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Evaluation of the liquid-chromatographic resolution of indenoindolic racemic compounds on three protein-based chiral stationary phases*

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Abstract: Three liquid-chromatography columns, containing immobilized proteins as chiral stationary phases (CSPs) were investigated for the direct separation of enantiomers of racemic indenoindolic compounds. By using an experimental design the effects of column temperature, pH, ionic strength, and type and concentration of organic solvent in the mobile phase on retention and resolution of racemic substances were systematically studied. The three CSPs investigated consisted of alpha(1)-acid glycoprotein (Chiral-AGP), bovine serum albumin (BSA-DSC) and ovomucoid (Ultron ES-OVM). The two enantiomers of all studied compounds could be separated on at least one of the three CSPs, which have different enantioselective properties.

Keywords: *Alpha (1)-acid glycoprotein; bovine serum albumin; chiral stationary phase; enantiomer separation; indenoindole; liquid chromatography; ovomucoid.*

Introduction

Methods for separating stereoisomers of drugs are of great interest as the isomers may have drastically different pharmacological potencies or actions. Several kinds of proteins have been immobilized on porous silica particles, and selective molecular recognition of such proteins has been utilized in chromatographic resolution of racemic compounds [1–3]. Diastereomeric complexes are formed between the enantiomeric analytes and the chiral selector on the stationary phase. Proteins like AGP, BSA and OVM are polymers with high molecular weight, which reversibly bind small molecules. The CSPs of protein type have low sample capacities but are useful for analytical separations. They are used with aqueous mobile phases, and the enantioselectivity and retention can be regulated by changing the mobile phase composition. In this study the resolution of six racemic indenoindolic compounds has been studied on three different chiral columns by systematically varying five chromatographic parameters.

Experimental

Chemicals and reagents

H 290/51, as hydrochloride, and H 290/30, H 266/86, H 301/20, H 325/10 and H 294/43 (Fig. 1) were synthesized at Medicinal Chemistry, Astra Hässle AB (Mölndal, Sweden). Acetonitrile and 2-propanol were of HPLC grade from Rathburn (Walkerburn, UK) and ethylenediaminetetraacetic acid (EDTA) was of analytical grade (Merck, Darmstadt, Germany). Water was purified through an ELGA purification system, ELGA (Wycombe, Bucks, UK).

Chromatographic system

The chromatographic system comprised a model 2248 LKB pump, (Pharmacia Biotech Norden AB, Sweden), an ISS-200 refrigerated autosampler (Perkin–Elmer, Überlingen, Germany) and a Waters M460 electrochemical detector (Millipore, Milford, MA, USA) at a potential of +0.80 V. The analytical columns Chiral-AGP (5 μm, 100 × 4.0 mm i.d.), BSA-DSC (7 μm, 125 × 4.0 mm i.d.) [4] and Ultron

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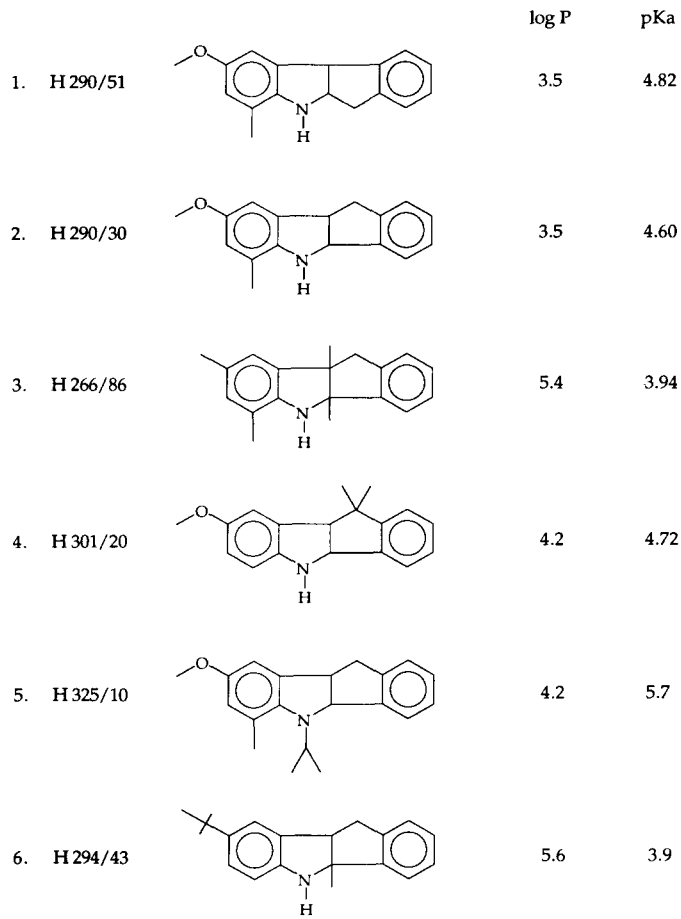

Figure 1
Structures of indenoindoles.

