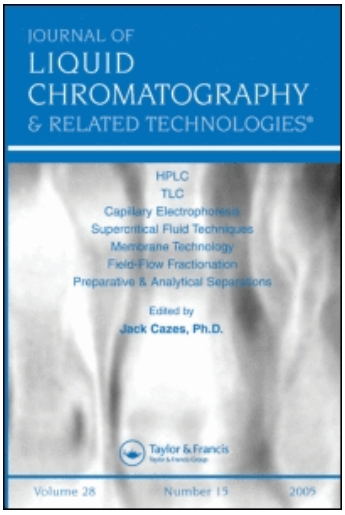

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HPLC-EC ASSAY FOR THE INODILATOR EXP513 AND ITS APPLICATION IN A DOG PHARMACOKINETIC STUDY

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ABSTRACT

An HPLC-EC assay was developed for EXP513, a substituted dihydropyridine inodilator. Inodilators are intended for congestive heart failure by increasing myocardial contractility and increasing peripheral vasodilation by blockage of vascular α-adrenergic receptors. The assay was linear between 5 and 2500 ng/mL in dog plasma and the CV for the intraday reproducibility at 5 ng/mL averaged 10.2%. The recovery from dog plasma averaged 67%. A pharmacokinetic study in dogs after PO and IV dosing showed that EXP513 was rapidly absorbed from the GI tract, highly distributed into tissues and was quickly eliminated. The bioavailability of EXP513 at 30 mg/kg administered as a solution was 13.5%. The half-life of EXP513 in dogs after IV and PO administration averaged 2.8 and 2.5 hr, respectively.

INTRODUCTION

Heart failure is characterized by increased sympathetic nervous system activity and a decreased contractility of the heart (1). Inodilators are compounds intended to improve contractility (positive inotropic effect) and reduce peripheral vascular resistance (vasodilation) (2). EXP513 has been shown to have a combination of calcium agonism which increases myocardial contractility and α-adrenergic receptor antagonism which induces peripheral vasodilation (3,4).

Reported assay methods for the determination of dihydropyridines in biological fluids include UV detection using ion-pair chromatography for nitrendipine (5), gas chromatography/mass spectrometry using selected ion monitoring for NB-818 (6), and electrochemical (EC) detection for nifedipine and MCP1304 (7,8). This report describes a sensitive and reproducible HPLC-EC assay for the new dihydropyridine inodilator, EXP513.

MATERIALS AND METHODS

Chemicals

HPLC grade methylene chloride and acetonitrile were purchased from Burdick and Jackson (Muskegan, MI). Water was triple distilled using a milli-Q-reagent water system from Continental Water System (El Paso, TX). Phosphoric and hydrochloric acids were obtained from Mallinckrodt (Paris, KY). Bicarbonate, carbonate, and potassium phosphate buffers and polyethylene glycol 400 were purchased from J. T. Baker (Phillipsburg, NJ). Sodium EDTA was obtained from Kodak Chemical Co. (Rochester, NY). EXP513 and its internal

standard (EXP510) were synthesized at the Du Pont Merck Pharma- ceutical Co. (Wilmington, DE). Methyl cellulose was obtained from Sigma Chemical Co. (St. Louis, MO). Ethyl alcohol was purchased from Pharmco Products, Inc. (Dayton, NJ).

Chromatographic System and Conditions

The HPLC system consisted of a Model 6000A high pressure pump and a WISP® automatic injection system from Waters Associates (Milford, MA). The effluent was monitored using a dual LC4B amperometric detector with a glassy carbon electrode from Bioanalytical Systems (BAS), Inc. (W. Lafayette, IN). A cyclic voltammagraph scan showed 1.0 volts oxidation to be the voltage maximum for EXP513, but 0.9 volts was chosen to avoid excessive background noise. A μBondapak C_{18} column (15 cm by 3.9 mm ID) from Phenomenex, Inc. (Torrance, CA) or a Zorbax® C_{18} column (15 cm by 4.6 mm ID) from MacMod, Inc. (Chadds Ford, PA) was used to chromatograph the compounds. The mobile phase consisted of acetonitrile, 0.02 M potassium phosphate monobasic buffer and 1 M phosphoric acid (30/69/1, v/v/v) and 50 mg/L EDTA with a flow rate of 1.5 mL/min.

