



Development of an automatic solid phase extraction and liquid chromatography mass spectrometry method by using a monolithic column for the analysis of Cyclosporin A in human plasma

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ABSTRACT

A sensitive and specific and automated liquid chromatography–electrospray mass spectrometric (LC–ESI–MS) assay for the quantification of Cyclosporin A in human plasma was developed. Following a simple protein precipitation step, the supernatant was extracted on-line and directly injected into the system LC–ESI–MS. A relatively new type of monolithic column consisting of a silica rod with bimodal pore structure was used to achieve a retention time of 2.4 min with a very low backpressure at a flow rate of 1 ml/min. The assay was linear from 0.050 to 1.000 µg/ml. The mean recovery was 91%. The mean inter-day and intra-day precisions were 1.85% and 2.83%, respectively. The combination of the automated solid phase extraction and the low retention time achieved with this columns increase the throughput and decrease the time of analysis of each sample. This technology is useful in order to improve the efficiency of the bioanalytical studies.

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1. Introduction

Monolithic materials for the use in HPLC are one of the tools for bioanalysis [1,2]. Different monolithic-type HPLC columns based on silica for fast separation have developed achieving speed, sensitivity and selectivity with low backpressure.

The use of the Merck Chromolith columns is described in this paper. These columns are prepared according to a sol–gel process, which is based on the hydrolysis and polycondensation of alkoxysilanes in the presence of water-soluble polymers [3,4].

The method leads to rods made of one piece of porous silica with a defined porous structure. The main feature of silica rods columns is a higher total porosity, about 15% higher than for conventional particulate HPLC columns. The column pressure drop is therefore much lower, allowing to operate at higher flow rates including flow gradients. Consequently, the high speed and good separation can be achieved at the same time using this type of monolithic column. The important feature of this is its relevance in high-throughput bioanalysis [5]. The method described has been used to screen over 4000 compounds thus far and has met the sensitivity and reproducibility criteria on over 97% of the compounds tested. The advantage of this method is that development time is no longer needed to investigate new drug candidates, and little, if any, sam-

ple preparation time is required. The result is increased productivity through higher sample throughput.

As a direct result of the short analysis times offered by these monolithic columns, sample preparation has become the rate-limiting step. Much effort has also been devoted in order to automate the sample preparation step [6]. Another possible approach to overcome the problem of high pressure associated with small particles is to fabricate a column made of one piece of a porous solid with small-sized skeletons and relatively large through-pores which could provide both low pressure drop and high column efficiency. The skeletons can be meso-porous or microporous to have double-pore structures, or even nonporous. The most important features are high mechanical stability of beds and the freedom in the ratio of through-pore size to skeleton size. Several examples of such monolithic columns made of an organic polymer have been reported recently.

The Cyclosporin A (CsA) (Fig. 1) has been used widely as an autoimmune drug in organ transplantation, treatment of autoimmune disorders and psoriasis [7]. The analysis of CsA in biological samples by high-performance liquid chromatography has been reported before [8–10]. The most sensitive method was achieved by coupling liquid chromatography to mass spectrometry (LC–MS) [11] or tandem mass spectrometry (LC–MS–MS) [12].

A first side-by-side comparison of the two main types of commercially available monolithic nano-LC column used in reversed-phase liquid chromatography by using them to analyze a real-life proteomics sample (*A. thaliana* chloroplast) has been

