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Short communication

A rapid and sensitive LC–MS/MS assay to quantify yonkenafil in rat plasma with application to preclinical pharmacokinetics studies

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ABSTRACT

A novel method for the quantitation of yonkenafil, a new synthetic phosphodiesterase V inhibitor, in rat plasma using high-performance liquid chromatography/tandem mass spectrometry (LC–MS/MS) has been developed. The analyte and internal standard (diazepam) were extracted from plasma (100 μl) by liquid–liquid extraction and separated on a C_{18} column using 10 mM ammonium acetate buffer: methanol (15:85, v/v) as mobile phase in a run time of 3.0 min. The detector was a Q-trap™ mass spectrometer with an ESI interface operating in the multiple reaction monitoring (MRM) mode. The assay was linear over the concentration range 1.0–1000 ng/ml with a limit of detection of 0.20 ng/ml. Intra- and inter-day precision (as relative standard deviation) were both within 8.45% with good accuracy. The method was successfully applied to a preclinical pharmacokinetic study of yonkenafil in rat after sublingual, oral and intravenous administration. The results demonstrate that the sublingual route gives a higher bioavailablity than the oral route and may represent a useful alternative route of yonkenafil administration.

1. Introduction

Yonkenafil (2-(2-ethoxy-5-(4-ethylpiperazin-1-yl sulfonyl) phenyl)-5-methyl-7-propyl-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one), a novel synthetic phosphodiesterase V inhibitor and analogue of sildenafil, is a promising drug for the treatment of impotence and male erectile dysfunction [1–3]. It is more potent than sildenafil (IC50: 2.0 nM compared to sildenafil 4.5 nM) with better acceptability and fewer gastrointestinal side effects. It is suitable for low-dose and short-term administration and is now undergoing preclinical trials in China [4].

Appropriate analytical methods for the assay of drugs are essential to carry out the pharmacokinetic studies required by the Chinese State Food and Drug Administration [5–12]. LC/MS based techniques are now the mainstay for such pharmacokinetic studies [13] particularly as the cost of the instruments has declined significantly over the past few years. In fact LC–MS/MS has become the dominant tool for bio-analysis due to its speed and selectivity. This paper reports on a rapid and sensitive LC–MS/MS method for the determination of yonkenafil in rat plasma using diazepam as internal standard (I.S.). The method requires only 100 μl of rat plasma, employs simple sample preparation and is completed in a run time of only 3 min. The method was successfully applied to a pharmacokinetic study of yonkenafil after sublingual, oral and intravenous (i.v.) administration.

2. Experimental

2.1. Chemicals and reagents

All chemicals from commercial sources were used as received except where stated otherwise. Reagents and sources were yonkenafil hydrochloride (purity 99.40%) from Tianjin Tasly Company Ltd. (Tianjin, P.R. China); Diazepam (purity 99.0%) from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China); methanol (HPLC grade) and other chemicals (analytical grade) from Corcond Technology (Tianjin) Company Ltd. (Tianjin, P.R. China). Blank rat plasma (drug free and anticoagulated with heparin sodium) was prepared in our laboratory. Distilled water, prepared from demineralized water, was used throughout the study.

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2.2. Stock solutions

All concentrations of yonkenafil refer to the free base. A stock solution of yonkenafil was prepared in methanol at a concentration of 1.0 mg/ml. Standard solutions (1.0, 3.0, 10, 30, 100, 300 and 1000 ng/ml) and QC solutions (3.0, 100 and 800 ng/ml) were prepared by serial dilution of the stock solution with methanol: water (50:50, v/v). A stock solution of diazepam (1.0 mg/ml) was also prepared in methanol and then diluted with methanol:water (50:50, v/v) to a final concentration of 250 ng/ml. All solutions were stored at 4 °C and used within one month after preparation.

2.3. LC–MS/MS analysis of yonkenafil

An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an Applied Biosystems Sciex Q-trap™ mass spectrometer (Concord, Ontario, Canada) via an electrospray ionization (ESI) source was used for analysis. Applied Biosystems Analyst software version 1.3.2 package was used to control the LC–MS/MS system and for data acquisition and processing.

