UPLC-MS/MS for the Screening, Confirmation and Quantification of 32 Drugs illegally added to Herbal/Dietary Supplements for the Enhancement of Male Sexual Performance

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Figure-5: Photographs of six positive samples. Forty-three suspected samples were analyzed in this study; 18 were found to be adulterated with ED drugs.

ABSTRACT

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 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. A spectral library for 32 compounds was generated. In addition, a screening protocol was suggested which permitted the detection of new, unknown ED analogues by generation of common fragmented ions. In parallel, a highly sensitive and selective MS/MS method was developed for confirmation and quantification using two multiple reaction monitoring (MRM) transitions for each compound.

INTRODUCTION

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.

We present a simultaneous procedure for screening of a wide range of known ED drugs and their analogues, together with a protocol for the detection of new, unknown ED analogues. To the best of our knowledge, this is the first description of this capability in a single analytical procedure. A description of the method is provided in addition to a confirmatory method based on MRM analysis.

MATERIALS & METHODS

MS Conditions

MS System:	Waters TQ Detector
Capillary Voltage:	3.0 kV
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 Voltage; 20V, 40V, 60V, 80V, 100V) Full scan MS in ESI- *m/z* 200 – 680 (Cone Voltage; 105V)
Confirmation Analysis:	MRM (see Table 2 for transitions and parameters)

Inlet Conditions

Inlet System:	Waters Acquity UPLC
Column:	Waters HSS C18 2.1 x 100mm, 1.8µm
Column Temp:	45 °C
Sample Temp:	8 °C
Weak Wash:	20:80 = Acetonitrile/Water with 0.1% Formic acid
Strong Wash:	80:20 = Acetonitrile/Water
Injection Volume:	10 µL
Mobile Phase A:	3 mM Ammonium Formate pH 2.9 ±0.03, using Formic Acid
Mobile Phase B:	Acetonitrile with 0.1% Formic acid
Flow Rate:	0.35 mL/min
Gradient Elution:	See Table-1

Time (min)	MPA (%)	MPB (%)
Initial	80.0	20.0
2.10	80.0	20.0
6.50	60.0	40.0
9.50	40.0	60.0
11.00	40.0	60.0
11.30	2.0	98.0
12.80	2.0	98.0
13.00	80.0	20.0
15.00	80.0	20.0

Table-1: UPLC Gradient

Sample Preparation

Sample powder, equivalent to one capsule or tablet/pill was weighed (in case of honey, 2g was used) and transferred to a 20mL volumetric flask. Fifteen millilitres of methanol/water (75:25) was added and the sample was sonicated for 20 min., the mixture was then made up to its 20mL graduated mark with methanol/water (75:25). The mixture was shaken for 5 min. and then centrifuged at 4000 rpm for 8 min. Samples were then filtered using a 0.45 µm PVDF syringe filter.

The filtered samples were diluted using water with 0.1% formic acid/ACN (70:30). For screening analysis a 50-fold dilution was used; for the confirmation analysis, samples were further diluted 400-fold to 1000-fold range. Liquid samples *e.g.*, herb drinks, juices and oral sprays were simply diluted 50-fold and 400 to 1000-fold respectively.

Calibration Solutions for MS/MS Confirmation and Quantification

Mixed calibrators comprising 32 reference compounds (0.2 ng/mL – 1000 ng/mL) were prepared both in the presence and absence of matrix. Three different matrices were evaluated *i.e.*, capsules/tablets/pills; honey and herbal drink. For an assessment of precision (%CV), mixed solutions of 2 ng/mL, 10 ng/mL and 100 ng/mL in herbal matrices were prepared.

RESULTS & DISCUSSION

For the purpose of screening , a spectral library for known ED drugs and analogues was prepared. Owing to recent reports of increased availability of 'all-in-one'/ 'combination' herbal products[2], we also added 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-1).