ES-OVM (5 μm, 150 × 4.6 mm i.d.) came from ChromTech AB (Hägersten, Sweden), Stig Allenmark (University of Göteborg, Sweden) and Rockland Technologies (Newport, DE, USA), respectively. The temperature was controlled by a RM 6 Lauda water bath (Lauda–Königshofen, Germany), the flow-rate was 0.75 ml min^{-1} and the injection volume 50 μl, containing ca 90 μmol l^{-1} of the analyte in mobile phase. Chromatographic data were collected and processed by a Multichrom chromatographic data system (VG Data Systems, Altrincham, UK).

Statistical experimental design

A fractional factorial design [5, 6] with four centre points was used to study the influence of the mobile phase components on retention and enantiomeric resolution of the analytes. The fractional design, which requires comparatively few experimental runs, uses a linear mathematic model to relate the responses (y-variables) to the factors (x-variables). Partial least squares regression was used to estimate the coefficients of the terms in the model. The design aims at a regular spread of the experiments. For a full factorial design with five factors, comprising all the possible combinations of the factor levels, there are 32 (2^5) possible combinations. The experiment was reduced to a (2^{5-2}) fractional design with a total of 12 experiments (mobile phases) including four centre points. Reduction of a design implies, that main effects are confounded with higher-order interactions. However, in comparison with the main effects, these higher-order effects were considered to be small. The program used for generation and evaluation of the statistical experimental design was a PC-windows program, Modde 2.0, by Umetri AB (Umeå, Sweden). For each of the three chiral columns (AGP, BSA, OVM) the same design was used, i.e. all 12 mobile phases were evaluated, and for each mobile phase six racemic substances were chromatographed. Only one column for each CSP was used. The

following five mobile phase parameters were varied, (1) type (Sol) and (2) concentration (*Co*) of organic modifier, (3) pH, (4) ionic strength (*I*) and (5) column temperature (*T*). The qualitative first factor was acetonitrile or 2-propanol, both modifiers having hydrogen-accepting properties and 2-propanol hydrogen-donating, as well. Each of the quantitative factors (2–5) were studied at two levels, for factor (2) 10 and 20% of organic modifier, factor (3) pH 5 and 7, factor (4) ionic strength 0.02 and 0.10 and for factor (5) 10 and 30°C.

In order to achieve a measure of the repeatability of the procedure four centre points, two experiments for each type of organic modifier, were performed at a level in-between, i.e. at 15% of modifier, pH 6, $I = 0.06$ and 20°C. The worksheet generated for the experimental design is shown in Table 1. All experiments were run in randomized order. The mobile phases at pH 5 and 5.8 contained citrate buffer and those at pH 7 phosphate buffer. Influence of the buffer type on the retention was examined for the BSA column by comparing citrate and phosphate buffer pH 6.0 in a mobile phase with 15% acetonitrile, $I = 0.10$ and 25°C. Identical retention times were obtained for the analytes. The mobile phases were ultrasonicated prior to use and some of them were added EDTA, 0.3 mmol l^{-1}, to keep the background low. There was no influence on retention by this addition.

Responses measured were the capacity factors ($k'A$ and $k'B$) of the enantiomers, the column efficiency, expressed as the dimensionless reduced plate-height (h) and the asymmetry factors (Asf). k' is defined as (t_R/t_0)-1, where t_R is the retention time for the analyte and t_0 is the elution time for an unretained solute. The reduced plate-height was calculated according to the equation $h = L/(N \times dp)$, where L is the column length, dp the particle size and N the number of theoretical plates. N was calculated using the peak-width at half the height ($W_{0.5}$), using equation $N = 5.54 \times (t_R/W_{0.5})^2$. Asf was calculated according to $Asf = b/a$, where a is the front and b the back half of the band-width, measured at one-tenth of the peak-height [7].

Log P was theoretically calculated using Hansch's fragment-constants [8].

Results and Discussion

Enantiomeric resolution

The experimental results obtained from the chromatographic separation of the enantiomers were illustrated graphically by plotting k' for the first enantiomer (A) vs k' for the second one (B), which, for each substance, gave 36 datapoints, one for each mobile phase and column (Fig. 2). As the elution order of the two enantiomers was different depending on column and type of organic modifier, $k'A$ was defined as the first-eluted enantiomer of each substance on the AGP column with a mobile phase containing acetonitrile and $k'B$ as the second-eluted enantiomer. For symbols above the diagonal line, peak A eluted before peak B, and for symbols below the line the elution order was reversed. The filled symbols are chromatographic systems, where baseline separation was obtained and unfilled symbols, on or close to the line, illustrate systems where the enantiomers were not completely separated. The figures in the graphs relate to the

Table 1
Worksheet of the experimental design

Mobile phase	Random order	Solvent type	Solvent conc.	pH	Ionic strength	Column temp.
1	3	1*	10	4.9	0.10	30
2	9	2†	10	5.0	0.02	10
3	2	1	20	5.0	0.02	30
4	5	2	20	4.9	0.10	10
5	7	1	10	7.1	0.10	10
6	4	2	10	7.2	0.02	30
7	12	1	20	7.2	0.02	10
8	8	2	20	7.1	0.10	30
9	1	2	15	5.8	0.06	20
10	10	2	15	5.8	0.06	20
11	6	1	15	5.8	0.06	20
12	11	1	15	5.8	0.06	20

*Solvent Type 1: Acetonitrile.
†Solvent Type 2: 2-Propanol.