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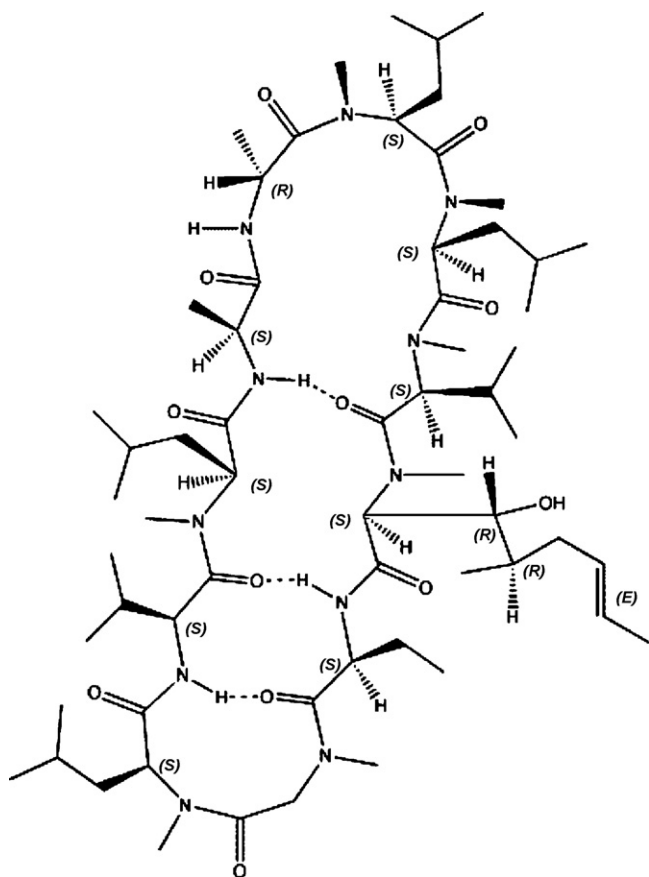


Fig. 1. Chemical structure of Cyclosporin A.

studied [14]. Superior separations were difficult to achieve on PS-DVB when strict MS-targeted conditions were adhered to (i.e. no additional ion-pairing agents were permitted in the mobile phase). In such cases, the conclusion was that nano-LC columns made from monolithic silica outperform those made from polystyrene in terms of separation efficiency and number of peptides collected. In addition, the higher permeability of the monolith allowed higher flow rates to be used, which enabled an increase in the information throughput. This should be of particular interest to those who wish to couple RP-HPLC with separation methods without sacrificing analytical throughput.

The aim of this work was to develop a new LC-MS method for CsA determination in human plasma by using the monolithic-type columns (Chromolith Performance) in combination with an automatic solid phase extraction (SPE) system in order to approach for optimizing its extraction and quantitation, after oral administration, achieving fast separation and high throughput.

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ, USA). Ultra-pure water was obtained from Milli-Q water purification system (Millipore, Milford, MA, USA). Analytical grade ammonium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA). CsA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Blank human plasma (Sodium heparin as anticoagulant) was used to prepare calibration and quality control samples (Valley Biomedical).

2.2. Instrument

The HPLC equipment consisted of an HP 1100 Series with a ChemStation (Hewlett Packard, Waldbronn, Germany) with electrospray, positive mode (API-ES). The separation column was Chromolith Performance RP-18e (10 mm × 4.6 mm) from Merck (Merck KGaA, Darmstadt, Germany) was eluted by 90% acetonitrile/10% ammonium acetate buffer pH 5.1 with a flow rate of 1 ml/min. The injection volume was 50 µl and the column temperature was maintained at 43 °C.

The LC-API-ES-MS conditions were set as follows: the fragmentor voltage was 250 V, the nitrogen gas flow was maintained at 11 l/min, the nebulizer pressure was set up at 40 psig, the positive capillary voltage was 3500 V and the drying gas temperature was 350 °C. The quantification is based on the total peak area of the CsA in SIM chromatogram.

2.3. Automatic sample preparation

The extractions were performed in an automatic Gilson ASPEC XL (West Beltline, Hwy, USA). The system is designed for the automation and optimization of SPE in order to provide a more efficient sample preparation. In addition the system is fitted with an injection valve for performing SPE with on-line injection onto the system.

Plasma samples (500 µl) were loaded onto the extraction cartridges (Oasis HLB, Waters Corporation, Milford, MA, USA), which had been preconditioned sequentially using 500 µl of methanol and 500 µl of water. The cartridge was then washed using 500 µl of water followed by elution with 500 µl of methanol with 2% of HCl. The eluted samples were directly injected into the LC-API-ES-MS system.

