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Optimized analytical method for cyclosporin A by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

The Micromass Platform LCZ mass detector parameters were optimized for simultaneous recording of the protonated ($CsA{\sim}H^+$), sodium adduct ($CsA{\sim}Na^+$) and potassium adduct ($CsA{\sim}K^+$) of cyclosporin A eluted from a Symmetry Shield RP8 column. The optimized procedure allows a precise analysis of CsA in whole blood or serum without removal of salts prior to analysis. The ratio of the three forms of CsA varied depending on the assay condition and the types of specimens being analyzed. The summation of three ionic forms of CsA detected by LC–ESI-MS is a reliable and simple method to assess CsA concentration in the blood.

Keywords: Cyclosporin A

1. Introduction

Cyclosporin A (CsA) has been used widely as an immunosuppressive drug in organ transplantation, treatment of autoimmune disorders, and psoriasis [1]. It is used in our hospital to prevent rejection in corneal transplantation. The immunosuppressive action of CsA is dose dependent. The concentration of CsA in the blood is an important parameter to assess if a patient has sufficient CsA prior to surgery [2,3]. The analysis of CsA by immunochemical methods [4–6] and high-performance liquid chromatography have been reported by others [7–12]. The most sensitive method was achieved by coupling liquid chromatography to mass spectrometry (LC–ESI-MS) [13] or tandem mass spectrometry (LC–MS/MS) [14,15].

In recent years the procedure for mass spectrometry has been highly simplified. The equipment cost has been markedly reduced. LC–ESI-MS has become increasingly popular for microanalysis. This study is directed to optimize the analytical method for CsA by LC–ESI-MS. One major problem we encountered is the tendency of CsA to form Na and K adducts. The ratio of the protonated CsA ($CsA{\sim}H^+$), sodium adduct ($CsA{\sim}Na^+$) and potassium adduct ($CsA{\sim}K^+$) varied under different instrumen-

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tal settings and differed among different specimens. The variation of the three ionic forms between standard and samples causes problems in quantitative analysis based on one form of CsA. Others have introduced a solid-phase extraction step to remove Na^+ and K^+ in order to convert all CsA to the protonated form before analysis [14,15]. The solid-phase extraction step introduced loss of CsA varying from sample to sample. Internal standard was needed to correct the variable loss during the solid-phase extraction step. This study was carried out to study in detail the LC–ESI-MS parameters on the magnitude of ion current produced from CsA~H^+, CsA~Na^+ and CsA~K^+, factors which influence the ratio of the three forms of CsA, and determine if the summation of the three forms is a reliable method for quantitative analysis of total CsA in a specimen.

2. Experimental

2.1. Materials

2.1.1. Chemicals

HPLC grade methanol was purchased from Fisher Scientific. Ultra-pure water was obtained from a Milli-Q water purification system (Millipore, Milford, MA). CsA was a gift from Novartis Pharma AG, Basel, Switzerland. Immunoassay kit was supplied by Abbot Laboratories (Abbot Park, IL).

2.1.2. Whole blood

Whole blood was stored at −60°C. Before analysis, the blood specimen was thawed. One part of the blood specimen was subjected to vortex with 9 parts of methanol for 3 min. The coagulated proteins were removed by centrifugation at 10 000×g for 15 min. The supernatant fraction was analyzed by LC–ESI-MS method.

2.1.3. Serum

Fresh blood specimens were allowed to clot in the refrigerator. The clotted blood sample was centrifuged for 15 min at 10 000×g to remove blood cells. An aliquot of 100 μl of serum was subjected to vortex with 400 μl of methanol for 1 min. Coagulated proteins were removed by centrifugation at 10 000×g for 15 min. The supernatant fraction was transferred to an autosampler vial for LC–ESI-MS analysis.

2.2. Instrument

Waters 2690 solvent delivery system including an auto-sampler, photodiode array detector and Micomass Mass Spectrometer (platform LCZ) were used for chromatographical separation and detection of CsA. The separation column, RP_8 (3.5 μm, 4.6× 75 mm supplied by Waters Associates) was eluted by 90% methanol/10% water at 0.5 ml/min.

