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Evaluation of essential parameters in the chromatographic determination of cyclosporin A and metabolites using a D-optimal design

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Abstract

The extensive use of routine monitoring of cyclosporin A (INN, ciclosporin) whole blood levels of patients undergoing such therapy has resulted in a wide variety of chromatographic conditions for analysing this drug. The aim of this study was to evaluate the importance of essential parameters in the chromatographic determination of cyclosporin A and its main metabolites, AM1, AM9 and AM4N. A D-optimal design was used to evaluate the effect of type and amount of organic modifier, temperature, flow rate, pH and gradient steepness. The optimal chromatographic conditions were determined by multi-linear regression. In the final chromatographic method separation of the compounds was carried out on a reversed phase C_8 column maintained at 80 °C. The mobile phase consisted of a linear gradient with two mobile phases containing acetonitrile and water. The flow rate was set at 0.8 ml/min. UV detection was carried out at 214 nm. Validation of the analytical method showed linearity over the range 25–1000 ng/ml ($r > 0.997$). The detection limits of cyclosporin A, AM1, AM9 and AM4N were 1.3 pmol on column. The within-day and between-day relative standard deviations were $< 15\%$ for cyclosporin A at all concentrations and for the metabolites at 250 and 1000 ng/ml, and $< 21\%$ for the metabolites at limit of quantification (25 ng/ml).

Keywords: Cyclosporin A; Metabolites; Reversed-phase chromatography; D-optimal design; Multivariate analysis

1. Introduction

The use of cyclosporin A (CsA) (INN, ciclosporin A) as an immunosuppressive agent in the treatment of transplanted patients has significantly increased graft survival, but is limited by its potentially nephrotoxic side effects which appear to be dose related [1–3]. A wide interindividual

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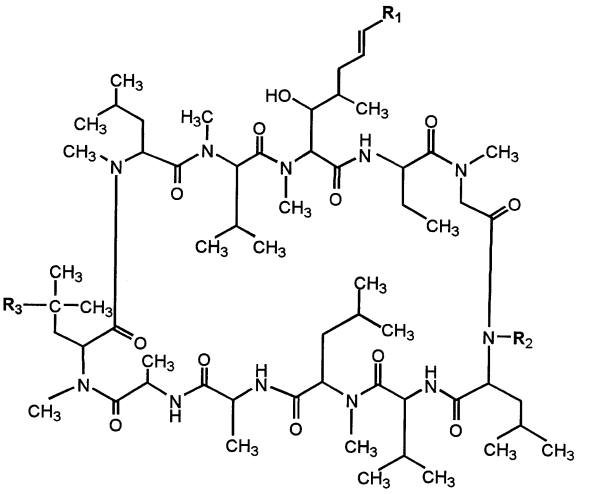
variability in the disposition of the drug has resulted in monitoring of CsA blood levels of the patients receiving CsA based immunosuppressive therapy to decrease the risk of underdosage and toxicity [4].

Immunoassay techniques are widely used in routine monitoring of CsA whole blood concentrations [5]. However, all immunoassay methods lack the specificity of chromatographic methods, and chromatographic determination is therefore the method of choice for the determination of CsA metabolites and also as the preferred reference method in the growing number of comparisons between the different immunoassay techniques [6]. This has resulted in numerous HPLC methods and a wide variety of conditions for analysing CsA.

The chemometric approach has previously been used to solve a variety of problems within analytical chemistry, mainly in sample preparation and optimisation of analytical methods [7]. This approach provides a tool to find an optimum of a selected response (or responses) or an adequate compromise in cases of conflict (for example baseline separation versus total time of analysis) from a limited number of experiments.

In the present study we investigated the importance of essential parameters in the chromatographic determination of CsA and its metabolites. The main challenge in the chromatographic determination of these compounds includes separation of the two metabolites AM1 and AM9, which only differ in the position of hydroxylation (Fig. 1). Also, the need for elevated temperatures in the separation of CsA and metabolites [8] adds to the number of parameters affecting the chromatographic determination of these compounds. A literature screening of previously published HPLC methods for the determination of CsA was conducted prior to selection of the variables to be investigated (Table 1) [9–25]. The aim of the study was to apply a D-optimal design to evaluate the importance of selected parameters in the chromatographic determination of CsA, AM1, AM9 and AM4N. The application of a chemometric approach allowed us to determine the optimal conditions of the chromatographic analysis of CsA and its metabolites.



	R_1	R_2	R_3
CsA	CH_3	CH_3	H
AM4N	CH_3	H	H
AM1	CH_2OH	CH_3	H
AM9	CH_3	CH_3	OH

Fig. 1. Chemical structure of CsA and the metabolites AM1, AM9, and AM4N.

2. Experimental

2.1. Chemicals and reagents

Novartis (Basel, Switzerland) kindly granted CsA, the metabolites AM1, AM9, AM4N and the internal standard cyclosporin C (CsC). Purity of the compounds was 98% or more (data sheet, Novartis). All other chemicals used were of analytical grade.

2.2. Preparation of standards

Standard solutions of CsA, CsC, AM1, AM9 and AM4N were prepared in methanol. EDTA drug free whole blood was obtained from Ullevål Hospital (Oslo, Norway). Whole blood samples were prepared by spiking drug free whole blood with aliquots of the standard solutions.

