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Article

DRUG FORMULATIONS

Application of Design Space, Uncertainty, and Risk Profile Strategies to the Development and Validation of UPLC Method for the Characterization of Four Authorized Phosphodiesterase Type 5 Inhibitors to Combat Counterfeit Drugs

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Abstract

Background: Counterfeit medicines are an increasing scourge that are difficult to identify and they have become industrialized and widespread through highly organized illegal channels.
Objective: This research aims to develop a robust method to determine four phosphodiesterase type-5 inhibitors in counterfeit drugs based on ultra-performance liquid chromatography.
Method: Experimental design methodology (DOE) and design space (DS) recommended by ICH Q8 were used side-by-side in the development phase to define the optimal parameters as well as the robustness of the chromatographic method. Moreover, both the uncertainty and risk profile derived from the β-content and γ-confidence tolerance interval were investigated during the validation phase to examine the performance of this method.
Results: Successful chromatographic results, in a high resolution between the four active ingredients and an optimal analysis time of less than 1.6 min, were achieved at the end of the optimization phase. In addition, validation results show a low risk of future measurements outside acceptance limits set at 5%.
Conclusions: Our procedure was successfully applied in the routine phase to identify 23 illicit formulations of an erectile dysfunction drug.
Highlights: An efficient method for the characterization of 4 authorized phosphodiesterase in less than 1.6 min was established. A DS approach was applied to test the performance of this analytical method during analytical development. A risk profile was then carried out to approve the validity of the analytical method through the uncertainty profile approach.

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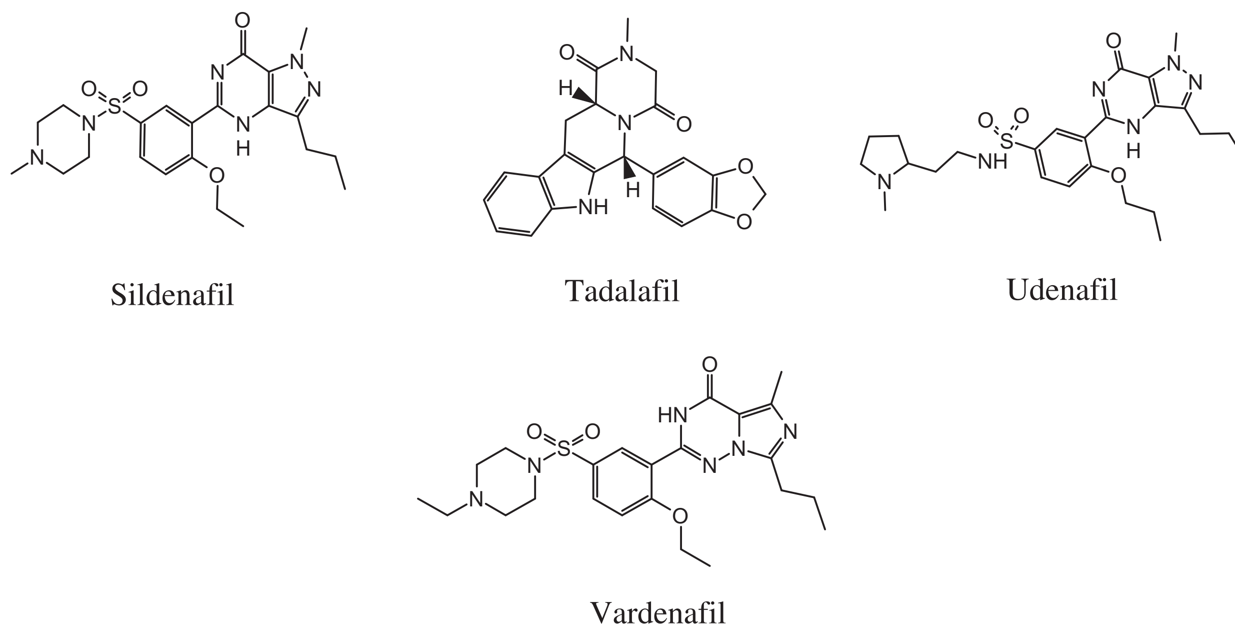

Figure 1. Chemical structure of Phosphodiestérase type 5 inhibitors.

Counterfeit medicines (CM) are an increasing scourge that are difficult to identify and they have become industrialized and widespread through highly organized illegal channels (1). This phenomenon takes different forms and affects both the drug and its packaging and is spreading into different therapeutic classes to focus on products that affect serious diseases (2).

According to the World Health Organization, accessibility to CM, including regular consumption of such medicines, has serious consequences for patient health. Counterfeit products can harm patients and do not treat the diseases for which they are intended. Moreover, these products are, by nature, very difficult to detect as they are often designed to appear identical to the authentic product (3).

Phosphodiesterase type five inhibitors (PDE-5i) (Figure 1), designed for the treatment of erectile dysfunction, are a scientific breakthrough because of their medicinal implications in different areas (4). Outside of the formal health system, illicit PDE-5i have become quite popular and widespread in many countries (5).

Pharmaceutical laboratories, in collaboration with health authorities, must implement preventative measures and technical solutions, not only to protect the authenticity of pharmaceutical products but also to ensure the health of patients. To achieve these objectives, new analytical methods have been developed.

Recently, several analytical techniques (LC-UV, LC-MS, IR, NMR, X-ray diffraction) have been used to identify the characteristics of counterfeited drugs. Some techniques, such as near-infrared spectrometry and Raman spectroscopy (6) are used to identify the chemical fingerprint of the product, while X-ray powder diffraction (7), NMR (8), liquid chromatography–electrospray ionisation mass spectrometry (9), gas chromatography–mass spectrometry (10), high performance liquid chromatography (HPLC) (11), and ultra-high performance liquid chromatography (9) are designed to determine the composition of the active ingredient, toxic compounds, and impurities. In particular, liquid chromatography coupled with various detectors has been frequently used for the analysis of these substances (12–14).

