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Optimisation of the reversed phase liquid chromatographic separation of atovaquone, proguanil and related substances

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Abstract

The optimisation of the separation of the antimalarial drugs, proguanil and atovaquone and six related compounds was obtained by two independent optimisation steps; the optimisation of the mobile phase composition and the optimisation of the pH. This was done using window selection diagrams (WSD) and a mixture design. The optimal conditions allow the identification of the six related compounds down to 0.1%. © 1997 Elsevier Science B.V.

Keywords: Chemometrics; Optimisation; Mixture design; Window selection diagram; High performance liquid chromatography; Antimalarial drugs

1. Introduction

Since the early 1940s, proguanil hydrochloride has been used as a prophylactic antimalarial agent [1–4]. Recently, it has been used in combination with atovaquone. The related substances include two isomers so the separation of all compounds is difficult. The method used until now to detect these substances, called hereafter the starting method [5]: (i) cannot completely resolve the isomers from proguanil; (ii) does not allow the substances to be monitored down to 0.1% (0.025% for 4-chloroaniline, which is one of the related substances of proguanil); and (iii) sometimes gives a poor peak shape for atovaquone.

It was our aim to start from the above mentioned method and improve the separation of all compounds whilst changing the starting method as little as possible, i.e. changing in the first instance only the mobile phase composition. Once the best possible separation was achieved, it was verified whether, under the optimal conditions, it was possible to detect all substances down to 0.1% (0.025% for 4-chloroaniline).

In this paper a reversed-phase liquid chromatographic (RPLC) method that allows all compounds to be identified and detected in low concentrations is proposed. Firstly a window selection diagram (WSD) derived from the Snyder's solvent selectivity triangle concept and a mixture design [6,7] was used to optimise the mobile phase composition. This procedure has been widely ap-

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plied in chromatography and some other applications of it can be found in [8–13]. Secondly, to improve the separation of the substances, and to avoid undesired mobile phase compositions (Section 3), another WSD was applied using pH as a variable.

The quantification of the substances should be verified in a further step, that is, the validation of the method. However, this is not the aim of this paper.

2. Experimental

2.1. Optimisation of the mobile phase composition

The method available as the starting point used a mobile phase composed of methanol (MeOH), acetonitrile (ACN), water and orthophosphoric acid (H_3PO_4) (160:480:360:5). The method is performed on a 15 cm × 4.6 mm i.d. Spherisorb 5 ODS1 column. The flow rate of the mobile phase is 3.0 ml min^{-1} and the detection wavelength 220 nm. During all experiments the column was thermostated at 30°C.

2.1.1. Standards and reagents

Proguanil, atovaquone and six related substances were available (GlaxoWellcome). Stock solutions of all compounds were prepared in 0.1 M methanolic NaOH, with the following concentrations: 100 mg ml^{-1} of atovaquone and proguanil, and 20 mg ml^{-1} of all others. From these stock solutions two new ones were prepared in MeOH–H_2O (1:1). The first, solution A, contained 100 μg ml^{-1} of atovaquone, 10 μg ml^{-1} of the related compounds, 40 μg ml^{-1} of proguanil and 4 μg ml^{-1} of 4-chloroaniline. The second, solution B, contained 100 μg ml^{-1} of atovaquone and 0.1 μg ml^{-1} of the related compounds, 40 μg ml^{-1} of proguanil and 0.01 μg ml^{-1} of 4-chloroaniline.

Solution A was used to optimise the separation and solution B to verify if under the optimal conditions selected, it was possible to detect 4-chloroaniline down to 0.025% and the other related substances down to 0.1%. These limits are required to be able to guarantee the quality of the product.

2.2. Optimisation of the pH

2.2.1. Standards and reagents

Two mobile phases, one at pH 1.0 and another at pH 5.0, composed of methanol MeOH, ACN and a phosphoric buffer with ionic strength 0.1 (188:458:354) were prepared. These solutions were used to find an optimum pH using a WSD.

The column, the flow rate and the detection wavelength are the same as those described in Section 2.1.

