Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2014 March 15; 0: 86–91. doi:10.1016/j.jchromb.2014.01.031.

A sensitive and fast LC-MS/MS method for determination of β -receptor agonist JP-49b: Application to a pharmacokinetic study in rats

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Abstract

Ocular administration of the beta (β)-adrenergic receptor agonist JP-49b prevents retinopathy-like damage in a preclinical rat model of diabetes. Importantly, JP-49b did not induce characteristic β -adrenergic agonist-related side effects (*e.g.*, left ventricular damage), which led to the hypothesis that JP-49b systemic exposure was minimal following ocular administration. To test this hypothesis, a sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed to study the preclinical pharmacokinetics of JP-49b in rats. Animals received either a single periocular or intravenous injection of JP-49b (10 mg/kg) and plasma and tissue samples were obtained. JP-49b and fenoterol hydrobromide (internal standard, IS) were isolated by liquid–liquid extraction and extracts were analyzed by reversed-phase liquid chromatography on a C₁₈ column using a gradient elution (acetic acid in water and methanol). A triple quadrupole mass spectrometer operating in the positive electrospray ionization mode with multiple reaction monitoring was used to detect JP-49b and IS transitions of m/z 346.4 $\rightarrow$ 195.1 and 304.1 $\rightarrow$ 134.9. The method was validated for selectivity, linearity, accuracy, and precision in rat vitreous humor, tissue homogenates, and plasma. Following intravenous administration, JP-49b was found to have a rapid clearance (36 ± 5.8 L/h/kg), high volume of distribution (244 ± 51.5 L/kg) and a terminal half-life of 4.8 ± 1.6 h. JP-49b was rapidly absorbed and extensively distributed into ocular tissue following topical administration. However, JP-49b was undetectable in heart tissue 24h after ocular administration. High local drug concentrations coupled with minimal systemic exposure following ocular administration supports further testing of JP-49b as a localized therapy for diabetic retinopathy.

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Keywords

LC-MS/MS; β -receptor agonist; JP-49b; rat pharmacokinetics

1. Introduction

Diabetic retinopathy is the leading cause of blindness in working age adults in the United States [1, 2], accounting for 12,000-24,000 new cases of blindness each year (JDRF website). Previous work has demonstrated that loss of sympathetic neurotransmission to the retina produces changes in the retina that mimic those noted in diabetic animal models[3, 4]. Because loss of β -adrenergic receptor signaling led to apoptosis of retinal endothelial cells (REC) [5–7] and retinal Müller cells [8], we hypothesized that restoration of β -adrenergic receptor signaling may prevent many of the deleterious changes noted in the diabetic rat retina. To test this hypothesis, topical isoproterenol (50 mM) was applied daily to rats with streptozotocin-induced diabetes for 2 and 8 months. Topical isoproterenol was effective in preventing the formation of degenerate capillaries, pericyte ghosts, cell loss in the ganglion cell layer, and functional changes in the electroretinogram [9, 10]. However, increased collagen content in the left ventricle of isoproterenol-treated diabetic rats suggested significant systemic exposure following topical isoproterenol administration.

To address the deleterious cardiovascular effects of isoproterenol [11, 12], a novel β -adrenergic receptor agonist, JP-49b, was designed. Within the structure of JP-49b, there is an N-substituted *p*-methoxyphenyl ring, which approaches the other ring to allow π - π electron interactions with the catechol aromatic ring lock the conformation (Figure 1). This stable conformation limits the conformational and orientational freedom within the β -adrenergic receptor binding pocket. In screening experiments, it was found that JP-49b increased PKA activity at 1mM vs. 50mM required for isoproterenol. Subsequent preclinical efficacy studies demonstrated that topical JP-49b (1 mM) was as effective as topical isoproterenol (50 mM) in preventing retinopathy-like changes in the diabetic rat [13]. We had previously found that topical isoproterenol reduced collagen staining in the left ventricle, suggesting isoproterenol did reach the systemic circulation (unpublished observations). A key finding for JP-49b was that it did not alter diabetes-induced changes to the left ventricle, suggesting it did not reach the systemic circulation. To address the pharmacokinetic properties of JP-49b and to measure levels of JP-49b in the vitreous humor of rats, we developed the following assays which demonstrated that JP-49b is cleared from the plasma rapidly, whether administered topically or intravenously at a dose 10-fold higher than the proposed therapeutic dose. Vitreous humor levels peak prior to 1 hour after topical delivery.

