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Journal of Chromatography B, 763 (2001) 201–206

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

# Determination of cyclosporin A in human and mouse plasma by reversed-phase high-performance liquid chromatography

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Received 17 May 2001; received in revised form 14 August 2001; accepted 22 August 2001

**Abstract**

An isocratic reversed-phase high-performance liquid chromatographic method with ultraviolet detection at 227 nm has been validated for the determination of cyclosporin A in human and mouse plasma. The cyclosporin D analog PSC 833 was used as internal standard. Plasma samples were pretreated by liquid–liquid extraction with diethyl ether. A good chromatographic separation between cyclosporin A, the internal standard and two potentially interfering endogenous peaks was achieved using a stainless steel column packed with 5 μm Nova-Pak phenyl material operated at 72°C, and a mobile phase consisting of acetonitrile–methanol–water (20:52:28, v/v/v). The calibration curve for cyclosporin A in human plasma was linear over the tested concentration range of 0.11 to 5.34 μ*M*. Murine plasma samples (200 μl) were diluted up to a total volume of 500 μl with blank human plasma and the concentrations were read from the calibration curve prepared in human plasma. The lower limit of quantitation was 0.11 μ*M* using 500 μl of human plasma and 0.28 μ*M* using 200 μl of mouse plasma. The validation data showed that the assay is sensitive, selective and reproducible for determination of cyclosporin A. The applicability was demonstrated in a pharmacokinetic experiment where mice received oral cyclosporin A.


*Keywords:* Cyclosporin A

## 1. Introduction

Cyclosporin A (CsA, Fig. 1) is an effective immunosuppressant drug used to prevent rejection after organ transplantation. Currently, this agent is also used in (pre-)clinical studies as an inhibitor of P-glycoprotein (P-gp). This membrane localized drug efflux pump transports a broad range of substrates, including many cytotoxic drugs [1]. P-gp was initially discovered by its ability to confer multidrug resistance in mammalian cancer cells [2]. Later, it was found to be present in many normal tissues as

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0378-4347/01/$ – see front matter 
PII: S0378-4347(01)00389-9

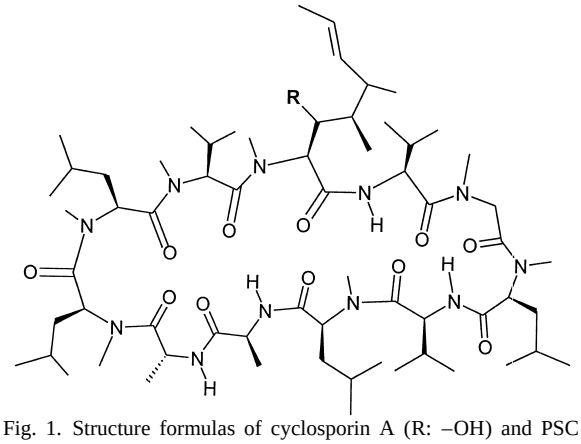


Fig. 1. Structure formulas of cyclosporin A (R: –OH) and PSC 833 (R: =O).

well, in particular those that have a barrier function, like the epithelial cells lining the intestinal wall and the endothelial cells in the brain forming the blood brain barrier [3,4]. The potential protective function of P-gp, for example by limiting the gastro-intestinal uptake of xenobiotics after oral ingestion, has now been well established [5]. We are currently investigating the oral route for administering paclitaxel and docetaxel, two anticancer agents which have very low oral bioavailabilities due to their affinity to P-gp [6]. We have shown that the oral bioavailability of paclitaxel can be increased significantly in mice [7,8] as well as in humans [9,10] by co-administration of CsA or the cyclosporin D analog PSC 833 (Fig. 1).

