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Research Article

High-sensitive LC-MS/MS method for the simultaneous determination of mirodenafil and its major metabolite, SK-3541, in human plasma: Application to microdose clinical trials of mirodenafil

A high-sensitivity LC/MS/MS method was developed and validated for the simultaneous determination of mirodenafil and its major metabolite, SK-3541, in human plasma. Mirodenafil, SK-3541, and udenafil as an internal standard were extracted from plasma samples with methyl tert-butyl ether. Chromatographic separation was performed on a Luna phenyl-hexyl column (100 × 2.0 mm) with an isocratic mobile phase consisting of 5 mM ammonium formate and ACN (23:77, v/v) at a flow rate of 0.35 mL/min. Detection and quantification were performed using a mass spectrometer in selected reaction monitoring mode with positive ESI at m/z 532.3 → 296.1 for mirodenafil, m/z 488.1 → 296.1 for SK-3541, and m/z 517.3 → 283.2 for udenafil. The calibration curves were linear over a concentration range of 2–500 pg/mL using 0.5 mL plasma for the microdose of mirodenafil (100 μg). Analytical method validation of the clinical dose (100 mg), with a calibration curve range of 2–500 ng/mL using 0.025-mL plasma, was also conducted. The other LC-MS/MS conditions were similar to those used for the microdosing. Each method was applied successfully to pharmacokinetic studies after a microdose or clinical dose of mirodenafil to six healthy Korean male volunteers.

Keywords: Clinical dosing / High sensitivity / Microdosing / Mirodenafil
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1 Introduction

Innovative methods have been explored to improve productivity and cost of the new drug development. A microdose clinical trial, which can accelerate the selection of promising candidates early in drug development, is a new experimental approach to clinical drug development [1–4]. The term "microdose" is defined as less than 1/100th of the dose calculated to yield a pharmacological effect of the new drug candidate, while not exceeding 100 μg [5, 6]. As only microdose levels of the drug are used, extremely sensitive analytical technology is crucial in microdosing studies. Accelerator mass spectrometry (AMS), which is used to determine pharmacokinetic properties, is the most common method for microdose analysis due to its high sensitivity with the limits of quantification at the femtogram or attogram per milliliter level [7–9]. Conversely, the in vivo distribution or receptor binding of new drug candidates can be explored using positron emission tomography [10]. However, these two methods have limitations. Drug concentration measurements using AMS and positron emission tomography require the synthesis of radiolabeled drugs, such as ^{14}C or ^{11}C, which can be costly and time consuming. Furthermore, AMS involves time-consuming sample processing and high operating costs [2–4].

Recently, with improvements in the analytical sensitivity of LC-MS/MS instruments, there is growing interest in the applicability of LC-MS/MS without radiolabeling to support human microdosing studies [3, 4, 11–15].

Mirodenafil, 2-(5-(4-(2-hydroxyethyl)piperazin-1-ylsulfonyl)-2-*n*-propoxyphenyl)-5-ethyl-7-*N*-propyl-3,5-dihydro-4*H*-pyrrolo[3,2-d]pyrimidin-4-one (SK3530, Fig. 1), is a newly developed phosphodiesterase type 5 (PDE5) inhibitor marketed in Korea since July 2007 to treat male erectile dysfunction [16–18]. The usual dose of mirodenafil tablets is 100 mg. In a Phase I clinical study, mirodenafil was absorbed rapidly, T_{max} = 1.25 h, and eliminated with a terminal

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Abbreviations: **AMS**, accelerator mass spectrometry; **$AUC_{0-\infty}$**, the total area under the plasma concentration–time curve from time zero to infinity; **C_{max}**, peak plasma concentration; **IS**, internal standard; **LLOQ**, lower limit of quantification; **QC**, quality control; **T_{max}**, time to reach C_{max}

