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Short communication

High-performance liquid chromatographic bio-analysis of PSC 833 in human and murine plasma

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Abstract

We have developed a rapid, sensitive and selective method for the determination of the cyclosporin analog PSC 833 in human and mouse plasma using cyclosporin A as internal standard. The assay uses liquid–liquid extraction with diethyl ether for sample clean-up followed by reversed-phase high-performance liquid chromatography with UV detection at 210 nm. Good peak shapes were obtained using a NovaPak Phenyl column operating at 72°C. Good selectivity from endogenous compounds was achieved using a mobile phase composed of methanol–acetonitrile–water (34:34:32). The retention times of cyclosporin A and PSC 833 were approximately 7.8 and 11.7 min, respectively, with two major endogenous peaks at 9.2 and 16.7 min. Selective decreasing of the retention times of cyclosporin A and PSC 833 relative to these interferences occurring upon aging of the column was balanced by increasing the percentage of methanol relative to acetonitrile. No other late eluting peaks were present, resulting in a total analysis time of 20 min per sample. The assay performance in human plasma was good. The absolute recovery of PSC 833 after the sample clean-up step was 48±6%. The lower limit of quantitation was 0.05 μ*M* using 500 μl of sample. Within the linear dynamic range of the assay (0.10–5.0 μ*M*) the accuracy was close to 100% and within-day and between-day variation less than 7%. Because of the limited availability of blank mouse plasma, the concentration in samples from mice were determined using calibration curves constructed in human plasma. The lower limit of quantitation in mouse was 0.25 μ*M* using 200 μl of sample. Overall, the performance of the assay in mouse plasma was somewhat less than in human plasma but accuracy and precision were within the ranges that are considered acceptable for bio-analytical assays.

Keywords: PSC 833

1. Introduction

PSC 833 (Fig. 1) is a non-immune suppressive cyclosporin D analog, which has been developed during the search for more effective and selective blockers of P-glycoprotein function. P-glycoprotein is a membrane associated drug transporting protein with a wide substrate specificity, including many cytotoxic and non-cytotoxic drugs [1]. Its (over) expression is known to confer multidrug resistance in

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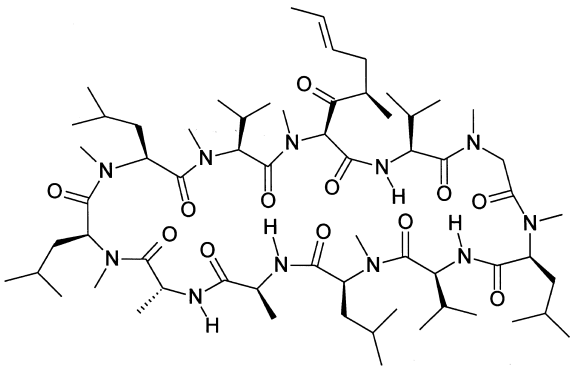

Fig. 1. Molecular structure of PSC 833.

mammalian cancer cells and the presence of this drug transporting protein in tumors of patients is believed to play a role clinical in multidrug resistance. The observation that chemical entities like verapamil were able to restore drug the sensitivity of drug resistant cells in vitro by inhibiting P-glycoprotein function prompted the search for more potent less toxic agents [2]. PSC 833 has emerged from these efforts and several promising results obtained in preclinical investigations have been reported [3,4]. As a result PSC 833 is currently under clinical evaluation [5,6].

Based on in vitro data, modulation of P-glycoprotein function in vivo is believed to require PSC 833 plasma levels of at least 1.0 μ*M* (approximately 1.2 mg/l). Consequently, an assay suited for the routine monitoring of PSC 833 levels should have a lower limit of quantitation (LLQ) of 0.5 μ*M* or less. Recently, a high-performance liquid chromatography (HPLC) method for PSC 833 in whole blood has been reported [7]. The reported method uses solid-phase extraction for sample clean-up and gradient elution and the LLQ of this assay was 0.8 μ*M* using 1 ml of sample. This relatively high LLQ was due to the very poor peak shape of PSC 833. PSC 833 eluted as two peaks, which was postulated to occur as a result of keto-enol isomerization at the 3′ position of the amino acid MeBmt at position one of the undecapeptide [7]. In order to obtain an acceptable peak shape gradient elution was essential, which resulted in a very long run time (60 min). To perform pharmacokinetic studies with this agent we required a rapid, sensitive and selective method for the determination in human plasma. Furthermore, because of our concomitant studies in small laboratory animals (mice) an additional requirement was that the required sensitivity (0.5 μ*M*) of the assay should already be met when using only small (≤200 μl) sample volumes. We here present a new rapid analytical procedure based on liquid–liquid extraction with diethyl ether and isocratic HPLC separation.

