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Development and validation of a ultra-high-performance liquid chromatography-UV method for the detection and quantification of erectile dysfunction drugs and some of their analogues found in counterfeit medicines

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ABSTRACT

Pharmaceutical counterfeiting is a permanently growing problem. Control laboratories are constantly analysing counterfeit medicines. In industrialised countries, one of the main counterfeited class of medicines are erectile dysfunction drugs. This paper describes the development and validation of a fast method to detect and quantify the three authorised phosphodiesterase type 5 inhibitors and five analogues. The method is based on the use of a sub-2 microns polar-embedded column with a gradient using acetonitrile as organic modifier and 10 mM ammonium formate buffer (pH 3.5) as aqueous component of the mobile phase. The separation was achieved in less than 4.5 min. The method has also been compared to the registered HPLC method for the assay of Viagra® which was considered as the reference method. The method is also compatible with on-line coupling mass spectrometry and will significantly reduce analysis times and solvent consumption.

1. Introduction

The number of cases of pharmaceutical counterfeiting is constantly growing since the first cases were detected in the early 90s [1]. In industrialised countries, one of the most counterfeited classes of medicines is the phosphodiesterase type 5 inhibitors (PDE5-i) [2]. Among them only three drugs are approved and marketed: sildenafil citrate (Pfizer), tadalafil (Eli Lilly) and vardenafil hydrochloride (Bayer). These drugs are used in erectile dysfunction disorders (Viagra®, Cialis® and Levitra®). Sildenafil citrate is also used in pulmonary arterial hypertension (Revatio®).

Due to the taboo associated with erectile dysfunction, PDE5-inhibitors are widely sold over the internet as both counterfeited medicines and illegal adulterants in herbal dietary supplements. In the latter the biggest diversity of analogues was found [2–4]. 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. Their chemical structures are shown in Fig. 1. These compounds are representative of what is commonly found in illegal preparations.

All of these analogues have already been found in illegal preparations. These preparations have been analysed using different analytical systems (LC-UV, LC-MS, IR, NMR, X-ray diffraction, etc.) [5–28]. The presented validated method allows a fast separation and quantification of the three authorised PDE5-i and five of their analogues. This method may constitute a good basis for the analysis of illegal erectile dysfunction medicines by official control laboratories.

The present paper describes a method enabling the separation and quantification of nine PDE5 inhibitors in a single run: sildenafil, tadalafil, vardenafil and some of their analogues and impurities (trans-tadalafil [16]). A full validation using spiked placebo validation samples has been performed using the "total error" approach [31–38]. The robustness of the method has also been investigated. The precision and accuracy for the quantification of sildenafil