To date, different chromatographic methods for the simultaneous characterization of PDE-5i have been developed (12, 15, 16). Most authors developed their method without providing any information about the technique used to optimize the experimental parameters. An overview of the literature (Supplemental File 1) has shown that the proposed chromatographic methods focus solely on the identification of three PDE-5i and their analogues, while no HPLC or ultra performance liquid chromatography (UPLC) method has been reported for the simultaneous determination of sildenafil, tadalafil, vardenafil, and udenafil in pharmaceutical preparations or in CM.

In this context, the main objective of this work is to develop a new analytical strategy to fight the counterfeiting of erectile dysfunction drugs. Our goal is to combine a set of chemometric tools to develop a chromatographic method that will serve as an analytical device to fight against CM. Through this process, it is hoped that a procedure can be created to accurately detect counterfeit tablets of the various PDE-5i, in particular, sildenafil, tadalafil, vardenafil, and udenafil.

Initially, several factors were selected such as pH, flow rate, and percentage of organic modifier to maximize the chances of identifying optimal separation. Box-Behnken design was then carried out in order to fix the optimal values of each influencing factor. This is a chemometric tool that is usually applied to the optimization of analytical methods (17).

In order to combine the purpose of the developed method with the guiding principles of the International Conference on Harmonization (ICH) Q8 (18), a design space (DS) was established. A key component of Quality by Design, it provides assurance of quality by defining the robustness zone of the optimized method (19). This DS makes it possible to better assimilate the critical parameters of the UPLC method and understand its operating mode. As a result, scientific evidence of this space can be used as a basis for quality risk management in the future use of the routine method.

However, the DS alone is unable to predict the analytical performance of the method when applied in the routine phase; it must be supported by analytical validation. At this stage, the chromatographic method has been subjected to an evaluation of its analytical performance through two holistic validation approaches recently proposed by our laboratory, called the

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uncertainty profile and risk profile (20). These two profiles are derived from the tolerance interval, and the concept of uncertainty was used to assess the efficiency of the measurements generated by the UPLC procedure. Finally, we combined theory with practice through the analysis of 23 counterfeit samples containing four PDE-5i, to reflect on the strengths and weaknesses of our chromatographic procedure.

METHOD

Instrument Parameters

Optimization and validation experiments were performed on an Acquity UPLC® H CLASS system. The system is comprised of a quaternary solvent manager, a sample manager with flow-through needle and a photodiode array detector. Data processing was done with Empower 3 software. Ultrapure water was obtained from an ELGA LabWater Classic UV. The pH of the buffer solution was controlled by Schott ProLab 3000 (Germany).

Analytical Condition

Two stationary phases were investigated during preliminary trials, namely: ACQUITY UPLC ® HSS T3 (2.1 mm × 50 mm, 1.8 μm) and ACQUITY UPLC ® BEH C18 (2.1 mm × 50 mm, 1.7 μm). Results using ACQUITY UPLC ® HSS T3 (2.1 mm × 50 mm, 1.8 μm) showed poor resolution, therefore the separations were carried out on a column, ACQUITY UPLC ® BEH C18 (2.1 mm × 50 mm, 1.7 μm), at a temperature between 40°C and 50°C in isocratic mode. The experiments were carried out with a flow rate of less than 1mL/min and the injection volume was 1 μl. The mobile phase consisted of a mixture of a buffer solution: ammonium formate acid (10 mM) with a pH 3.3, a flow rate 0.635, and acetonitrile in the proportion of (69:31, v/v). The analytes were monitored at an optimum wavelength of 280 nm while chromatographic data were recorded from 210 to 400 nm for all the studied experimental conditions.

Standards and Reagent

Reference standards of udenafil, tadalafil, sildenafil citrate, and vardenafil were provided by the National Laboratory of Medicines Control (LNCM). Acetonitrile (HPLC grade), formic acid (98%), and ammonium formate were supplied by LNCM. The matrix used in the validation phase was comprised of functional excipients to assimilate the required properties of the pharmaceutical form. It contains the following compounds: corn starch, sodium lauryl sulphate, colloidal silicon dioxide, lactose, hydroxypropyl cellulose, talc, magnesium stearate, microcrystalline cellulose, croscarmellose sodium, and Instacoat.

Sample and Standard Preparation

Sample medicines preparation

Ten tablets of each sample were pulverized. An amount of the pulverised tablets was accurately weighed and diluted in an ACN/water mixture (70/30) in a 50 mL flask. Then, 1 mL of this solution was transferred into a 25 mL flask containing 10 mL of the same mixture, this was stirred for 5 min in the ultrasonic bath and the flask was then filled to the mark with eluent. The final solutions were directly filtered through 0.45 μM filters.

Herbal sample and syrup preparation

An amount of 500 mg of each sample was accurately weighed and diluted in a mixture of ACN/water (70/30), these solutions were magnetically stirred for 30 min and sonicated for 10 min. The final solutions were directly filtered through 0.45 μM filters.

For candies and honey, the preparation was similar to the previous preparation, but included adding the quantity of each sample to a dilute mixture.

Standard preparation

A mixture of four PDE-5i was used and prepared as follows: in a 25 mL flask, 5 mg of the sildenafil, vardenafil, tadalafil, and udenafil standards were dissolved in the dilution phase. Then, 1 mL of the stock solution was diluted in a 50 mL flask with ACN/water (70/30).

