compounds have previously been tested regarding enantioselective retention [10] on Hypercarb using a chiral ion-pairing mobile phase.

As the highest column efficiencies were obtained when the analytes were chromatographed uncharged, sodium hydroxide dissolved in methanol was used as mobile phase. As mentioned above the use of an alkaline mobile phase is not a problem as the porous graphitic carbon is stable at all pH values from 0 to 14.

Addition of methylene groups between the asymmetrical carbon atom and the nitrogen atom had a dramatic effect on retention (Fig. 6A). As expected for reversed-phase chromatography retention increased with increasing number of methylene groups. Three solutes with different alkyl groups attached to the nitrogen atom were also chromatographed on Hypercarb (Fig. 6B). Surprisingly, for reversed-phase chromatography the most polar compound, the *n*-propyl substituted compound, eluted last and the most apolar compound, the *tert*.-butyl substituted eluted first. It seems that increasing bulkiness at the nitrogen atom prevents a close interaction with the flat porous graphitic carbon surface and therefore the retention order was *tert*.-butyl<isopropyl<*n*-propyl. Metoprolol and its two positional isomers were baseline separated using Hypercarb as the stationary phase (Fig. 6C). The retention order was *meta*< *para*<*ortho* whilst the unsubstituted compound eluted first.

The compounds tested above have previously been

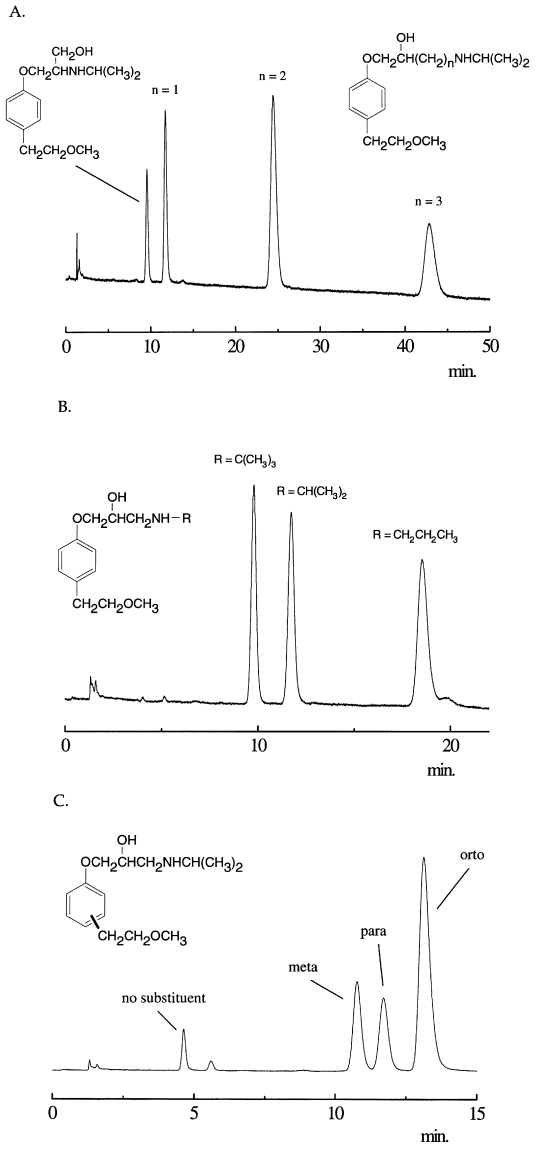

Fig. 6. Selectivity of metoprolol and closely related substances on Hypercarb. Mobile phase: 20 m*M* sodium hydroxide in methanol. Column temperature: 30°C. (A) Addition of methylene groups. (B) Different alkyl substituents. (C) Positional isomers.

chromatographed on reversed-phase silica-based materials. Of the positional isomers the *meta* and *para* forms eluted close to each other and baseline separation was not possible. The same observation was made for the *n*-propyl and isopropyl substituted amines.

Previously, versions of the porous graphitic carbon

material have shown rather low column efficiencies. However, this new 5 μm material gave about the same column efficiency (60 000 plates/m) as silica-based reversed-phase materials (Fig. 6A–C). Therefore, Hypercarb, with its unique selectivity and excellent stability when using alkaline mobile phases, is a complement to the traditional silica based materials.

4. Conclusion

The robustness of two different LC systems that could be used for both the assay of alprenolol and for the analysis of related substances in quality control were tested using experimental design and statistical evaluation. Hibar LiChrosorb RP-8 as solid-phase and a counter-ion dissolved in an acidic mobile phase was one of the methods while Hypercarb with an alkaline mobile phase was the other system. For the silica-based support, mobile phase pH, content of acetonitrile and concentration of counter-ion, were tested as variables and capacity and separation factors as responses. The same responses were tested on the porus graphitic carbon material, Hypercarb, at different column temperatures and with varying concentration of sodium hydroxide. The Hypercarb system was superior to the silica system and contrary to the Hibar LiChrosorb RP-8 no change in retention order was observed for the four tested compounds.

The ability of the Hypercarb system to separate metoprolol and closely related substances was also tested. Baseline separations were obtained with high column efficiencies.

In conclusion, Hypercarb, with its unique selectivity, excellent stability against alkaline mobile phases and high column efficiency, is a proper complement to the traditional silica-based reversed-phase supports.

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