Development and validation of a UHPLC-UV method for the detection and quantification of erectile dysfunction drugs and some of their analogues found in counterfeit medicines.

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Introduction :

Due to the taboo associated with erectile dysfunction, phosphodiesterase type 5 (PDE5) inhibitors are widely sold over the internet as both counterfeited medicines and illegal adulterants in herbal dietary supplements [1].

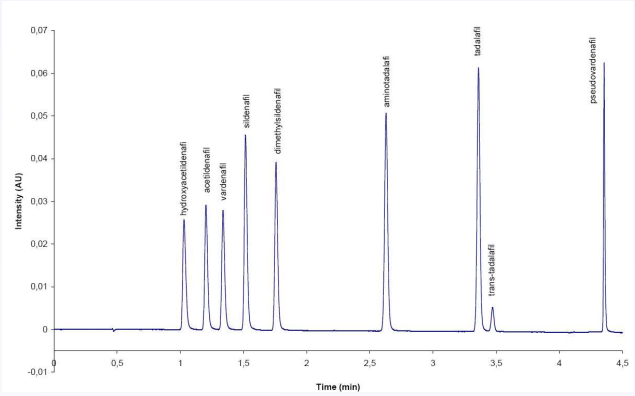
For this study, three analogues of sildenafil (acetildenafil, hydroxyacetildenafil and dimethylsildenafil), one of vardenafil (pseudovardenafil), one of tadalafil (aminotadalafil) and the bioactive diastereoisomer of tadalafil (trans-tadalafil) have been chosen. A full validation has been performed using the "total error" approach [2].

The method is also compatible with on-line coupling mass spectrometry and will significantly reduce analysis times and solvent consumption.

Chromatographic conditions :

The method has been first developed on HPLC and then transferred to an Acquity UPLC™ system with a diode array detector. The separation is performed on an Acquity™ BEH Shield RP18 (100mm x 2.1mm I.D., 1.7µm particle size) column.

The final method is a gradient elution with pH3.5 formate ammonium buffer (10mM) as aqueous phase (solvent A) and acetonitrile as organic modifier (solvent B).



Time (min)	Flow rate (ml/min)	% A	% B
0	0.55	75	25
2.5	0.55	65	35
3.5	0.55	55	45
3.8	0.55	30	70
4.5	0.55	30	70
5.0	0.55	75	25

Injection volume: 1.5 µl
Temperature: 40°C
Detection UV: 285 nm

Validation :

The compounds were divided in two groups according to their UV absorbance at 285 nm.

Group 1 contains hydroxyacetildenafil, acetildenafil and tadalafil. The second group contains vardenafil, sildenafil, dimethylsildenafil, aminotadalafil and pseudovardenafil.

All solutions were prepared in a mixture of H_2O/ACN (50:50, v/v).

Linearity:

For each substance, a calibration line has been prepared. Calibration lines cover the concentration ranges of 3 – 32 µg/ml for group 1 and 9 – 96 µg/ml for group 2. The calibration model was an unweighted linear regression.

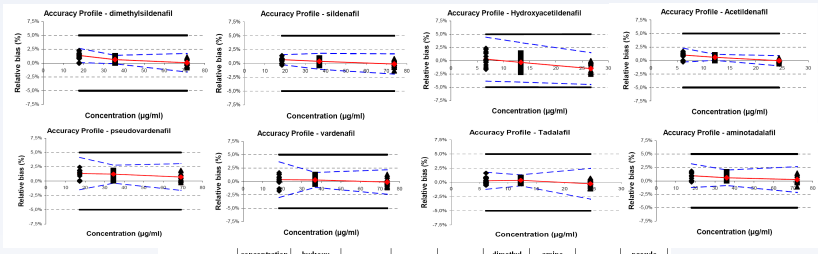
For all of the eight compounds the relationship was linear as the r^2 were all > 0.99 and the equation was close to y = x.

