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Evaluation of a cyano stationary phase for the determination of tacrolimus, sirolimus and cyclosporin A in whole blood by high-performance liquid chromatography–tandem mass spectrometry

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Abstract

The potential of a cyano HPLC column for the analysis of three immunosuppressants is investigated. Tacrolimus, sirolimus and cyclosporin A, were used to probe differences in the retention and efficiency of a cyano column compared to the more widely used C_{18} column. The cyano column showed comparable retention for all three compounds, whereas the C_{18} column showed stronger retention, especially for cyclosporin A. Furthermore, the efficiencies at 50 °C were up to 12 times higher on the cyano column. As a result, a baseline separation was achieved in less than three minutes with the cyano column, using an isocratic mobile phase of 52/48 (v/v) acetonitrile/water at 0.45 mL/min. The analysis of immunosuppressant drugs in human whole blood was performed with the cyano column using a selected reaction monitoring (SRM) method for each analyte with negative ion mode electrospray ionization on a triple quadrupole mass spectrometer. Detection limits were 0.05 ng/mL for sirolimus, 0.1 ng/mL for cyclosporin A and 0.2 ng/mL for tacrolimus. Calibration curves were linear over three orders of magnitude. Good agreement was obtained with the actual levels of immunosuppressant drugs in patient samples with an average error of less than 10%.
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Keywords: Immunosuppressants; Cyano column; Negative ions

1. Introduction

The success of organ transplantation is highly dependent on the dose of immunosuppressant drugs administered to the patient. In turn, proper dosing requires rapid and reliable quantification of the immunosuppressants present in the patient's blood. The primary reasons for this are a narrow therapeutic range, variations in intra- and inter-individual pharmacokinetics and the lack of a reliable correlation between dose and drug exposure [1–3]. Consequently, the dose of immunosuppressants needs to be tailored to the individual patient, necessitating therapeutic drug monitoring.

Therapeutic drug monitoring has been traditionally performed with immunoassays, primarily due to their ease of use and speed of analysis [4,5]. Immunoassays often produce erroneous results, however, as a result of cross-reactivity with inactive metabolites of the parent drug. High-performance liquid chromatography (HPLC) has been advocated as a highly selective method for the analysis of immunosuppressants, particularly when combined with mass spectrometric detection (LC–MS) [1–4,6,7]. This does not, however, mean that LC–MS analyses are not without pitfalls [8–11]. Most notably, there exists a common perception that the use of mass spectrometric detection, and in particular, tandem mass spectrometry guarantees the specificity of a particular analysis. As a result, the separation step in many bioanalytical assays is compromised, particularly in laboratories that are in need of high sample throughput. This practice can often lead to errors in quantification due to ion suppression or enhancement, and can have profound consequences in hospital laboratories that routinely analyze patient samples. Clearly, the speed and selectivity of mass spectrometry is greatly complemented by rapid separations that achieve the resolution

