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Journal of Chromatography A, 763 (1997) 115–123

JOURNAL OF CHROMATOGRAPHY A

Influence of chromatographic descriptors on enantioresolution of a dihydropyridine and structurally related compounds

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Abstract

The possibility to separate the enantiomers of a pharmacological active dihydropyridine and some of its possible by-products/metabolites was mapped using experimental design and multivariate analysis. All the tested racemates were enantioseparated using Chiral-AGP as the chiral stationary phase. All the investigated descriptor variables, mobile phase pH, organic modifier(s) and column temperature influenced the chiral recognition. The separation factor of the aprotic compound of pharmacological interest, H 324/38 (Solute No. 1), increased by increasing mobile phase pH, indicating changes in the conformation of the immobilized protein. The enantiomers of the major compound, a potential internal standard and the most important degradation product as well as the major metabolite, H 152/81 (Solute No. 5), could be separated simultaneously using a mix of two organic modifiers in the mobile phase. The presented work emphasises the use of experimental design and statistical multivariate evaluation. However, knowledge about the chromatographic system and the physical properties of the analytes, e.g., pK_a values, are of highest importance in order to derive accurate conclusions.

Keywords: Chemometrics; Experimental design; Enantiomer separation; Organic modifiers; Dihydropyridines

1. Introduction

The two enantiomers of a specific racemate can interact quite differently with biological systems and a major part of human metabolism is stereoselective. Therefore, it is well recognized that the contribution of a pharmacological effect might be unevenly divided between individual enantiomers in racemic drugs. This emphasizes the need for methods capable of chiral separation with subsequent quantification in the pharmaceutical industry [1].

Column liquid chromatography (LC) has proven to be a convenient way to resolve enantiomers and was used already in 1938 by Henderson and Rule [2]. Since then several liquid chromatographic techniques have been established to directly separate enantiomers [3–5]. Resolution of enantiomers can be obtained using either a chiral mobile phase additive [6] or by using a stationary phase with an immobilized chiral selector [7]. In the latter case enantioselective retention is obtained due to differences in adsorption properties of the two enantiomers to the chiral stationary phase.

In the present paper the direct enantioseparation approach of two anti-hypertensive drugs, i.e., H 324/38 (1) and felodipine (6) [8] and some of their chiral by-products were studied (structures as depicted in Fig. 1). Compound 1 is a new short-time acting anti-hypertensive substance that can be used to decrease the blood-pressure for a short and limited time during, e.g., surgery. The α_1-acidglycoprotein, immobilized to silica and commercially available as Chiral-AGP, was used as the chiral discriminator [9]. This protein column has previously been used to

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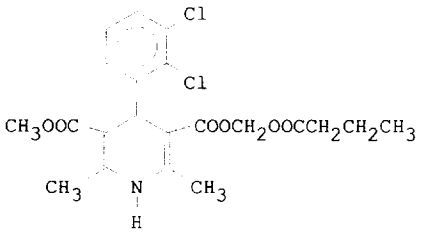
PII S0021-9673(96)00701-7



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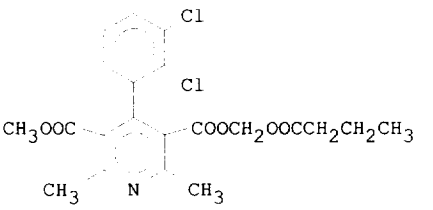

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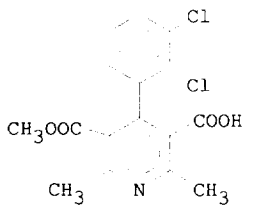
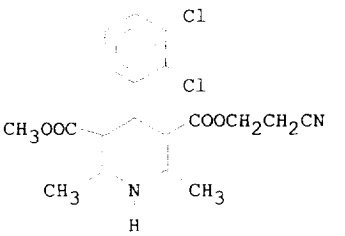
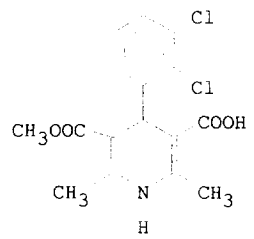


H 152/66 (Solute No. 3)



H 152/80 (Solute No. 4)