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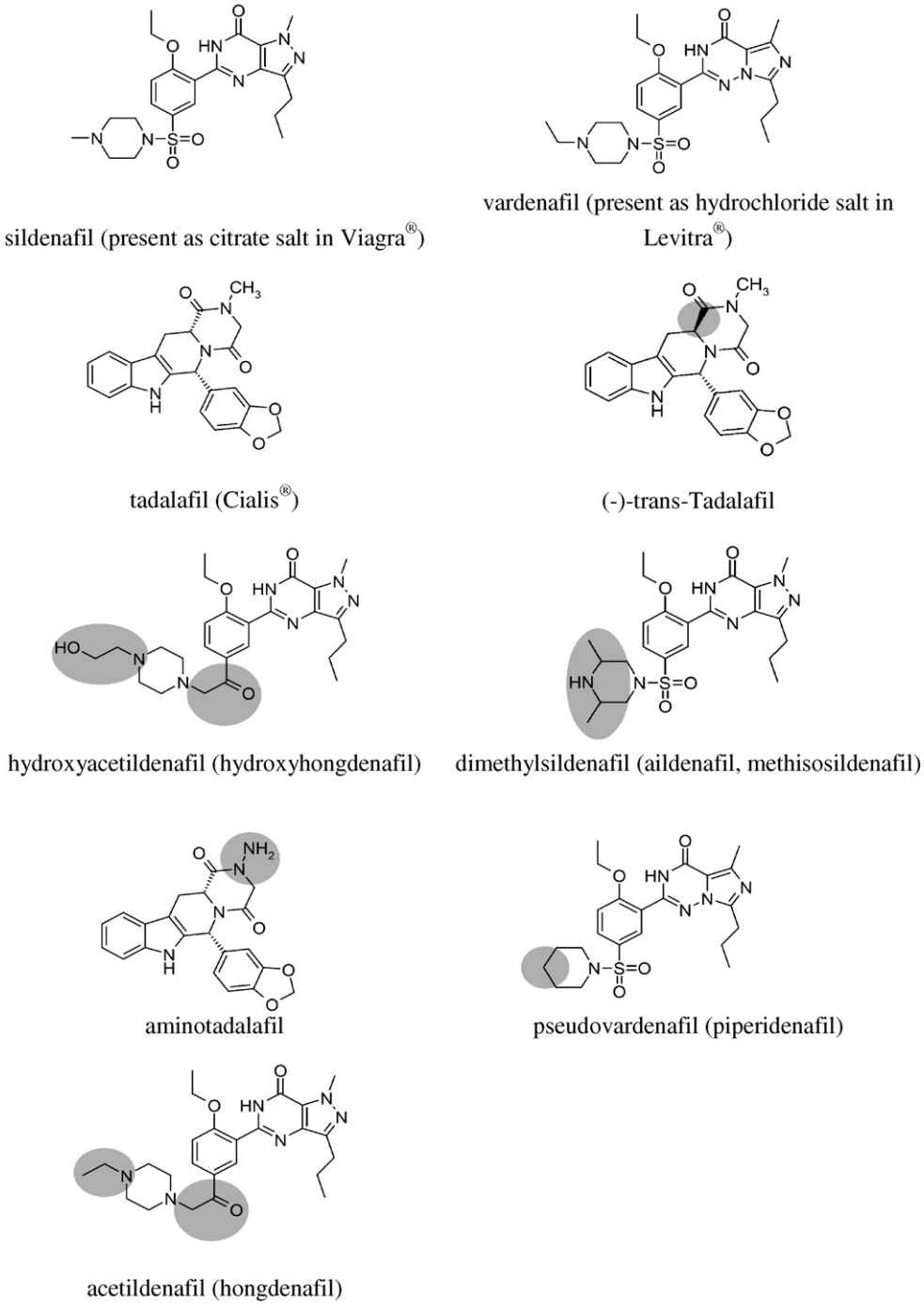

Fig. 1. Chemical structures of the studied compounds. The structural differences with the registered APIs are indicated in grey. Reproduced from [2].

citrate in Viagra® tablets has been compared to the HPLC method from the Viagra® registration dossier set as reference method. The method described here can be used as routine method for the analysis of PDE5-inhibitors and can be coupled in principle to a mass spectrometer for identity confirmation or structure elucidation.

The proposed method allows a faster and more environmental friendly high throughput analysis of both illegal and legal preparations containing PDE5-inhibitors.

2. Materials and methods

2.1. Standards

The reference standards of sildenafil citrate (batch 904958), tadalafil (batch RS0480) and vardenafil dihydrochloride trihydrate (batch BXR3835 R-1013-02B) were kindly provided by Pfizer SA/NV (Belgium), Eli Lilly SA/NV (Benelux) and Bayer SA/NV (Belgium), respectively.

Table 1
Concentrations of the calibration standards and the validation samples. These concentrations are based on the basic form of each compound.

Concentration levels	Calibration standards group 1 (μg/ml)	Calibration standards group 2 (μg/ml)	Validation samples group 1 (μg/ml)	Validation samples group 2 (μg/ml)
1	3	9	6	18
2	10	30	12	36
3	12	36	24	72
4	14	42		
5	32	96		

Reference standards of hydroxyacetildenafil (batches 1068-005A2 and 1068-013A2), acetildenafil (batch 1046-011A2), dimethylsildenafil (batch 1035-122A1), aminotadalafil (batch 1034-001A1) and pseudovardenafil (batch 1070-002A2) were purchased from TLC Pharmachem (Ontario, Canada).

2.2. Samples

Pfizer SA/NV (Belgium) kindly provided one batch of each different dosage of Viagra® (25 mg, 50 mg, 100 mg). Two other batches of each dosage were purchased in a local pharmacy in Belgium.

2.3. Reagents

HPLC-grade acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands), formic acid and sodium hydroxide were obtained from VWR International (Leuven, Belgium) and ammonia solution 25% was purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid was purchased from Sigma Aldrich (Saint Louis, USA). The water used was produced by a milliQ-Gradient A10 system (Millipore, Billerica, USA). The herbal matrix used to realize the validation samples comes from a placebo dietary supplement received for PDE5 inhibitors screening.

