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Development of a HPLC method for the determination of cyclosporin-A in rat blood and plasma using naproxen as an internal standard

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Abstract

An isocratic reversed phase high-performance liquid chromatographic (HPLC) method with ultraviolet detection at 205 nm has been developed for the determination of cyclosporin-A (CyA) in rat blood and plasma. Naproxen was successfully used as an internal standard. Blood or plasma samples were pretreated by liquid–liquid extraction with diethyl ether. The ether extract was evaporated and the residue was reconstituted in acetonitrile–0.04 M monobasic potassium phosphate buffer (pH 2.5) solvent mixture. After washing with *n*-hexane, 30 μl of the reconstituted solution was injected into HPLC system. Good chromatographic separation between CyA and internal standard peaks was achieved by using a stainless steel analytical column packed with 4 μm Nova-Pak Phenyl material. The system was operated at 75 °C using a mobile phase consisting of acetonitrile–0.04 M monobasic potassium phosphate (pH 2.5) (65:35 v/v) at a flow rate of 1 ml/min. The calibration curve for CyA in rat blood was linear over the tested concentration range of 0.0033–0.0166 M with a correlation coefficient of 0.989. For rat plasma, the range of the concentrations tested were between 0.002 and 0.0166 M and showed linearity with a correlation coefficient of 0.953. The intra- and inter-run precision and accuracy results were 1.24–21.87 and 3.1–12.23%, respectively. The low volume of blood or plasma needed (200 μl), simplicity of the extraction process, short run time (5 min) and low injection volume (30 μl) make this method suitable for quick and routine analysis.

Keywords: Cyclosporin A; Naproxen; Reversed phase high-performance liquid chromatography; Rat blood and plasma

1. Introduction

Cyclosporin-A (CyA) is a cyclic undecapeptide of fungal origin [1]. This drug has been used as a potent immunosuppressive agent, valuable in preventing rejection of transplanted organs and

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in treating certain autoimmune diseases [2,3]. More recently, this drug is being used in preclinical studies as an inhibitor of P-glycoprotein. CyA is bound to the lipoprotein fraction of plasma and is extensively taken up by erythrocytes [4]. It is believed that it acts through inhibition of proliferation of certain types of lymphocytes by interfering with the synthesis of certain lymphokines, including interleukin-2 (IL-2) and the expression of IL-2 receptors [4]. Over the past 20 years, CyA has produced profound changes in the outcome of organ transplantation. Its success has been well documented [5,6]. Use of this drug, however, requires rigorous monitoring since there is a poor correlation between dose administered and clinical response. The therapeutic window is narrow and the consequences of therapeutic and toxic levels are severe graft rejection on one hand and nephrotoxicity on the other [7]. Therapeutic monitoring of CyA is of critical importance because of large inter subject variability in bioavailability and metabolism of the drug [5]. There are conflicting reports about the biological matrix to be used and appropriate HPLC method that can provide best information for effective drug monitoring [8–10]. Some reports showed that whole blood is the preferred matrix [6,11]. However, others argued that plasma should be used as the preferred matrix to achieve better therapeutic monitoring of the drug [12]. To date, most of the HPLC methods available for quantitation of CyA in biological fluids use internal standards that are not available commercially. Cyclosporin C [13], D [14], D analog [12] or G [15] has been used as an internal standard. Further, the investigators have to go through complicated paperwork to get a small amount of internal standard from the company which has a patent on cyclosporine derivatives. Additionally, some assay methods use solid phase extraction [16,17], complicated [18], time consuming [19] or gradient elution [20] methods.

The main purpose of this study was to develop a simple and reliable method to quantitate CyA in a relatively short time with high linearity using a commercially available internal standard.

2. Experimental

2.1. Materials

CyA was purchased from Abbott Laboratories (Chicago, IL). Naproxen was purchased from Sigma (St. Louis, MO). Monobasic potassium phosphate was purchased from J.T. Baker (Phillipsurg, NJ). Diethyl ether was purchased from Fisher (Fisher Scientific, NJ). Other organic solvents were of HPLC grade and were purchased from Merck KgaA (Dermstadt, Germany). All other chemicals were of analytical grade and were used as received. Blood was collected from the tail of anesthetized male Sprague–Dawley rats (Charles River, Raleigh, NC) in heparinized tubes and placed in ice bucket. Some blood samples were centrifuged to separate the plasma. Both blood and plasma samples were later stored at −20 °C until further analysis.