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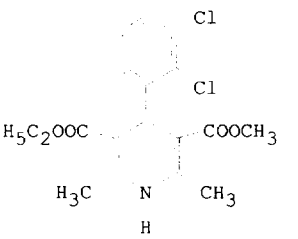
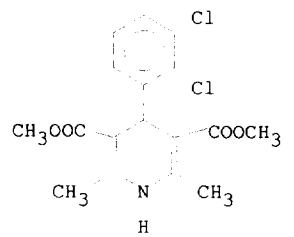
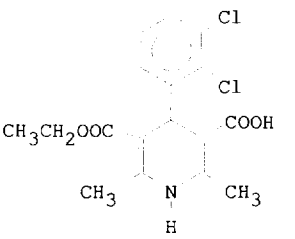


Felodipine (Solute No. 6)



H 161/33 (Solute No. 7)



H 172/99 (Solute No. 8)

Fig. 1. Structures of the investigated solutes.

separate and determine enantiomeric purity of the enantiomers of felodipine in bulk substance [10].

The aim of the study was to investigate the influence of chromatographic descriptor variables on the enantiodiscrimination system and to find useful conditions for routine enantioselective separation of the pharmacologically active substance, 1, in the anti-hypertensive drug. This is because several regulatory agencies require pharmacological and pharmacokinetic studies not only for the racemate but

also for the respective enantiomer. The influence on enantioselecetive retention for solutes with different types of substituents, e.g., acidic, basic and aprotic functionalities, formed by degradation of the dihydropyridine, was also examined. Typical variables such as column temperature, pH and organic modifier additions to the mobile phase, were selected and the chromatographic output (responses) was defined as capacity (k') and separation (α) factors. The influence of the descriptor variables on the responses was evaluated using chemometrics.

2. Experimental

2.1. Chemicals

H 324/38 (1), H 324/78 (2), H 152/66 (3), H 152/80 (4), H 152/81 (5), felodipine (6), H 161/33 (7) and H 172/99 (8), the structures in Fig. 1, were all synthesized by Astra Hässle AB. Sodium-phosphates and ortho-phosphoric acid were purchased from Merck (Darmstadt, Germany). Methanol (HPLC-grade) and acetonitrile (HPLC-grade S) were from Fisons (Loughborough, UK) and Rathburn (Walkerburn, UK) respectively.

2.2. Apparatus

The chromatographic system comprised a Pharmacia LKB HPLC pump 2248 (Uppsala, Sweden), a Waters 717 autosampler (Milford, USA) connected to a Spectra-Physics Analytical UV–Vis detector (Spectra 100, San Jose, USA). The Chiral-AGP columns (100×4.0 mm) were from ChromTech AB (Stockholm, Sweden). The chromatographic temperature was thermostatted by an RM 20 Lauda water bath (Königshofen, Germany). All pH estimates were done prior to the addition of organic modifier (-s) using a PHM83 pH meter (Radiometer, Denmark).

2.3. LC methods

The injection volume was 20 μl solute with concentrations of approximately 10 μM. The solutes were detected at 242 nm at constant flow (1 ml/min). The mobile phase buffer concentration was 0.01 M, composition and pH as indicated in tables and legends. The capacity factor, k' was defined as $k'=t_R/t_0-1$ where t_0 was the transport time from injection to the detector cell by an unretained component. The t_0 was calculated from the first disturbance of the baseline obtained after injection (0.7 min). The separation factor α was calculated by the k' for the later eluting enantiomer over k' for the faster enantiomer.

2.4. Statistical methods

Both full and fractional factorial designs were used [11]. All designs were produced by the Modde software (Version 2.1, Umetri AB, Umeå, Sweden) and center-point experiments in triplicate were included in order to estimate the precision of the method used. The data were evaluated by multivariate analyses using partial least squares [12,13] by the Modde software. The statistical models were validated by cross validation [14].

3. Results and discussion

3.1. Validation of projections

Partial least squares (PLS) is a multivariate regression method [12,13] that provides overview to large data sets. It is most certainly rewarding on data deriving from experimentation according to factorial design, since such combination guarantees accurate estimates of the coefficients in the regression equation [11], and thus inherently illuminating and reliable loading structures projected to the individual principal components (PC) [13], provided they are significant [14]. The rationale of employing PLS can be to use the regression equation for predictions [12], but interpretations from plots deriving from the various estimates from the individual PCs can also be informative [12,15] (see below).

