Journal of Chromatographic Science, 2021, Vol. 59, No. 1, 30–39
doi: 10.1093/chromsci/bmaa073
Advance Access Publication Date: 27 October 2020
Article

Article

UHPLC for Quality Evaluation of Genuine and Illegal Medicines Containing Sildenafil Citrate and Tadalafil

Naialy Fernandes Araújo Reis[1], Luiz Felipe Gomes da Silva[1], Mateus Araújo Castro e Souza[1], Sílvia Ligório Fialho[2], Carolina Paula de Souza Moreira[2], Marcus Vinicius de Oliveira Andrade[3], Washington Xavier de Paula[4], Maria Beatriz Abreu Gloria[1], Gerson Antônio Pianetti[1], and Christian Fernandes[1]

[1]Laboratório de Controle de Qualidade de Medicamentos e Cosméticos, Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, [2]Serviço de Desenvolvimento Tecnológico Farmacêutico, Diretoria de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, Belo Horizonte, MG, Brazil, [3]Setor Técnicoe Científico, Superintendência de Polícia Federal, Minas Gerais, Belo Horizonte, MG, Brazil, and [4]Instituto de Criminalística, Polícia Civil, Minas Gerais, Belo Horizonte, MG, Brazil

*Author to whom correspondence should be addressed. Email: cfernandes@farmacia.ufmg.br

Received 23 March 2020; Editorial Decision 6 September 2020

Abstract

One of the highest incidences of illegal drug products is related to phosphodiesterase-5 inhibitors, used in treatment of erectile dysfunction, including those containing sildenafil citrate and tadalafil. In this context, comprehensive evaluation of the quality of genuine and illegal medicines was performed. A simple and rapid ultra-high performance liquid chromatography (UHPLC-UV) method to quantify sildenafil and tadalafil in the presence of six degradation products was developed and validated. Sildenafil and tadalafil were submitted to forced degradation. The separation was carried out on a Kinetex C_{18} (50 × 2.1 mm; 1.7 μm) column with mobile phase composed of acetonitrile and aqueous triethylamine solution. The calibration curves were linear in the range of 14–126 μg mL^{-1} for sildenafil citrate and 4–36 μg mL^{-1} for tadalafil and the method proved to be selective, precise, accurate and robust. Sildenafil degraded in oxidative media, whereas tadalafil degraded in acidic, alkaline and oxidative environment. The chemical structures and the mechanisms for the formation of the main degradation products were proposed by UHPLC coupled to tandem mass spectrometry. The UHPLC-UV method was applied in the pharmaceutical analysis of genuine and seized medicines. Some of them did not meet quality standards, mainly due to contents below specifications and the large variation on contents between units within a batch.

Introduction

According to the World Health Organization, in the period from 2013 to 2017, lifestyle drugs, including those for erectile dysfunction, were the fourth largest class among substandards or counterfeit products (1). As presented by Brazilian police, drug products containing phosphodiesterase-5 inhibitors, including sildenafil (SLD) citrate (Figure 1a) and tadalafil (TAD) (Figure 1b), used to treat male erectile dysfunction, are the most commonly falsified in the country, followed by anabolic steroids (2).

The reference drugs Cialis (TAD in the base form) and Viagra (SLD in the citrate form), as well as generic and similar drugs, containing these active ingredients, have been marketed as tablets for oral administration. These drugs have similar mechanisms of action, differing mainly in terms of enzyme inhibition potency and pharmacokinetic properties, such as absorption rate, plasma half-life and duration of the effect (3).

Studies have been described in the scientific literature for the analysis of organic and inorganic compounds in illegal medicines containing SLD and TAD. These studies have indicated that some of these drugs do not have the active substance, they have the incorrect amount of the active substance, they claimed to contain SLD but, instead, they had TAD or a mixture of these active ingredients and/or they contained high amounts of contaminants (4–9).

In addition to being a criminal justice issue, the manufacturing and commercialization of illegal health products poses a serious threat to public health. This is so, because, as they are produced without the needed quality and safety standards, illegal medicines can worsen patients' health due to the absence of the active pharmaceutical ingredient (API) or to its inadequate content. It can also cause problems due to the presence of impurities and to unknown substances that can be added by the clandestine manufacturer, which may remain in the final product.

In this context, during development of analytical methods, especially those that may be used in the analysis of illegal medicines, it is important that they are selective for the analytes and also for their potential degradation products.

Some studies investigated, individually, the stability of SLD (10–12) and TAD (13–15). There have been also some methods by high performance liquid chromatography with UV detection (HPLC-UV) (16, 17) and ultra-high performance liquid chromatography (UHPLC-UV) (18) that do not consider products from SLD and TAD degradation, but allow the simultaneous quantification of these drugs in pharmaceutical preparations. However, a rapid and effective method for simultaneous quantification of SLD and TAD in the presence of all their potential degradation products using UHPLC-UV has not yet been described. In addition, a study that carried out a detailed verification of the pharmaceutical characteristics of illegal medicines was also not found in the peer-reviewed literature.

In order to determine SLD, TAD and their degradation products simultaneously in illegal and genuine medicines, the stability of these APIs was evaluated under acidic, neutral, alkaline, oxidative, heat and light conditions and in the presence of metal ions. Next, a simple and fast UHPLC-UV method using fused core column for quantification of SLD and TAD in the presence of their degradation products was developed and validated. The chemical structures and mechanisms of formation for the main degradation products were proposed using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). Finally, medicines seized by the Brazilian Police Authorities were evaluated in terms of identity, drug content, uniformity of dosage units and dissolution profile.

