ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD FOR CYCLOSPORINE A QUANTIFICATION IN BIOLOGICAL SAMPLES AND LIPID NANOSYSTEMS

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ABSTRACT

Cyclosporine A (CyA) is an immunosuppressant cyclic undecapeptide used for the prevention of organ transplant rejection and in the treatment of several autoimmune disorders. An ultra high performance liquid chromatography-tandem mass spectrometry method (UHPLC-MS/MS) to quantify CyA in lipid nanosystems and mouse biological matrices (whole blood, kidneys, lungs, spleen, liver, heart, brain, stomach and intestine) was developed and fully validated. Chromatographic separation was performed on an Acquity UPLC® BEH C18 column with a gradient elution consisting of methanol and 2 mM ammonium acetate aqueous solution containing 0.1% formic acid at a flow rate of 0.6 mL/min. Amiodarone was used as internal standard (IS). Retention times of IS and CyA were 0.69 min and 1.09 min, respectively. Mass spectrometer operated in electrospray ionization positive mode (ESI+) and multiple reaction monitoring (MRM) transitions were detected, *m/z* 1220.69→1203.7 for CyA and *m/z* 646→58 for IS. The extraction method from biological samples consisted of a simple protein precipitation with 10% trichloroacetic acid aqueous solution and acetonitrile and 5 µL of supernatant were directly injected into the UHPLC-MS/MS system. Linearity was observed between 0.001 µg/mL-2.5 µg/mL ($r \geq 0.99$) in all matrices. The precision expressed in coefficient of variation (CV) was below 11.44% and accuracy in bias ranged from −12.78% to 7.99% including methanol and biological matrices. Recovery in all cases was above 70.54% and some matrix effect was observed. CyA was found to be stable in post-extraction whole blood and liver homogenate samples exposed for 6 h at room temperature and 72 h at 4 ^{o}C. The present method was successfully applied for quality control of lipid nanocarriers as well as *in vivo* studies in BALB/c mice.

Keywords: Cyclosporine A, UHPLC-tandem mass spectrometry, lipid nanocarriers, pharmacokinetics, biodistribution

1. INTRODUCTION

Cyclosporine A (CyA) is a neutral cyclic peptide consisting of 11 aminoacid residues, widely used for the prevention of transplant organ rejection and also for the treatment of autoimmune disorders such as psoriasis, rheumatoid arthritis and nephrotic syndrome. The importance of this drug as immunosuppressant was due to its selective lymphocyte inhibition action. The molecule was first isolated from the fungal extract of *Tolypocladium inflatum* [1]. Its structure and its high molecular weight (1203 Da) confer poor biopharmaceutical properties on this substance such as low water solubility and low permeability through biological barriers (gastrointestinal tract, skin and cornea). It is therefore a challenge to formulate an appropriate delivery system that improves its bioavailability and thus its efficacy. To date, lipid nanosystems (LN) seem to be a promising strategy to overcome the limitations associated with certain drug characteristics including low solubility, poor permeability, instability in the gastrointestinal medium, P-glycoprotein efflux and presystemic drug metabolism [2]. Scientific efforts have therefore been employed to design novel delivery systems based on LN for CyA oral administration leading to better alternatives to those currently available on the market, which will be capable of enhancing its oral bioavailability and thus its efficacy [3-5].

In the field of novel dosage form development, it is important to have a suitable drug quantification method that allows us to evaluate its behavior in animal models and its determination in the new nanosystems as quality control. Several analytical techniques for monitoring CyA levels, including immunoassays and chromatography methods, have been reported in the literature so far [6-11]. Although immunoassays offer rapid analysis and easy handling, there are many concerns about these methods because of the cross-reactivity of the antibodies used with inactive CyA metabolites that may result in overestimation of the drug values [12-14]. For this reason, chromatography-based methods such as high performance liquid chromatography with ultraviolent detection (HPLC-UV), high performance liquid chromatography coupled to mass spectrometry detection (HPLC-MS) and ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), are considered the best options for the quantification of CyA in biological matrices [15-17]. HPLC-