Table 1
Previously published HPLC methods for the determination of CsA selected from extensive literature screening

Reference	Analytes	Temperature (°C)	Mode of chromatography	Column	Mobile phase	Flow (ml/min)	Detection
[9]	CsA	72	Reversed phase gradient elution	C_8 125 × 3 mm 5 μm	H_2O:ACN:MeOH. A: 600:200:200 B: 50:750:200	1.5	UV 210 nm
[10]	CsA	50	Reversed phase isocratic elution	CN 30 × 4.6 mm	ACN:phosphate buffer (pH 7) 43:57	1	UV 210 nm
[11]	CsA	50	Normal phase isocratic elution	CN 150 × 4.6mm 3 μm	Hexane:isopropanol 85:15	0.6	UV 212 nm
[12]	CsA	70	Reversed phase isocratic elution	C_{18} 250 × 4 mm 5 μm	ACN:H_2O 71:29	1.2	UV 212 nm
[13]	CsA	70	Reversed phase Isocratic elution	C_{18} 40 × 3.2 mm 3 μm	ACN:MeOH:10mM phosphate (pH 6.5) 50:11:39	1.5	UV 220–230 nm
[14]	CsA AM9 AM1	NM	Reversed phase gradient elution	C_8 150 × 1 mm 3μm	A: H_2O:MeOH 80:20 B: ACN:MeOH 80:20	NM	MS
[15]	CsA	70	Reversed phase isocratic elution	C_{18} 50 × 4.6 mm 5 μm	ACN:THF:1% H_3PO_4 52:4:44	NM	UV 208 nm
[16]	CsA	75	Reversed phase isocratic elution	C_{18}	ACN:MeOH:H_2O 55:15:30	1	UV 210 nm
[17]	CsA AM19 AM1 AM9 AM1c AM4N	70	Reversed phase	C_8 250 × 4.6 mm	ACN:MeOH:H_2O 51:20:29	0.6	UV 214 nm
[18]	CsA	70	Reversed phase isocratic elution	C_{18}	ACN:H_2O 70:30	0.8	UV 210 nm
[19]	CsA	65	Reversed phase isocratic elution	C_8 200 × 4.6 mm	ACN:MeOH:H_2O:isopropanol 57:18:25:1.5	1.4	UV 208 nm

Table 1 (*Continued*)

Reference	Analytes	Temperature (°C)	Mode of chromatography	Column	Mobile phase	Flow (ml/min)	Detection
				7 μm			
[20]	CsA AM1	80	Reversed phase gradient and isocratic elution	C_{18} various types (comparative study)	Various mobile phases (comparative study)	1–2.5	UV 210 nm
[21]	CsA AM1 AM9 AM4N	50	Normal phase isocratic elution	CN 250 × 4.6 mm 5 μm	Hexane:isopropanol 90:10	1.45	UV 212 nm
[22]	CsA	70	Porous graphite phase isocratic elution	PGC 100 × 4.6 mm 5 μm	*t*-Butylether:MeOH 50:50	1.5	UV 206 nm
[23]	CsA	60	Reversed phase isocratic elution	C_1 50 × 4.6 mm 5 μm	ACN:MeOH:H_2O 20:45:35	1	UV 214 nm
[24]	CsA AM9 AM19 AM1 AM1c AM4N	70	Reversed phase isocratic elution	C_8 100 × 2.1 mm 5 μm	MeOH:NH_4Ac (pH 5.1) 72:28	0.3	MS/MS
[25]	CsA	70	Reversed phase isocratic elution	C_{18}	ACN:H_2O 70:30	1	UV 210 nm MS

Abbreviations: ACN, acetonitrile; MeOH, methanol; THF, tetrahydrofuran; NH_4Ac, ammonium acetate.

2.3. Instrumentation

Sample preparation was carried out by solid-phase extraction using C_{18} cartridges (1 ml sample volume, 100 mg sorbent), Bond Elut®, Varian (Harbor City, CA, USA). The cartridges were placed on a vacuum-manifold, Visiprep®24, Supelco (Sigma-Aldrich). The HPLC apparatus consisted of a quarternary pump, autosampler and column oven with preheater, all Series 200 LC, Perkin Elmer (Norwalk, CT, USA). Detection of the compounds was carried out on a 785A Programmable Absorbance Detector, Applied Biosystems with a deuterium lamp operated at 214 nm. Turbocrom®, Version 4.1 (Perkin Elmer) software was used to operate the HPLC system. Separation of the compounds was carried out on a C_8 analytical column (150×4.6 mm, 5 μm), Hypersil (Runcorn, UK).

2.4. Sample preparation

Blood samples were collected in EDTA-containing tubes and frozen (−20 °C). Sample preparation of whole blood samples was performed using a previously reported solid-phase extraction procedure [24]. All samples in the D-optimal design were drug-free blood samples spiked with CsA, AM1, AM9 and AM4N.

2.5. Chromatographic conditions

During the optimisation of the analytical separation a C_8-column was used. The mobile phase consisted of a linear gradient with final elution strength 2.08 (Eq. (I)). Composition and elution strength of the starting mobile phase, flow-rate, gradient parameters (steepness and length) and temperature were varied in the design. UV detection was carried out at 214 nm.

2.6. Validation of the final chromatographic method

The concentration of the compounds in whole blood samples was determined from peak heights using CsC as an internal standard. Linearity was tested in the range of 25–1000 ng/ml. Drug-free whole blood was spiked with CsA, AM1, AM9 and AM4N (25, 250 and 1000 ng/ml) ($n = 5$) for determination of accuracy and precision of within-day and between-day runs. Recovery was measured at 50 and 1000 ng/ml by comparing the response of extracted samples spiked before extraction with the response of extracted blank samples spiked immediately before injection. Recovery of CsC was determined using CsA as internal standard. The stability of the compounds was evaluated by repeated freeze–thaw cycles ($n = 3$) at 100 and 750 ng/ml, and the results were evaluated by ANOVA at a level of statistical significance of $P < 0.05$. Limit of detection was determined at a signal-to-noise ratio of 3/1 and limit of quantification at a signal-to-noise ratio of 10/1.

