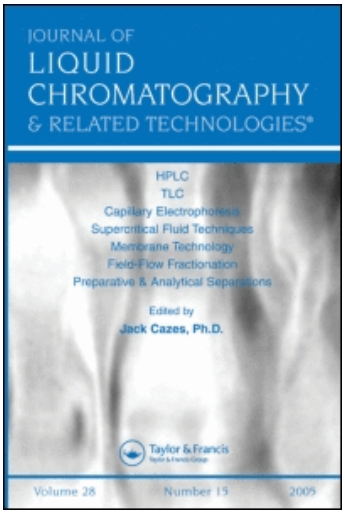

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DETERMINATION OF A SYNTHETIC DECAPEPTIDE IN THE PLASMA OF MONKEYS AND RATS BY NARROWBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Analytical methodology utilizing solid-phase extraction and reversed phase narrowbore high-performance liquid chromatography is described for the specific determination of the decapeptide RWJ-47428-099 in the plasma of monkeys and rats. Assay validation included the demonstration of specificity, recoverability of added standard, stability, accuracy, and precision in both matrices. Linearity was demonstrated over the concentration range of 0.031 - 3.0 μg/mL in monkeys and 0.047 - 9.20 μg/mL in rats. This methodology was subsequently implemented in support of preclinical drug metabolism investigations.

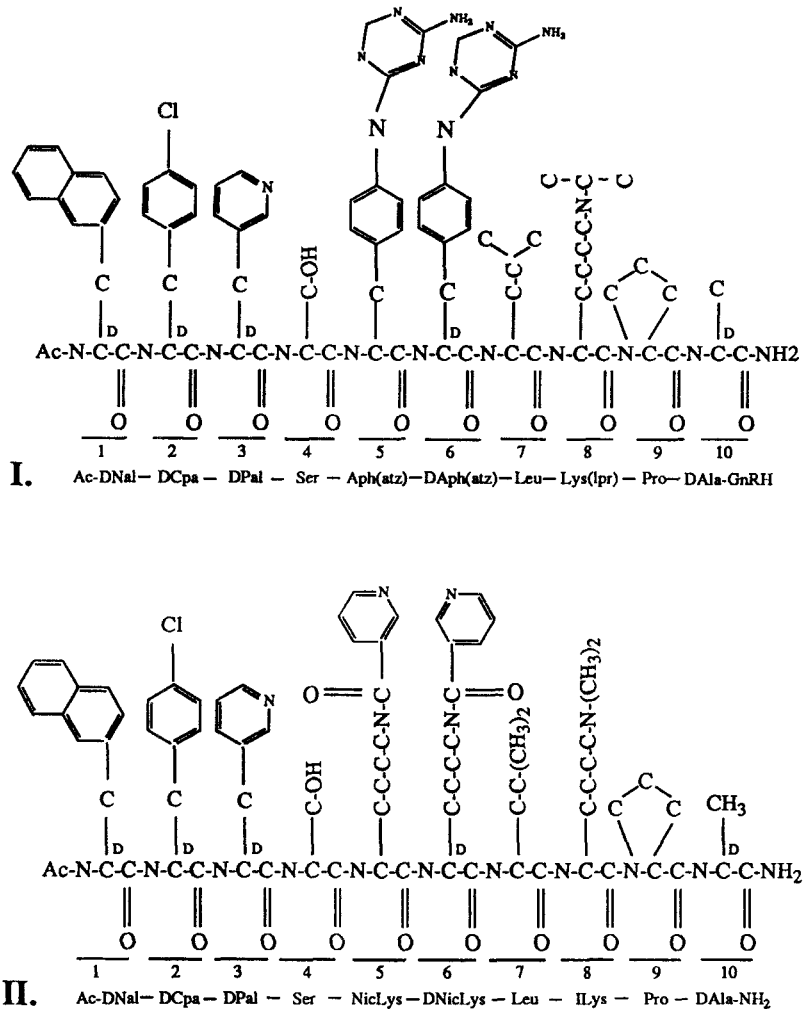

Figure 1. Primary structure of RWJ-47428-099 (I) and the internal standard (Antide; II).

