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High-performance liquid chromatographic analysis of cyclosporin A in rat blood and liver using a commercially available internal standard

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Abstract

All the available HPLC assays of cyclosporin A (CyA) use internal standards that are not commercially available. Our purpose was to develop an HPLC assay for measurements of CyA in rat blood and liver using a commercially available internal standard (I.S.). After the addition of tamoxifen (I.S.), blood (0.25 ml) or the liver homogenate (1 ml) samples were extracted into a mixture of ether:methanol (95:5). The residue after evaporation of the organic layer was dissolved in 200 μl of an injection solution and washed with 1 ml of hexane before analysis. The separation was achieved using an LC-1 column (70 °C) with a mobile phase of methanol–acetonitrile–0.01 *M* KH_2PO_4 (50:25:25, v/v) and a flow-rate of 1 ml/min. Detection was at 205 nm. Cyclosporin A and I.S. eluted at 5 and 7 min, respectively, free from endogenous peaks. Linear relationships ($r>0.98$) were observed between the CyA:I.S. peak area ratios and the CyA concentrations within the range of 0.2–10 μg/ml for blood and 0.1–4 μg/ml for the liver homogenates. The intra- and inter-run C.V.s and errors for both the blood and liver samples were <15%. The extraction efficiency ($n=5$) was close to 100% for both CyA and I.S. in both blood and liver homogenates. The lower limit of quantitation of the assay was 0.2 or 0.1 μg/ml based on 250 μl of blood or 1 ml of liver homogenate, respectively. The assay was capable of measuring blood and liver concentrations of CyA in a rat injected intravenously with a single 5-mg/kg dose of the drug.

Keywords: Cyclosporin A

1. Introduction

Cyclosporin A (CyA) is an immunosuppressive drug, which has been used to prevent rejection in solid organ transplantation and to treat autoimmune disorders [1]. The drug exhibits variable pharmacokinetics, resulting in different plasma and tissue concentrations in individuals receiving the same doses [2]. Therefore, therapeutic monitoring of the drug has routinely been carried out in most patients.

Several assay methodologies [3,4], including monoclonal and polyclonal immunoassays and chromatographic methods, have been reported for the measurement of CyA in whole blood, plasma, or serum of humans. Additionally, some of these methods have been used for characterization of the pharmacokinetics and pharmacodynamics of the drug in animals [5–8]. Because CyA undergoes substantial metabolism to a significant number of metabolites [9], the use of more specific methods such as

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HPLC is more appropriate than non-specific methods such as polyclonal immunoassays. Additionally, because the accumulation of the drug in red blood cells is substantial and temperature-dependent [10], the measurement of the drug concentrations in whole blood, as opposed to plasma or serum, has been advocated [9].

Many HPLC assays have been reported for the measurement of CyA concentrations in biological samples. However, almost all of the reported HPLC assays with internal standards use compounds such as cyclosporin C [11–13] or cyclosporin D [14–22], which are not commercially available. Therefore, the use of these assays by independent investigators is restricted. The main purpose of this study was to develop and validate an HPLC method for measurement of CyA concentrations in small volumes of rat blood and in rat liver tissue using a commercially available internal standard. The application of the assay was also demonstrated after a single i.v. dose of CyA administered to a rat.

2. Experimental

2.1. Chemicals

Cyclosporin A (CyA) and internal standard (I.S., tamoxifen) were obtained from Sigma (St. Louis, MO, USA). For extraction, diethyl ether was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). For chromatography, HPLC-grade acetonitrile and methanol were obtained from EM Sciences (Gibbstown, NJ, USA). All other reagents were analytical grade and obtained form commercial sources.

2.2. Standard solutions

Stock solutions of CyA (40 μg/ml) were prepared by dissolving 4 mg of CyA powder in 5 ml of methanol, followed by the addition of 95 ml distilled water. This solution was stored at 4 °C. Further dilution of the stock solution for preparation of calibration standards was carried out daily using water. Tamoxifen stock solutions (2.5 or 10 μg/ml for the blood or liver assays, respectively) were prepared by dissolving tamoxifen powder in methanol and were stored at 4 °C.