2. Experimental

2.1. Materials and reagents

PSC 833 was a gift of Dr. Lemaire (Novartis), and cyclosporin A (CsA) was purchased from Novartis, Basel, Switzerland as the pharmaceutical product formulated in Cremophor EL–ethanol (65:35, v/v, Sandimmune). 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. Water purified by the Milli-Q plus system (Millipore, Milford, MA, USA) was used throughout. Blank human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. HPLC instrumentation and conditions

The HPLC system was composed of a SF400 solvent delivery system (Kratos, Ramsey, NJ, USA), a Model 360 autosampler (Kontron, Basel, Switzerland) provided with a 100-μl sample loop, a Model SF757 UV–Vis detector (Kratos) operating at a wavelength of 210 nm and a Model SPH99 column thermostat (Spark, Emmen, The Netherlands) set at 72°C. Chromatographic separations were performed using a stainless steel column (150×3.9 mm) packed with 4 μm Nova-Pak Phenyl material (Millipore). The initial composition of mobile phase was a mixture of methanol–acetonitrile–water (34:34:32, v/v/v). However upon aging of the column this composition was adjusted as required (see Section 3.3). The mobile phase was delivered at a flow-rate of 1 ml/min. Peak recording and integration were performed on an SP4600 Datajet integrator connected to a WINner/8 system provided with WINner

for windows software. (ThermoSeparations Products, Fremont, CA, USA).

2.3. Stock solutions of drug and internal standard

Approximately 40 mg of SDZ PSC 833 was accurately weighed and dissolved in 25 ml of methanol. The final concentration of 1.310 m*M* was stored at −20°C and was stable for at least one year. CsA was used as internal standard. The pharmaceutical product of CsA containing 50 mg/ml was diluted 1000-fold in acetonitrile–water (40:60, v/v) and stored at −20°C.

2.4. Preparation of calibration standards and quality control samples

A calibration standard of approximately 5.00 μ*M* was prepared by dilution of 380 μl of PSC 833 stock solution with blank human plasma to yield a final volume of 100 ml. Aliquots were stored at −20°C. For each run, a set of calibration samples were prepared containing PSC 833 at concentrations of approximately 0.10, 0.25, 0.50, 1.00, 2.00 and 5.00 μ*M* by serial dilution of the 5.00 μ*M* standard using drug-free human plasma.

Quality control specimens in human plasma were prepared by dilution of the 5.00 μ*M* standard in drug free plasma to final concentrations of 0.25, 1.00 and 5.00 μ*M*.

Quality control samples in mouse plasma were prepared by mixing blank mouse plasma, calibration standard (st. 0.50 or 5.00 μ*M*) and drug free human plasma according to the following scheme:

Concentration (μ*M*)	Blank mouse plasma (μl)	Calibration standard (μl)	Blank human plasma (μl)
0.25	200	100 (st. 0.50)	200
0.50	200	200 (st. 0.50)	100
2.00	200	80 (st. 5.00)	220
5.00	200	200 (st. 5.00)	100

2.5. Sample pretreatment

A volume of 500 μl of human plasma sample or 200 μl of mouse plasma supplemented with 300 μl of blank human plasma was pipetted into a polyethylene PONY vials (Packard Instruments, Groningen, The Netherlands). Volumes of 50 μl of the internal standard and 3 ml of diethyl ether were added and the vials were mixed vigorously for 5 min. After centrifugation for 5 min at 2000 *g*, the aqueous layer was frozen by dipping the vials in bath of ethanol–solid carbon dioxide. The upper organic layer was decanted into a glass tube and evaporated at 37°C under a gentle stream of nitrogen. The residue was reconstituted by sonication for 5 min in 200 μl of acetonitrile–water (40:60, v/v). The solution was transferred into an Eppendorf vial and placed in the HPLC autosampler.

2.6. Assay validation

A full validation of the analysis of PSC 833 in human plasma and mouse plasma was performed. Statistical analyses were done with the computer program SPSS (version 6.1.3; SPSS, Chicago, IL, USA).

2.7. Linearity

The linear regression of the ratio of the peak areas of PSC 833 and 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.

2.8. Accuracy and precision

Control samples in human and mouse plasma were processed and analyzed in five-fold in each run. The accuracy was calculated by dividing the observed concentration and the nominal concentration and multiplied by 100%. An estimate of the between-day precision was obtained 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%) for each quality control sample was calculated using the formula:

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

The between-day precision (BDP) was calculated by the formula:

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

where n = the number of replicates in each run. If DayMS is smaller than ErrMS this means that no additional variation was observed by performing the analyses in different runs.