2.7. Experimental design

In cases were some of the factors can only be varied over a restricted area, and thereby create an irregular experimental domain in which orthogonality cannot be achieved, application of a D-optimal design would be appropriate [26]. In this case composition of the mobile phase was subject to such restrictions, and on this basis a D-optimal design was applied in this study. The amount and type of organic modifier in the mobile phase, temperature, flow-rate, pH of the mobile phase and steepness of gradient were included as variables in the D-optimal design. The settings of the design variables were determined from literature (type of organic modifier, temperature, flow rate and pH) (Table 1) and screening (amount of organic modifier and steepness of gradient) and are listed in Table 2. A total of six design variables were studied at two levels plus additional centre points, which due to restrictions in the composition of the mobile phase (Table 2) resulted in an initial irregular experimental matrix. The D-optimal strategy [26] was then applied to detect the best subset of experiments from the candidate points. This resulted in a final experimental matrix consisting of 38 experiments (Table 3), which was sufficient to study main effects ($p = 6$), all two-factor interactions ($p = 15$) and all square effects ($p = 6$) separately.

Table 2
Ranges and restrictions of variables for a D-optimal design

Variable	Lower limit	Upper limit
Acetonitrile	0%	65%[a]
Methanol	0%	80%[a]
Temperature	50 °C	80 °C
Flow	0.8 ml/min	2 ml/min
Hydrochloric acid	0 mM	10 mM
Gradient steepness	0.015 S_t/min	0.04 S_t/min
Restriction 1	0.026 (% methanol) + 0.032 (% acetonitrile) − 1.69 ≥ 0	
Restriction 2	−0.026 (% methanol) − 0.032 (% acetonitrile) + 2.08 ≥ 0	

[a] 80% methanol or 65% acetonitrile results in elution strength of 2.08 (Eq. (I)). S_t, solvent strength.

In this study both acetonitrile and methanol were selected as organic modifiers as they exhibit different selectivity. To correct for their difference in elution strength Eq. (I) was used to calculate the amount of each of the constituents in the mobile phase [27].

$$S_t = s_m \Phi_m + s_a \Phi_a + s_w \Phi_w \quad \text{(I)}$$

where S_t is solvent strength for the mixed solvent; s_x is solvent strength for methanol ($s_m = 2.6$), acetonitrile ($s_a = 3.2$) and water ($s_w = 0$); and Φ_x is volume fraction of methanol (m), acetonitrile (a) and water (w).

The components of the mobile phase cannot be varied independently as this would result in extremes where the obtained information from the experiment would be of little value. Thus, two restrictions concerning the composition of the mobile phase were made; the total elution strength of the mobile phase must be higher than 1.69 (i.e. 65% methanol or 53% acetonitrile) (restriction 1) and lower than 2.08 (i.e. 80% methanol or 65% acetonitrile) (restriction 2) as this will result in retention times for both CsA and the metabolites within a range that enables interpretation (Table 2). These ranges were pre-determined from screening. The remaining part of the mobile phase consisted of the aqueous component (containing 0, 5 or 10 mM hydrochloric acid).

Fixed conditions included stationary phase and injection volume. In the separation of CsA and its metabolites C_8, C_{18} and CN stationary phases are used (Table 1). Although good separation can be obtained with CN columns, this type of stationary phase appears to be less robust. From earlier work in our laboratory a large change in retention time and separation ability was seen between CN columns from the same supplier. Stationary phases consisting of polystyrene–divinylbenzene give high efficiency and low tailing. However, the lack of polar groups makes this type of stationary phase unsuitable for the separation of AM1 and AM9 (data not shown). To separate the metabolites AM1 and AM9, the accessibility of free silanol groups in the stationary phase is of principal importance, and on this basis silica based reversed phase stationary phase is preferred for the separation of the compounds in this study. C_8 was selected on the basis of its less hydrophobic properties compared to C_{18}, resulting in a reduction in the consumption of organic modifier. In addition, the free silanol groups are better accessible on the C_8 stationary phase and a better separation of AM1 and AM9 can therefore be achieved. The injection volume was set at 200 μl.

Resolution between AM1 and AM9, retention time of AM1/AM9 and retention time of CsA were selected as response variables. As the importance of the response variables differed, the following priority of the response variables was made in cases of conflict and where a compromise had to be made: (1) resolution of AM1 and AM9, (2) retention time of AM1/AM9, and (3) retention time of CsA.

2.8. Multivariate analysis

Multi-linear regression (MLR) was used to evaluate the effects of the selected variables after the experiments were performed. Both MLR and PLS2 models were developed, but MLR was preferred above PLS2 because of its lower error of prediction (RMSEP) and better R^2 and Q^2 for all response parameters for this data set. The observed effects were tested for significance using ANOVA. Non-significant regression coefficients ($\alpha > 0.05$) were excluded from the models. The Unscrambler® 7.11 (CAMO ASA, Oslo, Norway) was used for experimental design, statistical evaluation and response surface plots.