Yonkenafil and the I.S. were separated on a 5 μm Zorbax Extend C_{18} column (150 × 4.6 mm I.D. from Agilent Technologies) maintained at 30 °C. The mobile phase consisted of 85% methanol and 15% 10 mM ammonium acetate buffer delivered at a flow rate of 1.0 ml/min. A two phase switching valve was used to divert the pre-eluent from entering the ion source. An approximately 1:1 split of the column eluent was included so that only 0.50 ml/min enters the mass spectrometer.

The mass spectrometer was operated in the positive ESI mode with multiple reaction monitoring (MRM) at unit resolution. Nitrogen was used as the nebulizer, heater and curtain gas as well as the collision activation dissociation (CAD) gas. The precursor-to-product ion transitions were monitored at m/z 488.4 → 99.2 and m/z 488.4 → 310.3 for yonkenafil and at m/z 285.2 → 193.2 for diazepam. Mass spectrometer instrumental parameters were tuned to maximize the generation of precursor and fragment ions by infusion of a solution of yonkenafil and I.S. into the ESI source at 10 μl/min. Optimum parameters were as follows: nebulizer (GS_1), heater (GS_2) and curtain gas flow rates 55, 40 and 15 units, respectively; ionspray needle voltage 1500 V; heater gas temperature 450 °C; collision gas (N_2) medium; declustering potential 85 V; collision energies 83 eV (m/z 488.4 → 99.2) and 39 eV (m/z 488.4 → 310.3) for yonkenafil and 44 eV for diazepam.

2.4. Sample preparation

After thawing at room temperature for 30–45 min, plasma samples were vortexed and an aliquot (100 μl) mixed with 100 μl I.S. solution, 100 μl methanol:water (50:50, v/v) (or a standard or QC solution) and 50 μl NaOH (1 M) in a 10 ml capped tube. The mixture was vortexed for 10 s and extracted with 3.0 ml diethyl ether: dichloromethane, 60:40, v/v). After shaking for 10 min and centrifuging at 3500 × g for 5 min, the upper organic phase was carefully transferred to another tube and evaporated at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl mobile phase and 20 μl injected into the LC–MS/MS system. Samples with concentrations exceeding that of the highest standard (1000 ng/ml) were diluted with blank rat plasma prior to analysis.

2.5. Assay validation

Linearity was assessed by weighted linear regression ($1/x^2$) of analyte-internal standard peak area ratios based on three independent calibration curves prepared on each of three separate days. Accuracy (relative error) and intra- and inter-day precision [as relative standard deviation (R.S.D.)] were assessed by assay of six replicate QC samples on three different days.

Recovery of yonkenafil was determined by comparing peak areas of extracted QC samples with peak areas of post-extraction plasma blanks spiked at corresponding concentrations. Matrix effects for yonkenafil were evaluated by comparing peak areas of post-extraction blank plasma spiked at concentrations of QC samples with the areas obtained by direct injection of corresponding standard solutions. Stability of yonkenafil was evaluated using QC samples subjected to three freeze-thaw cycles, stored at −20 °C for one month and at room temperature for 12 h. Stability in stock solu-