2.9. Selectivity

Blank human plasma from six different healthy donors and two different mouse strains (BALB/c and FVB) were processed and analyzed to determine whether endogenous plasma peaks co-eluted with PSC 833 or the internal standard. As PSC 833 will be given in combination with cytotoxic drugs we have tested plasma of mice receiving 10 mg/kg of paclitaxel, 6 mg/ml of vinblastine or 5 mg/kg of doxorubicin to check for interferences.

2.10. Lower limit of detection

The LLQ was determined by spiking the blank plasma samples from the six healthy donors with PSC 833 at concentrations of 0.05, 0.10 and 0.20 μM. The LLQ was established when both the accuracy was within the 100±20% range and the BDP% was smaller than 20%.

2.11. Recovery

The extraction recovery was determined by comparing the slopes of the processed calibration curves to standard curves prepared by dilution of the PSC 833 stock solution in acetonitrile–water (40:60, v/v). The recovery of the internal standard was calculated from the mean peak area of processed samples versus an aliquot of internal standard diluted four-fold in acetonitrile–water (40:60, v/v).

2.12. Stability

Due to the limited availability of blank mouse plasma the stability of PSC 833 was only checked in human plasma spiked with 1.0 μM of PSC 833. Aliquots were stored at 4°C and at room temperature for 16 h. A set of quality control samples was subjected to two freeze–thaw cycles before analysis to study the impact of this procedure. The stability of PSC 833 in the processed specimens kept at room temperature was checked for 64 h.

3. Results and discussion

3.1. Chromatography

Several analytical methods for CsA using reversed-phase chromatography have previously been described [8]. Cyclosporins are known to be difficult compounds to analyze. Typically, HPLC separations need to be performed at elevated temperatures because of poor column performance at ambient temperature [9] and due to the absence of a suitable chromophor for UV detection in the low-wavelength range is required. Recently, a method for PSC 833 has been published [7]. This report showed that the chromatographic behavior of PSC 833 resulted in poor peak shapes, even under conditions that are successfully applied for CsA. The poor peak shapes and double peaks, which may be due to keto-enol isomerization [7] markedly reduces the sensitivity of the assay. We experienced similar results when testing various types and brands of columns and packing materials for the analysis of PSC 833. However, relatively good chromatographic performance was obtained with the NovaPak Phenyl packing material. So far, we have utilized three different NovaPak Phenyl columns and the column-to-column performance was good.

3.2. Sample pretreatment

Although whole blood samples have been used by others, we prefer to use plasma. Sample pretreatment was performed by using once-distilled diethyl ether, as we occasionally experienced problems with additional interfering peaks when using the commercially available solvent as purchased. The absolute recovery of the sample pretreatment procedure was 86% for CsA (internal standard), which is in agreement with the recovery reported in previous studies using liquid–liquid extraction [10]. The recovery for PSC 833 was 48±6% which was higher than obtained to the reported method based on solid-phase

extraction on Sep-Pak C_{18} cartridges [7]. Since the recovery was considered relatively low for a bio-analytical method, we have tested the usefulness of a second repeated extraction of the aqueous layer to further increase the recovery. Although the recovery in the combined ethereal layers was further increased, we found that the height and number of potentially interfering peaks increased to a much greater extent. Consequently, since it was possible to meet the required LLQ using a single-step liquid–liquid extraction step (see Section 3.5), we have made no further attempts to increase the recovery.

3.3. Selectivity

A typical chromatogram of a blank human plasma sample is depicted in Fig. 2. Apart from the peaks that eluted close to the solvent front, two additional endogenous peaks (designated X1 and X2) were present in most human plasma samples. The mobile phase composition was optimized to obtain a good separation of PSC 833 and CsA from these two endogenous peaks. On new columns, the optimum mobile phase composition was methanol–acetonitrile–water (34:34:32). However, upon aging the PSC 833 peak shifted in the direction of peak X1. Increasing the amount of methanol relative to acetonitrile caused a selective retention of PSC 833 relative to X2. Consequently, the mobile phase composition was adjusted when necessary. Typically, the mobile phase composition using an older column was methanol–acetonitrile–water (55:15:30). Besides these two peaks no other potentially interfering peaks were detected. Moreover, no late eluting peaks were present, which allowed a total run time of less than 20 min per sample. The cytotoxic drugs paclitaxel, vinblastine and doxorubicin did not cause interfering peaks in the chromatograms.

3.4. Stability

PSC 833 was stable in plasma kept at ambient temperature and 4°C for at least 16 h. Two freeze–thaw cycles had no effect on the PSC 833 levels. Processed samples were stable at ambient temperature for at least 64 h.

