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Microscale high-performance liquid chromatography–electrospray tandem mass spectrometry assay for cyclosporin A in blood

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Abstract

To facilitate quantitative analysis of cyclosporin A in low volume blood samples we developed a sensitive and specific microscale reversed-phase HPLC–electrospray tandem mass spectrometry assay. Blood samples (100 μl) were prepared by acetonitrile precipitation and C_{18} solid-phase extraction. Detection was by multiple-reactant monitoring. The method was linear over the range 5–1000 μg/l ($r \geq 0.997$) with accuracy between 95.4 and 102.0% over this range. Total imprecision was 11.1% at 10 μg/l and 2.8% at 800 μg/l. Absolute recovery of cyclosporin A and internal standard was 72.5 and 73.3%, respectively. When this method was evaluated against a conventional HPLC with UV detection, in patient samples, they were interchangeable ($y=0.988x+10.0$, $r=0.996$). This HPLC–ESI-MS–MS method will be applicable to therapeutic monitoring in paediatric transplant patients and multiple point pharmacokinetic studies in animals and humans. © 1998 Elsevier Science B.V.

Keywords: Cyclosporin A

1. Introduction

Frequent and accurate monitoring of cyclosporin A (CSA) concentrations is essential for optimal use of the drug in transplant patients [1]. Several techniques [2] have been developed to measure CSA, the two most commonly used being immunoassays and HPLC with ultraviolet detection (HPLC–UV). These immunoassays are limited by non-specificity due to cross-reactivity of CSA metabolites with the assay antibody [3–5]. HPLC–UV assays are limited by the low maximum absorptivity of CSA, 196 nm [6], which makes its detection difficult. Therefore HPLC–UV methods [7–9], typically require 1 ml of sample and extensive clean-up to allow detection at such wavelengths. The limits of quantification (LOQ) of these methods generally range from 25 to 50 μg/l, although improved sensitivity may be achieved using a photodiode array detector.

The development of the HPLC system interfaced to a mass spectrometer via an electrospray interface has revolutionised the analysis of biological samples

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[10,11]. The mass spectrometer, as a detector, provides superior sensitivity and specificity compared to traditional UV. For example, we have previously reported a method for the measurement of the immunosuppressant drug tacrolimus (FK506) in blood using this technique [12]. The limit of quantification of this method (0.3 μg/l) contrasts with 50–100 μg/l for HPLC–UV [13]. Whitman et al. [14] reported an assay for CSA and several of its metabolites that used single-ion monitoring mass spectrometry. Their detection limit was 100 pg/ml of CSA, three orders of magnitude lower than for their corresponding HPLC–UV method. We report here a microscale HPLC–electrospray tandem mass spectrometry assay (HPLC–ESI-MS–MS) for CSA in blood using multiple reactant monitoring (MRM) and the evaluation of the method in patients against our previously reported HPLC–UV assay [9].

2. Experimental

2.1. Materials

HPLC-grade acetonitrile and methanol were purchased from EM Science (Gibbstown, NJ, USA). Reagent-grade deionised water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA). CSA and the cyclosporin analogue, dihydrocyclosporin C (I.S.), were a gift from Sandoz Australia (Sydney, Australia). The cyclosporin metabolites (AM9, AM19, AM1, AM1c and AM4N) were a kind gift of Dr W.T. Lui (Dept. of Clinical Biochemistry, The Toronto Hospital, Ontario, Canada). All other chemicals were AR grade.

2.2. HPLC apparatus and conditions

The HPLC system consisted of a 616 pump with a 600S controller, a column oven with a temperature control module (Waters, Milford, MA, USA) and an IS200 autosampler (Perkin-Elmer, Danbury, CT, USA). The HPLC column was an Alltima C_8 column (100×2.1 mm I.D., 5 μm, Alltech, Deerfield, IL, USA), maintained at a temperature of 70°C. The mobile phase consisted of 72% methanol and 28% 50 m*M* ammonium acetate buffer (pH 5.1). The system operated at a flow-rate of 0.3 ml/min with approximately 1/10 of the flow split post-column into the MS.