The optimized settings in the MS detector were as follows. The nitrogen gas flow was maintained at 500 l/h. The capillary and cone voltage was set to 4.0 and 10, respectively. The source temperature and desolvation temperature was set to 150 and 375°C, respectively. The mass spectrum was recorded under a full scan operation for positive ions, with a scan range from m/z 500 to 1400. The quantification was carried out with the selected-ion recording (SIR) mode by monitoring the protonated molecular ion (m/z=1203), sodium ion adduct (m/z=1225) and potassium ion adduct (m/z=1241) simultaneously. The quantification is based on the total peak areas of CsA~H^+, CsA~Na^+ and CsA~K^+ in SIR chromatogram.

2.3. Methods

2.3.1. Optimization of LC–ESI-MS experimental conditions

The sensitivity of the CsA signal is largely dependent on LC–ESI-MS experimental parameters. In order to identify the optimized condition, the following MS parameters were tuned: cone voltage (5 to 90 V), capillary voltage (2.5 to 4.0 V), desolvation temperature (250 to 400°C) and nitrogen gas flow-rate (200 to 600 l/h).

2.3.2. Calibration curve

The determination of CsA was based on the external standard method. Six-point calibration curves (triplicate injections) were created for the range from 0.02 to 10 ng by plotting the summation of the peak areas of CsA~H^+, CsA~Na^+ and CsA~K^+ against the amount of CsA injected into the column.

2.3.3. Recovery, precision and accuracy

The recoveries were determined by comparing the peak areas of a serum specimen premixed with a known amount of CsA with that of an equivalent amount of standard CsA dissolved in pure methanol. For intra-assay precision, spiked serum samples with three different concentrations (40, 200 and 650 μg/l) were analyzed. For the inter-assay precision, the above samples were analyzed on three subsequent days. Accuracy was measured using CsA standard samples (40, 200, 400, 650 and 1000 μg/l) and calculated as the deviation from the theoretical values.

3. Results and discussion

The detection of a selected molecular size by a mass detector allows a precise quantitative measurement of a compound co-eluted with a group of compounds having different molecular sizes. In this way, the time consuming effort to establish the optimal chromatographic separation of all compounds is not needed. The large molecular sizes of CsA, 1202, is very different from physiological compounds in the blood. The major interference materials in the blood are proteins. Proteins form highly heterogeneous ion masses producing false positive peaks of a selected ion size. After removing proteins by methanol, the soluble fraction is ready for analysis.

The three forms of CsA can be recorded simultaneously according to their ion sizes even though they were eluted together. The intensity of the detection signals is dependent on the conditions of the mass detector. The optimized instrumental setting for quantitative analysis of CsA in human serum, and the comparison of the present method to the immunochemical method routinely used in clinical laboratories are described in the following.

3.1. Optimized mass detector conditions for analytical results

In order to determine the concentration of CsA in whole blood, serum and tissues with high sensitivity, the ESI interface parameters including cone voltage, capillary voltage, desolvation temperature and nitrogen gas flow-rate was examined.

3.1.1. Cone voltage and capillary voltage

The peak area of CsA~H^+, CsA~Na^+, CsA~K^+ and the summation of the three peak areas varied depending on the cone voltage (Fig. 1). CsA~H^+ began to decrease after the cone voltage was increased above 10 V. CsA~Na^+ increased with voltage up to 50 V. Above 50 V, CsA~Na^+ reduced with increased cone voltage. There was little change in CsA~K^+ at different cone voltage. The summation of the three peak areas was constant within the range of 10 to 30 V. Total CsA concentrations began to reduce as cone voltage increased above 30 V. Capillary voltage does not affect the sensitivity significantly.