Table 3
The experiments in the D-optimal design

	MeOH (%)	ACN (%)	Temperature (°C)	Flow (ml/min)	HCl (mM)	Gradient (S_t/min)
1	0	65	50	0.80	0	0.015
2	80	0	50	2.00	0	0.015
3	80	0	50	0.80	10	0.015
4	0	65	50	2.00	10	0.015
5	80	0	80	2.00	10	0.015
6	80	0	50	2.00	0	0.04
7	0	65	50	0.80	10	0.04
8	0	52.81	50	0.80	10	0.015
9	65	0	50	2.00	10	0.015
10	0	52.81	80	0.80	0	0.04
11	65	0	80	2.00	0	0.04
12	0	52.81	50	2.00	10	0.04
13	0	52.81	50	0.80	5	0.04
14	0	52.81	50	1.40	0	0.015
15	0	58.91	80	0.80	0	0.015
16	0	65	80	0.80	10	0.0275
17	0	52.81	80	2.00	10	0.0275
18	0	65	80	2.00	5	0.04
19	0	65	80	1.40	10	0.015
20	0	52.81	65	2.00	0	0.015
21	72.5	0	50	0.80	0	0.015
22	65	0	50	0.80	0	0.0275
23	80	0	50	1.40	10	0.04
24	80	0	80	0.80	0	0.0275
25	65	0	80	0.80	5	0.015
26	80	0	80	0.80	5	0.04
27	65	0	65	0.80	10	0.04
28	40	32.5	50	0.80	0	0.04
29	32.5	26.41	50	2.00	0	0.04
30	40	32.5	80	2.00	0	0.015
31	0	58.91	50	2.00	0	0.0275
32	0	65	65	1.40	0	0.04
33	72.5	0	65	0.80	0	0.04
34	36.25	29.45	80	0.80	10	0.04
35	40	32.5	65	2.00	10	0.04
36	36.25	29.45	65	1.40	5	0.0275
37	36.25	29.45	65	1.40	5	0.0275
38	36.25	29.45	65	1.40	5	0.0275

3. Results

For each of the response parameters a second-order regression model was developed. A summary of the statistical evaluation is shown in Table 4. No quadratic or interaction terms were of significance to any of the response variables (Table 4). The MLR analysis gave $R^2 > 0.850$ and $Q^2 > 0.776$ for t_R of AM1/AM9 and t_R of CsA. For the response parameter resolution of AM1/AM9 R^2 and Q^2 were 0.500 and 0.269, respectively (Table 4). All models were highly significant ($P < 0.001$) (Table 4). The relatively low values of R^2 and Q^2 for the resolution of AM1/AM9 are probably caused by increased noise in these data. The determination of the resolution factor was subject to inaccuracy, as it was difficult to carry out an accurate measurement due to overlapping peaks in some of the chromatograms. All parameters, except for addition of hydrochloric acid, were

Table 4
Predicted equations for each response variable

Response variable	Equation	R^2	Q^2	p_{reg}
t_R CsA	$Y = b_0 - 0.83x_1 - 0.67x_2 - 0.068x_3 - 4.9x_4 - 91.9x_5$	0.881	0.820	< 0.0001
t_R AM1/AM9	$Y = b_0 - 0.48x_1 - 0.37x_2 - 3.3x_4$	0.850	0.776	< 0.0001
R_s AM1/AM9	$Y = 1.49 - 0.042x_1 - 0.031x_2 + 0.024x_3$	0.500	0.269	< 0.001

R^2, the fraction of variation of the response explained by the model; Q^2, the fraction of variation of the response predicted by the model; p_{reg}, the p-values for regression; x_1, concentration of acetonitrile; x_2, concentration of methanol; x_3, temperature; x_4, flow; x_5, gradient steepness.

found to be of significance to one or more response variables (Table 4).

3.1. Resolution of AM1 and AM9

A resolution factor (R_s) of 1.5 is favourable as this means complete baseline separation of the compounds. The temperature is the single most important factor affecting the separation of the metabolites AM1 and AM9 (Table 4). Increasing the temperature leads to increased R_s-value as the peaks become narrower. Furthermore, from the response surface plot (Fig. 2) showing R_s values of AM1 and AM9 as a function of acetonitrile and methanol concentrations, it is clear that low amount of organic modifier also gives high R_s values. Along the line of 52.8% acetonitrile–65%methanol (elution strength constant) of the response–surface plot R_s has acceptable values.

3.2. Retention time of AM1/AM9

The retention time of AM1/AM9 should be larger than the system delay time (4.5 min in the case of 1 ml/min flow) for the compounds to be eluted on the gradient. The retention time of AM1/AM9 is significantly affected by the amount of organic solvent at the start of the gradient and flow rate (Table 4). The retention time of AM1/AM9 increases when the amount of organic modifier (methanol and/or acetonitrile) and/or the flow rate are decreased. From the response surface plot of the retention time of AM1/AM9 as a function of acetonitrile concentration and flow (Fig. 3), it is clear that the acetonitrile concentration should not exceed 64% when analysing at low flow rates (0.8 ml/min), and 56% when analysing at high flow rates (2.0 ml/min), to achieve a retention volume larger than system delay volume (4.5 ml).

3.3. Retention time of CsA

The retention time of CsA is significantly affected by the same factors as the retention time of AM1/AM9; amount of organic modifier at the start of the gradient and flow rate. In addition, gradient steepness and temperature also significantly affect the retention time of CsA (Table 4).

3.4. Final chromatographic conditions

The optimal chromatographic conditions determined from the D-optimal design consisted of a mobile phase containing acetonitrile and water delivered as a linear gradient of 0.04 S_t/min, with starting and final concentrations of acetonitrile of 52.8 and 73%, respectively. The flow was set at 0.8 ml/min.