2. Experimental

2.1. Reagents and Chemicals

JP-49b was synthesized in the laboratory of Dr. Duane Miller at University of Tennessee Health Science Center. The purity of JP-49b was verified to be greater than 96% by nuclear magnetic resonance spectroscopy. Fenoterol hydrobromide (internal standard; IS) was purchased from Sigma-Aldrich. All mobile phase solvents and water of HPLC grade were purchased from Fisher-Scientific (Pittsburgh, PA, USA). Drug-free rat vitreous humor and plasma were obtained from Pelfreez Biologicals (Rogers, AR, USA).

2.2. Animals

The study was conducted in strict adherence with the Guide for the Care and Use of Laboratory Animals. Male Lewis (LEW/Crl) rats were received from an accredited closed

colony (Charles River UK) and maintained in the animal facility at University of Tennessee Health Science Center. Rats were fed *ad libitum* on normal laboratory diet and water. A 12 h light-dark cycle and ambient temperature of 25 °C were maintained. Rats bearing jugular vein catheters were used for the peripheral pharmacokinetic study after intravenous bolus injection. The patency of the jugular vein catheters was maintained every other day with heparinized glycerol solution (500 IU heparin/mL final solution in 75% glycerol) according to vendor's instructions. Rats were allowed to recover from the surgical procedure overnight with free access to water and food at all times.

2.3. LC-MS/MS Parameters

The LC-MS/MS system comprised an Applied Biosystems (AB) Sciex (Foster City, CA) API 3000 tandem mass spectrometer, equipped with a Turboionspray™ ionization interface in positive ion mode. Chromatographic separation of analytes was carried out using a Phenomex Synergi Hydro C₁₈ analytical column (2.5 µm, 2.1x 50 mm, Waters Corporation, Milford, MA) maintained at 40°C using Shimadzu (Columbia, MD) LC-10ADvp pumps with a Leap (Carrboro, NC) HTS PAL autosampler. The mobile phase (A: 0.01% acetic acid in Milli-Q water, B: 100% methanol) was eluted at a flow rate of 0.3mL/min. The gradient started at 10% of mobile phase B and linearly rose to 20 % B over 1.2 min. Subsequently, the eluent composition was maintained for 7.7 min before it was decreased to 10 % mobile phase B for re-equilibration. Then returned to the initial condition (10% B) in 0.1 min and equilibrated for 3.0 min. The total run time was 12 min. A switching valve directed the mobile phase to the MS system between 5.8–9.0 min only. The electrospray ion source was operated in a positive ionization mode for all the experiments. The typical ion source parameters were: capillary 3.5 kV, declustering potential (DP) 100 V, entrance potential (EP) 10 V, collision energy (CE) 20 eV, collision cell exit potential (CXP) 15 V for JP-49b, 12 V for IS, channel electron multiplier (CEM) 2200 V, source temperature 325°C. Quantification was performed in the multiple reaction-monitoring mode (MRM), monitoring the transition of the m/z 346.1→195.1 product ion for JP-49b and m/z 304.1→134.9 product ion for IS. The analytical data were processed using the software program Analyst (Version 1.3). The run time for a single sample was 12 min with JP-49b eluting at 6.85 minutes, and IS eluting at 8.13 minutes.

2.4. Preparation of calibration standards and quality control samples

JP-49b and IS stock solutions (1 mg/mL) were prepared in high purity water. Working solutions of JP-49b were prepared by further diluting the stock solution in water: acetonitrile (1:1 v/v). Blank lung homogenates, homogenized in saline (1:3, wt/vol), were used as a surrogate matrix for JP-49b calibration standards and QC in lung, heart and spleen samples. Calibration standards and QC samples were prepared by mixing 10µL working solution in 90 µL drug free rat plasma, vitreous humor, or tissue homogenates, resulting in matrix concentrations of 2.5-1000 ng/mL, 40-2000 ng/mL and 40-4000 ng/g. IS were prepared in precipitation solution to a concentration of 200 ng/mL. Blank and zero samples were prepared using blank plasma, vitreous humor and tissue homogenates. All samples were stored at -80°C prior to use.