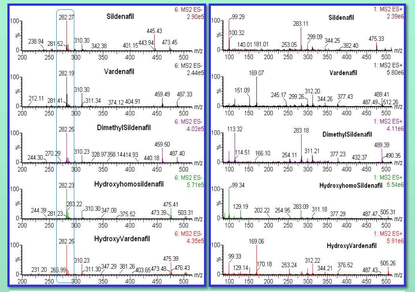

Figure-1: Mass spectra of five drugs, MS scans in ESI positive at cone voltage 100V (right) and in ESI negative at 105V (left). Note the fragment ion of *m/z* 282 (ESI negative) which is common to all analogues of sildenafil and vardenafil.

Waters MassLynx™ software v4.1 was used for data acquisition and the 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 positive and ESI negative modes, under multiple cone voltage conditions, along with RT provides high confidence in the identification.

Keeping abreast of emerging ED analogues is challenging. Previously, precursor ion scanning of *m/z* 283 (in ESI positive 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-1 and Figure-2). Similarly, under these conditions, tadalafil and its respective analogues demonstrates a common fragment at *m/z* 232 (data not shown).

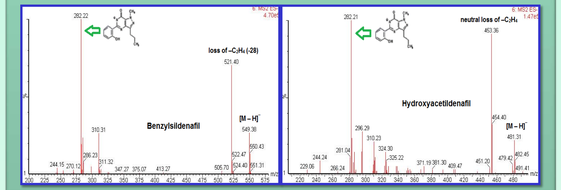

Figure-2: Screening for the 'unknowns'. MS scan in ESI negative mode (cone voltage=105V) leads to the formation of parent ion [M – H] with the loss of $-C_2H_4$ (-28) and formation of the common fragment ion *i.e., m/z* 282 (common in all analogues of sildenafil, vardenafil and acetildenafil).

Table 2: List of 32 compounds with retention times, and optimised MRM transitions parameters.

Substance	Retention Time (min.)	Precursor Ion (*m/z*)	Cone Voltage (V)	Quantifier Ion (*m/z*)	CE (V)	Qualifier Ion (*m/z*)	CE (V)	Dwell (ms)	LOQ (ng/mL)	R^2	%CV (*n*=4) 2 ng/mL	10 ng/mL	100 ng/mL
Yohimbine	2.77	355.2	41	144.1	28	212.2	25	50	0.2	0.9989	2.00	3.35	2.92
Acetylvardenafil	3.28	467.3	65	151.1	48	111.1	35	50	1.0	0.9980	4.80	7.05	1.98
Carbodenafil	4.23	453.2	61	339.2	25	311.2	32	10	0.2	0.9994	4.85	10.1	3.95
Hydroxyvardenafil	4.50	505.1	68	151.1	46	312.1	38	20	0.5	0.9990	3.20	1.23	4.46
Hydroxyacetildenafil	4.53	483.2	59	127.1	35	143.2	32	08	0.5	0.9983	9.73	4.69	3.29
Nor-acetildenafil	4.70	453.3	60	97.2	34	113.2	36	10	0.2	0.9989	7.77	3.54	2.04
Vardenafil	4.73	489.0	66	151.1	52	312.1	40	10	1.0	0.9988	26.3	2.83	3.19
Acetildenafil	4.93	467.2	60	111.2	35	127.2	40	08	0.2	0.9982	9.84	2.94	1.78
Piperiacetildenafil	5.42	438.0	63	98.2	38	297.1	42	20	0.2	0.9980	4.74	5.21	3.29
Icariin	5.53	677.2	42	531.2	15	369.2	33	08	2.0	0.9975	22.1	4.02	6.62
Hydroxyhomo sildenafil	5.55	505.1	65	99.2	38	112.2	33	20	0.5	0.9989	2.17	1.14	0.85
Avanafil	5.60	484.3	50	155.2	40	375.2	28	20	0.2	0.9986	9.12	7.3	2.2
Sildenafil	5.65	475.1	64	100.2	32	283.1	40	20	1.0	0.9995	15.6	4.30	1.86
Homo sildenafil	5.83	489.2	64	99.2	38	113.2	34	25	0.2	0.9996	10.3	3.85	3.25
Dimethyl sildenafil	6.05	489.1	62	99.2	39	113.2	35	25	0.2	0.9999	2.97	6.42	1.54
Aminotadalafil	6.38	391.2	28	269.1	12	169.1	35	08	2.0	0.9970	44.8	8.18	5.20
Udenafil	6.42	517.0	70	112.3	40	283.0	55	08	0.2	0.9974	3.22	5.24	2.12
Nor-tadalafil	6.58	376.1	31	254.2	11	135.1	22	20	5.0	0.9985	nd	10.8	6.70
Tadalafil	7.28	390.2	30	268.2	12	135.1	22	08	1.0	0.9962	29.2	10.5	5.43
Benzamidenafil	7.38	390.1	23	151.2	12	107.1	56	08	1.0	0.9989	4.13	3.08	1.23
Dapoxetine	7.53	306.2	29	261.2	13	157.1	23	08	0.2	0.9980	3.93	2.44	2.95
Benzyl sildenafil	7.73	551.2	60	91.2	35	134.1	50	08	1.0	0.9995	3.79	1.47	3.32
Hydroxythiohomo sildenafil	8.00	521.0	57	99.2	39	129.2	34	20	0.5	0.9995	2.48	1.17	1.71
Testosterone	8.06	289.3	49	97.2	22	109.2	22	20	0.5	0.9989	7.63	1.18	4.16
Thiosildenafil	8.11	491.0	61	100.2	37	341.2	32	20	1.0	0.9996	3.19	6.51	0.87
Thiohomo sildenafil	8.35	505.3	56	113.2	33	99.2	40	08	0.2	0.9991	6.61	2.92	2.23
Thiodimethyl sildenafil	8.50	505.2	58	113.2	35	327.2	40	08	0.2	0.9988	5.78	5.32	1.78
Methyltestosterone	8.64	303.3	50	97.2	25	109.2	22	08	0.5	0.9965	9.52	3.36	9.32
Gendenafil	8.76	355.2	56	285.3	32	327.3	27	08	1.0	0.9981	19.3	9.98	4.75
Pseudo vardenafil	9.17	460.0	62	151.1	44	312.1	40	20	0.2	0.9982	2.32	5.29	0.61
Norneo sildenafil	10.65	460.0	73	283.3	41	299.1	39	50	0.5	0.9991	1.52	6.17	0.84
N-octyl-nortadalafil	12.15	488.2	45	366.4	15	135.1	23	50	0.2	0.9985	4.87	3.74	2.88