2.3. Apparatus

A Merck Hitachi liquid chromatograph with an isocratic pump L6000, equipped with a Rheodyne injection valve (20 μl sample loop) was used to carry out the RPLC measurements. Detection was performed with a Perkin Elmer LC90-UV-detector. Chromatograms were recorded with a Hitachi D-2500 Chromato-Integrator.

2.4. Chromatographic experiments

Retention times and peak widths at 13.5% of the peak height, were measured and capacity factors (k') calculated. The retention time of the first eluting peak, that is the solvent peak, was used as the dead time. All experimental values are the average of at least two measurements.

3. Results and discussion

3.1. Optimisation of the mobile phase composition

A chromatogram obtained for solution A under the conditions of the starting method can be seen in Fig. 1. The numbers in the figure correspond to each compound in the mixture. Peaks 2 and 3 are isomers of compound A.

One can see that peaks 2 and 4 are completely overlapped on this occasion and that there is no baseline separation between these two and peak 3, as well as between peaks 5 and 6. Moreover, the resolution of peak 1 from the solvent is quite poor. However, the solvent has adequate solvent strength as the analysis time is acceptable. There-

fore it was decided to optimise the composition at this solvent strength.

The transfer rules described in the literature [9] were used to determine the volume fractions for the isoeluotropic mixtures of MeOH–H_2O–H_3PO_4 (751:249:5), ACN–H_2O–H_3PO_4 (610:390:5), THF–H_2O–H_3PO_4 (434:566:5) that have the same elution power as the mixture of the starting method. These three mixtures represent the vertices of the solvent triangle describing the isoeluotropic domain and will from now on be referred to as MeOH, ACN and THF, respectively.

3.1.1. WSD

The relationship between retention and mobile phase composition is curved but can be approximated by a empirical linear relationship Eq. (1) as used in the WSD approach.

$$\ln k' = \ln k'_o - S\Phi \quad (1)$$

where k' is the capacity factor of the solute in the mobile phase with a volume fraction of organic solvent Φ, k'_o and S are constants that depend on the type of organic solvent and the solute. According to Snyder [14], Eq. (1) can in a restricted domain be generalised to

$$\ln k' = A - BX \quad (2)$$

where X is any variable, such as the percentage of organic solvent, pH, temperature, etc.

In a first step it was tried to optimise the separation using mixtures of two of the three above defined mobile phases (vertices), applying a WSD approach [15–17].

(i) For the mixtures containing MeOH and ACN the best result obtained is presented in Fig. 2. Visual inspection verifies that, compared to the chromatogram in Fig. 1, the optimum found can resolve peaks 5 and 6 and decreases slightly the analysis time. However, the isomers, peaks 2 and 3 still elute together with proguanil.

(ii) Mixtures of ACN and THF did not give a good separation.

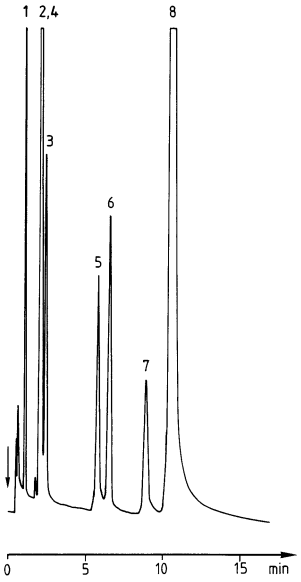

Fig. 1. Chromatogram obtained for solution A using the starting method conditions. The peak numbers correspond to 1, 4-chloroaniline; 2, A(1); 3, A(2); 4, proguanil; 5, B; 6, C; 7, D; and 8, atovaquone.

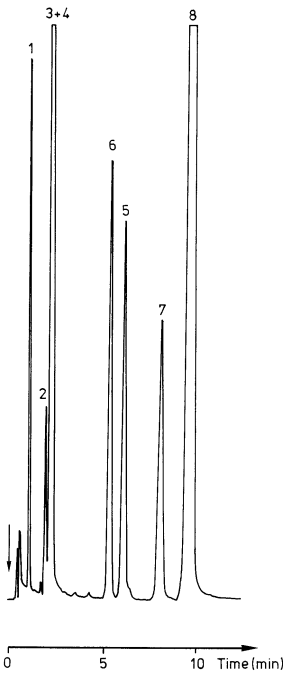

Fig. 2. Chromatogram obtained for solution A with the optimal conditions from the mixtures between MeOH and ACN at a flow rate of 3 ml min^{-1}. The mobile phase composition is MeOH–ACN–H_2O–H_3PO_4 (188:458:354:5).