2.3. MS apparatus and conditions

Mass spectrometric detection was performed on an API III triple quadrupole instrument (PE-Sciex, Thornhill, Toronto, Canada) using MRM. An ionspray (pneumatically assisted electrospray) interface was used in positive ionisation mode. The orifice potential was set at 100 V to produce predominantly protonated species of the analytes. The interface heater was set at 60°C. For collision-activated dissociation, argon was used as the collision gas at a thickness of 300×10^{12} molecules cm^{-2}. Peak area ratios obtained from MRM of CSA (m/z 1203.0→425.4) and I.S. (m/z 1221.0→425.4) were used for quantification. Standard curves (5, 20, 50, 100, 200, 500 and 1000 μg/l) were constructed using weighted ($1/x^2$) linear least-squares regression. Data were collected and analysed on a Macintosh computer operating RAD and MACQUAN software (PE-Sciex).

2.4. Extraction procedure

Standards, controls, and patient samples (100 μl) were treated with 200 μl acetonitrile–water (70:30, v/v), containing 18.2 ng of I.S., in 1.5-ml polypropylene centrifuge tubes. Samples were vortex-mixed for 1 min and centrifuged (5 min, 850 *g*). The supernatants were applied to 100-mg C_{18} solid-phase extraction cartridges (Waters) which had been preconditioned with methanol (5 ml) and water (5 ml). The loaded cartridges were washed sequentially with water (5 ml), 50% methanol–water (2 ml) and heptane (2 ml). The washed cartridges were placed under full vacuum for 15 min. The analytes were eluted with 50% isopropyl alcohol–heptane (1 ml) and the eluents evaporated under air flow (60°C). Samples were redissolved in 80% methanol–water (50 μl) and a 10-μl aliquot injected.

2.5. Linearity, imprecision, accuracy, and recovery studies

Linearity was tested by analysing blood standards containing known (weighed-in) amounts of CSA at concentrations of 5, 20, 50, 100, 200, 500 and 1000

μg/l. The method's accuracy and inter-day imprecision, over the analytical range, were determined from the back-calculated results of the above linearity study ($n=7$). Further, the imprecision of the method was determined by assaying spiked blood controls (10, 250 and 800 μg/l) in batches of four on each of 3 days. Intra-day, inter-day and total imprecision were derived from analyses of variance of the assayed controls using the method of Krouwer and Rabinowitz [15]. Accuracy was determined by expressing the mean assayed result for the control samples ($n=12$) as a percentage of the weighed-in concentration. Absolute recovery of the analytes was determined by comparing the peak areas of extracted samples, from 10 different subjects, spiked with CSA and I.S. before and after extraction.

2.6. Evaluation of HPLC–ESI-MS–MS against HPLC–UV

The HPLC–ESI-MS–MS methodology was evaluated against our previously reported HPLC–UV method [9]. A total of 90 blood samples, collected in EDTA vacutainer® tubes from transplant patients, were assayed by both methods and the results subjected to linear regression analysis.

3. Results

Fig. 1 shows the effect of orifice potential on the formation of precursor ions of CSA. The use of a high orifice potential (100 V) was necessary to produce predominantly the pseudomolecular ion $[M+H]^+$ of CSA and the I.S. (Fig. 1A). CSA monitored at a lower orifice potential (35 V) produced single and double charge species (Fig. 1B), which in MRM resulted in loss of signal, compared to that of 100 V, and variability in results. Collision-induced dissociation spectra were obtained for the analytes. The fragmentation of the precursor ion of CSA (m/z 1203) is shown in Fig. 2. The predominant product ion (m/z 425.4) was utilised in MRM.