The final concentration was 0.004 mg/mL of each substance studied. Successive dilutions were performed to obtain five concentration levels for standards calibration (SC). Three repetitions for each concentration level were replicated over 3 different days.

For standards validation (SV), a stock solution containing all the active ingredients was prepared in the same way as the SC and the excipients of each pharmaceutical formulation were added to the stock solution. The calibration range covers an interval of 50% to 150% of the nominal value according to the ICH standard.

SV was prepared in the matrix to simulate the drug formulation as much as possible. For each level of concentration, three independent repetitions ($n = 3$) were performed for 3 different days ($p = 3$).

Calculation

JMP 7 software was used for the statistical data treatment of the experimental design as well as the drawing of the DS plot. The validation treatment of the uncertainty profile, including the estimation of the uncertainty, was obtained with Matlab R2006a software.

Development and Robustness

The method was optimized by Box-Behnken design taking into consideration resolutions between peaks and retention time of the last peak. Based on preliminary studies, three factors were involved in the experimental design. As concerns the experimental space investigated, flow rate of the mobile phase ranged from 0.55 to 0.65 mL/min; percentage of the organic modifier from 31 to 37%; pH from 3.3 to 3.7. The risk associated with the development of an analytical method was evaluated through the DS.

Validation of the Method

The developed method for the simultaneous determination of the presence of sildenafil, vardenafil, udenafil, and tadalafil was fully validated using the uncertainty profile (21). This included the evaluation of the following parameters: system suitability, specificity, precision and accuracy, response function and uncertainty profile, limit of quantification, linearity, and risk profile.

Results and Discussion

Analytical Development

The Box-Behnken design was used to look for the optimal values of the most influential factors, thus, to define a well-adapted model. The experimental design consists of 15 tests,

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comprising three points in the center of the experimental domain.

Analysis of variance results of the quadratic models calculated for four responses are presented in (Supplemental File 2).

The sum of the squares due to the error is very small compared to the sum total. This indicates that the model is well adjusted. The analysis of the variance proposed here is a detailed analysis because the sum of the squares, due to the residues, has been decomposed in sum due to the lack of fit and the pure error. The replicas executed in the center of the experimental domain bring out the pure error, consequently, they enable the lack of fit test.

The values (Supplemental File 3) indicate that the test does not detect any mismatch of the fit, therefore, it is possible to test hypothesis H0" H0 (bi = 0, $\alpha = 0.05$) smaller level of importance leading to rejection of the hypothesis", that the model is well adjusted on average.

The data suggests that the most significant factors are under 0.05 (Supplemental File 3).

In Supplemental File 3, it is noted that the proportion of acetonitrile is the most significant factor affecting all the responses, however, the flow rate affects the retention time and resolution 1, while the pH affects resolutions 1 and 2. In addition, the quadratic terms also have a significant effect such that the quadratic term X_1^2contributes significantly to the four responses and the X_3^2 affects resolutions 1 and 2. In parallel, the interaction X_1X_3 has a significant effect on the resolution 1, and X_2X_3 has an effect on the retention time.

The %ACN, flow rate, and pH take simultaneously the symbols X1, X2, and X3Responses were modelled using the least squares regression method, non-significant terms (p-value > 0.05) are removed from these models. The equations of the adjusted models are:

$$Y_1 = 1.095 - 0.382X_1 - 0.072X_2 + 0.073X_1^2 - 0.049X_2X_3 \quad (1)$$

$$Y_2 = 3.081 - 0.71X_1 - 0.019X_2 - 0.106X_3 + 0.101X_1^2 - 0.030X_2^2 + 0.038X_1X_2 \quad (2)$$

$$Y_3 = 6.314 - 1.997X_1 - 0.135X_3 + 0.206X_1^2 + 0.149X_2^2 \quad (3)$$

$$Y_4 = 5.268 + 0.342X_1 - 0.313X_1^2 \quad (4)$$

The model calculated for resolution 3 accounts for about 94% of the variance of the experimental results. The models calculated for retention time, resolution 1, and resolution 2 exceed 99%.

In order to find the optimal conditions for the variation of each factor, a desirability function was carried out. The desirability value is equal to 1, these results lead to well-resolved chromatographic peaks of the order of 4.00, 8.86, and 4.60 with good symmetry and a minimum retention time equal to 1.573 min.

Robustness and DS

Based on the predictive statistical models of retention time and resolutions, a DS was established. Two actions were realized, the definition of final experimental conditions and the evaluation of the robustness on the experimental domain.

Maintaining certain potential conditions is essential to build the DS (the first requirement for the method). A good resolution (Rs) assumes that Rs ≥ 1.5, while a second consideration is the shorter retention time (Tr). Finally, a parameter is considered robust only if the obtained result is flexible and durable in its experimental domain.

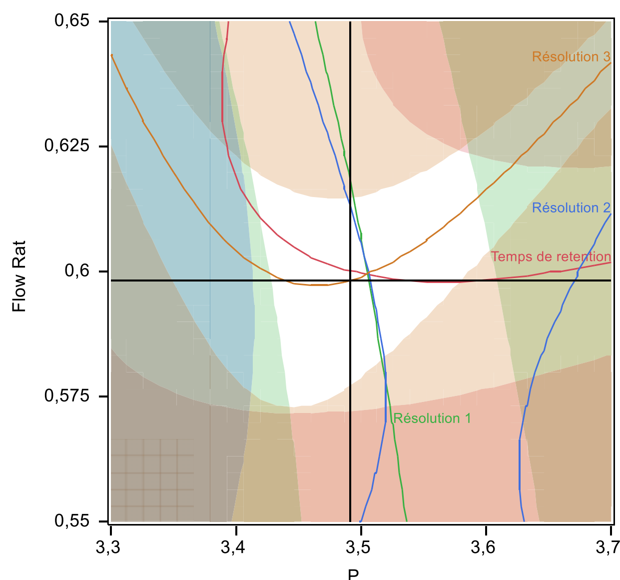

Figure 2. DS of the robust area of all selected responses against three independent factors (the pH of the buffer solution X1 and the flow rate X3), with % ACN equal to 31.