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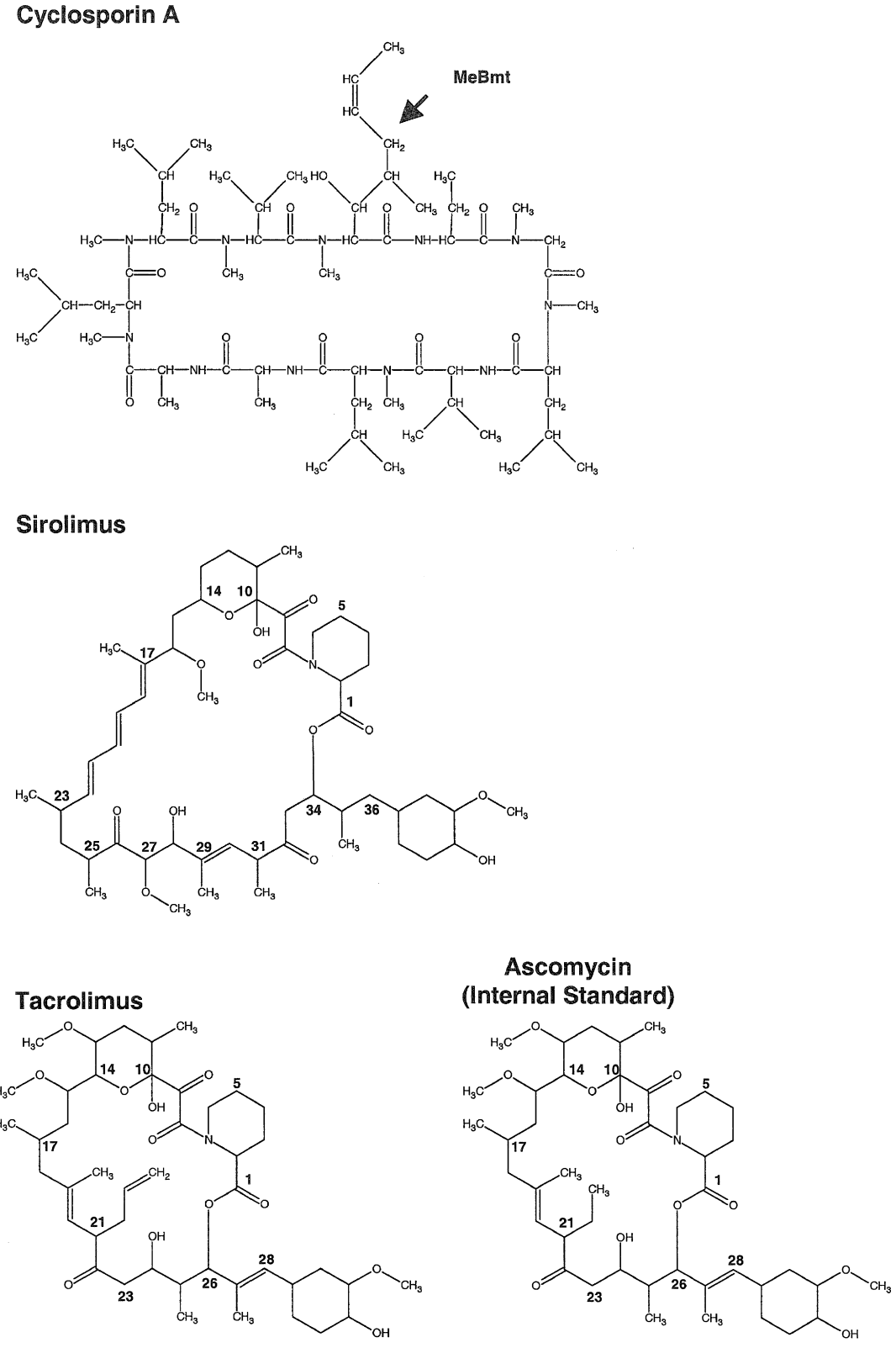

Fig. 1. Structures of cyclosporin A, sirolimus, tacrolimus and ascomycin. MeBmt (4R-[(E)-2-butenyl)]-4, *N*-dimethyl-L-threonine).

of all analytes from each other, and from interfering matrix components.

The purpose of this study is to examine the HPLC separation of three immunosuppressant drugs in detail. In particular, a change in column selectivity, through the use of a cyano HPLC column rather than the more commonly used C_8 or C_{18} columns, is evaluated for the baseline separation and rapid analysis of tacrolimus, sirolimus and cyclosporin A. Their structures are shown in Fig. 1. These immunosuppressants can be administered separately or in combination to an organ-transplant patient. The potential exists for a synergistic immunosuppressive effect when sirolimus is used in combination with tacrolimus or cyclosporin A [7,12,13]. This stresses the need for methodology that can assay all three drugs simultaneously. Previously, Keevil et al. [14] and Khoschsorur et al. [15] have employed cyano columns for the analysis of cyclosporin A, however, no detailed investigation into the retention and efficiency of cyano columns was made. Finally, after optimization of the mass spectrometric parameters is presented, the applicability of a cyano column for the analysis of whole blood samples containing tacrolimus, sirolimus and/or cyclosporin A is examined.

