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Dependency of cyclosporine tissue distribution and metabolism on the age and gender of rats after a single intravenous dose

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Abstract

In a previous study we demonstrated the dependency of cyclosporine (CyA) pharmacokinetics on the age and gender of Wistar rats given 10 mg/kg intravenously. The present study has been conducted under the same experimental conditions (10 mg/kg as a single intravenous dose) to identify the mechanisms behind such differences. On the one hand, drug distribution was studied by measuring the CyA levels in blood, liver, kidney, spleen, adipose tissue, skin and muscle at 48 h post-treatment by using a specific fluorescence polarization immunoassay (*m*-FPIA, Abbott Laboratories). Drug blood and tissue levels in male rats were significantly higher than the female counterparts except for adipose tissue where the concentrations were 2-fold higher in females. In males, the highest CyA concentrations were observed in the liver, followed in rank order by kidney and spleen, fat, skin, muscle, then blood. On the contrary, females showed the highest drug levels in fat, followed by liver, kidney, spleen, skin, muscle and blood. Age exerted a significant influence on CyA tissue levels in males but no effect was observed in females. The potential differences in drug metabolism were established by measuring (HPLC) the amounts of CyA and its metabolites accumulated in faeces after hepatic biotransformation and biliary excretion. The amounts of circulating metabolites in blood as well as those accumulated and excreted in the liver and urine were also estimated by using specific (*m*-FPIA) and non-specific fluorescence polarization immunoassay (*p*-FPIA, Abbott Laboratories), respectively. The analysis of faeces revealed that AM9 was the major identified metabolite with females excreting lower amounts of unchanged CyA than males. In addition, the comparison of the AUC values corresponding to parent CyA and total CyA derivatives suggested that blood concentrations of CyA metabolites were higher in females indicating higher biotransformation rates. Therefore, both CyA distribution and metabolism are responsible for the sex-associated differences in drug pharmacokinetics previously found in rats.

Keywords: CyA; Metabolism; Pharmacokinetics; FPIA; Rats

1. Introduction

Cyclosporine is a potent immunosuppressive drug widely used to inhibit organ rejection in

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transplant patients and for the treatment of autoimmune disorders (Fahr, 1993). When orally administered, its poor and variable bioavailability has been attributed to a large number of physiological factors affecting drug absorption, distribution and metabolism in humans (Lindholm, 1991; Fahr, 1993). In 1994, a microemulsion pre-concentrate was introduced into clinical practice in order to improve drug absorption, however, despite of more reproducible pharmacokinetic profiles the higher C_{max} and shorter t_{max} values obtained were not always associated to larger AUC values and they may have implications for therapy, as well as for toxic adverse effects (Friman and Bäckman, 1996). Furthermore, some authors recommend CyA therapy with Neoral only in those patients with erratic and nonstable pharmacokinetics (Bennett et al., 1996; Johnston and Holt, 1996; Pollard, 1996). Therefore, many research groups are still focused to develop alternative formulations of CyA, which are initially assayed in rodents to test their potential advantages over other existing preparations. Rats have also been used in several studies in order to develop pharmacokinetic models that could be easily scaled-up to humans (Sangalli et al., 1988; Bernareggi and Rowland, 1991; Kawai et al., 1998). Hence, the pharmacokinetic studies carried out on animal models are crucial for the rational design of new formulations and allow for the identification of potential sources of pharmacokinetic variability that afterwards, must be carefully evaluated to establish whether they may be relevant for humans.

In recent years, data on human kidney transplant patients and experimental heart and skin transplantation in rats have evidenced that there is a small but obvious sex-associated difference in the survival time of the graft, with female recipients generally rejecting organ grafts in shorter time than males (Hirasawa and Enosawa, 1991; Hirasawa and Kamada, 1992; Takami et al., 1995). This phenomenon has been observed for different immunosuppressive treatments including CyA and the differences attributed to sex-related organs and steroid hormones but no data were reported on drug blood or plasma levels. Several studies indicate that female rats clear CyA faster than males independently of the administration route or dosing regime (Takami et al., 1995; Brunner et al., 1996; Molpeceres et al., 1998). Then, the gender-associated difference of graft survival in experimental transplantation in rats may be partly related to the sex dependency of CyA pharmacokinetics in this species. In spite of this, no reports on the mechanisms responsible for such pharmacokinetic differences have been published.

Upon administration to humans or rats, CyA undergoes extensive distribution and metabolism. CyA is slowly and extensively distributed in blood components (red blood cells and lipoproteins) and tissues (Bernareggi and Rowland, 1991; Kawai et al., 1998; Napoli et al., 1998), being the tissue distribution dependent on the drug partition within the blood components (Lemaire et al., 1988; Lindholm, 1991). The volume of distribution of a drug also depends on its affinity for a tissue compartment and on tissue size and permeability. Therefore, the lipophilicity of CyA suggests that its partition into adipose tissue should play an important role in drug pharmacokinetics. However, our pharmacokinetic studies in rats (Molpeceres et al., 1998) evidenced that female rats, which usually have a higher fat content, exhibited lower volumes of distribution than males. Moreover, separate experiments in humans have demonstrated comparable volumes of distribution in both obese and normal-weight individuals (Blouin and Warren, 1999). Hence, tissue permeability appears as a major determinant of CyA tissue distribution in accordance to the results obtained by Kawai et al., (1994, 1998) with CyA and its derivative, SDZ IMM 125. In this respect, the multidrug-specific membrane transporter, P-glycoprotein, has been involved in CyA cellular disposition because it prevents drug accumulation in the intracellular space. This protein is present in several tissues, such as the liver, kidneys, intestine and blood brain barrier with male rats consistently expressing several fold lower levels than females (Furuya et al., 1994; Salphati and Benet, 1998). Then, it appears feasible that the differential expression of the P-glycoprotein among male and female rats may play a significant role in CyA tissue distribution.