The DS models the simultaneous influence of three parameters of the critical response (Rs and Tr). The method's zone of robustness can be visualized by the white zone (Figure 2), in which the critical resolutions cannot be lower than the fixed value for a retention time less than 1.6 min.

Figure 2 shows a DS (flow-pH-%ACN) in which the robustness can be defined as flow rate 0.6 ± 0.025 mL/min, pH 3.5 ± 0.1, and a constant level of % ACN equal to 31.

To challenge these results, we performed a robustness test using the Plackett-Burman design (22). The choice of factors and their levels of variation, as well as the selection of the experimental design and the responses, were made on the basis of the DS parameters elaborated above. After the execution of the experiments and obtaining the results, we evaluated the effects of the factors on the quantitative answers by browsing two analytical tools, namely the statistical test and graphical approach.

The effect of each factor was calculated by the following equation:

$$E_x = \frac{\sum Y(i)}{N/2} - \frac{\sum Y(j)}{N/2} \quad (5)$$

where $\Sigma Y(i)$ = the sum of the responses when factor x is at the levels +1, $\Sigma Y(j)$ = the sum of the responses when factor x is at the levels −1, and N = the number of experiments of the design.

The significance of the effects of each factor was statistically evaluated by comparison with the critical threshold (critical effect or $E_{critical}$) derived from Student's t-law (23):

$$E_{critique} = t_{critique} \cdot (E_{er}) \quad (6)$$

where E_{er} = the estimated standard error and $t_{critique}$ = the value of the related Student's t-law.

A significant effect is considered when its absolute value is greater than that of the critical effect (where the corresponding p-value is less than 0.05). The standard error (E_{er}) is estimated from the repetitions performed at the center of the experimental domain.

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Regarding the results of the statistical calculations (Supplemental File 4), we noticed that our results are not affected by small deliberate changes in the analytical parameters.

In addition, the effects of the factors can also be interpreted graphically so as to assess the significance of each factor; the distribution of the estimated effects was carried out. The difference between the mean values of the responses obtained at the two levels of the experimental design, −1 and +1, indicates the effect of each factor on the response. Markers comprised of factors with small or insignificant effects (the difference observed between the two means is small) on the response will describe (approximately) a straight line on the half-normal probability plot. As a result, several graphs can be drawn for quick visual evaluation.

From the half-normal probability plot (Supplemental File 5), we found that no factor affects the determination of PDE-5i. Considering these results, the different experimental conditions lead to obtaining an optimal separation with reduced retention time.

In a very innovative framework, the experimental design methodology was used in conjunction with the DS in order to simultaneously optimize the chromatographic analysis and to estimate the robustness of the method on the experimental domain.

Analytical Validation

System suitability

The aptitude test of the analytical system is evaluated by comparing the coefficients of variation of a determination method obtained with the reference values. The United States Pharmacopeia emphasizes that the coefficient of the variation of a method of assay should be less than or equal to 2% (24). Note that the coefficients of variation of the retention time and the coefficients of variation of the peak areas do not exceed 2% for six repetitive injections (Table 1).

Table 1. System suitability test (CV < 2%)

	Coefficient of variation (CV%)		
Compounds	Retention time	Pick area	Resolution
Vardenafil	0.19	0.5	......
Sildenafil	0.22	0.8	3.74
Tadalafil	0.15	0.9	3.51
Udenafil	0.17	0.5	7.25

Selectivity

The selectivity of the method was evaluated by comparing the typical chromatograms obtained by analyzing a blank solution (the mobile phase), a calibration standard solution (standard), and a standard validation solution (standard + excipients). The results obtained indicate the absolute absence of peaks or interference at the retention time corresponding to the peaks of four substances (Figure 3).

Response function

From the calibration standards, several regression models were calculated to demonstrate the relationship between the concentrations (PDE-5i) and the response (peak area). For each measurement series, and based on the regression models found, the concentrations of validation standards were back-calculated.

Using the previous calculation, we obtained the average relative bias for all concentration levels, as well as the tolerance interval of future measurements for a proportion, β, equal to 95% with a confidence level, γ, equal to 95%. The uncertainty profiles were then plotted according to these results (Figure 4).

The acceptance limits of the dosage of active ingredient in a pharmaceutical form were set at 5%. We examined the uncertainty profiles obtained for four substances. Considering the simple linear regression model, the relative uncertainty limits (LIR) do not exceed the acceptance limits (LA) of 5% for the four substances (Figure 4). Since the LIR does not exceed the LA at different levels of concentration, the purpose of the method is still achieved. This objective is based on characterizing the PDE-5i in counterfeit samples with a probability of future measurements that do not exceed the 5% acceptance limit. As a result, the simple linear regression model was the most suitable for