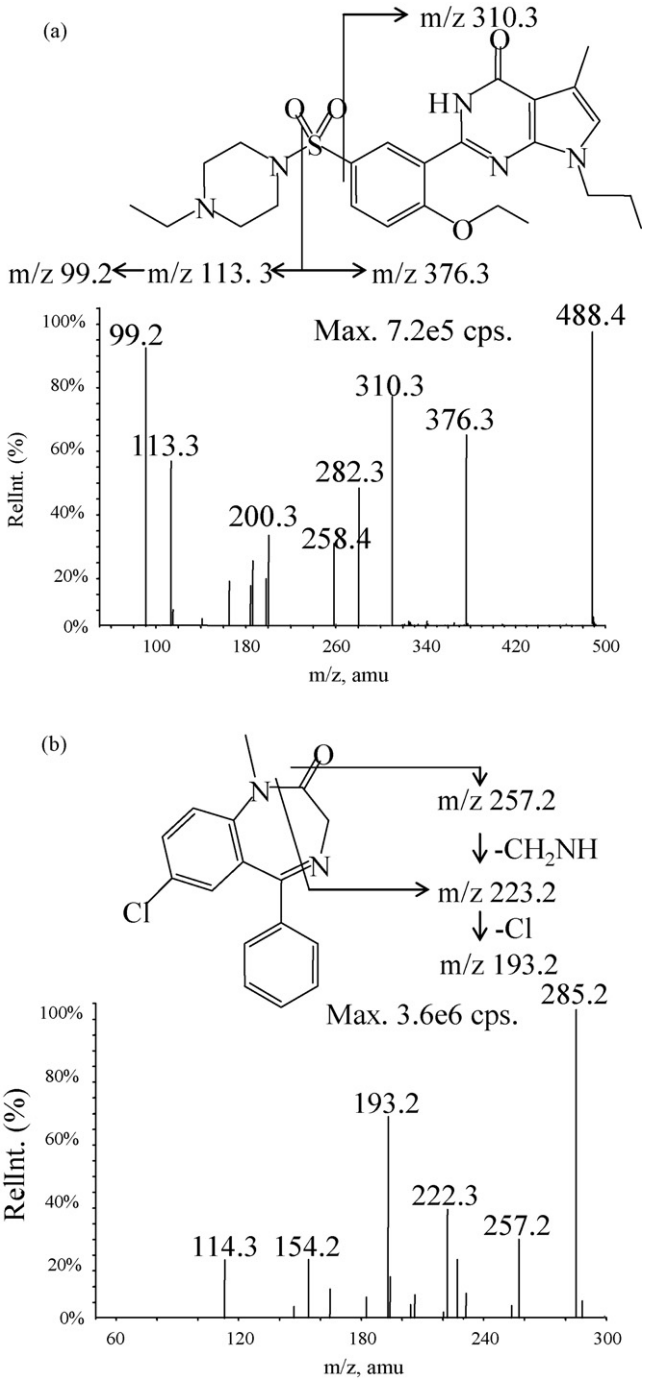

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation pathways for (a) yonkenafil and (b) diazepam.