We have previously reported an analytical procedure for PSC 833 using CsA as an internal standard [11]. This assay was based on available methods for determination of cyclosporin compounds [12,13] and utilized liquid–liquid extraction with diethyl ether and isocratic high-perfromance liquid chromatography (HPLC) separation with UV detection. The use of a high column temperature is mandatory due to the chromatographic behavior of cyclosporins [14]. Moreover, the choice of the HPLC packing material and the exact composition of the mobile phase turned out to be critical factors [11]. To support our pharmacokinetic studies with CsA, we have adapted the assay for PSC 833 to allow quantification of CsA. The biological matrix of our choice was plasma, although whole blood is routinely used for monitoring CsA levels in patients [13]. In general, the unbound levels of inhibitors of P-gp are considered to be indicative for activity. Moreover, plasma concentrations are used for quantification of all other inhibitors of P-gp as well. The assay, which is presented here, has been validated with a lower limit of quantitation (LLQ) of 0.11 μ*M* using a 500 μl sample of human plasma and 0.28 μ*M* using a 200 μl sample of mouse plasma. The sensitivity is considered to be sufficient for therapeutic drug monitoring both in studies using CsA as P-gp inhibitor as well as using it in organ transplantation.

## 2. Experimental

### *2.1. Chemicals*

Cyclosporin A [Sandimmune, 50 mg/ml in Cremophor EL–ethanol (1:1, v/v)] originated from Novartis (Basel, Switzerland). PSC 833 (dry powder) was a gift from Dr. Lemaire (Novartis). All other chemicals were from E. Merck (Darmstadt, Germany) and were used as supplied, except for diethyl ether, which was glass distilled once before use. Filtered and deionized water (Milli-Q Plus system, Millipore, Milford, MA, USA) was used in all aqueous solutions. Blank human plasma originated from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands) and was obtained from healthy donors.

### *2.2. HPLC equipment and conditions*

The HPLC equipment consisted of a Spectroflow SF400 solvent delivery system (Kratos, Ramsey, NJ, USA), a Model MSI660 autosampler (Kontron, Basel, Switzerland) provided with a 100-μl sample loop, a Spectroflow SF757 absorbance detector (Kratos) set at 210 nm and a Model 345 solvent recycler (Alltech, Deerfield, IL, USA). A stainless steel analytical column (150×3.9 mm) packed with 5 μm Nova-Pak phenyl material (Millipore Waters, Milford, MA, USA) was used and was kept at 72°C by a Model SPH99 column thermostat (Spark Holland, Emmen, The Netherlands). The mobile phase consisted of acetonitrile–methanol–water (20:52:28, v/v/v) and was delivered at a flow-rate of 1 ml/min.

Peak detection and integration was done using a Model SP4600 DataJet integrator connected to a WINner on Windows Version 2.0 data station (Thermo-Separations Products, Fremont, CA, USA).

### 2.3. Drug stock solutions and internal standard

A stock solution of CsA with a concentration of 1.67 m*M* was prepared by diluting 1.0 ml of the 50 mg/ml pharmaceutical solution of CsA 25-fold in methanol. PSC 833 was used as internal standard. A stock solution of 1.31 m*M* was prepared by dissolving approximately 40 mg of accurately weighed PSC 833 in 25 ml of methanol. The internal standard working solution was a 25-fold dilution of the PSC 833 stock solution in acetonitrile–methanol–water (20:52:28, v/v/v). Aliquots of all solutions were stored at −20°C.

### 2.4. Preparation of calibration standards and quality control samples

A calibration standard of 5.34 μ*M* was prepared by two successive dilution steps of the CsA stock solution in drug-free human plasma. Aliquots were stored at −20°C. With each run, a set of calibration standards were prepared in duplicate containing CsA at concentrations of 5.34, 2.14, 1.07, 0.53, 0.21 and 0.11 μ*M* by serial dilution of the 5.34 μ*M* calibration standard using drug-free human plasma. Quality control samples in human plasma were prepared by dilution of an independently prepared CsA stock solution of 1.67 m*M* in drug-free human plasma to final concentrations of 0.27, 1.07 and 5.34 μ*M*. To prepare quality control samples in mouse plasma, the 1.67 m*M* CsA stock solution was first diluted to 50 μ*M* in drug-free human plasma. Next, this solution was diluted with drug-free mouse plasma to final concentrations of 5.01 and 0.50 μ*M*.