Calibration standards were prepared daily by spiking rat blood or liver homogenate with stock solutions of CyA to produce final concentrations of 0 (blank), 0.2, 0.4, 1, 2, 4, and 10 μg/ml for blood and 0 (blank), 0.1, 0.25, 0.5, 0.75, 1, 2, and 4 μg/ml for the liver homogenates. Blank blood and liver used for preparation of calibration standards were collected from drug-free male Sprague–Dawley rats.

2.3. Sample preparation

To 250 μl of blood in a glass tube were added 50 μl of I.S. solution (2.5 μg/ml), 1.75 ml of deionized water, and 200 μl of 1 *M* sodium hydroxide. The drug and I.S. were then extracted into 8 ml of an ether–methanol (95:5) solution by vortex-mixing for 30 s. After centrifugation at 3800 *g* for 5 min, the organic layer was removed and evaporated under a nitrogen stream at 50 °C. The residue was then reconstituted in 200 μl of acetonitrile–0.5% (v/v) phosphoric acid (65:35). After washing the injection solution with 1 ml of hexane for 5 s and a brief (1 min) centrifugation (3800 *g*), 50 μl of the injection solution was injected into the system.

Liver samples were first homogenized in deionized water (1:19) using a laboratory homogenizer (Biohomogenizer, Biospec Products, Bartlesville, OK) at a speed of 10 000 rpm for 2 min. To 1 ml of the homogenate in a glass tube were added 50 μl of I.S. (10 μg/ml), 1 ml of deionized water, and 200 μl of 1 *M* sodium hydroxide. The extraction and hexane wash procedures, identical to those described above for the blood samples, were then carried out.

2.4. Chromatography

Samples were analyzed using a 15-cm×4.6-mm analytical, reversed-phase (C1) column (Supelcosil LC-1, Supelco; Bellefonte, PA, USA), preceded by a 2-cm×2-mm guard column (Upchurch Scientific, Oak Harbor, WA, USA) packed with pellicular C_{18} media (Pellicular ODS; Whatman, Clifton, NJ, USA). Both the analytical and guard columns were kept at 70 °C in a column heater (Brinkmann Instruments, Westbury, NY, USA). The mobile phase consisted of methanol–acetonitrile–0.01 *M* KH_2PO_4

(50:25:25, v/v) and was pumped at a flow-rate of 1.0 ml/min. For blood samples, an equilibration delay of 10 min was used before the next sample was injected. However, this equilibration delay was not absolutely necessary because in its absence, some samples showed only a moderate change in the baseline levels without a significant impact on the integration of peaks.

The HPLC instrument (Waters; Milford, MA) consisted of a 501 pump, a 717 plus Autosampler, and a 486 UV detector set at a wavelength of 205 nm. Calibration curves were constructed using the Millennium software (Waters) by plotting the peak area ratio of CyA to I.S. versus the CyA concentrations in blood or liver homogenate. The data was weighted by $1/\text{concentration}^2$.

2.5. Extraction efficiency

The efficiency of the extraction method to recover CyA and I.S. from both blood and liver homogenates was tested using samples containing 1.0 μg/ml CyA and appropriate concentrations of I.S. These samples were then subjected to the sample preparation procedure explained above. The peak areas of CyA and I.S. in these extracted samples ($n=5$) were then compared with those of unextracted samples ($n=5$) containing equivalent concentrations of CyA and I.S. in the injection solution.

Further experiments were carried out to determine the effects of variation in the hematocrit of blood samples on the recovery of CyA and I.S. Pooled blood (hematocrit of 0.44) from rats was diluted with isotonic phosphate buffer (pH 7.4) by a factor of 2, and the original blood and diluted samples were subjected to the extraction procedure ($n=5$ for each group). The recoveries of the analytes from diluted blood were compared with those from the original undiluted samples.

To determine the effects of pH on the extraction recovery of CyA and I.S., samples ($n=5$) were extracted under both basic and acidic conditions after the addition of 200 μl of 1 *M* sodium hydroxide or hydrochloric acid, respectively. The peak areas of each analyte extracted under acidic and basic conditions were then compared.

The possibility of the transfer of CyA and/or I.S. into the hexane wash was also studied by evaporating the hexane wash and analyzing the contents of the samples for residual CyA and I.S. ($n=5$).