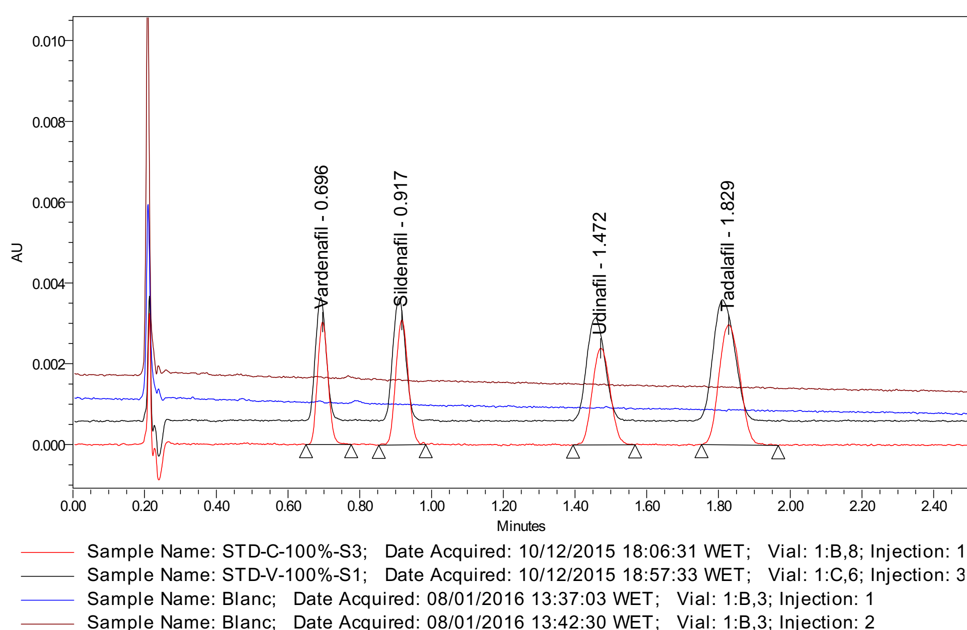

Figure 3. Typical chromatogram obtained after successive injection of standard calibration, standard validation, and mobile phase.

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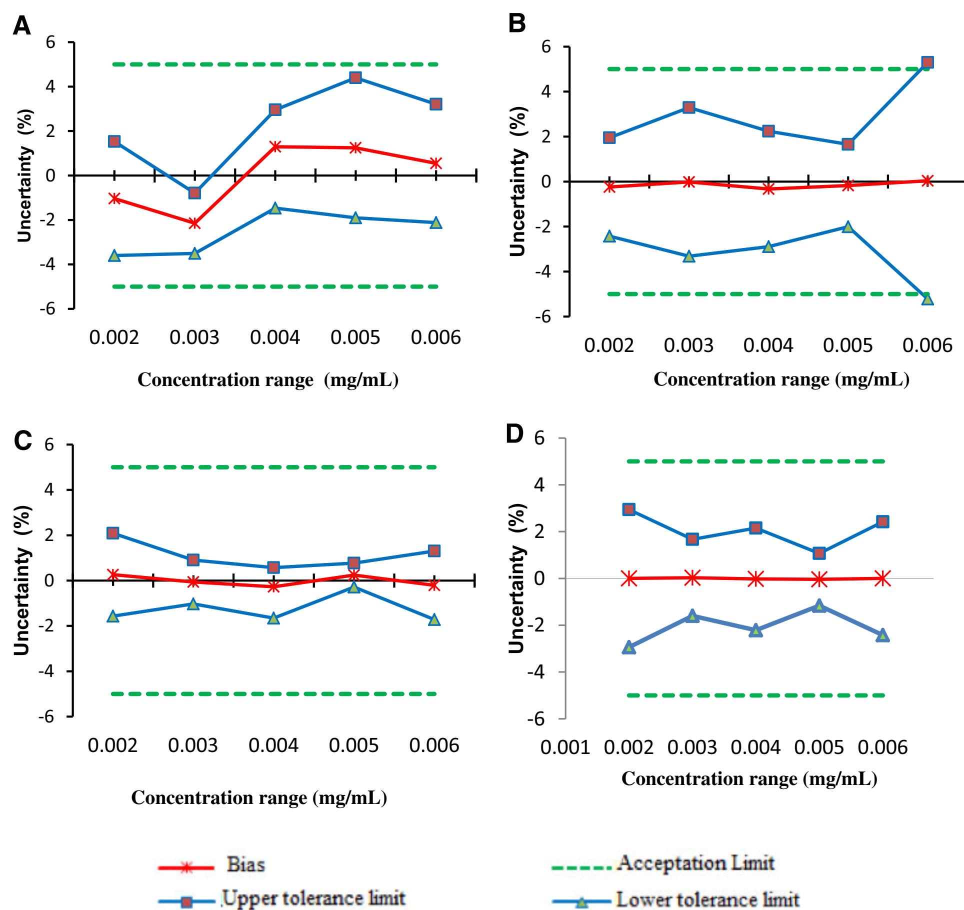

Figure 4. Uncertainty profile plot of the assay of (PDE-5i) by UPLC-DAD in a pharmaceutical matrix. [(A) Sildenafil, (B) Vardenafil, (C) Udenafil, and (D) Tadalafil], (Response function: simple linear), the acceptance limits set to 5%.

inverse prediction; it has provided reliable results that are consistent with the purpose of this method.

Accuracy

Accuracy is a considerable criterion in analytical validation as it evaluates the systematic error of an analytical method. It is represented by relative biases, which are determined from the concentrations found. The relative biases obtained are less than 2% for all the substances except for sildenafil at concentration level 2 near 2% according to Table 2.

Considering these results, the accuracy of the method is acceptable.

Precision

Precision is an indication of a random error. It has been estimated at two levels, namely repeatability and intermediate precision, considering each level of concentration used in validation. Table 2 summarizes the coefficients of the variation of repeatability and intermediate precision. The results do not exceed the value of 1.4% and they have a maximum Horwitz ratio of less than 0.5 (25). These values indicate homogeneity of the validation samples, therefore, an acceptable fidelity.

