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Application of high-performance liquid chromatography with charged aerosol detection for universal quantitation of undeclared phosphodiesterase-5 inhibitors in herbal dietary supplements

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ABSTRACT

Incidents of detecting novel analogues of phosphodiesterase 5 (PDE-5) inhibitors in illicit dietary supplements for erectile dysfunction are constantly reported. However, little is known about their content in a single dose, mainly due to the poor availability or inaccessibility of pure reference standards. This study presents a new strategy of quantitative analysis of unknown and recently identified compounds. Charged aerosol detector (CAD), described as "universal detector", combined with high-performance liquid chromatography (HPLC) system proved to be a useful tool for fast and simple quantitation of PDE-5 inhibitors' analogues in a complex herbal matrix without individual reference standards available. Universal calibration was employed for calculations. Two easily obtainable reference materials – sildenafil and tadalafil – were selected as universal standards and the content of analogues was estimated with respect to their response. The error of proposed indirect determination was found to be ±3%, which is less than enough to obtain a reliable result of the content. The elaborated method was applied for quantitative analyses of PDE-5 inhibitors and 10 analogues detected in 22 illicit dietary supplements and two bulk powdered herbal materials. All target analogues were identified using time-of-flight mass spectrometry with electrospray ionization. Obtained results indicate that the quantity of PDE-5 inhibitors in all tested samples is considered to be pharmacologically relevant.

1. Introduction

A significant part of all reported cases of illicit supplements in Europe concerns herbal aphrodisiacs adulterated with phosphodiesterase 5 (PDE-5) inhibitors [1]. Such contaminated and mislabelled products may be the source of serious medical incidents especially for patients with atherosclerosis, hypertension or diabetes, for whom PDE-5 inhibitors are contraindicated. Because they are composed of natural ingredients, herbal supplements are perceived as a safe alternative to synthetic active substances. Preferred distribution channels for counterfeit supplements for erectile dysfunction include the Internet, bazaars, oriental medicine shops and sexshops. Nevertheless fraudulent dietary supplements have also been reported on legal markets [1]. In order to avoid detection of such forgery in screening tests, patent infringement or coming under Pharmaceutical Law, analogues of PDE-5 inhibitors or their mixtures are frequently seen as adulterants. Contamination with analogues generates remarkably greater health risk than contamination with approved drugs. There are no systematic studies on the potency, toxicity, possible adverse reactions or safety profiles of the majority of the analogues. The use of a mixture of different analogues reduces the content of individual compounds in a single dose, often below the detection limit of the screening method. Still, cumulative action of all compounds may be sufficient to induce pharmacological effects.

There are many papers reporting the identification of novel PDE-5 inhibitors analogues [2–15]. New structures are continually elucidated [3]. However, research rarely presents a quantitative analysis with specific data on the content of analogues in herbal products. The predominantly used method is mass spectrometry (MS) which allows for simultaneous identification and quantification of analyte. For quantitative measurements, multiple reaction monitoring (MRM) scan mode is generally applied [4–7]. Moreover, PDE-5 inhibitors are determined in wastewater and sewage sludge [8] as well as in human plasma using a tandem mass spectrometry method [9,10] or HPLC with amperometric detection [11]. Quantitative evaluations of dietary supplements have also been performed using NMR [12], UHPLC-UV [13] or by combination of two methods: UV and MS [2,14] or UV and NMR [15]. The reference

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standard of a detected analogue was necessary in the majority of quantitative analyses mentioned above. Problems arise when reference materials do not exist or are inaccessible for researchers. In this case, the following solutions are applied. Usually, the content of active compounds is measured by ^{1}H NMR using TSP-d_4 (trimethylsilyl-2,2′,3,3′-tetradeuteropropionic acid) as internal reference [15]. In other published studies, the content of analogue is estimated using UV response of the approved PDE-5 inhibitor's standard [2,16]. However, this approach is justifiable only when the analogue and the reference standard possess an identical chromophore. It cannot be applied to the quantification of all analogues, as the recorded response could differ even for very similar compounds depending on the chromophoric group.

The present study focuses on the introduction of an alternative analytical method for the quantitative analysis of PDE-5 inhibitors or their analogues detected in counterfeit herbal supplements for erectile dysfunction. The method must meet the following requirements:

- allow quantitative analysis even for a small amount of sample (as most products are distributed as single-dose – one or two capsules),
- enable determination of the active compound content without available reference standards (as over 40 analogues have already been identified in supplements and novel structures continually appear, changing adulterants scene and making laboratories incapable of obtaining specific reference standards. Furthermore, they are often commercially unavailable)
- ensure appropriate sensitivity (to cope with samples adulterated with a mixture of low contents of several PDE-5 inhibitors or their analogues in a complex matrix).