Assay Procedure

Plasma standards were prepared by adding 50 μL of EXP513 (0.1 to 50 μg/mL in acetonitrile/water, 1:1 mixture), 50 μL internal standard (10 μg/mL of EXP510 in acetonitrile/water, 1:1 mixture), and 50 μL 0.1 M sodium carbonate/ bicarbonate buffer (pH 10) to 950 μL plasma. Plasma samples from the oral and intravenous dog studies were processed by spiking 50 μL of both the internal standard and 0.1 M sodium carbonate/bicarbonate buffer in 1 mL plasma. All samples

were then extracted with 10 mL of methylene chloride by mixing on a mechanical shaker for 10 minutes. After centrifugation at 3000 rpm, the upper aqueous layer was aspirated to waste and the bottom methylene chloride layer was transferred to a clean centrifuge tube and dried using nitrogen gas. A 300 µL aliquot of mobile phase was used to reconstitute the sample. After mixing, a 100 µL aliquot was injected onto the HPLC.

Animals

Male mongrel dogs were obtained from Bar Wan Labs (Crocker, MO). All animals were fasted overnight prior to dosing. The experiments were conducted in a research facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

IV Bolus Studies

Four male mongrel dogs were administered IV bolus doses of either 3 or 10 mg/kg EXP513. The compound was dissolved in ethanol/polyethylene glycol (PEG) 400/distilled water (30/60/10, v/v/v). Blood samples were collected from a cannulated saphenous vein using heparinized syringes at pre-dose, 1, 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, and 300 minutes post-dose. The plasma was obtained after centrifugation and stored at -20°C until analysis.

Oral Studies

Three male mongrel dogs were administered 30 mg/kg EXP513 in 0.1 N HCl/PEG 400 (20/80, v/v). Blood samples were collected from a cannulated saphenous vein using heparinized syringes at pre-dose, 5, 10, 20, 30, 45, 60, 90, 120, 150, 180,

240, 300, and 360 minutes postdose. The plasma was obtained after centrifugation and stored at -20°C until analysis.

Data Analyses

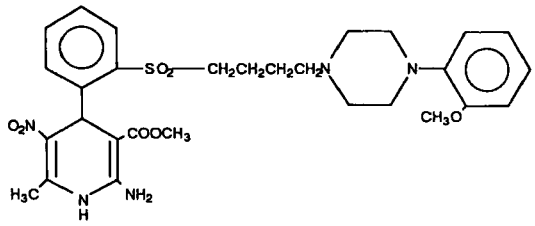
The HPLC peaks were identified by retention time with reference to standard compounds and quantitated using peak area ratio with reference to the standard curve. A Hewlett Packard 3357 laboratory computer system (Avondale, PA) was used for data acquisition.

The maximum plasma concentration (Cmax) was the observed peak concentration after a PO dose. Tmax was the corresponding time when Cmax was observed. The elimination rate constant (Kel) was determined by linear regression of the terminal phase of the logarithm plasma concentration-time curve. The plasma elimination $T_{1/2}$ was calculated by dividing 0.693 by Kel. The area under the curve from time zero to the last time point (AUC_t) was determined using the linear trapezoidal rule. The area from time zero to infinity (AUC) was determined by the sum of AUC_t and the area extrapolated from the last time point to infinity ($Cp_{,\ last}$/Kel). Systemic clearance (CL) was calculated from the dose divided by AUC. The volume of distribution (V) was determined from intravenous data by dividing dose by (AUC•Kel). Oral bioavailability (F) was determined from the ratio AUC_{PO}/AUC_{IV} where AUC was normalized for dose.