CyA is metabolized in the liver and the intestine of rats and humans through CYP450 3A mediated biotransformation; then, the drug and its metabolites are excreted in bile and accumulate in the faeces. Hence, alterations in hepatic metabolism of CyA may lead to changes in drug pharmacokinetics. Despite female rats cleared the drug faster than males (Molpeceres et al., 1998), no levels of 3A1 or 3A2 isoforms have been detected in uninduced female rats suggesting other isoenzymes should be involved. Recently, a female specific CYP450 3A isoform (3A9) was characterized in rat liver (Wang and Strobel, 1997), although CyA metabolism by this isoenzyme has not yet been proven. Thus, the different proteins involved in CyA biotransformation among male and female rats might also be responsible for the sex-dependency of CyA pharmacokinetics.

Due to rats having been used in several studies to predict CyA pharmacokinetics in humans (Sangalli et al., 1988; Bernareggi and Rowland, 1991; Kawai et al., 1998), the investigation of the mechanisms responsible for the sex-associated changes in CyA pharmacokinetics in rats may help in the interpretation of pharmacokinetic data obtained from humans. Therefore, the objective of this study was to analyze the dependency of drug distribution and metabolism on the sex and the age of Wistar rats in order to elucidate the basis for the pharmacokinetic differences previously found in rats.

2. Materials and methods

2.1. Chemicals

The IV solution of CyA was kindly supplied by the Ciudad Sanitaria La Paz, Madrid, Spain. All other reagents used were of analytical grade and the solvents of HPLC grade.

2.2. Experimental design and sample processing

A total of 20 Wistar rats were obtained from the Central Stabulary of the University (Homologation N° EC 28005-22A) and divided into four groups (five animals each) by age (10 and 40 weeks old) and gender. These rats were used for drug distribution studies and for measuring CyA compounds accumulated in the liver and excreted in faeces. Two additional groups ($n = 5$) of weight matched (250–300 g) males (10 weeks old) and females (40 weeks old) were used for pharmacokinetic evaluation in order to determine drug excretion in urine and the amounts of metabolites in blood. The rats were maintained in metabolical cages 24 h before the experiments, with a preserved 12 h dark–light cycle and free access to standard food and tap water. Before treatment, the animals were fasted overnight and had access to water ad libitum. CyA administration (Sandimmun® IV solution 50 mg/ml diluted with 0.9% saline NaCl to a final concentration of 12.5 mg/ml) was always carried out within the 09:00–10:00 h period to avoid chronopharmacokinetics effects. A bolus dose of 10 mg/kg was punctured into the right jugular vein of lightly isoflurane anaesthetized rats. A volume of 150 μl whole blood samples from the weight matched males and females (five animals each) were withdrawn from the left jugular vein of animals at 0 (pretreatment), 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 24, 26, 28, 32 and 48 h post-administration. Samples were collected in polyethylene vials over 20 μl disodium EDTA (22 mg·ml − 1), thoroughly mixed, splited into two fractions of 100 and 50 μl and frozen at − 20°C until further analysis within 2 days of the study. Urine was collected from under each cage; the urine volume was recorded and a 1.5 ml aliquot stored at − 20°C until drug content assay. Whole blood samples in the rest of animals were withdrawn from the abdominal aorta by insertion of a catheter 48 h post-administration. The total amount of faeces excreted as well as those retained in the intestine were collected and allowed to dry under ambient temperature. Drug levels in the liver, kidney, spleen, adipose tissue, skin and muscle 48 h post-administration were determined after tissues were perfused with 60 ml isotonic NaCl at 37°C via the catheter inserted in the abdominal aorta for blood collection. Once residual blood was eliminated, the organs were excised and a 300 mg portion (fresh weigth) from each one was homogenated in 2 ml MeCN to extract CyA. A volume of 120 μl TCA (10% in MeOH) was also added to complete protein precipitation.

After vortexing, the samples were centrifuged at 4500 rpm during 20 min and the supernantants (2 ml) evaporated under a nitrogen stream and mild heating (maximum 40°C) and stored at −20°C until analyzed. Finally, the dried extracts were resuspended in variable volumes of MeCN (500 μl for the liver and 300 μl for the rest of tissues) for drug analysis. Drug content in faeces was determined by using the procedure described for tissues excepting that the totality of dried faeces were extracted. Thus, appropriate amounts of MeCN were added to achieve the same sample matrix to solvent ratio as for organs but on the basis that higher amounts of drug and metabolites were expected, 5 ml of MeCN were used to redissolve the CyA derivatives from the final residue. All samples were centrifuged at 4500 rpm during 10 min before HPLC injection.

