A COMPREHENSIVE UPLC-MS(/MS) BASED APPROACH FOR SCREENING AND CONFIRMATION OF ILLEGALLY ADDED ERECTILE DYSFUNCTION (ED) DRUGS IN NATURAL HEALTH PRODUCTS.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Authors: Salman Azimi[1], Nayan Mistry[2] and Michelle Wood[2]
Affiliations: [1]Drug Quality Control Laboratory, Supreme Council of Health, Doha, Qatar, [2]Waters Corporation, Atlas Park, Wythenshawe, Manchester, M22 5PP, UK.

INTRODUCTION

The adulteration of herbal/dietary supplements with erectile dysfunction (ED) drugs and their analogues is reported worldwide and is an increasing problem[1]. The sale of so-called 100%, 'all-natural' products has become a highly profitable business for online pharmacies, however these products can pose a serious threat to consumers owing to the undisclosed presence of approved/ prescription drugs or the unknown safety and toxicity profile of unapproved ED drugs.

The Drug QC Laboratory, Qatar, has been involved in the testing of adulterated and counterfeit products for a number of years. Each year brings countless new warnings and alerts over the adulteration of products which are illegally advertised for the enhancement of male sexual performance. Consequently the identification of ED drugs and their analogues in these products is of great interest.

OBJECTIVE

The aim of this study was to develop a comprehensive method for screening, confirmation and quantification of illegally added ED drugs, and their analogues in herbal and dietary products that are marketed to improve male sexual performance, and imported to Qatar.

Figure 1. Waters ACQUITY UPLC and the TQ Detector

METHODS

Instrumentation

Waters® ACQUITY UPLC® system in combination with the TQ Detector Mass Spectrometer (Figure 1).

UPLC conditions

Column:	Waters HSS C18 2.1 x 100mm, 1.8µm
Column Temp:	45 °C
Injection Volume:	10 µL
Mobile Phase A:	3 mM Ammonium Formate pH 2.9
Mobile Phase B:	Acetonitrile with 0.1% Formic acid
Gradient elution:	20-98 % B
Flow Rate:	0.35 mL/min

MS conditions

Source Temp:	150 °C
Desolvation Temp:	375 °C
Desolvation Gas:	700 L/hr
Screening Analysis:	Full scan MS in ESI+ (*m/z* 55–680)
Cone voltages:	20 V, 40, 60, 80 and 100 V
	Full scan MS in ESI- (*m/z* 200-680)
Cone voltage:	105 V
Confirmation Analysis:	MRM analysis - quantifier and qualifier transitions monitored

RESULTS AND DISCUSSION

SCREENING: A spectral library for 32 compounds including 28 ED drugs and their analogues was prepared. Owing to recent reports of increased availability of 'all-in-one'/'combination' herbal products[2], we included the naturally-occurring substances Icariin and yohimbine, the synthetic, dapoxetine (used for premature ejaculation) and testosterone. The library was created according to a previously-described approach[3] *i.e.,* UPLC was used in conjunction with full scan MS analysis, which was performed under multiple cone voltage conditions (in-source collision–induced dissociation: CID), to generate both spectral and retention time (RT) information (Figure 2).

ChromaLynx™ application manager was used for data processing; ChromaLynx automatically examines the chromatograms produced at each cone voltage, detects the components and calculates the average spectral match factor (MF) against the library[4]. Screening in both ESI+ and ESI- modes, under multiple cone voltage conditions, along with RT provides high confidence in the identification.

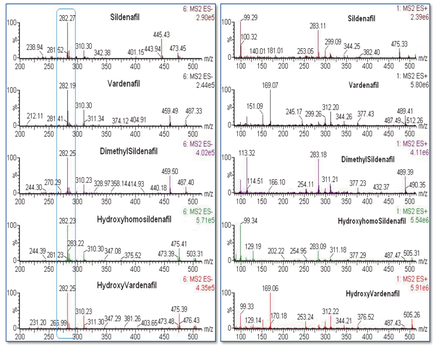

*Figure 2. Full mass spectra of five drugs, MS scans in ESI+ at cone voltage 100 V (**right**) and in ESI- at 105 V (**left**). Note the fragment ion of m/z 282 (ESI-) which is common to all analogues of sildenafil and vardenafil.*

Keeping abreast of emerging ED analogues is challenging. Previously, precursor ion scanning of *m/z* 283 (in ESI+ mode) has been described for screening for potential unknown analogues of sildenafil and vardenafil[5], however this product ion is not so abundant in vardenafil and its designer drugs[6]. Consequently, we proposed inclusion of an extra scanning function, performed simultaneously in negative ionisation mode at high energy. Under these conditions, a characteristic fragmentation pattern was observed for sildenafil, acetildenafil and vardenafil and their respective analogues (Figure 2 and Figure 3). Similarly, under these conditions, tadalafil and its respective analogues demonstrates a common fragment at *m/z* 232 (data not shown).