We have attempted to convert all three forms of CsA into one form by varying the cone voltage. When the cone voltage was increased to 50 V (Fig. 1), the CsA~Na^+ peak increased to 90% of total CsA. However, increased cone voltage reduced the sensitivity.

3.1.2. Desolvation temperature

The peak areas for all three ions increased proportional to desolution temperature. The summation of the three ions approached the maximal peak area as desolvation temperature was raised to 375°C (Fig. 2).

3.1.3. Nitrogen gas flow-rate

Fig. 3 shows the peak area of CsA~H^+, CsA~Na^+, CsA~K^+ and their sum as a function of

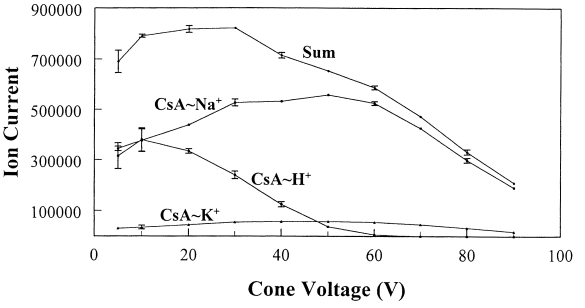


Fig. 1. The CsA~H^+, CsA~Na^+, CsA~K^+ and sum of these three peak areas as a function of cone voltage (V) in the mass detector.

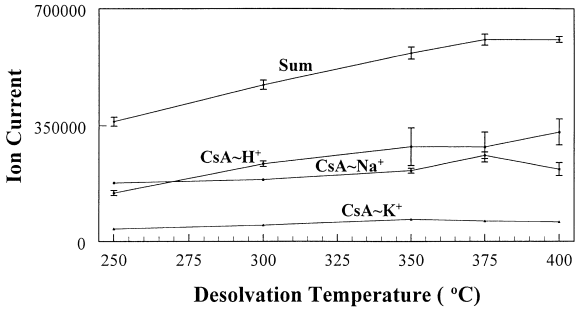


Fig. 2. The CsA~H^+, CsA~Na^+, CsA~K^+ and sum of these three peak areas as a function of desolvation temperature (°C) in mass detector.

nitrogen gas flow-rate. The optimal condition is 500 l/h.

3.2. Linearity, accuracy, recovery and imprecision studies of the LC–ESI-MS method

The typical mass spectrum of CsA standard sample (10 ng) is shown in Fig. 4A. The single ion chromatograms for each ion are shown in Fig. 4B. The standard curve was created by assaying 0.02, 0.1, 0.2, 1.0, 2.0 and 10.0 ng of CsA. The peak area increased linearly over the range from 0.02 to 10 ng analyzed (correlation coefficient=0.9977). Detailed accuracy data obtained by analysis of a set of spiked serum samples are listed in Table 1. The coefficients of variance (C.V.) were less than 3% over the whole analytical range and the deviations were less than 5.3%. When CsA was added to normal human serum, over 90% was recovered in the methanol extraction

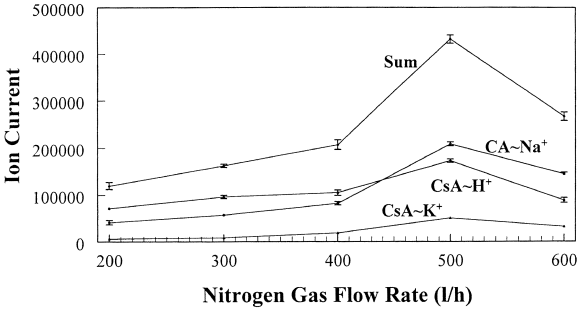
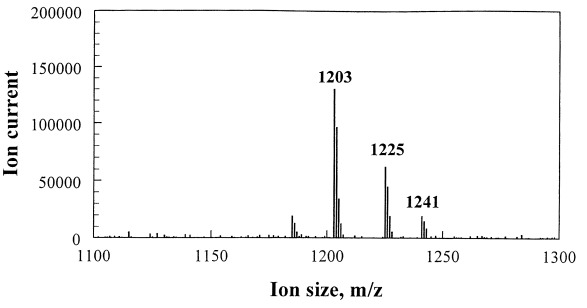
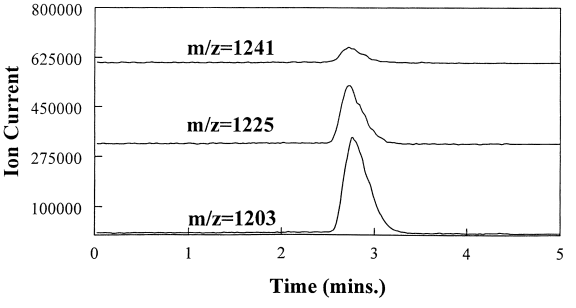