In the present study we aimed at explaining the correlation structure for pH, organic modifier and the temperature as descriptors to chromatographic output, such as capacity factors (k'), Table 1, and selectivity factors (α), as responses. In addition, the PLS regression equation was used to predict optimal chromatographic conditions for the intended sepa-

Table 1
Experimental design

Mobile phase composition				k'												
Number	Temp.	pH	% Methanol	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b	7
1	20.0	2.03	20.0	73.29	78.57	23.61	41.14	28.36	59.54	13.73	16.44	13.45	17.64	40.93	49.21	23.51
2	30.0	2.03	20.0	36.29	36.29	12.78	20.52	22.08	42.43	7.67	9.03	8.22	9.85	22.34	26.36	13.38
3	20.0	7.04	20.0	96.43	147.60	38.43	65.43	0.22	0.22	17.70	21.34	0.81	0.81	50.43	50.43	27.07
4	30.0	7.04	20.0	49.43	68.57	20.08	31.78	0.28	0.28	9.93	11.61	0.63	0.63	28.45	28.45	15.63
5	19.8	2.03	30.0	14.31	14.31	5.42	8.01	14.33	23.54	3.65	3.97	4.04	5.22	8.71	8.71	5.73
6	30.0	2.03	30.0	6.84	6.84	3.07	4.16	10.49	15.73	2.43	2.43	2.76	3.18	5.10	5.10	3.55
7	20.2	7.04	30.0	12.42	20.13	5.63	8.81	0.21	0.21	3.74	4.15	0.34	0.34	7.70	8.30	5.49
8	30.0	7.04	30.0	6.64	9.35	3.18	4.53	0.18	0.18	2.36	2.50	0.41	0.41	4.68	5.00	3.39
9	25.2	4.51	25.0	26.85	33.68	8.86	12.90	2.44	3.42	5.95	7.00	7.16	7.16	14.33	14.33	8.93
10	24.8	4.51	25.0	27.00	33.48	9.16	13.36	2.75	3.93	6.00	7.04	7.19	7.19	14.40	14.40	9.01
11	25.2	4.51	25.0	27.14	33.29	8.95	12.90	2.65	3.80	5.87	6.93	7.02	7.02	14.26	14.26	8.84
12	24.8	7.04	25.0	23.96	35.82	10.18	16.34	0.19	0.19	5.79	6.54	0.48	0.48	14.07	15.00	8.91
13	24.8	2.03	25.0	21.24	21.24	7.63	11.55	16.41	29.14	4.96	5.63	5.48	6.85	12.99	14.45	8.34

ration. The performance of the PLS-modeling, in the present study, was followed by the explained variance (by regression and cross validation). It was obvious that the k' data were more readily explained after logarithmic transformation [15], which might be explained by a more homoscedastic distribution of the uncertainty level for k' estimation with time. All k' were explained in the range 95–99% by regression coefficient (r^2) and >70% by cross validation Q^2, Table 2, when modeled by PLS. Also the separation factors for individual enantiomer pairs were used as responses for PLS-modeling providing explained variance of 90–98% by regression, Table 3. The cross validation was lower, from 40% for solute No. 5 and ranging 60–80% for the others, Table 3. For the enantiomers of 5, only small changes in separation were found in the results from the designed experiments. This rendered higher relative noise level and lower correlation coefficients in the regression in comparison to the other solutes. However, cross validation is a conservative estimate of fit with designed data, since the idea of design is to provide useful information from every single experiment. Thus, the true grade of explanation, by PLS, of the experimental data in Table 1 was presumably closer to the r^2 than the Q^2.