UV has some limitations related to specificity and sensibility. The lack of chromophores in CyA structure implies the use of short-wavelength light detection (e.g. 205 nm, 210 nm) where many molecular species also absorbs, yielding to interferences with the quantification of the drug studied [18]. Consequently, laborious and time-consuming extraction procedures and chromatographical separations are required to improve the limit of drug quantification. During the past decade mass detection has attracted more interest since quantification is based on the relationship of mass/electric charge of the molecule and thus all compounds with different mass to the target do not interfere in the measurement [16]. This leads to both reduced sample preparation and chromatographical separation efforts in complex matrices. Currently, UHPLC-MS/MS has become an attractive alternative to the quantification of this immunosuppressant because it offers multiple advantages: shorter analysis time, lower solvent consumption, minimal sample pre-treatment and also a higher range of measurement and throughput of samples. In addition, this technique is considered the best choice in pharmacokinetic studies due to its sensitivity to detect low concentrations of drug after a unique dose and in a long period of time and its specificity to detect it in complex biological matrices [19]. There are so far no validated UHPLC/MS-MS methods that allow the assessment of CyA tissue distribution in animal models when the drug is administered encapsulated in novel delivery nanosystems. Based on this, the main objective of the present research work was to develop and validate a simple, rapid, sensitive and specific UHPLC/MS-MS method to quantify CyA in lipid matrices and also in biological samples (whole blood, kidneys, lungs, spleen, liver, heart, brain, stomach and intestine) using the same analysis conditions, and to study the pharmacokinetic and biodistribution behavior of the drug *in vivo* as well as the quality control of the new lipid nanocarriers developed.

2. EXPERIMENTAL

2.1. Chemicals and reagents

CyA was provided by Roig Farma S.A. (Barcelona, Spain). Amiodarone hydrochloride (the internal standard, IS) and formic acid 99% were obtained from Sigma-Aldrich (Madrid, Spain).

Methanol and acetonitrile (both HPLC grade) were supplied by Merck (Barcelona, Spain). Ammonium acetate (HPLC grade) was purchased from Scharlau (Sentmenat, Spain). Trichloroacetic acid was obtained from Panreac Quimica S.A. (Barcelona, Spain). Sandimmun Neoral® 100 mg/mL oral solution and Sandimmun® 50 mg/mL intravenous solution were provided by Novartis Pharmaceutical (Barcelona, Spain). Polyvinyl alcohol (PVA) (87%-89% hydrolyzed, typical MW 13,000-23,000) was obtained from Sigma-Aldrich (Madrid, Spain). Precirol® ATO 5 was a gift from Gattefossé (Lyon, France). Other reagents employed for analysis were of analytical grade. Type I deionized water (18.2 MΩ resistivity) was obtained using a water purification system (Wasserlab, Pamplona, Spain). Nitrogen gas (ultra-pure, >99%) was produced by a Domnick Hunter LCMS series (Madrid, Spain). Argon gas (ultra-pure, >99.9%) was provided by Praxair (Madrid, Spain).

2.2. Instruments and analysis conditions

The UHPLC system consisted of an Acquity UPLC™ system (Waters Corp., Milford, USA) with thermostatized autosampler and a column compartment. Chromatography was performed on an Acquity UPLC® BEH C18 column (50 mm x 2.1 mm, 1.7 µm; Waters, USA) equipped with an Acquity UPLC® BEH C18 VanGuard™ precolumn cartridge (5 mm x 2.1 mm, 1.7 µm; Waters, USA), using a gradient elution program. The mobile phase system consisted of 2 mM ammonium acetate aqueous solution with 0.1% formic acid (A) and methanol (B). The gradient program was: initial-0.6 min 80% B; 0.61-2 min 95% B, 2.1-3 min 80% B with a flow rate of 0.6 mL/min. Total run time was 3 min. Column temperature was maintained at 50 ^{o}C and the autosampler was thermostatized at 4 ^{o}C. The volume injected was 5 µL.

Mass spectrometric detection was achieved on an Acquity™ TQD (Triple Quadrupole Detector) mass spectrometer (Waters Corp., Milford, USA) with an electrospray ionization (ESI) interface. The mass spectrometer was operated in positive mode and set up for multiple reaction monitoring (MRM) to monitor the transition of *m/z* 1220.69→1203.7 for CyA and the transition of *m/z* 646→58 for amiodarone with the dwell time of 0.1 s per transition. To optimize the mass parameters, standard solutions of both the analyte and the IS were infused into the mass

spectrometer at a concentration of 10 µg/mL. The following optimized mass parameters were employed: 135 °C source temperature, 420 °C desolvation temperature, 2.5 kV capillary voltage and 30 V cone voltage for both CyA and IS. Nitrogen was used for the desolvation and as cone gas at a flow rate of 600 L/h and 60 L/h, respectively. Argon was used as the collision gas. The optimized collision energy was 20 eV and 50 eV, for CyA and IS, respectively. Under these conditions, CyA and IS were eluted at 1.09±0.02 min and 0.69±0.02 min, respectively. Data acquisition and analysis were performed using the MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, USA).