Fig. 3. The CsA~H^+, CsA~Na^+, CsA~K^+ and sum of these three peak areas as a function of nitrogen gas flow-rate (l/h) in mass detector.



A



B

Fig. 4. (A) A typical mass spectrum of cyclosporine A pure standard sample in electrospray positive mode. m/z 1203 is protonated CsA $[CsA+H]^+$, m/z 1225 is sodium ion adduct of CsA $[CsA+Na]^+$, m/z 1241 is potassium ion adduct of CsA $[CsA+K]^+$. (B) The Selected ion recording (SIR) chromatogram of m/z=1203, m/z=1225 and m/z=1241 which corresponds to $[CsA+H]^+$, $[CsA+Na]^+$ and $[CsA+K]^+$, respectively. The quantification is based on SIR signals.

and analyzed by the optimized procedure (Table 2). The limit of detection at a signal-to-noise ratio of 3:1 was 10 pg/assay. The intra-day and inter-day imprecision for 40, 200 and 650 μg/l are less than 5% (Table 3). The run time of one sample was 5 min.

The present method eliminates the major analytical problem due to occurrence of three forms of CsA: CsA~H^+, CsA~Na^+ and CsA~K^+. The proportions of the three forms varied depending on the analytical condition (Fig. 1), amount of CsA injected to the column (Table 4), and types of specimens being analyzed. We proposed to use the summation of the three forms of CsA to evaluate total CsA in a blood specimen. The analysis for three ion forms avoids the necessity of removing Na^+ and K^+ from the specimens by a solid-phase extraction step prior

Table 1
Accuracy of the LC–ESI-MS method[a]

Theoretical concentration (μg/l)	Mean±SD Measured concentration (μg/l)	C.V. (%)	Accuracy (%)	Deviation (%)
40	41.9±0.61	1.5	104.8	+4.8
200	199±3.56	1.8	99.5	−0.5
400	408±8.58	2.1	102.0	+2.0
650	652±5.12	0.8	99.7	−0.3
1000	947±8.49	0.9	94.7	−5.3

[a] $n=6$; SD, standard deviation; C.V., coefficient of variance. Accuracy was expressed as a percentage of the mean measured concentration over the theoretical concentration.

Table 2
Recovery (%) of CsA ($n=6$) from serum

Concentration (μg/l)	Recovery (%±SD)
40	91.4±1.98
500	91.8±2.83
800	90.0±0.40

Table 3
Imprecision of the LC–ESI-MS method[a]

Theoretical concentration (μg/l)	Imprecision (%)	
	Intra-day	Inter-day
40	1.69	4.83
200	0.36	3.66
650	1.17	2.68

[a] Imprecision was expressed in terms of coefficient of variation ($n=6$).

to analysis. Without the solid-phase extraction step, there is no need to use internal standard to correct for the uncontrollable loss in the sample preparation step.