Experimental

Chemicals, reagents and materials

SLD citrate and TAD reference standards were purchased from the European Pharmacopoeia (Strasbourg, France). SLD citrate and TAD APIs were from Amphora (Belo Horizonte, MG, Brazil) and Vivence (São Roque do Canaã, ES, Brazil), respectively. Reference and generic tablets containing 50 mg SLD and 20 mg TAD were obtained from a drugstore located in Belo Horizonte, MG, Brazil. Ten batches of seized samples were provided by the Brazilian Police Authorities. Microcrystalline cellulose, croscarmellose sodium, titanium dioxide, magnesium stearate, anhydrous dibasic calcium phosphate, hydroxypropylcellulose, hypromellose, lactose monohydrate, sodium lauryl sulfate, yellow iron oxide and triacetin were obtained from Valdequímica (São Paulo, SP, Brazil). Opadry blue and transparent Opadry were from Colorcon (West Point, PA, USA). Ultrapure water was obtained from a Direct-Q 3 Millipore system (Bedford, MA, USA). Acetonitrile HPLC grade was purchased from J. T. Baker (Xalostoc, Mexico), Merck (Darmstadt, HE, Germany) and Honeywell (Muskegon, MI, USA). Methanol HPLC grade was from Sigma-Aldrich (St Louis, MO, USA). Ammonium formate was from Merck (Darmstadt, HE, Germany), sodium dodecyl sulfate, cupric sulfate pentahydrate, hydrochloric acid and triethylamine were purchased from Sigma-Aldrich (St Louis, MO, USA), acetic acid from Neon (Suzano, SP, Brazil), trifluoroacetic acid from Tedia Company (Fairfield, CT, USA), hydrogen peroxide and sodium hydroxide from Synth (Diadema, SP, Brazil). All of the reagents were of analytical grade, except HPLC solvents. Disposable polyvinylidene fluoride syringe filters (13 mm diameter, 0.2 µm pore size) were purchased from Agilent Technologies (Santa Clara, CA, USA).

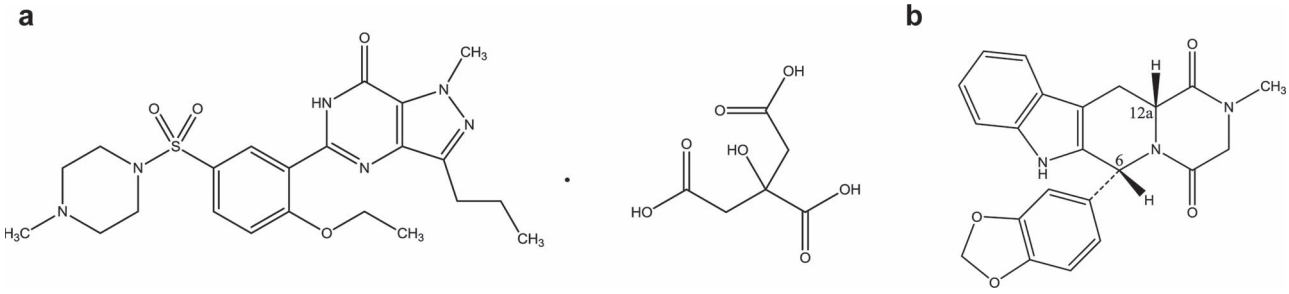

Figure 1. Chemical structures of (a) sildenafil (SLD) citrate and (b) tadalafil (TAD).

Instrumentation and analytical conditions

Sample preparation was performed using a Branson 3210R-MT ultrasound bath (Danbury, CT, USA) and pH of the mobile phase was measured using a Metrohm 827 pH Lab (Herisau, Switzerland) pH meter.

The UHPLC-UV system consisted of an ACQUITY UPLC coupled to an ultraviolet detector, both from Waters (Milford, MA, USA). Empower PRO 3.0 software was used for data acquisition and analysis. The chromatographic separation was performed on a Kinetex C18 (50 × 2.1 mm i.d.; 1.7 µm particle size) column from Phenomenex (Castel Maggiore, BO, Italy). The column was maintained at 35°C. The mobile phase was consisted of acetonitrile (A) and 0.2% (v/v) aqueous triethylamine solution (TEA), adjusted to pH 3.0 with formic acid (B) at a flow rate of 0.3 mL min^{-1}. The gradient elution program was 15–25% A from 0 to 0.5 min, 25% A from 0.5 to 2.0 min, 25–30% A from 2.0 to 2.5 min, 30% A from 2.5 to 6.5 min and 30–15% A from 6.5 to 7.0 min. For column re-equilibration, 15% A was maintained from 7.0 to 9.0 min. UV detection was performed at 285 nm and the injection volume was 5 µL.

Dissolution was carried out on a Hanson SR8-Plus dissolution system (Chatsworth, CA, USA).