2. Experimental

2.1. Materials

Tacrolimus, sirolimus and cyclosporin A were purchased with a minimum purity of 98% from LC Laboratories (Woburn, MA, USA). Ascomycin was used as the internal standard for this work and was purchased with 95% purity from Alexis Biochemicals (Lausen, Switzerland). Uracil (98% purity, Sigma–Aldrich, Oakville, ON, Canada) was the void time marker for the HPLC columns used in this study. Formic acid (96% ACS Reagent) was obtained from Sigma–Aldrich and glacial acetic acid from Caledon (Georgetown, ON, Canada). Acetonitrile (HPLC grade, Caledon), methanol (HPLC grade, Caledon) and Milli-Q organic free water (Millipore, Bedford, MA, USA) were used as HPLC solvents and for all solutions. Pre-analyzed blood samples from patients who were administered immunosuppressant drugs were obtained from the Queen Elizabeth II Health Sciences Centre (Halifax, NS, Canada). Samples were identified with a coded number and were completely anonymous.

2.2. Liquid chromatography

High-performance liquid chromatography (HPLC) was performed using an Agilent 1100 (Palo Alto, CA, USA) binary liquid chromatography system. HPLC separations were carried out on either an Atlantis^{TM} C_{18} column (150 mm × 2.0 mm i.d.) with 3 μm particle size (Waters, Milford, MA, USA) or a YMC^{TM} CN column (150 mm × 2.0 mm i.d.) with 3 μm particle size (Waters). Separations were performed isocratically using acetonitrile/water mobile phases at a flow rate of 0.45 mL/min and an injection volume of 20 μL, unless otherwise specified. The column compartment of the Agilent 1100, in conjunction with the mobile phase pre-heater, was used to maintain the column temperature at 50 °C. Mobile phases contained 0.1% acetic acid (negative ion mode) or 0.1% formic acid (positive ion mode) to enhance electrospray response [16,17]. The overall chromatographic run time was approximately 4 min, including 1 min for the injection.

2.3. Mass spectrometry

Experiments were performed on an MDS Sciex (Concord, Ontario, Canada) API 4000 triple quadrupole mass spectrometer with electrospray ionization. Experiments were conducted using a selected reaction monitoring (SRM) method for each analyte in either positive or negative ion mode; however, most experiments presented used the negative ion mode. Data was acquired with Analyst 1.3.1 software (MDS Sciex). The mass spectrometer was operated at a spray voltage of −4200 V and a declustering potential of 80 V. The Turbo-V heat injectors were heated to 350 °C. Nitrogen was used as the collision gas with a CAD gas setting of 12 (arbitrary units) and at a collision-offset voltage of −40 V. The SRM transitions for each analyte in positive ion mode were: cyclosporin A $1225^+/1114^+$, sirolimus $937^+/409^+$ and tacrolimus $827^+/616^+$. The SRM transitions for each analyte in negative ion mode were: cyclosporin A $1201^-/1089^-$, sirolimus $913^-/591^-$ and tacrolimus $803^-/561^-$. All SRM transitions used a 150 ms dwell time, a 5 ms pause, low resolution for Q1 and high resolution for Q3. Product ion scans were acquired from *m/z* 200 up to the precursor ion mass in 2 s using unit resolution on Q1 and Q3.

2.4. Sample preparation

A 1 mg/mL stock solution of each immunosuppressant was prepared in methanol and dilutions were made as needed. Standards for calibration curves were prepared in human whole blood containing 50 ng/mL ascomycin as internal standard. Sample cleanup consisted of protein precipitation of 100 μL of blood with 300 μL of acetonitrile. Samples were then vortexed and centrifuged at 14000 × *g* for 10 min. One hundred microlitres of the supernatant were evaporated using a Pierce Reacti–Therm Heating Module (Pierce, Rockford, IL, USA) and were reconstituted in 100 μL of mobile phase. The linear range of calibration curves was assessed from 1000 to 1 ng/mL. Calibration curves used for quantification were prepared over smaller ranges, e.g., tacrolimus and sirolimus used standards at 0, 1, 3, 6, 9, 12, 15, 20, 25, 35, and 50 ng/mL and cyclosporin A used standards at 0, 50, 150, 300, 450, 600, 750, 1000, 1750, and 2500 ng/mL. Analyses were performed in triplicate.