RESULTS AND DISCUSSION

The chemical structure of EXP513 and its internal standard, EXP510, is shown in Figure 1. Representative chromatograms of an extracted blank dog plasma sample (Figure 2A) and an extracted 250 ng/mL EXP513 spiked plasma

EXP513



EXP510

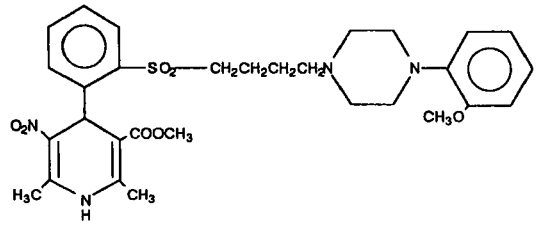


Figure 1. Chemical structure of EXP513 and the internal standard, EXP510

sample (Figure 2B) are shown. The HPLC retention times of EXP513 and EXP510 were 3.2 and 5.5 minutes, respectively. The intraday assay reproducibility and the plasma recovery data are shown in Table 1. The intraday variation was 10.2% for 5 ng/mL and approximately 30% or less for all other concentrations. Extraction recovery from dog plasma averaged 76.5, 64.2, 63.9, and 64.0% at 10, 25, 250, and 500 ng/mL, respectively. The assay was linear between 5 and 2500 ng/mL plasma and the correlation coefficient of the calibration curves exceeded 0.999. The compound was stable in dog plasma stored at -20°C for at least 35 days. The peak height ratios (n=4) for EXP513 and the internal standard were 1.12, 1.06, and 1.08 for Days 1, 7, and 35, respectively.

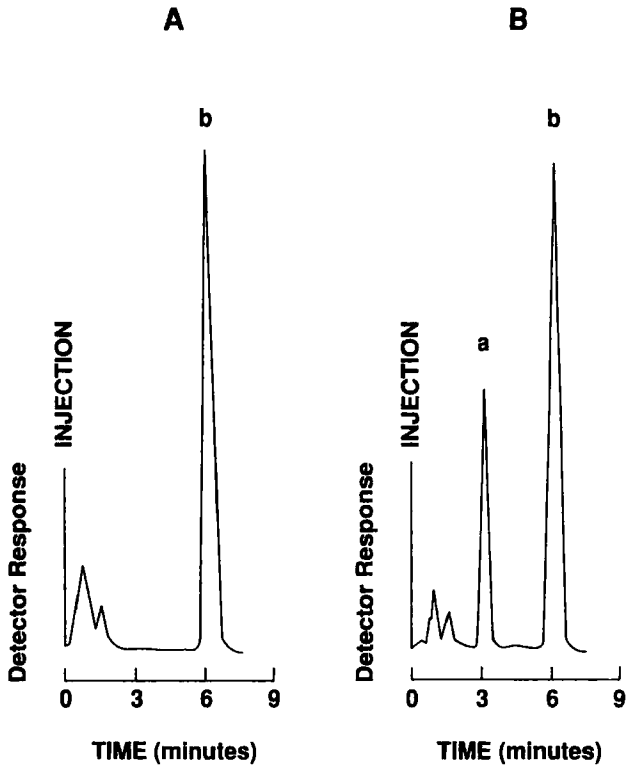


Figure 2. Typical chromatograms of extracted blank dog plasma (2A), and extracted plasma sample spiked with 250 ng/mL of EXP513 (2B). The HPLC retention times were 3.2 (a) and 5.5(b) minutes for EXP513 and its internal standard (EXP510), respectively.

TABLE I

Intraday Assay Reproducibility and Recovery in Dog Plasma

Intraday Reproducibility (N=6)		Plasma Recovery (N=4)	
Conc. (ng/mL)	Peak Height Ratio ± SD (CV)	Conc. (ng/mL)	% Recovery ± SD
5	0.049 ± 0.005 (10.2)	10	76.5 ± 11.8
10	0.085 ± 0.026 (30.6)	25	64.2 ± 4.33
100	0.625 ± 0.013 (2.10)	250	63.9 ± 4.63
500	3.060 ± 0.055 (1.80)	500	64.0 ± 5.23