2.6. Assay validation

The intra- and inter-run precision and accuracy of the assay ($n=5$) were determined by percent C.V. and percent error values, respectively, based on reported guidelines [23]. Briefly, each set of quality control samples containing the lowest, midpoint, and highest concentrations in the calibration curves was run along with a calibration curve. The concentrations of the quality control samples were then determined against the calibration curve and used for the calculation of percent C.V. and percent error values. The percent error values were calculated by the following equation:

$$\text{Percent error} = \frac{(\text{Calculated conc.} - \text{Added conc.})}{\text{Added conc.}} \times 100$$

The quality control samples were run at the CyA concentrations of 0.2, 2, and 10 μg/ml for blood and 0.1, 1.0 and 4 μg/ml for liver homogenates.

2.7. Application of the assay

To demonstrate the application of the assay, the left jugular vein of a 270-g male, Sprague–Dawley rat was cannulated under ketamine–xylazine (80:12 mg/kg, i.p.) anesthesia. After overnight recovery, a single 5-mg/kg dose of CyA (Cyclosporine Injection, USP, 50 mg/ml; Bedford Laboratories, Bedford, OH, USA) was injected into the catheter. Blood samples (~250 μl) were collected in heparinized microcentrifuge tubes at 0 (before drug administration), 5, 10, and 20 min and at 1, 2, 4, 6, 8, and 12 h. At the end of the sample collection period, the rat was euthanized using CO_2 and the liver was collected. Both blood and the liver samples were stored at −80 °C until analysis using the above method.

3. Results

3.1. Blood samples

Chromatograms of rat blood samples obtained before and 12 h after the intravenous administration

of a single 5-mg/kg dose of CyA and a standard containing 2.0 μg/ml of CyA are illustrated in Fig. 1. Under the stated chromatographic conditions, CyA and I.S. eluted at approximately 5 and 7 min, respectively, free from endogenous peaks (Fig. 1).

The statistical characteristics of five calibration curves obtained during the inter-run validation of the assay for blood samples are listed in Table 1. Linear relationships ($r > 0.98$, Table 1) were found between peak area ratios of CyA:I.S. and blood concentrations in the range of 0.2–10 mg/ml. Furthermore, the slopes of the calibration curves were very similar with less than 6% difference between the lowest (0.354) and the highest (0.375) values (Table 1). Except for the calibration no. 5, the intercepts of the lines were close to zero for all the inter-run curves (Table 1).

The intra- and inter-run assay validation data for blood are reported in Table 2. The accuracy and precision of the blood assay are demonstrated by error values of ⩽14% and C.V. values of ⩽11%, respectively, for both intra- and inter-run data (Table 2). Based on these data, the lower limit of quantitation of the assay, using only 250 μl blood, is 0.2 μg/ml.

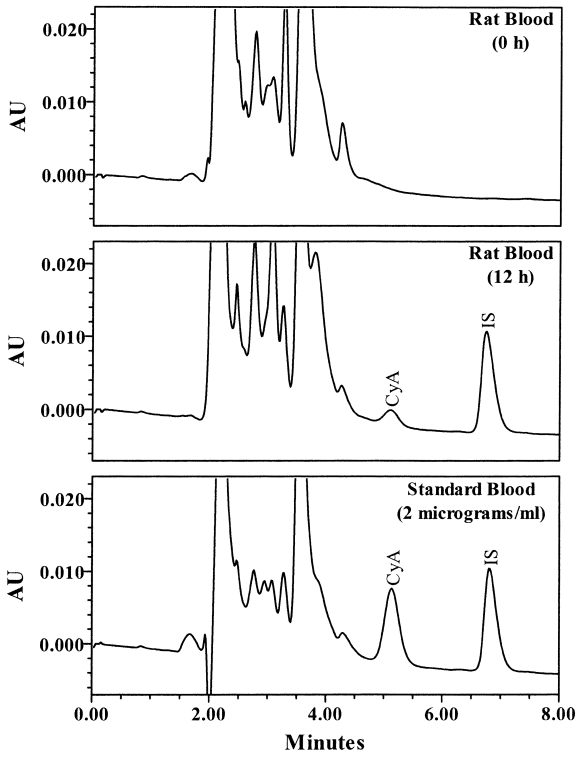

Fig. 1. Chromatograms of rat blood samples taken before (top) and 12 h (middle) after the i.v. injection of a single 5-mg/kg dose of cyclosporin A and a standard containing 2 μg/ml of the drug (bottom). The 12-h sample contained 0.417 μg/ml CyA.