INTRODUCTION

The synthetic decapeptide RWJ-47428-099 is a novel GnRH antagonist discovered at the Salk Institute and currently under investigation by The R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ for the treatment of endometriosis, uterine fibroids and other reproductive disorders.[1-6] During

initial pharmacological investigations, RWJ-47428-099 has demonstrated both increased potency and low anaphylactoid potential relative to other GnRH antagonists.

This report describes a sensitive and reliable method for the measurement of plasma RWJ-47428-099 concentrations. This method was used to support several preclinical investigations.

EXPERIMENTAL

Materials and Reagents

(RWJ-47428-099;N-acetyl-D-b-Nal-p-Cl-D-Phe-3-Pyridyl-D-Ala-Ser-Aph (atz)-Aph (atz)-Leu-N-e-Isopropyl-Lys-Pro-D-Ala-NH_2) was obtained from the Salk Institute (La Jolla, CA). The internal standard(Antide; N-Acetyl-D-b-Nal-p-Cl-D-Phe-3-Pyridyl-D-Ala-Ser-N-e-Nicotinoyl-Lys-N-e-Nicotinoyl-D-Lys-Leu-N-e-Isopropyl-Lys-Pro-D-Ala-NH_2 was obtained from Bachem California (Torrance, CA). The primary structures of both RWJ-47428-099 and the internal standard are shown in Figure 1. Note regarding abbreviations. IUPAC rules are used for nomenclature except for the following: Aph (atz), 4-[N-[5'-(3'-amino-1H-1', 2', 4'-triazolyl) amino] phenylalanine; Nic Lys, nicotinoyl lysine. All solvents (methanol, methyl-t-butyl ether, and acetonitrile) were HPLC grade (Fisher Scientific, Fairlawn, NJ). Trifluroacetic acid hydrochloric acid, potassium hydroxide, sodium phosphate dibasic and sodium phosphate monobasic were analytical grade (Baker, Phillipsburg, NJ or EM Science, Gibbstown, NJ).

The buffer (pH 7) was prepared by combining 41 parts sodium phosphate monobasic (0.07M) and 59 parts sodium phosphate dibasic (0.08M). Plasma samples were obtained from fasted Cynomolgus monkeys and Sprague Dawley rats which were housed in the vivarium of The R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ. Stock solutions of both RWJ-47428-099 and the internal standard were prepared in water containing 0.1% trifluoroacetic acid.

Working standards were prepared by addition of appropriate volumes of RWJ-47428-099 stock solution of concentration ~90 μg/mL to plasma and subsequent serial dilution of the spiked plasma to afford calibrators of 0.031, 0.063, 0.125, 0.250, 0.751, 1.5, and 3.0 μg/mL in monkeys and 0.047, 0.080, 0.160, 0.319, 0.638, 2.3, 4.5, and 9.0 μg/mL in rats.

Additionally, from separate weighings of compound, quality control samples were prepared at concentrations of 0.065, 0.725, and 2.17 μg/mL in monkeys and 0.080, 0.638, and 4.6 μg/mL in rats. These quality control samples were stored at -20 °C and were subsequently analyzed in triplicate during each validation run.

Instrumentation

All experiments were carried out at ambient temperature (approximately 21 °C). Solid-phase extractions were accomplished using bifunctional C_{18}CN solid phase extraction columns (Chemical Separations, PA).