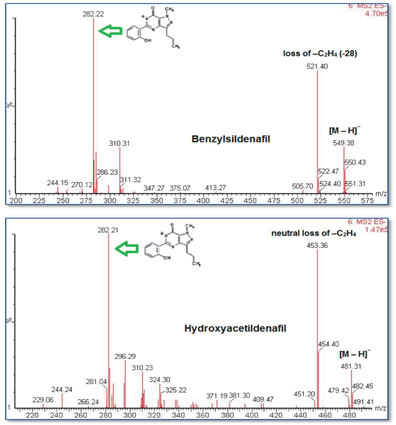

Figure 3. Screening for the 'unknowns', MS scan in ESI- (cone voltage 105 V) leads to the formation of parent ion [M – H]- with the loss of –C_2H_4 (-28) and formation of the common fragment ion i.e., m/z 282 (common in all analogues of sildenafil, vardenafil and acetildenafil).

CONFIRMATION: For subsequent quantitative analysis, a MRM method was also developed and validated for three alternative matrices *i.e.,* capsules/tablets/pills; honey and herbal drink. Calibration curves were constructed over the range of 0.2 – 1000 ng/mL. The coefficient of determination (R^2) for all compounds in this study was ≥ 0.995.

The precision, measured as coefficient of variation (% CV), was < 11% at 2 ng/mL for 26 compounds and < 10% at 10 ng/mL and 100 ng/mL concentrations for all compounds when the standard mix solutions were spiked into herbal matrices. The limit of quantification (LOQ) was ≤ 1.0 ng/mL for 29 compounds based on a signal-to-noise ratio of ≥ 10:1 for both quantifier and qualifier ions.

Figure 4. Photographs of six positive samples. Forty-three suspected samples were analyzed in the study; 18 were found to be adulterated with ED drugs.

The developed method was applied to 43 suspected samples, 18 of which were found to be adulterated with ED analogues (Figure 4, Table 1). Two samples that screened positive for thiodimethylsildenafil also gave matches for thiohomosildenafil, however chromatographic separation permitted clear differentiation between these two isobaric substances. In the same sample, sildenafil and dimethylsildenafil were also detected as minor compounds due to the hydrolysis of thio analogues of sildenafil (*e.g.,* thiosildenafil ⟶ sildenafil)[7].

Sample	Candidates	RT Sample	RT Actual	RT Match	Avg. Match Factor	Status	Amount
Royal Honey [7]	Thiosildenafil	8.10	8.11	✓	786	+	4 mg/pack
	Thiodimethylsildenafil	8.50	8.50	✓	835	+	65 mg/pack
	Thiohomosildenafil	8.50	8.35	*	758	–	X
Yunna 500mg Capsules [7]	Thiosildenafil	8.09	8.11	✓	828	+	48 mg/cap
	Thiodimethylsildenafil	8.47	8.50	✓	858	+	20 mg/cap
	Thiohomosildenafil	8.47	8.35	*	809	–	X
Chinese Pills	Sildenafil	5.62	5.65	✓	860	+	27 mg/pill
Cialis 20mg (counterfeit)	Sildenafil	5.64	5.65	✓	854	+	42 mg/tab
	Tadalafil	7.25	7.28	✓	830	+	11 mg/tab
Unknown Blue Tablets	Homosildenafil	6.07	5.83	*	776	–	X
	Dimethylsildenafil	6.07	6.05	✓	832	+	81 mg/tab
SATIBO	Sildenafil	5.62	5.65	✓	869	+	67 mg/cap
Roman Viagra (black)	Sildenafil	5.68	5.65	✓	882	+	117 mg/tab
Unknown Tablets	Aminotadalafil	6.36	6.38	✓	791	+	18 mg/tab
Korean Royal Jelly	Sildenafil	5.63	5.65	✓	824	+	6 mg/gm
MAXMAN Capsule	Sildenafil	5.66	5.65	✓	876	+	108 mg/cap

Table 1. Summary of results for ten adulterated herbal/dietary samples. The screening results, including spectral match factors, RT data and final screening status (+ = positive or - = negative) are presented, in addition to the quantitative data from the subsequent confirmatory analysis.