2.5. Ion suppression/enhancement

The possibility for ion suppression/enhancement with our methodology was measured using a method described in the literature [8,18,19]. Briefly, a 5 ng/mL solution of tacrolimus, sirolimus and cyclosporin A was infused post-column through an UpChurch zero dead volume tee (UpChurch Scientific, Oak Harbor, WA, USA) using a Cole Parmer 74900 Series syringe pump (Anjou, QC, Canada). Blank protein-precipitated blood samples were then injected (20 μL) onto the analytical column. Effluent from the HPLC column combined with the infused analytes and entered the electrospray source. The resulting "chromatogram" was monitored for any deviations from baseline, which would indicate ion suppression (negative deviation) or enhancement (positive deviation).

2.6. Data analysis

Data analysis was performed using Microsoft Excel 2000 software (Microsoft Corporation, Seattle, WA, USA). The *Regression* function in the *Data Analysis Tool Pak* was used to obtain coefficients for the linear regressions performed in this work. Calibration curves were prepared by plotting the ratio of the area of the analyte peak to the area of the internal standard peak against the concentration of the analyte. The effect of organic modifier on retention was studied by plotting the logarithm of the retention factor against the fraction of organic modifier in the mobile phase, according to the equation:

$$\log k = \log\left(\frac{t_r - t_o}{t_o}\right) = X + S\Phi \quad (1)$$

where k is the retention factor, t_r the retention time, t_o the void time of the column, X and S the constants and Φ the fraction of organic modifier in the mobile phase [20]. Retention times for severely tailing peaks were determined by calculating the peak's first moment using the following formula:

$$\text{First moment} = \frac{1}{A}\int_0^\infty tC\,dt \quad (2)$$

where A is the area of the peak (zeroth moment), t time and C the concentration at any time t [21]. In using Eq. (2), it was assumed that the detector response at time t is equivalent to C. Peak efficiencies were calculated with the equation:

$$N = \frac{41.7(t_r/w_{0.1})^2}{A/B + 1.25} \quad (3)$$

where N is the efficiency, t_r retention time, $w_{0.1}$ the width at 10% height and A/B the asymmetry factor for the peak [22]. Use of this equation takes into account asymmetry in the peak.

3. Results and discussion

3.1. Retention on CN compared to C_{18}

Most liquid chromatography–mass spectrometry (LC–MS) methods for the analysis of tacrolimus, sirolimus and/or cyclosporin A in blood employ a C_8 or C_{18} silica stationary phase [1,2,4,6,7,23,24]. These methods are highly robust and have found wide range applicability in hospital labs around the world. However, there are a couple of disadvantages inherent with the use of C_{18} columns for the analysis of these drugs. Firstly, column temperatures of at least 60 °C have to be employed with C_{18} or C_8 stationary phases in order to avoid excessive band broadening of chromatographic peaks. This band broadening stems from the partial separation of the conformers of individual immunosuppressants on HPLC stationary phases [1,25–27]. Such high column temperatures lead to a significant reduction in the lifetime of silica-based columns due to the instability of silica at elevated temperatures [28]. One way to circumvent this problem would be to use ultra-stable zirconia phases, which have been routinely used at temperatures as high as 150 °C [28]. Secondly, C_{18} stationary phases strongly retain tacrolimus, sirolimus and cyclosporin A necessitating gradient elution. However, the time necessary for column re-equilibration (usually 10 column volumes of mobile phase) can add to the sample turnaround time and negatively impact on sample throughput. These observations prompted us to search for an improved LC–MS method for tacrolimus, sirolimus and cyclosporin A through a change in column selectivity. Thus, we studied a cyano (CN) stationary phase for the separation of these drugs. It should be stressed here that we chose columns of identical particle size and dimensions from the same manufacturer so as to render any comparison between CN and C_{18} stationary phases as objective as possible.

Fig. 2 shows the effect of organic modifier on the retention of tacrolimus, sirolimus and cyclosporin A on a CN and C_{18} column. Linear regression analysis was performed based on Eq. (1). Significant linear relationships were obtained for all three immunosuppressants at the 95% confidence level (n = 5, $r^2 > 0.98$). Clearly, all immunosuppressants are more strongly retained on the C_{18} column than on the CN column. Furthermore, Fig. 2 clearly shows that the retention of the C_{18} column is substantially different for cyclosporin A than for either tacrolimus or sirolimus. A separation with baseline resolution of all three drugs would require gradient elution so that cyclosporin A is not excessively retained on the column. However, the CN column does not show radically different retention for cyclosporin A compared to either tacrolimus or sirolimus. Therefore, it is possible to resolve the three immunosuppressant drugs without the use of gradient elution as is shown in Fig. 3. This demonstrates one advantage of the CN column over the C_{18} column for the analysis of tacrolimus, sirolimus and cyclosporin A. Ascomycin, the internal standard used for quantification, elutes on the shoulder