2.3. Drug analysis in blood and urine

To evaluate the influence of gender on CyA biotransformation, the concentrations of CyA and its metabolites in whole blood and urine from weight matched males (10 weeks old) and females (40 weeks old) (five animals each) were determined by using monoclonal (m-FPIA) and polyclonal antibodies (p-FPIA) coupled to fluorescence polarization immunoassay (TDx, Abbot Laboratories).

p-FPIA was only performed in blood samples obtained at 0.5, 2, 4, 10, 24, and 48 h post-administration. Calibration curves (0–1500 ng/ml for m-FPIA and 0-2000 ng/ml for p-FPIA) were obtained each time a set of samples was analyzed and their quality assessed by analyzing the control samples provided by the manufacturer. Under these conditions, percent recovery of CyA in the samples ranged from 95.22 to 102.16% and the within-day and between-day coefficients of variation did not exceed 4.66% for the same batch of reagents. The limits of quantification were 25 and 65 ng/ml for the specific and non-specific immunoassay, respectively.

2.4. Drug analysis in different tissues

Recently, the m-FPIA method for CyA determination in whole blood was confirmed as an alternative method to HPLC for quantifying CyA in animal tissues (Napoli et al., 1998). Hence, parent drug levels in the liver, spleen, kidney, fat, skin, muscle and faeces were also determined by fluorescence polarization immunoassay using monoclonal antibodies (m-FPIA, TDx, Abbot Laboratories). For this purpose calibration curves in each tissue or sample matrix were developed. The recovery compared to drug solutions in the absence of tissue or faeces was $83.62 \pm 12.15\%$. The analysis of drug levels in fat, skin and muscle were only performed in weight matched males (10 weeks old) and females (40 weeks old). CyA is mainly metabolized by the liver and its major metabolites are excreted and accumulate in faeces, thus the amounts of total CyA compounds in both sites were also determined. Because of the high extraction yields, the results were calculated assuming 100% recovery (Wagner et al., 1987). CyA levels in the liver were analyzed by p-FPIA while faeces were also analyzed by HPLC as described previously (Vickers et al., 1992). CyA and its metabolites were detected with UV–vis monitoring (Waters 484, Milford, MA) and identified according to the known retention times and by comparison of reference standards containing CyA and metabolites AM9, AM1 and AM4N (Novartis, Basel, Switzerland). The amount of CyA compounds determined by HPLC was calculated from external calibration curves of CyA considering that the molar extinction coefficients of CyA and its metabolites are equal (Christians and Sewing, 1993). Cyclosporine concentrations were converted to ng/g wet weight for comparisons to blood concentrations. Tissue to blood partition coefficients (K_p) were calculated according to the following equation (Bernareggi and Rowland, 1991):

$$K_p = \frac{C_t}{C_b(1-E)}$$

where C_t (ng/g) and C_b (ng/mL) are the tissue and blood concentrations, respectively, and E stands for the tissue extraction ratio. The E value was calculated from Cl/Q_H, where Q_H represents the hepatic blood flow (11.80 ml/min) (Bernareggi and Rowland, 1991). As the liver is the major determinant for drug elimination, E was set equal to zero in the remaining tissues.

2.5. Pharmacokinetic analysis

The pharmacokinetic parameters associated to each animal were estimated by noncompartmental methods. The zero-order moment area under the curve (AUC_T), was determined to the last experimentally measured concentration $C_n(t_z)$ at time t_z by the linear trapezoidal rule and extrapolated to infinity by adding the term $C_n(t_z)/\lambda_z$, where $C_n(t_z)$ denotes the concentration estimated at time t_z from the curve fitting and λ_z is the slope of the terminal phase estimated by log-linear regression of the last four to five experimental blood concentrations. The mean disposition residence time (MDRT) was derived from the ratio $AUMC_T/AUC_T$, where $AUMC_T$ is the area under the curve for the plot of the product of concentration and time versus the time from time zero to infinity. The total body clearance CL, the volume of distribution associated with the terminal phase V_β, and that at steady state V_{ss} were calculated by using the following equations: $CL = Dose/AUC_T$, $V_\beta = CL/\lambda_z$ and $V_{ss} = CL \cdot MDRT$.

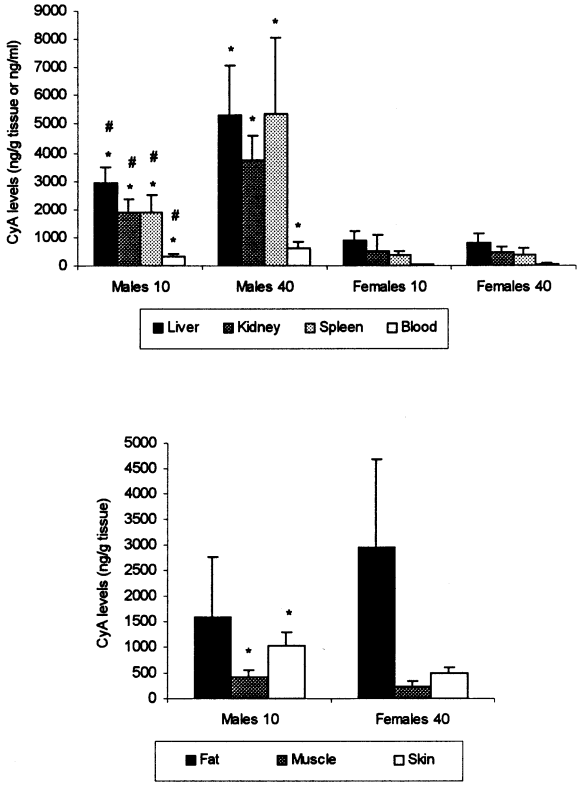

Fig. 1. CyA blood and tissue levels achieved at 48 h following a single 10 mg/kg IV dose administration. Upper: liver, kidney, spleen and blood of young adult (10 weeks old) and adult (40 weeks old) males and females. Lower: fat, muscle and skin of weight matched males and females (10 and 40 weeks old, respectively). $*P < 0.05$ males versus females. $\#\ P < 0.05$ 10 versus 40 weeks old males.