3.5. Validation of the final chromatographic method

The analytical method with optimal conditions found with the D-optimal design was validated. Validation of the analytical method showed linearity over the range 25–1000 ng/ml ($r > 0.997$) (Table 5). The intercept was not statistically different from zero for any of the calibration curves ($n = 5$). The detection limits (LOD) and limit of quantification (LOQ) of CsA, AM1, AM9 and AM4N were 8 and 25 ng/ml, respectively. Within- and between-day RSD values were $< 15\%$ for CsA at all concentrations, and for the meta-

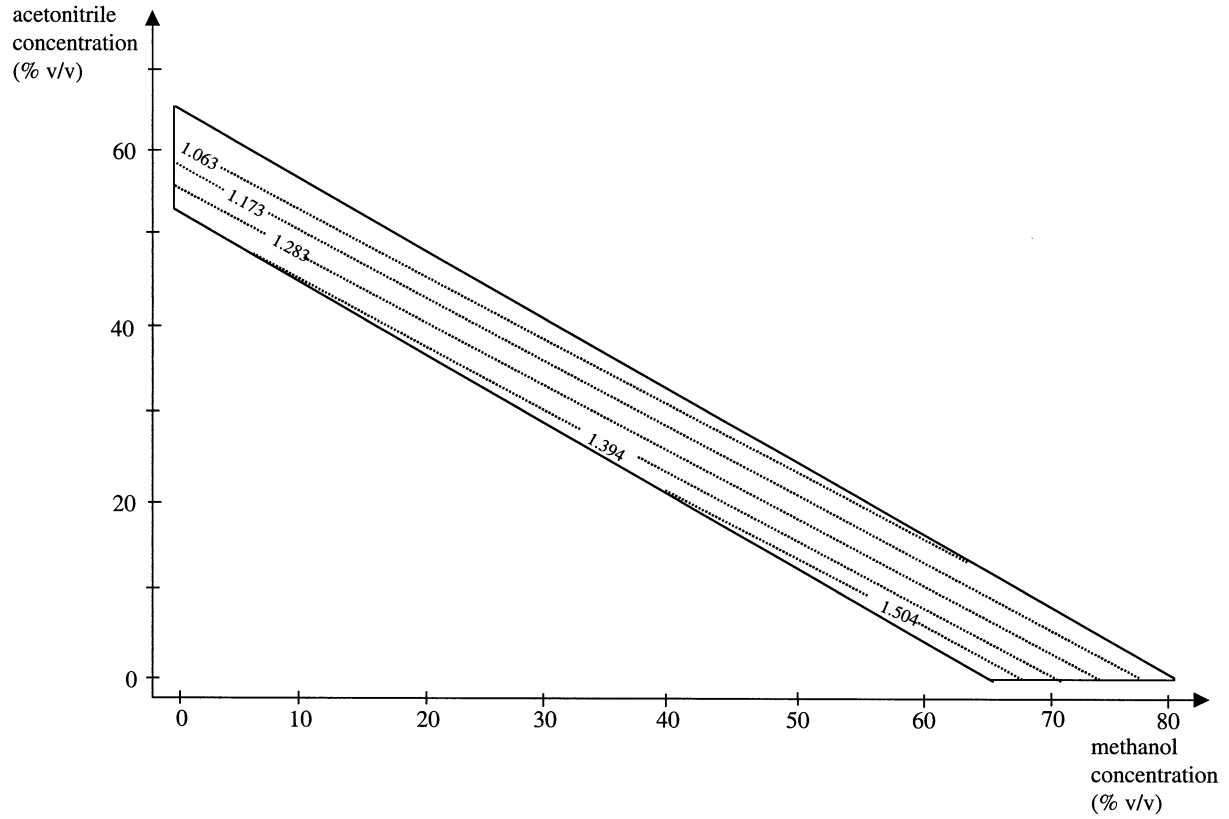


Fig. 2. Response surface plot showing R_s values of the separation of AM1 and AM9 as a function of acetonitrile and methanol concentrations. Temperature 80 °C, flow 0.8 ml/min, gradient steepness 0.04 S_t/min and no addition of hydrochloric acid (for restrictions in the experimental domain, see Table 2 and text).

bolites at 250 and 1000 ng/ml (Tables 6 and 7). At LOQ the within- and between-day RSD values were $< 21\%$ for the metabolites. Bias was $< 20\%$ for all compounds except for AM9 at LOQ (24.4%) (Tables 6 and 7). Recovery was highly reproducible, and 73.7% (RSD < 5.7) for CsA and more than 50% (RSD < 7.8) for all metabolites at both concentrations. Recovery of CsC was 80.4% (RSD < 2.1). The specificity of the method was tested by analysing blank whole blood from five individuals and no significant interference was shown at the retention times of the compounds of interest (Fig. 5). The stability of the compounds was not affected by repeated freeze–thaw cycles.

4. Discussion

To meet all of the criteria of a good chromatographic separation of CsA and its main metabolites the optimal chromatographic conditions should be determined. In cases of conflict between the selected response parameters optimal conditions for the more important response parameter (i.e. separation of AM1 and AM9) was selected.