Uncertainty profile

Tolerance intervals were established according to the Mee method (26) where the β and γ values were set at 95%. The calculated uncertainty limits are expressed in relative values (Table 2). The profiles obtained in Figure 4 show that no limit of uncertainty has exceeded the acceptable limit of ± 5%. Similarly, according to the results in Table 2, the values of the relative uncertainty limits do not exceed the acceptance limit of ± 5%. This method was successfully validated.

Limit of quantification

The limit of quantification (LOQ) is the smallest amount of substance analyzed under well-defined experimental conditions (27) and was estimated using the following relation:

$$LQ = 10 \times \frac{S_{a0}}{a_1} \quad (7)$$

where S_{a0} = the standard deviation of the response and a_1 = the slope of the calibration curve.

Table 3 provides an overview of the estimated LOQ for each of the substances under consideration.

Linearity

The linearity of an analytical procedure is its ability to obtain, within a certain assay interval, results directly proportional to the concentration of the test substance in the sample. The choice of linear range from 0.002 mg/mL was based principally on the experimental condition. Indeed, we couldn't measure less than 5mg of each calibration standard, because the balance could not provide accurate value. In parallel, with the quantity

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Table 2. Results of analytical validation of assaying (PDE-5i) by UPLC-DAD using uncertainty profile

Estimated parameters	Concentration levels	Sildenafil	Vardenafil	Udenafil	Tadalafil
Relative bias, %	1	−1.05	−0.23	0.22	0
	2	−2.18	−0.01	−0.03	0.03
	3	1.30	−0.32	−0.25	−0.02
	4	1.26	−0.17	0.22	−0.04
	5	0.53	0.037	−0.22	0
Repeatability	1	0.68	0.78	0.17	0.37
	2	0.58	1.15	0.40	0.25
	3	0.54	1.08	0.28	0.25
	4	1.30	0.57	0.22	0.07
	5	1.19	0.55	0.12	0.27
Intermediate precision	1	1.02	0.86	0.41	0.45
	2	0.58	1.30	0.43	0.26
	3	0.95	1.08	0.43	0.34
	4	1.30	0.69	0.22	0.22
	5	1.19	1.20	0.38	0.38
Relative uncertainty limits	1	[−3.6, 1.5]	[−2.4, 1.9]	[−1.98, 2.42]	[−2.94, 2.94]
	2	[−3.5, −0.8]	[−3.3, 3.3]	[−2.72, 2.65]	[−1.60, 1.67]
	3	[−1.5, 3.0]	[−2.9, 2.2]	[−2.92, 2.42]	[−2.21, 2.15]
	4	[−1.9, 4.4]	[−2.0, 1.7]	[−0.88, 1.33]	[−1.16, 1.07]
	5	[−2.1, 3.2]	[−5.2, 5.3]	[−2.16, 1.71]	[−2.42, 2.42]
Relative expanded uncertainty, %	1	5.97	5.46	2.20	2.94
	2	2.84	8.34	2.68	1.64
	3	5.72	5.47	2.67	2.18
	4	6.85	4.49	1.11	1.11
	5	4.13	6.69	1.94	2.42
Risk, %	1	0.92	0.13	0.36	0.009
	2	0.10	1.08	0.00	0.00
	3	2.37	0.27	0.07	0.008
	4	1.48	0.08	0.00	0.14
	5	0.52	4.14	0.42	0.02
Decision		Valid	Valid	Valid	Valid

Table 3. Estimated value of limit of quantification of the 4 compounds

Compounds	LQ, µg/mL
Sildenafil	0.00034
Vardenafil	0.00093
Udenafil	0.00218
Tadalafil	0.00115

upper than 5 mg, the preliminary trials give insignificant areas and heights peak.

For each standard, 5 mg allows the determination of the appropriate peaks. Indeed, after two successive dilutions, namely 10% and 4%, the final concentration was 0.004 mg/mL. According to Huber et al. (28), the concentration levels that can be used for the determination of a chemical substance (active ingredient) in a pharmaceutical specialty (matrix) includes three levels, namely 120%, 100%, and 80%.

In order to demonstrate the ability of our method and to cover a large concentration range weenlarged the range from 50% to 150%, obtaining the linear range of 0.002–0.006 mg/mL.

In this context, we applied the approach based on absolute tolerance intervals. For a method to be demonstrated as linear, the absolute tolerance limits should be within the predefined acceptance limits. Figure 5 clearly shows that the absolute tolerance limits are within the acceptance limits of ± 5%, so the linearity of the developed UPLC method is demonstrated.

Risk profile

To provide a minimum reliability probability needed for the subsequent routine application of the method, a risk assessment was examined to consider each level of concentration used in the validation. It is a recently recommended tool for acquiring diverse information to understand and improve analytical procedures.

This approach consists of estimating the probability to get a result outside these acceptance limits. It was first adapted from Dewé et al. (29), then by Govaerts et al (30).

Recently, El Hajji et al. (20) proved the relationship between the tolerance interval and measurement uncertainty and they give an innovative formula for calculating the risk based on the measurement of uncertainty:

$$\pi_i^{Risk} = P\left[t(\nu) > \frac{\lambda(\%) - bias(\%)}{u(\%)}\right] + P\left[t(\nu) < \frac{-\lambda(\%) - bias(\%)}{u(\%)}\right] \quad (8)$$

where λ = the acceptance limit, $bias(\%)$ = the relative bias of the method, u (%) = the relative uncertainty of the method, and $t(\nu)$ = the quantile b of the Student's t-law distribution with f degrees of freedom.