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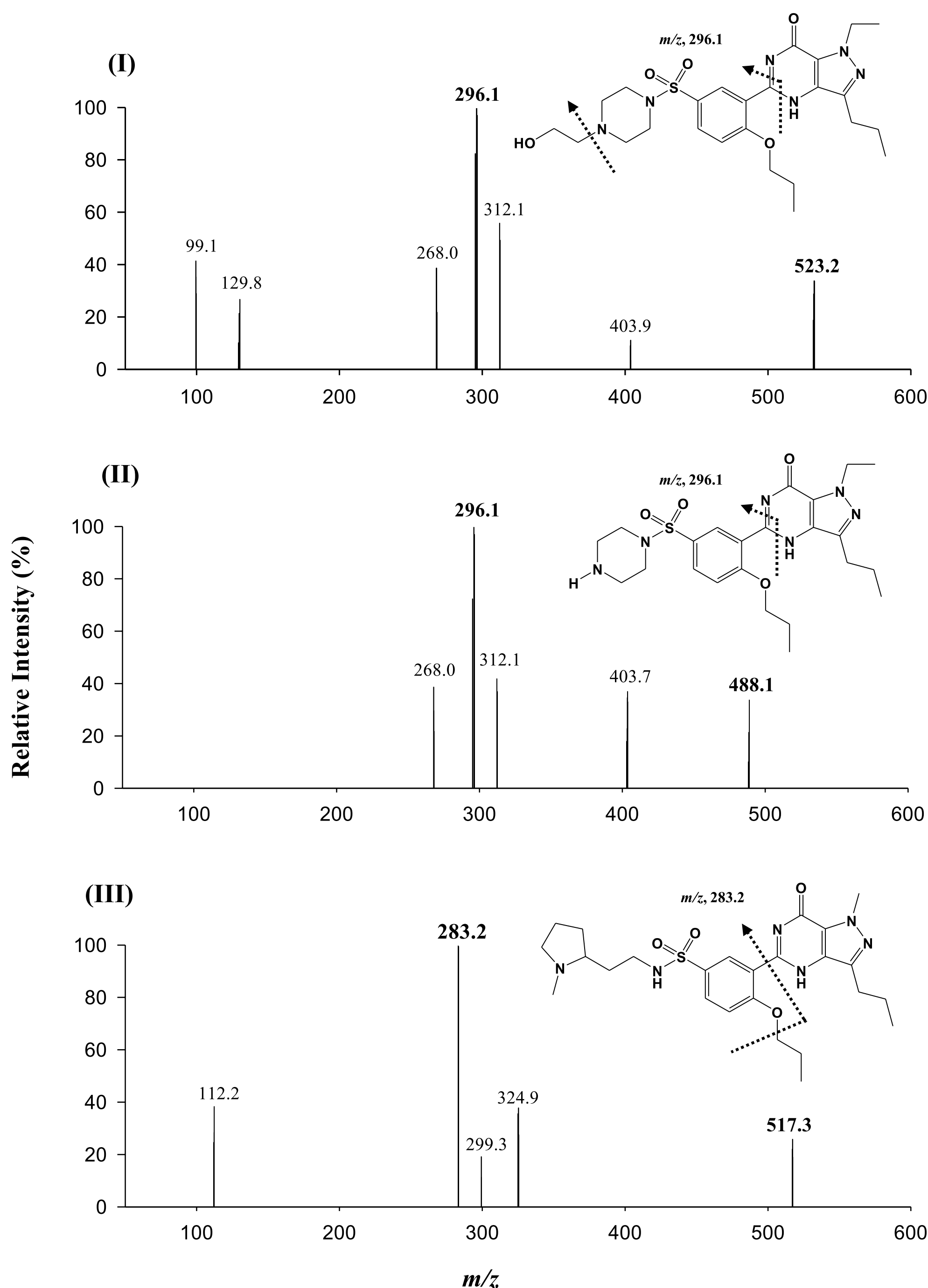

Figure 1. Product ion mass spectra of mirodenafil (I), SK-3541 (II) and udenafil (IS; III) with $[M+H]^+$ at *m/z* 532.3, 488.1, and 517.3 as the precursor ions.

half-life of 2.5 h after oral administration [17]. SK-3541 (*N*-dehydroxyethyl mirodenafil, Fig. 1) is the main metabolite in human plasma, with an *in vitro* pharmacological effect 1/10th that of mirodenafil in terms of PDE5 inhibitory activity [19]. Mirodenafil is metabolized mainly into SK-3541 by CYP3A4 and, to a minor extent, CYP2C8 and CYP2D6 in vitro [19].

In this study, we developed a highly sensitive LC-MS/MS method for the simultaneous determination of mirodenafil and its major metabolite, SK-3541, in human plasma and applied it to nonradiolabeled microdosing studies of mirodenafil (100 μg). The established method was validated and successfully applied to a mirodenafil microdosing study in six healthy Korean male volunteers. We also performed another analytical method validation using higher calibration ranges for pharmacokinetic studies of mirodenafil at a clinical dose of 100 mg and compared the pharmacokinetic profiles of the clinical dose with those of the microdose.

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2 Materials and methods

2.1 Materials and reagents

Mirodenafil and SK-3541 (Fig. 1) were kindly donated from SK chemical (Suwon, Korea). Udenafil (Fig. 1) was obtained from Dong-A Pharmaceutical (Yongin, Korea). HPLC-grade ACN, methanol, and methyl tert-butyl ether were purchased from Merck (Darmstadt, Germany). Ammonium formate was obtained from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals were of the highest analytical grade available. The drug-free human heparinized plasma was obtained from the Clinical Trial Center of Busan Paik Hospital (Busan, Korea).

2.2 Preparation of standards and quality controls (QCs)

Stock solutions of mirodenafil, SK-3541, and udenafil were prepared by dissolving the compounds in methanol at 1 mg/mL.