Both the organic modifiers in this study showed satisfactory effect on the resolution of AM1 and AM9 and could be used in the chromatographic determination of CsA and metabolites. However, acetonitrile was preferred, as it seemed to show

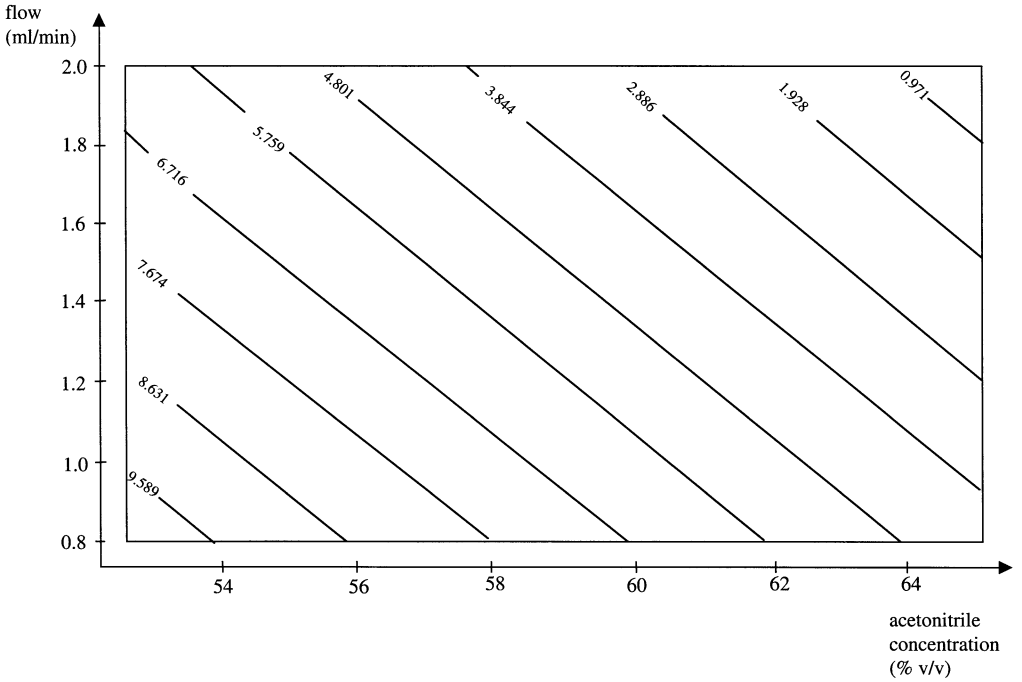


Fig. 3. Response surface plot showing the retention time of AM1/AM9 as a function of acetonitrile concentration and flow. Mobile phase consisting only of acetonitrile and water with no addition of methanol or hydrochloric acid, temperature 80 °C and gradient steepness 0.04 S_t/min (for restrictions in the experimental domain, see Table 2 and text).

Table 5
Standard calibration curves of CsA, AM1, AM9 and AM4N ($n = 5$)

	r	a (SE)	b
CsA	0.998	0.0072 (0.00025)	0.058
AM1	0.998	0.0079 (0.00037)	−0.00055
AM9	0.997	0.0067 (0.00055)	−0.054
AM4N	0.998	0.0069 (0.00063)	0.035

r, correlation coefficient; a, slope; b, intercept.

slightly better effect on the separation of the two metabolites, on the basis of visual inspection of the chromatograms. In addition, a low starting concentration of organic modifier in the mobile phase was needed to separate AM1 and AM9 (Fig. 2). R_s of M1 and M9 did not increase with a combination of the two organic modifiers compared to either acetonitrile of methanol alone.

Addition of hydrochloric acid did not significantly affect any of the response parameters selected in this study. Thus, pH adjustments seem to be of no importance in the chromatographic determination of these compounds. The final method consisting of a mobile phase not containing hydrochloric acid or other pH adjusting compounds is a simplification compared to many of the existing methods (Table 1).

Elevated temperature has previously been shown to be of principal importance in the chromatographic determination of CsA [8,28], and we are not aware of any reports on the separation of CsA and its metabolites at room temperature. This was also shown in this study where the temperature was the single most important factor affecting the separation of AM1 and AM9 (Table 4). Increasing the temperature probably leads to a decrease in the contribution of mass-transport in mobile phase and stationary phase to band broadening. In addition, it has been suggested that CsA undergoes temperature-related conformational changes, possibly related to intramolecular hydrogen-bonding, that could alter solubility and contribute to the narrow peak

Table 6
Intra-assay variation in the determination of CsA, AM1, AM9 and AM4N in whole blood samples prepared by SPE, expressed as mean ± SD and relative standard deviation (RSD) ($n = 5$)

Analyte	Concentration added (ng/ml)	Measured concentration (mean ± SD) (ng/ml)	RSD (%)	Bias (%)
CsA	25	22.4 ± 1.27	5.7	−10.3
	250	289 ± 9.03	3.1	+15.6
	1000	935 ± 63.8	6.8	−6.5
AM1	25	21.9 ± 4.55	20.8	−12.5
	250	256 ± 7.90	3.1	+2.4
	1000	956 ± 40.2	4.2	−4.4
AM9	25	31.1 ± 4.10	13.2	+24.4
	250	274 ± 19.2	7.0	+9.6
	1000	867 ± 60.1	6.9	−13.3
AM4N	25	20.2 ± 1.57	7.8	−19.2
	250	267 ± 6.15	2.3	+6.8
	1000	916 ± 73.6	8.0	−8.4

shape and high resolution observed with increasing temperatures [8]. On the basis of their similar structure this would also most likely be the case for the metabolites. Increasing the temperature also gives a shorter retention time of CsA, probably due to decreased viscosity and the increased solubility of CsA in the mobile phase. The effect of increased temperature is, however, not of significant influence on the retention time of AM1/AM9, possibly because the total retention time of the metabolites is relatively short.