2.6. Statistical evaluation

CyA tissue levels in the four groups were compared by analysis of variance (two-way ANOVA) at the 0.05 significance level (EPISTAT software package). When statistical significant differences were found, the least significant difference test (LSD) was applied. It is the simplest and more powerful approach to a posteriori comparisons.

3. Results

3.1. Tissue distribution

As shown in Fig. 1, parent CyA was extensively distributed into many organs reaching higher tissue concentrations than blood at 48 h post-treatment, which is consistent with the high lipid solubility of CyA. Drug blood and tissue levels in male rats were higher ($P < 0.05$) than the female counterparts (Fig. 1, upper), except for adipose tissue where the average CyA levels in females were 2-fold higher than males (Fig. 1, lower). However, the differences in the latter case did not achieve statistical significance due to the variability observed. Age significantly altered CyA levels in males as shown by the two-fold higher CyA tissue and blood levels achieved in adult males as compared to young adults. On the contrary, the age of female rats did not exert a significant influence on CyA tissue and blood levels. The liver showed the highest CyA concentrations in most cases, with the exception of adult females where adipose tissue exhibited the highest concentrations (3-fold higher than liver). In order to normalize the results, the tissue-to-blood level ratios (K_p) were calculated and shown in Fig. 2. Despite the different CyA tissue concentrations

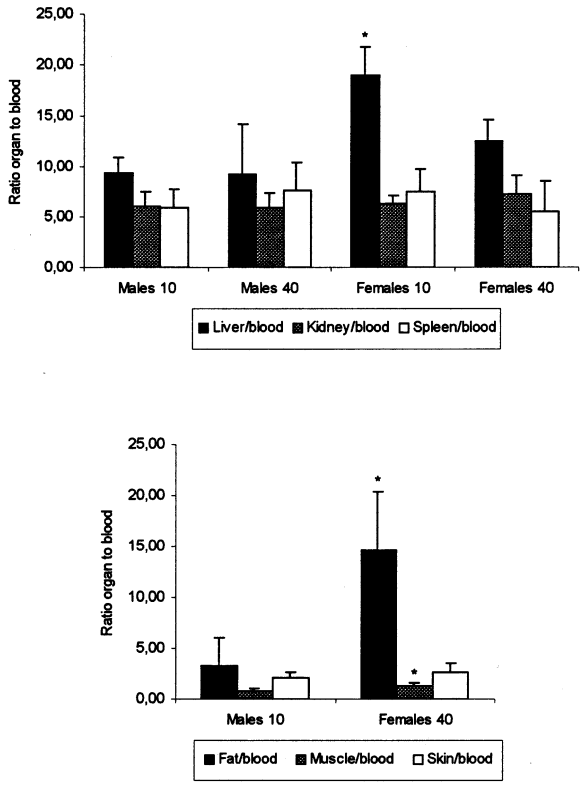

Fig. 2. Tissue-to-blood levels (K_p) achieved at 48 h following a single 10 mg/kg IV dose administration. Upper: liver, kidney and spleen of young adult (10 weeks old) and adult (40 weeks old) males and females. Lower: fat, muscle and skin of weight matched males and females (10 and 40 weeks old, respectively). $*P < 0.05$ males versus females.

achieved in each group (Fig. 1), similar drug tissue affinities were found in adult and young adult males. With respect to females, non significant differences in the K_p values corresponding to the skin, the spleen and the kidneys were found between males and females (Fig. 2). The skin showed an average of 2 to 3-fold higher concentrations than blood while the average CyA levels achieved in the spleen and the kidneys were between 6- and 7-fold above blood levels, showing no dependency on the age or the gender of rats. In respect to the rest of tissues, female rats presented a more marked tendency to accumulate parent CyA in the liver, fat and muscle tissue ($P < 0.05$). Indeed, CyA hepatic levels were all about ten-fold higher than blood levels in males regardless of the age whereas CyA concentrations in the liver of adult and young adult female rats were 13-fold and 19-fold higher than blood levels, respectively (Fig. 2, upper). Likewise, K_p values for adipose and muscle tissue in females (14.62 ± 5.73 and 1.28 ± 0.34) were higher than those found for males (Fig. 2, lower).