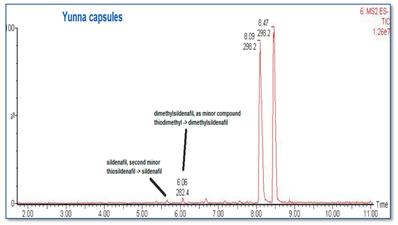

Figure 5. TIC of seized sample, thiosildenafil and thiodimethylsildenafil as major compounds; producing minor compounds sildenafil and dimethylsildenafil after the hydrolysis of thiocarbonyl group to a carbonyl group (C = S ⟶ C = O).

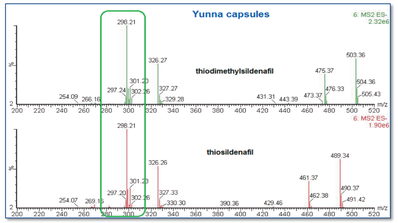

Figure 6. ESI negative spectra of seized sample (Figure 5), formation of common fragment ion m/z 298 due to the presence of thiocarbonyl group in thio analogous of sildenafil.

CONCLUSION

- **We have developed a novel screening method that is suitable for both the detection of known and unknown ED drugs and analogues. This is the first description of a single analytical method with this capability.**
- **Full scan data is collected simultaneously in both ESI negative and ESI positive modes, under multiple energy conditions, yielding comprehensive spectral data which are automatically compared to a prepared library of known drugs.**
- **The high energy fragmentation patterns generated in negative ESI mode are used specifically to facilitate identification of new and currently unknown analogues of ED drugs.**
- **Furthermore, a quantitative confirmatory method for 32 compounds including 28 ED drugs and their analogues has been developed and validated. This UPLC-MS/MS method is sensitive, accurate and demonstrates excellent linearity.**
- **These procedures have been applied to the analysis of 43 samples received by our laboratory between the periods 10/2010 – 08/2011. Eighteen samples were found to contain unauthorised substances.**

References

1. Tainted Sexual Enhancement Products, www.fda.gov, last accessed 25th January 2012
2. J Dean, R Klep and J Aquilina; Counterfeit dapoxetine sold on the internet contains undisclosed sildenafil. Int. J. Clin Pract.64 (9) (2010)
3. L Humbert, F Grisel, C Richeval and M Lhermitte. Screening of xenobiotics by ultra-performance liquid chromatography-mass spectrometry using in-source fragmentation at increasing cone voltages: library constitution and an evaluation of spectral stability. Journal of Analytical Toxicology 34 (2010)
4. R Lee, M Roberts, A Paccou and M Wood. Development of a new UPLC-MS method for systematic toxicological analysis. Waters Corp. Application Note 720029058EN (2009)
5. M. Dusek, M Sanda, P Culra and S Barsova; Silmultaneous LC-MS/MS determination of sildenafil and related analogues added illegally to herbal products intended for the treatment of erectile dysfunction. 3rd Int Symposium on Recent Advances in Food Analysis, Prague, November 7-9, (2007)
6. CS Ng, TY Law, YK Cheung, P Ng and KK Choi; Development of a screening method for the detection of analogues of sildenafil and vardenafil by the use of liquid chromatography coupled with triple quadrupole linear ion trap mass spectrometer. Anal. Methods 2 (2010)
7. JC Reepmeyer and DA D'Avignon; Use of a hydrolytic procedure and spectrometric methods in the structure elucidation of a thiocarbonyl analogue of sildenafil detected as an adulterant in an over-the-counter herbal aphrodisiac. J. AOAC Int. 92 (2009)
8. H Lee; BJ Lee; A novel approach to simultaneous screening and confirmation of regulated pharmaceutical compounds in dietary supplements by LC/MS/MS with an information-dependent acquisition method. Food Additives and Contaminants 28 (2011)