2.4. Sample preparation

2.4.1. Preparation of standards

According to their absorbance, the substances were divided into two groups. Group 1 contains hydroxyacetildenafil, acetildenafil and tadalafil. Stock solutions of each compound of group 1 were prepared in double, diluting 10.0 mg of pure substance (basic form) with 50.0 ml of a mixture of H_2O/ACN (50:50, v/v) (final concentration of 0.2 mg mL^{-1} of the basic form) The second group contains vardenafil, sildenafil, dimethylsildenafil, aminotadalafil and pseudovardenafil. Stock solutions of each compound of group 2 were prepared in double diluting 30.0 mg of pure substance (basic form) with 50.0 ml of a mixture of H_2O/ACN (50:50, v/v) (final concentration of 0.6 mg mL^{-1} of the basic form).

Calibration standards were then prepared by diluting the stock solutions to obtain the concentrations indicated in Table 1. All solutions were prepared in a mixture of H_2O/ACN (50:50, v/v).

2.4.2. Preparation of spiked placebo validation samples

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 min, sonicated during 10 min and diluted to obtain the three concentration levels presented in Table 1. 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.

2.4.3. Preparation of samples for the comparison of methods

Five tablets of each dosage form of different batches of Viagra® samples were pulverised. An amount of the pulverised tablets of 25 mg, 50 mg and 100 mg was accurately weighed and diluted in a mixture of H_2O/ACN (50:50, v/v) to obtain the concentration levels 1, 2 and 3 respectively. Concentrations of sildenafil at the levels 1, 2 and 3 were 16 μg mL^{-1}, 36 μg mL^{-1} and 72 μg mL^{-1}, respectively. Three different samples were weighed daily for each concentration level and were analysed three times per day for seven consecutive days (see Section 3.3).

2.5. Equipment and chromatographic conditions

The HPLC experiments were performed on an Alliance 2690 HPLC system (Waters, Milford, USA) coupled to a 996 PDA detector (Waters). Data acquisition and treatment were performed with the Empower2 software (Waters).

The method optimisation and validation were performed on an Acquity UPLC™ system (Waters). This system is composed of a binary solvent manager, a sample manager and a PDA detector. Data acquisition and treatment were also performed with the Empower2 software (Waters).

The initial method was developed in HPLC with a XTerra™ RP18 (150 mm × 4.6 mm, 5 μm particle size) column (Waters). The optimisation and validation of the UHPLC gradient were performed on an Acquity™ BEH Shield RP18 (100 mm × 2.1 mm, 1.7 μm particle size) column. Mobile phase A consisted of a 10 mM ammonium formate buffer (pH 3.5) and mobile phase B was acetonitrile. The gradient conditions are presented in Tables 2 and 3. After each injection, the systems were reconditioned for 10 min for HPLC and 4 min for UHPLC (Ultra High Pressure Liquid Chromatography).

Table 2
HPLC and initial UHPLC gradient conditions. For more details see Section 2.5.

HPLC conditions				Initial UHPLC conditions			
Time (min)	Flow rate (ml/min)	% A	% B	Time (min)	Flow rate (ml/min)	% A	% B
0	1.0	70	30	0	0.55	70	30
5.0	1.0	65	35	2.2	0.55	65	35
8.0	1.0	55	45	2.7	0.55	55	45
9.0	1.0	20	80	2.9	0.55	20	80
11.0	1.0	20	80	3.5	0.55	20	80
12.0	1.0	70	30	4.0	0.55	70	30
Injection volume:		20 μl		Injection volume:		2.8 μl	
Column temperature:		30 °C		Column temperature:		40 °C	