For the mirodenafil and SK-3541 microdosing analysis (clinical dosing analysis), stock solutions were serially diluted with methanol and added to drug-free plasma to obtain final concentrations of 2, 5, 10, 20, 50, 200, and 500 pg/mL of both mirodenafil and SK-3541 (2, 5, 10, 20, 50, 200, and 500 ng/mL of both mirodenafil and SK-3541 for clinical dosing). The udenafil stock solution was diluted further to 1 ng/mL (10 ng/mL for clinical dosing) in methanol for routine use as an internal standard (IS). In addition, stock solutions for quality control (QC) samples were serially diluted with methanol and added to drug-free human plasma to achieve final concentrations of 2 (lower limit of quantification; LLOQ), 8 (low QC), 100 (medium QC), and 400 (high QC) pg/mL of both mirodenafil and SK-3541 (2, 8, 100, and 400 ng/mL of both mirodenafil and SK-3541 for clinical dosing).

On the days of their respective analyses, calibration graphs for mirodenafil and SK-3541 in human plasma were derived from their peak area ratios relative to that of udenafil using linear regression with 1/x as a weighting factor. The respective QC samples were assayed along with each batch of plasma samples. The QC samples were used to evaluate the intra and interday precision and accuracy of the method. All prepared plasma samples and stock solutions were stored at −80°C (Revco ULT 1490 D-N-S; Western Mednics, USA). Both the analytical method validation of mirodenafil and SK-3541 for the microdosing and clinical dosing were conducted following the procedures described above.

2.3 Sample preparation

First, 500 μL aliquots of plasma samples for the microdosing study or 25 μL aliquots of plasma for the clinical dosing study were transferred into a glass tube (Eppendorf tube for the clinical dosing study) and spiked with 20 μL IS solution containing udenafil 1 ng/mL (clinical dosing, 10 μL IS solution containing udenafil 10 ng/mL). The mixture was extracted with 5 mL (1 mL for clinical dosing) of methyl tert-butyl ether by vortexing for 5 min. After centrifugation at 3000 × *g* for 10 min, the organic layer was transferred into a clean glass tube (clinical dosing, Eppendorf tube) and evaporated until dry under a gentle stream of nitrogen gas at 40°C. The residue was reconstituted with 100 μL (clinical dosing, 500 μL) of mobile phase, and a 5 μL (clinical dosing, 2 μL) aliquot was injected into the LC–MS/MS system for analysis.

2.4 LC-MS/MS conditions

The microdosing and clinical dosing samples were analyzed using the same LC-MS/MS conditions. The LC-MS/MS system consisted of an API 5500 Q-Trap mass spectrometer (AB SCIEX, USA) equipped with a 1290 HPLC system (Agilent Technologies, USA) in ESI mode to protonated molecular ion $[M+H]^+$. The compounds were separated with a phenyl-hexyl column (Luna, 100 × 2.0 mm id, 3.0 μm particle size; Phenomenex) in an isocratic mobile phase consisting of 5 mM ammonium formate and ACN (23:77, v/v) at a flow rate of 0.35 mL/min. The column and autosampler temperatures were maintained at 40 and 4°C, respectively. The total run time was 4 min per sample.

The optimized ion spray voltage and temperature were set at 5500 V and 500°C, respectively. The operating conditions, which were optimized by flow injection of mirodenafil, SK-3541, and udenafil, were declustering potential, entrance potential, collision energy, and collision cell exit potentials of 211, 10, 50, and 8 V, respectively. Nitrogen gas was used as the nebulizer, curtain, and collision-activated dissociation gas, set at 55, 22, and 55 psi, respectively. The selected reaction monitoring mode with positive ESI used m/z 532.3 → 296.1 for mirodenafil, m/z 488.1 → 296.1 for SK-3541, and m/z 517.3 → 283.2 for udenafil. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed using Analyst software (ver. 1.5.2; Applied Biosystems, USA).

2.5 LC-MS/MS analytical validation

The validation parameters obtained were the selectivity, linearity, sensitivity, accuracy, precision, recovery, and stability of mirodenafil and SK-3541 in human plasma in accordance with the USFDA guidance for bioanalytical method validation [20]. The analytical method for both the microdose and clinical dose of mirodenafil in human plasma were validated using the following procedures.

2.5.1 Selectivity

Selectivity was obtained by comparing chromatograms of six different batches of blank plasma obtained from six subjects to ensure that no interfering peaks were present at the

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retention times of both mirodenafil and SK-3541 at the LLOQ (microdose, 2 pg/mL; clinical dose, 2 ng/mL) level.

2.5.2 Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of mirodenafil or SK-3541 to IS versus the nominal concentration (x) at ranges of 2–500 and 2–500 pg/mL for the microdosing and clinical dosing, respectively. The calibration curves were constructed with the weighted (1/x) least-square linear regression method. The LLOQs for mirodenafil and SK-3541 in human plasma samples from their respective method were defined as the lowest concentration with at least a 5× S/N ratio, acceptable accuracy (80–120%), and sufficient precision (within 20%); this was verified by the analysis of ten replicates.