2.3. Preparation of standard and quality control (QC) solutions

2.3.1. Stock and working solutions

Stock solutions of CyA and IS were prepared in methanol at 100 µg/mL. Further dilutions in methanol were performed to obtain working solutions of CyA at 0.005, 0.0125, 0.025, 0.5, 2.5, 5 and 12.5 µg/mL. Quality controls were prepared in the same way as calibrators at four different concentrations of 0.005, 0.015, 7.5 and 10 µg/mL (Low Limit of Quantification (LLOQ) and low, medium and high concentration, respectively) from independent stock solutions. The working solution of IS was obtained by diluting the stock solution with methanol until a concentration of 0.25 µg/mL. Lastly, the working solutions and QC were stored at 4 °C and were brought to room temperature before use.

2.3.2. Standard solutions and QC samples for whole blood and tissue samples

Calibration samples were prepared by spiking 20 µL of each CyA and IS working solution to 100 µL of mouse blank whole blood or tissue homogenate, therefore providing drug concentrations of 0.001, 0.0025, 0.005, 0.1, 0.5, 1 and 2.5 µg/mL. QC samples were prepared in the same way as calibration samples at concentrations of 0.001 µg/mL (LLOQ), 0.003 µg/mL (low), 1.5 µg/mL (medium) and 2 µg/mL (high). The solutions obtained (calibrators and QC) were then processed following the extraction procedure described in section 2.4.

2.3.3. Lipid nanoparticles

Calibration samples were prepared by diluting the working solutions of CyA in methanol in order to obtain drug concentrations of 0.001, 0.0025, 0.005, 0.1, 0.5, 1 and 2.5 µg/mL. Next, an aliquot of 20 µL of IS working solution (0.25 µg/mL) was added. QC samples were prepared in the same way as calibration samples, obtaining concentrations of 0.001 µg/mL (LLOQ), 0.003 µg/mL (low), 1.5 µg/mL (medium) and 2 µg/mL (high). Finally, an aliquot of 5 µL of calibrators and QC samples were injected into the UHPLC system.

2.4. Sample preparation

2.4.1. Whole blood samples

Whole blood samples from BALB/c mice were collected in EDTA-K3 surface-coated tubes, stored at -80 ^{o}C until analysis and thawed to room temperature before use. To an aliquot of 100 µL of whole blood was added 20 µL of methanol and 20 µL of IS working solution. After vortex mixing for 30 s, 10 µL of 10% trichloroacetic aqueous solution were added and vortex-mixed for 30 s for protein precipitation. Then, 50 µL of acetonitrile were added to the mixture and vortex-mixed for 1 min. Samples were then centrifuged at 14,500 x *g* for 15 min, and the supernatant was filtered (0.22 µm centrifugal filter, Millipore Corp, Billerica, USA). Last, 5 µL were injected into the UHPLC system.

2.4.2. Tissue samples

Tissue samples were weighed and homogenized in 1 mL of Phosphate Buffered Saline 10 mM (PBS) using a Mini-bead Beater (BioSpect Products, Inc, Bartelsville, USA) and centrifuged at 10,000 x *g* for 10 min. The supernatant was separated, stored frozen at −80 ^{o}C until analysis and thawed to room temperature before use. To 100 µL of tissue homogenate were added 20 µL of methanol and 20 µL of IS working solution and votex-mixed 30 s. Then, 10 µL of 10% trichloroacetic aqueous solution were added to the mixture and vortex-mixed for 30 s. An aliquot of 50 µL of acetonitrile was added to the mixture. After vortex-mixing for 1 min and

centrifuging at 14,500 x *g* for 10 min the supernatant was filtered (0.22 µm centrifugal filter, Millipore Corp, Billerica, USA) and 5 µL were injected into the UHPLC system.

2.4.3. Lipid nanoparticles

A 500 µL aliquot of chloroform was added to 5 mg of lyophilized nanoparticles. Then, 1.5 mL of methanol were added to the mixture. After vortex-mixing for 30 s and centrifuging at 21,000 x *g* for 10 min, to an aliquot of 100 µL of the supernatant was added 900 µL of methanol. Finally, 100 µL of this final solution were mixed with 20 µL of IS working solution (0.25 µg/mL) and 80 µL of methanol and 5 µL aliquot were injected into the UHPLC system for analysis.

