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Rapid detection and identification of counterfeit of adulterated products of synthetic phosphodiesterase type-5 inhibitors with an atmospheric solids analysis probe

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The market success of the three approved synthetic phosphodiesterase type-5 (PDE-5) inhibitors for the treatment of erectile dysfunction has led to an explosion in counterfeit versions of these drugs. In parallel a large market has developed for herbal products claimed to be natural alternatives to these synthetic drugs. The herbal products are heavily advertised on the internet and are freely available to purchase without prescription. Furthermore, adulteration of these supposed natural medicines is a very common and serious phenomenon. Recent reports have shown that the adulteration has extended to the analogues of the three approved synthetic PDE-5 inhibitors.

An Atmospheric Solids Analysis Probe (ASAP) was used for the direct analysis of the counterfeit pharmaceuticals and herbal products. Using the ASAP combined with time-of-flight mass spectrometry (TOF MS) it was possible to detect fraudulent counterfeit tablets. The physical appearance of the pills resembled the pills from the original manufacturer but contained the wrong active pharmaceutical ingredient (API). Detecting adulteration for five herbal supplements marketed as natural alternatives to PDE-5 inhibitors was also possible using the ASAP. Three types of adulteration were found in the five samples: adulteration with tadalafil or sildenafil, mixed adulteration (tadalafil and sildenafil), and adulteration with analogues of these drugs.

Keywords: phosphodiesterase type-5 inhibitors; UPLC; MS/MS; sildenafil citrate; vardenafil hydrochloride; tadalafil; counterfeit; adulterated; atmospheric pressure; ASAP

Introduction

Pharmaceutical counterfeiting is a global phenomenon and the number of detected cases continues to grow.[1–4] The Center for Medicine in the Public Interest predicts that counterfeit medicine sales will reach approx. €55.5 billion globally by 2010.[4] The market success of the three approved synthetic phosphodiesterase type-5 (PDE-5) inhibitors for the treatment of erectile dysfunction (ED) has made them a prime target for counterfeiting and caused a corresponding explosion in the number of detected cases. The chemical structures of these compounds (sildenafil citrate, vardenafil hydrochloride and tadalafil) are shown in Fig. 1.

In addition to the reported detection of counterfeit tablet forms of these products, herbal dietary supplements (HDS) that are claimed to be entirely natural alternatives to the PDE-5 inhibitors are advertised on the internet. Recently, there have been reports that these supposed 'natural alternatives' to the drugs used to treat ED have been illicitly adulterated with approved synthetic products, or their structurally modified analogues.[5–15] When an HDS is labeled as natural, the underlying implication is that buyers will believe that it is safer. Given that the HDS may contain undeclared synthetic drugs and can be obtained freely over the internet without prescription, there is a genuine threat to public health. There is a need for a rapid and effective screening method to confirm the identity of the dosed active ingredient to ensure patient safety. For the legal manufacturer of these products a method that is able to quickly profile these 'counterfeit medicines' and HDS would confirm the integrity of their own product in relation to the counterfeit.

In the pharmaceutical industry the high selectivity of liquid chromatography and liquid chromatography hyphenated with mass spectrometry (LC/MS) and nuclear magnetic resonance (NMR) have become techniques of choice for profiling and quantification of pharmaceutical compounds and their impurities. However, the time taken for sample extraction and chromatographic separation by LC/MS and gas chromatography (GC)/MS can create a bottleneck. In recent years the use of novel ambient desorption ionisation techniques for surface analysis of solid and liquid samples with subsequent MS detection have been reported.[16–18] These ambient techniques are faster because sample preparation is often minimal or unnecessary. The total analysis time can be decreased significantly due to the elimination of the chromatographic separation allowing remarkably high sample throughput. These techniques include desorption electrospray ionisation[16] (DESI), direct analysis in real time[17] (DART) and atmospheric solids analysis probe[18]