2.5.3 Precision and accuracy

The intraday precision and accuracy were determined by analyzing six replicates of the LLOQ sample and three different QC samples (2, 8, 100, and 400 pg/mL for microdosing and 2, 8, 100, and 400 ng/mL for clinical dosing) on the same day. The interday precision and accuracy were also evaluated by analyzing ten replicates of the LLOQ sample and three different QC samples (2, 8, 100, and 400 pg/mL for microdosing and 2, 8, 100, and 400 ng/mL for clinical dosing) on five different days (two replicates per day). The precision was expressed as the relative standard deviation (RSD,%) and the accuracy was expressed as:

$$[(\text{mean observed concentration})/(\text{nominal concentration}) \times 100\%]$$

The concentrations of QC including LLOQ samples were determined from the standard calibration curve and analyzed on the same day.

2.5.4 Matrix effect and extraction recovery

Two different QC samples, 8 and 400 pg/mL for microdosing and 8 and 400 ng/mL for clinical dosing, and drug-free plasma oriented from six different sources were used to evaluate matrix effects and the extraction recovery of mirodenafil and SK-3541. All assays were performed in triplicate. Using the analyte peak areas obtained by direct injection of diluted (or neat) standard solutions as *A*, the corresponding peak areas of diluted (or neat) standard solutions spiked into plasma extracts after extraction as *B*, and the peak areas of diluted (or neat) standard solutions spiked into plasma before extraction as *C*, the matrix effects and extraction recovery were calculated as [21]:

$$\text{Matrix effect (\%)} = \text{B/A} \times 100$$

$$\text{Extraction recovery (\%)} = \text{C/B} \times 100$$

The matrix effects and extraction recovery of the IS were evaluated using the same method.

2.5.5 Stability

Since the two analytical methods for microdosing and clinical dosing used overlapping procedures for sample preparation and MS/MS parameters, we conducted a stability test using only microdosing plasma samples and stock solutions. The stability of mirodenafil or SK-3541 in human plasma was assessed by assaying three replicate samples spiked with 8 and 400 pg/mL, respectively, of mirodenafil or SK-3541, under five conditions: (i) short-term storage for 12 h at room temperature, (ii) long-term storage for 90 days at −80°C, (iii) three freeze–thaw cycles, (iv) post-treatment storage for 6 h at room temperature, and (v) post-treatment storage for 48 h at 4°C. The concentrations obtained were compared with the nominal values of the QC samples. The stabilities of the stock solutions of mirodenafil, SK-3541, and IS were evaluated after 2 months at 4°C and after 4 months at −80°C, by comparison with a freshly prepared solution of identical concentrations.

2.6 Clinical application: Microdosing and clinical dosing studies

This study was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea) and was performed according to the Korean Good Clinical Practice. Six Korean healthy male volunteers (23–29 years old; body weight 63–76 kg; height 168–180 cm) who gave written informed consent were enrolled in this study. Health problems, drug or alcohol abuse, and abnormalities in laboratory screening values were the exclusion criteria. This study was designed as a single-center, open-labeled, randomized, three-treatment-period, crossover study with a 1-week washout in six healthy male adults. The three treatments consisted of 100 μg of nonradiolabeled mirodenafil (treatment I), 100 μg of ^{14}C-radiolabeled mirodenafil (treatment II), and 113.8 mg of nonradiolabeled mirodenafil dihydrochloride (equal to 100 mg of mirodenafil; treatment III) while fasting. The subjects received an oral solution formulation of the respective amount of powdered drug in 240 mL of tap water. In this study, only the samples from treatments I and III were analyzed. Approximately 6-mL blood samples were collected via the median cubital vein: predose and 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, and 10 h after administration. The blood samples were centrifuged immediately (2000 × *g*, 10 min) at 4°C and the plasma samples were stored at −80°C until the LC-MS/MS mirodenafil and SK-3541 analysis.

The pharmacokinetic parameters were calculated by a noncompartmental analysis (WinNonlin Professional ver. 5.2, Pharsight, Mountain View, CA, USA) for determining the following: the total area under the plasma concentration–time curve from time zero to infinity ($AUC_{0-\infty}$) or the last measured time (total area under the plasma concentration–time curve from time zero to the last measured time), and apparent oral clearance (CL/F). The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were taken directly from the experimental data.

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3 Results and discussion

3.1 LC-MS/MS optimization

In positive ion mode, both mirodenafil and SK-3541 yielded protonated molecular ions, $[M+H]^+$, as the major species. The fragmentation patterns of the protonated molecular ions were evaluated by increasing the collision energy. The product ion spectra and fragmentation patterns for mirodenafil, SK-3541, and the IS are shown in Fig. 1. The greatest intensities occurred at *m/z* 296.1, 296.1, and 238.1 for mirodenafil, SK-3541, and the IS, respectively. The mass parameters were optimized by observing the maximal response of the product ions.