The samples stock solutions were prepared the same way as the reference standards with the addition of 200 mg herbal matrix to the pure substances. These solutions were magnetically stirred for 30 minutes, sonicated during 10 minutes and diluted to obtain three concentration levels.

These levels were chosen with a ratio 0.5/1/2 to cover a large concentration range and to take into account the differences in concentration of the approved medicines. These final solutions were filtered with 0.2 µm PTFE filters before injection.

concentration levels	validation samples group 1	validation samples group 2
1	6 µg/ml	18 µg/ml
2	12 µg/ml	36 µg/ml
3	24 µg/ml	72 µg/ml

Trueness, precision, accuracy and uncertainty assessment :



	concentration level	hydroxy acetildenafil	acetildenafil	vardenafil	sildenafil	dimethyl sildenafil	amino tadalafil	tadalafil	pseudo vardenafil
trueness									
relative bias (%)	1	0.30	1.02	0.33	0.68	1.38	0.97	0.29	1.31
	2	-0.31	0.59	0.28	0.38	0.62	0.58	0.37	1.21
	3	-1.48	-0.04	-0.11	-0.10	0.05	0.24	-0.24	0.71
intra-assay precision									
repeatability (RSD%)	1	0.55	0.39	0.66	0.38	0.44	0.48	0.54	0.35
	2	0.49	0.23	0.43	0.27	0.26	0.34	0.34	0.26
	3	0.26	0.17	0.51	0.40	0.45	0.43	0.15	0.46
between-assay precision									
intermediate precision (RSD %)	1	1.37	0.50	1.10	0.38	0.52	0.78	0.64	0.80
	2	1.24	0.24	0.55	0.47	0.31	0.51	0.41	0.51
	3	1.00	0.31	0.82	0.66	0.64	0.79	0.77	0.76
accuracy									
β-expectation tolerance limits (%)	1	[-3.85;4.45]	[-0.27;2.30]	[-3.01;3.68]	[-0.22;1.58]	[0.08;2.67]	[-1.25;3.19]	[-1.22;1.79]	[-1.52;4.14]
	2	[-4.03;3.42]	[0.04;1.14]	[-1.11;1.68]	[-1.06;1.82]	[-0.14;1.39]	[-0.85;2.02]	[-0.60;1.33]	[-0.36;2.78]
	3	[-4.47;1.52]	[-0.98;0.91]	[-2.39;2.18]	[-1.94;1.74]	[-1.64;1.74]	[-2.17;2.65]	[-2.95;2.48]	[-1.62;3.04]
uncertainty									
relative expanded uncertainty (%)	1	2.99	1.09	2.41	0.81	1.12	1.73	1.35	1.78
	2	2.67	0.60	1.18	1.04	0.66	1.12	0.87	1.13
	3	2.16	0.68	1.78	1.43	1.38	1.74	1.71	1.68

The concentrations were back-calculated using the calibration lines. These concentrations were used to determine the relative bias, the repeatability, the intermediate precision and the β-expectation tolerance intervals at the 95 % probability level.
The RSD values of repeatability and intermediate precision are said acceptable since they are inferior to two-thirds of the Horwitz value [3] (1.76 % for sildenafil in Viagra®, 2.06 % for tadalafil in Cialis® and 1.86 % for vardenafil in Levitra®).

Conclusions :

The presented fully validated method enables the detection and the quantification of authorised PDE5 inhibitors and some of their analogues in less than 4.5 minutes. This rapidity associated to a low flow rate permits the analysis of a large number of samples with a reduced cost and associated solvent consumption.

The method has already been applied to real samples and showed no interference with common other substances present as yohimbine (retention time of 0.77 min) and caffeine (retention time of 0.57 min).

The main problem with counterfeit medicines is that their chemical composition is unknown. This is why they represent a real danger for public health. The method permits the detection of all PDE5 inhibitors even new ones as it covers a wide range of polarity. The elucidation of structures and the confirmation of identity may be performed by UHPLC-MS systems since the mobile phase is compatible.

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