2.4. Methods

2.4.1. Optimization of LC-API-ES-MS experimental conditions

In order to set up the optimized conditions, the following parameters were tuned: the fragmentor voltage (50–250 V), the capillary voltage (1000–4000 V), the nebulizer pressure (40–60 psig) and the drying temperature (200–350 °C).

2.4.2. Calibration curve

The determination of CsA was based on the external standard method. Five point calibration curves (triplicate injections) were created at 0.05, 0.125, 0.250, 0.500 and 1.000 µg/ml.

2.4.3. Recovery, precision and accuracy

The recoveries were determined by comparing the peak areas of plasma premixed with a known amount of CsA with that of an equivalent amount of standard CsA dissolved in pure methanol. For intra-day precision, spiked plasma samples with three different concentrations (0.050, 0.250 and 1.000 µg/ml) were analyzed. For the inter-day precision, the above samples were analyzed on three subsequent days.

Accuracy was measured using CsA standard samples (0.050, 0.125, 0.250, 0.500 and 1.000 µg/ml) and calculated as the deviation from the theoretical values.

3. Results and discussion

3.1. Optimization of the LC-API-ES-MS conditions

In order to measure the concentration of CsA in plasma with high sensitivity the API-ES interface parameters including the fragmentor voltage, the positive capillary voltage and the drying gas temperature were tested.

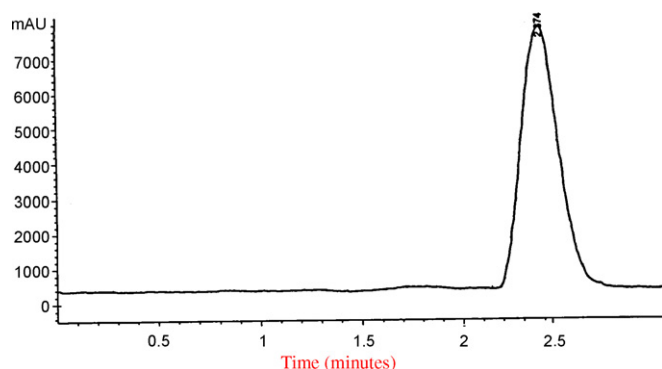


Fig. 2. Chromatographic profile of Cyclosporin A (CsA) after optimization of the chromatographic system.

The fragmentor was set up at 250 V. The positive capillary voltage was raised at 3500 V, and the optimal conditions for the drying gas temperature was approached at 350 °C.

With the optimal conditions the peak quality is shown in Fig. 2. As can be seen in Fig. 2 the retention time obtained is ≈ 2.4 min, due to the high porosity of the monolithic silica rod columns which possess a higher total porosity, allowing the use of a high flow rate achieving a low backpressure. The decrease on retention time is evident in comparison with previous studies. Salm et al. [13] obtained a retention time of 17 min for CsA by using a 10 μ m Bondepak C₁₈ column (3.9 mm \times 300 mm I.D., 10 μ m particle sizes, Waters, Milford, USA). The mobile phase was a mixture of acetonitrile–methanol–deionized water (55:15:30) (v/v/v). The use of other type of particulate column (phenyl type) is also described in the bibliography for CsA samples [10] achieving a retention time of 8 min.

3.2. Linearity, accuracy and recovery of the LC–API–ES–MS method

The LOD, defined as the minimum analyte concentration that produced an instrumental signal significantly different from that of the blank, was calculated in accordance with the IUPAC's criterion, i.e. as the analyte concentration giving a signal exceeding that of the blank (y_B) by 3 times its standard deviation (S_B). The LOQ, defined as the minimum analyte concentration needed to ensure precise quantitative measurements, was determined similarly, using 10 times the standard deviation for the blank instead. Thus, LOD and LOQ were calculated from the following expression:

$$\text{LOD(LOQ)} = \frac{y_B + 3(10)S_B}{b}$$

where b is the slope of the calibration curve. The limit of detection (LOD) and the limit of quantification (LOQ) for the CsA in plasma were 0.0004 and 0.015 μ g/ml, respectively.

