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Solid phase extraction and liquid chromatographic determination of sildenafil and *N*-demethylsidenafil in rat serum with basic mobile phase

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Abstract

HPLC method for the determination of sildenafil and its metabolite (*N*-demethylsidenafil) in rat serum has been developed. The technique included a solid phase extraction of the serum samples on a [poly(divinylbenzene-co-*N*-vinylpyrrolidone)] solid phase extraction sorbent. After conditioning, the cartridge was loaded with 0.5 mL of buffered serum containing internal standard. Elution was made with 1 mL of acetonitrile. After evaporation of the eluates to dryness and reconstitution with methanol, the samples were analyzed on Kromasil C_{18} column phase with phosphate buffer 0.05 M/acetonitrile: 54/46, pH 8. Detection was carried out using a photodiode array detector. For sildenafil and demethylsildenafil, full validation of the proposed method was provided (linearity range, calibration curves, average extraction efficiency; average intra-day and interday variabilities, limit of detection, limit of quantification, specificity). The proposed method was successfully utilised to quantify sildenafil and *N*-demethylsidenafil in rat serum for a pharmacokinetic study.

Keywords: Column liquid chromatography; Basic mobile phase; Solid phase extraction; Photodiode array detector; Sildenafil; Demethylsildenafil

1. Introduction

Sildenafil (1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine) is used as an oral agent to treat male erectile dysfunction [1–3]. It is a selective inhibitor of cyclic guanosine monophosphate (cGMP) specific phosphodiesterase type 5 (PDE5). Sildenafil citrate is metabolized and demethylated to give UK-103, 320 having a similar potency to sildenafil and hence may contribute to pharmacological effects. Therefore, it is essential to evaluate the two compounds in serum.

Many techniques were used to determine sildenafil citrate in pharmaceuticals, such as spectrophotometric methods [4] or HPLC. The determination of sildenafil citrate in pharmaceuticals was made with HPLC using C_{18} [5] or monolithic columns [6]. Daraghmeh et al. [7] reported an HPLC technique for the determination of sildenafil citrate and its related substances. Recently, Abd-Elbary et al. [8] proposed an HPLC procedure for the determination of sildenafil citrate in bulk and in formulations. Structure elucidation of sildenafil analogues in herbal products was made by Blok-Tip et al. [9].

There are some papers in the literature reporting the determination of sildenafil in pharmaceuticals [10] or in serum samples using micellar electrokinetic [11,12], capillary zone electrophoresis [13], gas chromatography [14,15] or HPLC [16,17]. A high-performance liquid chromatographic (HPLC) analysis of sildenafil and one of its metabolite UK-103, 320 (*N*-demethylsildenafil) in serum was reported using automated sequential trace enrichment of dialysates (ASTED) system to prepare serum samples [18]. Another method using narrow-bore column switching has also been used to this for the simultaneous determination of sildenafil and its active metabolite in serum [19]. Liquid chromatographic method of sildenafil with a detection limit of 10 ng mL^{-1} was proposed by Sheu et al. [20]. Liquid chromatography–tandem mass spectrometry was used for simultaneous assay of sildenafil and desmethylsildenafil in human serum [21–23].

It is important to note that both sildenafil and its demethylated metabolite UK-103, 320 (Fig. 1) have basic functional groups with a pK_a, value of 8.7 [18]. It is well known that difficulties may arise during the analysis of compounds with basic properties due to the adsorption exposed residual silanols of silica HPLC

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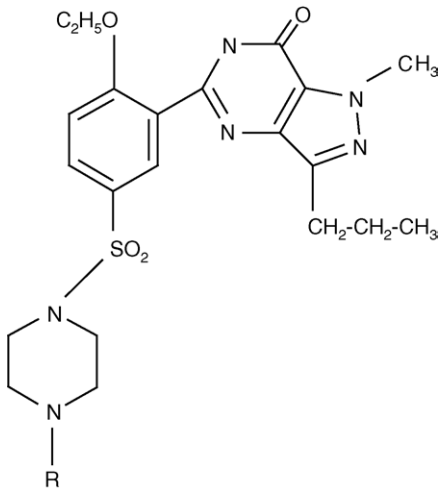

Fig. 1. Chemical structure of sildenafil (R = CH_3) and *N*-demethylsidenafil (R = H).

column. These interactions induce peak tailing, which can affect resolution, sensitivity, and reproducibility. A new approach consists in developing new stationary phases, especially designed to avoid secondary interactions between basic compounds and free silanols. Some of these supports even if silica based, are chemically stable over a wide pH range.

