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アプリケーションノート

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

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Abstract

A novel screening method is presented which, for the first time, permits the simultaneous detection of both known and currently unknown ED drugs and analogues in a single analytical procedure.

Benefits

For the first time, a novel analytical screening method for the detection of both known and unknown erectile dysfunction (ED) drugs and their analogues is successfully completed. The screening method is complemented by a highly sensitive and selective MS/MS confirmation assay.

- The full scan MS screening approach accurately detects known and currently unknown ED drugs and analogues
- This method encompasses the largest number of ED drugs in a single analytical procedure
- Full scan data allows for retrospective data processing
- A robust and highly sensitive and selective MS/MS confirmation complements this method

Introduction

An estimated 150 million men worldwide suffer from erectile dysfunction (ED), a number that is expected to double by 2025.[1] Viagra (sildenafil citrate) was the first drug approved by the U.S. Food and Drug Administration (FDA) for the treatment of the condition. Since its launch in 1998, Viagra is considered one of the most successful drugs ever introduced. To date, however, there are still relatively few ED drugs on the market. FDA approved drugs include: Levitra (vardenafil hydrochloride), Cialis (tadalafil), Staxyn (an orodispersible formulation of vardenafil), and Stendra (avanafil). In other regions of the world, additional drugs are available, such as udenafil (Zydena) in Korea and in other Asian countries. The efficacy, toxicity profiles, and drug interactions of these approved medications are known and documented. In most countries, they are available under medical supervision by prescription only.

The use of herbal remedies and dietary supplements for the treatment of ED has recently increased in popularity. Although some compounds derived from natural products, such as yohimbine and icariin, have been reported to increase sexual performance, a significant number of these so-called ‘all natural’ products are in fact adulterated with undeclared ED drugs or their unapproved structural analogues.

Adulteration is reported worldwide and is an increasing problem.[2] These products pose a serious threat to the health of consumers, due to the undisclosed presence of approved prescription drugs and the unknown safety and toxicity profile of unapproved ED drugs. Countless new warnings are raised each year concerning the adulteration of products that 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.

Experimental

LC conditions

System:	ACQUITY UPLC
Column:	ACQUITY UPLC HSS C_{18} 2.1 x 100 mm, 1.8 µm
Column temp.:	45 °C

Flow rate:	350 µL/min
Mobile phase A:	3 mM ammonium formate, pH 2.9 (with formic acid)
Mobile phase B:	acetonitrile with 0.1% formic acid
Initial conditions:	80% mobile phase A
Gradient:	Gradient increasing to 98% mobile phase B
Analysis time:	15 min
Weak wash:	20:80 acetonitrile/water (0.1% formic acid)
Strong wash:	80:20 acetonitrile/water
Injection volume:	10 µL

MS conditions

Mass spectrometer:	ACQUITY TQD
Ionization modes:	ESI+ and ESICapillary
voltage:	3.0 kV
Cone voltage:	ESI+ 20 V to 100 V (in 20-V increments) ESI- 105 V
Desolvation temp.:	375 °C
Desolvation gas:	700 L/h

Source temp.:	150 °C
Screen:	UPLC-MS: ESI+ *m/z* 55 to 680 ESI- *m/z* 200 to 680
Confirmation/quantification:	UPLC-MS/MS Multiple reaction monitoring (MRM), shown in Table 1

Sample description

Samples were received in the form of capsules, tablets, and pills, as well as honey and herbal drinks.

One hundred milligrams of sample powder was weighed (in the case of honey, 2g was used) and transferred to a volumetric flask with 15 mL of 75:25 methanol/water. Following sonication for 20 min, the volume was increased to 20 mL, and the sample was shaken for an additional 5 min. After centrifugation at 4000 rpm for 8 min, the supernatant was filtered using a PVDF syringe filter.

Prior to analysis, the samples were further diluted using a mixture of mobile phases A and B (80:20). A 50-fold dilution was used for the screening analysis with 400-fold to 1000-fold dilutions used for the confirmatory analysis.

Results and Discussion

Screening procedure: UPLC-MS

For the purpose of screening, a spectral library of known ED drugs and structural analogues was prepared. Based on recent reports of increased availability of 'all-in-one' / 'combination' herbal products,[3] naturally occurring substances such as icariin and yohimbine, the synthetic, dapoxetine (used for premature ejaculation), and testosterone were included.