The specificity of the assay is illustrated in Fig. 3, with blank blood (A), blank blood with CSA (B) and blank blood with I.S. (C), showing no interfering peaks. Further, an extract of blank blood spiked with the five major metabolites of CSA (approx. 500

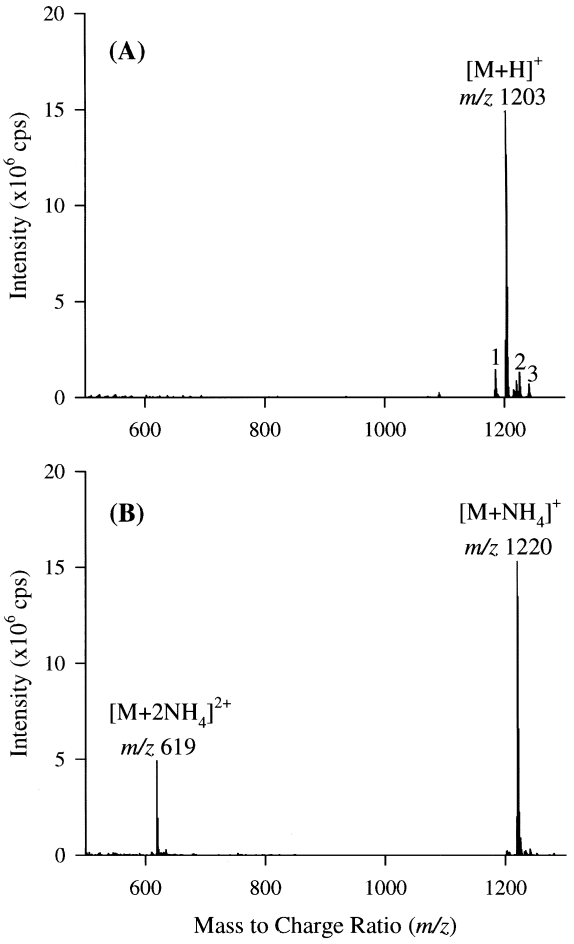

Fig. 1. Mass spectra showing the effect of orifice potential (OR) on the formation of CSA precursor ions: (A) OR=100 V and (B) OR=35 V). Minor ions shown in (A) are: 1, m/z 1185, $[M+H-H_2O]^+$; 2, m/z 1225, $[M+Na]^+$; 3, m/z 1241, $[M+K]^+$.

μg/l) shows no interference with the analytes (Fig. 4A). Typical chromatograms of an extract of a blood standard (5 μg/l) and a patient sample (272 μg/l) are shown in Fig. 4B and Fig. 4C, respectively. The retention times of CSA and I.S. are 11.8 and 10.0 min, respectively.

The assay is linear over the range 5–1000 μg/l ($r\geq0.997$, $n=7$). The method accuracy and inter-day imprecision, over the analytical range, are 95.4–102.0% and 1.3–6.2%, respectively (Table 1). As shown in Table 2, all between-day, within-day and total coefficients of variation are <12% at weighed-in concentrations of 10, 250 and 800 μg/l. The accuracy at these three concentrations ranged from

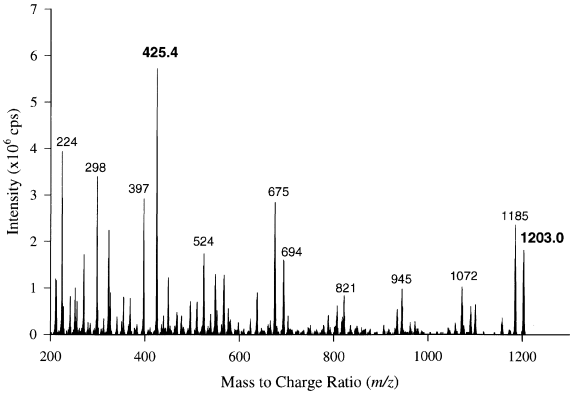

Fig. 2. Collision-induced dissociation spectrum of CSA (m/z 1203), using argon as the collision gas. The major product ion (m/z 425.4) was used for multiple-reactant monitoring.