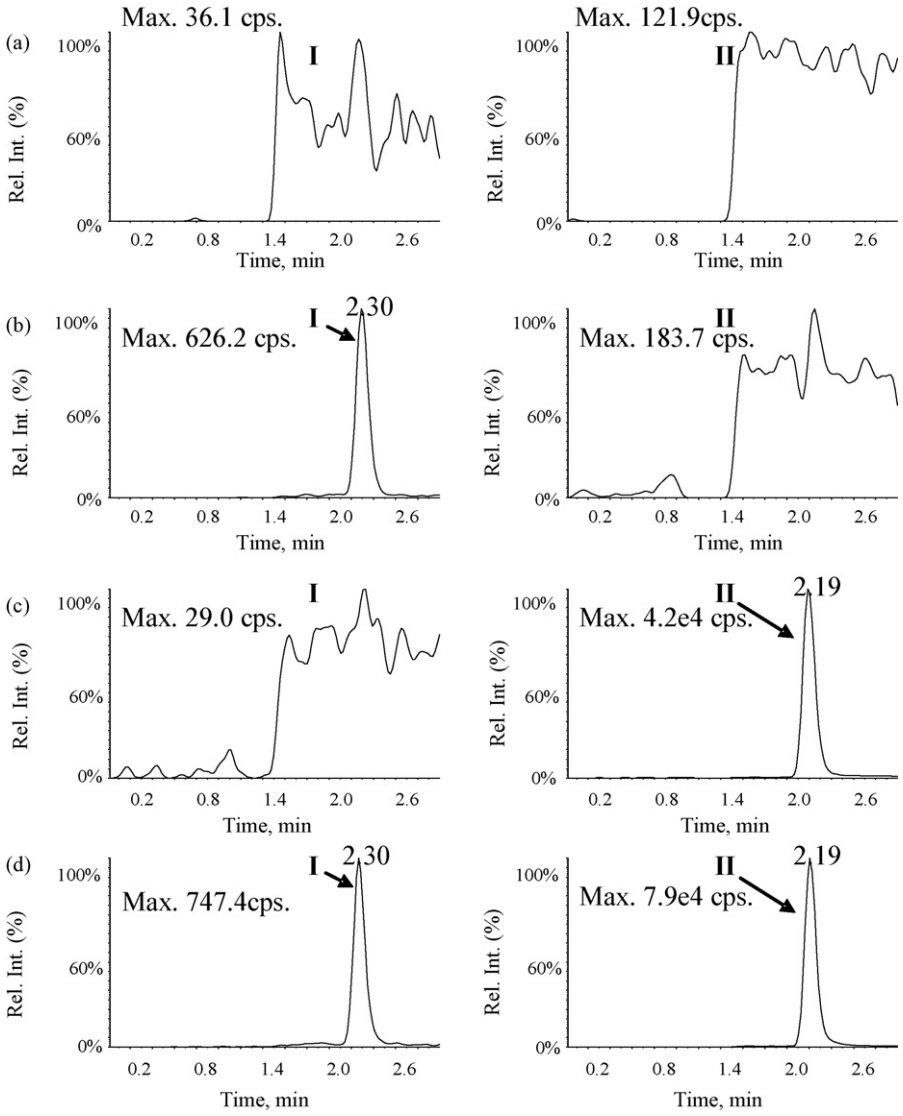

Fig. 2. Typical chromatograms of (a) blank plasma, (b) blank plasma spiked with yonkenafil at 1.0 ng/ml, (c) blank plasma spiked with I.S. and (d) a study sample containing a low yonkenafil concentration. Peak I, yonkenafil; Peak II, diazepam.

tions (4 °C) and mobile phase on storage in plastic autosampler vials under autosampler conditions for 12 h were also assessed.

2.6. Pharmacokinetic study

After a 12 h fast, 18 male Wistar rats (Gaoxin Laboratory Animal Center, Changchun, P.R. China), weight 200–250 g, were randomly divided into three equal groups and administered yonkenafil (2 mg/kg) by the sublingual, oral and i.v. routes. Blood samples (approximately 0.3 ml) were collected into heparinized tubes before the dose and at 0.08, 0.17, 0.33, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0 and 12 h after the dose. Plasma samples were obtained by centrifugation at 3000 × *g* for 10 min and stored at −20 °C until analysis. Plasma yonkenafil concentration-time data were analysed by a noncompartmental method using the TopFit 2.0 program.

3. Results and discussion

3.1. Method development

Sildenafil was first considered as I.S. but the signal suppression of yonkenafil relative to that of sidenafil increased with increasing yonkenafil concentration, potentially affecting the linearity of the assay. Diazepam was selected as the I.S. because its chromatographic behavior and extraction efficiency were similar to those of yonkenafil.

Table 1
Precision and accuracy for the determination of yonkenafil in rat plasma (based on assay of 6 replicates per day on 3 different days)

Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	Intra-day RSD (%)	Inter-day RSD (%)	Accuracy (%)
3.00	3.09 ± 0.09	4.60	2.50	102.9
100	98.9 ± 3.4	5.04	3.10	98.9
800	793 ± 33	8.45	3.27	99.1

Table 2
Pharmacokinetic parameters for yonkenafil after sublingual, oral and i.v. administration (2 mg/kg)

	$AUC_{0\text{-}t}$ (ng h/ml)	$AUC_{0\text{-}\infty}$ (ng h/ml)	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/ml)
i.v.	2241 ± 323	2273 ± 335	1.79 ± 0.73	–	–
Sublingual	1917 ± 167	1944 ± 154	2.54 ± 1.58	0.28 ± 0.08	1078 ± 142
Oral	617 ± 58	651 ± 96	3.34 ± 1.20	0.44 ± 0.09	212 ± 10

As regards mass spectrometer detection, both yonkenafil and diazepam produced strong signals in the positive ion mode due to the presence of basic groups in their structures. The ion spray voltage was limited to 1500 V to reduce in-source dissociation and the source temperature for GS_2 to 450 °C to avoid thermal degradation of analyte and I.S. Other parameters were adjusted to optimize ionization.

Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation pathways of yonkenafil and diazepam are shown in Fig. 1. The transition m/z 488.4 → 310.3 was chosen for quantitation of yonkenafil and m/z 488.4 → 99.2 was used as the qualifier. This allows the method to be easily applied to the assay of yonkenafil in other biological matrices [14].