HPLC analysis was performed on a Model 172 Microbore HPLC system consisting of a Model 140B solvent delivery system and a Model 785A programmable absorbance detector (ABI-Perkin Elmer, Applied Biosystem Division, Foster City, CA). A Model SIL-10A autosampler was equipped with a 20 mL fixed loop and a Model SIL-10A system controller (Shimadzu Scientific Instruments, Tokyo, Japan). PEEK (polyetheretherketone) biocompatible tubing (Upchurch Scientific, Oak Harbor, WA) was used in place of standard stainless steel tubing. An AQ ODS guard column (5, 100 x 2 mm i.d.) was fitted with an AQ ODS guard column (2.0 mm i.d., YMC, Inc, Wilmington, NC).

Data acquisition and processing were accomplished on a Model LAS 3350A laboratory automation system (Hewlett Packard, Avondale, PA).

Extraction Procedure

Plasma samples (250 μL) were spiked with internal standard solution (100 mL) and phosphate buffer, pH (250 μL). The solid-phase columns were preconditioned with methanol and washed with water and phosphate buffer prior to sample application. Upon application of the plasma samples, the columns were washed with one column volume of water and methyl-t-butyl ether. For the extraction of monkey plasma, RWJ-47428-099 and the internal standard were eluted from the column with 1% (v/v) hydrochloric acid in methanol. For the extraction of rat plasma, 1% trifluroacetic acid in methanol was used as the elution solvent. The eluents were evaporated in a TurboVap LV evaporator (Zymark, Corp., Hopkinton, MA) at 40 °C under nitrogen. After reconstitution of the extracts in 200 mL of 6 mM hydrochloric acid, aliquots (30 μL) were injected into the HPLC system.

Chromatographic Conditions

HPLC mobile phase was a mixture of two solutions: Solution A was hydrochloric acid (6 mM) and solution B was a 50:50 (v:v) mixture of hydrochloric acid (6 mM) and acetonitrile. Following the extraction of monkey plasma, RWJ-47428-099 and the internal standard were eluted from the column using a 15 minute linear gradient starting at 70% solution A: 30% solution B and ending at 20% solution A: 80% solution B. Following the extraction of rat plasma, RWJ-47428-099 and the internal standard were eluted from the column using a 15 minute linear gradient starting at 70% solution A: 30% solution B and ending at 34% solution A: 66% solution B. In both cases, the flow rate was 0.2 mL/min. Uv detection was accomplished at 214 nm.

Assay Validation

Assay linearity over the usable standard concentration range was assessed in plasma standard curves via linear regression analysis of RWJ-47428-099/internal standard peak area ratio versus RWJ-47428-099 concentration. Standard curves were run in duplicate on at least 3 separate days. Accuracy and precision were assessed in quality control samples of monkey and rat plasma. Accuracy was expressed as the percent deviation from theoretical or target concentration. Precision or relative standard deviation, was expressed as the coefficient of variation (%CV).

The recovery or extraction efficiency was determined by comparing the amount of RWJ-47428-099 extracted from plasma to the same amount from stock solution injected directly into the HPLC system.

Additionally, the stability of RWJ-47428-099 in both matrices was assessed in spiked quality control samples which were: (a) stored frozen at -20 °C and (b) subjected to 3 freeze-thaw cycles. These samples were analyzed with freshly prepared quality control samples.

Data Reduction/Acceptability Criteria

The calculation of drug concentration in unknown samples was based on a weighted $\left(\frac{1}{x^2}\right)$ least squares regression of plasma calibration standard concentrations against peak are ratios. The peak area ratios were obtained by dividing the peak area of RWJ-47428-099 by that of the internal standard.