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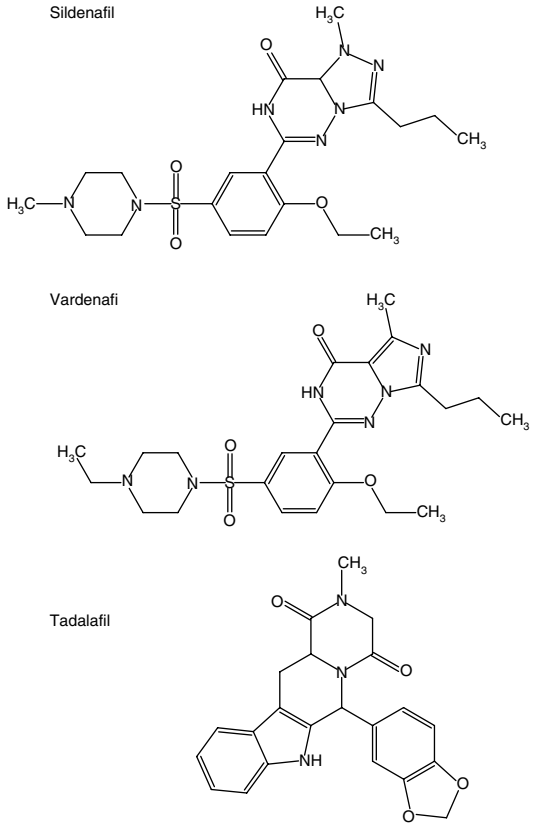

Figure 1. Structures of tadalafil, sildenafil and vardenafil.

(ASAP). In DESI, which is related to electrospray ionisation (ESI), a charged mist of liquid is directed at a surface onto which a sample has been deposited. Gaseous ions from the sample are generated by the impact of the charged particles on the surface. The ions are detected by MS at ambient pressure and DESI can be used on untreated surfaces.[19] DESI has been interfaced with various mass spectrometers and used in the analysis of counterfeit drugs,[20–22] forensic samples,[23] pharmaceuticals,[24,25] and explosives.[26]

DART is related to atmospheric pressure chemical ionisation (APcI) and employs an electrical discharge to create a plasma. Reactive ionising species are produced from metastable gas ions such as He and N_2, reacting with ambient water, oxygen or other atmospheric components to produce hydronium ions. Protons are then transferred to the analyte molecules. The DART technology has been used in conjunction with MS for a wide range of polar and non-polar compounds on various surface types,[17] analysis of counterfeit antimalarials,[20] reaction monitoring in drug discovery,[27] and quantification of drugs in biological matrices.[28]

The Atmospheric Pressure Solids Analysis Probe (ASAP) was invented by McEwen *et al*. and can be used to analyse volatile or semi-volatile solid or liquid samples using atmospheric pressure ionisation[18] (API). The sample is loaded onto a glass melting point capillary and inserted into a heated stream of gas (100–500 °C) which vaporises the sample. A corona discharge is used for ionisation (Fig. 2). The probe is fitted to an API source by replacing the ESI or APcI probe and installing a corona pin. The probe consists of two parts, an outer assembly and an inner probe, which holds the melting point capillary securely in place. Once the probe is installed the sample is completely enclosed within the source.

