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Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 43–49

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Enantiomeric separation of verapamil and norverapamil using Chiral-AGP® as the stationary phase

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Received 9 September 1998; received in revised form 13 January 1999; accepted 26 January 1999

Abstract

Simultaneous enantiomeric separation of verapamil and its main metabolite norverapamil was achieved using Chiral-AGP as the stationary phase. The optimized chromatographic system was obtained using statistical experimental design with partial least squares as regression method. The three variables studied were buffer pH, content of acetonitrile and column temperature. A high buffer pH favors enantioselectivity as well as the selectivity between (*S*)-verapamil and (*R*)-norverapamil. The concentration of the organic modifier in the mobile phase was a compromise as a high content of acetonitrile decreased enantioselectivity but increased the selectivity mentioned above. Increased column temperature increased the separation between (*S*)-verapamil and (*R*)-norverapamil with only a slight decrease in enantioresolution.

Keywords: Column temperature; Direct separation; Experimental design; Mixed organic modifiers; Statistical evaluation

1. Introduction

Liquid chromatography is one of the most important and most used techniques for the separation of enantiomers. Enantioresolutions have been achieved and used for bioanalysis [1] as well as for the determination of enantiomeric purity [2] in pharmaceutical formulations using chiral phases where proteins have been immobilized to silica particles. A wide applicability has been shown using α_1-acid glycoprotein as the chiral selector, and anionic [3], cationic [4] and aprotic [5] racemates have successfully been resolved using the Chiral-AGP® column.

Direct separation of the enantiomers of verapamil have previously been performed using straight phase [6] or reversed phase [7] liquid chromatography. In both these studies coupled achiral–chiral chromatography was used to separate verapamil from its main metabolite, the demethylated form, norverapamil. For bioanalysis purposes the two enantiomers of verapamil were successfully separated using α_1-acid glycoprotein immobilized to silica particles as the resolving stationary phase [7,8].

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0731-7085/99/$ - see front matter
PII: S0731-7085(99)00093-X

In this work, the enantiomers of verapamil and norverapamil were simultaneously separated in the direct mode using Chiral-AGP and statistical experimental design [9]. The multivariate analysis calculation was made using the partial least squares method (PLS) [10]. Model predictability was estimated by cross-validation [11]. The optimized chromatographic system has been used for the bioanalysis of verapamil and norverapamil [12,13].

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a Binary LC pump 250 (Perkin Elmer, Norwalk, CT), an AS-3000 autosampler (Spectra-Physics Analytical, San Jose, California, USA), and an LC-detector Chrompack UV-VIS (Chrompack, Netherlands). The Chiral-AGP® column (150 × 4.0 mm, 5 μm particle size), consisting of α_1-acid glycoprotein as the immobilized protein, was purchased from ChromTech (Stockholm, Sweden). The temperature of the column and solvent reservoir was maintained by a waterbath (Grant LTD 6; Cambridge, UK). The mobile phase flow rate was kept constant at 1.0 ml min^{-1}. The analyte solutions, injected twice, and the mobile phases were all freshly prepared. The solutes were detected at 272 nm unless otherwise stated. The injection volume was 20 μl and the sample concentration varied from 0.01 to 0.02 mM.

2.2. Chemicals

Methanol (LiChrosolv quality), 1-propanol (p.a.) acetonitrile (gradient quality), 2-propanol (p.a.) sodium acetate (p.a.), acetic acid (p.a.), sodium dihydrogen phosphate (p.a.) and disodium hydrogen phosphate (p.a.) were obtained from Merck (Darmstadt, Germany). Ethanol was bought from Kemetyl (Stockholm, Sweden). (*R*,*S*)-, (*R*)- and (*S*)-verapamil, (*R*)-norverapamil and (*R*,*S*)-norverapamil were kind gifts from Knoll AG (Darmstadt, Germany).