3.5. Validation

The LLQ was established by spiking blank human plasma of six different individuals with 0.05, 0.10 and 0.20 μ*M* of PSC 833. The accuracy and precision at the 0.05 μ*M* concentration level already met the requirements (Table 1). With only 200 μl of mouse plasma being used for the analysis instead of 500 μl as for human plasma, the LLQ in mouse plasma would be 0.125 μ*M*. Since we considered an LLQ of 0.10 and 0.25 μ*M* in mouse and human plasma, respectively, appropriate for our pharmacokinetic purposes, we have selected a dynamic range for the calibration curve from 0.10 to 5.00 μ*M*. Calibration curves within this range were linear. The optimum weight factor for curve fitting was $1/x^2$ (reciprocal of the square of the concentration).

The results for accuracy and precision of quality control samples prepared in human plasma were well within the ranges that are considered acceptable for bio-analytical purposes (Table 2). The validation result in mouse plasma samples was also acceptable, although it was not as good as achieved in human plasma. Within the tested concentration range, except for the concentration at the LLQ, the accuracy was approximately 90%, indicating that the recovery of PSC 833 was slightly reduced by the presence of mouse plasma. Although this discrepancy could, in theory, be solved by adding a similar amount of blank mouse plasma to each of the calibration samples, this was not an option, because each calibration curve would require the sacrifice of seven to eight mice. Moreover, although the precision in mouse samples was less than in human plasma, it was within the 15% limits required for bio-analytical assays.

When using the assay for routine analysis over four months (eight runs), while adapting the mobile phase composition if necessary, the reproducibility

Table 1
Assay validation: determination of the lower limit of quantitation

Nominal concentration (μ*M*)	Measured concentration (μ*M*)	Accuracy (%)	Precision (%)
0.050	0.043	86.0	8.0
0.100	0.092	92.0	7.0
0.199	0.204	102.1	10.7

Table 2
Assay validation

Nominal concentration (μM)	Measured concentration (μM)	Accuracy (%)	Within-day precision (%)	Between-day precision (%)
Human				
0.249	0.260	104.4	6.2	4.3
0.996	1.027	103.1	2.3	4.3
4.973	5.070	101.9	3.2	–[a]
Mouse				
0.249	0.258	103.1	11.1	7.8
0.497	0.438	88.0	4.9	14.0
1.992	1.796	90.2	13.2	8.0
4.973	4.492	90.2	6.1	0.6

[a] No additional variation was observed by performing the analyses in different runs.

of the quality control samples assayed in duplicate in each series was better than 11.2%.

3.6. Applicability

The applicability of the assay was demonstrated in samples from mice receiving 25 mg/kg of PSC 833 by oral administration (Fig. 2c and d). So far the applicability in human plasma could not be tested as no human study samples were available. However, given the results of the validation in human plasma it is reasonable to assume that the assay is suited for human pharmacokinetic studies.

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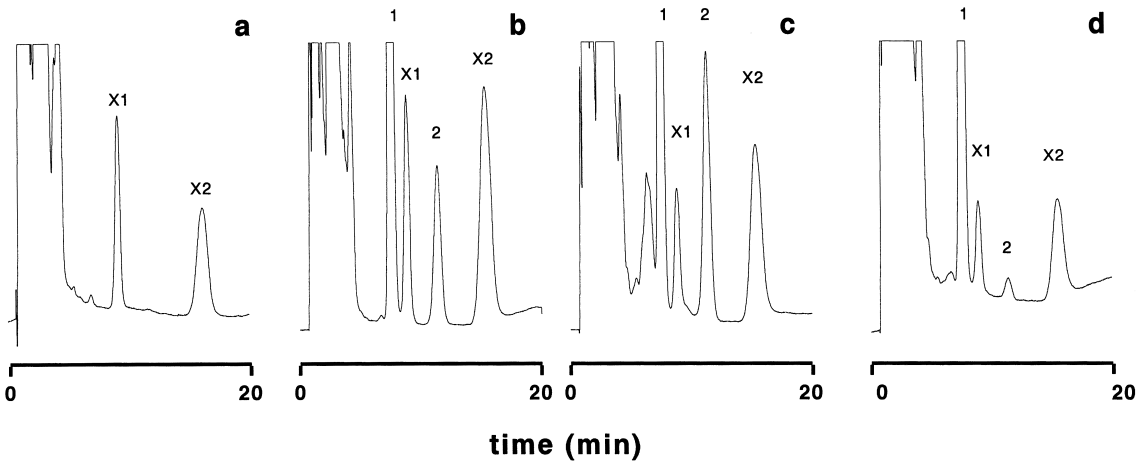

Fig. 2. Typical chromatograms of blank human plasma (a), human plasma spiked with 1.0 μM of PSC 833 (b) and plasma samples from mice obtained at 1 (c) and 24 h (d) following the oral administration of 25 mg/kg of PSC 833, containing 4.32 and 0.25 μM of PSC 833, respectively. Peaks: 1=CsA (internal standard); 2=PSC 833; X1 and X2 are endogeneous substances.

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