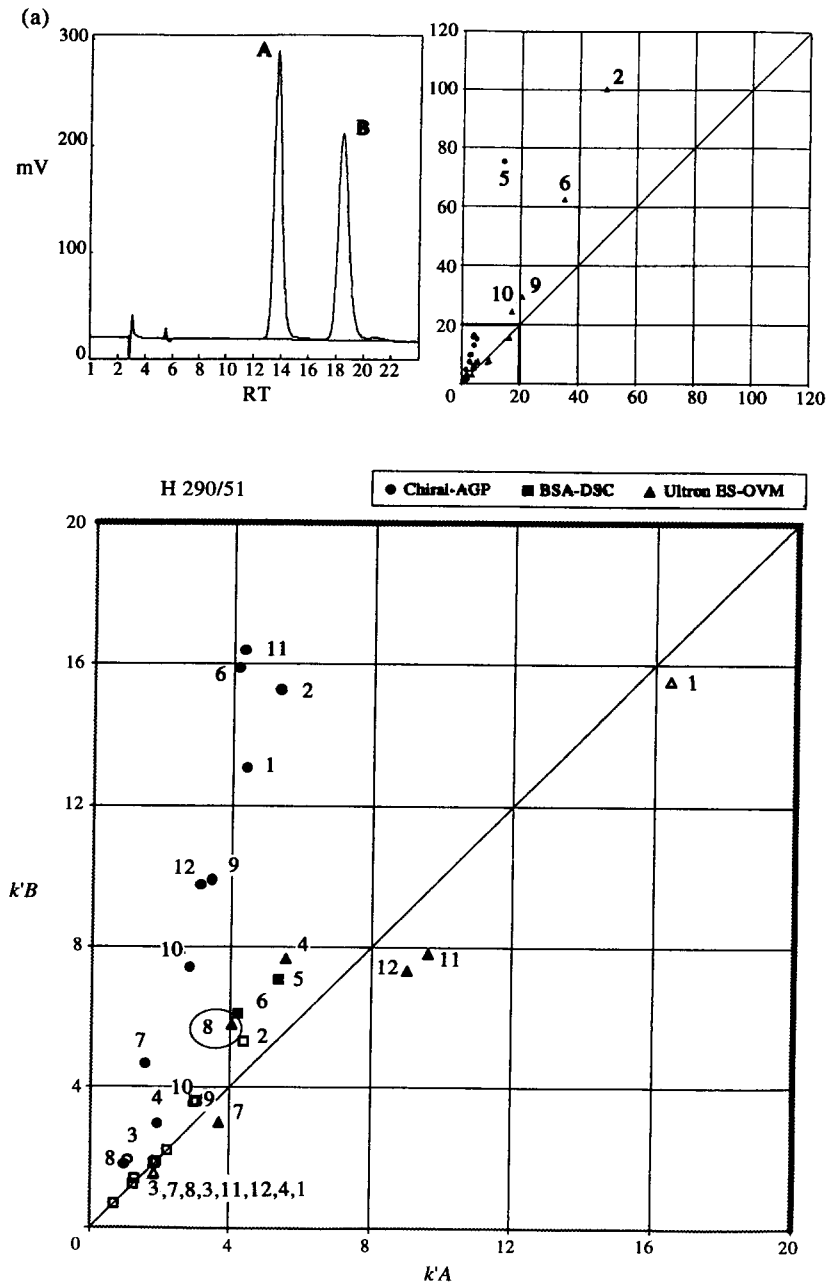

Figure 2
Graphs showing $k'A$ vs $k'B$ for (a) H 290/51, (b) H 290/30, (c) H 266/86, (d) H 301/20, (e) H 325/10 and (f) H 294/43 with 12 mobile phases (Figs 1–12 in Table 1) on three columns (● = AGP, ■ = BSA, ▲ = OVM). One chromatogram per substance is shown; corresponding system encircled in the graph. A = First-eluted enantiomer with a mobile phase containing acetonitrile on the AGP column. B = Second-eluted enantiomer.

different mobile phases, described in Table 1. One chromatogram for each substance is shown in the figure, the corresponding chromatographic system being encircled in the graph.