High gradient steepness only affected the retention time of CsA (Table 4), which is not strictly limited to a certain length, as long as the sample preparation is done off-line. High gradient steepness was anyhow selected to limit the total time of analysis.

Although the flow rate significantly affected the retention time, it did not have a significant influence on the resolution of AM1 and AM9 (Table 4). Both the lowest (0.8 ml/min) and the highest flow rate investigated (2.0 ml/min) (other conditions optimal, i.e. no addition of hydrochloric acid, 80 °C, gradient steepness 0.04 S_t/min, starting concentration of acetonitrile 52.8%) give acceptable results for resolution and retention time of AM1/AM9 (Fig. 4A and B). The former experiment was carried out as part of the original

Table 7
Inter-assay variation in the determination of CsA, AM1, AM9 and AM4N in whole blood samples prepared by SPE, expressed as mean ± SD and relative standard deviation (RSD) ($n = 5$)

Analyte	Concentration added (ng/ml)	Measured concentration (mean ± SD) (ng/ml)	RSD (%)	Bias (%)
CsA	25	20.6 ± 2.01	9.8	−17.6
	250	258 ± 29.1	11.3	+3.2
	1000	1003 ± 0.27	3.5	+0.3
AM1	25	20.7 ± 2.52	12.2	−17.2
	250	267 ± 31.9	11.9	+6.8
	1000	1082 ± 116.1	11.7	+8.2
AM9	25	28.1 ± 4.83	17.2	+12.4
	250	271 ± 32.5	12.0	+8.6
	1000	1040 ± 145.4	14.0	+4.0
AM4N	25	21.3 ± 2.01	19.2	−14.7
	250	269 ± 24.7	9.2	+7.5
	1000	1029 ± 96.5	7.1	+2.9

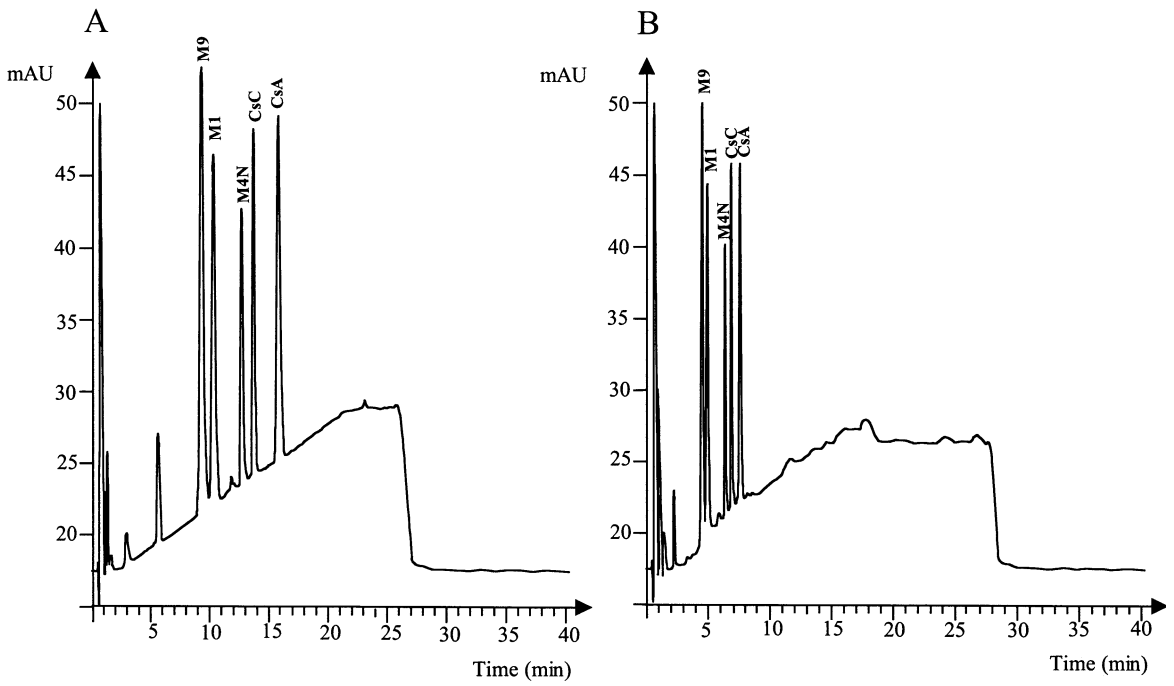


Fig. 4. Chromatograms of drug-free human whole blood samples spiked with 500 ng/ml of CsA and the metabolites AM1, AM9 and AM4N, with a flow rate of (A) 0.8 ml/min and (B) 2.0 ml/min, other experimental conditions identical. For entire chromatographic conditions, see text.

D-optimal design, while the latter was carried out after evaluating the data. The retention time of CsA at a flow rate of 0.8 and 2.0 ml/min (other conditions optimal) is approximately 16 and 9 min, and the retention volumes at the same flow rates are 12.8 and 18 ml, respectively. Although high flow rates give lower retention times it also causes a larger consumption of organic modifier (approximately 30%) compared to analysing at low flow rates. In addition, low flow rates give a better separation from the front. This could be of principal importance in the determination of CsA and metabolites in samples from patients undergoing multiple drug therapy. However, if a high throughput is of major importance, high flow rates will be suitable, but when this is not the case, a low flow rate will be preferred.