The UHPLC–MS/MS analyses were carried out on an Agilent Technologies system (Santa Clara, CA, USA), composed of an Agilent 1290 Infinity LC system and an Agilent 6540 UHD Accurate-Mass quadrupole time-of-flight liquid chromatography with mass spectrometry, equipped with an electrospray ion source. Agilent MassHunter software was used for data acquisition and analysis. The mobile phase was acetonitrile containing 0.5% (v/v) formic acid (A) and 10 mM ammonium formate, adjusted to pH 3.0 with formic acid (B), at a flow rate of 0.3 mL min^{-1}. The gradient elution program, column, temperature and injection volume were the same used for the UHPLC-UV method. UV detection was performed from 190 to 400 nm. Mass spectrometry was performed in the positive mode. The mass spectra were obtained under the following conditions: spray, fragmentor, skimmer and octapole voltages of 3000, 175, 75 and 750 V, respectively; drying gas temperature of 300°C; flow rate of 8 L/min and pressure of 35 psi. Total ion chromatograms (TIC) of solutions subjected to forced degradation were acquired using full-scan MS mode. For structural characterization of the major degradation products, MS/MS spectra were obtained for each observed chromatographic peak in the range of 90–1000 Da. The energies for collision-induced dissociation experiments were set at 15 eV for TAD and its degradation products and 35 eV for SLD and its degradation products.

Standard solutions

Stock standard solutions of SLD citrate at 700 µg mL^{-1} and TAD at 200 µg mL^{-1} both using diluent 1 (acetonitrile and water 70:30 v/v) were prepared. Aliquots of the stock standard solutions were diluted with diluent 2 (acetonitrile and water 15:85 v/v) to obtain standard solutions at the following concentrations: 14, 70 and 126 µg mL^{-1} of SLD citrate and 4, 20 and 36 µg mL^{-1} of TAD. These standard solutions were used to determine drugs content.

Sample preparation

A chemometric approach was employed for the optimization of sample preparation. A complete factorial design 2^4 was used considering the variables that could affect sample preparation: diluent composition (acetonitrile and water or acetonitrile and 0.2% TEA, pH 3.0), ultrasound time (10 or 20 min), procedure for removing particules (filtration or centrifugation) and solutions' stability (at initial time and 24 h after).

Forced degradation and preparation of sample solutions

Forced degradation studies were performed by submitting SLD citrate and TAD APIs to several stressfull conditions (acid, alkaline, neutral, metal ions, heat, light and oxidant). And it was performed with both the isolated drugs and the APIs together in the same solution.

SLD and TAD were submitted to high temperature (60°C in oven) for 30 days—heat stress studies. The light stress studies were performed by subjecting the samples to UV (600 watt.h/m^2) and VIS (3.6 million lux.h) lights in a photostability chamber. After exposure, solutions at 70 µg mL^{-1} of SLD citrate and at 20 µg mL^{-1} of TAD were prepared.

For the preparation of samples submitted to hydrolytic degradation (acidic, alkaline and neutral), oxidation (with hydrogen peroxide) and degradation by metal ions, stock solutions of the isolated and mixed APIs containing 1400 µg mL^{-1} of SLD citrate and 400 µg mL^{-1} of TAD were prepared. Subsequently, the degradation solutions were prepared by adding 5 mL of the stressing agents (ultrapure water, 2 M hydrochloric acid, 0.02 M sodium hydroxide, hydrogen peroxide 6% (v/v) and 0.1 M copper sulfate) to 5 mL of stock solutions. These samples were prepared in duplicate; one maintained at room temperature and the other in a water bath at 50 °C. After exposure for a specific time to allow decrease between 10% and 30% of the APIs content, an aliquot of 1 mL of the degraded solutions was transferred to a 10 mL volumetric flask, which was filled up to the mark with diluent 2. These solutions were filtered and injected into the chromatograph. The chromatographic conditions were adjusted to provide adequate separation between SLD, TAD and their degradation products.

Placebo

The mixture of excipients was prepared according to the qualitative composition provided by the supplier of Viagra and Cialis and the amounts of excipients were those usually employed in tablets (19).

The amount of powder of excipients present in 1/4 of the average tablet weight was accurately weighed and transferred to a 50 mL volumetric flask, which was filled to the mark with diluent 1. An aliquot of 1 mL of the solution was transferred to a 10 mL volumetric flask. The flask was filled to the mark with diluent 2.

Method validation

The UHPLC-UV method was validated following the Brazilian Guideline RDC No 166/2017 (20), the ICH Guidance for Industry Q2 (R1) Validation of Analytical Procedures: Text and Methodology (21), INMETRO Guideline on validation of chemical test methods DOQ-CGCRE-008 (22) and Souza and Junqueira (23). The following parameters were evaluated: selectivity, linearity, precision, accuracy, robustness, quantification and detection limits.

Finally, to prove that the validated analytical method for the quantification of SLD and TAD in tablets could also be used for quantification of these analytes in dissolution test, selectivity was assessed by using different diluents such as 0.01 M hydrochloric acid and 0.5% sodium dodecyl sulfate, which are the media described in