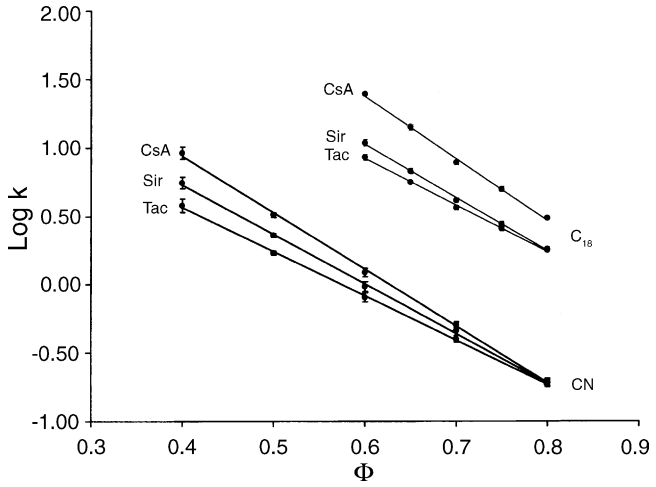

Fig. 2. Retention on the C_{18} and CN column as a function of the fraction of organic modifier in the mobile phase. Experimental conditions: acetonitrile/water mobile phases, 20 μL injection, 0.45 mL/min, and 50 °C.

of the tacrolimus peak as shown by the arrow in Fig. 3. Baseline separation of ascomycin from tacrolimus could not be achieved since their structures are almost identical.

The effect of temperature on retention was studied for the CN and C_{18} columns by means of van't Hoff plots [29]. The retention of all immunosuppressants decreased with increasing temperature on both columns. Furthermore, no significant selectivity changes were observed as a function of temperature on either column. This is usually the case in reversed-phase liquid chromatography [29].

Fig. 3 demonstrates the separation of tacrolimus, sirolimus and cyclosporin A in less than three minutes. There are two reasons for the short separation time. Firstly, the organic modifier and temperature for the separation were optimized to obtain baseline separation in a minimum amount of time. Secondly, the drop in mobile phase viscosity at elevated temperature was exploited to increase the flow rate of the separation as much as possible [30], while still maintaining an operating pressure below 200 bar. Although high-throughput methods exist in the literature [31,32], to the best of our knowledge, such a rapid, high-resolution separation of immunosuppressant drugs is unprecedented. An interesting extension to our methodology, however, would be the use of on-line solid phase extraction for enhanced sample cleanup and completely automated analysis [1,32].

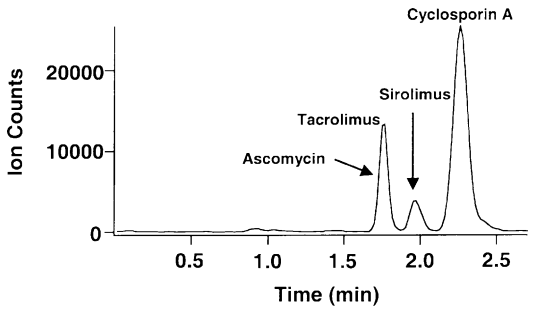

Fig. 3. Separation of tacrolimus, sirolimus and cyclosporin A. Experimental conditions: CN column, 52/48 (v/v) acetonitrile/water, 20 μL injection, 0.45 mL/min (172 bar system backpressure), and 50 °C. Chromatogram was reconstructed from the individual SRM traces. Tacrolimus and sirolimus at 10 ng/mL and cyclosporin A at 75 ng/mL.

Table 1
Chromatographic efficiency of immunosuppressant drugs and progesterone on a C_{18} and CN column

	Chromatographic efficiency (C_{18})	Chromatographic efficiency (CN)
Progesterone	7500	6100
Tacrolimus	800	3300
Sirolimus	700	2100
Cyclosporin A	200	2500

Experimental conditions: Cyano column, 50/50 (v/v) acetonitrile/water, 20 μL injection, 0.45 mL/min, and 50 °C. For C_{18} column, 80/20 (v/v) acetonitrile/water, 20 μL injection, 0.45 mL/min, and 50 °C. Mobile phases gave comparable retention on both columns. Efficiencies are expressed as plates per column.