If both the enantiomers had not eluted after 1, 2 or 5 h (corresponding k'-values 25, 95 and 110) on the BSA, AGP and OVM column, respectively, the data for that particular compound were omitted. This was the case for H 325/10 and H 294/43 with several mobile phases on the BSA column and for H 325/10 on the OVM column. With mobile phase no. 5 results were omitted for all substances on the OVM column and for H 325/10 and H 294/43 on the AGP column. On the AGP column all enantio-

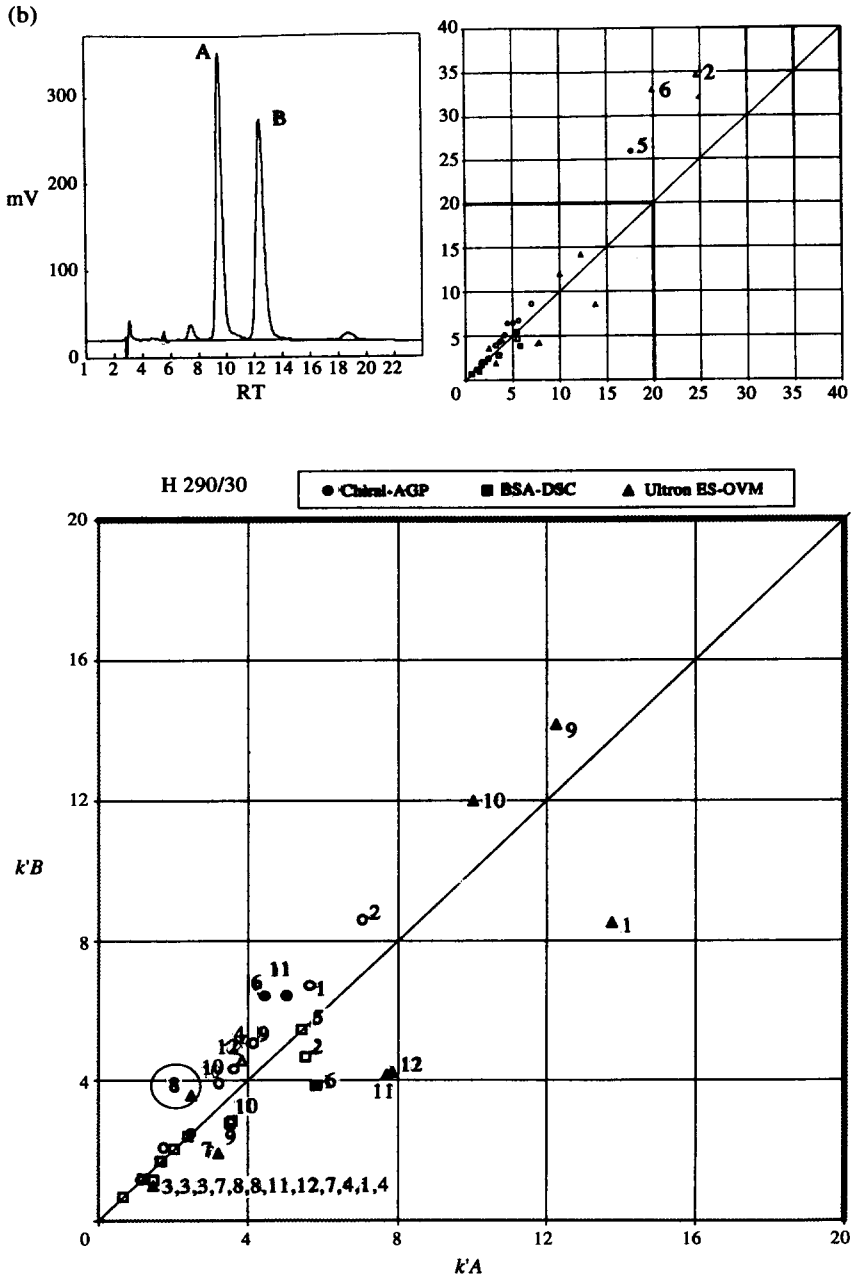

Figure 2
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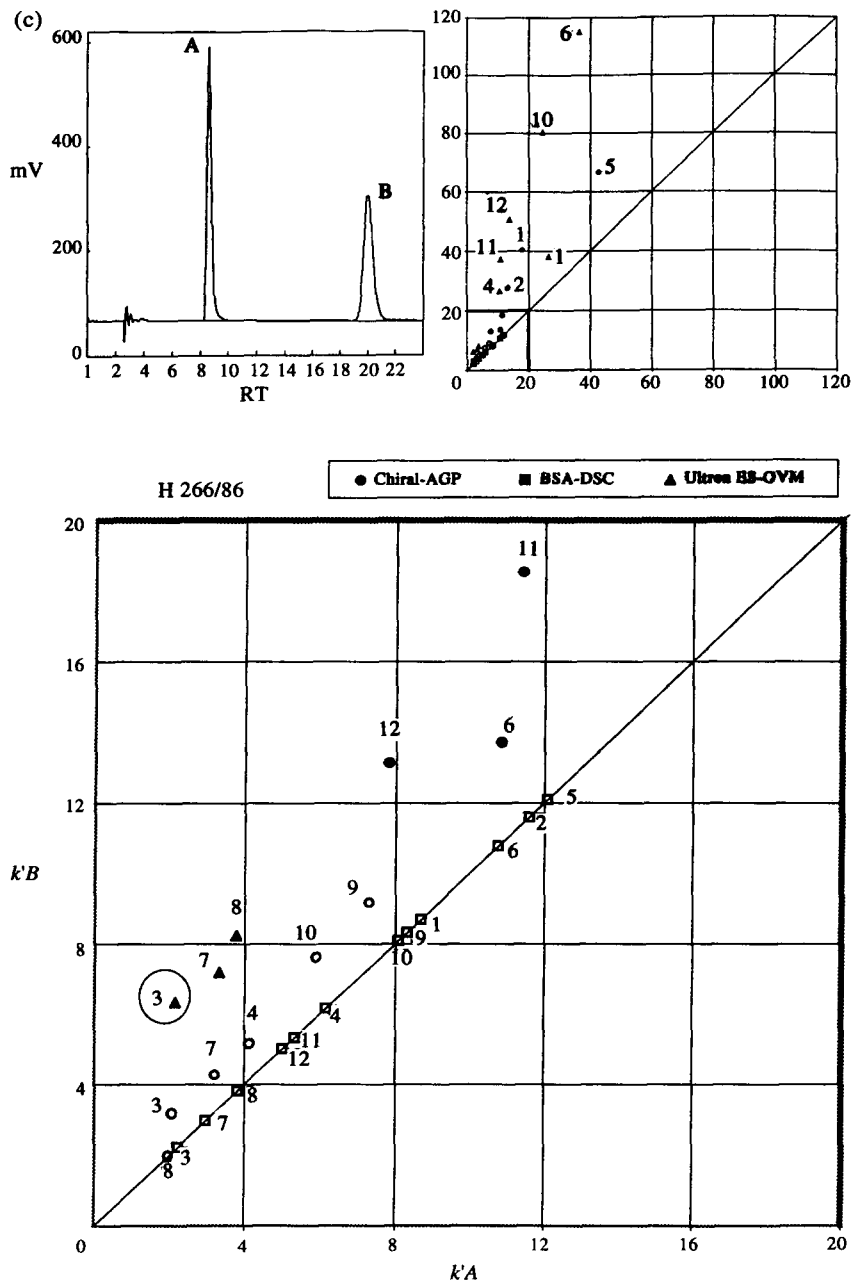
mers eluted in the same order, irrespective of acetonitrile or 2-propanol being used as organic modifier. For some substances reversed elution order, i.e. peak B before peak A, was shown both for the OVM and the BSA column. However, the systems giving reversed elution order differed between the two columns. Reversal of the elution order has earlier been described [9].