2.2. Preparation of stock solutions

Stock solution of CyA with a concentration of 0.083 M was prepared by dissolving 10 mg of CyA in 100 ml of mixture of acetonitrile–0.04 M monobasic potassium phosphate (pH 2.5) (65:35 v/v). Different volumes were taken from this solution and added to blood or plasma to prepare the concentrations needed for calibration. A stock solution of the internal standard was prepared by dissolving 10 mg of naproxen in 100 ml of the same solvent mixture used for the drug.

2.3. Sample extractions

Aliquots of 200 μl of whole blood or plasma were dispensed in 10 ml clean glass tubes using a micro pipette. Different volumes of CyA standard stock solutions containing known concentrations of CyA were added to different tubes. 100 μl of internal standard stock solution was added to each sample. The tubes were vortexed for 15 s and 5 ml of diethyl ether was added to each tube for drug extraction. The tubes were again vortexed for 5 min and left to stand for 1 min at room temperature to allow complete extraction. The extracts were centrifuged at 3000 rpm for 5 min. Four

milliliter of supernatants were then transferred to clean glass tubes. The ether extracts were evaporated using a gentle stream of air. The extract residues were reconstituted using 1 ml of acetonitrile–0.04 M monobasic potassium phosphate (pH 2.5) (65:35 v/v) and washed twice with 3 ml of *n*-hexane. The volume of the washed solution injected into the HPLC column was 30 μl.

2.4. Instrumentation and conditions

The high-performance liquid chromatography (HPLC) system consisted of Waters 510 pump, Waters 484 Tunable absorbance detector, and Waters 715 Ultra Wisp sample processor. A stainless steel analytical column (3.9 × 150 mm) packed with 4 μm Nova-Pak Phenyl material (Millipore Waters Inc., Milford, MA) was used for the chromatographic analysis. The column was kept at 75 °C by a waters temperature control module. The mobile phase consisted of acetonitrile–0.04 M monobasic potassium phosphate (pH 2.5) (65:35 v/v). The flow rate was adjusted to 1 ml/min and the pump pressure was adjusted to 700–800 psi. The detection wavelength was 205 nm and the AUFS was 0.001. Peak detection and integration was done using Star 5.3 ADCB 16 Chromatographic System (Varian Inc, Walnut Creek, CA).

2.5. Linearity

Linearity was tested by assaying whole blood or plasma spiked with known volumes of CyA stock solution at concentrations 0.0033, 0.0066, 0.0099 and 0.0166 M for blood sample or 0.0020, 0.0041, 0.0083 and 0.0166 M for plasma sample. The ratio of the peak area of CyA and internal standard versus concentration were plotted and the correlation coefficients were calculated.

2.6. Precision and accuracy

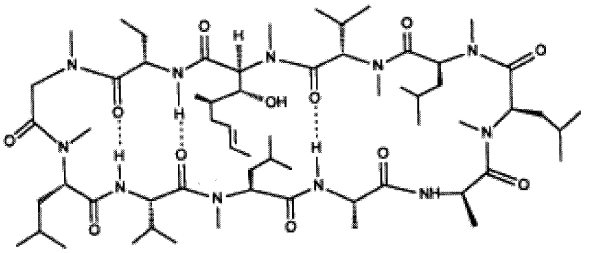
Assay precision was determined by spiking whole blood or plasma with varying concentrations of CyA and fixed concentration of internal standard. CyA concentrations were determined in batches of three on the same run and on different runs. Intra and inter-run assay precision was determined by calculating standard deviation (S.D.) and coefficient of variation (CV) of the obtained data.

Relative accuracy was determined by calculating the percent accuracy using the equation,

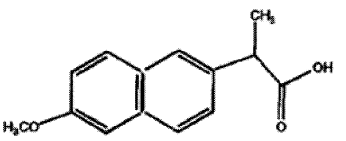
$$\%\ \text{Accuracy} = \frac{\text{observedconcentration}}{\text{nominalconcentration}} \times 100$$

3. Results and discussion

Several HPLC methods for determination of CyA in blood or plasma have been published [21] but most of them need relatively expensive and complicated techniques. In addition, most of the methods described in the literature use cyclosporine analogs as internal standard [22]. The unavailability of internal standard other than cyclosporine analogs is another problem to put these methods in application. Recently, an internal standard, which is structurally different from CyA, has been used in the HPLC determination of CyA [23]. The method used in the present study provides a simple and reliable procedure for determination of CyA in rat blood and plasma using inexpensive and commercially available internal standard. Although there is no similarity between CyA and naproxen (Fig. 1), yet there is a tremendous similarity in the solubility behavior of



Cyclosporin A



Naproxen

Fig. 1. Chemical structure of CyA and naproxen.