3.2. Chromatographic efficiency of CN compared to C_{18} column

Table 1 shows the chromatographic efficiency of tacrolimus, sirolimus and cyclosporin A on the C_{18} and CN columns at 50 °C. Clearly, the CN column outperforms the C_{18} column for all three immunosuppressants by at least a factor of three. In the case of cyclosporin A, the cyano column achieves an efficiency approximately 12 times higher than the C_{18} column. The lower efficiencies on the C_{18} column are not due to column degradation as evidenced by the efficiency for progesterone, a small molecule frequently used to probe column efficiency. Rather, as has been proposed by a number of authors, the poor efficiency is due to the partial separation of immunosuppressant conformers that results in broad peaks [1,25–27]. The CN column is thought not to be able to separate the individual conformers to the same extent as the C_{18} column, which results in narrower peaks and higher efficiencies [1,27]. It could be argued that at a higher temperature (e.g., 70 °C) the C_{18} column would provide equal or perhaps better efficiency than the CN column. However, this would result in a significant decrease in column lifetime, particularly in the case of the C_{18} column used in this study, which should not be used routinely at temperatures higher than 50 °C.

3.3. Mass spectrometry of immunosuppressant drugs

The analysis of immunosuppressant drugs with mass spectrometry is most often performed with positive ion mode electrospray ionization (ESI) although atmospheric pressure chemical ionization (APCI) [7] and matrix-assisted laser/desorption ionization methods (MALDI) [33,34] exist in the literature. Our preliminary experiments with positive ion mode electrospray ionization revealed that all immunosuppressants are primarily ionized by sodium attachment even when conditions are employed to promote ammoniated

molecules. Unfortunately, sodiated molecules proved to be difficult to fragment, thus requiring unusually high collision energies (collision offset voltage greater than 70 V) to generate structurally diagnostic fragment ions for selected reaction monitoring. Furthermore, the production of a structurally diagnostic fragment ion was accompanied with the generation of several lower intensity fragment ions. This is not the ideal scenario for sensitive selected reaction monitoring as the intensity of the precursor ion is distributed over a wide range of different product ions, thus sacrificing sensitivity.

Previously, Hogge et al. [35] employed negative ion mode electrospray ionization for the analysis of cyclosporin A. They discovered that in negative ion mode, a single structurally diagnostic product ion is formed upon collision induced dissociation, which is the ideal situation for selected reaction monitoring. For this reason, we explored the potential of negative ion electrospray ionization for the simultaneous analysis of tacrolimus, sirolimus and cyclosporin A. Fig. 4 compares product ion spectra for each drug studied in positive and negative ion mode. Deprotonated molecules were obtained for all three drugs in negative ion mode. Chlorinated molecules were also observed, albeit to a much lesser extent. We obtained a structurally diagnostic fragment ion at *m/z* 1089 for cyclosporin A, which is in agreement with Hogge et al. [35]. Although the product ion spectra of sirolimus and tacrolimus show more than one product ion, there are fewer product ions in negative ion mode than in positive ion mode. Thus, improved sensitivity can be expected in negative ion mode for all three immunosuppressant drugs.

Using the work of Jegorov et al. as a guide [36], we propose that the fragment ion at *m/z* 1089 in the product ion spectrum of cyclosporin A results from cleavage of the side chain of MeBmt (4R-[(E)-2-butenyl)]-4, *N*-dimethyl-L-threonine) shown in Fig. 1. Furthermore, the sirolimus fragment ion at *m/z* 591 could result from cleavage at position 34 and 27 (Fig. 1), and similarly, the tacrolimus (and ascomycin) fragment ion at *m/z* 561 could result from cleavage at position 26 and 23 [37].

