Validation of an LC-ESI-MS/MS method for the quantitation of phosphodiesterase-5 inhibitors and their main metabolites in rat serum and brain tissue samples

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Abstract

This work proposes a liquid chromatography-electrospray ionization ion trap mass spectrometry (LC-ESI-ITMS) method, for the quantification of sildenafil (SDF), tadalafil (TDF) and vardenafil (VDF) and their metabolites N-desmethylSDF, O-desethylSDF and N-desethylVDF, preceded by a sample preparation step based on protein and phospholipid elimination. A C8 column (150 mm x 4.6 mm, 5 µm) with ammonium formate (20 mM) and acetonitrile as the mobile phase components have been used. This method has been validated, obtaining limits of quantification ranged from 1 to 2.5 ng/mL and 2 to 5 ng/g in serum and brain tissue respectively, while limits of detection ranged from 0.3-0.9 ng/mL in serum and 0.6-1.9 ng/g in brain tissue. Assay recoveries for low level QC samples were higher than 83 % and the matrix effect ranged between 91-108 % in serum and between 98-107 % in brain tissue. The method has been applied to the quantification of these compounds in the serum and brain tissue of rats treated intraperitoneally with 10 mg/kg of SDF, TDF or VDF.

Keywords: Phosphodiesterase-5 inhibitors; metabolites; LC-ESI-ITMS; rat serum; rat brain tissue.

1. Introduction

Erectile dysfunction (ED) is a widespread medical condition that has a worldwide prevalence of 52% in men from 40 to 70 years old. Since the 1970s, different options have been developed for ED treatment, but after approval by the FDA in 1998, sildenafil (Viagra®), a phosphodiesterase-5 (PDE5) inhibitor, became the first-line treatment for ED. During 2003, two other PDE5 inhibitors, tadalafil (Cialis®) and vardenafil (Levitra®) were approved for use in ED treatment. Despite the efficacy of PDE5 inhibitors in the treatment of erectile dysfunction, experimental data obtained in rodents show that SDF and VDF also have potent behavioural modification action in rats, and there is now a general consensus towards evaluating the possibility of using PDE5 inhibitors as therapeutic agents in the treatment of cognitive and depressive disorders [1,2]. Although many studies have shown neurochemical and behavioural effects of acute and chronic SDF treatment in rats, they do not present data on the distribution of the drug and its metabolite in the serum or brain of the treated animals. Because brain disposition studies of PDE5 inhibitors in animals may help in understanding their mechanism of action and facilitate the extrapolation of biochemical findings to humans, we determined the brain levels of these drugs and their main active metabolites after the acute administration of these drugs at a dose (10 mg/kg) reported as necessary for the evocation of centrally mediated responses [1-4].

To date, the determination of SDF, VDF or TDF has been focused on plasma or serum samples by LC-MS [5-8]. Most of the sample pretreatment methods proposed for biological samples are based on liquid-liquid extraction [6-8] and to a lesser extent, on solid phase extraction [5]. The objective of this study was the development of a LC-ESI-MS method for the quantification of SDF, VDF, TDF, desmethylSDF, O-desethylSDF and desethylVDF in rat serum and brain tissue samples.

2. Materials and methods

2.1 Chemicals and reagents

The ammonium formate (99%) used in the mobile phase was supplied by Acros Organics (Morris Plains, New Jersey, USA), and acetonitrile was obtained from Scharlab (Barcelona, Spain). The formic acid used in the sample clean-up was from Sigma-Aldrich (St. Louis, MO, USA). SDF, desmethylSDF, desethylSDF, VDF, desethylVDF and TDF and the deuterated internal standards with purities greater than 98% were obtained from LGC Standards (East Greenwich, Rhode Island, USA).

2.2. LC-ITMS analysis

The liquid chromatographic system used was an Agilent 1100-series binary pump system. The column used was a Zorbax Eclipse XDB-C8 column (150 x 4.6 mm, 5 μm) and the mobile phase consisted of a mixture of ammonium formate (20 mM, eluent A) and acetonitrile (eluent B) with a flow rate of 0.6 mL/min. The separation was performed with the following solvent gradient: 0-2 minutes 67:33 (A:B, v/v); 2-7 min linear gradient to 20:80 (A:B, v/v); 7-8 min gradient to 67:33 (A:B, v/v) maintained for 2 min.