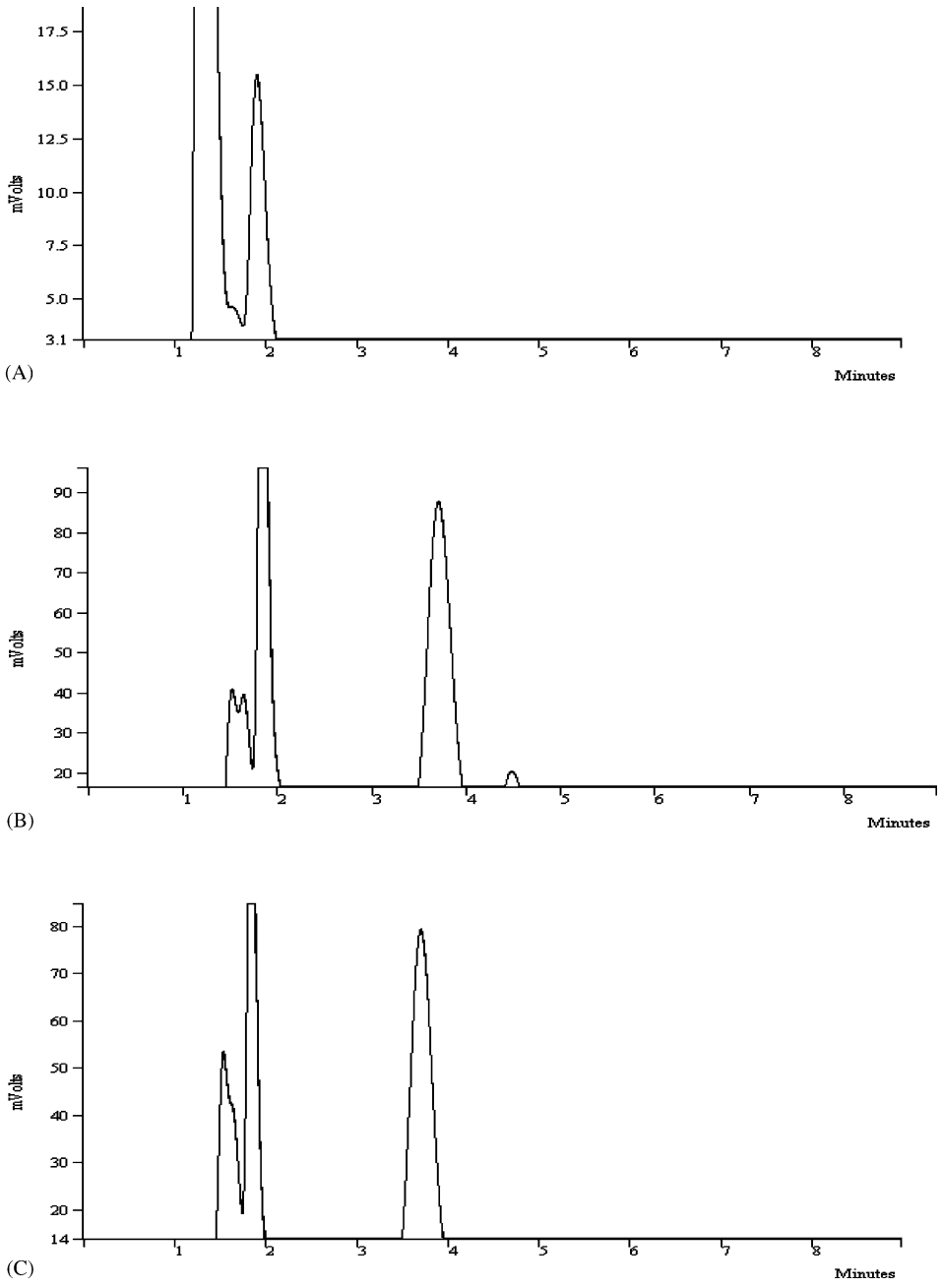


Fig. 2. Chromatograms of the extract of 200 μl rat blood spiked with internal standard (A), 200 μl rat blood spiked with IS and CyA (B), and 200 μl rat plasma spiked with IS and CyA (C).