To optimize the chromatographic conditions, a variety of columns, such as C_{18}, C_8, phenyl-hexyl, hilic, and CN columns, and various compositions of mobile phases with good resolution, high sensitivity, and symmetric analyte peak shapes, as well as suitable retention times, were examined. The Luna phenyl-hexyl column (100 × 2.0 mm, id; 3 μm particle size) and a mobile phase consisting of 5 mM ammonium formate and ACN (23:77, v/v) yielded good peak shapes and responses. Initially, protein precipitation for sample preparation was attempted for its convenience, but significant matrix effects and lower sensitivity hindered further development. Next, a liquid–liquid extraction procedure was investigated. Several organic solvents, including ethyl acetate, ether, dichloromethane, acetone, chloroform, methyl tert-butyl ether, and their mixtures were evaluated. Ultimately, methyl tert-butyl ether was found to be optimal, producing a clean chromatogram for blank plasma samples, the best recovery, and the fewest matrix effects. Although stable isotope-labeled ISs are the first choice, they are not economical and deuterium-labeled compounds can show unexpected behavior, such as different retention times or recoveries, compared to the analyte [22]. Furthermore, it was not feasible for use in the microdosing study. Therefore, we investigated several compounds to find a suitable IS and chose a compound structurally and chemically similar to mirodenafil, udenafil, as the IS in this study.

3.2 LC-MS/MS method validation

3.2.1 Selectivity

There were no interfering peaks from endogenous substances at the elution times for mirodenafil (1.2 min), SK-3541 (1.7 min), or udenafil (2.6 min) and no cross-talk phenomena were observed among MS/MS channels. Representative chromatograms of zero blank plasma; drug-free plasma spiked with the IS, a plasma sample at LLOQ (2 pg/mL) for the microdosing analysis, and a plasma sample collected at 12 h after a single oral administration of mirodenafil (100 μg) from a volunteer are shown in Fig. 2. The total run time was 4 min per sample.

3.2.2 Linearity and sensitivity

The calibration curves in human plasma provided reliable responses from 2–500 pg/mL for microdosing and 2–500 ng/mL for clinical dosing for both mirodenafil and SK-3541. The best linear fit and least-squares residuals for the two respective calibration curves in the microdosing and clinical dosing were achieved using a 1/x weighing factor. In terms of validation of these two methods in human plasma, the calibration curves exhibited good linearity. The correlation coefficients (r) of mirodenafil and SK-3541 for both calibration curves were >0.995. For the microdosing, the back-calculated results for all mirodenafil calibration standards were <14.8% RSD (clinical dosing, 7.24% RSD) and −6.17 to 3.12% relative error (clinical dosing, −7.57 to 2.70% relative error) and the corresponding values for SK-3541 were <12.6% RSD (clinical dosing, 11.6% RSD) and −12.4 to 11.9% relative error (clinical dosing, −4.27 to 4.24% relative error). The LLOQs for both mirodenafil and SK-3541 were 2 pg/mL in the microdosing and 2 ng/mL in clinical dosing experiments, which were sufficient for pharmacokinetic studies of mirodenafil and SK-3541.

3.2.3 Precision and accuracy

The intra and interday precision and accuracy of the method were measured by assaying the LLOQ and three different QC samples (2, 8, 100, and 400 pg/mL for microdosing and 2, 8, 100, and 400 ng/mL for clinical dosing) on five different days and are summarized in Table 1. Both the precision and accuracy were well within the 15% acceptance range and at the LLOQ level within precisions of 20% and accuracies of 80–120%. In the microdosing study, the CVs for the intra and interday precision were <7.17% (clinical dosing, <12.1%) and <14.6% (clinical dosing, <14.7%), respectively. The intra and interday accuracies were 95.9–117% (clinical dosing, 94.0–105%) and 94.1–105% (clinical dosing, 97.1–107%), respectively. These results satisfied the criteria.

3.2.4 Matrix effect and extraction recovery

Two QC samples, 8 and 400 pg/mL for the microdosing and 8 and 400 ng/mL for the clinical dosing, and drug-free plasma, were used to evaluate the effects of the sample matrix on mirodenafil and SK-3541 ionization; i.e. the degree of ion suppression or enhancement caused by matrix components. The percentages of the matrix effect were 85–115%, indicating no significant matrix effect for any of the analytes (data not shown). In the microdosing study, the extraction recoveries in human plasma were 103.5 ± 5.68 and 91.8 ± 6.57% at 8 and 400 pg/mL for mirodenafil, and 90.7 ± 4.68 and 89.4 ± 3.71% at 8 and 400 pg/mL for SK-3541, respectively.