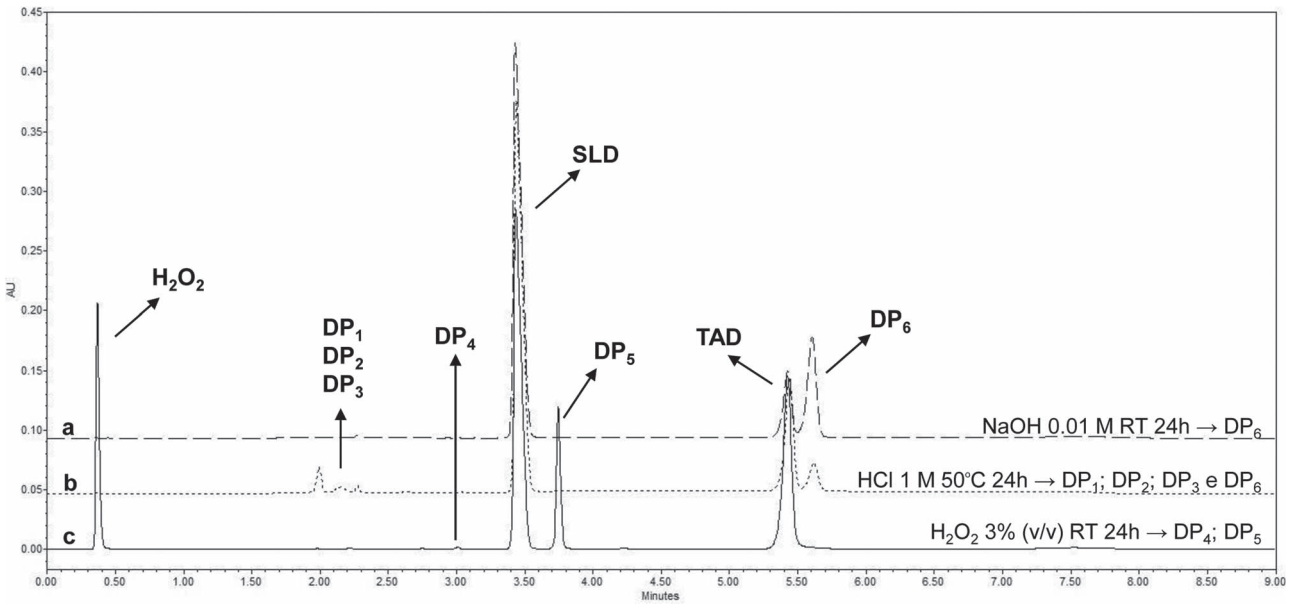

Figure 2. Chromatograms of SLD and TAD obtained after degradation under (a) alkaline (0.01 M NaOH, 24 h room temperature), (b) acidic (1 M HCl, 24 h 50°C) and (c) oxidative (H_2O_2 3% v/v, 24 h room temperature) conditions.

the Pharmacopeia monographs in the dissolution tests for SLD and TAD tablets, respectively (24).

Structure elucidation of degradation products

The chemical structures of the degradation products were obtained by UHPLC–MS/MS. The chromatograms of the degraded solutions were obtained first by using full-scan MS mode to determine the precursor ions and then the product ion scans method was used.

Application of the method: quality evaluation of genuine and illegal medicines

Batches of illegal and genuine tablets were submitted to the following physicochemical quality control tests: identification, assay, uniformity of dosage units, dissolution test and dissolution profile.

For identification and assay, the developed and validated UHPLC-UV method was used to identify and quantify SLD and TAD in the tablets. The results were compared to the amount in the label and it should not be <90.0% and not >110.0% (24).

The uniformity of dosage units test was performed by the content uniformity method. The individual content of each tablet was evaluated and an acceptance value (AV) was determined for each batch and it should be ≤15.0 (24).

The dissolution test was performed according to the USP dissolution methods for SLD and TAD tablets (24). The dissolution profile test was performed as recommended by RDC Resolution No. 31/2010 (ANVISA) (25).

For SLD tablets, the parameters were 900 mL of 0.01 M hydrochloric acid as dissolution medium and apparatus 1 (baskets) at 100 rpm. The samples were withdrawn at 2, 5, 10, 15, 30, 45 and 60 min. The results obtained at the collection time of 15 min were used in the dissolution test. SLD dissolved must be at least 85% (Q = 80%) (24). The parameters for tablets containing TAD were 1000 mL of 0.5% sodium dodecyl sulfate as dissolution medium and apparatus 2 (paddles) at 50 rpm. The samples were withdrawn at 5, 10, 15, 20, 30, 45, 60 and 75 min and the results obtained at the collection times of 10 and 30 min were used for the dissolution test. Dissolved TAD must be at least 45% (Q = 40%) after 10 min and 85% (Q = 80%) after 30 min (24).

Results

Forced degradation and UHPLC-UV method

According to the results of forced degradation, SLD was only susceptible to oxidative degradation with hydrogen peroxide, whereas degradation products from TAD were obtained in acidic, alkaline and oxidative media.

The chromatograms obtained with the UHPLC-UV method (described in 'Instrumentation and analytical conditions') are shown in Figure 2. Under the optimized condition, DP1, DP2, DP3, DP4, SLD, DP5, TAD and DP6 eluted at 2.0, 2.1, 2.3, 3.0, 3.4, 3.8, 5.4 and 5.6 min, respectively. As observed, eight analytes were separated in <6 min.

Sample preparation

After carrying out the 16 treatments according to multivariate analysis, recoveries of SLD and TAD were obtained. The main and second order effects were calculated and the error was estimated. The significance of the main effects was obtained, with 95% of confidence, using Microsoft Excel and STATISTICA software. Second order effects were not significant for analyte recovery.

When analyzing the effects of the main variables and the Pareto chart concerning the recovery of SLD and TAD (Figure 3), diluent composition was significant for both analytes and the method of particle removal was significant for TAD. Thus, during the sample solutions preparation, a mixture of acetonitrile and water was used as a diluent; an ultrasound time of 10 min should be used for complete APIs solubilization; before being diluted, the stock solutions must be filtered using 0.45 µm pore size syringe filters and sample solutions can be used within 24 h after preparation.

Method validation

System suitability was assessed injecting standard solution containing SLD and TAD from the same vial six times. The relative standard deviations (RSD) between the peak areas and retention times of SLD and TAD were <1%; the tailing factor was 1.85 for SLD and 0.85 for TAD; retention factors were 8.2 for SLD and 13.7 for TAD; and the number of theoretical plates were 16038 and 36693 for SLD and TAD, respectively. Thus the results were satisfactory.