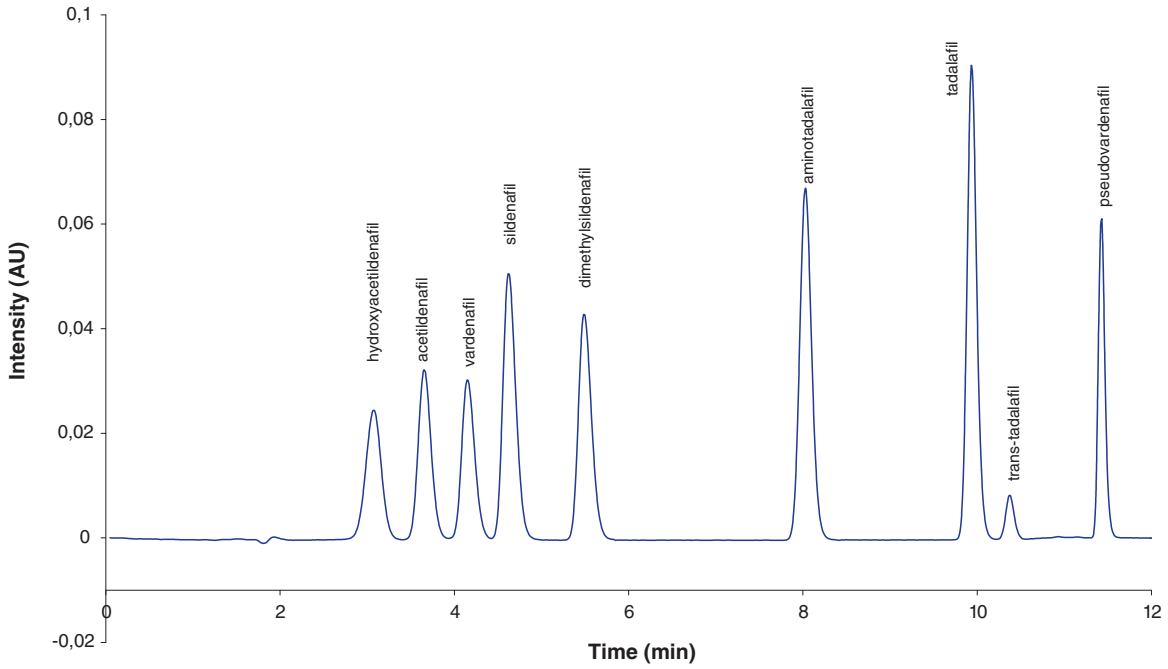

Fig. 2. Typical chromatogram obtained by applying the gradient conditions of the HPLC method.

Table 3
Final UHPLC gradient conditions. For more details see Section 2.5.

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	
Column temperature:		40 °C	

The quantitative results of the developed UHPLC method were compared to the results obtained with the reference method used in our lab for the assay of the Viagra® samples. For confidentiality reasons, the method is not described in this paper. The statistical comparison was performed using the method described by Kuttatharmmakul et al. [29].

2.6. Method transfer

Fig. 2 illustrates a chromatogram obtained by applying the initial HPLC gradient conditions presented in Table 2. These HPLC conditions were then adapted to obtain a UHPLC method by using the Waters Acquity UPLC™ column calculator 1.0. This software optimizes the UHPLC parameters based on the HPLC conditions (for HPLC parameters see Section 2.5) and column dimensions. The deduced conditions are presented in Table 2. The sub-2 micron polar-embedded stationary phase was chosen as the closest to the chemistry of the XTerra™ RP18 material column. These initial gradient conditions were modified to obtain a greater resolution between the peaks corresponding to vardenafil and acetildenafil which led to a more robust method. The final conditions are illustrated in Table 3. Fig. 3 shows the corresponding chromatogram.

2.7. System suitability testing

System suitability testing was performed on the validation standard with the medium concentration. The acceptance criteria were a relative standard deviation (RSD) values for areas and retention times of less than 1.0% for 8 replicate injections.

2.8. Method validation

This method has been validated using the "total error" approach in accordance with the validation requirements in the ISO-17025 norm and the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP) [30–38].

The "total error" approach adds the systematic error (bias or trueness) and the random error (precision or standard deviation) to know the difference between the observed result and the true value. Thus, the total error estimation of an analytical method shows the biggest errors that may be encountered while using it.

The goal of the validation of an analytical method is to guarantee that a chosen proportion (set at 95% during this study) of future samples will fall between the acceptance limits fixed a priori (for pharmaceutical specialties, [−5%;5%]). This proportion is evaluated by the β-expectation tolerance intervals (well described in [33]) at each concentration level studied. If the β-expectation tolerance intervals are comprised within the acceptance limits then the expected proportion of results will be included within these limits.

The results obtained during the validation process are plotted with their β-expectation tolerance intervals and the acceptance limits, allowing a simple and fast evaluation of the present and future accuracy of the method.

2.9. Robustness

Robustness was performed on a standard solution prepared by mixing 25.0 ml of the validation standard solution of groups 1 and 2 at the medium concentration.

The diastereoisomer of tadalafil, trans-tadalafil, was prepared for the robustness testing. Sodium hydroxide was added to a solution of tadalafil in a mixture of H_2O/ACN (50:50, v/v). After mixing for 30 min, the solution was neutralized with trifluoroacetic acid. An aliquot of 3.0 ml of this solution was added to the 50.0 ml standard solution used for the robustness test.

