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Original Article Short Communication

Validated HPLC Method for the Determination of SK3497 in Rat Plasma and its Application to a Comparative Pharmacokinetic Study of the Free Base and Hydrochloride Salt Forms of SK3497.

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Abstract

In this study, a sensitive and reliable method for the quantitation of SK3497 in rat plasma was developed and validated using high performance liquid chromatography (HPLC). The pharmacokinetics of 2 forms of SK3497, namely the base form and the hydrochloride salt form, were investigated in rats. The 2 forms were orally administered to rats and the plasma concentrations of SK3497 were determined using HPLC. The SK3497 base and hydrochloride salt forms showed similar pharmacokinetic profiles in terms of their maximum plasma concentration (C_{max}) and area under the concentration-time curve (AUC). The time to peak concentration (T_{max}) of the base form was slightly greater than that of the salt form, but this difference was not statistically significant. These results suggest that the SK3497 base and hydrochloride forms are pharmacokinetically equivalent in rats, and thus the base form could be used in various SK3497 formulations as a substitute for the existing SK3497 hydrochloride form.

Keywords: SK3497; HPLC; Rat; Pharmacokinetics.

1. Introduction

Male erectile dysfunction, the present inability to achieve or maintain an erection for satisfactory sexual performance, is a common and important medical problem (Hatzimouratidis & Hatzichristou, 2008). 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 (Lincoln, 2004; Aversa, Bruzziches, Pili, & Spera, 2006). However, PDE-5 inhibitors have common adverse reactions such as headache, flushing, nasal congestion, and dyspepsia (Broderick, 2003). Thus, SK3497 (Fig. 1), a new PDE-5 inhibitor, was developed to alleviate drawbacks of above common side effects of PDE-5 inhibitors (Kim, Ji, Park, & Kim, 2014). SK3497 appears to be safe and effective in the treatment of male erectile dysfunction. The log partition coefficient (octanol / water) of SK3497 was approximately 3.59. The solubilities of SK3497 in methanol, acetonitrile, and distilled water were 150, 195, and 1.12 mg/ml, respectively, at 20±5^{o}C. The IC_{50} value of SK3497 (0.235 nM) for the inhibition of PDE-5 was smaller than other PDE-5 inhibitors such as sildenafil and tadalafil (an internal report). SK3497 of which the active pharmaceutical ingredient (API) is SK3497 hydrochloride salts is about to enter the clinical study. Generally, drugs in salt forms are preferable as they have higher dissolution rate, which facilitates absorption from the gastrointestinal tract (Engel, Farid, Faul, Richardson, & Winneroski, 2000). However, the salt forms of drugs can have limitations when they are used in some formulations, such as gels, patches, films, or chewable

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tablets, due to their greater volume compared with free base forms. Furthermore, salt forms may cause corrosion to equipment used in the manufacturing process. With the aim of developing a new SK3497 formulation, free base form of SK3497 was selected and used as the API instead of the existing salt form. The free base form negated the disadvantages of the salt form mentioned above but was also expected to improve the taste of the product, which is one of the important properties of drugs (Dixit & Puthli, 2009; Goel, Rai, Rana, & Tiwary, 2008). In this study, the pharmacological equivalence of 2 forms of SK3497, the free base and hydrochloride salt forms, was compared in the context of the development of SK3497 new formulation that use SK3497 free base as the API. We determined the SK3497 concentration in the plasma after the administration of SK3497 free base and hydrochloride salt forms to rats by using HPLC and compared their pharmacokinetic properties.

2. Materials and methods

2.1. Materials

SK3497 free base and hydrochloride salt (SK3497·2HCl) were provided by Jungwon university (Chungbuk, Korea) with a chemical purity of more than 99%. Sildenafil, an internal standard for the HPLC analysis of SK3497 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.

2.2. Preparation of SK3497 dosing solutions

SK3497 free base and SK3497·2HCl were dissolved in distilled water with 0.5% sodium carboxymethylcellulose to a final concentration of 3 mg/mL as free base.

2.3. 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^{o}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 hr 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.

2.2.2. Preparation of calibration standards and quality control samples

Stock solutions of SK3497 (1 mg/mL) were prepared in methanol. Appropriate dilutions of the stock solutions of SK3497 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 SK3497 in rat plasma were prepared by spiking with an appropriate volume (10 □ L/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.