Through equation (8), several response functions were evaluated to calculate the probability that future measurements generated by our chromatographic method will exceed the acceptance limits.

The risk profile was examined considering each level of concentration used in validation. Several response functions were

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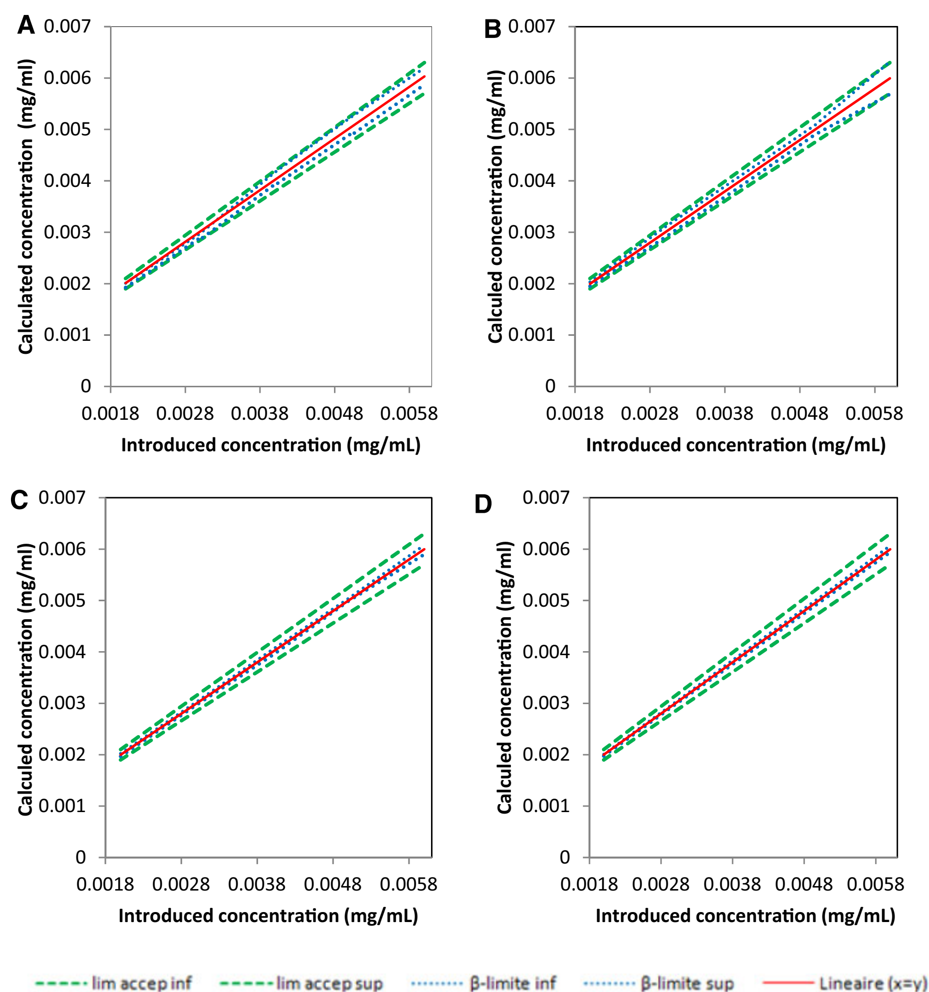

Figure 5. Linearity profile plot of the UPLC method for the determination of: (A) Sildenafil, (B) Vardenafil, (C) Udenafil, and (D) Tadalafil.

evaluated to calculate the probability that future measurements generated by our chromatographic method will exceed the acceptance limits.

Only three response functions are used to estimate the risk of future measurements within the acceptance limits, namely simple linear, weighted linear, and the model after a square root transformation (Figure 6). However, considering the last two models, the risk of having future measurements outside the 5% acceptance limits is important for vardenafil at a concentration level of 0.006 mg/mL. In contrast, the simple linear regression model is capable of providing future measurements included in the acceptance limit. We considered the simple linear regression model to establish the risk profile in order to justify the results obtained with the uncertainty profile and to retain the uniqueness of the applied model.

Figure 6 shows that the risk of future measurements outside the 5% acceptance limits is practically low for all concentration levels examined. These results are in perfect agreement with those obtained by the uncertainty profile. Considering the simple regression model, the risk does not exceed 5% for the four substances; it is concluded that the method is valid.

Routine application

The proposed method was applied to the analysis of 23 actual samples including dietary supplements, counterfeit medicines, and herbal preparations for erectile dysfunction. The preparations of these samples have been adapted so that the concentrations are in the dosing intervals.

The concentrations of four PDE-5i detected in the counterfeit samples are presented as recovery rates compared to the nominal quantities claimed (Table 4).

The results showed that 91% of the samples contained at least one of the targeted compounds. Of the samples studied, sildenafil was found in 78% of samples at concentrations ranging from 0.18 mg to 400 mg per dose unit. Tadalafil was found in 17% of samples with a variation of less than 1mg per sample, whereas the composition of a single sample was negative.