3.2. Metabolism and excretion

The dependency of CyA metabolism on rat gender was initially estimated from the concentrations of circulating metabolites in blood. To this end, the pharmacokinetic profiles corresponding to unchanged CyA and CyA plus some metabolites were determined. Plots showing the mean concentration–time data after IV administration of CyA to weight matched males (10 weeks old) and females (40 weeks old) are shown in Fig. 3. The upper part of Fig. 3 shows the blood levels of parent CyA and the lower part those corresponding to CyA plus metabolites. The mean pharmacokinetic parameters derived from a noncompartmental analysis are presented in Table 1. The terminal elimination half-lives ($\lambda_z - HL$) corresponding to parent CyA showed gender-associated differences and their mean values amounted to 26.97 ± 5.59 h and 14.59 ± 3.45 h for males and females, respectively. Total exposure to the parent drug (AUC_T) showed a strong dependency on gender ($P < 0.05$) and the extrapolated AUC contributed on average of $7 \pm 3\%$ for females and $22 \pm 7\%$ for males to the total AUC indicating an appropriate sampling period. The parameter V_{ss} was used to evaluate the differences in drug distribution, since, changes in V_β were also associated with changes in λ_z values. The higher V_{ss} found for males (3.37 ± 0.78 l/kg) suggest that unchanged CyA distributed significantly more abundantly in male rats than females ($V_{ss} = 2.49 \pm 0.12$ l/kg). Finally, females showed larger values of total body clearance (165.7 ± 37.8 ml/h·kg) than males (109.2 ± 15.7 ml/h·kg) and shorter MDRT (15.7 ± 3.80 vs. 31.2 ± 8.01 h) (Table 1).

Parent CyA blood levels in males and females were very close to the values obtained by using the non-specific method (p-FPIA) suggesting that

the amounts of circulating metabolites were small or that only small amounts of CyA metabolites reacted with the polyclonal antibody. Consequently, noncompartmental analysis of the pharmacokinetic profiles corresponding to CyA plus metabolites led to the same conclusions obtained with unchanged drug (Table 1) indicating that CyA metabolites follow the same behavior as parent drug within the body. However, the analysis of blood from females by using p-FPIA resulted in a significantly lower volume of distribution ($V_{ss} = 2.06 \pm 0.22$ l/kg) than that calculated when m-FPIA was used ($V_{ss} = 2.49 \pm 0.13$ l/kg) suggesting that unchanged drug has stronger tissue affinity than its metabolites in this group. The same result was obtained when the elimination half-lives were analyzed (11.63 ± 1.09 vs. 14.59 ± 3.45 h for females and 22.81 ± 6.16 vs. 26.97 ± 5.59 h for males, respectively) which showed slightly decreased values but the differences with those derived from parent CyA did not achieve statistical significance. The differences among the AUC values corresponding to parent drug and CyA plus metabolites represent a rough estimation of drug metabolism in vivo. In agreement with the small amounts of metabolites observed in blood, minor changes were detected for these parameters. Nevertheless, the average difference obtained in the AUC for females (7753 ng·h/ml) with respect to parent CyA was about 13%, whereas the one corresponding to male rats (5524 ng·h/ml) represented a 7% increase, suggesting that females metabolized the drug to a higher extent. This fact is consistent with the lower AUC values, shorter elimination half-lives and faster clearance rates described for females either by using m-FPIA or p-FPIA. Due to the fact that the liver plays a major role in CyA biotransformation and it contains proteins able to covalently bind CyA metabolites in rats, a second approach was taken to evaluate drug metabolism involving the indirect measurement of CyA metabolites at the hepatic level. Table 2 shows the concentrations of parent CyA and CyA plus metabolites present in the liver at 48 h post-treatment for each group. It becomes evident that the amount of extractable metabolites was greater ($P < 0.05$) in the liver of males showing no dependency on the age of the rats, although the ratio between unchanged CyA and CyA plus metabolites was slightly lower for older males (0.38 ± 0.11).

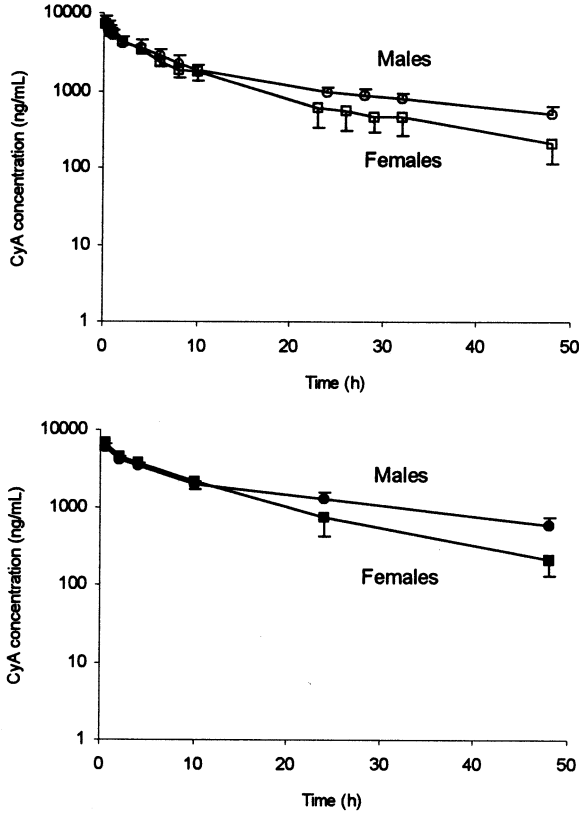

Fig. 3. Whole blood concentration-time profiles of unchanged CyA (upper) and total CyA derivatives (lower) (mean ± S.D., $n = 5$) following a single 10 mg/kg IV dose administration to weight matched males and females (10 and 40 weeks old, respectively).