2.3. Statistical methods

A 2^3 full factorial design with centerpoints was used to examine the influence of the descriptor variables on the chromatographic responses for the two analytes. With three variables, the experimental design included eight experiments and in addition centerpoint experiments were replicated three times. All experiments were performed in random order, except for the centerpoint replicates which were run in the beginning, in the middle and at the end of the experiments.

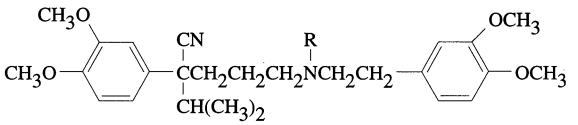
Design and evaluation of the statistical model were performed using the software MODDE version 3.0 (Umetri AB, Sweden) using PLS as regression method. PLS is a multivariate regression method [10,14] that provides overview of large data sets. It is most certainly rewarding on data deriving from experimentation according to factorial design, since such combination guarantees accurate estimates of the coefficients in the regression equation [9], and thus inherently illuminating and reliable loading structures projected to the individual principal components (PCs) [10], provided they are significant [14]. The rationale of employing PLS can be to use the regression equation for predictions [11], but interpretations from plots deriving from the various estimates from the individual PCs can also be informative [14,15]

Validation of the chemometric model was made with cross-validation [11]. 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. In addition a test experiment was performed and its outcome was compared to predicted data.

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:

$$R^2 = \frac{SS_{REG}}{SS} \quad (1)$$

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



verapamil (R = methyl)

norverapamil (R = H)

Fig. 1. Solute structures.

where SS_{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.

3. Results and discussion

3.1. Design and statistical evaluation

The strategy in the present study was to optimize the enantioresolution of verapamil and its main metabolite, norverapamil (Fig. 1), on Chiral-AGP by using statistical experimental design. Previous results found in the literature showed difficulties in separating the enantiomers of verapamil and norverapamil simultaneously when using α_1-acid glycoprotein immobilized to silica particles as the resolving stationary phase [8]. In order to find the appropriate ranges in the experimental design, mobile phase pH and type of modifier were studied. The tested organic modifiers were acetonitrile, methanol, 2-propanol, 1-propanol and ethanol. As acetonitrile gave the highest enantioselectivities for verapamil and norverapamil, this modifier was chosen for further studies, despite the fact that higher selectivities between (*S*)-verapamil and (*R*)-norverapamil were observed when using methanol, 1-propanol, 2-propanol and ethanol as organic modifier instead of acetonitrile (Table 1).

The complete design, with the values of the descriptor variables, mobile phase pH (6.6–7.6), column temperature (Te = 20–40°C) and acetonitrile content (AC = 15–25%, v/v), as well as the responses, the capacity factors (k) and the selectivity factor (α) are given in Table 2. MODDE software (version 3.0; Umetri AB, Sweden) was used to create the experimental design (Table 2) and to perform the multivariate analysis. The multivariate analysis calculation used in this software is the PLS method. Model predictability was estimated by cross-validation. The statistical model, i.e. correlation between descriptor variables and the responses, was optimized to create the best fitting.

The optimization of the statistical model is given in Table 3. With the default settings in the statistical program, i.e. the linear and the interaction terms, a rather high explanation (R^2) of the

Table 1
Influence of organic modifier on selectivity[a]

Response[b]	Acetonitrile (22%, v/v)	Methanol (40%, v/v)	2-Propanol (22%, v/v)	1-Propanol (22%, v/v)	Ethanol (30%, v/v)
$k_{(RV)}$	4.90	4.08	6.16	3.08	4.56
$k_{(SV)}$	5.62	4.79	6.16	3.08	4.56
$k_{(RN)}$	6.18	7.68	8.66	5.13	7.36
$k_{(SN)}$	6.64	7.82	8.66	5.13	7.36
α_1	1.15	1.17	1.00	1.00	1.00
α_2	1.07	1.02	1.00	1.00	1.00
α_3	1.10	1.60	1.41	1.67	1.61

[a] Mobile phase: phosphate buffer (pH 7.6, $I = 0.01$). Column temperature: 20°C.