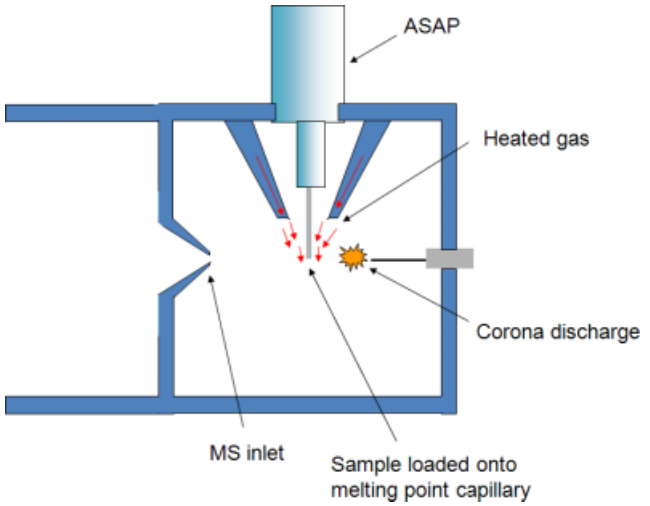

Figure 2. Illustration of ionisation using ASAP. Reproduced with the permission of the American Chemical Society from S. Yu, E. Crawford, J. Tice, B. Musselman, J. Y. Wu, *Anal. Chem*. **2009**, *81*, 193.

In this paper we present the use of an ASAP for the rapid determination of synthetic phosphodiesterase type-5 (PDE-5) inhibitors in counterfeit tablet samples and adulterated herbal supplements.

Experimental

Sample procurement

Authentic brand sildenafil citrate, vardenafil hydrochloride and tadalafil tablets were obtained from reputable pharmaceutical wholesalers for comparison with the presumed counterfeit samples.

Counterfeit tablet samples

Imitation 'brand' and 'generic' samples of these drugs were obtained from internet pharmacies. There are currently no approved generic equivalents for the three synthetic PDE-5 inhibitors on the market.

The samples arrived with customs declarations that did not reflect the contents of the packages. Some samples were labeled as 'natural foods', 'candy' or 'gifts'. A few of the samples were packaged using boxes and logos that appeared to come from the originator. Twenty-one products were obtained either from outside of continental USA or from online pharmacies, all without prescription. Patient information leaflets were present in only 7 out of 21 packages. If present only two packages did not have spelling and other typographical mistakes in them. Only one of the tablet samples purchased from the internet pharmacies were packaged to look like authentic sildenafil citrate, vardenafil hydrochloride and tadalafil tablets from the authorised manufacturers.

Herbal dietary supplements (HDS)

Five products were obtained over the internet and analysed using the ASAP with high-resolution mass spectrometry (MS). Four samples with capsules and one sample with tablet supplement were purchased. All five were found to be adulterated, containing one or both of sildenafil and tadalafil, or else analogues of these drugs, none of which were declared on the box or in the enclosed information.

Mass spectrometry conditions

Mass spectrometric analysis was performed on a Waters® Micromass® LCT Premier™ XE orthogonal time-of-flight mass spectrometer or on a Xevo quadrupole time-of-flight (QTOF) mass spectrometer. The instruments were operated in combined ESI/APcI mode, named ESci, which alternates rapidly between ESI and APcI, thus keeping the data from each mode discrete. This enabled the acquisition of analyte data in APcI mode and reference data in ESI mode using a capillary voltage (ESI) of 3.0 kV with a corona current (APcI) of 5 µA. The source conditions were optimised to use a cone voltage of 40 V, desolvation temperature of 100–500 °C, desolvation gas 500 L/h and a source temperature of 120 °C. The MS data was acquired over a range of 100–1000 *m/z* with an acquisition of 0.5 s, using leucine enkephalin as the lock mass reference (ESI), for a period of 1 min per sample. A collision energy ramp from 35–55 eV was used. The unbiased mode of fragment ion acquisition, called 'MS to the E' (MS^E), allowed the simultaneous acquisition of MS and product ion fragmentation data. The MS^E mode of acquisition has further advantages in providing quantitative data and requires no pre-knowledge of the precursor ions.[29]

Sample preparation and loading

The tablet samples were loaded onto the glass capillary by first exposing the inside of the tablet and making physical contact between the capillary and the inner pill. A separate capillary was used for each sample and therefore no contamination of the samples was possible.

The herbal supplement samples were predominantly capsules containing fine powder. The solid probe tip was brushed against the herbal products then the tip wiped clean with a lint-free tissue before insertion into the source for a data measurement. Once the sample had been applied to the glass capillary, excess was then removed using a stream of nitrogen. Between each measurement a blank sample was run to ensure that there was no between-sample contamination in the instrument.

