Validated HPLC Method for the Determination of JWU1497 in Rat Plasma and Its Application to a Comparative Pharmacokinetic Study of the Free Base and Hydrophosphate Salt Forms of JWU1497

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Abstract

In this study, a sensitive and reliable method for the quantitation of JWU1497 in rat plasma was developed and validated using high performance liquid chromatography (HPLC). The pharmacokinetics of 2 forms of JWU1497, namely the base form and the hydrophosphate salt form, were investigated in rats. The 2 forms were orally administered to rats and the plasma concentrations of JWU1497 were determined using HPLC. The JWU1497 base and hydrophosphate salt forms showed similar pharmacokinetic profiles in terms of their maximum plasma concentration (Cmax), area under the concentration-time curve (AUC), and other pharmacokinetic parameters.

Keywords: JWU1497; HPLC; Rat; Pharmacokinetics

Introduction

Male erectile dysfunction, the present inability to achieve or maintain an erection for satisfactory sexual performance, is a common and important medical problem [1]. Recently, phosphodiesterase type 5 (PDE-5) inhibitors are used to improve erectile dysfunction by binding cyclic guanosine monophosphate and maintaining sufficient cellular levels in the smooth muscles [2,3]. However, PDE-5 inhibitors have common adverse reactions such as headache, flushing, nasal congestion, and dyspepsia [4]. Thus, JWU1497 (Figure 1), a new PDE-5 inhibitor, was developed to alleviate drawbacks of above common side effects of PDE-5 inhibitors. JWU1497 appears to be safe and effective in the treatment of male erectile dysfunction. The log partition coefficient (octanol / water) of JWU1497 was approximately 3.59. The solubilities of JWU1497 in methanol, acetonitrile, and distilled water were 150, 195, and 1.12 mg/ml, respectively, at 20 ± 5°C. The IC50 value of JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). JWU1497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report).

Generally, drugs in salt forms are preferable as they have higher chemical purity of more than 99%. Sildenafil, an internal standard for the determination of JWU1497, the free base and hydrophosphate salt forms, was compared in the context of the development of JWU1497 new formulation that use JWU1497 free base as the API. We determined the JWU1497 concentration in the plasma after the administration of JWU1497 free base and hydrophosphate salt forms to rats by using HPLC and compared their pharmacokinetic properties.

Materials and Methods

JWU1497 free base and hydrophosphate salt (JWU1497·HPO4) were provided by Jungwon university (Chungbuk, Korea) with a chemical purity of more than 99%. Sildenafil, an internal standard for the chromatographic analysis of JWU1497 were purchased from Sigma–Aldrich Corporation (St. Louis, MO). Acetonitrile and methanol were products...
from Burdick & Jackson (Muskegon, MI, USA). Other chemicals were of reagent grade or HPLC grade.

Preparation of JWU1497 dosing solutions
JWU1497 free base and JWU1497-HPO₄ were dissolved in distilled water with 0.5% sodium carboxymethylcellulose to a final concentration of 3 mg/mL as free base.

Animal experiments
Male Sprague–Dawley rats, 6-8 week old and weighing 220-300 g, were purchased from the Samtako Bio Korea (Osan, South Korea). Rats were maintained in a Clean room at a temperature of between 23 ± 2°C with 12-h light (07:00-19:00) and dark (19:00-07:00) cycles, and a relative humidity of 55% ± 5%. Rats were housed in metabolic cages (Tecniplast, Varese, Italy) under filtered pathogen-free air and with food (Sam Yang Company, Pyeongtaek, South Korea) and water available ad libitum. The rats were fasted overnight before drug administration and for 4 h after dosing. The rats were placed in a restrainer and were orally administered a dose of 30 mg/kg with a catheter. Blood was collected in a heparinized tube at the pre-dose stage, and at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, and 12 h after p.o. administration. Plasma was harvested after centrifugation at 3,000 rpm and 4°C for 10 min and stored frozen at -70°C until it was analyzed.

Preparation of calibration standards and quality control samples
Stock solutions of JWU1497 (1 mg/mL) were prepared in methanol. Appropriate dilutions of the stock solutions of JWU1497 were made with methanol (0.003, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, or 1 mg/mL). Standard solutions of JWU1497 in rat plasma were prepared by spiking with an appropriate volume (10 µL/mL of plasma) of the diluted stock solutions, giving final concentrations of 0.03, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, or 10 µg/mL for plasma. The IS working solution was prepared by dissolving sildenafil in acetonitrile to give a final concentration of 10 µg/mL.

Preparation of plasma samples
A 50 µL aliquot of sample was deproteinized with a 75 µL of acetonitrile containing 10 µg/mL sildenafil (an IS). After vortex-mixing and centrifugation at 3,000 rpm for 10 min, the supernatant was transferred into a vial and a 20 µL aliquot was injected directly onto the HPLC column.