[b] (RV), (SV), (RN) and (SN) correspond to the (*R*) and (*S*) forms of verapamil and norverapamil, respectively. $\alpha_1 = k_S/k_R$ (verapamil); $\alpha_2 = k_S/k_R$ (norverapamil); $\alpha_3 = k_{(RN}/k_{(SV)}$.

Table 2
The statistical experimental design[a]

Te	AC	pH	$k_{(RV)}$	$k_{(SV)}$	$k_{(RN)}$	$k_{(SN)}$	α_1	α_2	α_3
20	15	6.6	9.17	11.1	10.3	11.7	1.21	1.13	0.93
40	15	6.6	7.14	8.22	8.04	8.91	1.15	1.11	0.98
20	15	7.6	24.7	29.5	28.0	33.8	1.20	1.21	0.95
40	15	7.6	17.3	20.2	22.0	24.2	1.17	1.10	1.09
20	25	6.6	2.47	2.69	2.90	2.90	1.09	1.0	1.08
40	25	6.6	2.41	2.41	2.77	2.77	1.0	1.0	1.15
20	25	7.6	4.50	4.96	5.94	6.26	1.10	1.05	1.20
40	25	7.6	3.16	3.35	5.01	5.01	1.06	1.0	1.50
30	20	7.1	5.11	5.73	5.94	6.34	1.12	1.07	1.04
30	20	7.1	4.97	5.57	5.79	6.21	1.12	1.07	1.04
30	20	7.1	4.93	5.57	5.78	6.21	1.13	1.07	1.04

[a] Te, temperature in °C; AC, content of acetonitrile (%, v/v); pH, buffer pH. For other abbreviations, see Table 1.

model was obtained but the predictability of this model was rather low (Q^2) (Table 3). Values of (R^2) and (Q^2) close to 1 indicate high explanation and predictability of the statistical model [10]. The predictability of the model increased when the interaction terms Te × pH, Te × AC and AC × pH were withdrawn, however, with a minor decrease in explanation of the mathematical model. By transforming the capacity factors to their 10-logarithm and excluding the interaction terms the optimized model was obtained, $Q^2 = 0.92$ and $R^2 = 0.83$. The descriptor variables giving statistical significant effects are listed in Table 4. All capacity factors increased with increased buffer pH and decreased with higher content of acetonitrile. The enantioresolution of verapamil and norverapamil decreased by increasing the column temperature and using high content of acetonitrile. However, the most critical separation (α_3), between (*S*)-verapamil and (*R*)-norverapamil increased when increasing all the variables (Table 4). In fact reversal of retention order of these compounds was observed (Table 2). Due to these results a high mobile phase pH should be used and pH 7.6 was chosen for the phosphate buffer. Fig. 2 shows the modeled effects of content of acetonitrile and column temperature on the enantioselectivity of verapamil (α_1) and the selectivity between (*S*)-verapamil and (*R*)-norverapamil (α_3). Fig. 2; the data in Table 4 show that the variables affected the two separations in opposite directions.

3.2. Optimized chromatographic system for separation of the enantiomers of verapamil and norverapamil

In order to separate simultaneously the enantiomers of verapamil without co-elution between (*S*)-verapamil and (*R*)-norverapamil the content of acetonitrile must be chosen with care. As a compromise between the two separations, (α_1) and (α_3), the content of acetonitrile was set to 22% (v/v). The column temperature was set to 30°C and the mobile phase buffer pH was 7.6. The enantioseparations using the chosen chromatographic system are given in Fig. 3. The obtained chromatographic data were compared with data

Table 3
Optimization of the statistical model[a]

Response	Descriptors	R^2	Q^2
k and α	Linear Te × pH Te × AC AC × pH	0.85	0.56
k and α	Linear	0.75	0.63
log k and α	Linear Te × pH Te × AC AC × pH	0.90	0.60
log k and α	Linear	0.92	0.83

[a] For abbreviations see Table 2.