The library was created according to a previously described approach,[4] using UPLC in conjunction with full scan MS analysis performed under multiple cone voltage conditions (in-source collision – induced dissociation: CID), to generate both spectral and retention time (RT) information, as shown in Figure 1.

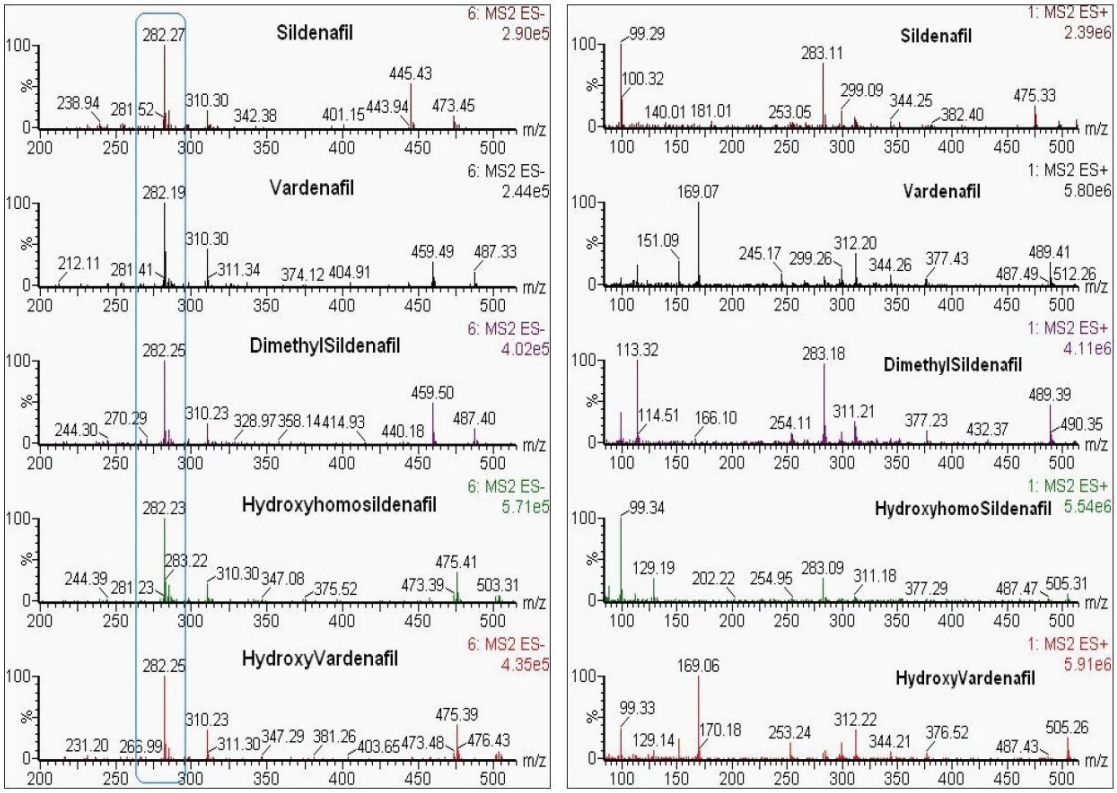

Figure 1. Mass spectra of five drugs. MS scans in ESI positive at cone voltage 100 V (right) and in ESI negative at 105 V (left). Note the fragment ion of m/z 282 (ESI negative) which is common to all structural analogues of sildenafil and vardenafil.

ChromaLynx Application Manager was used for automated data processing. ChromaLynx examines the chromatograms produced at each cone voltage, detects the components, and calculates an average spectral match factor (MF) against the library. Screening in both ESI positive (ESI+) and ESI negative (ESI-) modes, under multiple cone voltage conditions along with RT provides high confidence identification.

Tracking emerging ED analogues is a challenge; therefore, we proposed inclusion of an extra scanning function that was performed simultaneously in ESI- mode at high energy. Under these conditions, a characteristic fragmentation pattern was observed for sildenafil, acetildenafil, vardenafil, and their respective analogues, as shown in Figures 1 and 2. Similarly, these conditions demonstrated that tadalafil and its respective analogues had a common fragment at *m/z* 232, while the thiosildenafil analogues yielded a common fragment at *m/z* 298, as shown in Figure 3. Thus, the presence of any of these common, characteristic ions could be indicative of a new structural analogue.