Table 1

Standard Curve Characteristics

Matrix	Theoretical Value (μg/mL)	Mean Value n=6 (μg/mL)	SD	CV (%)	Dev (%)	Linear Regression Analysis
Monkey	0.0310	0.0312	0.0023	7.46	0.57	Day 1
Plasma	0.0630	0.0671	0.0022	3.25	6.51	$r^2 = 0.9976$
	0.126	0.123	0.0098	7.99	-2.34	
	0.250	0.244	0.0275	11.3	-.2.35	Day 2
	0.751	0.738	0.054	7.41	-1.74	$r^2 = 0.9954$
	1.50	1.47	0.101	6.88	-2.29	
	3.00	3.05	0.203	6.64	1.730	Day 3
						$r^2 = 0.990$
Rat	0.0470	0.0497	0.0015	3.05	5.67	Day 1
Plasma	0.080	0.0780	0.0062	7.97	-2.18	$r^2 = 0.9989$
	0.160	0.162	0.0102	6.30	1.30	
	0.319	0.322	0.0229	7.10	1.07	Day 2
	2.30	2.27	0.0553	2.43	-1.09	$r^2 = 0.9993$
	4.60	4.70	0.159	3.38	2.22	
	9.20	9.20	0.343	3.73	0.00	Day 3
						$r^2 = 0.9990$

Each calibration curve had to demonstrate a coefficient of determination (r^2) value of at least 0.99. The accuracy had to be within ±15% of the theoretical/target value and the precision had to be below 15% over the range of the standard curve.

RESULTS AND DISCUSSION

Antide was chosen as the internal standard for this assay as it eluted without interference from either RWJ-47428-099 or coextracted endogenous plasma components. The respective retention times of RWJ-47428-099 and its internal standard were approximately 11.5 and 13.5 minutes in monkey plasma and 16 and 18 minutes in rat plasma.

The back calculated values of standard curves in both monkey and rat plasma are summarized in Table 1. Over the concentration ranges of 0.031 - 3.0 μg/mL (monkey plasma) and 0.047 - 9.2 μg/mL (rat plasma), linear regression analysis of RWJ-47428-099/internal standard peak area ratios versus RWJ-47428-099 concentration afforded a mean r^2 (coefficient of determination) value of 0.990 or greater on each day of analysis.

Table 2

Precision and Accuracy for RWJ-4728-099

		Monkey Plasma Target Conc'n (μg/mL)			Rat Plasma Target Conc'n (μg/mL)		
		0.065	**0.725**	**2.17**	**0.080**	**0.638**	**4.60**
Day 1[1]	Mean	0.067	0.766	2.21	0.076	0.603	4.81
	SD	0.000	0.024	0.121	0.034	0.012	0.074
	%CV	14.09	3.07	5.49	4.44	1.99	1.53
	Accuracy	2.62	5.70	1.84	-5.46	-5.43	4.49
Day 2[1]	Mean	0.073	0.682	1.98	0.077	0.622	4.54
	SD	0.001	0.011	0.057	0.004	0.004	0.140
	%CV	1.16	1.55	2.87	4.55	0.65	3.08
	Accuracy	12.72	-5.93	-8.60	-3.39	-2.56	-1.30
Day 3[1]	Mean	0.066	0.650	2.12	0.075	0.641	4.68
	SD	0.007	0.006	0.029	0.012	0.072	0.212
	%CV	10.12	0.85	1.36	15.89	11.19	4.53
	Accuracy	1.85	-10.3	-2.15	-6.42	0.52	1.81
Overall[2]	Mean	0.069	0.700	2.11	0.076	0.622	4.68
	SD	0.007	0.054	0.121	0.007	0.040	0.174
	%CV	9.76	7.67	5.76	8.58	6.42	3.73
	Accuracy	5.73	-3.51	-2.99	-5.08	2.52	1.68

[1]n = 3 determinations.
[2]n = 9 determinations
SD = standard deviation
Precision expressed as %CV
Accuracy expressed as [(Actual concentratin - Target concentration)/Target concentration] x 100

As shown in Table 2, overall interday precision was 10% or less in monkey plasma and 9% or less in rat plasma. Accuracy, expressed as percent deviation from theoretical concentration, was approximately ±6% in monkey plasma and ±5% in rat plasma.

The recovery was determined by calculating the ratio of the amount of extracted compound from plasma spiked with known amounts of RWJ-47428-099 to the amount of compound added at the same concentrations to water just prior to HPLC injection. In spiked quality control samples (0.065, 0.725, and 2.17 μg/mL in monkey plasma and 0.08, 0.648, and 4.60 μg/mL in rat plasma), the overall mean recoveries of RWJ-47428-099 over the usable assay ranges were approximately 91 and 78%, respectively.