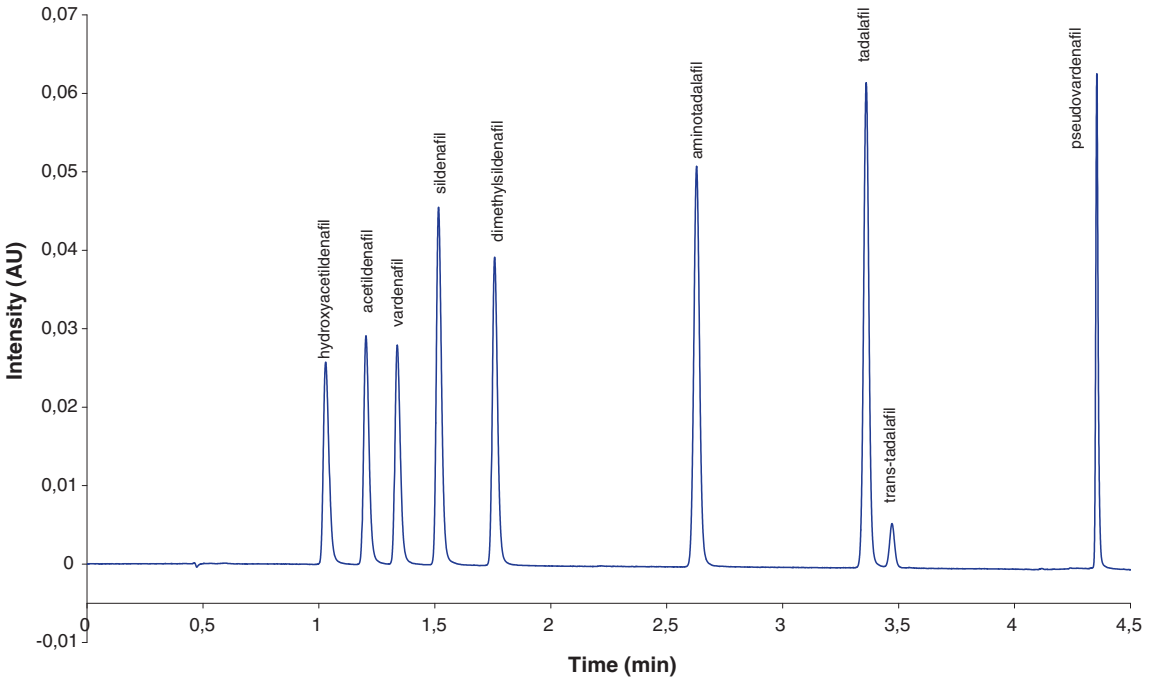

Fig. 3. Typical chromatogram obtained by applying the final gradient conditions for the UHPLC method.

2.10. Statistics

The statistics and computations were performed using Microsoft® Office Excel 2003.

The choice of the calibration model and the validation of the Excel results were performed with the E-noval™ software V3.0 (Arlenda, Liège, Belgium).

3. Results and discussion

3.1. Method development

3.1.1. Initial conditions selection

The separation method has been developed in HPLC with UV detection in order to be applicable by a large amount of control laboratories. Acetonitrile was chosen as organic modifier as it causes less back pressure and better baseline stability than methanol. A 0.1% formic acid aqueous solution (pH 2.8) was used as aqueous component of the mobile phase to be compatible with on-line mass spectrometry.

Initial HPLC conditions were a linear gradient starting from 5% acetonitrile to 100% in 27 min. The gradient time was calculated using the following equation considering 150 mm × 4.6 mm column dimensions:

$$\overline{k} \cong \frac{F \times t_G}{\Delta\phi \times V_m \times S} \quad (1)$$

where $\overline{k}$ is the mean retention factor (here set at 4), t_G is the gradient time (min), F is the flow rate (ml/min), $\Delta\phi$ is the difference between the final and initial percents of organic modifier divided by 100, V_m is the column dead volume (ml) and S is a constant (equal to 4 for small molecules).

The presented gradient conditions were used on different stationary phases (results not shown). The best results were obtained with a C_{18} polar embedded stationary phase such as an XTerra™ RP18.