Table 1
The relationship between the cyclosporin:internal standard peak area ratio and the added concentration for rat blood inter-run data [Peak Area Ratio = Intercept + (Slope × Conc.)]

Calibration no.	Intercept	Slope	r	SE
1	−0.004	0.374	0.990	0.0478
2	0.000	0.375	0.992	0.0438
3	−0.010	0.354	0.994	0.0378
4	−0.007	0.370	0.985	0.0600
5	−0.068	0.372	0.990	0.0539

The extraction recoveries of CyA and I.S. from blood samples were almost complete with efficiencies (mean±SD) of 100±7 and 95.1±1.4%, respectively. The hexane wash samples analyzed for residual contents of CyA and I.S. showed no peaks for I.S. However, small peaks, which were below the lower limit of quantitation (area <12% of that in the injection solution), were detected for CyA. Additionally, a substantial reduction of hematocrit by diluting the blood by a factor of 2 did not have any significant effect (unpaired *t*-test at a significance level of 0.05) on the recovery of CyA or I.S. from the blood. Regarding the pH effect on the recovery of analytes, CyA was extracted to the same extent in both acidic and basic pH values. However, the recovery of I.S. under acidic condition was approximately one-third of that observed after basic extraction. Therefore, for tamoxifen to be used as an I.S. for this assay, the extraction procedure should be conducted under basic condition explained under the sample preparation section.

3.2. Liver homogenates

The chromatograms of homogenates from a blank liver, a liver 12 h after the i.v. injection of CyA, and a standard containing 2.0 μg/ml of CyA are shown in Fig. 2. Similar to blood samples, CyA and I.S. eluted at approximately 5 and 7 min, respectively,

Table 2
Intra- and Inter-run accuracy and precision of the assay for blood samples ($n=5$)

Added conc. (μg/ml)	Calculated conc. (μg/ml)		% C.V.		% Error	
	Intra-run	Inter-run	Intra-run	Inter-run	Intra-run	Inter-run
0.20	0.228	0.184	7.76	5.43	14.1	−8.10
2.0	1.83	1.93	6.10	10.9	−8.31	−3.70
10	10.3	10.6	2.15	4.92	3.12	6.46

and were well separated from the endogenous and/or metabolite peaks (Fig. 2). The statistical characteristics of five calibration curves obtained during the inter-run validation of the assay for liver homogenates are presented in Table 3. Linear relationships ($r>0.99$, Table 3) were found between peak area ratios of CyA:I.S. and liver homogenate concentrations in the range of 0.1–4 mg/ml. Furthermore, the calibration curves had low intercepts and their slopes were <5% different from each other (Table 3).

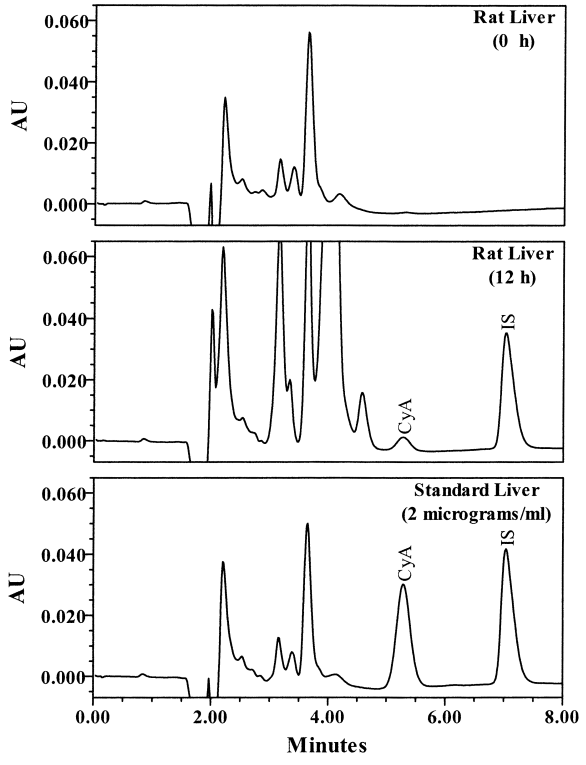

Fig. 2. Chromatograms of rat liver homogenates obtained from a drug-free rat (top), 12 h after the i.v. injection of a single 5-mg/kg dose of cyclosporin A (middle) and a standard containing 2 μg/ml of the drug (bottom). The 12-h sample contained 0.262 mg/ml CyA.