Table 2 compares the detection limits (calculated as three times the standard deviation of the blank) obtained in positive

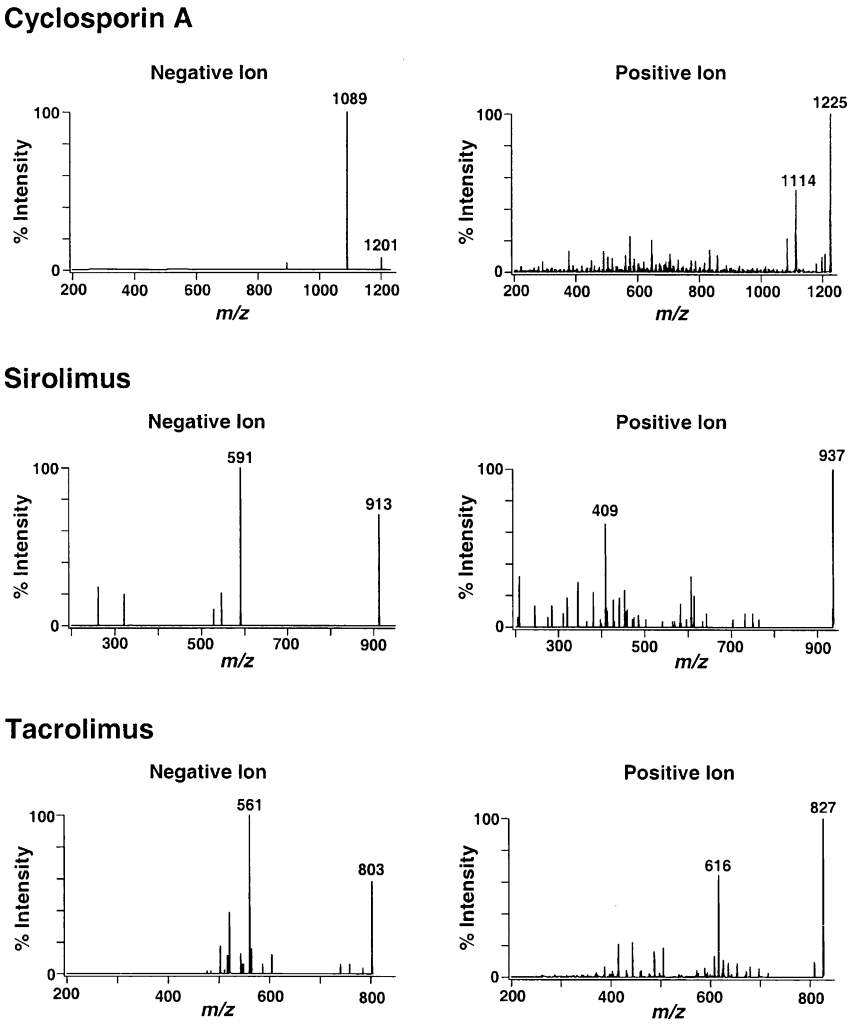

Fig. 4. Product ion spectra of cyclosporin A, sirolimus and tacrolimus in negative and positive ion mode.

Table 2
Detection limits obtained for three immunosuppressant drugs using positive and negative ion mode ESI-MS

	Detection limit (ng/mL)	
	Positive ion mode	Negative ion mode
Tacrolimus	1	0.2
Sirolimus	2	0.05
Cyclosporin A	5	0.1

Experimental conditions: CN column, 52/48 (v/v) acetonitrile/water, 20 μL injection, 0.45 mL/min, and 50 °C. SRM transitions for positive ion mode: cyclosporin A $1225^+/1114^+$, sirolimus $937^+/409^+$ and tacrolimus $827^+/616^+$. SRM transitions for negative ion mode: cyclosporin A $1201^-/1089^-$, sirolimus $913^-/591^-$ and tacrolimus $803^-/561^-$.

ion mode with those obtained in negative ion mode. Clearly, there is a considerable advantage in using negative ion mode electrospray ionization. Improvements in detection limits range from a factor of five for tacrolimus up to a factor of fifty for cyclosporin A. The linear range of calibration curves in negative ion mode was from 0.6 to 1000 ng/mL for tacrolimus ($n = 10$, $r^2 > 0.999$), 0.15 to 500 ng/mL for sirolimus ($n = 10$, $r^2 > 0.999$) and 0.3 to 1000 ng/mL for cyclosporin A ($n = 10$, $r^2 > 0.999$). The limit of quantification was set at three times the detection limit. Retention times for the immunosuppressants varied by 2.6 % relative standard deviation on a day-to-day basis.