2.5. Method validation

The analytical method was validated on biological matrices and methanol, including selectivity, linearity, precision and accuracy, recovery, matrix effect and stability according to FDA guidelines [20].

2.5.1. Selectivity

The selectivity of the method was evaluated by analyzing blank (whole blood, tissue homogenate and methanol) and spiked samples at LLOQ level to compare interferences at retention times of CyA and IS. Absence of the peaks at the retention times of the analytes in blank samples must be observed. In any case, the response does not exceed 20% of the LLOQ for CyA and 5% for the IS.

2.5.2. Linearity

The linearity of the present method was evaluated by analyzing calibration samples at concentrations ranging from 0.001 µg/mL to 2.5 µg/mL in duplicate on three different validation days, using the internal standard method. Standard curves were calculated using a linear weighted ($1/x^2$) least squares regression between the peak area ratios of the CyA to the IS

and the theoretical CyA concentrations on calibration samples. LLOQ was defined as the lowest concentration of CyA quantified with an acceptable precision and accuracy (less than 20%).

2.5.3. Precision and accuracy

Within-day precision and accuracy were assessed with the analysis of five determinations (n=5) of four QC samples at concentrations of 0.001 µg/mL (LLOQ), 0.003 µg/mL (low), 1.5 µg/mL (medium) and 2 µg/mL (high) on a single run. Between-day precision was also assessed with the analysis of four determinations of the four QC samples in five different validation days. Within- and between- day precisions were evaluated as a function of the coefficient of variation (CV), whereas accuracy was expressed as a function of deviation from theoretical values.

2.5.4. Dilution integrity

A dilution integrity experiment was performed in order to validate the dilution to be carried out on samples with CyA concentrations above the upper limit of quantification. Dilution integrity was evaluated by diluting at 1:15 and 1:3 a stock CyA solution in whole blood or stomach homogenate with blank matrix to give a theoretical concentration of 2 µg/mL. The precision and accuracy for diluted samples were determined by analyzing the samples against calibration curve standards. Dilutional integrity was considered acceptable if precision and accuracy of replicate (n = 5) values varied by less than 15%.

2.5.5. Recovery and matrix effect

The extraction recoveries and matrix effect were evaluated on whole blood and tissue samples in three replicate measurements (n=3) at three different concentration levels (0.003 µg/mL, 1.5 µg/mL and 2 µg/mL). Extraction recovery was calculated as the ratio of the mean peak areas from blank samples spiked with CyA before extraction and the mean peak areas of blank-processed samples spiked after extraction. Matrix effect was measured as the ratio of the mean of the peak areas obtained from blank-processed samples spiked with CyA after extraction and

the mean of the peak areas obtained from water and extraction solutions spiked with CyA corresponding to the equivalent amount injected on the system.

2.5.6. Stability

The stability study of CyA was performed in whole blood and liver homogenate samples after extraction. First, QC samples were prepared in triplicate at two levels of concentration (0.003 µg/mL and 2 µg/mL). Then, samples were stored under different conditions, at room temperature for 6 h and at 4 ^{o}C for 72 h. Finally, samples were analyzed and CyA was considered stable when accuracy biases of the stored QC samples were within ±15% of a freshly prepared standard curve.

2.6. Application of the method

2.6.1. Pharmacokinetic and biodistribution studies

To demonstrate the applicability of this validated UHPLC-MS/MS method, the quantification of CyA in whole blood and tissue samples from BALB/c mice was performed. Mice were divided in two groups (n=4) and treated with two commercial available formulations, Sandimmun® intravenously (i.v.) and Sandimmun Neoral® orally (p.o.). The administration in both cases was in a single dose equivalent to 10 mg of CyA per kg of body weight. Whole blood samples were withdrawn at 0 h (only for i.v. administration), 1, 2, 5, 8, 24 and 48 h post-administration, collected in EDTA-K3 surface-coated tubes and stored frozen (-80 ^{o}C) until analysis. Then the animals were sacrificed and kidneys, lungs, spleen, liver, heart, brain, stomach and intestine were collected, weighed and homogenized as previously described in section 2.4.2 in order to study CyA tissue distribution.