Results and Discussion

Sildenafil citrate, vardenafil hydrochloride and tadalafil tablet samples

The positive ion solid probe MS data acquired from the thirteen samples of legitimate products and the internet pharmacy samples are shown in Fig. 3. Here we can see that the data resembles a LC/MS chromatogram, with the thermal desorption of the tablet from the glass capillary giving rise to a Gaussian-shaped peak. The first three peaks (1–3) in Fig. 3 represent the authentic pharmaceutical products. The peaks labeled 4–13 were obtained from the analysis of the internet-purchased PDE-5 pharmaceuticals. Even in manual mode, data from thirteen samples can be acquired in less than 20 min. The mass spectra for the authentic, legitimate sildenafil, tadalafil and vardenafil are shown in Fig. 4. Here we can see that the sildenifil sample gave rise to a major peak at *m/z* 475.2128 corresponding to an elemental composition of $C_{22}H_{30}N_6O_4S$ and a fragment ion at *m/z* 410.2482. The tadalafil sample gave a peak with an *m/z* value of 390.1449 corresponding to $C_{22}H_{20}N_3O_4$ and the vardenafil sample gave a peak with an *m/z* value of 489.2289 corresponding to $C_{23}H_{33}N_6O_4S$. All mass measurements were within 1 ppm. Although the visual

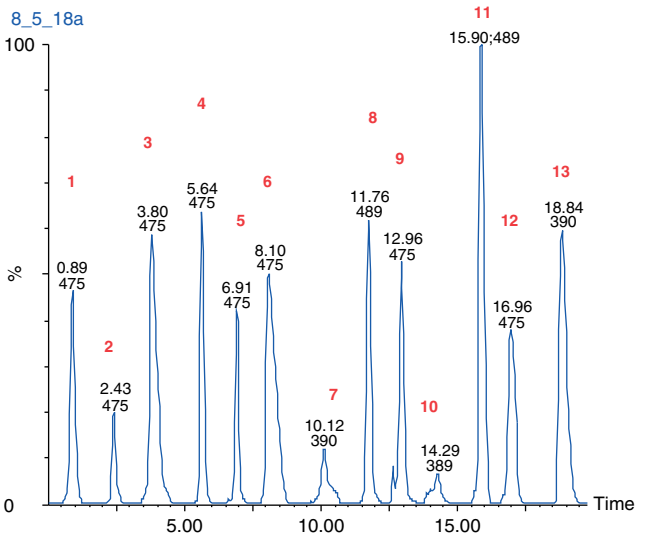

Figure 3. ASAP sample vaporisation profiles (desolvation temperature is ramped from 100–450 °C) for thirteen PDE-5 tablet samples obtained over the internet and authentic products obtained from reputable sources. Peak 1, genuine brand sildenafil; 2, internet pharmacy 'brand' sildenafil; 3, internet pharmacy A 'brand' sildenafil; 4, internet pharmacy A 'brand' vardenafil; 5, internet pharmacy A 'brand' tadalafil; 6, 'generic' sildenafil; 7, genuine brand tadalafil; 8, genuine brand vardenafil; 9, 'generic' sildenafil; 10, 'generic' tadalafil; 11, 'generic' vardenafil; 12, 'generic' sildenafil; 13, 'generic' tadalafil.