In the other hand, sildenafil and its metabolite are highly and weakly protein binding [24]. Solid phase extraction can give an alternative solution to isolate the basic SC and its metabolite.

Throught the literature [11,14,21,25–28], before solid phase extraction, it appeared that it was not necessary to carry out a special treatment of serum samples to unbound sildenafil and its metabolite. To isolate sildenafil and its metabolite from serum, liquid–liquid extraction [20] or solid–liquid extraction were used. Solid phase extractions were performed on cartridges C_8 [21] or C_{18} bonded silica [11,14,25–28]. The proposed SPE procedures were difficult to use and time consuming. This classical C_{18}-bonded silica phase cannot endure to strong apolar organic solvents, contrary to the polymeric sorbents. Modern porous polymer sorbents stable in a large pH range (pH 1–11) are generally copolymers of styrene and divinylbenzene processed to enhance their properties for SPE [29–32]. The large surface areas (700–1200 m^2/g) and the porous structure of the highly crosslinked polymers result in higher retention with a compatibility with aqueous and organic solvents. Extend of their properties can also be increased by light surface modification with polar functional groups [31,33]. The same goals are achieved by a macroporous poly(divinylbenzene-co-*N*-vinylpyrrolidone) polymer (Oasis HLB), which has a surface area of about 800 m^2/g [34]. Several references describing the successful biological applications of this sorbent such in the extraction of methadone and benzodiazepine [35], linezolid [36], epirubicin [37], metroprolol [38], lanzoprazole and its metabolites [39], celecoxib [40]. In this work, a SPE cartridges based on poly(divinylbenzene-co-*N*-vinylpyrrolidone) were used to direct extracting SC and its metabolite. HPLC technique with a basic mobile phase was developed.

2. Experimental

2.1. Reagents

Acetonitrile and methanol of chromatographic grade were from Fluka (Switzerland). Ultra pure water was made by the Milli-Q ultra pure system (Millipore, USA). Sildenafil and *N*-demethylsidenafil were kindly gift by Pfizer (USA), phenacetin (internal standard) was from Sigma (USA).

2.2. Chromatographic instrumentation

Waters chromatograph with a 600E pump, 7625i Rheodyne injector with 20 μL sample loop, Waters diode array detector 991 was used. Separations were carried out on a Kromasil C_{18} (250 mm × 4.6 mm) proceeded with a Kromasil C_{18} guard column 1 × 0.4 cm both from Interchim (France). Several mobile phases were tested in isocratic mode. They were made from phosphate buffer 0.05 M and acetonitrile. According to McCalley and Brereton [41], pH measurement of the aqueous buffer was made after to organic modifier addition because the modifier can have a considerable effect on buffer, solute and silanol ionisation.

Flow rate was fixed to 1 mL min^{-1}. Data were collected with Millenium 32 program (Waters). Quantitation was made at 300 nm.

2.3. Collection of the samples

Serum samples were collected from rats of Wistar race (mean weight, 200 g) which received orally 1 mg kg^{-1} of sildenafil suspension. After 0.5, 2, 3, 4, 6, 8, 11 and 24 h, blood samples were collected from the orbital venous plexus. Serum samples were separated by centrifugation at 6,000 rpm for 15 min. They were stored at −20 °C and allowed to defrost at 25 °C prior to use.

2.4. Sample extraction procedure

The solid phase extractions of the samples were carried on poly(divinylbenzene-co-*N*-vinylpyrrolidone) cartridge (Oasis HLB, 60 mg) from Waters. First, conditioning was made by flushing the cartridge with 1 mL of methanol and 1 mL of water. A mixture of 0.5 mL of serum containing phenacetin 20 μg mL^{-1} and 0.1 mL of K_2HPO_4 0.05 M was applied by allowing it to pass trough the bed with a minimal suction, the cartridge was washed with 1 mL of water. Elution was made with 1 mL of acetonitrile. The sample was then evaporated to dryness under nitrogen at 30 °C and reconstituted with 500 μL of methanol. An amount of 20 μL were injected into the chromatograph.

2.5. Extraction recovery

The extraction recoveries were determined by comparing the peak areas of the extracts of spiked serum samples with those