2.6.2. Determination of CyA encapsulated in lipid nanosystems

The present method was also used to evaluate the encapsulation efficiency and loading capacity of a lipid nanoparticle formulation (CyA-LN). The formulation was prepared by hot-homogenization followed by ultrasonication method previously described with slight

modifications [21]. Precirol® ATO 5 was used as lipid and a 2% PVA aqueous solution was used as surfactant. Triplicate samples were processed as described previously in section 2.4.3 and analyzed for drug quantification.

3. RESULTS AND DISCUSSION

3.1. Development of the analytical method

The main purpose of developing and optimizing the present UHPLC method was to quantify CyA, either in lipid nanoparticles or biological samples, under the same analysis conditions. Moreover, another intended purpose was to reduce, where possible, the lower limit of quantification for samples collected towards the end of the sampling schedule in pharmacokinetic and biodistribution studies after drug administration.

During the preliminary assays, different columns were tested including Zorbax C8, Kinetex HILIC and Acquity UPLC® BEH C18. Likewise, different mobile phases, organic solvents (methanol and acetonitrile) and aqueous solutions containing ammonium acetate with formic acid or acetic acid at different concentrations and different ratios were also tested. In addition, different flow rates, isocratic and gradient elutions and column temperatures were studied. All these conditions were assayed in order to obtain the best peak resolution and shorter retention time of CyA. The optimal chromatography conditions for CyA and amiodarone elution were achieved in an Acquity UPLC® BEH C18 column using as mobile phase methanol and 2 mM ammonium acetate aqueous solution with 0.1% formic acid, in the gradient profile described in section 2.2, a flow-rate of 0.6 mL/min and column temperature set at 50 ^{o}C. Similar mobile phase compositions have been successfully used previously in the literature [22,23]. High peak resolution and symmetry of CyA and IS were achieved. Although the retention times were 0.69±0.02 min and 1.09±0.02 min for IS and CyA, respectively, it was necessary to prolong the total run analysis for 3 minutes to stabilize the system pressure. Further, the chromatogram showed a rise in the baseline at about 1 min, as is commonly observed when using gradient elution. In the case of both lipid and biological matrices, no significant interferences were

observed in the retention times of the analytes in their respective transitions, *m/z* 1220.69→1203.7 for CyA and *m/z* 646→58 for IS (figure 1).

Moreover, since cyclosporine structural analogs are not commercially available, it was necessary to investigate other substances with physicochemical properties similar to CyA. Three different analytes, including rifampicin (MW 823), anidulafungin (MW 1140) and amiodarone (MW 645), were tested as internal standards. Evaluation of the fragmentation was performed by the infusion of each analyte into the mass spectrometer at a concentration of 10 µg/mL. Amiodarone showed the highest mass spectrometric response after the molecule fragmentation in the conditions assayed. Moreover, amiodarone has been used as an internal standard in previous studies where CyA was analyzed using HPLC-MS [24].

3.2. Mass spectrometry

Maximum sensitivity was observed by monitoring the fragmentation of CyA ammonium adducts $[M+NH_4]^+$, which by deamination, at low collision energy (20 eV), forms the protonated molecule $[M+H]^+$ in high abundance. The transition monitored was *m/z* 1220.69→1203.7. Moreover, a higher collision energy for amiodarone was needed (50 eV) in order to obtain the fragmentation of the ammonium adduct $[M+NH_4]^+$, resulting in the abundant fragment ion *m/z* 58 attributed to two ethyl groups attached to the tertiary nitrogen present in the molecule. In this case the transition monitored was *m/z* 646→58.

3.3. Sample preparation

Pre-treatment of the biological matrices is important since an inadequate procedure can, firstly, shorten the life time of the column when a large amount of samples are analyzed and, secondly, interfere with endogenous compounds present in the sample. Even though liquid-liquid extraction was widely used in the past for CyA extraction [11,24,27-29], the protein precipitation procedure was chosen among the extraction techniques, as it is a fast, simple sample preparation process. The first attempt in this study was to use acetonitrile as a unique precipitation reagent and this resulted in damage to the column. As a result, the novel aspect of

the sample preparation technique proposed in this paper is the addition of trichloroacetic acid as a precipitation reagent, which has not been previously published in CyA detection methods. It was observed that an aliquot of 10% trichloroacetic acid aqueous solution improved protein precipitation. This led to a reduction in the organic solvent volumes needed to precipitate proteins reported by other authors (methanol with zinc sulfate or acetonitrile) [7,9,18,25,26], enabling the direct injection of the final supernatant for analysis and consequently improved the lower limit of quantification. The use of trichloroacetic acid as precipitation reagent has not been previously published in CyA detection methods. In this case, CyA extraction method was a simple and fast protein precipitation procedure, avoiding time-consuming and labor-intensive extraction steps in solid phase extraction or liquid-liquid extraction (evaporation and reconstitution) used by other authors [11,24,27-29]. Also, the specificity of the MS/MS detector contributed to simplify sample preparation.