For subsequent quantitative analysis, a MRM method was 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.

The developed method was applied to 43 suspected samples, 18 of which were found to be adulterated with ED analogues (Table-3). Two samples that screened positive for thiodimethylsildenafil also gave matches for thiohomosildenafil, however chromatographic separation permitted clear differentiation between these two identical 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].

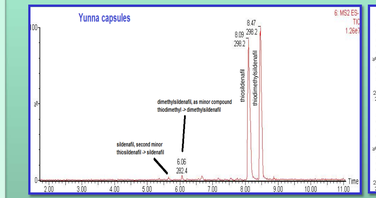

Figure-3: TIC of seized sample, thiosildenafil and thiodimethylsildenafil as major compounds; producing minor compounds sildenafil and dimethylsildenafil after the hydrolysis of thiocarbonyl group to a carbonyl group (C = S → C = O).

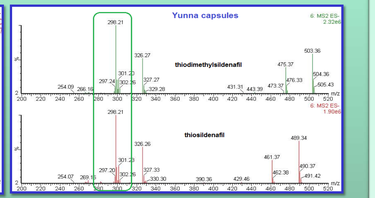

Figure-4: ESI negative spectra of seized sample (Figure-3), formation of common fragment ion *m/z* 298 due to the presence of thiocarbonyl group in thio analogous of sildenafil.

Table-3: Summary of results for eight 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.

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	8.49	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	Homosildenail	6.07	5.83	×	776	–	X
	Dimethylsildenail	6.07	6.05	✓	832	✚	81 mg/tab.
SATIBO	Sildenafil	5.62	5.65	✓	869	✚	67 mg/cap
Russian 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

CONCLUSIONS

- 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 ED drugs/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 period 10/2010 – 08/2011. Eighteen samples were found to contain unauthorised substances.

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