For the clinical dosing study, the extraction recoveries at 8 and 400 ng/mL for mirodenafil were 110 ± 7.99 and

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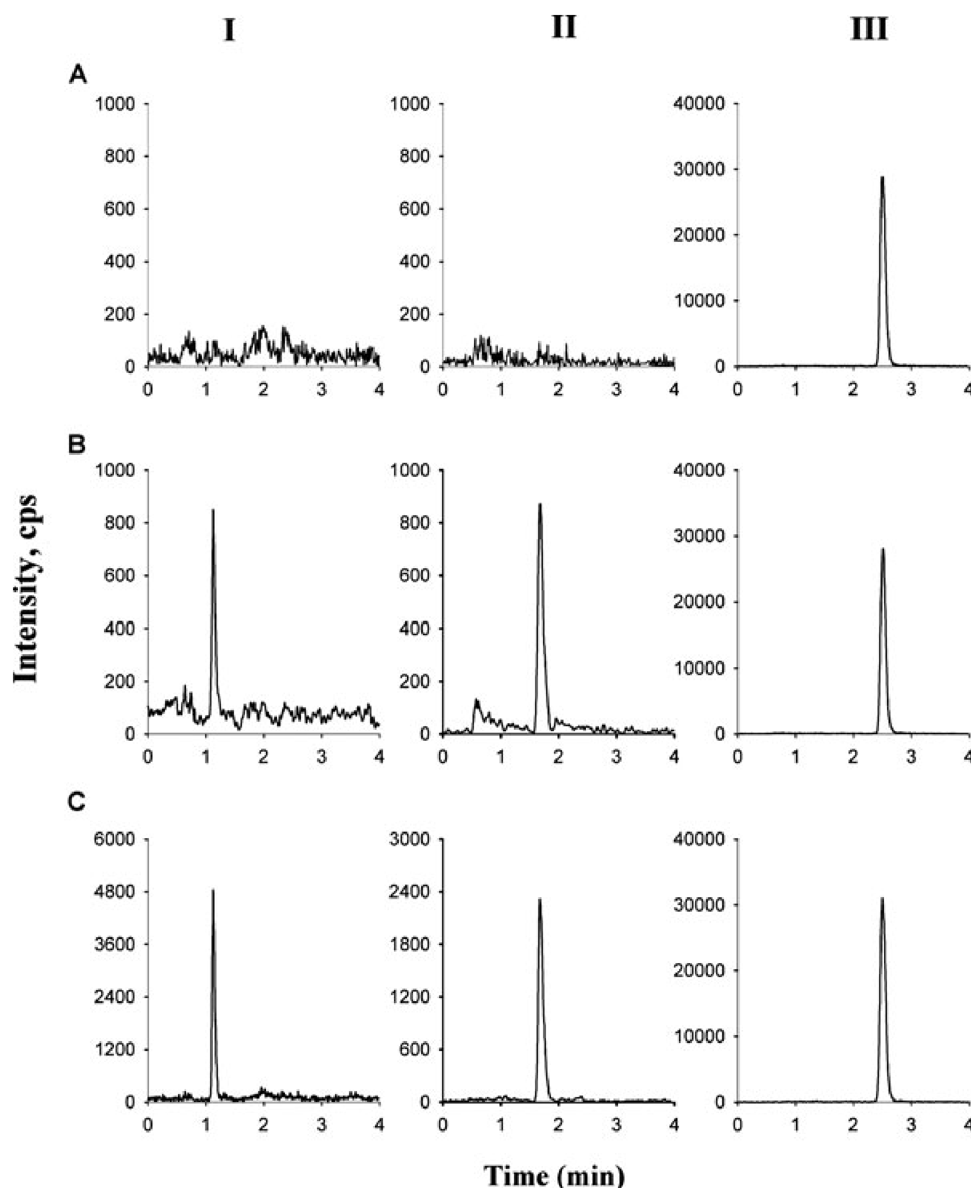

Figure 2. Representative chromatograms of mirodenafil (I), SK-3541 (II), and udenafil (IS) (III) for the micordose: (A) zero blank plasma; blank plasma spiked with the IS (1 ng/mL), (B) blank plasma spiked with mirodenafil (2 pg/mL) and SK-3541 (2 pg/mL) at LLOQ and the IS (1 ng/mL), and (C) a plasma sample from 6 h after microdosing of mirodenafil, 100 μg to a subject. The concentrations of mirodenafil and SK-3541 were 11.7 pg/mL and 5.34 pg/mL, respectively.

101 ± 8.10%, and the corresponding values for SK-3541 were 103 ± 9.01 and 99.7 ± 6.10%. For the IS, the extraction recoveries at 1 ng/mL (microdose) and 10 ng/mL (clinical dose) were 87.6 ± 3.41 and 87.0 ± 6.10%, respectively. The low matrix effects and highly reproducible recovery results demonstrated the reliability of the two methods for bioanalyses.

3.2.5 Stability

The stock solutions of mirodenafil, SK-3541, and udenafil in methanol were stable for 2 months at 4°C and 4 months at −80°C; we obtained 99.4 ± 8.67% and 92.7 ± 3.19% recoveries from samples spiked with stock solutions stored under these respective conditions. No significant degradation of the analytes in human plasma occurred after short-term storage for 12 h at room temperature, long-term storage for 90 days at −80°C, three freeze–thaw cycles, or post-treatment storage for 6 h at room temperature and for 48 h at 4°C, with ±15% deviation between the predicted and nominal concentrations (data not shown).