The ion trap mass spectrometer was an MSD Trap XCT Plus equipped with an electrospray ionisation source G1948A operating in positive ion mode (ESI+). The operating conditions of the ESI interface for the optimal ionization of all analytes were the following: drying gas (N_2) temperature of 350 ºC, 11.0 L/min drying gas flow (N_2), 60 psi nebulizer gas (N_2) pressure and -3500V of capillary voltage. Full-scan MS and MS/MS spectra were obtained by scanning in mass ranges from 100 to 500. The chromatographic separation was divided into three time segments. Multiple reaction monitoring (MRM) transitions, using as precursor ions the protonated molecular ions $[M+H]^+$, and MRM parameters are summarised in Supplementary Table S1.

2.3. Standards and quality control (QC) samples

Stock solutions containing 1 mg/mL of individual analytes or internal standards were prepared in methanol. Due to the solubility of TDF and TDF-d3, a 0.3 mg/mL or 0.2 mg/mL stock

solutions were respectively prepared in ethanol. Fresh calibration standards were prepared in triplicate in blank rat serum and tissue to yield concentrations ranged between 1 and 1000 ng/mL and 2 and 2000 ng/g respectively. QC samples were prepared at the lower limit of quantification (LLOQ) 1 or 2.5 ng/mL, 7 ng /mL (low QC), 50 ng/mL (middle QC) and 400 or 800 ng/mL (high QC) in serum and 2 ng/g or 5 ng/g (LLOQ), 15 ng/g, 100 ng/g and 800 or 1600 ng/g in brain tissue. To each calibration standard and QC sample, deuterated analogues were added. All solutions were stored at -42ºC upon processing.

2.4. Sample preparation

In order to minimize the ion suppression effect, a preparation step based on protein precipitation and phospholipid removal is proposed. 200 µL of serum sample was spiked with 20 µL of an internal standard solution containing 1 mg/L of the deuterated analogues. After vortexing in order to precipitate the proteins, 0.6 mL of 1% formic acid in acetonitrile was added, and the mixture was agitated again for 5 min and centrifuged at 10,000 rpm for 5 min at 4 ºC. The supernatant was transferred directly to a HybridSPE^{TM}-PPT cartridge (Supelco, Bellefonte, USA), which is recommended to removal of endogenous protein and phospholipid interferences from biological samples prior to LC–MS or LC–MS/MS analysis. The resulting eluent was evaporated to dryness under a nitrogen stream at room temperature and reconstituted in 100 µL of a mixture of ammonium formate and acetonitrile (67:33, v:v), and 20 µL was injected directly into the LC/MS system. For brain samples, 100 mg of sample were homogenised with a sonicator for 10 s. An internal standard solution (20 µL) containing 1 mg/ L of the deuterated standards and 0.6 mL of 1% formic acid in acetonitrile were added. The same extraction procedure was then carried out.

2.5. Animal treatment

The in vivo experimental procedures were approved by the Ethics Committee of the University of the Basque Country (UPV/EHU), according to the European legislation on the use

and care of laboratory animals (CEE 86/609). Nine male Sprague-Dawley rats (225-250g) bred by Harlan Ibérica (Barcelona, Spain) were divided into three groups (SDF, VDF and TDF). The drug dosage of 10 mg/kg was selected because studies in rats have demonstrated central pharmacological action for these PDE5 inhibitors [1-4]. SDF or VDF solutions were prepared by disolving Viagra™ (25 mg sildenafil citrate) or Levitra™ (10 mg vardenafil hydrochloride trihydrate) tablets in distilled water acidified with hydrochloric acid (0.1 N). SDF solution was adjusted to pH 4 with NaOH (1 M). TDF was prepared disolving Cialis™ (10 mg TDF) in 50% dimethyl sulphoxide (DMSO, v/v), and this solution was made up to final volume by addition of 0.9% NaCl. All drugs were injected in a volume of 1 mL/kg of body weight as a single intraperitoneal injection. Animals were anaesthetised with ether 60 min later and were killed by decapitation. Serum samples and brains were frozen on dry ice and stored at -80ºC until analysis.

