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Optimization of the separation of (6*R*)- and (6*S*)-leucovorin and evaluation of the robustness of the optimum

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ABSTRACT

The variable-size simplex approach was used to optimize the separation of (6*R*)- and (6*S*)-leucovorin diastereoisomers. The experimental parameters optimized were pH, ionic strength and percentage of propanol in the mobile phase. The optimization criterion was the valley-to-peak-ratio. Fourteen experiments were performed to obtain the optimum. The ruggedness of the optimum was evaluated by means of a partial factorial design for three factors.

INTRODUCTION

Leucovorin is a reduced folate that is used in combination with 5-fluorouracil for the treatment of colorectal and gastric carcinoma. Leucovorin contains two asymmetric carbon atoms. As L-glutamic acid is used for the chemical synthesis, racemic leucovorin consists of an equimolar mixture of 6*R* and 6*S* diastereoisomers. The 6*S* isomer is the biologically active form [1]. High-performance liquid chromatography (HPLC) can be used for the separation of stereoisomers, but stereoselective agents are necessary to perform these separations. Leucovorin diastereoisomers can be separated by means of a bovine serum albumin (BSA) chiral stationary phase [1]. An application to the analysis of plasma using column switching was reported by Wainer and Stiffin [2]. One of the columns was also a BSA column.

As the composition of the mobile phase influences the selectivity of the chromatographic system, it is important to optimize the different mobile phase parameters. Fell *et al.* [3] described the optimization of the enantioseparation of oxamniquine using a simplex design. Optimization procedures can be divided into two groups, sequential and simultaneous. Simultaneous optimization creates models for the chromatographic retention behaviour. In sequential optimization, one optimizes in a stepwise fashion without developing a model. The mobile phase composition in an experiment with such a procedure is determined from the results of the previous experiments. The simplex algorithm is a sequential optimization procedure. The rules for performing this algorithm have been fully described elsewhere [4].

This paper describes the optimization of the separation of (6*R*)- and (6*S*)-leucovorin on a BSA stationary phase by means of the simplex algorithm. The robustness of the optimum was evaluated by means of a saturated fractional factorial design.

EXPERIMENTAL

Apparatus

A Merck Hitachi L-6000 chromatograph with a Rheodyne loop injector (volume 100 μl) and a Merck Hitachi L-4000 variable-wavelength UV detector were used. The flow-rate of the mobile phase was 0.5 ml min^{-1} for all experiments. The detection

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wavelength was 290 nm. Chromatograms were recorded and integrated by a Shimadzu C-R6A Chromatopac.

The BSA column was a Resolvosil BSA-7 (150 mm × 4 mm I.D.), obtained from Macherey-Nagel (Düren, Germany).

Standards and reagents

1-Propanol was of HPLC quality and was supplied by Merck (Darmstad, Germany). Sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate and phosphoric acid were of analytical-reagent grade (Merck) and were used for the preparation of the phosphate buffers. Milli-Q-purified water was used for the preparation of the buffer solutions. These solutions were filtered through a membrane filter (0.2 μm) before being used for chromatography.

Racemic leucovorin and (6*R*)- and (6*S*)-leucovorin (as sodium salts) were obtained from Lederle Labs.

Computer program

A computer program for the simplex optimization procedure is available [5]. It is written in BASIC and runs on an IBM-PC computer.

RESULTS AND DISCUSSION

The influence of pH, ionic strength (phosphate buffer) and percentage of 1-propanol in the mobile phase on the separation of (6*R*)- and (6*S*)-leucovorin was evaluated. 1-Propanol is recommended as an organic modifier by the manufacturer of the BSA phase. A variable-size simplex was used to optimize these parameters. As three parameters were optimized, the resulting simplex contained four vertices and was thus a tetrahedron [4]. The algorithm is based on the rejection of the vertex giving the worst result. For a variable-size simplex, the search space is contracted or expanded [4]. To perform the optimization procedure, a criterion has to be chosen that expresses the quality of the separation. Therefore the expert system CRISE [6] was consulted. CRISE advises the valley-to-peak ratio (P_v) as a criterion. The calculation of P_v is illustrated in Fig. 1 [7]. If a complete resolution between two peaks is obtained, P_v becomes 1 as the height of the valley is zero. Fig. 1 shows that two P_v values can be calculated for a pair of peaks. Here, P_{v2} was calculated for each experiment, *i.e.*, the valley height is divided by the height of the second peak.