To determine selectivity in relation to the degradation products, SLD and TAD APIs were subjected to the acid, alkaline and oxidation treatments (1 M hydrochloric acid at 50°C for 24 h, 0.01 M sodium hydroxide at room temperature for 6 h and hydrogen peroxide 3% (v/v) at room temperature for 10 h, respectively). All peaks from the six degradation products were separated from SLD and TAD peaks, with a minimum resolution of 1.5. No interfering peaks were observed in the retention time of SLD, TAD and the degradation products in the runs performed with the reagents and diluents used in the forced degradation study. To confirm the absence of coelutions, standard and solutions of degraded samples were analyzed at different detection wavelengths along the ultraviolet wavelength range, namely: 220, 254, 270 and 350 nm. It was observed that, regardless of the detection wavelength used, there was a change only in the intensity of the peaks (the number and shape of the peaks were constant). Moreover, during the analyses using full-scan MS mode, no additional peaks were observed. There was no peak from the excipients eluting at the same retention time of the analytes of interest. In addition, no statistical difference was found between sample solutions with and without added excipients. Thus, the UHPLC-UV method was selective and able to determine SLD and TAD in the presence of their degradation products in tablets.

Linearity was evaluated at nine concentrations, corresponding to 20; 40; 60; 80, 100, 120, 140, 160 and 180% of the working concentrations (70 μg mL^{-1} of SLD citrate and 20 μg mL^{-1} of TAD). The outliers were excluded from the data set after statistical tests. The results obtained for the tests of normality (Ryan Joiner's test), homoscedasticity (modified Levene's test) and independency (Durbin–Watson test) of residuals showed that they were in agreement with all the least squares method assumptions. The residuals showed random distribution, the regression was statistically significant and lack of adjustment to the linear model was not observed. The equations of the calibration curves obtained were $y = (19.12 \pm 0.05)\,x + (9.32 \pm 4.04)$ for SLD and $y = (31.64 \pm 0.10)\,x + (0.69 \pm 2.15)$ for TAD. The correlation coefficients were >0.99 (20). Analysis of variance (ANOVA) F-test showed that angular coefficients were statistically different from zero. Thus calibration curves were linear in the range of 14–126 μg mL^{-1} for SLD citrate and 4–36 μg mL^{-1} for TAD.

Accuracy and precision were evaluated using the placebo spiking approach. SLD and TAD were added to the mixture of excipients of the tablets to obtain three working concentrations (20, 100 and 180%). The RSD values obtained in the intraday and inter-days precisions were <2.0%, showing the precision of the developed method. All recoveries were within specified limits, between 98.0 and 102.0% of the theoretical concentration, demonstrating accuracy (22). The average contents and RSD values obtained for SLD and TAD are demonstrated in Table I.

Method robustness was assessed through the Youden's test (26). The parameters investigated were: temperature (32 and 38°C), flow rate (0.25 and 0.35 mL min^{-1}), injection volume (4.5 and 5.5 μL), acetonitrile brand (Honeywell and J.T.Baker), pH of the aqueous eluent (2.8 and 3.2), detection wavelength (280 nm and 290 nm) and gradient variations. The obtained Daniel and Lenth graphs showed that no active effects were detected. Thus, the method was robust for all variations tested, for both SLD and TAD quantification.

Signal-to-noise ratios were calculated, and the detection and quantification limits obtained were 0.0014 and 0.0042 μg mL^{-1}, respectively, for SLD citrate, and 0.0022 and 0.0068 μg mL^{-1}, respectively, for TAD.

Chromatograms obtained from the injection of acetonitrile-water mixture, 0.01 M hydrochloric acid and 0.5% sodium lauryl sulfate presented the same profile and no peaks appeared from these diluents eluting at the SLD and TAD retention times. Furthermore, ANOVA F-test showed that there was no statistical difference between SLD sample solutions prepared with diluent consisting of acetonitrile and water and prepared with 0.01 M hydrochloric acid. Also, there was no statistical difference between TAD sample solutions prepared with diluent consisting of acetonitrile and water and prepared with 0.5% sodium lauryl sulfate. Therefore, the analytical method can be used for identification, assay and uniformity of dosage units and dissolution test of tablets containing SLD or TAD.

UHPLC–MS/MS analyses

UV and mass spectra of standard solutions containing SLD citrate and TAD at 100 μg mL^{-1} were recorded by UHPLC–MS/MS. The SLD protonated molecular ion $[M + H]^+$ presented *m/z* of 475.21 and the main fragment *m/z* of 100.10 (Figure 4a and b). For TAD, the protonated molecular ion $[M + H]^+$ presented *m/z* of 390.15 and the main fragment *m/z* of 268.11, as shown in Figure 5b.