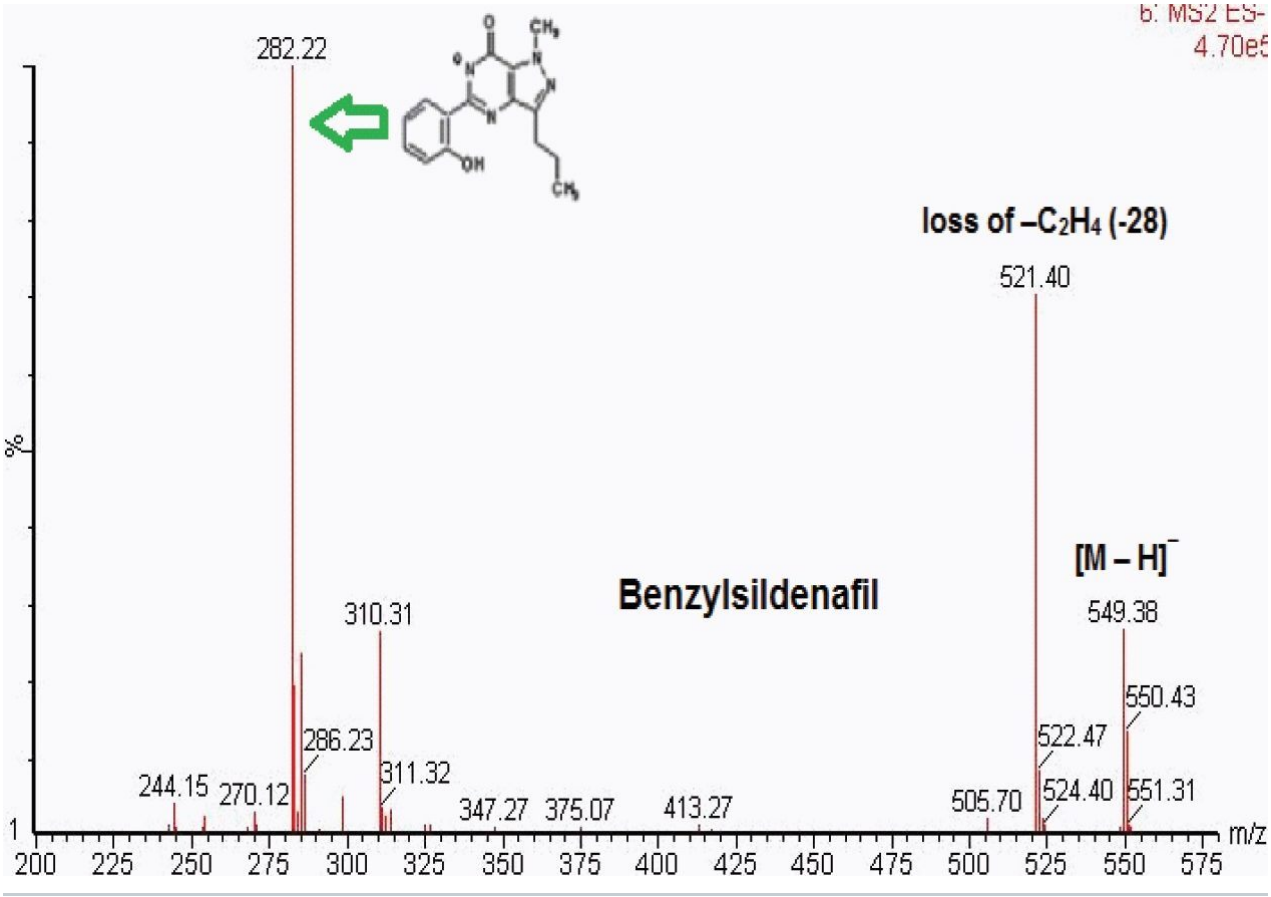

Figure 2. Screening for the 'unknowns.' MS scan in ESI negative mode (cone voltage = 105 V) leads to the formation of parent ion [M - H]⁻ with the loss of - C_2H_4 (-28), and formation of the common fragment ion i.e., m/z 282 (common in all analogues of sildenafil, vardenafil, and acetildenafil).