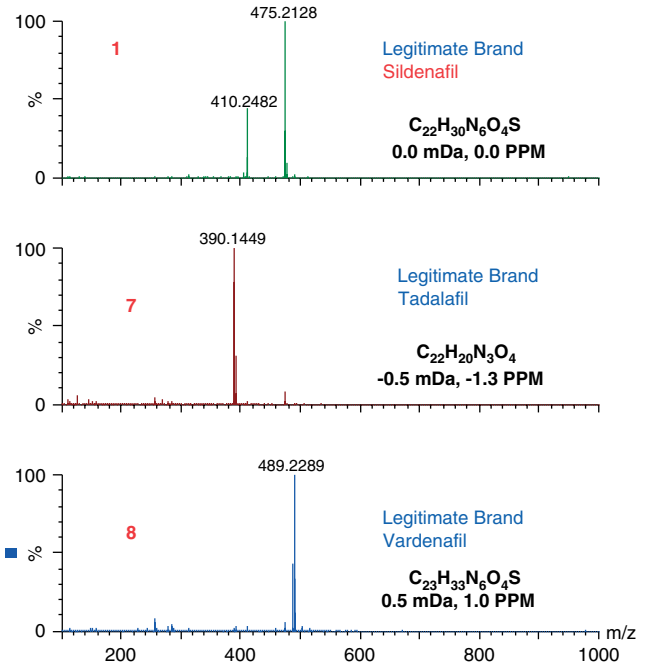

Figure 4. Elemental composition results for legitimate sildenafil, tadalafil and vardenafil.

appearance of the counterfeit tablets conformed to that of the genuine medicine in terms of color, size, shape and mass, when analysed using the ASAP the MS results indicated that they were clearly different. The sildenafil citrate tablets did indeed contain the correct active pharmaceutical ingredient (*m/z* 475.2128), but those labeled to contain vardenafil (*m/z* 489.2284) and tadalafil (*m/z* 390.1454) samples did not (Fig. 5). The vardenafil and tadalafil

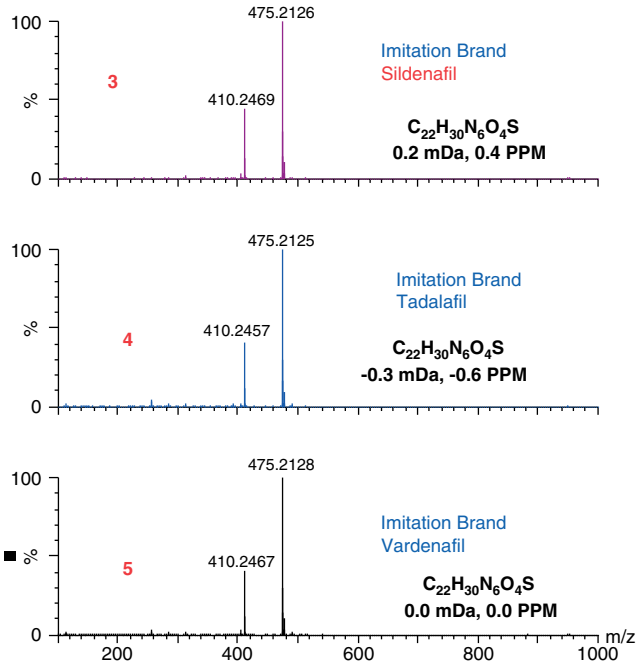

Figure 5. Elemental composition results for imitation brand sildenafil, tadalafil and vardenafil.

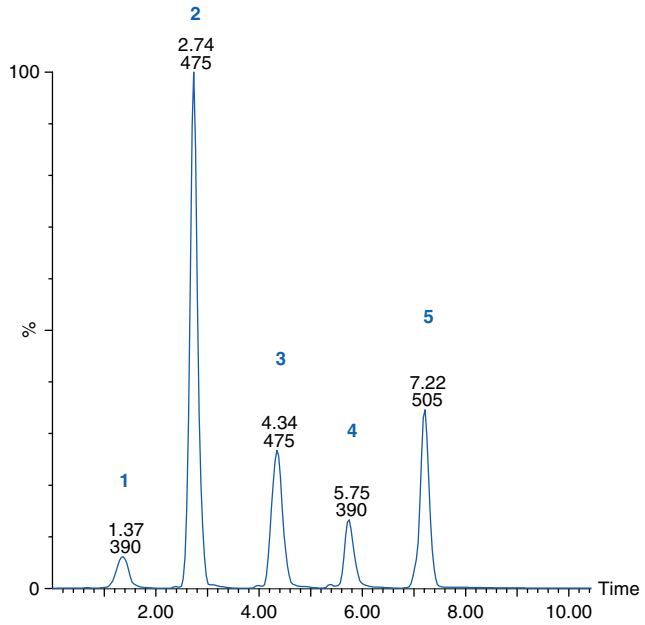

Figure 6. Vaporisation profiles of the five HDS samples.