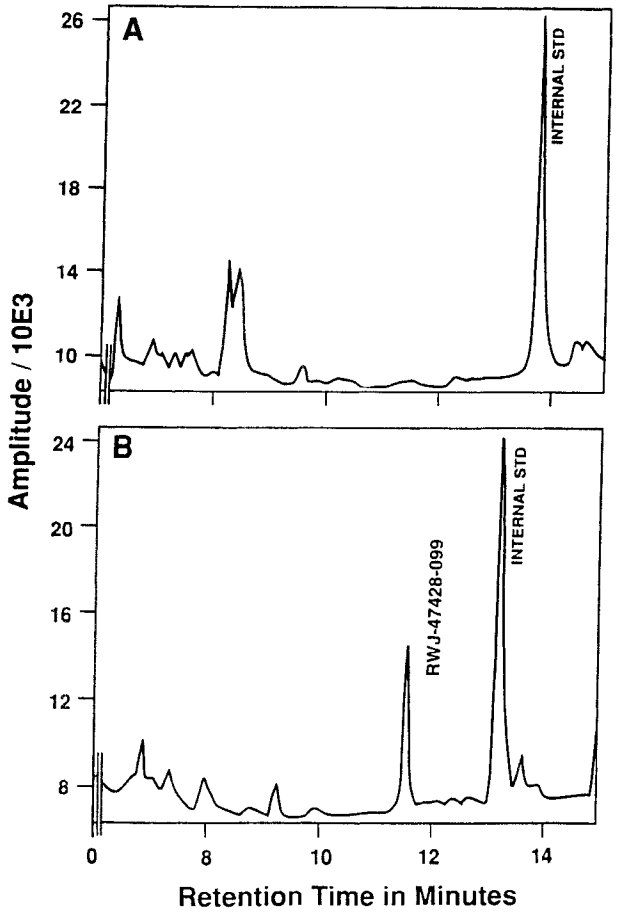

Figure 2. Elution profiles of RWJ-47428-99 and the internal standard (Antide) in: (a) blank monkey plasma, and (b) monkey plasma containing RWJ-47428-099 at a concentration of 1.33 μg/mL following subcutaneous administration of a single 6 mg/kg dose.

Analysis of these samples afforded concentrations which were nearly identical to concentrations obtained from the analysis of freshly prepared quality control samples, thus confirming that RWJ-47428-099 was stable in both matrices for at least 3 freeze-thaw cycles. Additionally, RWJ 47428-099 was stable frozen at -24 °C for at least 3 months in monkey plasma and 1 month in rat plasma.