HPLC analysis
The HPLC system consisted of a Gilson-234 autosampler (Gilson, Middleton, WI, USA), a Gilson 307 pump (Gilson), a Capcell PACK (C₈) column (250 mm × 4.6 mm, i.d.; particle size, 5 µm; Shiseido, Tokyo, Japan), a model UV-118 UV/VIS detector (Gilson), and a model Gilson unipoint system software (Gilson). The mobile phase, 0.02 M ammonium acetate buffer:acetonitrile (45:55, v/v), was run at a flow rate of 1.0 mL/min, and the column eluent was monitored using an ultraviolet detector at 254 nm at room temperature. The retention times of sildenafil (an internal standard) and JWU1497 were approximately 5.7 and 8.3 min, respectively.

Analytical method validation
The analytical method was validated with regards to its specificity, linearity, intra- and interday precision and accuracy, matrix effect, and stability according to the US Food and Drug Administration’s “Guidance for Industry, Bioanalytical Method Validation, 2001 [8].”

Pharmacokinetic and statistical analyses
The total area under the plasma concentration-time curve to the last time (AUCₜₐₜ), the maximum plasma concentration (Cₚ₈₉₉), the time to reach Cₚ₈₉₉ (Tₚ₈₉₉), and the half-life (T½) were estimated using non-compartmental calculations carried out within WinNonlin® 5.2 (Pharsight, Sunnyvale, CA,USA). All data are expressed as the mean ± standard deviation (SD). The statistical significance of the differences between the 2 groups was analyzed using Student’s t-tests carried out within SPSS (IBM, Yorktown Heights, NY, USA). A p value of <0.05 was considered statistically significant.

Results and Discussion
Development and validation of the HPLC method
The HPLC method for the determination of JWU1497 in rat plasma was developed and validated with regard to specificity, linearity, accuracy, and sensitivity. No interferences from endogenous substances were observed in the blank rat plasma samples. The retention times of sildenafil and JWU1497 were 5.7 and 8.3 min, respectively. The analytical method used was linear over the range of 0.03-10 µg/mL, with correlation coefficients (r values) greater than 0.9997. The lower limit of quantitation was 0.03 µg/mL with relative standard deviation (RSD) values less than 20% and relative errors within ± 20%. Intra- and inter-day accuracies (as relative error values) ranged between 1.0% and 11.5% and intra- and inter-day precisions (as RSDs) were 3.0-10.1% for all QC samples, with the result that they all met the criteria for bioanalysis method validation (Table 1). The matrix effect, recovery, and process efficiency values for JWU1497 and sildenafil in rat plasma are provided in (Table 2). The recovery was, on average, more than 90% for both compounds. JWU1497 was found to be stable under various conditions.

| Nominal conc. (ng/mL) | Measured conc. (ng/mL) | Coefficient of variation (%) | Relative error (%) |
|-----------------------|------------------------|-----------------------------|-------------------|
| Intra-day (n=6)        |                        |                             |                   |
| 30                    | 30 ± 2                 | 8.1                         | 0.0               |
| 100                   | 106 ± 6                | 5.2                         | 6.0               |
| 1000                  | 1109 ± 34              | 3.0                         | 10.9              |
| 7500                  | 7827 ± 315             | 4.0                         | 4.4               |
| Inter-day (n=18, 6 runs per day) |                  |                             |                   |
| 30                    | 32 ± 3                 | 10.1                        | 6.7               |
| 100                   | 112 ± 8                | 7.4                         | 12.0              |
| 1000                  | 1081 ± 55              | 5.1                         | 8.1               |
| 7500                  | 7428 ± 438             | 5.9                         | -1.0              |

Data represent mean ± SD. Coefficient of variation (%) = (SD/mean) × 100
Relative error (%) = ((Measured conc. - Nominal conc.) / Nominal conc.) × 100

Table 1: Intra- and inter-day precision and accuracy for JWU1497 in rat plasma QC samples.

| Concentration (ng/mL) | Matrix effect (%) | Recovery (%) | Process efficiency (%) |
|-----------------------|-------------------|--------------|------------------------|
| JWU1497               |                   |              |                        |
| 100                   | 72.5 ± 12.9       | 96.0 ± 2.9   | 69.3 ± 11.2            |
| 1000                  | 71.6 ± 4.3        | 93.9 ± 6.3   | 67.3 ± 6.0             |
| 7500                  | 82.6 ± 3.8        | 96.5 ± 2.1   | 71.4 ± 3.5             |

Sildenafil
10000                  | 73.8 ± 1.9        | 91.6 ± 2.6   | 67.6 ± 2.1             |

A, Peak area of analytes in mobile phase
B, Peak area of analytes spiked after extraction
C, Peak area of analytes spiked before extraction