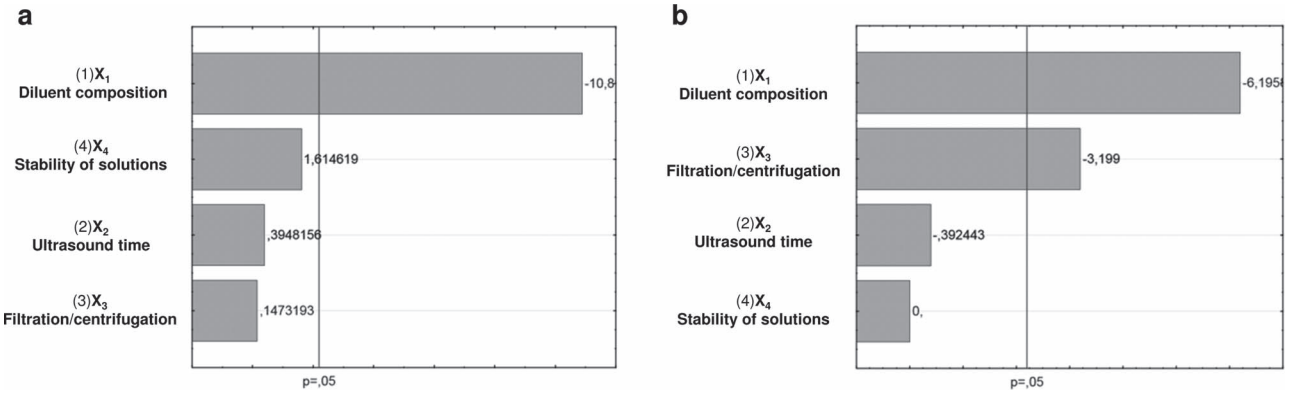

Figure 3. Pareto chart acquired from complete factorial design 2^4 plan for (a) SLD recovery and (b) TAD recovery.

Table I. Mean contents and RSD values obtained for SLD and TAD

Precision	Level[a]	SLD		TAD	
		Content (%)[b]	RSD (%)	Content (%)[b]	RSD (%)
Intraday (day 1)	20%	101.34	0.0725	99.99	0.4544
	100%	99.59	1.8490	100.05	0.2439
	180%	100.65	0.3716	100.21	0.4014
Intraday (day 2)	20%	99.60	1.6344	101.82	0.1088
	100%	101.72	0.5306	101.62	0.4227
	180%	98.89	1.8102	101.17	0.1532
Inter-days	20%	100.47	1.3955	100.90	1.1279
	100%	100.66	1.6733	100.83	0.9070
	180%	99.77	1.5098	100.69	0.5894

[a]Concentration level with respect to working concentration. [b]Average content ($n = 3$).

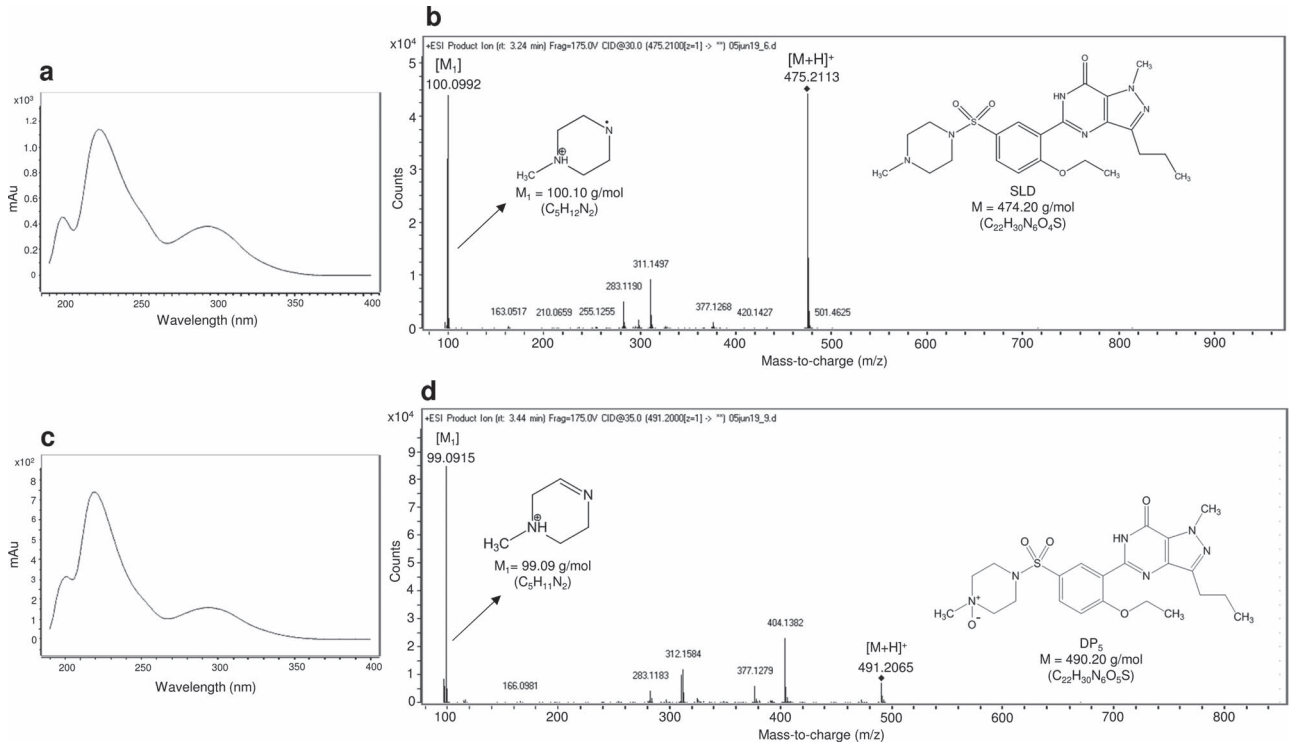

Figure 4. (a) UV SLD; (b) full-scan MS2 of SLD; (c) UV of DP5 and (d) full-scan MS2 of DP5.