3.3 Clinical application: Microdosing and clinical dosing studies

The two validated methods were applied successfully to a pharmacokinetic study of mirodenafil in human plasma after mirodenafil microdosing (100 μg) and clinical dosing (100 mg). The mean plasma concentration–time profile of mirodenafil and its main metabolite, SK-3541, after microdosing or clinical dosing of mirodenafil to six healthy

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Table 1. Intra and interday precision and accuracy of QC samples for the microdose (100 μg) and clinical dose (100 mg) in human plasma

Compound	Microdose (100 μg)				Clinical dose (100 mg)			
	Added (pg/mL)	Precision		Accuracy (%)	Added (ng/mL)	Precision		Accuracy (%)
		Measured (pg/mL)	RSD (%)			Measured (ng/mL)	RSD (%)	
Intraday ($n = 6$)								
Mirodenafil	2	2.26 ± 0.0870	3.85	113	2	2.03 ± 0.214	10.5	102
	8	8.79 ± 0.342	3.89	110	8	8.39 ± 0.845	10.1	105
	100	113 ± 3.21	2.83	111	100	99.5 ± 5.52	5.55	99.5
	400	417 ± 29.6	7.09	104	400	399 ± 47.4	11.9	99.7
SK-3541	2	2.35 ± 0.0173	0.739	117	2	1.98 ± 0.241	12.1	99.2
	8	8.44 ± 0.286	3.39	106	8	8.36 ± 0.696	8.33	104
	100	105 ± 7.55	7.17	105	100	94.0 ± 7.23	7.69	94.0
	400	384 ± 11.2	2.92	95.9	400	411 ± 29.4	7.16	103
Interday ($n = 5$)								
Mirodenafil	2	2.10 ± 0.210	9.97	105	2	2.03 ± 0.299	14.7	102
	8	7.98 ± 0.756	9.47	99.7	8	8.10 ± 0.709	8.75	101
	100	96.4 ± 10.2	10.5	96.4	100	103 ± 3.41	3.32	103
	400	388 ± 43.4	11.2	97.2	400	427 ± 17.9	4.18	107
SK-3541	2	2.16 ± 0.315	14.6	105	2	1.94 ± 0.211	10.9	97.1
	8	7.61 ± 0.418	5.50	95.1	8	7.96 ± 0.570	7.16	99.2
	100	94.1 ± 5.84	6.20	94.1	100	104 ± 5.36	5.16	104
	400	375 ± 32.8	8.75	94.7	400	433 ± 42.3	9.76	105

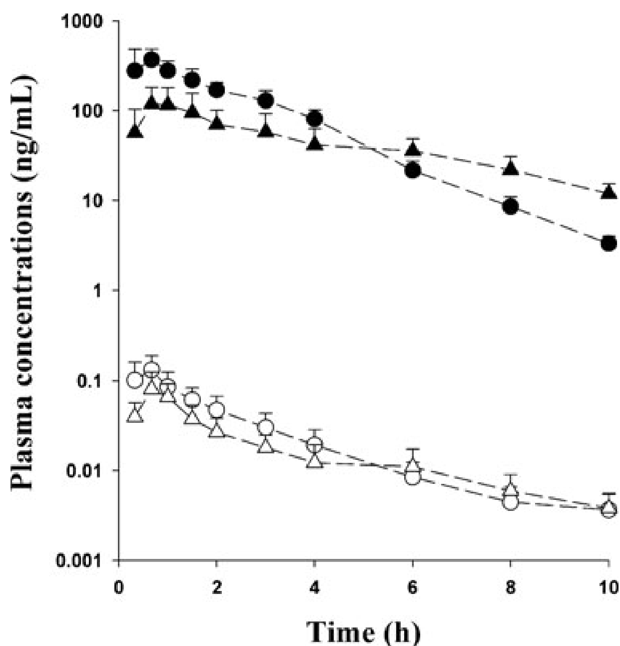

Figure 3. Mean plasma concentration–time profiles of mirodenafil (circle) and SK-3541 (triangle) after receiving microdosing of mirodenafil, 100 μg (opened), and clinical dosing of mirodenafil, 100 mg (closed), in six healthy Korean male volunteers. Vertical bars represent SD.

Korean male volunteers is shown in Fig. 3 and the relevant pharmacokinetic parameters are listed in Table 2.

The LLOQ in human plasma after microdosing was calculated based on the reported C_{max}, of 374 ng/mL at a 100 mg clinical dose level [18]. We calculated the 1/1000th value of the C_{max} with a clinical dose and assumed 374 pg/mL to be the C_{max} for microdosing. An approximately twentyfold lower value of 2 pg/mL was set as the LLOQ for the microdosing. For the clinical dosing, the LLOQ was 2 ng/mL for both mirodenafil and SK-3541 and could be sufficiently determined using only 25 μL of plasma.