(iii) The best results for binary isoeluotropic mixtures were achieved for the mixtures between MeOH and THF.

Applying a WSD for these mixtures suggested the mobile phase MeOH–THF–H_2O–H_3PO_4 (488:152:360:5) as optimum. The chromatogram using this mobile phase is shown in Fig. 3.

The initial conditions proposed by the starting method used a flow rate of 3 ml min^{-1}. However, for the mixtures of MeOH–THF, this flow rate increased the pressure too much and it had to be reduced to 2 ml min^{-1}. This was, of course, reflected in the analysis time. Despite this longer analysis time, it was clearly seen that under these conditions (MeOH–THF) (i) the separation of all substances is improved considerably compared to those of Figs. 1 and 2; (ii) the isomers are resolved, both from each other and from proguanil; and (iii) the resolution of peak 1 and the solvent peak is quite good.

Because the analysis time was considered relatively long it was decided to continue the experiments applying a mixture design for the three isoeluotropic solvents and to include the analysis time as an additional optimisation criterion.

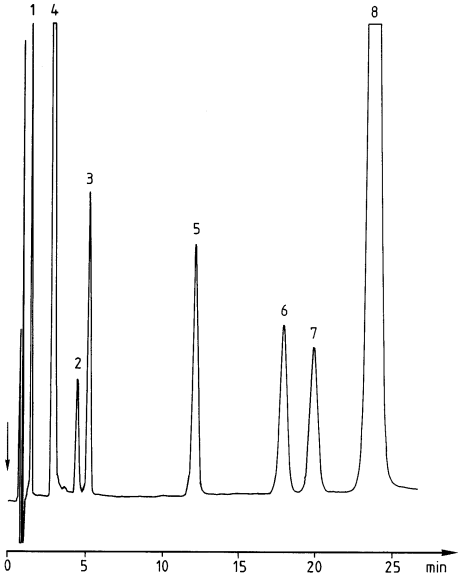

Fig. 3. Chromatogram obtained for solution A with the optimal conditions from the mixtures between MeOH and THF at a flow rate of 2 ml min^{-1}. The mobile phase composition is MeOH–THF–H_2O H_3PO_4 (488:152:360:5).

Table 1
Solvent compositions of the experiments in the mixture design

Experiment	MeOH ($X1$)	ACN ($X2$)	THF ($X3$)
1	1	0	0
2	0	1	0
3	0	0	1
4	0	1/2	1/2
5	1/2	1/2	0
6	1/2	0	1/2
7	1/3	1/3	1/3
8	2/3	1/6	1/6
9	1/6	2/3	1/6
10	1/6	1/6	2/3

3.1.2. Mixture design

A simplified cubic model was applied.

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (3)$$

In this model Y represents the analytical responses to be modelled, in our case: (i) the logarithm of the capacity factor of the substances; (ii) the peak width at 13.5% height; and (iii) the analysis time, expressed as the retention time (tr) of the last eluting peak. The regression coefficients are represented by b and the variables X_1, X_2 and X_3, are the relative amounts of the isoeluotropic solvents MeOH (X_1), ACN (X_2) and THF (X_3). Ten experiments were selected for the mixture design according to a 7 point simplex-centroid augmented with 3 internal points [18]. Their solvent composition can be seen in Table 1. The experiments were performed at a flow rate of 2 ml min^{-1}. The capacity factor (k'), the peak width (w) and the analysis time were modelled according to Eq. (3). Using the predictions obtained for k' and w, the resolution between two adjacent peaks (i and j) (Eq. (4)), could be calculated.

$$Rs = \frac{2(\mathrm{tr}_j - \mathrm{tr}_i)}{(w_i + w_j)} \quad (4)$$

Our goals were to maximise the minimal resolution, which is the resolution of the two nearest eluting peaks in a chromatogram, and to minimise the analysis time.