### 2.5. Sample pretreatment

To a volume of 500 μl of human plasma or 200 μl of mouse plasma diluted with 300 μl of human plasma, a volume of 50 μl of internal standard working solution and 3 ml of diethyl ether were added. After mixing vigorously for 5 min and centrifugation at 2000 *g* for 5 min, the aqueous layer was frozen in a bath of ethanol–dry ice. The organic layer was decanted into a glass tube, evaporated at 37°C under a gentle stream of nitrogen, reconstituted in 200 μl of acetonitrile–methanol–water (20:52:28, v/v/v), transferred into a polypropylene vial and placed into the autosampler for analysis.

### 2.6. Assay validation

A 3-day validation (2-day for mouse plasma) of the analysis of CsA was performed. Due to the limited availability, a limited validation was performed in drug-free mouse plasma. The statistical analyses were done with the software package SPSS for Windows (version 9.0; SPSS, Chicago, IL, USA).

#### 2.6.1. Linearity

The linear regression of the ratio of the peak areas of CsA and the internal standard versus the concentration were weighed by $1/x^2$ (reciprocal of the square of the concentration). The *F*-test for lack of fit ($\alpha = 0.05$) was used to evaluate the linearity of the calibration curves.

#### 2.6.2. Accuracy and precision

Quality control samples in human plasma were processed and analyzed (five replicates) in three runs and in mouse plasma (six replicates) in two separate runs. The accuracy was calculated by dividing the observed concentration and the nominal concentration and multiplied by 100%. The between-day precision was calculated by one-way analysis of variance (ANOVA) for each control sample using the run day as the classification variable. The day mean square (DayMS), error mean square (ErrMS) and the grand mean (GM) of the observed concentrations across run days were obtained. The within-day precision (WDP%) and the between-day precision (BDP%) for each quality control sample were calculated using the formulas:

$$\mathrm{WDP\%} = (\mathrm{ErrMS})^{0.5}/\mathrm{GM} \cdot 100\%$$

$$\mathrm{BDP\%} = [(\mathrm{DayMS} - \mathrm{ErrMS})/n]^{0.5}/\mathrm{GM} \cdot 100\%$$

where *n* is the number of replicates in each run. When the DayMS is smaller than ErrMS it means

that no additional variation was observed by performing the analyses in different runs.

*2.6.3. Selectivity*

Drug-free human plasma from six different healthy donors and pooled drug-free plasma from untreated FVB mice were processed and analyzed to determine whether endogenous plasma peaks co-eluted with CsA or the internal standard.

*2.6.4. Lower limit of quantitation*

The calibration standard of 0.11 μ*M* was processed and analyzed (6 replicates) in three separate runs to establish the accuracy and within-day precision. The LLQ was established when both the accuracy was within the 100±20% range and the WDP% was smaller than 20%.

*2.6.5. Recovery*

To determine the extraction recovery of CsA, the slopes of three independently processed calibration curves were compared to a standard curve prepared by dilution of the CsA stock solution in acetonitrile–methanol–water (20:52:28, v/v/v). The extraction recovery of the internal standard was calculated from the average peak area of a fourfold dilution of the internal standard working solution in acetonitrile–methanol–water (20:52:28, v/v/v) (two replicates) and the average of the areas of the internal standard peaks from the calibration standards of three separate runs.