Table 2
Explained variance for log k' for solutes as indicated according to regression coefficient (r^2) and cross validation (Q^2)

Capacity factor	r^2	Q^2
1a	0.9878	0.7271
1b	0.9809	0.8732
2a	0.9835	0.8280
2b	0.9869	0.8614
3a	0.9553	0.9151
3b	0.9553	0.9140
4a	0.9859	0.7591
4b	0.9901	0.7805
5a	0.9394	0.8945
5b	0.9615	0.9172
6a	0.9877	0.7310
6b	0.9909	0.7387
7	0.9927	0.7351

3.2. Descriptor to response correlation

The modeling of the k' revealed that pH and the organic modifier proportion of the mobile phase had close to orthogonal loadings (i.e., w for principal components 1 and 2) indicating that they influenced

Table 3
Explained variance for enantioselectivity (α) for solutes as indicated according to regression coefficient (r^2) and cross validation (Q^2)

Separation factor	r^2	Q^2
a1	0.9736	0.8172
a2	0.9136	0.6352
a3	0.9809	0.7327
a4	0.9549	0.7856
a5	0.9105	0.4156
a6	0.8739	0.5500

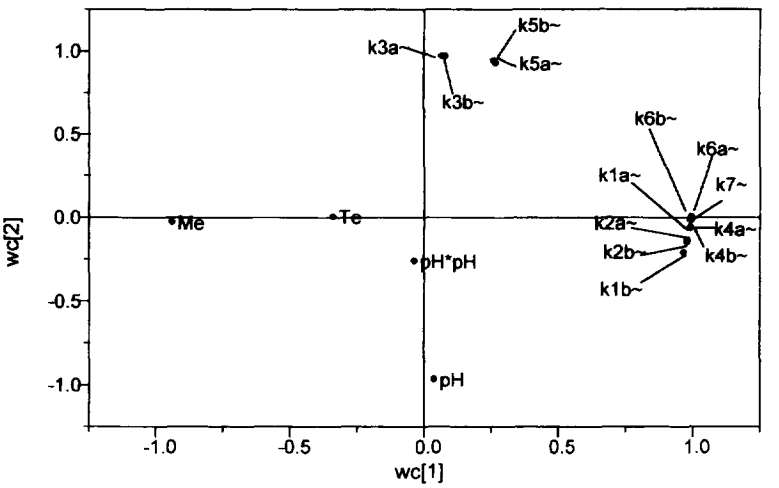


Fig. 2. Descriptors from mobile phases depicted in Table 1 (w) and response (c) variable inter-correlations and sizes (i.e., $w1$ versus $w2$ and $c1$ versus $c2$ superimposed). The responses were log k' for the solutes shown in Fig. 1. Abbreviations: Me, methanol; Te, temperature; pH, mobile phase pH; responses, a, first eluted and, b, last eluted enantiomer.

capacity- and separation-factors independently. The distribution of response loadings (c) suggested two groups of correlation structures for the solutes, Fig. 2. Increased temperature lead to lower k' which was expected [16,17] although the opposite has been reported by Jönsson et al. [18]. They suggested that new binding sites were exposed by the higher temperature which is possible if, e.g., protein conformation changes take place [19]. Further, change in enantioselective retention was also suggested from the modeling of α-loadings (i.e., correlation of separation factors to descriptors, see Fig. 3). The acidic substances, 3 and 5, correlated inversely with the mobile phase pH presumably due to altered possibilities for electrostatic interaction between the α_1-AGP and the respective enantiomers. However, the analyte No. 1 unexpectedly correlated positively with increasing pH since that substance is essentially

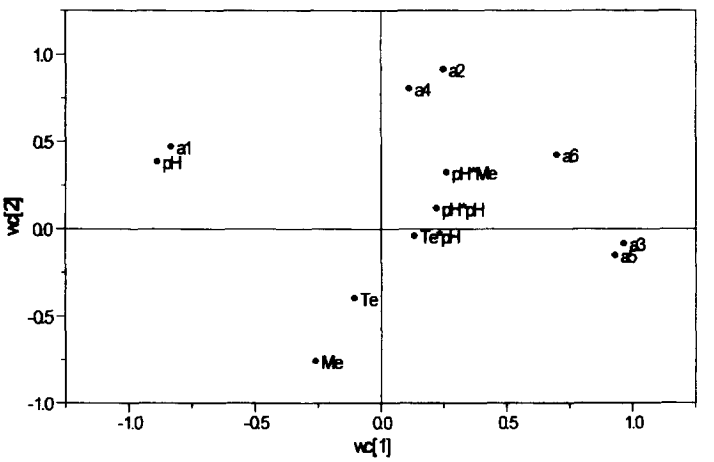


Fig. 3. Descriptors from mobile phases depicted in Table 1 (w) and response (c) variable inter-correlations and sizes (i.e., $w1$ versus $w2$ and $c1$ versus $c2$ superimposed). The responses were separation factors (α) for the enantiomer pairs of the solutes shown in Fig. 1. Abbreviations: Me, methanol; Te, temperature; pH, mobile phase pH; responses a1–a6.

aprotic in the pH range investigated. The mechanism for this must be found in conformational changes of the stationary phase α_1-AGP. As expected no variation in the separation factors of the weak amine, 2, and the aprotic compound, 4, were obtained when altering the mobile phase pH.