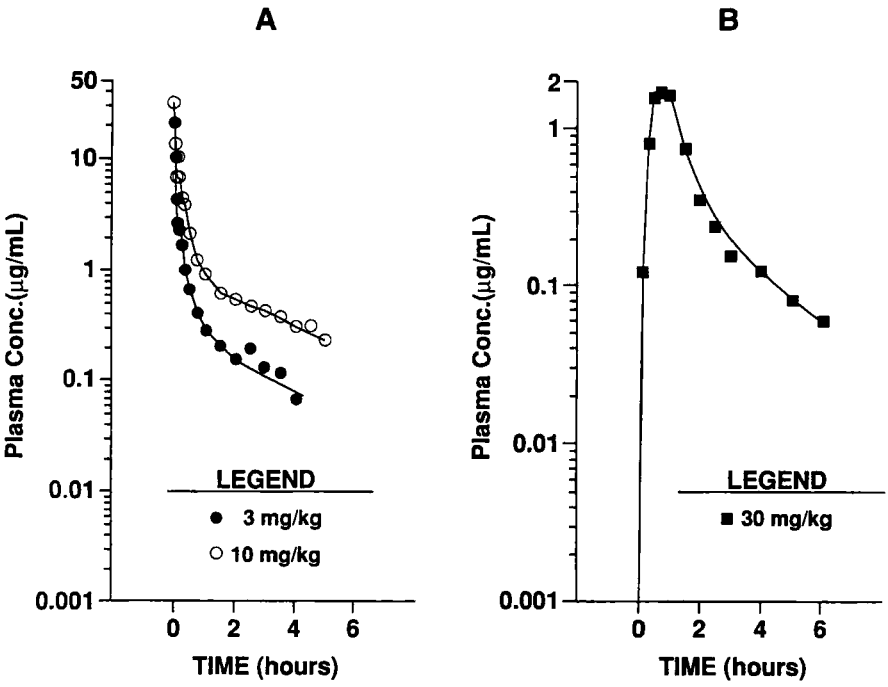


Figure 3. Mean Dog Plasma Concentrations of EXP513 Versus Time Following IV Bolus Injections of 3 and 10 mg/kg (A) and PO Administration of 30 mg/kg (B).

Figure 3 shows the 3 and 10 mg/kg IV bolus and 30 mg/kg PO plasma profile over time. The corresponding pharmacokinetic parameters are listed in Table 2. The mean clearance, volume of distribution and elimination half-life of EXP513 were 1.04 L/hr/kg, 4.33 L/kg, and 3.10 hr for the 3 mg/kg dose and 1.48 L/hr/kg, 5.30 L/kg, and 2.45 hr for the 10 mg/kg dose. The similar mean clearance and half-life values for the 3 and 10 mg/kg doses indicate that there were no significant dose-dependent changes in the pharmacokinetics of EXP513 over this dose range. EXP513 was rapidly absorbed from the gastro-

TABLE II

Pharmacokinetic Parameters Following IV Bolus and Oral Gavage Administration of EXP513 in Dogs

Pharmacokinetic Parameters	IV Bolus[a]		Oral Gavage[b]
Dose, mg/kg	3.00	10.00	30.00
CL, L/hr/kg	1.04	1.48	---
V, L/kg	4.33	5.30	---
T1/2, hr	3.10	2.45	2.47 ± 0.46
AUC, μg•hr/mL	3.03	6.96	2.82 ± 0.26
Cmax, hr	---	---	2.16 ± 0.49
Tmax, hr	---	---	0.75 ± 0.25
F, %[c]	---	---	13.50

a N=2.
b N=3, mean ± SD.
c F obtained by $\frac{[AUC_{PO}\ (30\ mg)]/3}{[AUC_{IV}\ (10\ mg)]}$

intestinal tract and the volume of distribution after IV administration indicates that this compound is highly distributed into tissues. The oral bioavailability of EXP513 was relatively low at 13.5%, but two other dihydropyridines, nitrendipine (5) and benidipine (9) also demonstrated low oral bioavailability in dogs.

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