The results of the assay validation for liver homogenates are reported in Table 4. The accuracy and precision of the assay are demonstrated by error and C.V. values of ⩽8 and ⩽14%, respectively, for all the quality control samples during intra- and inter-run analysis (Table 4). Consequently, the lower limit of quantitation of the assay is 0.1 μg/ml for the liver homogenates or, based on a 20-fold dilution of the liver tissue in homogenates, 2.0 μg/g for the liver tissue.

The efficiencies of the extraction of CyA and I.S. from the liver homogenates (mean±SD) were 107±3 and 96.9±3.3%, respectively.

3.3. Application

The blood concentration–time profile of CyA after a single 5-mg/kg i.v. injection of the drug to a rat is illustrated in Fig. 3. The blood concentrations of CyA could be measured for at least 12 h after the drug administration (Fig. 3). As expected, the decline in the plasma concentrations of CyA with time was multiexponential. Based on a two-compartment model (Fig. 3), the clearance, volume of distribution

Table 3
The relationship between the cyclosporin:internal standard peak area ratio and the added concentration for rat liver inter-run data [Peak Area Ratio = Intercept + (Slope × Conc.)]

Calibration no.	Intercept	Slope	r	SE
1	−0.002	0.408	1.00	0.0139
2	−0.006	0.412	0.999	0.0196
3	−0.001	0.398	1.00	0.0115
4	−0.009	0.416	1.00	0.0119
5	−0.004	0.404	1.00	0.0143

Table 4
Intra- and Inter-run accuracy and precision of the assay for liver homogenates ($n=5$)

Added conc. (μg/ml)	Calculated conc. (μg/ml)		% C.V.		% Error	
	Intra-run	Inter-run	Intra-run	Inter-run	Intra-run	Inter-run
0.10	0.100	0.0986	4.30	14.4	0.00	−1.40
1.0	0.930	1.06	4.28	7.43	−6.96	6.42
4.0	3.69	4.11	1.66	5.84	−7.75	2.65

of the terminal phase, and terminal half-life of CyA were 0.291 l/h per kg, 2.67 l/kg, and 6.35 h, respectively. The concentration of CyA in the liver tissue taken from the rat at 12 h was 5.24 μg/g, and the total amount of CyA recovered in the liver was 61.1 μg (4.2% of the administered dose).

4. Discussion

It is generally believed that the use of internal standards in HPLC analyses would improve the accuracy and precision of the assay. Consistent with this notion, when we initially used the current assay methodology in the absence of any internal standard, substantial variability in the results was observed. The large variability in these initial studies were most likely due to the fact that our sample preparation included an extraction procedure, requiring separation of organic and aqueous phases, and transfer of the organic layer to a different tube. Additionally, other factors such as partial evaporation of the injection solution for samples injected into HPLC later during the run and errors associated with the volume of injection may have contributed to this variability. Therefore, the use of an internal standard appeared necessary for improving the accuracy and precision of the assay.

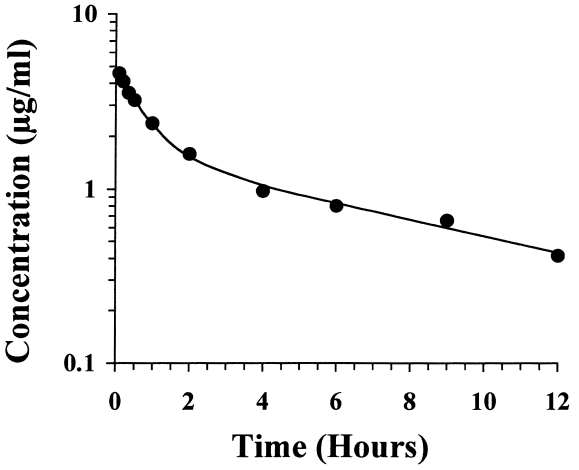

Fig. 3. Blood concentration–time course of CyA after the i.v. administration of a single 5-mg/kg dose of the drug to a rat. The symbols and the line represent the measured and fitted (two-compartment model) concentrations, respectively.