3.3. Prediction of k'

The polynomial functions derived from the PLS regression were generally performing well, as exemplified for the first capacity factor and the separation factor of solute No. 1 in Fig. 4. The polynomial functions were used to predict k' for putative mobile phases, e.g., one consisting a 0.01 M phosphate buffer, pH 2.9 mixed with 20% methanol (v/v) at a temperature of 20°C. The predictions performed well with an average prediction error of −3.5% (SEM, 2.0) for all structures except 3. The predicted k' of both enantiomers for this substance were approximately 50% below the actual k'. This was explained by a severe curvature in the k' dependence on pH in the low pH range (2–3.5), which the PLS could not adjust for. Thus knowledge of the pK_a of the enantioseparating solutes is useful for the understanding of the modeling of the capacity factors of 3,

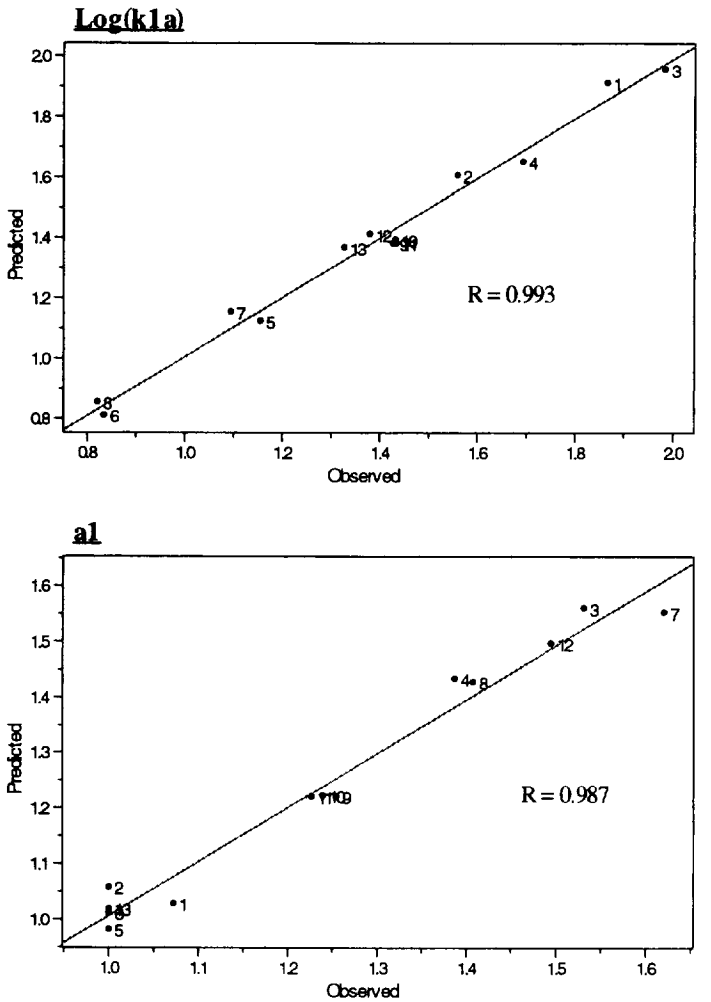


Fig. 4. Observed versus predicted capacity factors k1a and separation factors a1, by cross validation, for mobile phases as in Table 1. Numbers indicate mobile phases as in Table 1.