Fig. 4 shows a simplified contour plot obtained from the predictions for resolution and analysis time. The results show that taking into account only the resolution, the most interesting region is the one delimited by a dashed line inside the triangle. In this region the resolutions predicted are equal to or higher than 2.0. It is clear that to have a good separation either a mixture of MeOH–THF–ACN or MeOH–THF is needed. The closer the mixture is to the other vertex of the triangle (ACN), the worse the separation. The shortest analysis time can be obtained with mobile phase compositions in the direction of the MeOH↔ACN side in the triangle. Combining these observations and looking for an analysis time not longer than 20 min (tr_{limit}), an optimum region was defined which, in Fig. 4, is represented by the shaded area. In other words, any point inside this region fulfils the requirements established for resolution and analysis time. The tr_{limit} was selected as 20 min because it is the expected analysis time for the chromatogram in Fig. 1 with a flow rate of 2 ml min^{-1}. In the shaded region, a point around which the predicted resolutions

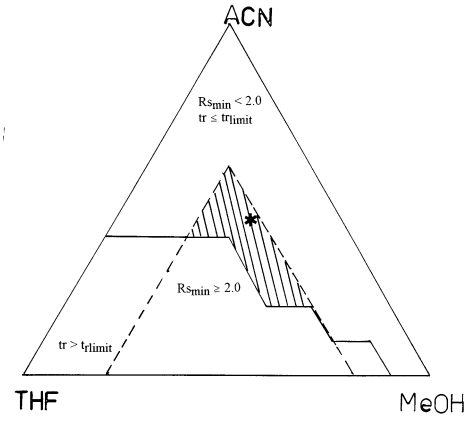

Fig. 4. Simplified contourplot of the experimental mixture design domain, showing the optimal experimental region.

Rs_{min} stands for minimum resolution, tr for retention time and tr_{limit} for the maximum accepted retention time. The region in which the predicted Rs_{min} is larger than 2.0 is situated under the dashed line whereas the region in which the retention time is smaller than tr_{limit} is defined by the upper part of the triangle, above the solid line. The * represents the experiment performed to which the experimental conditions were predicted as optimal.

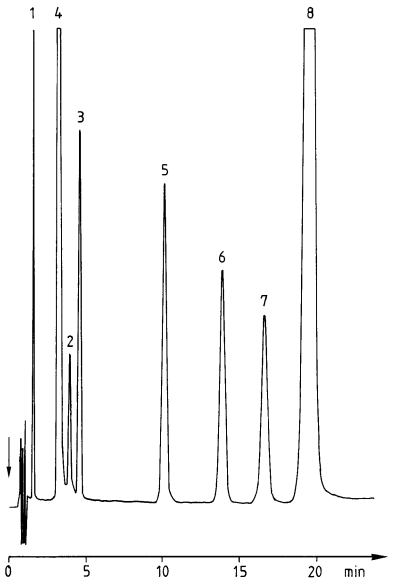

Fig. 5. Chromatogram obtained for solution A with the optimal conditions selected from the contourplot. The mobile phase composition is MeOH–THF–ACN–H_2O–H_3PO_4 (250:101:264:384:5) at a flow rate of 2 ml min^{-1}.

were similar (rugged area), was selected. It corresponds to the composition (MeOH–THF–ACN–H_2O–H_3PO_4) (250:101:264:384:5) (point '*' in the figure). The chromatogram obtained under these conditions is shown in Fig. 5. Visually comparing this chromatogram with the one of Fig. 3, it is clear that the analysis time is shorter and that the resolution of peak 1 with the solvent peak is good. The resolution between peaks 2, 3 and 4 becomes somewhat smaller. From the chromatogram in Fig. 5 the resolutions between peaks 2, 3, and 4 were calculated according to Eq. (4). They are $Rs_{42} = 3.0$, $Rs_{23} = 2.0$ and $Rs_{43} = 4.8$ and the Rs_{min} among them is $Rs_{23} = 2.0$. This is in agreement with the predictions of the model for the shaded region in the contourplot. The same can be said about the prediction of the analysis time for Fig. 5.