In most samples, sildenafil was found, but, as shown in Table 4, most of the products were somehow noncompliant. For example, the sildenafil content should be between 98% and 102% according to European Pharmacopoeia, compared to the declared nominal amount. For samples coded M1 → M9 (Table 4), the amount of sildenafil recovered varies between 6% and 392% relative to the claimed nominal quantity of 100 mg, whereas for the M6 sample, 98% of the required quantity for 50 mg was measured. The amount of other inhibitors measured in these samples was low or even inexistent. Given the underestimate/overestimate quantities claimed for the active ingredients, these samples are considered as noncompliant. Similarly, the other coded samples (M10 → M13, CA, and C) were

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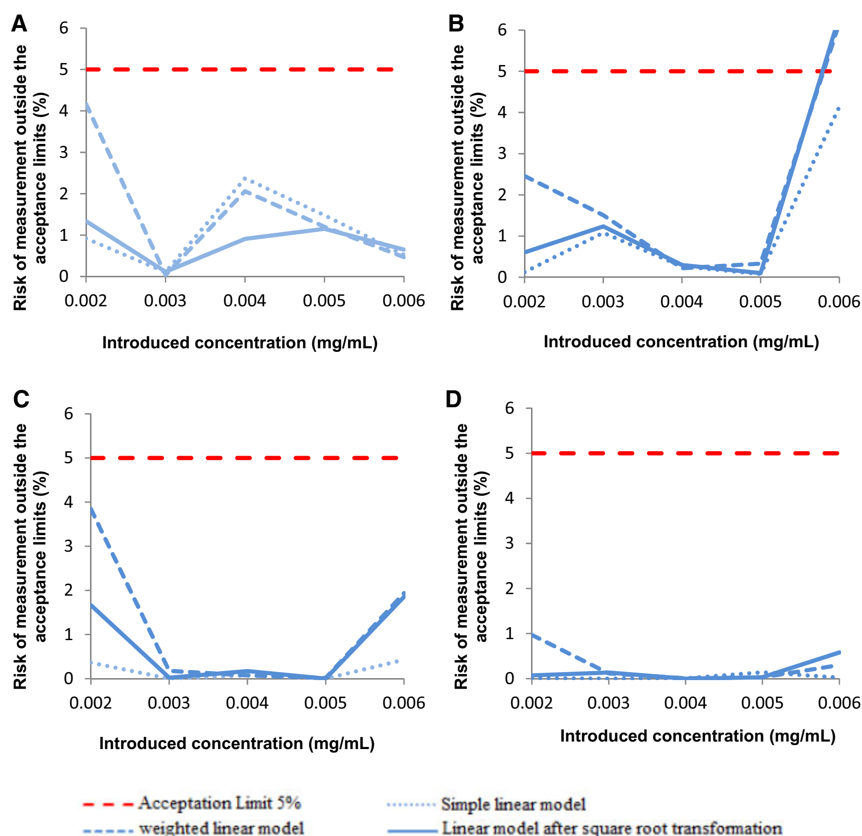

Figure 6. Risk profile plot of the method (acceptation limit 5%). Maximal tolerated risk is equal to 5%, [(A) Sildenafil, (B) Vardenafil, (C) Udenafil, and (D) Tadalafil].

Table 4. Recovery percentage of active ingredients in counterfeit samples (the nominal amount claimed in sildenafil equal to 100 mg; the recovery rate of a conforms sample is between 98% and 102%)

Samples[a]	Types	Recovery percentage of active ingredients, %				Unknown impurity
		Sildenafil	Tadalafil	Vardenafil	Udenafil	
M1	Tablet	147	–[b]	–	–	–
M2	Tablet	392	–	–	–	–
M3	Tablet	<1	–	–	–	+1[c]
M4	Tablet	176	–	–	–	–
M5	Tablet	113	–	–	–	–
M6	Tablet	50	–	–	–	–
M7	Tablet	142	–	–	–	–
M8	Tablet	156	–	–	–	–
M9	Tablet	5	–	–	–	–
M10	Capsule	52	–	–	–	–
M11	Capsule	<1	<1	<1	<1	–
M12	Syrup	1 mg per g	–	–	–	–
M13	Syrup	1 mg per g	–	–	–	–
CA	Dopped in honey	53 mg per (1 dose)	–	–	–	–
CA	Dopped in honey	41 mg per (1 dose)	–	–	–	–
CA	Dopped in honey	56 mg per (3 dose)	–	–	–	–
CA	Dopped in honey	56 mg per (3 dose)	–	–	–	–
CA	Herbalpreparation	–	<1	<1	–	–
CA	Herbalpreparation	<1	–	–	–	–
CA	Herbalpreparation	–	–	–	–	–
C1	Candy	<1	–	–	–	–
C2	Candy	<1	<1	–	–	+1
C3	Candy	–	<1	–	–	+5

[a] M = Medicines, CA = Dietary supplement, C = Candy.
[b] - = Not detected.
[c] + = At least one impurity detected.

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not compliant because the content of the four active ingredients was lower than the acceptance criteria.

Conclusions

In conclusion, we want to emphasise that this project was undertaken in order to offer analysts an appropriate analytical procedure to fight counterfeit erectile dysfunction drugs. To this end, we have paid particular attention to chromatographic techniques because they are the most powerful and robust for the quality control of pharmaceutical forms.

Drawing on previous work, including their weaknesses and limitations, we have created a unique UPLC method built from scratch using the methodology of the experimental design and the space design approach. Our separative technique served as an instrument capable of detecting illegal tablets through the various molecules of PDE-5i, in particular, sildenafil, tadalafil, vardenafil, and udenafil.

In order to avoid any failure of the implementation of our procedure in the routine phase, we began a validation process through two pioneering approaches, the uncertainty profile and risk profile. Both profiles reported compelling evidence that the uncertainty of future measurements generated by our method does not exceed the acceptance limits for risk of less than 5%. That is to say that the procedure is suitable for the routine control of counterfeit medicines.

As a result, we confirmed the validation results, from one end to the other, by the identification of 23 illicit drug formulations of erectile dysfunction in the routine phase.

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Supplemental Information

Supplemental information is available on the *J. AOAC Int.* website.

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