2.5. Sample preparation

Protein precipitation was used to extract JP-49b from rat plasma, vitreous humor and lung, heart and spleen tissue homogenates. An aliquot (50 µL) was added in precipitation solution (20% trichloroacetic acid:methanol, 1:1 v:v), vortexed for 30 seconds, and then centrifuged for 10 minutes at 4°C (circa 21000g). The supernatant was transferred to a 96-well plate and an aliquot (5 µL) was injected onto the LC-MS/MS system.

2.6. LC-MS/MS method validation

Method validation was conducted in accordance with the criteria suggested by the US Food and Drug Administration (FDA) Guidance for Industry – Bioanalytical Method Validation[14]. Calibration curves were constructed by plotting the peak area ratio of JP-49b: IS against the nominal concentration of calibration standards. For each batch of samples a calibration curve was constructed from the results of duplicate measurement of 9 concentrations of JP-49b. The peak area ratios of JP-49b to IS versus corresponding concentrations were used for the linear least-squares regression of the calibration lines and for determination of slopes (A), intercepts (B), and correlation coefficients. Unknown sample concentrations of JP-49b were calculated from the linear regression equation with a weighted factor of $1/x^2$. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a signal to noise > 5 over six independent runs with coefficient of variation % accuracy within 20% and precision within 20% for every run. The limit of detection (LOD) was not determined. Three quality control (QC) validation samples were prepared at 4.5, 90, 900 ng/mL in rat plasma, 40, 200, 1800 ng/mL in vitreous humor and 40, 400, 3600 ng/mL in tissue homogenates. All chromatograms were processed using the automatic integration software module in Analyst 1.3 followed by manual check to confirm proper integration of each peak area.

Method selectivity was evaluated by comparing the chromatograms of six blank plasma samples from six different untreated rats for interference at the retention times of JP-49b and IS. The intra- and inter-day precision and accuracy were estimated by parallel analytical runs performed on the same day or on four different days. Each analytical run consisted of a matrix blank, a set of calibration standards, six replicate LLOQ samples, and a set of three levels of QC samples. The acceptance criteria of the data included precision within 15% relative standard deviation and accuracy within $\pm 15\%$ deviation of the nominal value. Ion suppression via matrix effect was evaluated by adding three level JP-49b QC sample with and without IS to extracted blank plasma comparing JP-49b peak areas to neat solution samples. Stability of JP49b-spiked plasma samples under various conditions including freeze–thaw cycles and long-term storage at -80°C . Freeze–thaw sets of QC samples ($n = 3$) were thawed at room temperature then put back into the freezer for a minimum of 24 h. This process was repeated three times. Long-term stability QC samples were analyzed after 1 week. Post-preparative autosampler stability was determined by re-injection of samples 6 h following initial injection. The peak areas of the JP-49b and IS obtained at freshly prepared samples were used as the reference to determine the relative stability at subsequent points. Absolute recoveries ($n = 3$) using QC samples were assessed by comparison of the response ratio of extracted plasma QC samples with those of QC samples in neat solution (Millie Q water).

2.7. Pharmacokinetic study

Rats ($n = 5$) received a single dose JP-49b (hydrochloride, 10mg/kg, 2.5 mL/kg) in PBS administered through the jugular vein cannula followed by heparinized saline (1 mL) flush. Blood samples (0, 5, 15, 30, 45 min and 1, 2, 4, 8 16, and 24 h) were manually obtained through the cannula. Plasma was separated by centrifuge and stored at -80°C prior to analysis by (LC-MS/MS). After collection of the final blood sample, rats were sacrificed and the heart, lungs and spleen were removed, washed in saline, placed into plastic scintillation vials, weighed and then snap frozen. Tissue samples were homogenized in saline (1:3, wt:vol). The JP-49b concentration in tissue was calculated as mg/g. A separate cohort of rats ($n = 40$) received a single ocular dose of JP-49b (10 mg/kg) in PBS. Animals ($n = 5$ /each time point) were sacrificed at 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hours post-dose and blood samples were obtained. Eyes (*in situ*) were rinsed with ice-cold saline to remove residual surface dose and the vitreous humor from both eyes of each rat were collected and pooled.