During the optimization process, no satisfactory conditions were found with the 0.1% formic acid solution (pH 2.8) as aqueous phase. The problem comes from the fact that vardenafil co-eluted with hydroxyacetildenafil before the start of the gradient. Indeed, at pH 2.8, the three basic nitrogens of vardenafil are ionised decreasing the retention of the molecule in reversed phase conditions. It was then decided to set the pH at 3.5 to deprotonate partially vardenafil (pKa values of 8.8, 6.7 and 3.4). This pH value was obtained using a 10 mM ammonium formate buffer. The change of pH resulted in a higher retention of vardenafil. It was then possible to slightly adjust the gradient conditions to obtain the desired separation. These final gradient conditions are presented in Table 2.

3.1.2. Method transfer

The HPLC conditions were transferred to UHPLC as described in Section 2.6. The calculated initial UHPLC gradient conditions were slightly modified to obtain a better separation. Especially for the two critical pairs: acetildenafil/vardenafil and tadalafil/trans-tadalafil. The final UHPLC conditions (shown in Table 3) were then validated.

3.2. Method validation

3.2.1. Selectivity

The method selectivity was assessed by the constancy of the retention times and the UV spectrum of each component determined separately during the validation process.

3.2.2. Response function

Several response functions were tested. They are the unweighted linear regression, the linear regression after mathematic transformations (log, square root), the weighted linear regression ($1/X$, $1/X^2$) and the weighted quadratic regression ($1/X$, $1/X^2$). The unweighted linear regression model was chosen since it gives comparable results with the more complicated calibration models tested.

3.2.3. Linearity

The linearity of the relationship between the measured and theoretical concentrations was investigated over the concentration