The standard curve was created between 0.050 and 1.000 μ g/ml with a typical correlation coefficient of 0.9989

Table 1

Accuracy of the LC–ESI–MS method ($n = 3$).

Theoretical concentration (μ g/ml)	Measured concentration (μ g/ml)	C.V. (%)	Accuracy (%)	Deviation (%)
0.050	0.046	4.70	91.86	−8.14
0.125	0.124	2.07	99.88	−0.12
0.250	0.259	1.92	103.86	+3.86
0.500	0.497	3.60	99.43	−0.57
1.000	1.092	4.30	109.18	+9.18

($A = -5399.16 + 6511.32 \times C$), where A achieves the absorbance obtained and C de-concentration achieved expressed in μ g/ml. The peak area increased also linearly over the range from 0.015 to 5.000 μ g/ml (correlation coefficient 0.99). Detailed accuracy data obtained by the analysis of a set of spiked plasma samples are listed in Table 1. The coefficient of variance (C.V.) was less than 3% over the whole analytical range and the deviations were less than 10%. Detailed precision data obtained by the analysis of a set of spiked plasma samples are listed in Table 2. The coefficients of variance for both, intra-day and inter-day data were less than 3% over the whole analytical range.

When CsA was added to blank plasma, over 90% was recovered with the 2% HCl methanol extraction in the automatized ASPEC XL system and analyzed by the optimized procedure (Table 2).

Table 2 shows the inter-day and intra-day precisions which were less than 3% for 0.050, 0.125 and 1.000 μ g/ml samples.

The previous results show that the developed method has a good accuracy, recovery and precision, than other existing methods described before [15], but with the main advantage of the increased throughput due to the monolithic column which allows the decrease on the retention time (2.4 min) in comparison with other methods described before [10,11] and the automated SPE system in a sequential input. This input consists in a total extraction time of 6 min, while the LC–MS system is analyzing the previous sample. This total and real time of 6 min/sample allows the system to analyze 10 samples/h. At this point the blood samples under this study were analyzed following this possibility.

Other methods have been described in literature with the similar purpose [16]. It has been shown that the use of short monolithic silica columns coupled to mass spectrometry provides reduced analytical run times for the purpose of metabolite identification from different samples compared to conventional analytical chromatography, with no loss in chromatographic performance.

3.3. Application of the new method

Previous studies have shown that the absorption of oral CsA was shown to be slow and incomplete [17]. Peak blood CsA concentrations occurred between 1 and 8 h after dosing. The peak blood concentration ranged from 0.165 to 3.431 μ g/ml. Therefore our method is perfectly up to be used in pharmacokinetic studies after oral administration of CsA. This is the beginning for further studies that will be developed in the future.

Table 2

Recovery (%) of CsA ($n = 3$) from human plasma and precision of the LC–ESI–MS method.

Concentration (μ g/ml)	Recovery (% \pm SD)	Precision (C.V. %)	
		Intra-day (average)	Inter-day (average)
0.050	94.58 \pm 2.48	(1.68, 1.69, 1.73) 1.70	(2.62, 2.59, 2.64) 2.62
0.125	92.37 \pm 6.46	(2.06, 2.10, 2.06) 2.07	(3.10, 2.97, 3.21) 3.09
1.000	92.39 \pm 4.73	(1.93, 1.79, 1.80) 1.80	(2.86, 2.97, 2.60) 2.80
		Mean average (C.V. %)	
		1.84	2.81

4. Conclusions

The results shown in this report describe a simple and sensitive analytical method for the CsA in biological samples after oral administration. The monolithic-type column Chromolith decreased the retention time of the CsA from 12 min with phenyl columns [10] into 2.4 min, and in combination with the highly automated nature of the method significantly improved the sample analysis throughput (6 min/sample). The LC-API-ES-MS method can detect samples in the range of 0.015–5.000 µg/ml with linear response.

Added in proof

Salm et al. [18] have recently reported an lc-ms assay for CsA with a run time of 1.5 min per sample.

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