2.2.3. 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.

2.5. HPLC analysis

The HPLC system consisted of a Gilson-234 autosampler (Gilson, Middleton, WI, USA), a Gilson 307 pump (Gilson), a Capcell PACK (C_{18}) column (250 mm×4.6 mm, i.d.; particle size, 5 □ m; 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 SK3497 were approximately 5.7 and 8.3 min, respectively.

2.6. 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"

2.7. Pharmacokinetic and statistical analyses

The total area under the plasma concentration-time curve to the last time (AUC_{last}), the maximum plasma concentration (C_{max}), the time to reach C_{max} (T_{max}), and the half-life ($T_{1/2}$) were estimated using noncompartmental 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 SK3497 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 SK3497 were 5.7 and 8.3 min, respectively. The analytical method used was linear over the range of 0.03–10 □g/mL, with correlation coefficients (r values) greater than 0.9997. The lower limit of quantitation was 0.03 □g/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 SK3497 and sildenafil in rat plasma are provided in Table 2. The recovery was, on average, more than 90% for both compounds. SK3497 was found to be stable under various 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 SK3497 in plasma with acceptable specificity, linearity, accuracy, precision, recovery, and stability. On the basis of this HPLC method, SK3497 concentrations in rat plasma were determined.

Comparative pharmacokinetics of SK3497 free base and hydrochloride salt forms in rat plasma

Plasma samples were collected after the oral administration of the free base and hydrochloride salt forms of SK3497 and the concentrations of the API, SK3497, were determined using the validated HPLC method. Fig. 2 shows the mean plasma concentration-time curves for SK3497 after the oral administration of the 2 SK3497 formulations in rats; the pharmacokinetic parameters are presented in Table 4. The maximum plasma concentrations of SK3497 were achieved 1.1 and 0.6 hr after oral administration for the free base and hydrochloride forms, respectively. The C_{max} values were 6.31±2.90 and 6.25±2.85 □g/mL, and the AUC_{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_{max} and AUC_{last} values for these 2 groups were comparable. The free base form of SK3497 appeared to have been absorbed more slowly from the gastrointestinal tract than the hydrochloride salt form, but there was no statistically significant difference between the pharmacokinetic profiles of the 2 groups.

Conclusion

The HPLC method was developed and validated for the determination of SK3497 in rat plasma and developed method was successfully applied to a comparative

pharmacokinetic study of the free base and salt forms of SK3497. The pharmacokinetic profile of the free base form was comparable to that of hydrochloride 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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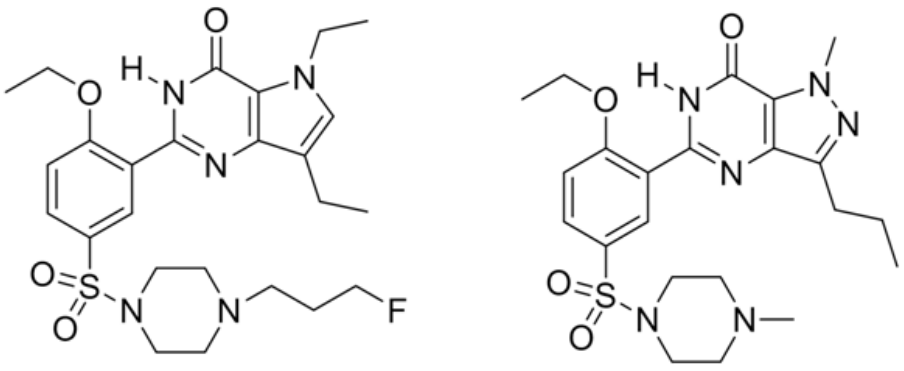

SK3497 **Sildenafil (IS)**

Fig. 1. Chemical structures of SK3497 and sildenafil (an IS).