3.2. Optimisation of the pH

The optimal mobile phase composition uses THF (Section 3.1), which can lead to technical problems in routine analysis since the detection

wavelength is very low (220 nm) and THF has a high cut-off value (e.g. transmission at 220 mn is between 10 and 20%). To avoid working with THF, the optimisation of the pH starting from the optimal mobile phase for the mixture MeOH↔ACN (Fig. 2) was tried [19]. In a first step of the pH optimisation, a phosphoric buffer was introduced in the mobile phase, instead of the water–H_3PO_4 solution used in the starting method.

Mobile phases with pH values of 1.0 and 5.0 were selected as the limits within which the optimum would be searched for. At each pH, a chromatogram was recorded using solution A. The WSD obtained suggests pH 2.3 as the best one to separate the compounds in the mixture.

The chromatogram obtained for solution A at pH 2.3 and at a flow rate of 3 ml min^{-1} can be seen in Fig. 6. Compared to Fig. 2, it is clear that peaks 2, 3 and 4 have a better separation and the analysis time is shorter. Compared to Fig. 3, the analysis time is much shorter, due partially to the higher flow rate used. However, the resolution between peaks 1, 2, 3 and 4 decreases considerably.

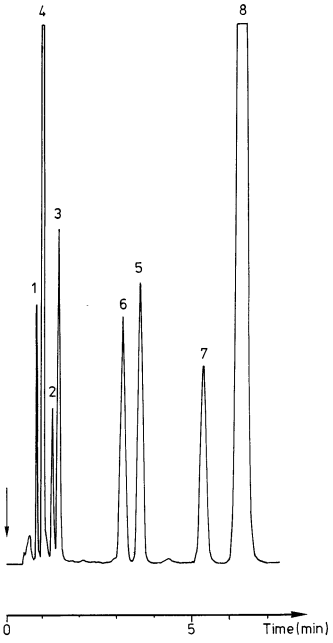

Fig. 6. Chromatogram obtained with the optimal pH conditions. The mobile phase composition of MeOH, ACN and buffer at pH 2.3 is (188:458:354). This chromatogram was recorded with solution A at a flow rate of 3.0 ml min^{-1}.

Fig. 7. Chromatogram obtained with the optimal pH conditions. The mobile phase composition of MeOH, ACN and buffer at pH 2.3 is (188:458:354). This chromatogram was performed with solution B at a flow rate of 3.0 ml min^{-1}.

To verify if there was another pH where the separation would be even better than the one proposed at pH 2.3, two new WSD were created using the data obtained at pH 2.3; one between the pH limits of 1.0 and 2.3 and another with pH limits between 2.3 and 5.0. This was done because the WSD approach supposes a linear behaviour of the retention function of the mobile phase composition and in a large pH range, such as the one used, this is not necessarily true.

For the first pH interval, the WSD suggests pH 2.1 as optimal and for the second interval, pH 2.3 was proposed. Because these proposals were very similar to the one suggested by the previous WSD (Fig. 6), pH 2.3 was maintained as the optimal one. Setting these as the optimal conditions, a chromatogram of solution B was recorded and the results are presented in Fig. 7. these conditions, despite the fact that one can identify all peaks, it becomes particularly difficult to de-

tect peak 2. It is required to detect the substances down to 0.1%. However, the isomers (peaks 2 and 3) were provided as a mixture with an unknown ratio between 2 and 3. Therefore, when preparing the solution of 0.1% of this mixture, one has actually a lower concentration of the separate substances. Even in a lower concentration it is possible to detect the isomers (peaks 2 and 3) which suggests that in the concentration desired (0.1%) it will be also possible and somewhat easier.

4. Conclusions

Compared to the starting method (Fig. 1), it was possible to improve the separation, especially for the isomers (peaks 2 and 3) and the peak shape of atovaquone (peak 8), either when optimising the mobile phase composition (Fig. 3) or the pH (Fig. 6).

The optimum found when optimising the pH, that is pH 2.3, ionic strength 0.1 and a mobile phase composition of MeOH–ACN–phosphoric buffer (188:458:354) is preferred both in terms of separation but also in terms of the analysis time which, is reduced by one third.

It is possible to detect the peaks down to the limits required, that is 0.1% and 0.025% for 4-chloroaniline.

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