3. Results and discussion

3.1. LC-MS/MS conditions

To optimize chromatographic conditions 150 x 4.6mm dimensions and 5 µm particle size Zorbax Eclipse XDB-C8 and XDB-CN columns and different composition mobile phases were assayed. A Zorbax Eclipse XDB-C8 column and a mobile phase containing an aqueous phase of 20 mM ammonium formate (pH=3) and acetonitrile as organic modifier were employed. Under isocratic elution conditions, the high retention time of TDF increased the analysis time to 20 min. However, a gradient method with a flow rate of 0,6 mL/min could achieve a satisfactory separation and resolution of the compounds in less than 10 min and no additional equilibration time was required for the sample injection (Figure 1).

ESI Interface and Ion Trap Mass Spectrometer parameters were optimized by infusing 20 µg/mL of each standard in control serum and brain tissue previously extracted following the sample preparation procedure indicated above. The infusion was performed directly in the ionization source at a flow of 10 µL/min with a syringe pump. The MS spectra of analytes showed the protonated

molecular ions [M+H]+ as precursor ions selected to generate MS/MS spectra. The optimum fragmentation amplitude for each analyte was determined by increasing the fragmentation amplitude until the precursor ion intensity was reduced to 5–20% of its major product ion response. The cut-off values (the minimal value of m/z ratio, for the ions with smaller values than these quantities not to be trapped by the IT) were set to the default value (27%) from the precursor ions m/z ratio.

3.2. Method validation

Blank serum and brain tissue samples were screened and no interfering signals were observed at the retention times and with the same mass ratios as the compounds of interest. All the analytes were fitted to one model linear calibration over the interval LLOQ-1000 ng/mL for serum samples and LLOQ -2000 ng/g for brain tissue samples except for SDF and desethylSDF, for which the linearity ranged from LLOQ to 500 ng/mL for serum samples and from LLOQ to 1000 ng/g for brain tissue (Table 1). Correlation coefficients (r^2) were higher than 0.988 in all the cases, and a weighting factor of $1/x^2$ was applied to the data. The LOD (Table 1) was defined as the lowest concentration with acceptable chromatography, and with the presence of precursor and product ions with the ion ratios within ±20% of standards. The LLOQ, defined as the lowest concentration used in the calibration with an accuracy and precision of 100 ± 20%, was validated in serum at 2.5 ng/mL for all the analytes except for TDF, which was validated at 1 ng/mL. In brain tissue, the validated LLOQ ranged from 2 ng/g for TDF to 5 ng/g for the rest of the compounds.

Related to the accuracy, the mean value should be within 85-115 % of the real value except at LLOQ, where the mean value should not deviate by more than 20%. As shown in Supplementary Table S2, accuracy fell within the acceptable range in this study. In the same way, precision, which should not exceed 15% of the RSD except for the LLOQ that should be lower than 20%, was also determined. The resulting RSD values, listed in Table S2 (supplementary data), were ≤17% at the LLOQ concentration level and ≤14% at the low, middle and high concentration levels.

As shown in Supplementary Table S3, the modification of the signals due to the matrix effect ranged between 91-108% in serum and between 98-107% in brain tissue. These data show that the matrix effect was corrected by performing the matrix-matched calibration curves and using stable isotope-labelled internal standards to correct the loss of analyte during the preparation process and the matrix effect in the suppression or enhancement during analyte ionization. Extraction recoveries for low level QC samples and for six different lots, which were higher than 83% for all the analytes, are presented in Supplementary Table S3.

The stock solutions are stable (90 and 109%) for at least 1 month at 4ºC and 3 months at -42ºC.The stability of the QC samples has been demonstrated after storage at room temperature for one day (85-114% in serum and 87-111% in brain tissue), at -42 ºC for 2 months (88-113% in serum and 90-107% in brain tissue) and after freeze and thaw cycles (87-115% in serum and 88-109% in brain tissue). The calculated concentrations at room temperature for the analytes in processed samples for one day fall within 82-112 % in serum and 86-113% in brain tissue.