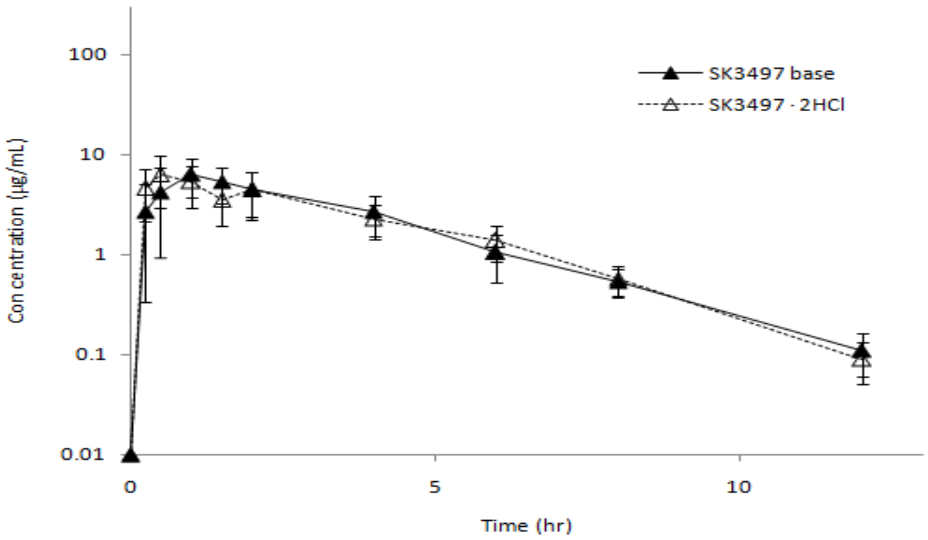

Fig. 2. Mean plasma concentration-time curves for SK3497 after oral administration of SK3497 free base and hydrochloride salt forms at a dose of 30 mg/kg as free base in rats. Each point represents the mean ± standard deviation (*n* = 5).

Table 1. Intra- and inter-day precision and accuracy for SK3497 in rat plasma QC samples.

Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Coefficient of variation (%)	Relative error (%)
	Intra-day (n=6)		
30	29.5 ± 2.4	8.1	-1.7
100	106.3 ± 5.5	5.2	6.3
1000	1109.2 ± 33.7	3.0	10.9
7500	7827.4 ± 314.9	4.0	4.4
	Inter-day (n=18, 6 runs per day)		
30	31.8 ± 3.2	10.1	6.0
100	111.5 ± 8.3	7.4	11.5
1000	1080.6 ± 55.2	5.1	8.1
7500	7427.9 ± 438.3	5.9	-1.0

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

Table 2. Matrix effect, recovery, and process efficiency data for SK3497 and sildenafil in rat plasma

Concentration (ng/mL)	Matrix effect (%) (B/A×100)	Recovery (%) (C/B×100)	Process efficiency (%) (C/A×100)
SK3497			
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	86.5 ± 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 3. Stability of SK3497 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	102.7 ± 6.5	2.7
1000		1030.5 ± 34.8	3.1
7500		7625.5 ± 323.8	1.7
Long-term stability (at -80°C)			
100	7 days	109.8 ± 10.1	9.8
1000		1076.2 ± 45.6	7.6
7500		7703.2 ± 418.3	2.7
Freeze and thaw stability			
100	3 cycles	105.4 ± 8.2	5.4
1000		1070.2 ± 40.7	7.0
7500		8010.3 ± 380.7	6.8
Auto-sampler stability (at 4°C)			
100	24 h	103.6 ± 8.0	3.6
1000		1053.7 ± 51.2	5.4
7500		7865.5 ± 638.8	4.9
Stock solution			
500	2 h at RT	510.23 ± 5.3	2.0
	11 days at 4°C	489.7 ± 3.6	-2.1

Data represent mean ± SD

Relative error (%) = ((Measured conc. - Nominal conc.) / Nominal conc.) x 100

Table 4. Pharmacokinetic parameters of SK3497 after a single oral administration of SK3497 base and hydrochloride salts (SK3497•2HCl) forms at a dose of 30 mg/kg (as a base form) to male rats.

Parameters	SK3497 base (*n*=5)	SK3497·2HCl (*n*=5)
AUC_{last} (□ ghr/mL)	22.25 ± 9.79	21.85 ± 8.95
C_{max} (□ g/mL)	6.31 ± 2.90	6.25 ± 2.85
T_{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 (□ ghr/mL).
C_{max}: Peak plasma concentration (□ g/mL)
T_{max}: Time to reach peak plasma concentration (hr)
$T_{1/2}$: Elimination half life (hr)

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