Early HPLC assays for CyA almost exclusively used cyclosporin D as an internal standard [14,15,20–22]. Cyclosporin D showed favorable chromatographic properties in both reversed-phase [14,15,20–22] and normal-phase [18,19] systems; in the reversed-phase systems, it eluted after CyA peak without significantly increasing the run time, and in the normal-phase systems, it eluted earlier in the chromatogram before the peaks of CyA and its metabolites [19]. Additionally, being structurally similar to CyA, cyclosporin D exhibited extraction recovery and UV absorbance properties similar to those of CyA [20,22]. However, when the provider of cyclosporin D (Sandoz Research Institute which is now part of Novartis Pharmaceuticals, East Hanover, NJ) stopped supplying it [12], some investigators [12] had to modify existing methods to switch to another internal standard, cyclosporin C, which was available from the same company.

Cyclosporin C also showed suitable characteristics for use as an internal standard for CyA measurement in HPLC assays [12]. However, because cyclosporin C eluted before CyA in reversed-phase chromatography, replacement of cyclosporin D with cyclosporin C resulted in longer chromatographic times and lower sensitivity in the modified assay [12]. Nevertheless, the use of cyclosporin C as an internal standard has the same disadvantage of cyclosporin D; the internal standard is available only through Novartis with some restrictions for independent

researchers for obtaining the compound. Therefore, we decided to develop an HPLC assay for cyclosporin which uses an internal standard other than those supplied by Novartis.

Cyclosporin A is a lipophilic compound, which is extensively retained on reversed-phase columns. Therefore, several lipophilic compounds with a history of strong retention on these columns were identified and tested as potential internal standards. Among compounds tested, acepromazine, clozapine, ethopropazine, pimozide, thioridazine, thiothixene, and reserpine were either eluted with solvent front or earlier in the chromatogram before the CyA peak. Because CyA undergoes significant metabolism [9], it was decided that a suitable internal standard would preferentially elute after CyA to prevent possible co-elution with the metabolites peaks. Search for a more lipophilic compound resulted in identification of tamoxifen, which originally eluted with a retention time similar to that of CyA when the strength of the aqueous portion (potassium dihydrogen phosphate) of the mobile phase was 0.02 *M*. A decrease in the molarity of the salt to 0.01 *M* in the final mobile phase, however, resulted in an increase in the retention time of tamoxifen and its baseline resolution from CyA (Figs. 1 and 2). Consequently, tamoxifen was chosen as the optimum internal standard for the assay.

A number of assays [7,14,18] have used solid-phase extraction procedure for the analysis of CyA in biological samples. This method of extraction is relatively labor-intensive and, at times, may result in low recoveries [18]. We used a liquid–liquid extraction method based on diethyl ether–methanol. Initially, our extraction solvent contained only ether. However, it was noticed that with some samples (especially the liver homogenates), the extraction recovery was not reproducible. Therefore, 5% (v/v) methanol was added to the solvent, resulting in quantitative (100%) and reproducible recovery of both CyA and I.S. from both blood and liver samples.

Many reported HPLC assays for CyA are developed for use with human samples, and, therefore, use blood volumes of 1 ml or higher. However, these methods may not be directly applicable to studies in small animals, such as rats, when smaller blood volumes are available after serial sampling. Alternatively, these methods have been used [7] only in destructive sampling designs (separate animals for each sampling point). The method reported here uses a 0.25-ml blood sample and, hence, allows characterization of the blood pharmacokinetics of CyA in a single rat (Fig. 3).

Similar to other reports on the HPLC analysis of cyclosporine, our chromatography was carried out at a relatively high-temperature (70 °C), which may have a detrimental effect on the column performance. We used the same column during the method development, validation process, and application studies without a significant change in the resolution of peaks in the chromatograms. However, with time, the retention times of CyA and I.S. tended to decrease slightly ($<$10%). The decrease in the retention times was readily reversible by a slight (2–3%) increase in the aqueous part of the mobile phase.

In conclusion, an isocratic, liquid–liquid extraction chromatographic method is reported for the determination of the cyclosporin A concentrations in blood and liver of rats. Unlike previously reported HPLC assays, this method uses an internal standard which can be purchased commercially without any restrictions.

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