Finally, CyA and its metabolites are excreted in the faeces and a small amount is also excreted in urine, therefore CyA plus metabolites were determined in both sites. Table 3 shows the amounts of CyA excreted in faeces for each group as well as the results corresponding to the analysis of urine by using m-FPIA and p-FPIA methods. Despite of the large variability found for the excretion of CyA compounds in faeces, the average amounts of parent drug excreted by males was higher than females, which is consistent with higher biotransformation rates in the female groups. The amounts of CyA metabolites excreted in urine

Table 1
Mean (± S.D.) pharmacokinetic parameters of CyA after noncompartmental analysis of kinetic data obtained by using monoclonal (m-FPIA) and polyclonal (p-FPIA) antibodies

Parameters	Males		Females	
	m-FPIA	p-FPIA	m-FPIA	p-FPIA
λ_z (h−1)	0.027 ± 0.01[a]	0.032 ± 0.01[a]	0.050 ± 0.01	0.060 ± 0.01
λ_z-HL(h)	26.97 ± 5.59[a]	22.81 ± 6.16[a]	14.59 ± 3.45	11.63 ± 1.09
AUC_n (ngh/ml)	72193 ± 9492[a]	77717 ± 8697	58259 ± 10559	66012 ± 13280
$AUC_{0\text{-}\infty}$ (ngh/ml)	92954 ± 1219[a]	98108 ± 17454[a]	62703 ± 13212	69460 ± 14844
$AUC_{n\text{-}\infty}$ (%)	22 ± 7[a]	20 ± 8[a]	7 ± 3	5 ± 1
MDRT (h)	31.21 ± 8.01[a]	29.46 ± 7.97[a]	15.70 ± 3.80	14.13 ± 2.34
V_{ss} (l/kg)	3.37 ± 0.77[a]	2.99 ± 0.48[a]	2.49 ± 0.13[b]	2.06 ± 0.22
V_β (l/kg)	4.22 ± 0.91	3.35 ± 0.62[a]	3.40 ± 0.60[b]	2.48 ± 0.39
Cl (ml/h per kg)	109.22 ± 15.70[a]	104.37 ± 17.28[a]	165.75 ± 37.80	150.30 ± 37.60

[a] $P<0.05$ males versus females.
[b] $P<0.05$ m-FPIA versus p-FPIA.

were also slightly higher in females although not significantly different. Fig. 4 represents a typical HPLC chromatogram of the faecal extract whereas the relative proportions of each identified peak in every group are shown in Table 4. A fair correlation between the content of CyA in faeces determined by HPLC and m-FPIA (slope = 1.09; $r^2 = 0.92$) (Tables 3 and 4) indicated the specificity of the m-FPIA method as previously reported (Napoli et al., 1998). Among the different metabolites previously found in faeces (Maurer et al., 1984), three primary (AM1, AM9 and AM4N) and one secondary (AM1c) metabolites were identified in this study. The relative proportions of CyA and its metabolites in faeces did not show any dependency on the age or gender of rats. In all cases the major metabolite was AM9 representing about 50% of the total CyA derivatives considered. A second important metabolic pathway involved the hydroxylation of CyA in aminoacid 1(AM1) and its subsequent cyclization (AM1c). The sum of these two metabolites amounted to 24% of the total CyA compounds except for young females where it reached an average value of 16%. Finally, the N-demethylation of aminoacid 4 (AM4N) constituted a minor pathway accounting for about 10% of the total CyA found in faeces.

Table 2
Mean hepatic levels (S.D.) of unchanged CyA and total CyA derivatives at 48 h post-treatment and liver weight (LW) to total body weight (BW) ratios in each group

Group	CyA m-FPIA (ng/g)	CyA + metab. p-FPIA (ng/g)	Ratio m-FPIA/p-FPIA	Ratio LW/BW
10 weeks	2929.01	6848.89	0.43	0.032
Males	(568.83)	(1570.62)	(0.05)	(0.003)
40 weeks	5348.56[b]	14051.78[b]	0.38	0.017[a,b]
Males	(1735.38)	(1971.72)	(0.11)	(0.003)
10 weeks	905.85[a]	1384.10[a]	0.78[a]	0.031
Females	(369.98)	(1058.94)	(0.27)	(0.003)
40 weeks	792.69[a]	1289.56[a]	0.64[a]	0.026
Females	(344.29)	(672.87)	(0.11)	(0.001)

[a] $P<0.05$ males versus females.
[b] $P<0.05$ 10 versus 40 weeks old males.

Table 3
Excretion of unchanged CyA and total CyA derivatives in urine and faeces 48 h post-treatment with a single intravenous dose (10 mg/kg) as determined by FPIA

Group	CyA Faeces (μg)	Total CyA faeces[a] (μg)	CyA metabolites in faeces (μg)	CyA urine (μg)	Total CyA urine (μg)	CyA metabolites in urine (μg)
10 weeks	27.71	186.80	159.09	30.97	66.30	29.12
Males	(12.37)	(80.10)	(71.36)	(5.71)	(8.14)	(5.76)
40 weeks	20.26	140.24	119.98	40.58	Nd [b]	Nd
Males	(26.54)	(133.19)	(127.17)	(10.26)		
10 weeks	19.88	125.21	105.33	11.62[c,d]	Nd	Nd
Females	(12.65)	(68.64)	(59.03)	(2.73)		
40 weeks	11.21	104.01	92.79	32.34	72.67	35.37
Females	(10.67)	(53.92)	(43.60)	(7.87)	(5.59)	(5.81)

[a] Determined by HPLC.
[b] Not determined.
[c] $P<0.05$ males versus females.
[d] $P<0.05$ 10 versus 40 weeks old females.