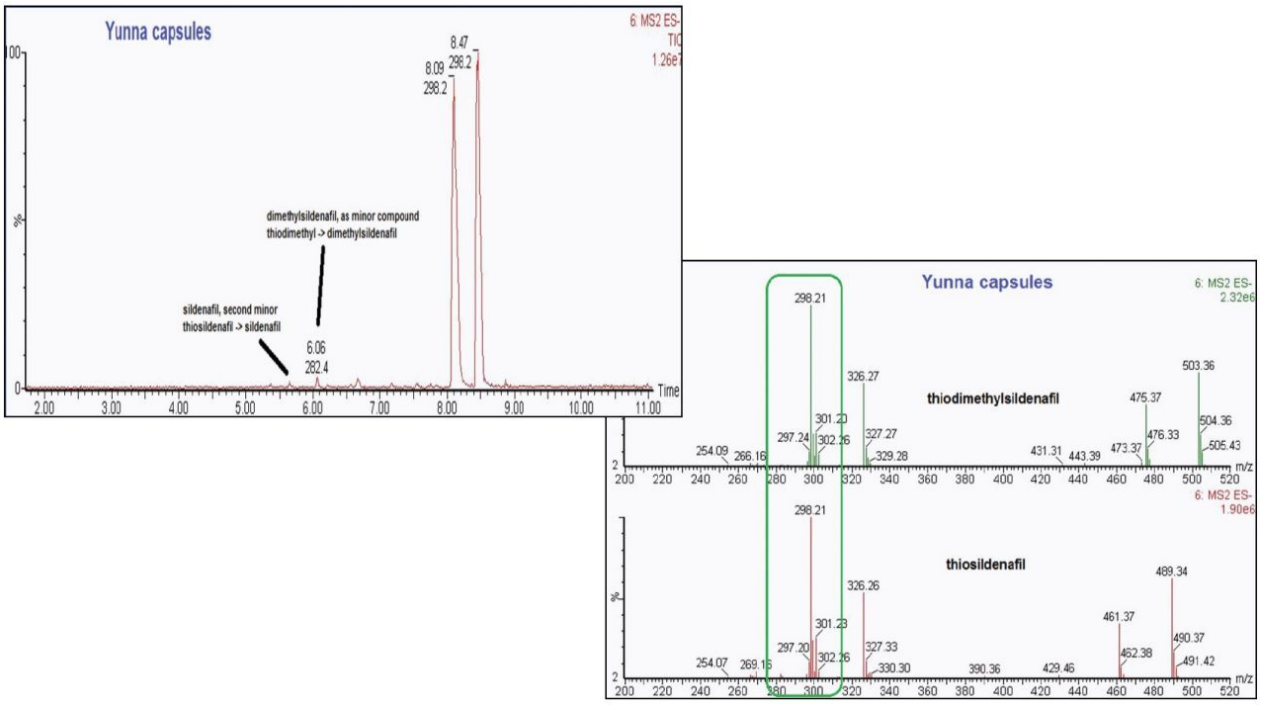

Figure 3. Analysis of seized yunna capsules. The upper figure shows the chromatogram (ESI negative) for the capsules which were found to contain thiosildenafil and thiodimethylsildenafil. A small amount of sildenafil and dimethylsildenafil were also observed. The latter were likely products of the hydrolysis of the thiocarbonyl group to a carbonyl group (C = S → C = O). The lower figure shows the underlying spectra for the same sample. Note the formation of the fragment ion m/z 298, an ion commonly observed with the thio analogues of sildenafil.

Confirmatory procedure: UPLC-MS/MS

For subsequent quantitative analysis, an MRM method was developed and validated for three alternative matrices, including capsules, tablets, pills, as well as honey and herbal drinks. Calibration curves were constructed over the range of 0.2 to 1000.0 ng/mL. The coefficient of determination (R^2) for all compounds in this study was ⩾0.995. The precision, measured as a 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, as shown in Table 1.