Pooled samples were weighed prior to snap freezing and stored at -80°C until LC-MS/MS analysis. No clinical signs (behavioral changes, any indications of ill health or reaction to treatment) were observed during the study. JP-49b plasma and vitreous humor concentration vs. time data were analyzed by WinNonlin (Version 7.0, Pharsight, Mountain View, CA).

2.8. Plasma protein binding

Plasma protein binding of JP-49b was determined using rapid equilibrium dialysis (Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. In brief, JP-49b (1 mg/mL in normal saline) was added to rat and human plasma (200 μL) to achieve final concentrations of 4.5, 90, and 900 ng/mL. Equilibrium between dialysis buffer and plasma was reached over 6 hr at 37°C with 100 rpm orbital shaking. JP-49b concentration was measured in aliquots (100 μL) of post-dialysis plasma and buffer by LC-MS/MS as described above. The unbound fraction of JP-49b was estimated by comparing the buffer to plasma concentration ratio.

3. Results and discussion

3.1. Method development and qualification

Fenoterol hydrobromide was chosen as the IS because of its similar basic structure, physicochemical property and mass spectrometric behavior to those of JP-49b. Optimization of MS first involved tuning of MS parameters in positive and negative ionization modes for JP-49b and IS using a 1 $\mu\text{g/mL}$ tuning solution in methanol. For both compounds, the response observed in positive mode was better than in the negative mode. Fig. 2 depicts the mass spectrum of JP-49b and IS in positive mode. Both mono-protonated molecular ions $[\text{M}-18]^+$ at m/z $346.5 \rightarrow 195.2$ and $[\text{M}+\text{H}]^+$ $304.4 \rightarrow 134.9$ were present as major peaks for JP-49b and IS, respectively. The identifications matched the theoretical molecular weights of JP-49b (363.4 Da, free) and IS (303.36 Da, free).

The mobile phase, elution type, flow rate, as well as type of chromatographic column were tested to optimize the best chromatographic conditions. A methanol-based mobile phase had lower background noise than acetonitrile. The inclusion of 0.01% acetic acid greatly enhanced the intensities of the peaks. It was also noted that gradient elution dramatically improved response intensity, resolution, and peak shape. Out of a number of commercially available C18 columns evaluated, the Phenomenex Synergi Hydro 50×2.1 mm, analytical column was found to give the most satisfactory chromatography, and the 5.0 cm column length helped to reduce the whole analysis time.

A simple protein precipitation (PPT) method was selected for simplicity and low cost. Method optimization initially focused on the selection of an appropriate organic extraction agent (methanol and acetonitrile). Methanol proved superior to acetonitrile when matrix effect, analyte recovery, and peak shape were considered. Addition of trichloroacetic acid (TCA 20%: methanol v/v) further increased analyte recovery as well as signal to noise ratio under the aforementioned mass spectrometry parameters.

3.2. Method validation

Selectivity, linearity, precision, accuracy, matrix effect, recovery and stability were assessed for method validation. Assay selectivity was assessed at the LLOQ using six drug-free samples from six different plasma lots. No interfering peaks at the retention times of either JP-49b or IS were observed in blank extracted plasma. Additionally, no chromatographic peaks corresponding to the retention times for either JP-49b or IS were detected in pre-dose rat plasma. Fig. 3 displays representative chromatograms of blank plasma and plasma spiked with JP-49b and IS.

Calibration curves for JP-49b were linear over the concentration range of 2.5–1000, 40–2000 ng/mL for plasma and vitreous humor, and 40–4000 ng/g for tissue (heart, lung, spleen) samples. The correlation coefficient (R^2) for all assays was greater than 0.999 with weighting ($1/x^2$). The LLOQ for JP-49b was 2.5, 20, and 40 ng/mL for the plasma, vitreous humor and tissue assays. The range of inter- and intra-day accuracy and precision values in plasma were 0.44–15.2% and 6.5–13.5% (Table 1). Absolute recovery of JP-49b from plasma ranged from 95.3–103% using high, mid and low QC samples (4.5–900 ng/mL). The matrix effects of JP-49b in plasma were from 85.3 % and 88.5%. JP-49b and IS were stable for 6 hours following precipitation at room temperature (Table 2). The degradation of JP-49b after long-term and three freeze-thaw investigation was less than 10%.