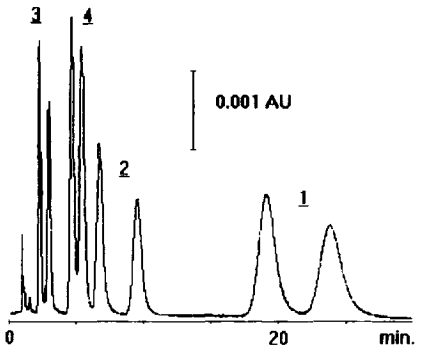


Fig. 5. Simultaneous chiral separation of 1, 2, 3, and 4. Solid-phase: Chiral-AGP. Mobile phase: phosphate buffer (pH 4.51)–methanol (3:1). Column Temperature: 25°C.

and a more extensive experimental design within the area of interest (i.e., pH 2–3.5) would have improved the accuracy of the PLS predictions.

Simultaneously enantioselective retention of four of the structurally related racemates are given in Fig. 5.

3.4. Influence of mixed organic modifiers in the mobile phase

The enantiomers of the pharmacologically interesting analyte 1 could easily be separated using methanol as the organic modifier. However, acetonitrile in the mobile phase improved the enantioselective retention by the enantiomers of the most important by-product and metabolite, 5, formed by hydrolysis.

There is seldom any selectivity to gain by mixing organic modifiers for reversed-phase LC [17]. However, protein structures, e.g. the α_1-AGP-conjugated stationary phase in the present study, may reconform in a selective way depending on the type and proportion of organic modifier it is incubated in [20,21]. Therefore, an additional series of mobile phases including both acetonitrile and methanol were investigated in order to simultaneously separate the enantiomers of 1, 5 and 8 (candidate as internal standard), Table 4. The loadings for the capacity factors are given in Fig. 6. The modeling by PLS for this data set rendered models with r^2 of >90%, and cross validation from 67–77%. However, when the α-factors were used as responses, r^2 was 32–65% and the predictability by cross validation was below 20%. Therefore, the indication of selective enantiomer retention capability by methanol and acetonitrile that was revealed by the PLS-loadings was interpreted with caution, and any conclusion had to be confirmed by additional experimentation. Using the combined information from both series of experiments, Tables 1 and 4, a mobile phase with 18% methanol and 4% acetonitrile, pH 5.5, temperature 25°C was confirmed to give higher chiral selectivity for the three compounds than corresponding mobile phases using only one of the two organic modifiers, Fig. 7A–C.

4. Conclusion

With the collected knowledge, as condensed by the projection methods of the present study, a mobile

Table 4
Experimental design-mixed modifiers

Mobile phase composition					k'					
Number	pH	Temp.	Acetonitrile (%)	Methanol (%)	1a	1b	5a	5b	9a	9b
1	2.0	20.0	2.0	10.0	291.9	291.9	42.00	43.93	105.1	123.6
2	7.0	20.0	2.0	20.0	54.57	76.07	0.69	0.69	0.57	0.57
3	2.0	30.0	2.0	20.0	23.45	23.45	6.21	7.03	11.93	11.93
4	7.0	30.0	2.0	10.0	207.6	247.6	1.14	1.57	1.14	1.57
5	2.0	20.0	10.0	20.0	4.38	4.38	2.44	2.44	3.47	3.47
6	7.0	20.0	10.0	10.0	21.46	24.74	0.68	0.68	0.71	0.71
7	2.0	30.0	10.0	10.0	11.10	11.10	4.35	4.35	7.28	7.28
8	7.0	30.0	10.0	20.0	3.21	3.21	0.45	0.45	0.45	0.45
9	4.5	25.0	6.0	15.0	26.67	26.67	6.33	8.22	11.16	14.15
10	4.5	25.0	6.0	15.0	27.35	27.35	6.44	8.36	11.54	14.58