Table 2: Matrix effect, recovery, and process efficiency data for JWU1497 and sildenafil in rat plasma.
Comparative pharmacokinetics of JWU1497 free base and hydrophosphate salt forms in rat plasma

Plasma samples were collected after the oral administration of the free base and hydrophosphate salt forms of JWU1497 and the concentrations of the API, JWU1497, were determined using the validated HPLC method. Figure 2 shows the mean plasma concentration-time curves for JWU1497 after the oral administration of the 2 JWU1497 formulations in rats; the pharmacokinetic parameters are presented in Table 4. The maximum plasma concentrations of JWU1497 were achieved 1.1 and 0.6 hr after oral administration for the free base and hydrophosphate forms, respectively. The $C_{\text{max}}$ values were 6.31 ± 2.90 and 6.25 ± 2.85 µg/mL, and the AUC$_{\text{last}}$ values were 22.25 ± 9.79 and 21.85 ± 8.95 µg·h/mL for the free base and salts forms, respectively. The $C_{\text{max}}$ and AUC$_{\text{last}}$ values for these 2 groups were comparable. The free base form appeared to have been absorbed more slowly from the gastrointestinal tract than the hydrophosphate salt form, but there was no statistically significant difference between the pharmacokinetic profiles of the 2 groups.

Conclusions

The HPLC method was developed and validated for the determination of JWU1497 in rat plasma and developed method was successfully applied to a comparative pharmacokinetic study of the free base and salt forms of JWU1497. The pharmacokinetic profile of the free base form was comparable to that of hydrophosphate salt form in rats. This suggests that these 2 forms could be used interchangeably to produce a variety of pharmaceutical preparations.

Acknowledgement

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conditions, whether in the plasma or in the stock solution, and the detailed stability data are presented in (Table 3). In summary, the HPLC method developed in the current study was found to be suitable for the quantification of JWU1497 in plasma with acceptable specificity, linearity, accuracy, precision, recovery, and stability. On the basis of this HPLC method, JWU1497 concentrations in rat plasma were determined.

### Table 3: Stability of JWU1497 in rat plasma and stock solutions (n=3).

| Nominal conc. (ng/mL) | Duration | Measured conc. (ng/mL) | Relative error (%) |
|----------------------|----------|------------------------|-------------------|
| Short- term stability (at room temperature, RT) |
| 100                   | 4 h      | 103 ± 7                | 3.0               |
| 1000                  |          | 1031 ± 35              | 3.1               |
| 7500                  |          | 7626 ± 324             | 1.7               |
| Long-term stability (at -80°C) |
| 100                   | 7 days   | 110 ± 10               | 10.0              |
| 1000                  |          | 1076 ± 46              | 7.6               |
| 7500                  |          | 7703 ± 418             | 2.7               |
| Freeze and thaw stability |
| 100                   | 3 cycles | 105 ± 8                | 5.0               |
| 1000                  |          | 1070 ± 41              | 7.0               |
| 7500                  |          | 8010 ± 381             | 6.8               |
| Auto-sampler stability (at 4°C) |
| 100                   | 24 h     | 104 ± 8                | 4.0               |
| 1000                  |          | 1054 ± 51              | 5.4               |
| 7500                  |          | 7866 ± 638             | 4.9               |
| Stock solution |
| 500                   | 2 h at RT | 510 ± 5                | 2.0               |
| 11 days at 4°C         |          | 490 ± 4                | -2.0              |

Data represent mean ± SD

Relative error (%) = $\frac{|\text{Measured conc.} - \text{Nominal conc.}|}{\text{Nominal conc.}} \times 100$

### Table 4: Pharmacokinetic parameters of JWU1497 after a single oral administration of JWU1497 base and hydrophosphate salts (JWU1497•HPO$_4^-$) forms at a dose of 30 mg/kg (as a base form) to male rats.

| Parameters | JWU1497 base (n=5) | JWU1497-HPO$_4^-$ (n=5) |
|------------|--------------------|-------------------------|
| AUC$_{\text{max}}$ (µg·h/mL) | 22.25 ± 9.79 | 21.85 ± 8.95 |
| $C_{\text{max}}$ (µg/mL) | 6.31 ± 2.90 | 6.25 ± 2.85 |
| $T_{\text{max}}$ (hr) | 1.10 ± 0.22 | 0.60 ± 0.22 |
| $T_{1/2}$ (hr) | 1.76 ± 0.69 | 1.52 ± 0.78 |

Data represent mean ± SD (n=5).

AUC: Area under the curve to the collected time point (µg·h/mL).

$C_{\text{max}}$: Peak plasma concentration (µg/mL).

$T_{\text{max}}$: Time to reach peak plasma concentration (hr).

$T_{1/2}$: Elimination half life (hr).

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