The solutions subjected to forced degradation in acidic, alkaline and oxidative environment were analyzed and the mass spectra of the degradation products were obtained. The fragmentation transitions for the main degradation products, DP5 and DP6, were *m/z* 491.21 → *m/z* 99.09 and *m/z* 390.14 → *m/z* 268.11, respectively. DP5 is the sildenafil N-oxide and DP6 probably corresponds to the diastereoisomer 6R,12aS of TAD.

Quality evaluation of genuine and illegal medicines

Seized drug products were numbered from illegal 1 to 10. Except for samples 4 and 9, all of them presented primary packaging. The packages, although not homogeneous in font, letter color, laminated aluminum foil printing and plastic film staining, were labeled as SLD (the exception was sample 3, which was labeled as TAD). Batches of genuine tablets containing SLD and TAD were randomly numbered.

After receiving the samples, they were stored in a desiccator under protection from light and controlled temperature of 25°C, until they were removed for analysis.

Together with assay, identification of the API was performed in each sample using retention time. Only one peak for API was observed in each sample analyzed, with no secondary peaks related to impurities, degradation products or contaminants (Table II).

Regarding the assay, all samples analyzed presented contents according to the Pharmacopeial specification, except for illegal sample 1, which had a content of 27.61% of the labeled value of SLD, i.e., 13.81 mg sildenafil, much lower than the specification (24). Some seized samples (illegal samples 4, 5 and 9), presented values close to the specification lower limit. On the other hand, genuine medicines presented levels very close to 100%, as can be seen in Table II.

With the exception of illegal sample 1, the tablets units analyzed (2, 3.6 and 10) had adequate API content and AV < 15.0. The content

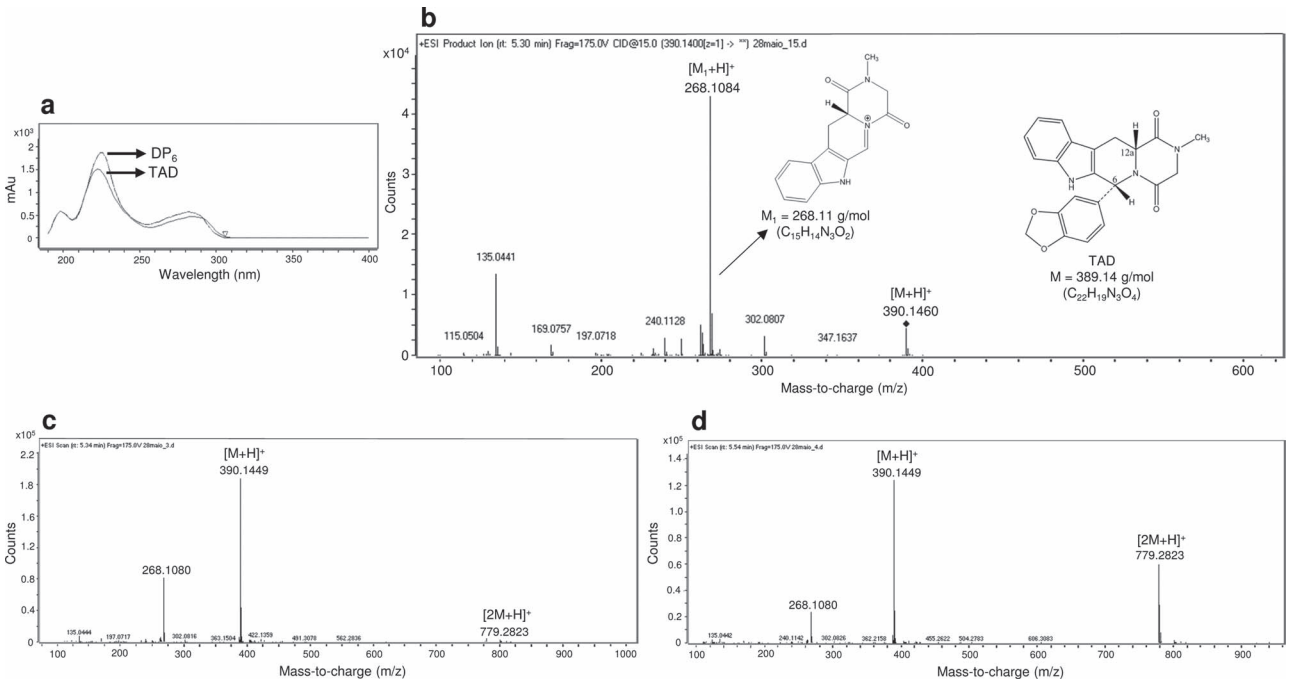

Figure 5. (a) UV of TAD and DP6; (b) full-scan MS2 of TAD; (c) full-scan MS1 of TAD and (d) full-scan MS1 of DP6.

Table II. API identification and assay of illegal and genuine medicines containing SLD citrate and TAD

Sample	API identified	Assay (%)
Illegal		
1	SLD	27.61
2	SLD	98.09
3	TAD	101.35
4	SLD	92.74
5	SLD	92.78
6	SLD	97.64
7	SLD	98.92
8	SLD	94.82
9	SLD	93.33
10	SLD	100.98
Viagra		
1	SLD	99.64
2	SLD	101.21
Generic SLD		
1	SLD	99.29
2	SLD	98.80
Generic TAD		
1	TAD	100.80
2	TAD	102.34
3	TAD	100.00

uniformity test was not performed with the illegal samples 4, 5, 7, 8 and 9, as there were not enough units. The tablets of illegal sample 1 showed a wide variation (AV = 78.3) among units tested.