Theoretically, Na^+ and K^+ could be removed by a special step as described by others [15]. In reality, total removal of Na^+ and K^+ from solvent is very difficult. The removal of Na^+ and K^+ from specimens varies depending on types of specimens used. A special method designed for the removal of metals in one specimen may not be adequate for another. Even for the same type of specimens, there is uncontrollable variation that requires a different way to purify the specimen. Furthermore, the method to separate CsA from ions introduced a step of uncontrollable loss of CsA. The affinity of CsA to ions is quite unpredictable. Theoretically, ions should be separated from CsA as a specimen moves down the column. In reality, some Na^+ bound to CsA move down from the column with CsA. Our experience showed unpredictable variation depending on the type of specimen being analyzed. Therefore, we recommend the summation of the three forms to obtain the total CsA concentration.

The limit of detection of our method (10 pg/assay) is in the same magnitude as other LC–MS methods (20 pg/assay, for example in Ref. [14]. Our

Table 4
$[CsA{\sim}H]^+$ ($m/z=1203$), $[CsA{\sim}Na]^+$ ($m/z=1225$) and $[CsA{\sim}K]^+$ ($m/z=1241$) peak areas in standard CsA samples with different concentration (three injections in each concentration)[a]

CsA amount (pg)	CsA~H^+%±SD	CsA~Na^+%±SD	CsA~K^+%±SD
20	100±0.00	0.00±0.00	0.00±0.00
100	71.79±1.60	28.21±1.60	0.00±0.00
200	66.03±1.31	33.97±1.31	0.00±0.00
1000	66.12±0.61	32.53±0.43	1.35±0.20
2000	64.73±0.51	32.91±0.32	2.36±0.19
10 000	57.93±0.49	34.79±0.28	7.28±0.20

[a] Normalized data, CsA~H^+, CsA~Na^+, CsA~K^+=100%.

method also shows good accuracy, recovery and precision, which is comparable with other existing LC–MS methods [13,14].

3.3. Comparison of LC–ESI-MS with the commercial immunoassay method

The immunoassay kit is currently used in many hospitals for the analysis of CsA in whole blood. The data obtained by the LC–ESI-MS method correlated very well to that by the immunoassay for CsA in whole blood. The blood specimens were obtained from three patients at different time intervals after oral administration of CsA in order to compare the two methods for samples containing different amounts of CsA. The CsA concentration in the whole blood detected by LC–ESI-MS correlated very well with that by the immunochemical method (Fig. 5, correlation coefficient R^2=0.97). Immunoassay kits were designed for the analysis of CsA in whole blood. They are not sensitive enough for CsA concentration in the serum. The LC–ESI-MS method is sensitive to both serum and whole blood.

3.4. Comparison of CsA in serum and whole blood

After oral administration, most CsA was present inside the blood cells. The amount of CsA in some serum specimens were too low to be detected by immunoassay. We used the LC–ESI-MS method to compare the concentration of CsA in the whole blood and serum. There was a good correlation between the CsA concentration in the serum to that in the whole blood. A regression was calculated resulting in the following equation: CsA (in serum)= 0.2258 CsA (in whole blood)−38.56 (μg/l), R^2= 0.976. However, the amount of CsA in the serum is only 16% of that in the whole blood.

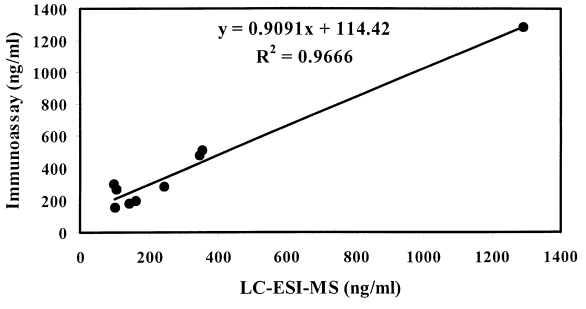


Fig. 5. Comparison of HPLC–ESI-MS with immunoassay using blood samples obtained from cornea transplant patients receiving CsA treatment (n=9).

Only the amount of CsA in the serum is available to the target organ. Therefore, CsA in the serum is more important than that in the whole blood to assess if the patient has sufficient CsA prior to surgery. The immunoassay kits are not sensitive enough for the detection of CsA in the serum.