H 290/51 and H 290/30 are closely related substances with the same molecular weight, having different configuration (Fig. 1). Nevertheless, this minor structural deviation highly effects the chiral selectivity. On the AGP column $k'B$ for H 290/51 was considerably higher than $k'A$, giving as high α-value ($k'B/k'A$) as 5.3 with mobile phase no. 5, while the corresponding value for H 290/30 was 1.5. H



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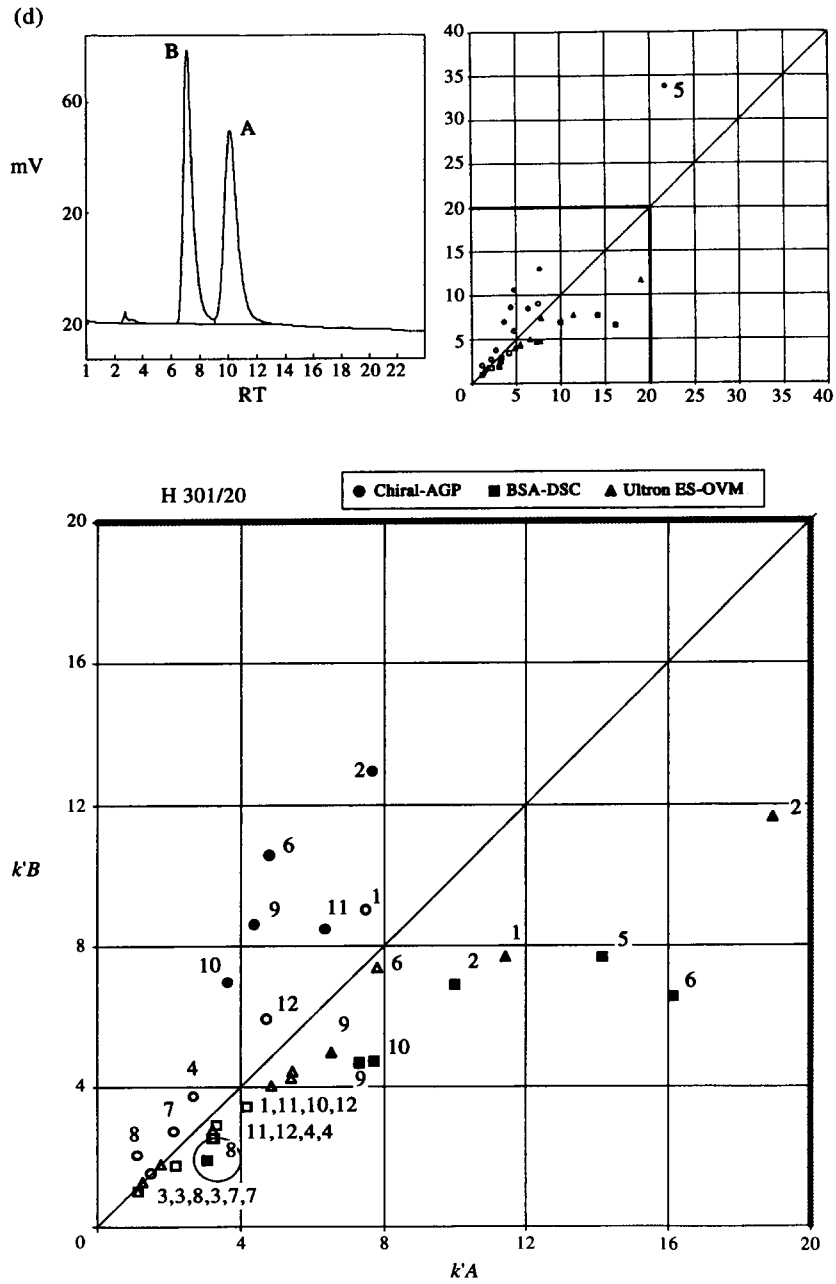
266/86 and H 294/43, the substances with the highest log *P* values and no methoxy group in the aromatic ring, eluted on the BSA column without any resolution of the enantiomers. The best separation on the BSA column was achieved for H 301/20, a compound without any shielding group around the nitrogen atom, giving α-values ranging from 1.1 to 2.5. According to the literature the BSA-DSC sorbent is particularly useful in resolving acidic compounds [4].