3.3. JP-49b Pharmacokinetics

The above validated LC-MS/MS method was used to study the ocular and systemic exposure of JP-49b in rats. The plasma concentration versus time profile for JP-49b following intravenous administration is shown in Figure 4. JP-49b was found to have a rapid clearance (36 ± 5.8 L/h/kg), high volume of distribution (Vd) (244 ± 51.5 L/kg) and a terminal half-life of 4.8 ± 1.6 hr. JP-49b extrapolated area under the curve (AUC) represented less than 10% of AUC from time zero to infinity. Individual pharmacokinetic parameters are presented in Table 3. JP-49b was extensively bound to plasma proteins with percent bound values of 94.2 ± 0.95 %, and 96.1 ± 0.45 % at concentrations of 90 and 900 ng/mL, respectively. The large volume of distribution coupled with the low unbound fraction of JP-49b in the plasma suggests high tissue binding of JP-49b as well.

3.4. Ocular Pharmacokinetics

The vitreous humor and plasma concentration-vs.-time profiles of JP-49b following periocular administration in rats are shown in Figure 5. Thirty minutes after periocular administration, JP-49b produced a mean peak vitreous humor concentration of 3858 ± 1297 ng/mL. In the vitreous humor, JP-49b concentration declined exponentially, with a terminal half-life of 6.9 ± 2.7 hours, the Vd was 15.6 ± 6.69 (L/kg), and the clearance was 1.3 ± 0.30 L/hr/kg. Following ocular administration, JP-49b plasma concentrations were undetectable after one hour (Figure 5B). A comparison of the plasma exposure ($AUC_{0 \rightarrow 1 \text{ hr}}$) following intravenous and ocular drug administration, revealed that plasma exposure after ocular administration was much lower than the plasma exposure following intravenous administration (35.2 ± 14.2 versus 286 ± 14.0 ng•hr/mL). JP-49b concentrations in lung and spleen were 0.23 ± 0.22 µg/g and 2.14 ± 1.05 µg/g 24 hr after ocular administration. JP-49b was undetectable in heart tissue 24h after ocular administration.

4. Conclusions

A very sensitive and simple quantification method based on LC-MS/MS to determine, JP-49b in rat plasma, vitreous humor and tissue homogenates was developed and validated. This is the first method capable of determining JP-49b concentrations in plasma samples after injection in rats. A simple protein precipitation procedure was used before samples were injected into the autosampler of the LC-MS/MS system. The method showed good precision, accuracy, high sensitivity (2.5ng/mL in plasma), low consumption of bio-sample volume (50 µL), as well as a wide linear range of 2.5–1000, 40–2000 ng/mL and 40–4000 ng/g for JP-49b in plasma, vitreous humor and tissue homogenates. Successful analyses of hundreds of bio-samples from rats demonstrated that the method is efficient, reliable, and suitable for preclinical and future clinical studies of JP-49b.

A potential concern with topically-applied β -adrenergic receptor agonists (*e.g.*, isoproterenol) is significant systemic exposure leading to cardiovascular toxicity (*i.e.*,

increased collagen content in the left ventricle) [11, 12]. Preclinical efficacy studies demonstrated that topical JP-49b (1 mM) was as effective as topical isoproterenol (50 mM) in preventing retinopathy-like changes in the diabetic rat [13]. A key finding was that isoproterenol reduced collagen staining in the left ventricle, whereas JP-49b did not. Consequently, we hypothesized that the lack of cardiovascular effects seen with topically-applied JP-49b was attributed to minimal systemic exposure. Indeed, in this study we demonstrate that JP-49b is rapidly eliminated from the systemic circulation resulting in minimal exposure compared to drug exposure in ocular tissue. Lack of observed cardiovascular effects coupled with minimal systemic exposure following topical administration demonstrates that JP-49b is an ideal candidate for further development for the treatment of diabetic retinopathy in humans.