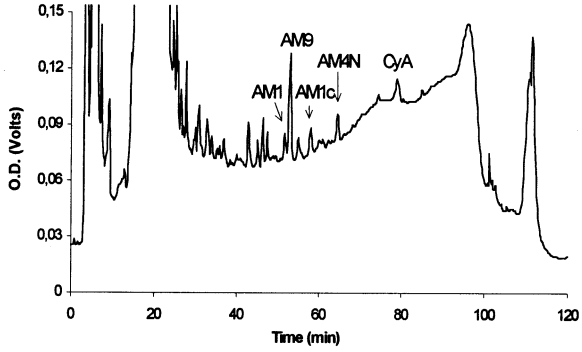

Fig. 4. Typical HPLC chromatogram of a faecal extract containing unchanged CyA and metabolites AM1, AM9, AM1c and AM4N.

4. Discussion

Recent evidence suggest that the dependency of CyA pharmacokinetics on rat gender may be partly responsible for the sex-associated rejection pattern found in experimental transplantation. However, the impact of these findings on human transplantation needs a careful examination. Rats have been used in several studies in order to develop pharmacokinetic models that could be easily scaled-up to humans (Sangalli et al., 1988; Bernareggi and Rowland, 1991; Kawai et al., 1998). Therefore, the knowledge of the mechanisms responsible for the pharmacokinetic differences previously reported in rats might help in the interpretation of data obtained from humans.

It is well documented that CyA undergoes extensive distribution into many tissues after intravenous administration to rats and humans. Due to its lipophilic nature, adipose tissue should be expected to play a significant role, however, several studies have demonstrated a non significant influence of obesity on CyA pharmacokinetics (reviewed in Blouin and Warren, 1999). Furthermore, previous (Molpeceres et al., 1998) and present results show a lower volume of distribution in female rats despite of the higher K_p value and body fat content. As previously reported, CyA distribution into adipose tissue, liver, muscle and skin is by far the major determinant of total volume of distribution estimated from blood samples (Bernareggi and Rowland, 1991). Then, the higher K_p values found for the liver, muscle and adipose tissue in female rats should be correlated with higher volumes of distribution since liver size is not affected by gender (Table 2) and the post-mortem examination of rats revealed higher amounts of dorsal and perirenal fat in females. Furthermore, no significant differences in the distribution of CyA within the blood compartment between males and females can be assumed (Molpeceres et al., 1998). Therefore, not only tissue size and affinity, but other important factors are involved in the tissue distribution of CyA in rats. Recent papers (Kawai et al., 1994, 1998) indicate that drug transfer kinetics between interstitial and intracellular spaces needs particular consideration. In the last few years, the P-glycoprotein has been shown to pump several drugs out of the cell thus avoiding their accumulation in several organs. Moreover, its expression shows a clear dependency on rat gender with males consistently expressing several fold lower levels than

Table 4
Relative proportions of CyA and metabolites AM1, AM9, AM1c and AM4N excreted in faeces of rats following a single bolus IV administration of 10 mg/kg

Group	CyA content (μg)	CyA (%)	AM1 (%)	AM9 (%)	AM1c (%)	AM4N (%)
10 weeks	29.02	15.74	8.52	48.82	15.26	8.83
Males	(10.52)	(1.34)	(2.43)	(7.49)	(6.81)	(4.22)
40 weeks	19.21	11.65	7.90	53.97	16.42	10.04
Males	(5.82)	(3.04)	(3.51)	(4.48)	(2.45)	(2.73)
10 weeks	21.45	19.86	5.94	54.02	10.27	9.91
Females	(7.51)	(5.38)	(2.39)	(4.39)	(2.03)	(4.24)
40 weeks	14.89	16.09	10.86	48.85	14.03	8.31
Females	(7.46)	(3.34)	(6.23)	(7.40)	(5.08)	(2.68)

females (Furuya et al., 1994; Salphati and Benet, 1998). CyA is a substrate for this membrane glycoprotein, which is expressed mainly in cells of the liver, intestine, kidneys and blood brain barrier. Hence, the lower CyA tissue concentrations found in female rats may be the result of a more efficient drug exsorption as a result of higher P-glycoprotein levels. Notwithstanding, the discrepancy between the lower volumes of distribution (V_{ss}) and higher K_p values found in females remains unexplained.