## 3. Results and discussion

### *3.1. Selectivity*

Typical chromatograms of blank human plasma (Fig. 2A) and blank mouse plasma showed, besides peaks eluted close to the solvent front, two additional endogenous peaks at approximately 6.7 and 10.6 min. By optimizing the composition of the mobile phase a good separation of CsA and the internal standard PSC 833 from these endogenous peaks was accomplished (Fig. 2B). During routine use of the method for determination of PSC 833 we noticed that upon aging of the column the PSC 833 peak shifted towards peak $X_1$ [11]. By increasing the percentage of methanol relative to acetonitrile the PSC 833 peak could be selectively shifted towards peak $X_2$. Consequently, the composition of the mobile phase needs to be adjusted when necessary as described by van Tellingen et al. [11]. The column needs to be replaced when separation between the analytes and the endogenous peaks is not longer possible by adjusting the mobile phase as described above or when peak shapes begin to deteriorate. No late eluting peaks were detected, allowing a run time of 15 min per sample.

### *3.2. Validation*

Calibration curves in human plasma were linear over the concentration range of 0.11 to 5.34 μ*M*. This range was considered acceptable for our phar-

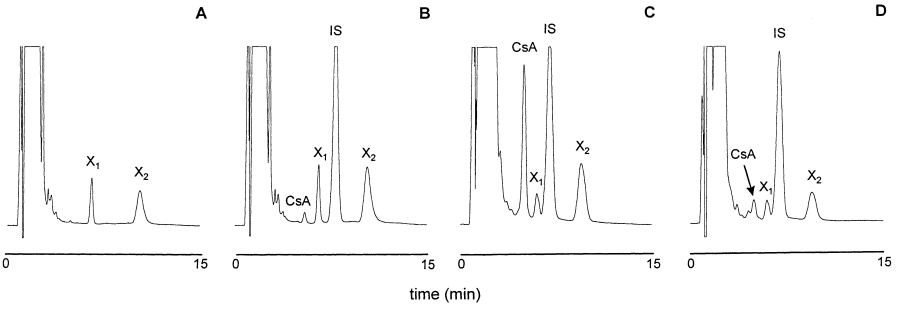


Fig. 2. Typical chromatograms of blank human plasma (A), human plasma spiked with 0.11 μ*M* of CsA (B) and plasma samples from mice obtained at 0.5 (C) and 24 h (D) following the oral administration of 50 mg/kg of CsA, containing 4.31 and 0.58 μ*M* of CsA, respectively. Peaks: CsA = cyclosporin A; I.S. = PSC 833 (internal standard); $X_1$ and $X_2$ are endogenous substances.

macokinetic studies. The slope, intercept and correlation coefficient of the calibration curve were $0.242 \pm 0.016$ l/μmol, $-0.608 \cdot 10^{-3} \pm 1.54 \cdot 10^{-3}$ peak-area ratio units and $0.9980 \pm 0.0010$ (mean±SD; $n=9$), respectively. The optimum weighting factor for fitting of the calibration curve was $1/x^2$ (reciprocal of the square concentration).

Based on preliminary results a LLQ of 0.1 μ*M* was considered appropriate for our pharmacokinetic purposes. Therefore, a calibration standard of 0.11 μ*M* was processed and analyzed in sixfold in three separate runs. A typical chromatogram is showed in Fig. 2B. The accuracy and precision were 88.8 and 8.1%, respectively, and met the requirements. This concentration could thus be used as the LLQ for human plasma samples. With only 200 μl of mouse plasma being used for analysis instead of 500 μl of human plasma, the LLQ in mouse plasma becomes 0.28 μ*M*. The upper limit of quantitation (ULQ) was considered to be equal to 6.1 μ*M* (115% of the concentration of the highest calibration standard).

Table 1 shows the accuracy and precision of the quality control samples prepared in human and mouse plasma. The accuracy of the quality control samples prepared in mouse plasma was approximately 110%, indicating a slightly increased extraction recovery of CsA in the presence of mouse plasma when compared to human plasma. However, all results were within the ranges that are acceptable for bio-analytical purposes. The absolute recovery of CsA and internal standard were 49.6±2.5% and 48.4±6.4% (mean±SD; $n=3$), respectively.