Table 1. Comparison of the ASAP and LC/MS approaches for the analysis of counterfeit tablet samples

Activity	Time taken ASAP	Time taken LC/MS/MS
Tablet dissolving	10 min	10 min
Filtering	NA	2 min/per sample
Data acquisition	1 min/per sample	12 min
Spectra for review	1 min/per sample	12 min/per sample
Isomer resolution	No	Yes
Sensitivity	≈ 500 ng/mL	<1 ng/mL

samples appeared to contain sildenafil in addition to the claimed active ingredient, as the spectrum showed a peak with an *m/z* ratio of 475.2128 (see Fig. 5).

This study highlights the risks a consumer takes when purchasing drugs from uncertified internet pharmacies. Results obtained from the TOF MS data, LC/MS/MS data and PDA spectral comparisons agree with the data obtained by ASAP for all of the samples analysed (data not shown). The data in Table 1 compares the analysis time and sample preparation time of the ASAP approach with that of standard LC/MS. Here we can see that there is a significant time saving from using the ASAP approach; there is no need to filter the tablet sample and the analysis time is significantly reduced, by a factor of 90%. However, with this approach it is not possible to distinguish isomers and the level of sensitivity is not that which is achieved by LC/MS.

Adulteration of HDS samples with synthetic PDE-5 inhibitors

Herbal or natural remedies are often considered by the general public to be 'safe' because they are derived from naturally occurring plants or minerals. Indeed some of the drugs we take today are derived from these natural products. Aspirin, acetylsalicylic acid, had its origin in the use of willow bark to treat fever and pain. Digoxin is derived from herbaceous perennials, shrubs, and biennials that are commonly called foxgloves. Some of these herbal remedies are marketed to people who cannot take a particular pharmaceutical medication due to contraindications. These can often be sold for many hundreds of dollars a sample. There have been many recent reports of these natural treatments being adulterated with actual pharmaceutical compounds and hence these present a real risk to human health.[30–33] The analysis of herbal supplements for non-original products is normally performed by a hyphenated chromatography spectroscopic or spectrometric technique such as LC/UV or GC/MS. To investigate the applicability of the ASAP probe for the analysis of natural products, five herbal products purchased on the internet were analysed using the ASAP with TOF MS detection.

The samples were analysed as before using positive ion ESCI (ESCI being the combination of both APCI and ESI on the same MS probe) and the direct analysis solids probe. The results produced are displayed in Fig. 6. All five of the herbal supplements were adulterated with tadalafil and sildenafil or suspected analogues.

Of the five herbal supplements tested, sample 1 was found to be adulterated with tadalafil (*m/z* 390), as indicated by the data in spectrum 1 in Fig. 7. According to the accompanying literature, this sample declared the presence of many natural ingredients including, *Dioscorea spinosina* (wolfberry fruit), *Glycyrrhiza glabra* (liquorice root) and others. Neither the patient information nor the packaging declared the presence of tadalafil.

In each case the principle component identified in the samples agreed with that determined when the same HDS samples were analysed by ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) with simultaneous ultraviolet (UV) detection. Sample 2 was adulterated with sildenafil and interestingly shared the same product name as sample 1 but shipped from a different geographical location (Europe and Asia, respectively).