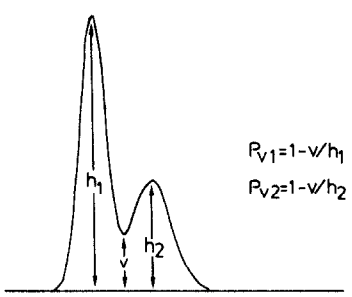

Fig. 1. Calculation of the valley-to-peak ratio, P_v [7].

The computer program calculated the experiments of the start simplex from the start and step values for each factor [5]. These values are shown in Table I. The start values represent a first guess, determined on the basis of preliminary experiments concerning the evaluation of the enantioselectivity of the BSA phase. The step values were chosen in order to make the search space as large as possible. For each factor, one also has to set the boundaries of the experimental domain. These can be determined from the advice of the column manufacturer (Table I). One should not exceed these limits in order to avoid denaturation of the protein.

Based on the P_v values of the first four experiments, the computer program calculated the mobile phase composition for the fifth experiment. Fourteen experiments were performed. The results of the experiments are summarized in Table II. Fig. 2 illustrates the movement of the simplex. The separate diastereoisomers were injected in each mobile phase: (6*S*)-leucovorin was the first-eluting diastereoisomer for each experiment. The optimization

TABLE I

START AND STEP VALUES AND LIMITS FOR EACH MOBILE PHASE PARAMETER

Factor	Start value	Step value	Limits
pH	6	2	5–8
Ionic strength	0.1	0.1	0.05–0.20
1-Propanol (%)	0	0.5	0–5

TABLE II

RESULTS OF THE SIMPLEX OPTIMIZATION

Experiment No.	pH	Ionic strength	1-Propanol (%)	Valley-to-peak ratio, P_v	Capacity factor of second peak
1	6.00	0.10	0	0.05	5.44
2	8.00	0.10	0	0	0.97
3	6.00	0.20	0	0.09	3.31
4	6.00	0.10	0.5	0.17	4.69
5	7.00	0.12	0.10	0.05	2.39
6	6.70	0.18	0.40	0	1.65
7	6.20	0.12	0.10	0.09	4.45
8	5.10	0.16	0.30	0.81	12.3
9	5.50	0.06	0.61	0.66	21.7
10	5.85	0.11	0.28	0.16	5.76
11	5.75	0.11	0.45	0.13	6.19
12	5.06	0.10	0.63	0.86	21.4
13	5.49	0.11	0.48	0.62	13.4
14	5.10	0.16	0.30	0.81	12.3