Mobile phase parameters influencing retention

Mobile phase parameters, causing effects on log k', were evaluated by the Modde program and are shown as bargraphs in Fig. 3. The factors were scaled and centred and an explanation of the variation of the response, adjusted



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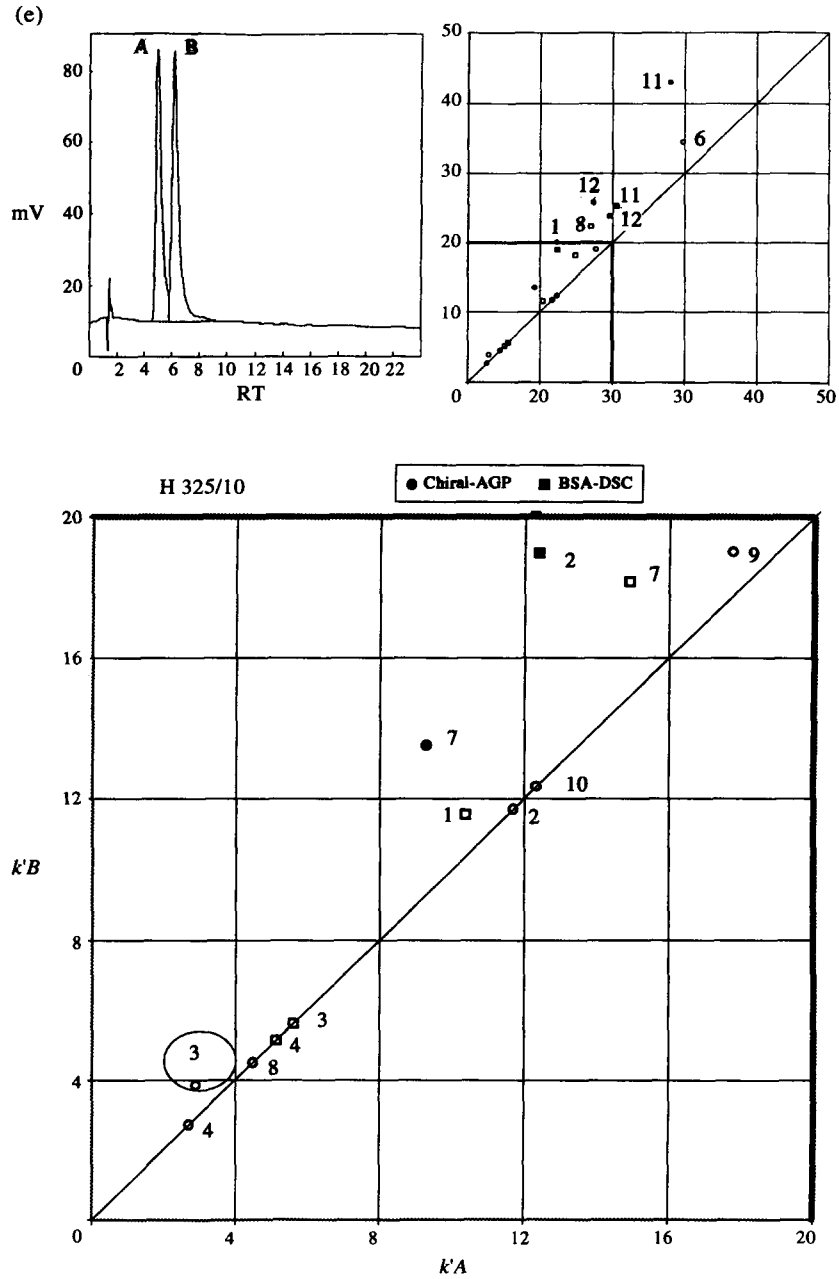
for degrees of freedom (R^2_{adj}), of ca 90% was obtained for all compounds except H 325/10, which showed 43%. H 325/10 is a tertiary amine, with a somewhat higher pK_a-value than the other indenoindoles (Fig. 1). When adding a square term (pH*pH) to the model the explanation degree increased for H 325/10 to 67%, indicating some curvature in the modeling for that compound. The effects, which are twice the coefficients in the mathematic model, are plotted in absolute (logarithmic) values. The 95% confidence intervals are drawn as upper and lower lines. Bars crossing the lines show significant effects on k'. A positive bar indicates increasing k', when changing from low to high factor level, and a negative bar decreasing value.