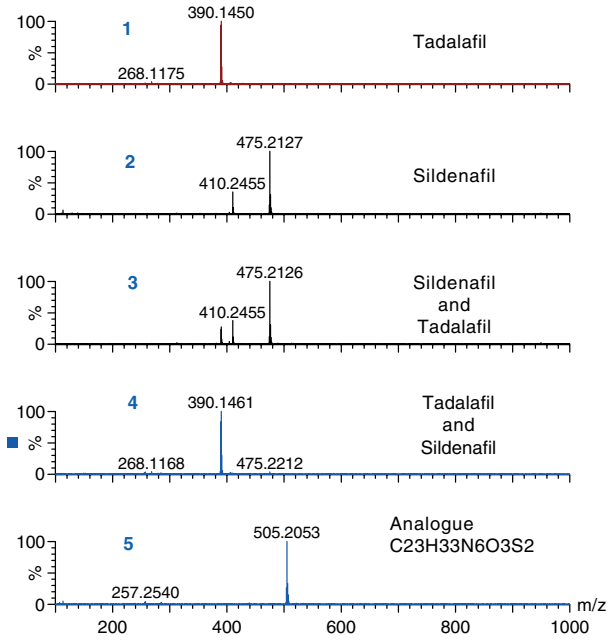

Figure 7. Spectra from the direct analysis of the five HDS samples.

Samples 3 and 4 were found to have been subject to adulteration with two or more pharmaceutical compounds. Sample 3 was adulterated with tadalafil and an even higher level of sildenafil. Sample 4 was found to contain tadalafil as the major adulterant with less sildenafil. Quantitation of the samples with LC/MS/MS using multiple reaction monitoring (MRM) detection revealed that the doses of the sildenafil and tadalafil are sufficiently high to be therapeutic. In all cases with samples 1–4 the accurate mass MS data acquired was within 2 mDa of that of an authentic standard.

The accompanying literature for sample 5 also declared it to be entirely natural, stating that the ingredients had helped to support 'male performance' for centuries. It claimed to contain wild yam extract, Siberia ginseng extract, jujube extract and cayenne extract, amongst others. In sample 5, the ASAP in combination with accurate mass MS detection identified the presence of a suspected PDE-5 analogue, with suggested elemental composition of $C_{23}H_{33}N_6O_3S$ (−0.3 mDa, −0.6 ppm). It is hypothesised that this analogue is thiohomosildenafil (*m/z* 505.2056) where an oxygen is substituted with sulfur and an ethyl group replaces the methyl group attached to the piperazinyl nitrogen.[13]

Figure 8 shows the spectra from the ASAP analysis of sildenafil drug substance and *m/z* 505.2056 from sample 5. An MS^E experiment was performed on the Waters Xevo QTOF. In this way MS information (low collision energy function) is collected in addition to fragmentation information induced by the high collision energy function. Major fragments observed for *m/z* 505.2060 were *m/z* 113.1072, 299.0968, 327.1280 and 393.1055. These fragments have been reported in certain analogues including thiohomosildenafil in the literature.[12–15]

The major advantage of the ASAP is that it requires no knowledge of chromatography and requires no complicated sample preparation. It is easy to use and the results can be obtained in a matter of minutes from sample receipt. Although the current process is a manual one, best suited to the analysis of a few samples quickly, it could easily be automated to provide