For this purpose, the NMR spectroscopy may be applied. It provides full structural information, and allows quantification of novel adulterant at the same time. In addition, no reference standard is needed. The sensitivity of this technique is sufficient to quantify pharmaceutically relevant levels. For low content determination, it can be enhanced by applying specific NMR techniques (gradient shimming techniques, inverse or cryo probes, microcoil technology, increasing the number of scans) [17]. When a mixture of synthetic or natural compounds is analyzed, it is difficult to obtain clearly separated signals. However, the implementation of hyphenated techniques such as LC–MS–NMR [18] or the application of relaxation, diffusion and other NMR methods may reduce signal overlapping and unravel the spectra of complex mixtures [19]. Despite its evident advantages, due to their high cost, NMR systems with advanced technological solutions suitable for investigating adulterated herbal supplements are often beyond the means of a single laboratory. As an alternative, an HPLC method with a universal detector which responds to every component in the column effluent except for the mobile phase may be considered appropriate. This can be achieved with the following detectors: mass spectrometer (MS), refractive index detector (RI), and aerosol-based detectors (evaporative light scattering detector – ELSD, condensation nucleation light-scattering detector – CNLSD, chemiluminescent nitrogen detector – CLND, charged aerosol detector – CAD). Although MS is very sensitive and, it provides identification data, its response depends on the ability of the ionization in specified settings. RI suffers from limited sensitivity and is not compatible with gradient elution. Mass-dependent aerosol-based detectors, which generate a uniform response regardless of the structure, spectral and physicochemical properties are more appropriate [20,21]. These detectors enable a universal calibration and quantitative analysis without reference standards of investigated compounds. Nevertheless, a CLND system can be applied only for nitrogen-containing analytes and a diminished response can arise from compounds with adjacent nitrogen atoms. CLND is also incompatible with nitrogenous chromatographic solvents and additives (such as acetonitrile and triethylamine) [22]. Comparing ELSD, CNLSD and CAD systems, the latter is considered to be the most sensitive detector with the widest dynamic range (4 orders of magnitude) [23]. The response of CNLSD is more dependent on analyte nature than CAD. The signal variability is caused by water solubility differences between chemical compounds. Measurements recorded by CAD are more precise than those obtained using ELSD and are less affected by the aerosol droplet size and its size distribution, hence a linear calibration fits can be used for quantitation over a narrow concentration range [20,24]. Thus, the charged aerosol detector was chosen for this method development.

The application of CAD for PDE-5 inhibitors determination has not been reported, however, this detector appears to be suitable for such quantification, when the NMR technique cannot be used. Moreover, HPLC-CAD methods are compatible with MS/MS-TOF detection. Therefore, all observed peaks may be easily identified even when standards are not available. Operating principles of CAD involve a nebulization of the eluent using a flow of nitrogen, followed by an evaporation of solvents and other volatile compounds. An aerosol of analyte particles produced in the previous step is charged by the opposite stream of positively charged nitrogen that has passed through a high-voltage platinum wire – corona discharge source. In the last step, charged particle flow is measured by a sensitive electrometer. The weakness of all detectors employing an evaporation and nebulization processes is that only non-volatile or semi-volatile analytes can be detected and the response is dependent on the composition of the mobile phase (increasing detector response while the amount of organic modifier increases across the gradient, as it changes the efficiency of the analyte delivered to the detector). Different strategies of overcoming such difficulties have been proposed. Górecki et al. [25] developed an empirical method of the mobile-phase gradient compensation. The mobile phase composition entering the detector was equalized by introducing an inverse gradient which was mixed with the column eluent. Another solution presented in existing literature was applied to quantify unknown impurities. A unified calibration function, which was a mean of a series of compound calibration functions, was determined. Afterwards, changes of detector response during a gradient elution were compensated by generating a correction function for calculating calibration slopes specific for a certain retention time and a percentage of organic modifier at the elution time. Obtained slope-corrected calibration functions were used for calculating the results [26]. Stojanovic et al. [27] decided on employing a step gradient elution mode. Two isocratic steps (40% ACN and 80% ACN), in which analytes were detected at constant percentage of organic modifier, were separated by the gradient step.

The aim of this work was to develop an isocratic method (as this kind of elution is unaffected by response variations) for the determination of analogues of PDE-5 inhibitors without using their reference standards. Several authors proved the similarity of CAD response to different non-volatile compounds [26–31]. Calibration curves prepared for two easily accessible standards – sildenafil and tadalafil – would be employed for content calculations. Obtained peak area values for both standards and analogues would be recorded for the identical percentage of organic component in mobile phase.

2. Materials and methods

2.1. Reagents

Methanol and acetonitrile from Rathburn Chemicals Ltd. (Walkerburn, UK), formic acid from Park Scientific Limited