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An LC–MS/MS method for the determination of five erectile dysfunction drugs and their selected metabolites in hair

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ABSTRACT

The abuse of sildenafil and its analogous, accelerated by their inappropriate or illegal distribution, is a serious social issue globally. However, no studies have been conducted to monitor these drugs simultaneously in hair, which can provide valuable information on chronic drug use. In the present study, an LC–MS/MS method was developed for the simultaneous determination in hair of five erectile dysfunction drugs having a high risk for abuse (mirodenafil, sildenafil, tadalafil, udenafil and vardenafil) and their selected metabolites (SK3541, desmethylsildenafil, DA8164 and desethylvardenafil). The novel method was fully validated after optimizing matrix effects and extraction efficiency. The optimized sample preparation included acidic methanol extraction followed by solid phase extraction using C18 mixed mode strong cation exchange polymeric cartridges. The prepared samples were analyzed by LC–MS/MS with electrospray ion source in the positive ionization mode. The validation results proved the method to be selective, sensitive, accurate and precise, with acceptable linearity within calibration ranges. LODs ranged from 0.05 (DA8164) to 1 ng/10 mg hair (tadalafil). LOQs were 1 ng/10 mg hair except for DA8164 and vardenafil, of which they were 2.5 ng/10 mg hair. No significant variations were observed by different sources of matrices in both human and rat hair, except for tadalafil, for which a stable isotope-labeled internal standard was effective. The animal study suggested hair pigmentation was a major factor for the incorporation of the drugs and metabolites into hair. However, a wide variation of the sildenafil-to-desmethylsildenafil ratios was observed in human hair samples. The developed method will be very useful for monitoring the abuse of erectile dysfunction drugs for both legal and public health aspects.

1. Introduction

Since the introduction of sildenafil as a medication for the treatment of erectile dysfunction in the late 1990s, the abuse of sildenafil and its analogs has rapidly increased globally. The abuse has been accelerated by the illegal distribution of counterfeit compounds through routes including the Internet [1–4]. A previous study in Korea documented the predominance of counterfeit Viagra in drugs seized by Korean authorities, with sildenafil as the main constituent followed by tadalafil [3].

Sildenafil has shown acceptable efficacy and safety for male impotence treatment in clinical studies [5,6]. Minor adverse effects include headache, flushing, dyspepsia, nasal congestion, urinary tract infection, abnormal vision diarrhea, dizziness and rash [7]. Nevertheless, sildenafil has received attention in forensic drug testing not only from the aspect of illegal possession, traffic or use of counterfeit tablets but also from the aspect of drug-related deaths [8,9] and drug-facilitated sexual assaults [10]. The autopsy findings in the previous two fatal cases showed severe coronary arteriosclerosis and signs of previous myocardial infarctions [8,9]; however, its cardiovascular risk, especially associated with sexual activity, is still in controversy.

Recently, hair becomes a more frequently and routinely analyzed specimen for forensic and clinical investigations, as a consequence of refinement in mass spectrometry [11]. Research findings have accumulated on the differentiation of external contamination and actual ingestion of drugs [12–14], dose–concentration relationship [15] and effect of hair pigmentation on drug incorporation

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into hair [15,16]. This knowledge has advanced the interpretation of analytical results of drugs and metabolites in hair. Even with the continuing scarcity of evidence concerning interpretation of quantitative results of drugs and metabolites in hair from the pharmacokinetic point of view, drug analysis in hair is widely accepted as an analytical tool to prove drug abuse.

In a previous study [17], an analytical method of sildenafil and its metabolite, desmethylsildenafil, in hair was developed using gas chromatography–mass spectrometry after acidic methanol extraction and solid phase extraction (SPE) followed by trimethylsilyl derivatization and applied to rat and human hair. The incorporation rate (the ratio of drug concentration in hair to the area under the concentration versus time curve in plasma) of sildenafil was 0.105 in male dark-agouti pigmented rats. Sildenafil was also determined in a hair sample from a postmortem case where the chronic use of sildenafil was proven [9]. In another study [10], the relationship of sildenafil to a case of drug-facilitated crime was established by segmental hair analysis. These studies highlight the value of the analysis of sildenafil and its analogs in hair in detecting their inappropriate use as well as understanding the association of their chronic use with forensic or clinical cases. However, simultaneous determination of sildenafil and its analogs, as well as their metabolites in hair has not been reported.

Even though the exact mechanism for drug incorporation into hair is not known, binding of drugs and metabolites to melanin or sulfhydryl-containing amino acids was suggested as a major mechanism [18]. Hair is mainly composed of protein (65–95%, keratin essentially) [18], which is considered as a complicated matrix for analysis. Maximizing the release of analytes from the hair matrix is an important point of sample preparation in hair analysis. Also, matrix effects should be considered at the initial step of method development using LC–MS/MS since different degrees of ion suppression or enhancement by matrices from different origins may significantly affect method sensitivity and reproducibility [19].