these two agents [24]. Based on the solubility behavior of the drug and the internal standard, we can argue that both of them could be extracted using diethyl ether and eluted with acetonitrile. The two solutions were detected at the same wavelength on UV analysis. Most of the reported

procedures for CyA analysis require 1–2 ml of blood or plasma. In this method, the volume of blood or plasma used to accomplish the extraction process was as low as 200 μl. There was no need for protein precipitation step preceding liquid–liquid extraction. In addition *n*-hexane was successfully employed to remove any interfering substances. In the extraction process, glass sample holders were used since it is reported that plastic sample holders give several interfering peaks on the chromatogram [19]. Peak broadening was one of the disadvantages of HPLC methods for CyA analysis [21] and was one of the reasons for poor resolution of CyA from other interfering peaks. The peaks obtained in this study were narrower and sharper when acetonitrile–0.04 M monobasic potassium phosphate buffer (pH 2.5) was used. Lowering the pH and maintaining the column at relatively high temperature (75 °C) were found to have some influence on the results. Fig. 2 illustrates the chromatograms for internal standard (A), CyA extracted from rat blood (B) and plasma (C). The retention time was 1.8 min for the internal standard and 3.5 min for CyA. Both peaks were well resolved and no interfering peaks were observed during the run time.

3.1. Linearity

Fig. 3 demonstrates the linearity of the assay method of CyA from rat blood. Linearity was tested by assaying blood or plasma spiked with

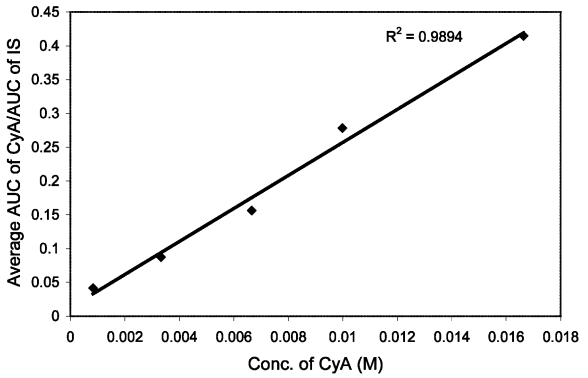


Fig. 3. Linearity of the assay of CyA in rat blood using naproxen as internal standard.

Table 1
Intra and inter-run accuracy and precision of determination of CyA in rat blood

Nominal concentration (M)	Intra-run				Inter-run				
	Observed mean concentration (M)	S.D.	CV (%)	Accuracy (%)	Observed mean concentration (M)	S.D.	CV (%)	Accuracy (%)
0.0033	0.0030	0.214	6.77	90.66	0.0038	0.361	10.10	115.36
0.0066	0.0059	0.507	8.07	89.15	0.0064	0.126	1.92	97.28
0.0099	0.010	0.645	6.20	109.13	0.0111	0.823	7.80	111.64
0.0166	0.0164	0.124	0.75	98.91	0.0163	0.195	1.18	98.31

Table 2
Intra and inter-run accuracy and precision of determination of CyA in rat plasma

Nominal concentration (M)	Intra-run				Inter-run				
	Observed mean concentration (M)	S.D.	CV (%)	Accuracy (%)	Observed mean concentration (M)	S.D.	CV (%)	Accuracy (%)
0.0020	0.0026	0.404	17.11	127.25	0.0018	0.07	3.84	89.85
0.0041	0.0049	0.53	1.72	118.07	0.0038	0.12	3.10	91.56
0.0083	0.0060	1.57	21.87	73.13	0.0096	0.47	5.27	116.02
0.0166	0.0168	0.209	1.24	101.74	0.0117	1.73	12.23	70.48

known volumes of CyA standard solution containing known concentrations of the drug. In rat blood, the range of spiked concentrations was between 0.0033 and 0.0166 M and the correlation coefficient was found to be 0.989. In rat plasma, the range of concentration of CyA was between 0.0020 and 0.0166 M and the correlation coefficient was found to be 0.953. Although the range of concentrations tested was within the acceptable limit for pharmacokinetic studies, the method can detect up to 0.0001 M with a per cent recovery of 80–102 when the injection volume was increased from 30 to 100 μl. Although the quantitation was accomplished by measuring the ratio of peak area of drug to that of internal standard, the peak height also showed linearity within the range of concentrations tested (data not shown).