Table 4
Descriptor variables giving statistical significant effects on the responses[a]

Response	Significant increase	Significant decrease
$k_{(RV)}$	pH	AC
$k_{(SV)}$	pH	AC
$k_{(RN)}$	pH	AC
$k_{(SN)}$	pH	AC
α_1	pH	Te and AC
α_2	pH	Te and AC
α_3	Te, pH and AC	–

[a] For abbreviations see Table 2.

predicted by the statistical model (Table 5). The experimental data were in accordance with the data predicted by the model and deviations less than 3% were obtained.

An increase in column temperature should further optimize the separation between (*S*)-verapamil and (*R*)-norverapamil according to Table 4 and Fig. 2. An increase in column temperature from 30 to 40°C increased the separation factor, α_3, from 1.2 to 1.4. However, at the same time the separation factor for the enantiomerer of verapamil, α_1, decreased from 1.10 to 1.07 (Fig. 3). The separation between (*S*)-verapamil and (*R*)-norverapamil could be further optimized by decreasing the ionic strength of the aqueous buffer in the mobile phase. At the same time the separation factors for verapamil and norverapamil increased slightly. The ionic strength in the final chromatographic mobile phase was set to $I = 0.01$ (for chromatogram see Fig. 4A).

As described above (data in Table 1), the selectivity between (*S*)-verapamil and (*R*)-norverapamil could probably be further increased using a mixture of organic modifiers. A mobile phase that contained a mixture of phosphate buffer (pH 7.6, $I = 0.01$)–methanol–acetonitrile (75:5:20) was used to demonstrate this effect (Fig. 4B). Enantioresolution of verapamil and norverapamil did not decrease by the minor addition of methanol to the mobile phase. Mixed modifiers have previously been used for the same reason to separate enantiomers of clevidipine and structurally related compounds [16].

When using the described separation method for bioanalytical applications fluorescence detection was used (excitation wavelength of 232 nm and emission wavelength 310 nm) [12,13]. A loop size of 50 μl was used and the limits of quantification (LOQ, ± S.D.) were 5.5 ± 0.3 ng ml^{-1} for (*R*)-verapamil, 5.5 ± 0.4 mg nl^{-1} for (*S*)-verapamil, 2.9 ± 0.2 ng ml^{-1} for (*R*)-norverapamil and 2.9 ± 0.1 ng ml^{-1} for (*S*)-norverapamil [12]. The linearity of the method was tested from the LOQ level up to a concentration of 3000 ng ml^{-1}, and correlation factors (r) > 0.998 were observed for the four stereoisomers.