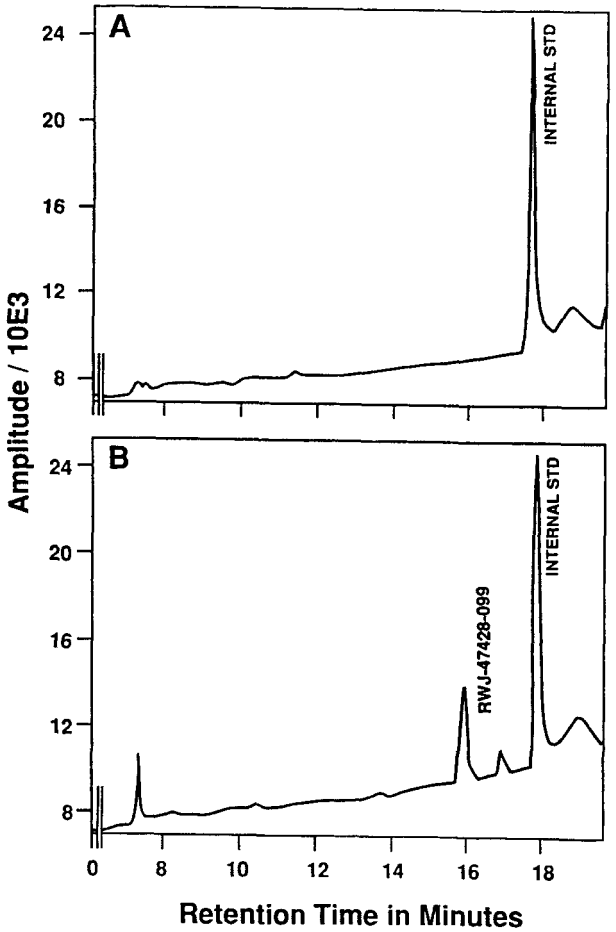

Figure 3. Elution profiles of RWJ-47428-99 and the internal standard (Antide) in: (a) blank rat plasma, and (b) rat plasma containing RWJ-47428-099 at a concentration of 0.683 μg/mL following subcutaneous administration of a single 6 mg/kg dose.

Numerous analytical methods have been developed for the measurement of peptides,[7-20] but most of the methods cited in the literature have either lacked sensitivity or required modification prior to application in biological fluids. The method described in this communication was readily applied to the analysis of samples from preclinical drug metabolism studies. Representative chromatograms of extracted plasma samples from monkeys and rats receiving subcutaneous doses of RWJ-47428-099 are shown in Figures 2 and 3,

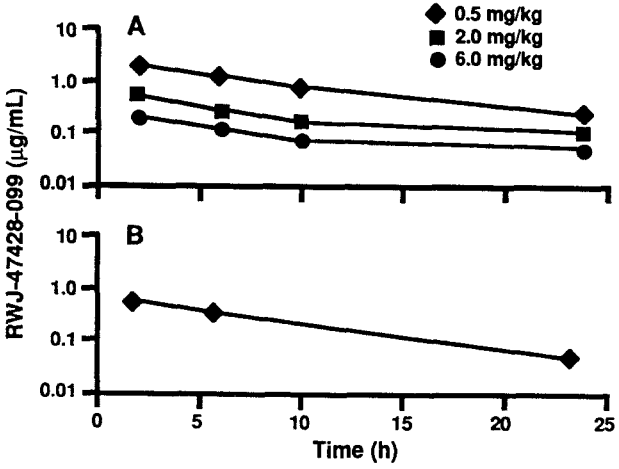

Figure 4. Mean plasma concentration (n=6) vs. Time profiles of RWJ-47428-99 in: (a) Cynomolgus monkeys following subcutaneous administration of single 0.5, 2, and 6 mg/kg doses, and (b) Sprague Dawley rats following subcutaneous administration of single 6 mg/kg doses.

respectively. Figure 4 shows representative plasma concentration-time profiles following subcutaneous administration of: (a) single 0.5, 2, and 6 mg/kg doses to Cynomolgus monkeys; and (b) a single 6 mg/kg dose to Sprague Dawley rats.

In general, sensitivity and chromatographic resolution are known to improve considerably upon decreasing the column internal diameter from the conventional analytical range (3.9 - 4.6 mm) through the narrowbore (2 - 3 mm) and microbore (1 - 2 mm) ranges. However, biofluid analysis presents a significant challenge especially with regard to the shortening of usable column life. It has been suggested that the narrowbore columns provide an effective combination of adequate chromatographic performance and column life.[9] Indeed, during development of the methodology described in this communication, the narrowbore columns sustained good performance over considerably larger numbers of sample injections than did the microbore columns using identical sample pretreatment conditions. Narrowbore columns presented an additional advantage over microbore columns in that they were compatible with conventional analytical systems, thus eliminating the need for specialized HPLC equipment. For these reasons, they were employed in a

number of bioanalytical support activities. As the methodology described here is rapid and reliable, it should generally prove useful in supporting further investigations in biological matrices.

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