Table 2. Pharmacokinetic parameters of mirodenafil and SK-3541 after receiving microdose of mirodenafil, 100 μg, and clinical dose of mirodenafil, 100 mg, in six Korean healthy male volunteers

Parameters[a]	Mirodenafil	SK-3541
Microdose (100 μg)		
$AUC_{0-\infty}$ (ng h/mL)	0.272 ± 0.107	0.184 ± 0.0777
AUC_{0-t} (ng h/mL)	0.263 ± 0.109	0.169 ± 0.07
Terminal half-life (h)	1.80 ± 0.737	2.44 ± 1.22
C_{max} (ng/mL)	0.135 ± 0.0564	0.0787 ± 0.0426
T_{max} (h)[b]	0.67 (0.33–0.67)	0.84 (0.67–1)
CL/F (L/h)	538 ± 360	
$AUC_{0-\infty}$ ratio (mirodenafil/SK-3541)	0.698 ± 0.276	
Clinical dose (100 mg)		
$AUC_{0-\infty}$ (ng h/mL)	888 ± 232	514 ± 212
AUC_{0-t} (ng h/mL)	882 ± 231	454 ± 205
Terminal half-life (h)	1.32 ± 0.0769	3.39 ± 1.03
C_{max} (ng/mL)	404 ± 148	122 ± 63.9
T_{max} (h)[b]	0.67 (0.33–0.67)	0.84 (0.67–1)
CL/F (L/h)	131 ± 46.4	
$AUC_{0-\infty}$ ratio (mirodenafil/SK-3541)	0.575 ± 0.192	

a) Values are mean ± SD.
b) Median (ranges).

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In the microdosing clinical trial, the mean C_{max} of mirodenafil was 135 ± 56.4 pg/mL, which occurred at T_{max} = 0.67 h (range 0.33–1 h). The terminal half-life and $AUC_{0-\infty}$ values of mirodenafil were 1.80 ± 0.737 h and 272 ± 107 pg h/mL, respectively. The mean C_{max} of SK-3541 was 78.7 ± 42.6 pg/mL occurring at T_{max} = 0.75 h (range 0.5–1 h) and the $AUC_{0-\infty}$ value was 184 ± 77.7 pg h/mL. The total area under the plasma concentration–time curve from time zero to the last measured time/AUC_{∞} ratio exceeded 80% for all subjects (mean value, 95.9 ± 3.50%). Therefore, plasma sample collection for up to 10 h could be used for microdosing studies of mirodenafil.

For the clinical dose study, the mean C_{max} of mirodenafil was 404 ± 148 ng/mL occurring at T_{max} = 0.67 h (range 0.233–0.67 h). The terminal half-life and $AUC_{0-\infty}$ values of mirodenafil were 1.32 ± 0.0769 h/mL and 888 ± 232 ng h/mL, respectively. The mean C_{max} of SK-3541 was 122 ± 63.9 ng/mL occurring at T_{max} = 0.84 h (range 0.67–1 h), and the $AUC_{0-\infty}$ value was 514 ± 212 ng h/mL. The pharmacokinetic parameters of mirodenafil and SK-3541 in the clinical dose study were similar to those reported in the literature [18, 23].

The QC samples for each method were assessed using the calibration curves, and were found to be within 15% of the nominal concentrations, meeting the US FDA acceptance criteria for the validation of bioanalytical methods [20].

To estimate the pharmacokinetic properties of a new drug candidate at the clinical dosing from its properties at the microdosing, linear pharmacokinetics of the compound must be guaranteed [14]. Based on our results, the nonlinearity of mirodenafil was observed between clinical dosing and microdosing. The values of apparent oral clearance (CL/F) of mirodenafil at the clinical dosing (131 L/h) were significantly lower than those at the microdosing (538 L/h) (Table 2). The dose-normalized area under the plasma concentration–time curve ($AUC_{0-\infty}$) values of mirodenafil increased in a dose-dependent manner and was 3.28-fold higher at the clinical dosing than at microdose (Table 2). However, the ratios of the $AUC_{0-\infty}$ value of the SK-3541 to that of the parent compound between clinical dosing (0.575) and microdosing (0.698) were comparable (Table 2). The nonlinear pharmacokinetics of mirodenafil might be caused mainly by the saturation of efflux transporters in the small intestine. Futher studies are needed to elucidate the mechanisms. Recently, it was reported that similar PDE5 class drugs to mirodenafil, vardenafil, and sildenafil are substrates of efflux transportes, i.e. P-gp, BCRP, and, MRP2 in the small intestine [24]. In addition, the nonlinear pharmacokinetics of quinidine and verapamil bewteen clinical dosing and microdosing were observed due to the saturation of MDR1 and/or CYP3A4 in the small intestine [25]. Further studies are needed to elucidate the mechanism.

4 Concluding remarks

A highly sensitive LC-MS/MS method for the simultaneous determination of mirodenafil and its major metabolite, SK-3541, in human plasma was described. This asssy exhibited a sensitivity (LLOQ, 2 pg/mL) sufficient to conduct a clinical microdose study of mirodenafil at a dose of 100 μg. Our data demonstrate the utility of the sensitive LC-MS/MS method for supporting clinical microdose trials of nonradiolabeled drugs.

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