Acknowledgments

This work was supported by NEI R01-EY022045 (JJS), NEI R01-EY022330 (JJS), JDRF (2-2011-597 to JJS), HEI Research to Prevent Blindness Award (PI: James C. Fleming), and NEI Vision Core Grant: PHS 3P30 EY013080 (PI: Dianna Johnson), and VA Merit Award IBX001585A (JJS). This technology has a US patent number (WO 2011112243 A2) and is owned by the University of Tennessee Research Foundation.

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Highlights

- JP-49b, beta agonist, is in preclinical development for diabetic retinopathy.
- We have developed/validated an LC-MS/MS method for JP-49b measurement.
- JP-49b was well-distributed into ocular tissues after ocular administration.
- JP-49b demonstrates minimal systemic exposure after ocular administration.

A)

(B)

Figure 1.
Structure of (A) JP-49b, (B) fenoterol hydrobromide

(A)

(B)

Figure 2.

Q1 full-scan mass spectrum of JP-49b (A) and IS (B) (1 $\mu\text{g/mL}$) obtained by positive ESI using direct syringe injection of 5 μL .

(A) Blank rat plasma

(B) Blank rat plasma spiked with 1 µg/mL JP-49b

(C) Rat plasma sample after JP-49b administration

Figure 3.
Identity of chromatographic peak used to generate sample data.

Figure 4.

Time-concentration profile of JP-49b in rat plasma. A single intravenous bolus dose of JP49b was used to determine plasma pharmacokinetics (10 mg/kg; n = 6). Data (-▲-) represent mean $\pm$ S.D.

Figure 5.

JP-49b concentration versus time profile in plasma and vitreous humor. JP-49b (10 mg/kg) was administered as a single periocular administration to rats. (A) Time-concentration profile of JP-49b in rat vitreous humor. (B) Time-concentration profile of JP-49b followed by serial sampling of the plasma (-*-) and vitreous humor (-▲-). Data are presented as mean $\pm$ S.D. (n = 5 at each time point).

Table 1

Accuracy, precision, and recovery of the method for determination of JP-49b in plasma.

Concentration (ng/mL)	Intra-day (n=5)		Inter-day (n=9)		Recovery (n=3)	Matrix effect (n=6)
	Accuracy	RSD	Accuracy	RSD		
4.5	9.1%	9.4%	0.4%	10.5%	95±14%	85.3±0.2%
90	13.5%	5.4%	15.2%	5.3%	101±6.8%	87.4±1.2%
900	6.5%	9.6%	11.7%	4.8%	103±6.9%	88.5±3.5%

Table 2

Stability results for JP-49b in rat plasma (n = 3).

stability conditions	Concentration (ng/mL)	RE (%)	RSD (%)
Long-term (1 week, -80 °C)	4.5	1.95	9.23
	90	-3.16	7.41
	900	-8.93	4.18
Post-preparative sample (6 h, room temperature)	4.5	12.5	14.5
	90	5.15	6.55
	900	8.68	7.25
Three freeze-thaw cycles (-80 °C)	4.5	13.8	10.5
	90	-2.36	8.77
	900	-6.82	10.2

Table 3

Plasma and vitreous humor pharmacokinetic parameters of JP-49b in rats

Parameter (units)	Plasma [#] n = 5 (mean ± SD)	Vitreous humor [†] n = 5/time point (mean ± SD)
C _{max} (ng/mL)	1051 ± 71	3858 ± 1297
t _{1/2} (hr)	4.8 ± 1.6	6.9 ± 2.7
AUC _{0-∞} (ng•hr/mL)	281 ± 47.8	7983 ± 2160
V _d (L/kg)	244 ± 51.5	15.6 ± 6.69
CL (L/hr/kg)	36 ± 5.8	1.3 ± 0.30

[#] Plasma pharmacokinetic parameters were determined following intravenous administration of JP-49b (10 mg/kg)

[†] Vitreous humor pharmacokinetic parameters were determined following ocular administration of JP-49b (10 mg/kg)