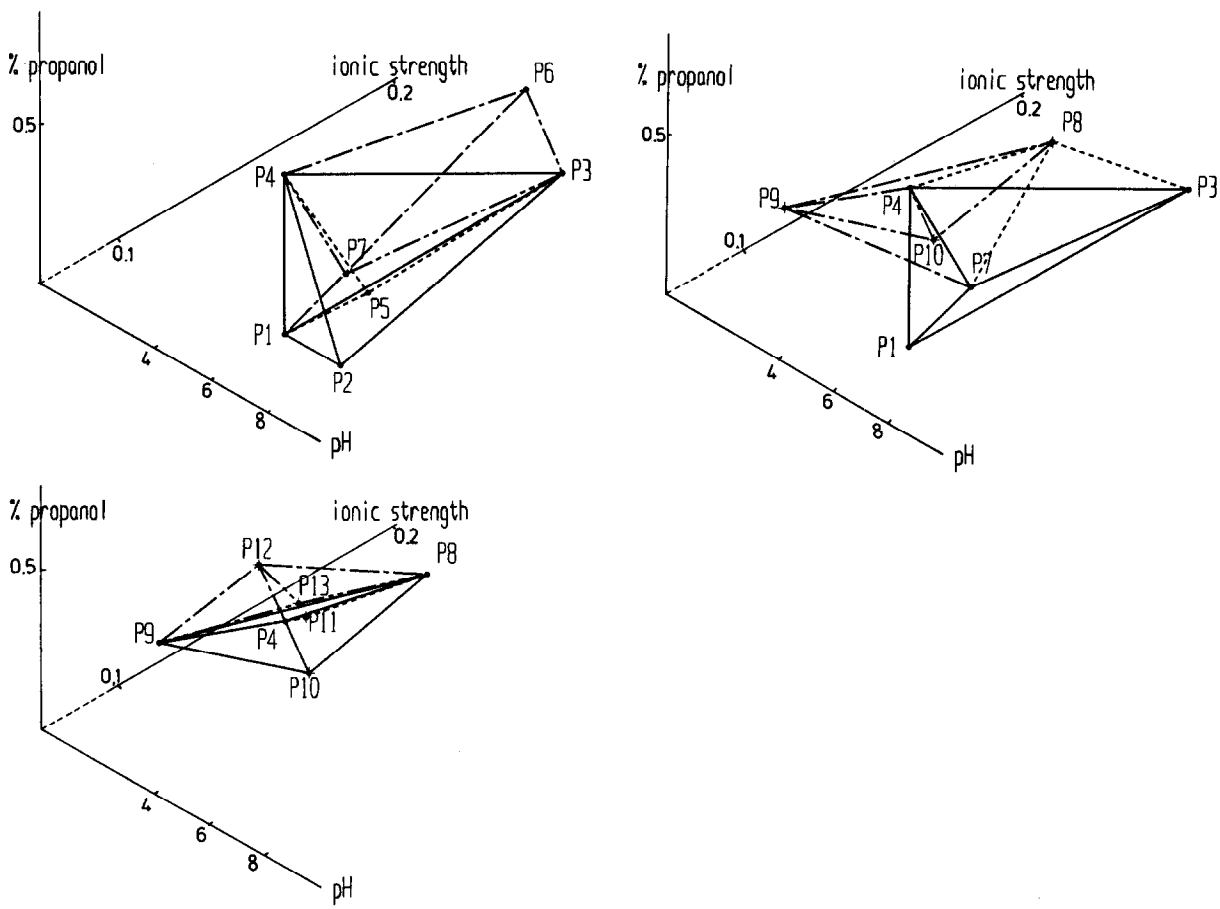

Fig. 2. Movement of the simplex.

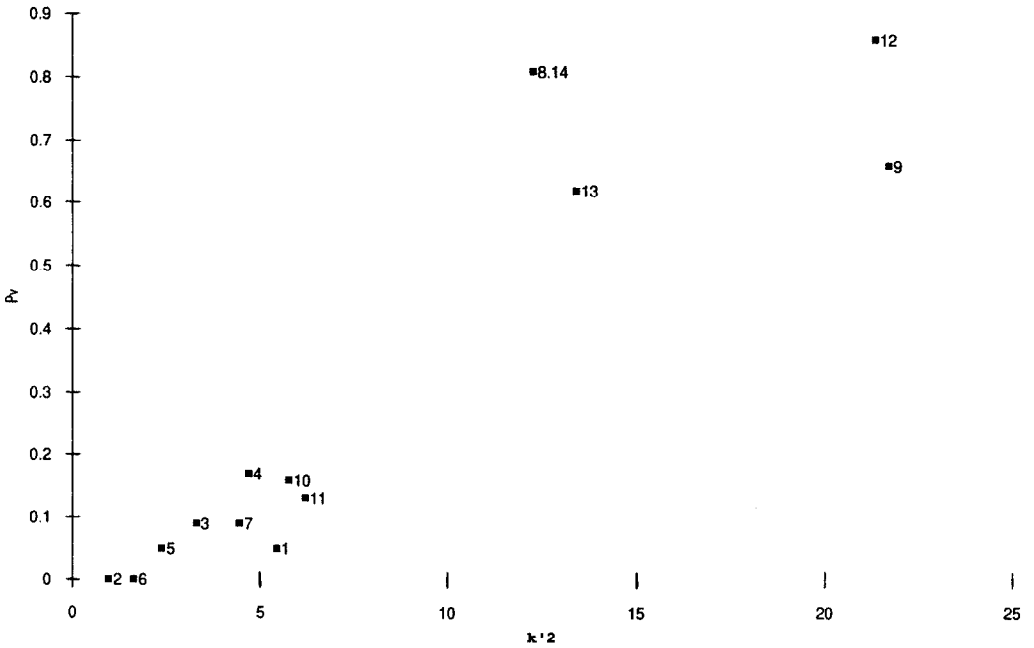

Fig. 3. Plot of P_v *versus* k'_2.

procedure was stopped as the mobile phase parameters for experiment fourteen were the same as those for experiment eight. The P_v value obtained for these experiments is 0.80 and approaches the maximum value of 1. Experiment 12 seems to give the best separation quality (P_v = 0.86). Resolutions calculated for these experiments are 1.29 and 1.33, respectively. The resolution of the separation described in the literature [1] was 1.65, but was achieved by coupling two BSA columns in series.