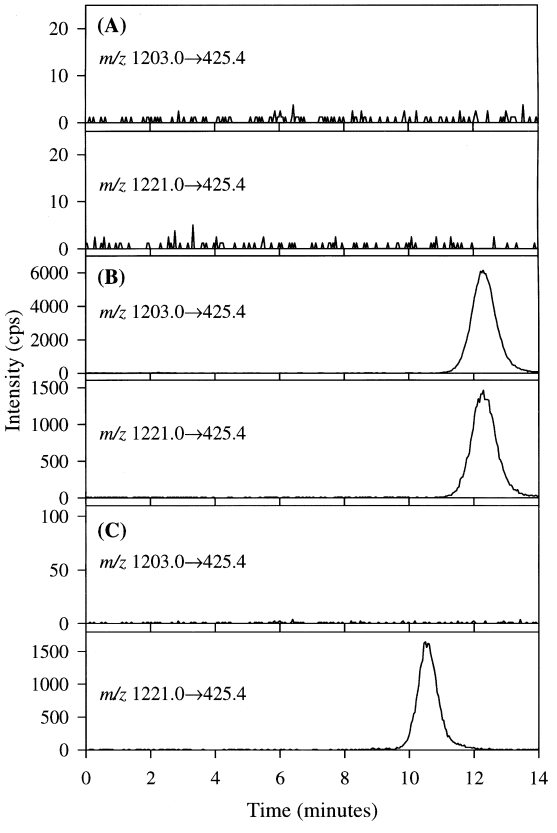

Fig. 3. Chromatograms of (A) blank blood, (B) a blood standard (1000 μg/l) with no I.S., and (C) blank blood spiked with I.S. Peaks: 1, CSA (m/z 1203.0→425.4); 2, I.S. (m/z 1221.0→425.4). Retention times are 11.8 and 10.0 min, respectively.

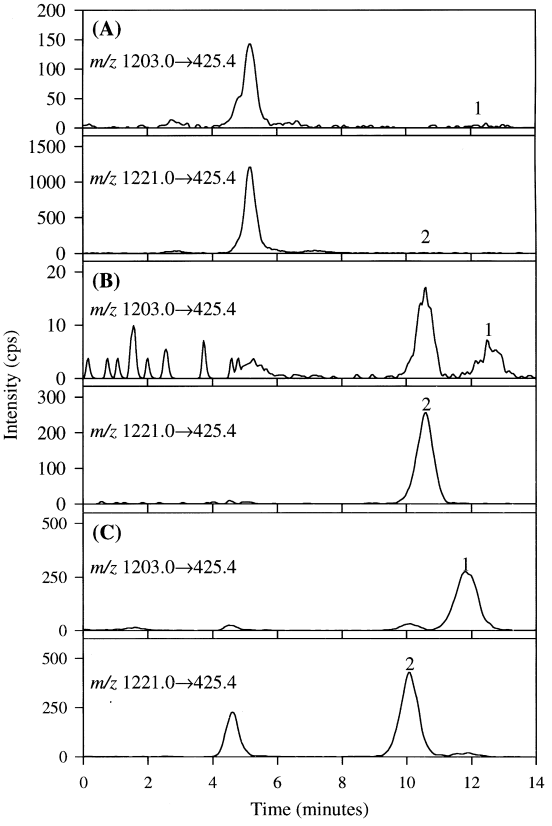

Fig. 4. Typical chromatograms of (A) blank blood spiked with the CSA metabolites AM9, AM19, AM1, AM1c and AM4N (approx. 500 μg/l), (B) a blood standard (5 μg/l), and (C) a patient sample (272 μg/l). Peaks: 1, CSA (m/z 1203.0→425.4); 2, I.S. (m/z 1221.0→425.4). Retention times are 11.8 and 10.0 min, respectively.