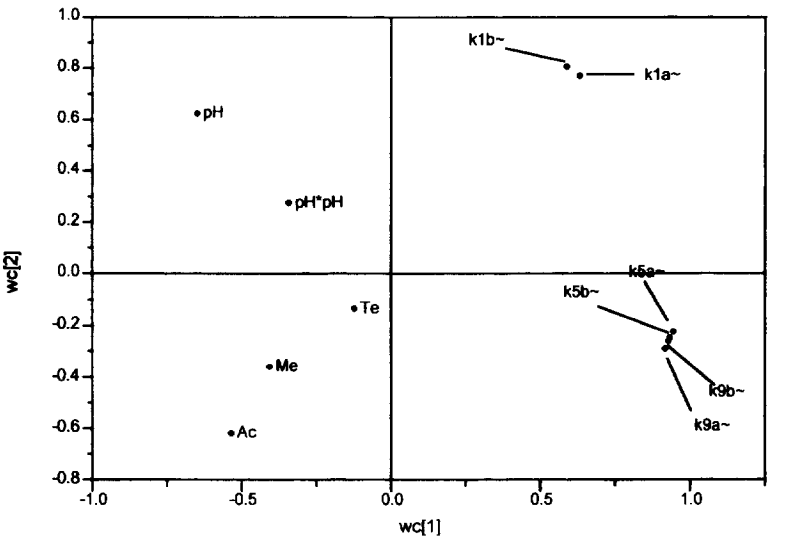


Fig. 6. Descriptors from mobile phases depicted in Table 2 (w) and response (c) variable inter-correlations and sizes (i.e., $w1$ versus $w2$ and $c1$ versus $c2$ superimposed). The responses were log k' for the indicated solutes (see Fig. 1). Abbreviations: Me, methanol; T, temperature; Ac, acetonitrile; pH, mobile phase pH; responses as in Fig. 2.

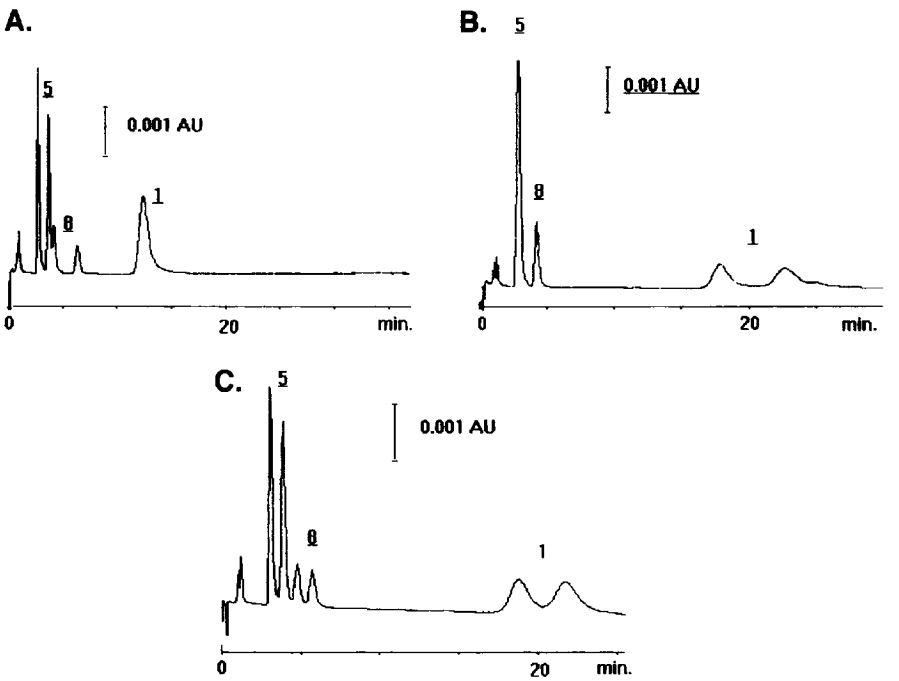


Fig. 7. Simultaneous enantioseparation of 1, 5 and 8 by using mixed organic modifiers. Solid-phase: Chiral-AGP. Mobile phase: Phosphate buffer (pH=5.5) with addition of: (A) Acetonitrile 15%. (B) Methanol 26%. (C) Acetonitrile 4%+Methanol 18%. All additions as (v/v).

phase could be designed that in one and the same injection could separate four pairs of enantiomers of structurally related compounds in less than 28 min. However, it must be emphasized that linear modeling without physical scrutiny may comprise pitfalls as shown for predictions of the enantiomers of solute No. 3. On the other hand an apparent difference in enantioselectivity between methanol and acetonitrile for chiral separations was indicated, and subsequently confirmed, by PLS-modeling. A mobile phase with a mix of two organic modifiers, i.e., methanol and acetonitrile made it possible to simultaneously separate the enantiomers of the major compound, the most important degradation product and a possible internal standard.

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