### 3.3. Applicability

The applicability of the assay was demonstrated in plasma samples of mice receiving 50 mg/kg of CsA by oral administration to enhance the oral absorption of the cytotoxic agent docetaxel which is a substrate of P-gp (Fig. 2C and D). So far, the assay has not been used to determine CsA levels in plasma from patients. However, given the results of the validation in human plasma it is reasonable to assume that the assay will also be suited for pharmacokinetic studies in humans.

## Acknowledgements

This work was supported by grant NKI98-1799 from the Dutch Cancer Society.

## References

[1] J.A. Endicott, V. Ling, Annu. Rev. Biochem. 58 (1989) 137.
[2] R.L. Juliano, V. Ling, Biochim. Biophys. Acta 455 (1976) 152.
[3] F. Thiebaut, T. Tsuruo, H. Hamada, M.M. Gottesman, I. Pastan, M.C. Willingham, Proc. Natl. Acad. Sci. USA 84 (1987) 7735.
[4] C. Cordon-Cardo, J.P. O'Brien, D. Casals, L. Rittman-Grauer, J.L. Biedler, M.R. Melamed, J.R. Bertino, Proc. Natl. Acad. Sci. USA 86 (1989) 695.
[5] O. van Tellingen, Toxicol. Lett. 120 (2001) 31.

Table 1
Validation parameters of cyclosporin A in human and mouse plasma

| Nominal concentration (μ*M*) | Measured concentration (μ*M*) | Accuracy (%) | Within-day precision (%) | Between-day precision (%) |
|---|---|---|---|---|
| *Human* | | | | |
| 0.27 | 0.23 | 86.2 | 1.6 | 11.2 |
| 1.07 | 0.99 | 92.5 | 6.7 | 2.2 |
| 5.34 | 5.15 | 96.3 | 1.9 | 6.7 |
| *Mouse* | | | | |
| 0.50 | 0.54 | 107.6 | 7.6 | 3.8 |
| 5.01 | 5.63 | 112.4 | 8.8 | –[a] |

[a] No additional variation was observed by performing the analysis in separate runs.

[6] A. Sparreboom, J. van Asperen, U. Mayer, A.H. Schinkel, J.W. Smit, D.K.F. Meijer, P. Borst, W.J. Nooijen, J.H. Beijnen, O. van Tellingen, Proc. Natl. Acad. Sci. USA 94 (1997) 2031.
[7] J. van Asperen, O. van Tellingen, M.A. van der Valk, M. Rozenhart, J.H. Beijnen, Clin. Cancer Res. 4 (1998) 2293.
[8] J. van Asperen, O. van Tellingen, A. Sparreboom, A.H. Schinkel, P. Borst, W.J. Nooijen, J.H. Beijnen, Br. J. Cancer 76 (1997) 1181.
[9] J.M. Meerum Terwogt, J.H. Beijnen, W.W. ten Bokkel Huinink, H. Rosing, J.H. Schellens, Lancet 352 (1998) 285.
[10] J.M. Meerum Terwogt, M.M. Malingre, J.H. Beijnen, W.W. ten Bokkel Huinink, H. Rosing, F.J. Koopman, O. van Tellingen, M. Swart, J.H. Schellens, Clin. Cancer Res. 5 (1999) 3379.
[11] O. van Tellingen, M. Kemper, F. Tijssen, J. van Asperen, W.J. Nooijen, J.H. Beijnen, J. Chromatogr. B 719 (1998) 251.
[12] M.G. Scott, K.G. Hock, D.L. Crimmins, P.M. Fracasso, Clin. Chem. 43 (1997) 505.
[13] H.G. McCoy, Pharmacotherapy 11 (1991) 165S.
[14] L.D. Bowers, S.E. Mathews, J. Chromatogr. 333 (1985) 231.