The metabolic capacity for scantly cleared drugs, (such as CyA), is usually very low compared with the hepatic blood flow, hence the CyA elimination is mainly dependent on drug liver uptake and metabolism. Several studies indicate potential gender-associated differences in the biotransformation of CyA by rats (Prueksaritanont et al., 1993; Sadrieh and Thomas, 1994). Female rats lack the isoforms (3A1 and 3A2) previously shown to metabolize the drug in this species but they express 10-fold higher levels than males of the recently identified CYP450 3A9 (Wang and Strobel, 1997). Then, drug metabolism appears as a second potential determinant for the sex-associated pharmacokinetics of CyA. Actually, although the differences observed between the AUC values corresponding to native CyA and total CyA derivatives were small, the larger values obtained for females suggest more extensive drug metabolism. Furthermore, slightly higher amounts of metabolites were also detected in the urine of females (Table 3). The small differences found between parent drug blood levels and CyA plus metabolites suggest formation rate-limited elimination of the metabolites, which are being eliminated as soon as they are formed from its precursor at the hepatic level. Sadrieh and Thomas (1994) reported that there were liver proteins which might bind the drug covalently to form its metabolites. Furthermore, female rats older than 7 weeks contained higher levels of these proteins than males. Hence, another possible explanation for the small amounts of circulating metabolites is their potential accumulation at the hepatic level, which was evaluated by analyzing the content of CyA derivatives in the liver. Indeed, the concentrations of metabolites in the liver exceeded by far those found in blood. However, the relative proportions of total CyA compounds and unchanged CyA (Table 2) indicated that more than 50% of the drug found in the liver of males were metabolites but only 30% of total CyA were metabolites in females. These results are partly in agreement to previous papers showing the predominance of native CyA over individual metabolites in rat blood and liver tissue; however, they do not support the finding that the concentrations of unchanged CyA and its metabolites in blood reflect their relative proportions in the liver (Wagner et al., 1987; Pell et al., 1988). Again, the P-gp appears as a feasible explanation for the different equilibrium between parent CyA and metabolites in the liver of females. It has been suggested that CyA metabolites may compete with CyA for protein binding. Hence, a higher excretion of CyA metabolites in the bile of females would lead to lower hepatic concentrations with a concomitant accumulation of parent compound corresponding to a higher m-FPIA/p-FPIA ratio (Table 2). Furthermore, this hypothesis also explains the higher Kp values observed for the liver of females. Since there seems to be liver proteins that may covalently bind CyA derivatives at higher amounts in the liver of females (Sadrieh and Thomas, 1994), it is also possible that part of the metabolites produced by the liver of females had not been extracted during sample processing. All the metabolites produced at the hepatic level are mainly excreted in faeces and a small amount in urine (Wagner et al., 1987). We have found that AM9 is the major primary metabolite identified in faeces according to previous reports (Maurer et al., 1984, Pell et al., 1988) followed by AM1 + AM1c and AM4N. Since no differences in the relative amounts of each metabolite excreted in faeces have been observed among males and females, it may be that metabolites other than those identified herein could account for the gender dependency of drug clearance in rats. In fact, Wagner et al. (1987) reported that after IV dosing, an average of 68 and 7% of the dose were excreted over 48 h as ^{3}H-CyA-derived radioactivity in faeces and urine, respectively. Our results (Table 3) indicate that the amounts of total CyA compounds identified in faeces and urine were

much lower (< 7 and 2.5% of the dose, respectively). p-FPIA mainly detects primary and even some secondary metabolites of CyA in blood. However, it was not possible to reliably assure the presence of metabolites other than the primary CyA derivatives in faeces. Phase II metabolites of CyA have been found in the urine of transplant patients (Christians and Sewing, 1993) although, no phase II metabolism has yet been proven in rats for this drug. Notwithstanding, the fact that gender seems to play a role in the genetic regulation and expression of these transferases (mainly glucuronidation) in rats (Strasser et al., 1997; Li et al., 1999) makes it tempting to speculate that phase II reactions may help in the interpretation of the results obtained, particularly in females. Moreover, Wagner et al. (1987) reported about the excretion of a polar unidentified CyA metabolite in the urine and bile of rats, which amounted to 39 and 24% of the sample radioactivity, respectively. On the other hand, the existence of CYP 450 3A and the P-gp in epithelial cells of the gastrointestinal tract may contribute to explain the results obtained with faeces. Native CyA and its metabolites excreted via biliary route may suffer further metabolism in the intestinal lumen but the P-gp has been associated to drug exsorption to the intestine after IV dosing (Arimori and Nakano, 1998). In this respect, previous studies (Wagner et al., 1987) showed that 12% of an IV dose was excreted as ^{3}H-CyA-derived radioactivity in the faeces of bile duct-cannulated rats.

In summary, our data are consistent with previous findings on the tissue distribution of unchanged CyA in rats (Wagner et al., 1987; Bernareggi and Rowland, 1991; Shibata et al., 1993; Yoshikawa et al., 1997; Kawai et al., 1998; Napoli et al., 1998). In males, the highest concentrations were found in the liver and the lowest in muscle. On the contrary, the highest tissue levels in females corresponded to adipose tissue. In females, CyA showed a higher affinity towards the liver, adipose and muscle tissue, but lower V_{ss} were calculated from blood data. Concerning drug metabolism, the amounts of circulating metabolites were very small but the differential AUC values obtained from unchanged CyA and total CyA compounds suggest more extensive biotransformation in females. The hepatic levels of CyA metabolites show evidence that the liver does not reflect the situation in blood indicating accumulation processes. Moreover, the lower proportion of metabolites in the liver of females does not support the hypothesis of higher metabolic rates in this sex. Among the three primary metabolites identified in faeces, AM9 was the major biotransformation product (more than 50% of CyA compounds) in accordance with previously reported data in rats (Maurer et al., 1984; Wagner et al., 1987; Vickers et al., 1992). Lower amounts of unchanged CyA were found in the faeces of female rats, although the relative proportions among native drug and its metabolites showed no gender-dependency. Consequently, metabolites other than those identified herein may account for the pharmacokinetic differences reported. Therefore, present in vivo results are controversial and no reliable conclusions can be inferred about the relative contribution of drug distribution and metabolism to the gender-associated pharmacokinetic differences previously found in rats. The complexity of CyA pharmacokinetics recommends that more in vitro and in vivo studies are needed to evaluate the relative importance of sex-related factors such as the expression of the P-gp or different drug metabolizing enzymes.

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