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)
Yohimbine	2.77	355.2	41	144.1	28	212.2	25	50	0.2
Acetylvardenafil	3.28	467.3	65	151.1	48	111.1	35	50	1.0
Carbodenafil	4.23	453.2	61	339.2	25	311.2	32	10	0.2
Hydroxyvardenafil	4.50	505.1	68	151.1	46	312.1	38	20	0.5
Hydroxyacetildenafil	4.53	483.2	59	127.1	35	143.2	32	08	0.5
Nor-acetildenafil	4.70	453.3	60	97.2	34	113.2	36	10	0.2
Vardenafil	4.73	489.0	66	151.1	52	312.1	40	10	1.0
Acetildenafil	4.93	467.2	60	111.2	35	127.2	40	08	0.2
Piperiacetildenafil	5.42	438.0	63	98.2	38	297.1	42	20	0.2
Icariin	5.53	677.2	42	531.2	15	369.2	33	08	2.0
Hydroxyhomo sildenafil	5.55	505.1	65	99.2	38	112.2	33	20	0.5
Avanafil	5.60	484.3	50	155.2	40	375.2	28	20	0.5
Sildenafil	5.65	475.1	64	100.2	32	283.1	40	20	1.0
Homo sildenafil	5.83	489.2	64	99.2	38	113.2	34	25	0.2
Dimethyl sildenafil	6.05	489.1	62	99.2	39	113.2	35	25	0.2
Aminotadalafil	6.38	391.2	28	269.1	12	169.1	35	08	2.0
Udenafil	6.42	517.0	70	112.3	40	283.0	55	08	0.2
Nor-tadalafil	6.58	376.1	31	254.2	11	135.1	22	20	5.0
Tadalafil	7.28	390.2	30	268.2	12	135.1	22	08	1.0
Benzamidenafil	7.38	390.1	23	151.2	12	107.1	56	08	1.0
Dapoxetine	7.53	306.2	29	261.2	13	157.1	23	08	0.2
Benzyl sildenafil	7.73	551.2	60	91.2	35	134.1	50	08	1.0
Hydroxythiohomo sildenafil	8.00	521.0	57	99.2	39	129.2	34	20	0.5
Testosterone	8.06	289.3	49	97.2	22	109.2	22	20	0.5
Thiosildenafil	8.11	491.0	61	100.2	37	341.2	32	20	1.0
Thiohomo sildenafil	8.35	505.3	56	113.2	33	99.2	40	08	0.2
Thiodimethyl sildenafil	8.50	505.2	58	113.2	35	327.2	40	08	0.2
Methyltestosterone	8.64	303.3	50	97.2	25	109.2	22	08	0.5
Gendenafil	8.76	355.2	56	285.3	32	327.3	27	08	1.0
Pseudo vardenafil	9.17	460.0	62	151.1	44	312.1	40	20	0.2
Norneo sildenafil	10.65	460.0	73	283.3	41	299.1	39	50	0.5
N-octyl-nortadalafil	12.15	488.2	45	366.4	15	135.1	23	50	0.2

Table 1. List of ED compounds with retention times and optimized MRM parameters.

Authentic samples

Full scan analysis was performed on 43 suspected samples received into the Drug Control Laboratory, Qatar, between the time period of October 2010 and August 2011. Eighteen were found to be adulterated and contained unauthorized substances, as shown in Table 2.

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 500 mg 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 20 mg (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
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
MAXMAN Capsule	Sildenafil	5.66	5.65	✔	876	+	108 mg/cap

Table 2. 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.

**Match Factor is the cutoff number for a yes/no decision.*

Conclusion

A novel screening method is presented which, for the first time, permits the simultaneous detection of both known and currently unknown ED drugs and analogues in a single analytical procedure.

Full scan data is simultaneously collected in both ESI+ and ESI- modes, under multiple energy conditions to yield comprehensive spectral data. The data is automatically compared to a prepared library of known drugs. The high energy ESI- data can be used to facilitate the identification of currently unknown ED analogues.

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 the Drug Control Laboratory, Qatar, between the time frame of October 2010 and August 2011. Analysis revealed that 18 samples were adulterated with unauthorized ED substances.

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A full validation by the user would be necessary prior to adoption in a laboratory.

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720004511, December 2012