3.2. Precision and accuracy

Tables 1 and 2 show the precision and accuracy of the assay method in rat blood and plasma, respectively. The precision (intra-run variability) and reproducibility (inter-run variability) were determined from the analysis of S.D. and CV of the obtained data. For blood analysis, the intra-run variability ranged from 0.75 to 8.07% and for inter-run variability, the CV ranged from 1.18 to 10.1%. For plasma analysis, the intra-run variability was between 1.24 and 21.87% and the inter-run variability was between 3.1 and 12.23%.

The percent accuracy was calculated to reflect the difference between the nominal and observed concentration. The percent accuracy was in the range of 89.15–115.36 for rat blood and in the range of 70.48–127.25 for rat plasma.

4. Conclusions

A simple and reliable HPLC method for measuring CyA in rat blood and plasma has been developed. Naproxen could be successfully used as an internal standard since it was extracted by the same way and measured at the same wavelength as CyA. The low volume of blood or plasma needed, the availability of the internal standard, the simplicity of the extraction procedure, the short

run time and the low volume of injection make this method suitable for quick and routine analysis. The intra-run and inter-run variability and accuracy results were in acceptable limit. Concentrations of CyA as low as 0.002 M in rat plasma and 0.0033 M in rat blood could be measured. The sensitivity is considered to be sufficient for therapeutic drug monitoring in experimental animals.

References

[1] R. Wenger, Transplant Proc. 22 (1990) 1104–1108.
[2] N. Talal, Transplant Proc. 20 (Suppl. 4) (1988) 11–15.
[3] S. Noble, A. Morkham, Drugs 50 (1995) 924–941.
[4] A. Hess, P. Colombani, Transplant Proc. 18 (Suppl. 4) (1986) 219–237.
[5] S. Carruther, D. Freeman, J. Koegler, W. Howson, P. Keown, A. Laupacis, C. Stiller, Clin. Chem. 29 (1983) 180–183.
[6] L. Shaw, L. Bowers, L. Demers, Clin. Chem. 33 (1987) 1269–1288.
[7] G. Yee, D. Gmur, M. Kennedy, Clin. Chem. 28 (1982) 2269–2271.
[8] W. Vine, M. Flye, P. Jatlow, Clin. Chem. 32 (1986) 1828–1831.
[9] G. Lensmeyer, D. Wiebe, I. Carlson, Clin. Chem. 33 (1987) 1841–1850.
[10] T. Rosano, Clin. Chem. 31 (1985) 410–412.
[11] E. Giesbrecht, S. Soldin, P. Wong, Ther. Drug Monit. 11 (1989) 332–336.
[12] H. Bardelmeijer, M. Ouwehand, J. Beijnen, J. Schellens, O. Tellingen, J. Chromatogr. B 673 (2001) 201–206.
[13] W. Liu, G. Levy, P. Wong, Ther. Drug Monit. 17 (1995) 479–486.
[14] H. Brozmanova, M. Grundmann, K. Safarcik, A. Jegorov, J. Chromatogr. B 749 (2000) 93–100.
[15] J. Mangold, L. Rodriguez, Y. Wang, Drug Metab. Dispos. 23 (1995) 615–621.
[16] A. Hamwi, M. Veitl, G. Manner, K. Ruzicka, C. Schweiger, T. Szekere, Am. J. Clin. Pathol. 112 (1999) 358–365.
[17] W. Awni, J. Malioney, J. Chromatogr. 425 (1988) 233–236.
[18] G. Hamilton, E. Rorh, E. Wallisch, F. Tichy, J. Chromatogr. 341 (1985) 411–419.
[19] R. Kates, R. Latini, J. Chromatogr. 309 (1984) 441–447.
[20] M. Kumar, S. Singhal, A. Singh, J. Pharm. Biomed. Anal. 25 (2001) 9–14.
[21] L. Bowers, S. Mathew, J. Chromatogr. A 333 (1985) 231–238.
[22] H.A. Bardelmeijer, M. Ouwehand, J.H. Beijnen, J.H. Schellens, O. van Tellingen, J. Chromatogr. B 763 (2001) 201–206.
[23] V.J. Wacher, W. Susan, H.T. Wong, J. Pharm. Sci. 91 (2002) 77–90.
[24] The Merck Index, 13th ed., Merck & Co., Inc. NJ, 2001.