3.4. Method validation

3.4.1. Selectivity

Free-drug whole blood and tissue samples from different sources were tested in order to observe if there were endogenous components that could interfere in the analysis. These chromatograms were compared with chromatograms obtained from blank whole blood and tissue samples spiked with IS and CyA at the concentration of the LLOQ. No significant interferences were observed at the retention times of CyA and IS. Figure 1 shows chromatograms of blank samples, spiked whole blood and tissue samples with IS and CyA at the LLOQ analyzed by the UHPLC-MS/MS technique.

3.4.2. Linearity

The calibration curves of CyA in methanol and biological matrices were linear in the concentration range of 0.001 µg/mL–2.5 µg/mL with r-values ≥0.99 (n=3). The mean ±SD of the regression curve equations for all the matrices are shown in table 1. Calibrator concentration calculated from these regression curve equations expressed in terms of coefficient of variation

did not exceed 15% in any case. The lower limit of quantification of the drug in all samples was 0.001 µg/mL, which is sensitive enough to allow the quantification of CyA in complete pharmacokinetic and biodistribution studies in mice or even in other species. The present method covers a larger range of concentrations compared to those recently published and also decreases the LLOQ [11,22,23,30].

3.4.3. Precision and accuracy

Between- and within- day precision and accuracy values obtained from the four QC samples tested in methanol and biological matrices are summarized in table 2. The precision varied from 1.44% to 11.44%, while the accuracy varied from −12.78% to 7.99% in all cases. This was within the FDA acceptance criteria (<15%).

3.4.4. Dilution integrity

Dilution integrity was evaluated at two dilution factors, 1:3 for stomach samples and 1:15 for whole blood samples, at five determinations for each factor. The precision for dilution integrity 1:15 and 1:3 were 7.26% and 6.14%, respectively, while the accuracy results were 10.35% and 9.64%, respectively, which are within the acceptance limit of 15%

3.4.5. Recovery and matrix effect

In general, high extraction recoveries of CyA were obtained from the biological samples studied. This was above 98.72% from whole blood samples, values comparable to those reported by other groups [22,31]. Recoveries from homogenate tissue samples were about 85.12±7.71% (table 3). Moreover, some matrix effect with variability among the same tissue was observed. Percentages up to 64.61% in whole blood and up to 25.60% in tissue homogenates were obtained, meaning an ion suppression effect (table 3). This matrix effect could be explained by the presence of undetectable co-eluting compounds with the analytes in the post-extraction sample and, in the case of tissues, also the PBS used to homogenize the

samples. Bogusz et al. found a signal suppression caused primarily by zinc sulfate used in deproteinizing solution [26]. However, in the present method CyA diluted in extraction solution was evaluated and showed higher peak intensity than that obtained from blank-processed samples spiked with the drug. Therefore, it was noted that reagents used to precipitate proteins were not responsible for the matrix effect. On the other hand, when a small portion of solvent (1:1) is used in protein precipitation methods endogenous compounds, including fatty acids, triglycerides, nucleotides and salts will be present in the post-treated sample and could interfere in the ionization of the analyte leading to ion suppression [32]. Furthermore, since CyA is mainly bound to erythrocytes, whole blood is the matrix of choice for drug determination [33], so that more interferences are present in the post-treated sample. As was expected, variability in matrix effect occurred because the validation was performed in biological matrices obtained from different subjects, and therefore it was impossible to generate samples containing the same endogenous compounds in the matrices after the CyA extraction [34]. Despite these factors, it was proved that the precision, accuracy, selectivity, sensitivity and even reproducibility of this developed method were not compromised by the matrix effect.