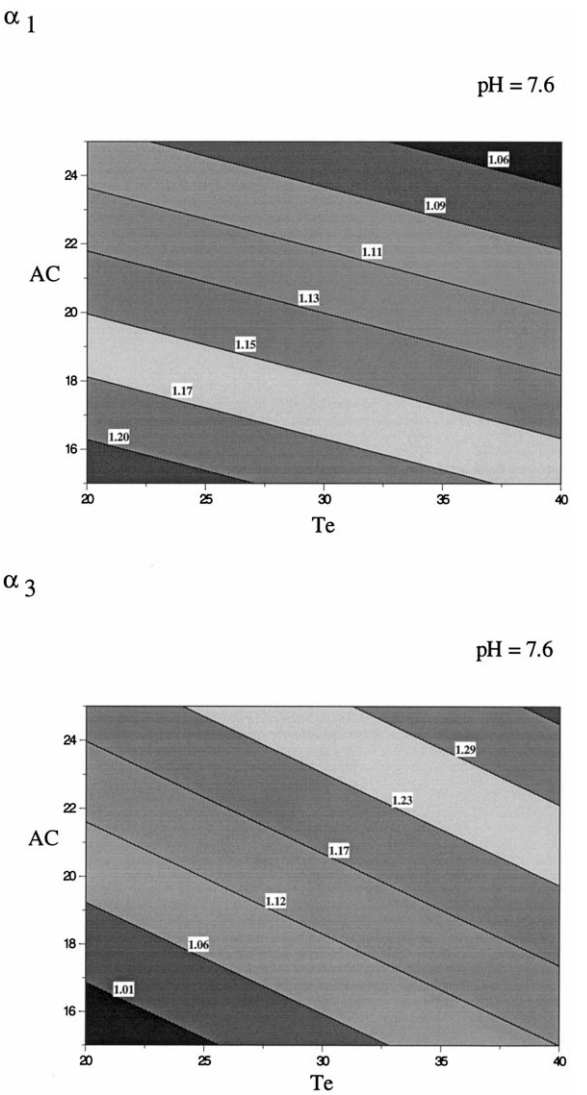


Fig. 2. Modeled effects of content of acetonitrile and mobile phase pH.

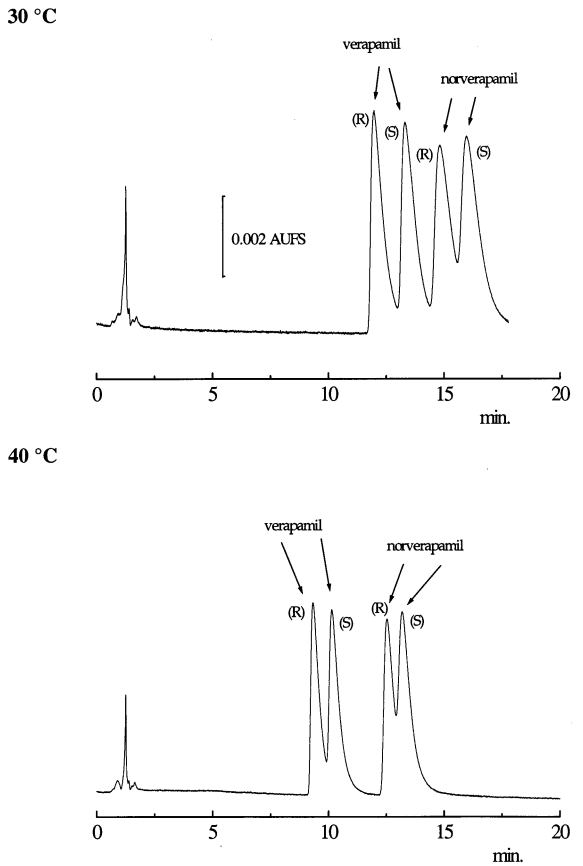


Fig. 3. Improved selectivity between the enantiomers of verapamil and norverapamil by increasing the column temperature. Solid phase: Chiral-AGP. Mobile phase: phosphate buffer (pH 7.6, $I = 0.05$)–acetonitrile (78:22).