As the criterion P_v only measures chromatographic selectivity and takes no account of analysis time, the P_v values are plotted against the retention time of the second peak in Fig. 3. The purpose of this pareto-optimality plot [8,9] is to find the experiment with optimum separation *versus* analysis time characteristics. From Fig. 3, one can conclude that experiment 8 represents this optimum: the P_v value approaches unity and the time required for analysis does not become too long. The mobile phase parameters used in experiment 8 are pH 5.10, ionic strength 0.16 and 0.30% 1-propanol. The chromatogram in Fig. 4 illustrates the separation obtained with these mobile phase conditions.

The robustness of this optimum was evaluated by means of a fractional factorial design [4]. This was done to evaluate the influence of small changes in experimental conditions on the optimum. In this way, one can determine how accurately the different mobile phase parameters have to be set to obtain reproducible results (P_v values).

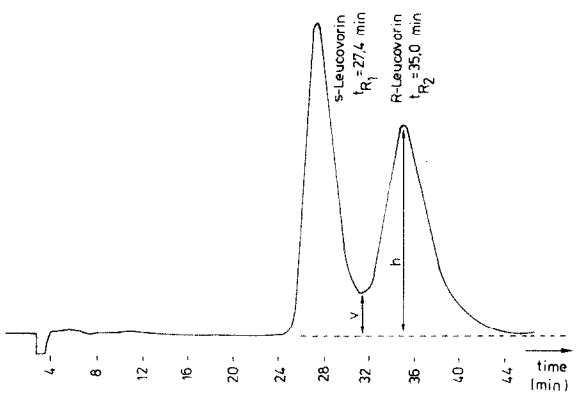

Fig. 4. Separation of (6*S*)- and (6*R*)-leucovorin with the optimum mobile phase (phosphate buffer pH 5.10, ionic strength 0.16, 0.30% 1-propanol).

TABLE III

TWO-LEVEL PARTIAL FACTORIAL DESIGN FOR THREE FACTORS

Experiment	Factor *A*	Factor *B*	Factor *C*
1	+	+	+
2	−	+	−
3	+	−	−
4	−	−	+

TABLE IV

PARTIAL FACTORIAL DESIGN TO EVALUATE THE ROBUSTNESS OF THE OPTIMUM

Experiment No.	Factor pH	Factor ion strength	Factor 1-propanol (%)	P_v
1	5.00	0.14	0.25	0.89
2	5.20	0.14	0.35	0.79
3	5.00	0.18	0.35	0.87
4	5.20	0.18	0.25	0.72

Here, a two-level partial factorial design in three factors was used to study the effect of small changes in mobile phase pH, ionic strength and percentage of 1-propanol on the P_v values. Each of the factors has two levels in the design, as indicated by + and − in Table III. Here, these levels were chosen so that the nominal value, *i.e.*, the exact value at which the optimum was obtained, was situated between them. The results of the experiments are shown in Table IV. The effect of factor *A* (D_A), for instance, was calculated as $[(P_{v1} + P_{v3})/2 - (P_{v2} + P_{v4})/2]$.

The main effect for each of the three factors was calculated and were as follows:

$$D_{pH} = \frac{(0.89 + 0.87)}{2} - \frac{(0.79 + 0.72)}{2} = 0.125$$

$$D_{\mu} = \frac{(0.89 + 0.79)}{2} - \frac{(0.87 + 0.72)}{2} = 0.045$$

$$D_{prop.} = \frac{(0.89 + 0.72)}{2} - \frac{(0.79 + 0.87)}{2} =$$

$$|-0.025| = 0.025$$

If changing the value of *A* has no effect on the results P_{vi}, one expects D_A to be close to zero. Factor *A* is regarded as significant if *D* exceeds $2s_D$ (s_D is the standard deviation of the difference between two averages, *i.e.*, the difference that expresses the effect of factor *A*) [4]. As the standard error of the mean of two measurements is $s/\sqrt{2}$, where *s* is the standard deviation of replicate measurements at the nominal level, s_D is equal to *s*.

The standard deviation *s* was determined by calculating the P_v values of eight experiments, performed at the exact nominal level for each factor. This standard deviation is 0.028. From the *D* values given above, on can conclude that only D_{pH} exceeds $2s = 0.056$. This means that the factor pH has to approach the nominal level more accurately than is the case with the two levels chosen here.

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