3.3. Application to real samples

The validated method was applied to the three experimental groups. The mean final concentrations found in rat serum and brain samples are shown in Table 2. For SDF, the serum mean metabolite-to-parent drug concentration ratio ranged from 2.7 to 0.7 for desmethylSDF and O-desethylSDF, respectively. Results that are supported by previous pharmacokinetic studies, in which SDF administered orally to rats showed that the principal route of metabolism of SDF is the N-demethylation mediated by cytochrome P450 3A1/2 and 2C11, with desmethylSDF as the major circulating metabolite [9,10]. However, our data also demonstrated the presence of O-desethylSDF, a metabolite of SDF that did not correspond with the rat plasma metabolites previously described. According to a recent investigation, O-desethylSDF has been found in urine samples [11]. With respect to the brain distribution of SDF, desmethylSDF and O-desethylSDF, our data show significant brain levels of SDF and desmethylSDF, while O-desethylSDF was not detected. These

results indicate the potential contribution of desmethylSDF to the central pharmacological action that has previously been reported after the intraperitoneal administration of SDF.

For VDF, the major circulating metabolite reported previously, the serum mean desethylVDF-to-VDF concentration ratio was around 0.2 (20%). However, the brain exposure to desethylVDF was too low to suggest any contribution of the metabolite to the central pharmacological action of VDF (Table 2). With respect to VDF, the brain levels were 4% of the serum counterpart. The [brain]/[serum] ratio for TDF was 10% (Table 2), at least twice those observed in the corresponding relationships obtained for both SDF and VDF at the same administered doses.

4. Conclusions

A new LC-MS/MS method has been developed for the simultaneous determination of SDF, VDF, TDF and metabolites desmethylSDF, O-desethylSDF and desethylVDF in rat serum and brain tissue. The proposed sample treatment demonstrated to be an alternative to liquid-liquid extraction since it is also accurate and reproducible and it is a time and solvent saving method. Besides, this method shows advantage of being suitable for serum and brain tissue samples. The method has been validated and meets the current requirements of bioanalytical method validation. To the best of our knowledge, our study has been the first to evaluate the rat brain concentrations of PDE5 inhibitors after their acute administration at a dose reported as necessary for the evocation of centrally mediated responses.

Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:

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Figure Captions

Figure 1. LC–MS/MS ion trap segmented chromatogram (MRM) and MS/MS spectra of 500 ng/mL of each analyte and 100 ng/mL of the corresponding deuterated IS. 1: desethylVDF-d8, 2: desethylVDF 3: VDF-d5, 4: VDF, 5: desmethylSDF-d8, 6: desmethylSDF, 7: SDF-d3, 8: SDF, 9: O-desethylSDF, 10 TDF-d3, 11: TDF (Analytes in blue, deuterated IS in red).

Table 1. Summary of linearity, detection and quantification limits for all the analytes in serum and brain tissue samples.