The retention on all three columns was



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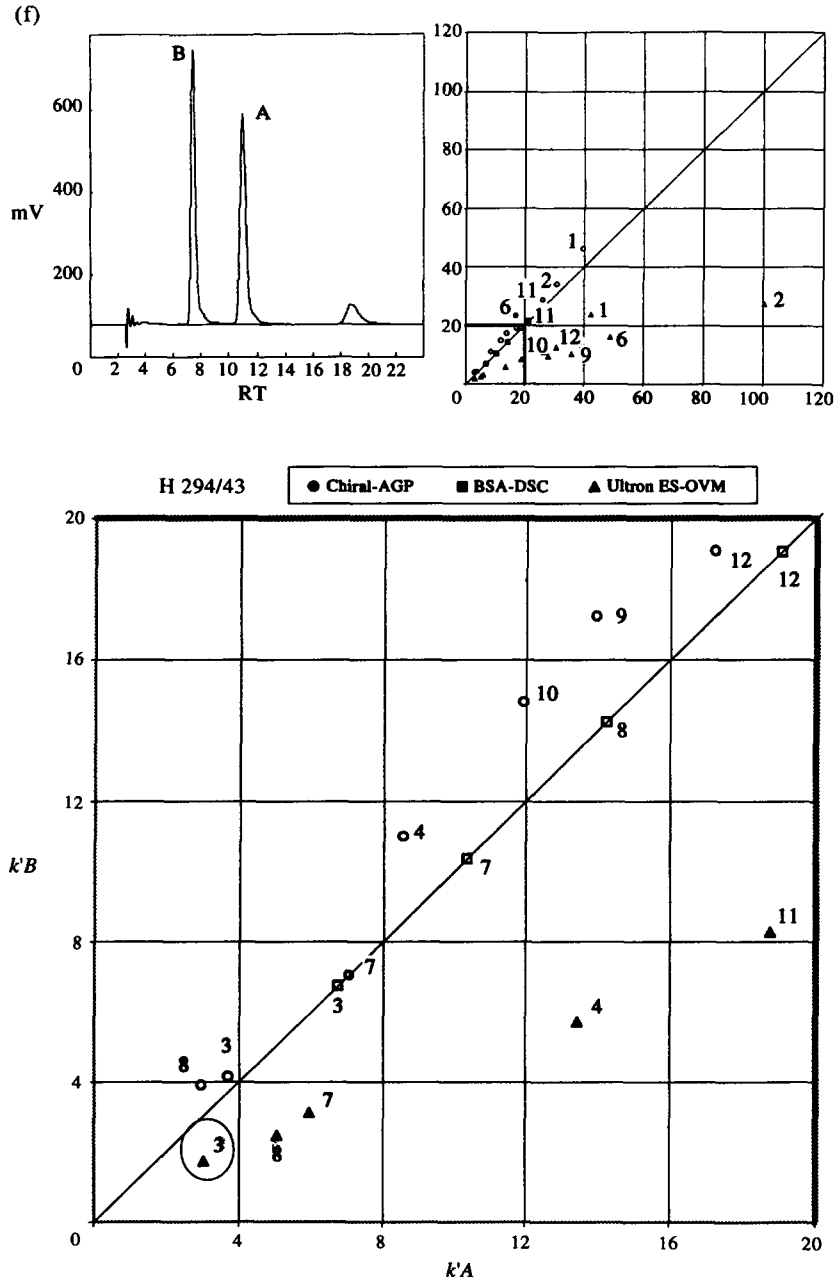
strongly affected by the concentration of organic modifier (*Co*), indicating hydrophobic interactions. Changing the type of modifier (Sol) had an impact on k' for the BSA and OVM columns. Increasing k' values were obtained going from acetonitrile to 2-propanol. The opposite was noticed for the AGP column, even though the effects were not statistically significant. This was in accordance with results by Enquist and Hermansson [10]. The influence of the temperature (T) on k' was most pronounced for the AGP and BSA column, giving decreased retention at elevated column temperature. Varying of the pH (5 to 7) and ionic strength (0.02 to 0.10) had minor effects on k' for the three CSPs. The amines studied are predominantly unprotonated in the pH range, why the retention was not significantly



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affected by pH of the eluent. However, for the tertiary amine, H 325/10, increased retention was observed at higher pH, which might be explained by increased hydrophobic interaction between the solute and the stationary phase when the amine became less protonated.