98.5 to 102.3% (Table 2). Absolute mean recoveries for CSA and I.S. were determined as 72.5 ($n=10$) and 73.3% ($n=10$), respectively. When HPLC–ESI-MS–MS was evaluated against HPLC–UV, the two methods were interchangeable ($y=0.988x+10.0$, $r=0.996$). A comparison of the results obtained by each method are shown in Fig. 5.

4. Conclusions

The HPLC–ESI-MS–MS method provides a selective, accurate and precise means of assaying CSA in blood using minimal sample volume (100 μl). The interchangeability of HPLC–ESI-MS–MS

Table 1
Linearity, accuracy and inter-day imprecision over the analytical range, 5–1000 μg/l

Day no.	Correlation coefficient	CSA concentration (μg/l)						
		5	20	50	100	200	500	1000
1	0.998	5.00	20.9	45.7	91.7	211	500	1069
2	0.998	4.88	22.5	47.6	96.6	195	509	987
3	0.999	5.05	19.4	48.6	99.8	204	522	986
4	0.997	4.94	21.9	44.4	95.8	214	488	1024
5	0.999	5.08	18.7	50.6	98.8	200	508	1034
6	0.999	5.02	19.8	47.9	106	201	494	990
7	0.999	5.03	19.9	49.0	92.7	204	538	993
Mean concentration		5.00	20.4	47.7	97.3	204	508	1012
Accuracy (%)		100.0	102.0	95.4	97.3	102.0	101.6	101.2
Inter-day imprecision		1.3	6.2	4.0	4.6	3.0	3.1	2.9

Correlation coefficient was determined by weighted ($1/x^2$) linear least-squares regression. Accuracy was determined as a percentage of the mean assayed concentration over the weighed-in concentration. Inter-day imprecision is expressed in terms of percentage coefficient of variation.

Table 2
Imprecision and accuracy of the HPLC–ESI-MS–MS assay, using control samples

Weighed-in concentration (μg/l)	Imprecision (%)			Accuracy (%)
	Intra-day	Inter-day	Total	
10	10.4	3.7	11.1	102.3
250	3.8	1.8	4.2	101.4
800	2.6	1.1	2.8	98.5

Imprecision was calculated by the method of Krouwer and Rabinowitz [15] and expressed in terms of coefficient of variation ($n=12$). Accuracy was determined as a percentage of the mean assayed concentration over the weighed-in concentration ($n=12$).

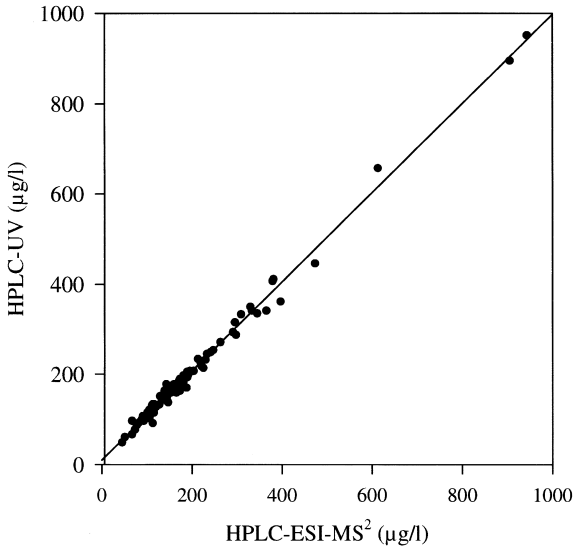

Fig. 5. Comparison of HPLC–ESI-MS–MS with HPLC–UV using blood samples obtained from transplant patients receiving CSA treatment ($n=90$).

with HPLC–UV means that both can be considered to be reference methods. The limit of quantitation of the HPLC–ESI-MS–MS method (5 μg/l) is lower than that of our previously reported HPLC–UV method (45 μg/l) [9]. Possible applications include therapeutic drug monitoring of paediatric transplant patients, multiple point pharmacokinetic studies in humans and animals, and analysis in biopsy samples which routinely comprise only 1–5 mg of tissue.

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