In the present study, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous determination of five erectile dysfunction drugs (mirodenafil, sildenafil, tadalafil, udenafil and vardenafil) and their selected metabolites [SK3541 (mifodenafil, a metabolite of mirodenafil), desmethylsildenafil (a metabolite of sildenafil), DA8164 (a metabolite of udenafil) and desethylvardenafil (a metabolite of vardenafil)] in hair was developed and fully validated, permitting application of the method to prove chronic use of erectile dysfunction drugs. Different sample preparation methods were compared to reduce matrix effects and increase extraction efficiency. The developed method was used to investigate the distribution of the drugs and metabolites in human and rat hair.

2. Materials and methods

2.1. Chemicals

All solvents were of high performance liquid chromatography grade. Desmethylsildenafil, sildenafil, sildenafil-d_8 and tadalafil-d_3 were obtained from TLC PharmaChem (ON, Canada). Desethylvardenafil, tadalafil and vardenafil were purchased from Santa Cruz Biotechnology (CA, USA). Mirodenafil and SK3541 were kindly provided by SK Chemicals (Gyeonggi-do, Republic of Korea) and udenafil and DA8164 by Dong-A Pharmaceutical (Seoul, Republic of Korea). For analytical study, 1 μg/mL and 100 ng/mL analyte mixture solutions and 1 μg/mL internal standard (sildenafil-d_8 and tadalafil-d_3) mixture solution were prepared in methanol and stored at −20 °C before analysis. Sildenafil dissolved in distilled water (2.5 mg/mL) and the same concentrations of tadalafil, vardenafil, mirodenafil and udenafil suspended in 2% Tween 80 (Sigma–Aldrich, MO, USA) were used for the animal study.

2.2. Human hair samples

Drug-free hair for the preparation of quality control samples were voluntarily provided by five staff in our laboratories. Authentic hair samples were collected from two illegal drug use suspects who possessed counterfeit sildenafil tablets and were submitted by the police.

2.3. Animals

Fifteen male lean Zucker rats (Orient Bio, Seoul, Korea), eight weeks old, were used after at least a one-week acclimation period in the laboratory animal facility. The rats were provided with tap water and a commercial diet ad libitum. The animal room was maintained at a temperature of 24 ± 2 °C and a relative humidity of 50 ± 20%, with a 12 h light/dark cycle. Each rat was housed in a separate metabolism cage to prevent urine and saliva contamination of hair.

2.4. Animal study

All procedures were approved by the Animal Care and Use Committee at the National Forensic Service. Fifteen rats were weighed and randomly assigned to five groups (n = 3 for each group) for oral administration of 10 mg/kg body weight of mirodenafil, sildenafil, tadalafil, udenafil or vardenafil. Before drug administration, pigmented and non-pigmented hair was shaved separately from the dorsal region using an electric shaver for use as drug-free samples for analysis. Beginning on day 3, the particular drug was administered to the rats once per day for ten consecutive weekdays. Six weeks after the initial treatment, newly grown pigmented and non-pigmented hair was shaved and collected separately. All shaved hair was stored at room temperature until analysis.

2.5. Human and rat hair sample preparation

Initially, three different sample preparation methods were attempted to extract sildenafil, its analogs and their metabolites in both human and rat hairs, as previously described [17,20,21] with some modification. The hair was washed twice with 2 mL of methanol followed by two washes with 2 mL of distilled water and two washes with 2 mL of methanol. After that, each sample was dried on filter paper at room temperature. The hair was then cut finely, weighed accurately (approximately 10 mg) and incubated for 16 h in 2 mL of a 20:1 solution of methanol and 5 M HCl (acidic methanol extraction) or methanol (methanol extraction). Twenty five microliters of the 1 μg/mL internal standard solution was added before incubation. The extract was evaporated to dryness under a stream of nitrogen at 45 °C. The residue was reconstituted in 100 μL of a solution of methanol and mobile phase A (1:1) and filtered through a 0.45-μm polyvinylidenefluoride microporous membrane. Finally, 5 μL was injected into the LC–MS/MS system.

Additionally, SPE using C18 mixed mode strong cation exchange polymeric cartridges (Bond Elut Plexa PCX; Agilent Technologies, CA, USA) was performed with the evaporated extract of acidic methanol extraction. The cartridge was preconditioned by adding 2 mL of methanol, 2 mL of distilled water and 2 mL of 0.1 M phosphate buffer (pH 6.0). The hair extract residue was dissolved in phosphate buffer and loaded onto the cartridge. After this, the cartridge was washed with 1 mL of distilled water followed by 250 μL of 10 mM acetic acid and was dried under vacuum for 20 min. The sample was eluted from the cartridge sequentially