Selection of a flow rate of 0.8 ml/min results in a total assay turn-around time of 30 min. However, the assay turn-around time could be significantly decreased if CsA was the only substance of interest. The experimental design approach gives sufficient information to modify the method with regard to this subject without any further experiments. Selection of different response variables, in this case solely retention time of CsA, enables determination of optimal conditions for analysing CsA, excluding the metabolites.

5. Conclusion

A D-optimal design was used to evaluate importance of selected parameters (amount and type of organic modifier in the mobile phase, temperature, flow rate, pH of the mobile phase and steepness of gradient) on the chromatographic determination of CsA and the metabolites AM1, AM9 and AM4N. The chemometric approach allowed us to determine the optimal conditions of this HPLC separation, given a number of selected response variables. pH adjustment of the mobile phase, by addition of hydrochloric acid, was shown not to influence on the separation or

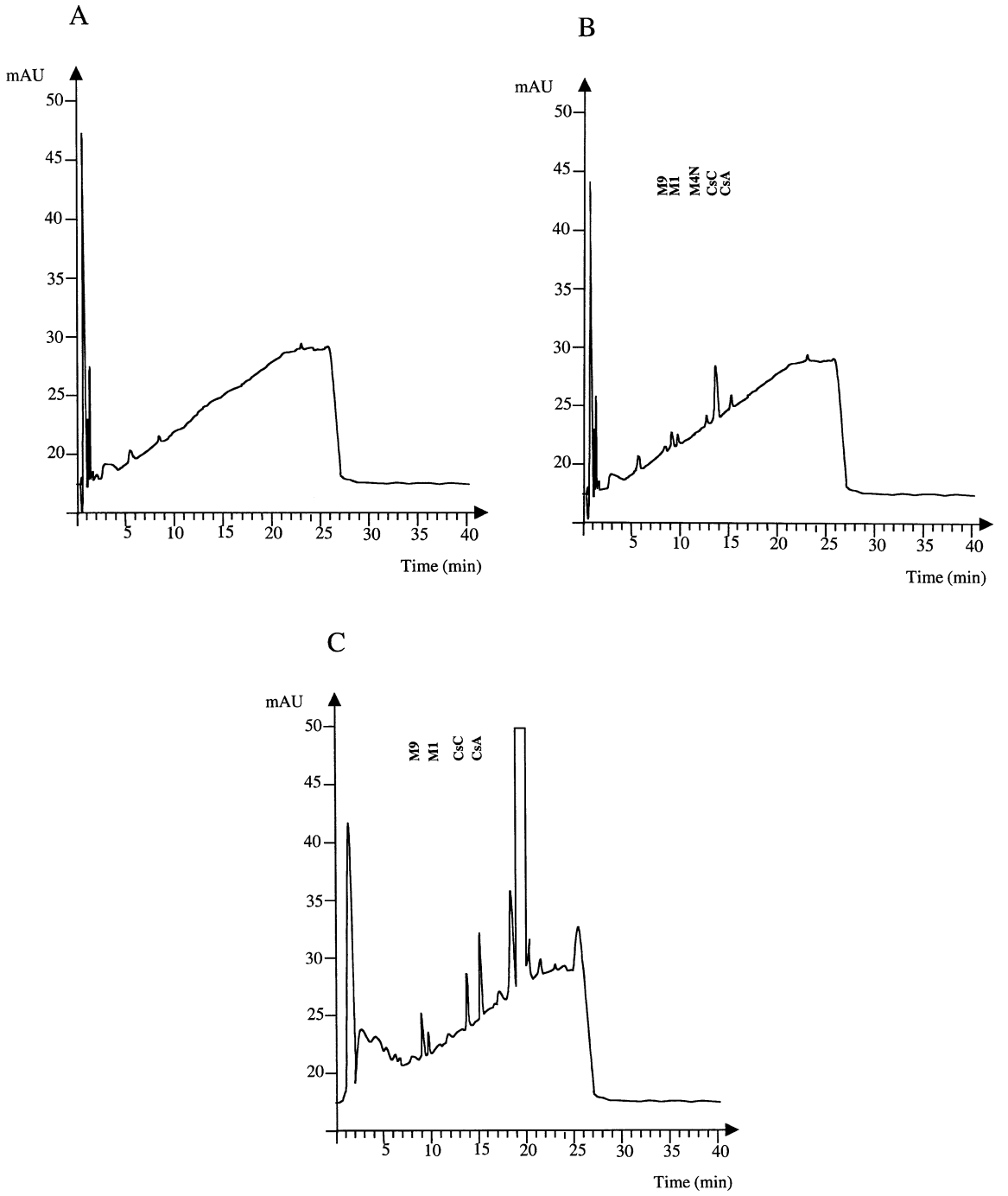


Fig. 5. Chromatograms of (A) drug-free human whole blood, (B) drug-free human whole blood spiked with CsA, AM1, AM9 and AM4N at LOQ (25 ng/ml) and (C) patient sample from a renal transplant recipient on CsA based immunosuppressive therapy, receiving 250 mg CsA (Sandimmun Neoral®) daily. The level of M4N is below LOQ in this sample (for chromatographic conditions, see text).

retention times of any of the compounds. All of the other design variables significantly affected one or more of the response variables.

The final method has been validated and was shown to be an accurate, precise and selective method for the determination of CsA and metabolites. The method has been used in the analysis of more than 500 blood samples from renal transplanted patients undergoing CsA therapy (Fig. 5C) and no significant interfering peaks have been observed despite the multiple drug therapy of these patients. The method described is currently being used in our laboratory for the analysis of patient samples from pharmacokinetical drug interaction studies and could also be used in routine monitoring of patient undergoing CsA therapy.

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