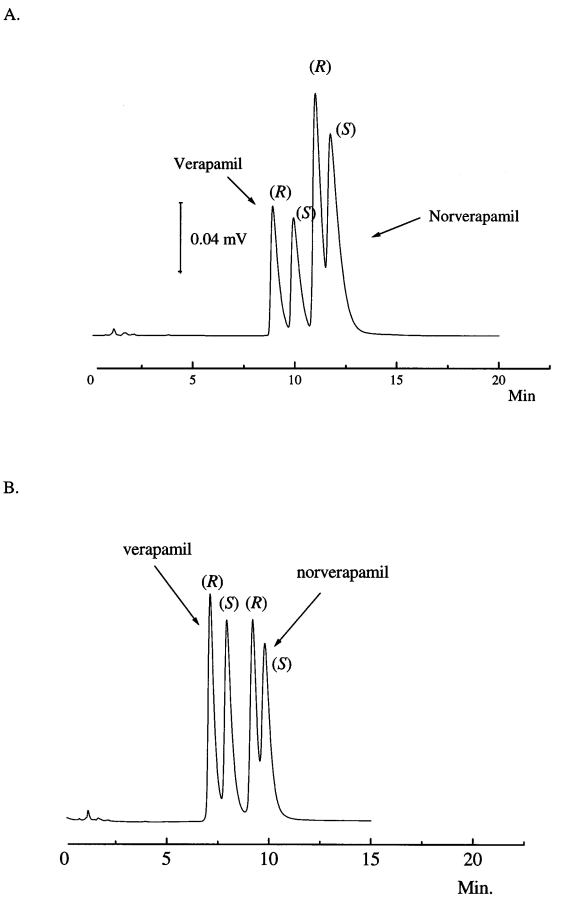


Fig. 4. Simultaneous enantiomeric separation of the enantiomers of verapamil and norverapamil. Solid phase: Chiral-AGP. Mobile phase: phosphate buffer (pH 7.6, $I = 0.01$) with (A) 22% (v/v) acetonitrile, (B) 20% (v/v) acetonitrile and 5% (v/v) methanol.

Table 5
Experimental and predicted chromatographic responses[a]

Responses[b]	Experimental	Predicted	Deviation (%)
$k_{(RV)}$	6.47	6.31	−2
$k_{(SV)}$	7.11	6.96	−2
$k_{(RN)}$	8.37	8.31	−0.7
$k_{(SN)}$	8.85	8.82	−0.3
α_1	1.10	1.10	0
α_2	1.06	1.06	0
α_3	1.18	1.21	3

[a] Solid phase: Chiral-AGP. Mobile phase: phosphate buffer (pH 7.6, $I = 0.05$)–acetonitrile (78:22). Column temperature: 30°C.

[b] For abbreviations see Table 2.

4. Conclusion

Statistical experimental design was used to optimize the enantioseparation of verapamil and norverapamil on Chiral-AGP. A strategy for optimization of the statistical model, using partial least square as regression method, is presented. A high mobile phase pH gave the best enantioseparation as well as the best separation between (*S*)-verapamil and (*R*)-norverapamil. The most critical separation between (*S*)-verapamil and (*R*)-norverapamil could be controlled by column temperature. An increased column temperature increased this

separation with only a minor decrease in enantioresolution. The enantiomers of verapamil and norverapamil could simultaneously be separated within 15 min using a mobile phase that consists of phosphate buffer (pH 7.6)–acetonitrile (78:22) and a column temperature of 20°C. The selectivity between the last eluted enantiomer of verapamil and the first eluted enantiomer of norverapamil could be further increased using a mixture of organic modifiers.

References

[1] D. Haupt, J. Chromatogr. 685 (2) (1996) 299–305.
[2] A. Karlsson, A. Aspegren, Chromatographia 47 (1998) 189–196.
[3] J. Hermansson, M. Eriksson, J. Liq. Chromatogr. 9 (1986) 621–639.
[4] G. Schill, I. Wainer, S. Barkan, J. Liq. Chromatogr. 9 (1986) 641–666.
[5] A. Karlsson, K. Pettersson, K. Hernqvist, Chirality 7 (1995) 147–153.
[6] A. Shibukawa, I.W. Wainer, J. Chromatogr. 574 (1992) 85–92.
[7] Y.-Q. Chu, I.W. Wainer, J. Chromatogr. 497 (1989) 191–200.
[8] H. Fieger, G. Blaschke, J. Chromatogr. 575 (1992) 255–260.
[9] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for Experimenters, Wiley, New York, 1978.
[10] A. Höskuldsson, J. Chemometr. 2 (1988) 211–228.
[11] S. Wold, Technometrics 20 (1978) 397–405.
[12] R. Sandström, A. Karlsson, L. Knutson, H. Lennernäs, Pharm. Res. 15 (1998) 856–862.
[13] R. Sandström, A. Karlsson, H. Lennernäs, J. Pharm. Pharmacol. 50 (1998) 729–735.
[14] P. Geladi, B.R. Kowalski, Anal. Chim. Acta 185 (1986) 1–17.
[15] A.-M. Andersson, A. Karlsson, M. Josefssson, J. Gottfries, Chromatographia 38 (1994) 715–722.
[16] J. Gottfries, P. Johansson, A. Karlsson, J. Chromatogr. 763 (1997) 115–123.