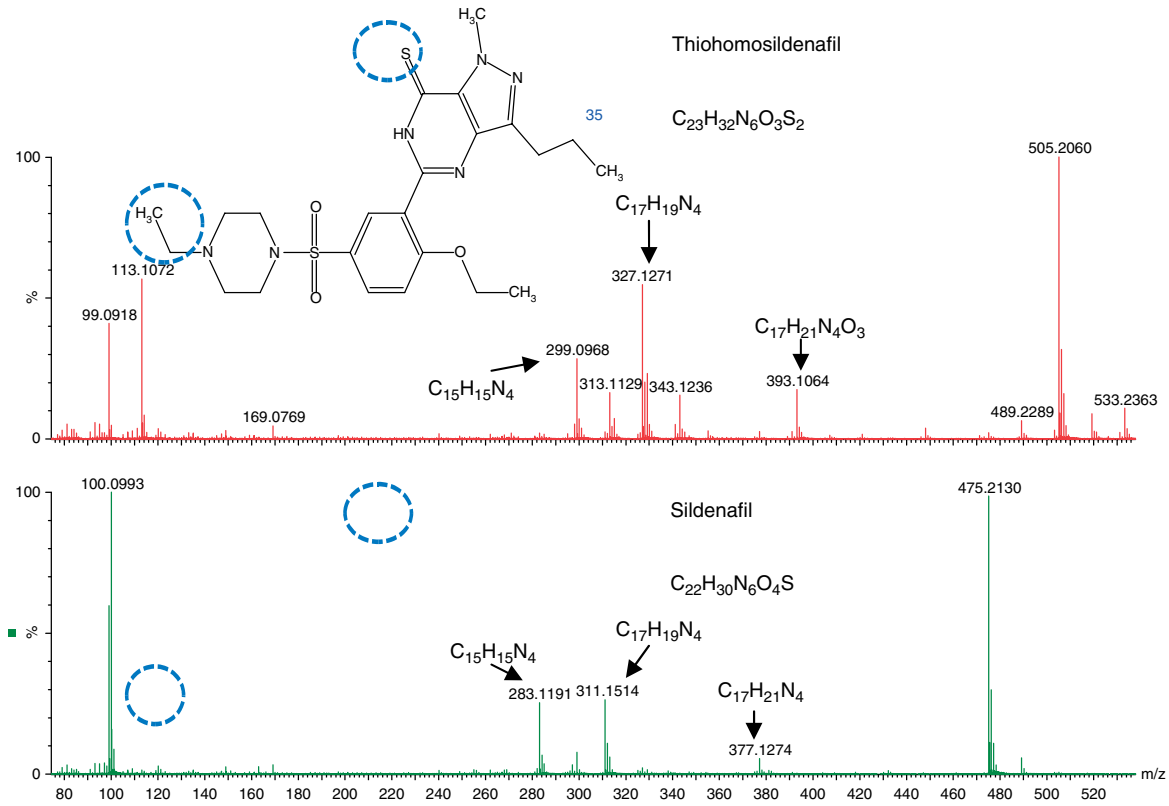

Figure 8. Spectra from the direct analysis of sample 5 (top) using a high collision energy experiment on the Waters Xevo QTOF mass spectrometer. Spectrum for sildenafil standard is also shown (bottom).

a rapid throughput analysis platform. In certain analyses, such as drug screening or sports doping, it is sometimes sufficient to get a yes/no answer from the analysis before the sample is passed for further characterisation. A secondary benefit of using the ASAP is that the long acquisitions times on the mass spectrometer allow for excellent sampling statistics and hence a mass accuracy between 0.2 and 0.5 ppm can be achieved across the compounds tested.

Conclusion

The counterfeiting of pharmaceuticals and adulteration of herbal dietary supplements with synthetic pharmaceuticals has been identified as a growing problem, with the number of detected cases rising every year. This has created a demand for high-throughput screening techniques.

The ASAP provides an appropriate technique because it does not require sample extraction or chromatographic separation. The ASAP can be applied to a sample with ingredients that are sufficiently volatile. When used in conjunction with TOF MS or MS/MS, the ASAP can rapidly detect and identify unknown compounds using exact mass measurement and elemental composition determination. Using ASAP sampling the turnaround time from sample receipt to structural identification and unknown compound determination for this application is accelerated. Chromatographic separation and sample extraction were not required to determine the results in this case. In a previous study, analysis of these samples was carried out by LC/MS; sample extraction using methanol/water followed by centrifugation was required to extract the tablets adding a sample processing time of 30 min to the experiment (data not shown). The absence of chromatographic separation does however have drawbacks: there is the possibility of ionisation suppression when a sample is presented to the source without prior LC separation. When an LC system is used multiple detectors are possible to add orthogonal information, e.g. MS, UV or evaporative light scattering detection (ELSD). Other concerns include the inability to detect non-volatile components in the sample.

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