4. Conclusion

This report describes a simple method to prepare the specimens for a simultaneous analysis of the three forms of CsA. The chromatographic step required only 5 min for each sample, and detects as low as 10 pg CsA per assay. The LC–ESI-MS method can detect a broad range of CsA concentration from 10 pg to over 100 ng, and is quite satisfactory for analysis of either serum or whole blood. The results obtained by the LC–ESI-MS method in the whole blood is comparable to the immunochemical method commonly used in the clinical chemistry laboratory. The immunochemical method is not sensitive enough for the analysis of CsA in serum.

Acknowledgements

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References

[1] D. Faulds, K.L. Goa, P. Benfield, Cyclosporin. A review of its pharmacodynamic and pharmacokinetics properties, and therapeutic use in immunoregulatory disorders, Drugs 45 (1993) 953.

[2] M. Oellerich, V.W. Armstrong, B. Kahan, L. Shaw, D.W. Holt, R. Yatscoff et al., Lake Louise Consensus Conference on cyclosporin monitoring in organ transplantation: report of the consensus panel, Ther. Drug Monit. 17 (1995) 642.

[3] C. Althaus, E. Dagres, T. Reinhard, U. Christians, R. Sundmacher, Cyclosporin A and its metabolites in the anterior chamber after topical and systemic application as determined with high-performance liquid chromatography–electrospray mass spectrometry, German J. Ophthalmol. 5 (1996) 189.
[4] M. Winkler, G. Schumann, D. Petersen et al., Monoclonal fluorescence polarization immunnoassay evaluated for monitoring cyclosporine in whole blood after kidney, heart and liver transplantation, Clin. Chem. 38 (1992) 123.
[5] G.L. Lensmeyer, D.A. Wiebe, D.H. Carlson et al., Three commercial polyclonal immunoassay for cyclosporine in whole blood compared. I.I. Cross-reactivity of the antisera with cyclosporine metabolites, Clin. Chem. 36 (1990) 119.
[6] R.W. Yatscoff, K.R. Copeland, C.J. Faraci, Abbott TDx monoclonal antibody assay evaluated for measuring cyclosporine in whole blood, Clin. Chem. 36 (1990) 1969.
[7] I. Fu, L.D. Bowers, Clin. Chem. 37 (1991) 1185.
[8] L. Sangalli, M. Bonati, Ther. Drug Monit. 25 (1988) 510.
[9] L.D. Bowers, Transplant Proc. 22 (1990) 1150.
[10] P. Salm, R.L.G. Norris, P.J. Taylor, D.E. Davis, P.J. Ravenscroft, Ther. Drug Monit. 15 (1993) 65.
[11] U. Christians, K.O. Zimmer, K. Wonigeit et al., Measurement of cyclosporine A and four metabolites in whole blood by high-performance liquid chromatography, J. Chromatogr. 413 (1987) 1219.
[12] U. Christians, K.O. Zimmer, K. Wonigeit et al., Liquid-chromatographic measurement of cyclosporine A and its metabolites in blood, bile and urine, Clin. Chem. 34 (1988) 34.
[13] G.I. Kirchner, C. Vidal, M. Winkler, L. Mueller, W. Jacobsen, A. Franzke, K.F. Sewing, LC/ESI-MS allows simultaneous and specific quantification of SDZ RAD and cyclosporine, including groups of their metabolites in human blood, Ther. Drug Monit. 21 (1999) 116.
[14] J. Simpson, Q.L. Zhang, P. Ozaeta, H. Aboleneen, A specific method for the measurement of cyclosporine A in human whole blood by liquid chromatography–tandem mass spectrometry, Ther. Drug Monit. 20 (1998) 294.
[15] P.J. Taylor, C.E. Jones, P.T. Martin, S.V. Lynch, A.G. Johnson, S.M. Pond, Microscale high-performance liquid chromatography–electrospray tandem mass spectrometry assay for cyclosporine A in blood, J. Chromatogr. B 705 (1998) 289.