A number of commercially available reversed phase HPLC columns and various mobile phases were evaluated for chromatographic behavior and the ionization responses of yonkenafil and I.S. Isocratic delivery of a mixture of methanol and 10 mM ammonium acetate gave the best response. A Zorbax Extend C_{18} (5 μm, 150mm × 4.6 mm I.D.) column with isocratic delivery of methanol: 10 mM ammonium acetate, (80:20, v/v) gave satisfactory chromatography with minimal matrix effects. A Nucleosil C_{18} column with this mobile phase adjusted to pH 7.5 with aqueous ammonia also gave satisfactory chromatography but was rejected to avoid potential chromatographic shifts due to volatilization of ammonia. Finally, the proportion of organic modifier in the mobile phase was increased to 85% to reduce the run time and enhance sample throughput capability of the assay.

In terms of sample preparation, yonkenafil and diazepam are sufficiently lipophilic to allow a one-step liquid–liquid extraction procedure to give satisfactory recovery (>80%).

3.2. Assay validation

The detection of yonkenafil and diazepam by MRM was highly selective with no interference. Typical chromatograms are shown in Fig. 2. The run time was only 3 min because full chromatographic separation was not necessary. The standard curve was linear in the range 1.0–1000 ng/ml with an LOQ of 1.0 ng/ml and an LOD of 0.20 ng/ml (S/N ratio of 3). A typical equation of the standard curve was $y = 0.00253x + 0.000161$, $r = 0.9987$. Precision and accuracy were satisfactory at the three concentrations studied (Table 1).

Several extraction solvents (diethyl ether, dichloromethane, ethyl acetate and hexane) were tested to optimize recovery. The best recovery was obtained with diethyl ether: dichloromethane (60:40, v/v). Recoveries of yonkenafil at 3.0, 100 and 800 ng/ml were 89.0%, 84.4% and 85.7%, respectively, and recovery of the I.S. was approximately 80%.

The stability of yonkenafil in drug-free plasma was found to be satisfactory (>92.7% of drug remaining under all the conditions examined). In general, matrix effects are a significant problem in LC–MS/MS analysis of biological samples, but in this assay, no significant signal suppression or enhancement was found.

The effect of dilution of samples containing yonkenafil at concentrations up to 50 μg/ml to a final concentration of 1000 ng/ml was investigated in view of the high concentrations encountered after i.v. administration. Six replicate dilutions of samples initially containing 5, 20 and 50 μg/ml were analysed and gave values that were 96.9–107%, 97.8–105% and 92.8–108%, respectively, of the nominal final concentration.

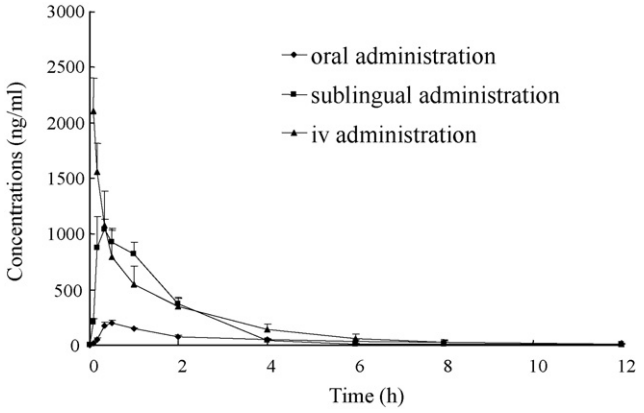

Fig. 3. Mean plasma concentration-time profile of yonkenafil after sublingual, oral and i.v. administration (2 mg/kg) (Data are means + S.D., $n = 6$).

3.3. Pharmacokinetic study

Mean plasma concentration-time profiles after sublingual, oral and i.v. administration are shown in Fig. 3. Pharmacokinetic parameters for these different routes of administration are listed in Table 2. The results show that yonkenafil undergoes extensive and rapid first-pass metabolism after oral administration, and that the sublingual route produces higher plasma levels than the oral route. Thus, the sublingual route may be advantageous in avoiding first pass metabolism and producing fewer gastrointestinal side effects.

4. Conclusion

A simple, sensitive and selective LC–MS/MS method for the determination of yonkenafil in rat plasma has been developed and shown to be suitable for comparing yonkenafil pharmacokinetics after different routes of administration. The results show that sublingual yonkenafil significantly increases the bioavailability of yonkenafil compared with oral administration and suggests the sublingual route may be advantageous in the treatment of male erectile dysfunction.

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