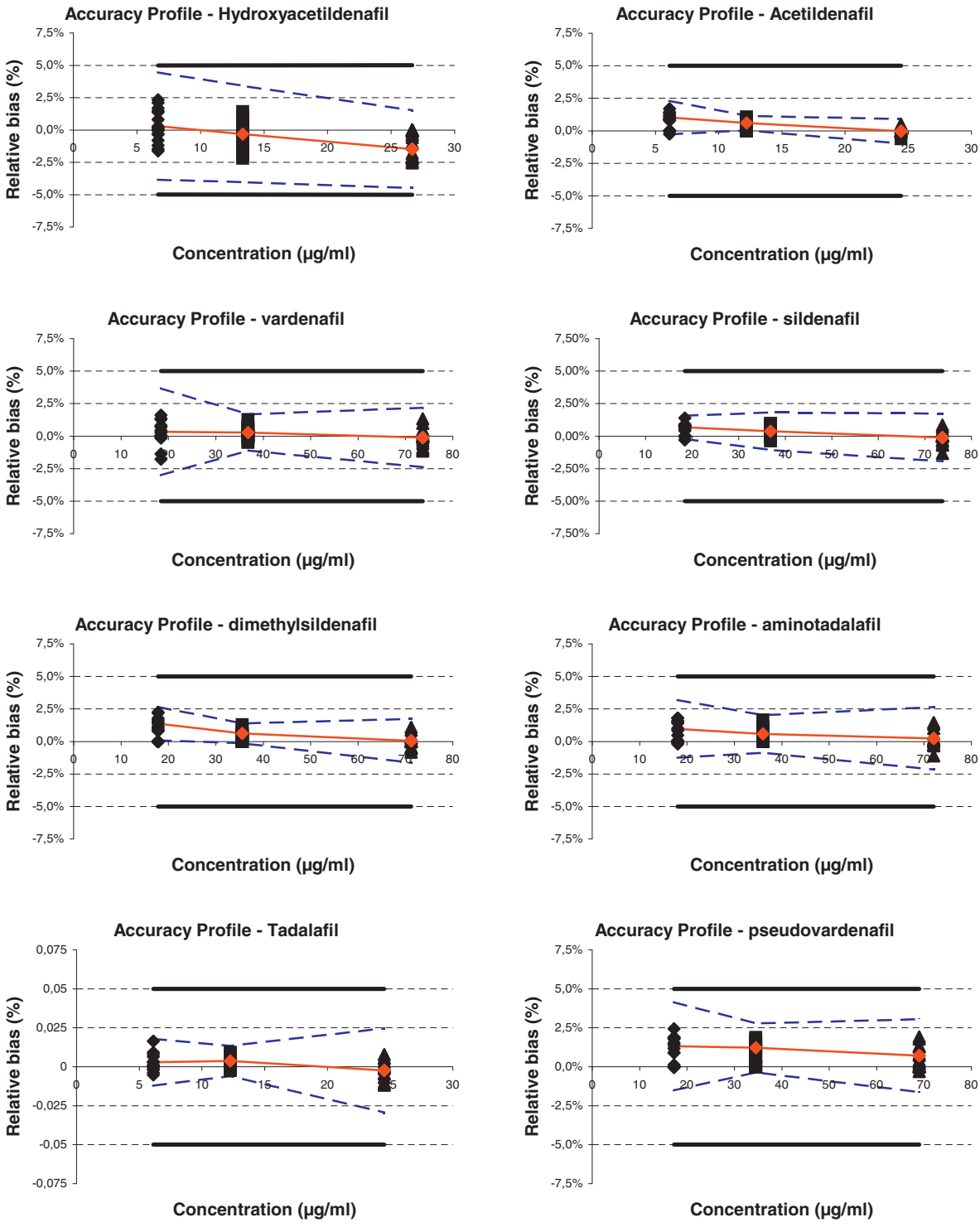

Fig. 4. Accuracy profiles of the studied compounds. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits (β = 95%) and the bold plain lines are the acceptance limits set at 5%. The dots represent the relative back-calculated concentrations of the validation samples, plotted with regards to their target concentration.

range described in Table 1. The measured concentrations were back-calculated using the selected calibration model. Validation results for tadalafil were both computed with an in-house Excel template and the E-noval software. The results obtained with Excel were comparable with those obtained with E-noval. The linearity of the results is expressed by the coefficient of determination (r^2). For the eight compounds the relationship was linear as the r^2 values were all >0.99 and the equation was close to $y = x$.

3.2.4. Trueness, precision, accuracy and uncertainty assessment

A statistical approach based on the "total error" profiles was applied to validate the method. All validation samples were analysed in triplicate for four consecutive days.

The concentrations were back-calculated using the calibration lines described in Section 2.4.1. 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 results are shown in Table 4.

Table 4
Trueness, precision, accuracy and uncertainty.

	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	1	1.37	0.50	1.10	0.38	0.52	0.78	0.64	0.80
(RSD %)	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	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]
tolerance limits (%)	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	1	2.99	1.09	2.41	0.81	1.12	1.73	1.35	1.78
uncertainty (%)	2	2.67	0.50	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 RSD values of repeatability and intermediate precision were inferior to 1% and 1.37%, respectively. These values are said acceptable since their maximal Horwitz ratio is inferior to 0.5 [39] (0.251 for sildenafil in Viagra®, 0.249 for tadalafil in Cialis® and 0.393 for vardenafil in Levitra®).

As the method will also be used for the analysis of registered medicines, the acceptance limits were set at ±5%. As shown in Fig. 4, the β-expectation tolerance intervals of each substance are within the acceptance limits with a probability of 95% except for the medium concentration level of acetildenafil and the highest concentration level of pseudovardenafil. However, the tolerance limits remain close to 5%.

The uncertainty of measurement [35] characterises the dispersion of the values that could reasonably be attributed to the analyte. The expanded uncertainty represents the interval around the results where the unknown true value can be observed at a confidence level of 95%. Relative expanded uncertainties (%) are obtained by dividing the corresponding expanded uncertainty by the corresponding concentration. The values are presented in Table 4 and are all below 3%.

3.2.5. Robustness

Robustness is the evaluation of the constancy of the results when variables inherent to the method of analysis are varied deliberately.

The test was performed by a three-factor three-level full factorial design [40]. The factors were the flow rate of the mobile phase, the column temperature and the pH of the ammonium formate buffer. The response was the resolution between tadalafil and trans-tadalafil (critical pair). The values were chosen to cover typical errors that could occur. Table 5 shows the experimental design. Each experiment was performed in triplicate and the mean value was used for computations.

The effect of each factor was calculated for its signification at 5% level using an ANOVA analysis. The regression is meaningful since the value of r^2 is 99.96%. From the ANOVA table, it can be seen that only the pH and the flow rate have a significant effect on the resolution (p-values <0.0001). However this effect is still very small since the resolution varies between 2.64 and 2.79.

The method can be considered as robust since only a very small change in resolution occurs.

3.3. Method comparison

The method was compared with a validated method considered as reference method.

The samples were prepared as described in Section 2.4.3 and analysed three times per day for seven consecutive days by applying UHPLC and HPLC methods. The minimum of days required and the comparison were performed according to the method described by Kuttatharmmakul et al. [29]. Results are shown in Table 6.