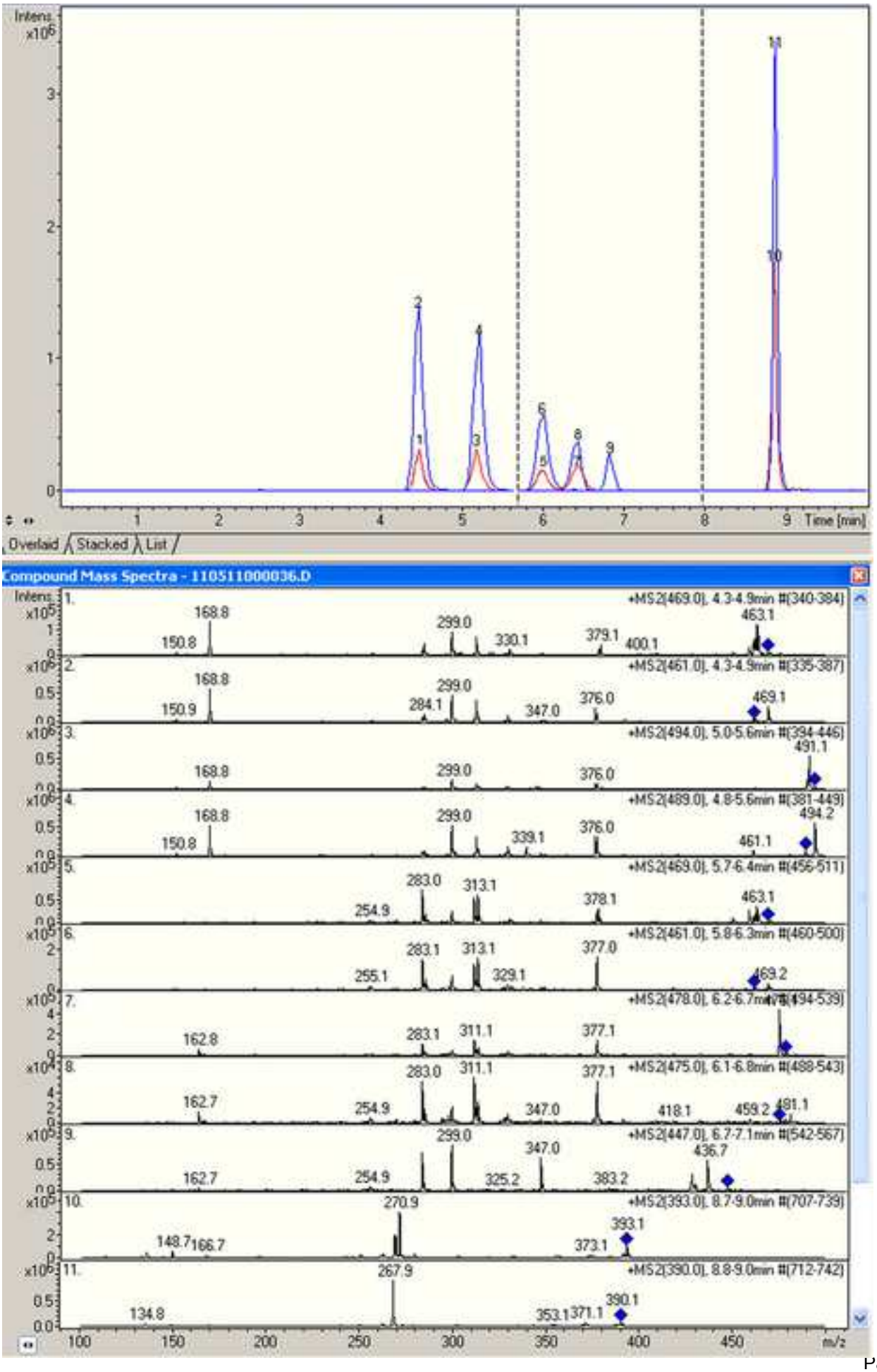
Compound IS	SERUM					BRAIN TISSUE*			
	Linear range (ng/mL)	r^2	LOD (ng/mL)	LOQ (ng/mL)	LOQ (other autors) (ng/mL)	Linear range (ng/g)	r^2	LOD (ng/g)	LOQ (ng/g)
DesethylVDF DesethylVDF-d8	LOQ-1000	0.994	0.7	2.5	0.5 [7]	LOQ-2000	0.996	1.6	5
VDF VDF-d5	LOQ-1000	0.996	0.8	2.5	0.5 [7]	LOQ-2000	0.999	1.7	5
DesmethylSDF DesmethylSDF-d8	LOQ-1000	0.988	0.7	2.5	0.5 [6], 1[5]	LOQ-2000	0.995	1.7	5
SDF SDF-d3	LOQ-500	0.988	0.8	2.5	1 [5, 6]	LOQ-1000	0.988	1.8	5
O-DesethylSDF SDF-d3	LOQ-500	0.988	0.9	2.5	-	LOQ-1000	0.988	1.9	5
TDF TDF-d3	LOQ-1000	0.996	0.3	1	10 [8]	LOQ-2000	0.993	0.6	2

* No data found by other authors in brain tissue

Table 2. Concentrations of the detected target analytes in rat serum and brain tissue.

Compound	SERUM (n=3)		BRAIN TISSUE (n=3)		BRAIN/SERUM (%)
	(ng/mL)	μM	(ng/g)	μM	
SDF	554 ± 49	1.17	28.4 ± 0.3	0.06	5
DesmethylSDF	1476 ± 358	3.20	46 ± 19	0.10	3
O-DesethylSDF	412 ± 473	0.92	n.d.	n.d.	-
TDF	13028 ± 2808	33.45	1325 ±171	3.40	10
VDF	1810 ± 8	3.70	78 ± 9	0.16	4
DesethylVDF	426 ± 36	0.92	7.5 ± 0.5	0.02	2

n.d.: not quantifiable, < LLOQ



- A new LC-ESI-ITMS method is established for the determination of PDE5 inhibitors
- A sample treatment using HybridSPE-Precipitation cartridges is proposed.
- The method permits the determination of analytes in serum and brain tissue
- The study has been the first to evaluate the rat brain levels of PDE5 inhibitors