3.4.6. Stability

Post-extraction stability of CyA in whole blood and liver homogenate samples was studied in a variety of situations, including being on bench top for 6 h and in the autosampler (4 ^{o}C) for 72 h. The drug was unaltered in extraction supernatant at room temperature for 6 h and at 4 ^{o}C for 72 h. All the QC samples both in whole blood and liver homogenate samples yielded accuracy biases within -12.5% and 8.7% of those of a freshly prepared standard curve (table 4). Previous studies confirmed CyA stability in methanol for 6 months at 4 ^{o}C, in blood during three freeze/thaw cycles, up to 24 h at room temperature and in a freezer at or below -15 ^{o}C [7].

3.5. Application of the method

As we have already mentioned, there are as yet no validated UHPLC/MS-MS methods that allow the assessment of CyA tissue distribution in animal models. Therefore, this is the first

time a method has been described that is sensitive enough to determine not only the biodistribution, but also the whole blood CyA concentrations needed to calculate the pharmacokinetic behavior of the drug.

3.5.1. Pharmacokinetic and biodistribution studies

The present method was successfully applied to quantify CyA in whole blood and tissue samples from BALB/c mice. Figures 2 and 3 depict the concentration *vs* time profile of CyA in mouse whole blood after a single 10 mg/kg drug administration intravenously and orally, respectively. As shown in figure 2, a typical i.v. drug profile was observed; maximum concentrations were achieved almost instantaneously and a steep decline in the curve was observed in the first two hours. Concentrations subsequently declined over time due to drug elimination. The mean Cmax was 21.24 µg/mL and the mean Cmin was 0.39 µg/mL during the 48 h-experiment. Figure 3 shows a typical concentration-time course when a drug is orally administered. In this case, the time to maximum concentration was influenced by the absorption and distribution rates. The mean Cmax 1.21 µg/mL was observed approximately 2 h post-administration and the mean Cmin was 0.15 µg/mL over the 48 h-experiment. This confirmed the applicability of the UHPLC method for the pharmacokinetic analysis of CyA after its oral administration. Figure 4 summarizes the CyA distribution to different mouse tissues after intravenous and oral treatment with 10 mg/kg 48 h post-administration. In all cases drug tissue levels were measurable by this method. Mean values ranged from 0.028 µg/g in brain (p.o.) to 3.315 µg/g in stomach (p.o.). CyA concentrations were higher in all organs after intravenous administration compared with oral, except in the stomach.

3.5.2. Determination of CyA encapsulated in lipid nanosystems

The CyA quantification method developed in this work was also employed to determine the drug encapsulated in Precirol® ATO 5 nanoparticles (CyA-LN), thus becoming the first validated UHPLC method applied to the quality control of the preparation of lipid based drug

delivery systems. After the analysis, the drug loading of the lipid nanosystem resulted in 11.94±0.35 µg of CyA per mg of formulation with an encapsulation efficiency of 79.72±2.35%.

4. CONCLUSION

An UHPLC-MS/MS method to quantify CyA was developed and validated. The method proved to be simple, rapid, reproducible, sensitive, selective, accurate and precise in methanol and biological matrices (whole blood, kidneys, lungs, spleen, liver, heart, brain, stomach and intestine) from BALB/c mice. The biological sample pre-treatment was found to be easy and reproducible. The method developed has demonstrated its suitability for pharmacokinetic and biodistribution studies and quality control of lipid nanosystems. This bioanalytical method could also be applied in other animal species or even in human matrices.

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Figure Legends

Figure 1. MRM transition chromatograms of blank samples at (A) *m/z* 1220.69→1203.7 and (B) *m/z* 646→58; MRM transition chromatograms of blank samples spiked with cyclosporine A at the LLOQ and amiodarone used as internal standard (0.25 µg/mL) at (C) *m/z* 1220.69→1203.7 and (D) *m/z* 646→58.

Figure 2. Whole blood concentration-time profile of cyclosporine A (CyA) over 48 h after a single intravenous administration (10 mg/kg) of Sandimmun® to BALB/c mice (error bars represent SD, n = 4).

Figure 3. Whole blood concentration-time profile of cyclosporine A (CyA) over 48 h after a single oral administration (10 mg/kg) of Sandimmun Neoral® to BALB/c mice (error bars represent SD, n = 4).

Figure 4. Tissue distribution of cyclosporine A (CyA) at 48 h after a single intravenous (i.v.) and oral (p.o.) administration (10 mg/kg) of Sandimmun® and Sandimmun Neoral®, respectively, to BALB/c mice (error bars represent SD, n = 4) measured by the UHPLC-MS/MS method.