Ion suppression/enhancement is a major concern with mass spectrometric methods, particularly when working with analytes present at ng/mL levels in biological samples. For this reason, the susceptibility of our methodology to ion suppression/enhancement was evaluated using the procedure described in the Section 2. Although five different blood samples were examined, Fig. 5 shows the results from one sample for the sake of simplicity. Equivalent results were obtained with the other samples. Fig. 5 shows that ion suppression/enhancement is not an issue with our methodology, at least for the five whole-blood samples examined in this work. All matrix components elute well before ascomycin, which is the first peak in the chromatogram. It could be argued that a higher percentage of organic modifier can be used to improve

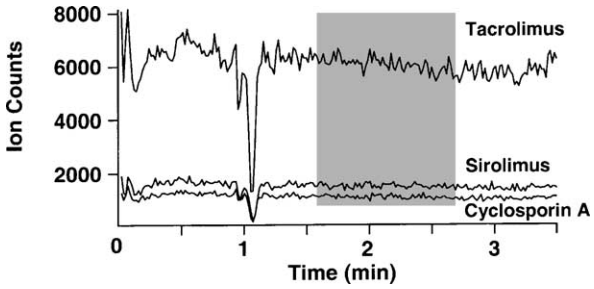

Fig. 5. Measurement of ion suppression/enhancement during separation of immunosuppressant drugs. Shaded area indicates region in chromatogram where immunosuppressant drugs elute. Experimental conditions: CN column, 52/48 (v/v) acetonitrile/water, 20 μL injection of matrix components, 0.45 mL/min, and 50 °C. A 5 ng/mL solution of tacrolimus, sirolimus and cyclosporin A was post-column infused at 5 μL/min into the mass spectrometer, while blank matrix was injected onto the CN column.

separation speed without the harmful effects of ion suppression/enhancement. However, it is important to realize that although no ion suppression/enhancement was observed for the five samples used in this test, ion suppression/enhancement may occur with another sample. Thus we feel that adequately resolving all analytes from matrix components and achieving baseline separation helps minimize the possibility of ion suppression/enhancement in future analyses.

Blood samples from organ-transplant patients that were administered tacrolimus, sirolimus and/or cyclosporin A were used in a blind test of the methodology described in this work. The results were compared to those obtained using a validated APCI-MS method that employed a C_{18} stationary phase at 70 °C [7]. Table 3 lists the results of the comparison. A *t*-test was used to compare the concentrations of immunosuppressant drugs determined by each method. The two methods are in general agreement at the 95% confidence level for all immunosuppressants in all samples, except for sirolimus in sample 1 and tacrolimus in sample 2. Unfortunately, we cannot say our method is statistically equivalent to the reference method, as a much larger number of samples would have to be analyzed.

Table 3
Results obtained for the quantification of immunosuppressant drugs in patient samples

	Tacrolimus	Sirolimus	Cyclosporin A
Sample 1			
Present method (ng/mL)	4.2 ± 0.2	6.5 ± 0.3	–
Accepted value (ng/mL)	4.6	8.0	–
Percentage difference	−9	−19	–
Sample 2			
Present method (ng/mL)	7.9 ± 0.4	8.9 ± 0.4	160 ± 10
Accepted value (ng/mL)	9.1	9.4	150
Percentage difference	−13	−5	6
Sample 3			
Present method (ng/mL)	–	16 ± 1	780 ± 40
Accepted value (ng/mL)	–	15	720
Percentage difference	–	7	8

Experimental conditions: CN column, 52/48 (v/v) acetonitrile/water, 20 μL injection, 0.45 mL/min, and 50 °C. A blank cell means that the patient was not administered that particular drug.

4. Conclusions

We have demonstrated the potential of a CN stationary phase for the analysis of immunosuppressant drugs. The favourable retention characteristics as well as the higher efficiency on the CN stationary phase allowed a separation of tacrolimus, sirolimus and cyclosporin A in less than three minutes. To the best of our knowledge, such a rapid, high-resolution separation of immunosuppressant drugs is unprecedented in the literature. When combined with negative ion mode ESI-MS detection, a sensitive method for immunosuppressant analysis in whole blood is obtained. Future work will examine the possibility for even higher sample throughput by means of alternative stationary phases and on-line sample cleanup.

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