Column efficiency and peak symmetry

The efficiency, was increased by ca 50% for all three columns, when changing from 2-propanol to acetonitrile. By changing column from BSA to AGP, h was diminished to half the value and further decreased twofold when applying OVM. Asf varied from 1 to 3 for all substances and all three columns except for H 301/20, giving 2–5 on the AGP column.

Conclusions

This way to optimize mobile phase com-

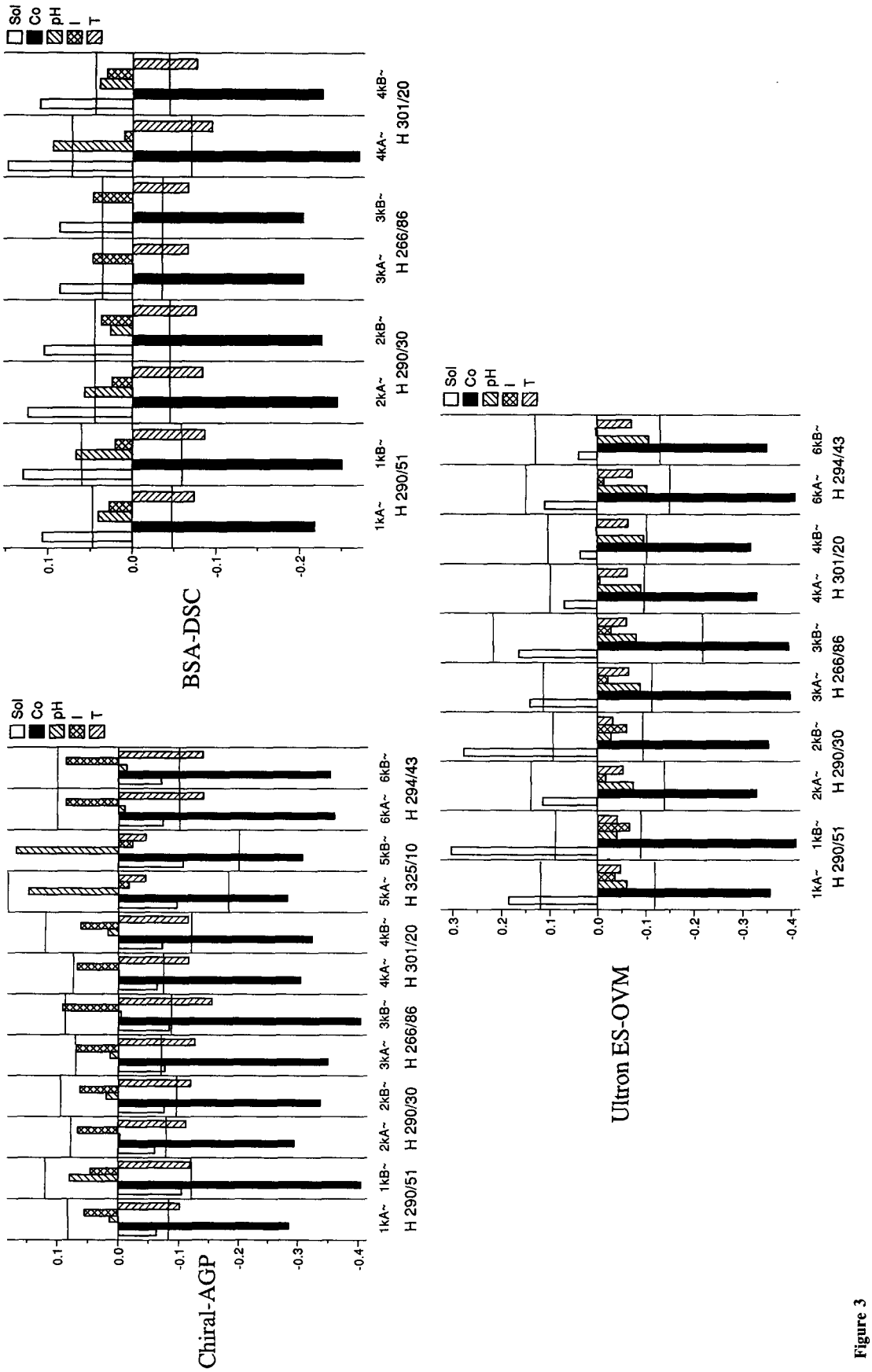

Figure 3
Bargraphs showing mobile phase parameters causing effects on k' on varying.

position and column temperature can improve the speed and enantiomeric resolution of a separation. All the studied enantiomers could be separated on at least one of the CSPs. Chiral-AGP and Ultron ES-OVM showed excellent enantioselective properties for most substances. Ultron ES-OVM showed the best column efficiency and lowest asymmetry and BSA-DSC showed the best long-term stability.

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