The variances of both methods were compared using a two-sided F-test at a significance level $\alpha = 0.05$ (6 degrees of freedom

Table 5
3-Factors 3-levels full factorial design performed for robustness evaluation.

pH	Temperature (°C)	Flow (ml/min)	Resolution tadalafil/trans-tadalafil
3.4	39	0.50	2.74
3.4	39	0.55	2.73
3.4	39	0.60	2.68
3.4	40	0.50	2.75
3.4	40	0.55	2.71
3.4	40	0.60	2.66
3.4	41	0.50	2.75
3.4	41	0.55	2.72
3.4	41	0.60	2.68
3.5	39	0.50	2.75
3.5	39	0.55	2.69
3.5	39	0.60	2.64
3.5	40	0.50	2.73
3.5	40	0.55	2.70
3.5	40	0.60	2.65
3.5	41	0.50	2.73
3.5	41	0.55	2.71
3.5	41	0.60	2.67
3.6	39	0.50	2.77
3.6	39	0.55	2.76
3.6	39	0.60	2.70
3.6	40	0.50	2.78
3.6	40	0.55	2.76
3.6	40	0.60	2.71
3.6	41	0.50	2.79
3.6	41	0.55	2.76
3.6	41	0.60	2.69

Table 6
Results of the comparison tests.

	Concentration level 1	Concentration level 2	Concentration level 3
Variance of the reference method (σ_A^2)	0.429	0.113	2.825
Variance of the new method (σ_B^2)	0.516	0.091	2.624
t-Statistics for bias between both methods	0.14	1.22	1.02
Interval hypothesis test (%)	[−1.06;1.25]	[−0.35;1.86]	[−0.74;2.75]

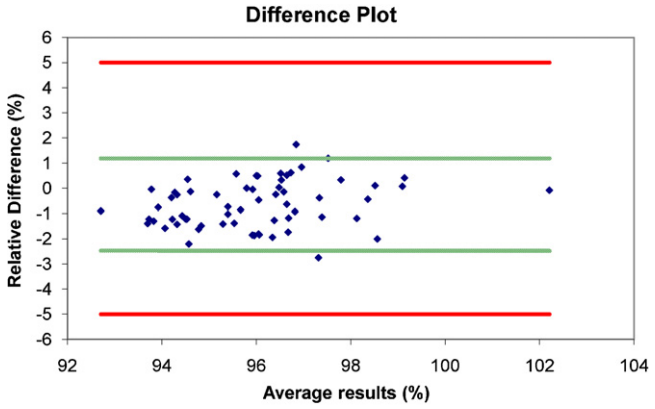

Fig. 5. Bland and Altman plot of the relative differences (%) of the results obtained by the HPLC reference method and the new UHPLC method against the average content of API (%) for the three concentration levels results of the two methods. *Dashed lines*: 95% agreement limits of the relative differences; *Continuous lines*: maximum acceptable relative difference between the two methods set at ±5%; *Dots*: relative differences.

for both methods). The variances were not shown as statistically different since all F-statistics are below the critical value of 5.82.

The bias between the methods was tested using a paired *t*-test comparing the grand means of both methods. The differences between the grand means was considered statistically non significant since their value is below the critical value of 2.18 ($\alpha/2 = 0.025$, 12 degrees of freedom for both methods).

The interval hypothesis test described by Hartmann et al. [41] was performed to be sure of not accepting a new method with an unacceptable bias. For the interval hypothesis test, a bias of 2% was said to be acceptable. As can be seen in Table 6, these requirements are fulfilled for the concentration levels 1 and 2. However, the highest concentration level has an unacceptable bias which means that the HPLC method is best suited at that concentration for the assay of sildenafil citrate in Viagra® tablets.

A Bland and Altman plot [42] is shown in Fig. 5. This plot represents the relative differences (%) between the HPLC reference method and the new UHPLC method against the average content of API (%) for the three concentration levels. As one can see, 95% of the relative differences are comprised between [−2.61%;1.18%]. Those results are comprised between the maximum acceptable relative differences between the two methods set at ±5%. It is finally concluded that the two methods gave comparable results.

4. Conclusion

This paper describes for the first time a fully validated method which enables the detection and the quantification of authorised phosphodiesterase type 5 inhibitors and some of their analogues in less than 4.5 min. 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 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 other new structurally related substances 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.

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).

An important point in counterfeit medicines detection is the cost of the UHPLC system and its applicability in developing countries. However, this is not really a problem since PDE5 inhibitors are mainly sold in rich and industrialised countries.

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