The dissolution test was not performed with the seized samples 4 and 9, as there were not enough units. Illegal, reference and generic products containing SLD released >85% of sildenafil in 15 min, except for the illegal sample 1. The units of the sample 1 presented dissolution lower than the specification and the RSD among the dissolution of units was also high (7.92%).

Illegal samples containing SLD can be divided into some groups according to dissolution profiles results obtained. Illegal samples 5, 6, 8 and 10 had overlapping profiles, sample 2 shows a profile very similar to this group; however, with slightly faster SLD release and dissolution. Sample 7 presents a slightly slower release profile than the others. But they all released virtually all of their SLD content within 30 min.

Tablets are manufactured by compressing a mixture containing the API and excipients. They may be formulated to have immediate release or modified release, depending on the excipients used and the manufacturing processes employed. All SLD containing tablets analyzed showed immediate release and, with the exception of seized sample 1, all samples dissolved an amount >85% within 15 min.

In relation to samples containing TAD, all units presented dissolution >45% after 10 min. However, all tablets analyzed in the seized sample 3 and two tablets of the generic TAD batch 3 showed dissolution < 85% in 30 min. Thus, these samples were not in agreement with the dissolution test specification (24).

Dissolution profiles evidence that generic TAD 1 and 2 have very similar dissolution profiles. Generic TAD 3 tablets presented a slightly slower dissolution than the previous ones and the illegal sample 3 presented even slower dissolution; also, they did not meet the dissolution test criterion, since the amount dissolved was <85% within 30 min.

Discussion

To optimize the UHPLC-UV method, samples from acidic, alkaline and oxidative degradation studies were used, since these were the stress conditions that lead to the formation of degradation products.

Overall, six products resulted from forced degradation conditions. Three minor (DP1, DP2 and DP3) and one major degradation products (DP6) were obtained from TAD in acidic condition;

however, only one product (DP6) was formed in alkaline stress. This degradation product had the same retention time of the major degradation product formed from TAD in acidic medium. In the oxidative medium, a minor degradation product from TAD was observed (DP4) and a different product from SLD (DP5) was observed on the experiment with hydrogen peroxide. Extracts containing SLD, TAD and these six degradation products were used in method development.

Initially, several isocratic methods were proposed, using different aqueous and organic eluents as mobile phases, flow rates, injection volumes, temperatures and detector acquisition frequencies. However, a satisfactory resolution between SLD, TAD and degradation products was not possible.

Then, several gradient elutions with different ratios of acetonitrile (A) and TEA pH 3.0 (B) were tested and the condition that allowed suitable resolution among the peaks of degradation products and analytes were those described in 'Instrumentation and analytical conditions'. Figure 2 shows the chromatogram obtained from the UHPLC-UV optimized method.

Regarding the optimization of the sample preparation, the results of the multivariate analysis showed that diluent composition was significant for both analytes. Thus, to maximize recovery, diluents consisting of acetonitrile and water should be used during sample preparation. Ultrasound time was not significant and, in order to reduce length of the sample preparation step, the lower level of this variable (10 min) was used. The results obtained for particle removal showed that recovery of SLD was not affected filtration or centrifugation, but for TAD, recovery was reduced when centrifugation is used. Therefore, filtration using 0.45 µm pore size syringe filters was chosen. Finally, the stability of the sample solutions for both analytes was considered adequate for 24 h.

The UHPLC-UV method was validated in a wide range of concentration (20–180% of the SLD and TAD working concentrations), since it is intended for quality control analysis of suspected medicines. Therefore, the samples may present different levels of analyte concentration. In addition, the analytical method can be used in the test of dissolution and uniformity of dosage units. The selectivity of the method was confirmed for quantification of SLD and TAD in the presence of all potentials degradants. Therefore, in addition to quality control tests, this method can also be applied to stability studies.

In relation of the identification of the main degradation products, in oxidative medium, SLD formed DP5 with m/z 491.20 → m/z 99.09, which is probably the sildenafil N-oxide, with molecular formula $C_{22}H_{30}N_6O_5S$, derived from the oxidation mechanisms of tertiary amines to amine oxides (27). Figure 4c show the UV spectrum of DP5 and Figure 4d show the mass spectra of its daughter ions and the probable structure of DP5 (28).

The main degradation product of TAD formed in acidic and alkaline condition (DP6), in addition to having the same retention time, presented the same mass spectrum in the UHPLC–MS/MS analyzes. The maximum wavelength of TAD and DP6 differed by 2 nm (Figure 5a) and the full-scan MS1 showed a difference in the ionization between these compounds, with DP6 showing significant $2\,M + H^+$ signal, whereas TAD presented low intensity of this signal (Figure 5c and d). However, DP6 and TAD cannot be distinguished by the MS2 spectrum. Thus, this degradation product is probably a diastereoisomer of TAD.

As can be seen from Figure 1b, TAD, with the chemical name (6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-[3,4-(methylenedioxy)phenyl] pyrazino [1′,2′:1,6] pyrido [3,4-b]indole-1,4-dione, is an optically active compound. It contains two chiral centers in the 6R,12aR configuration, and there may be two cis (6R,12aR/6S,12aS) and two trans (6R,12aS/6S,12aR) stereroisomers (29). Venhuis *et al.* (30) described a HPLC-DAD-MS method that was able to separate TAD from the trans-tadalafil diastereoisomer present in a falsified medicine. In addition, they measured the optical rotation of the diastereomeric mixture and concluded that the unknown diastereoisomer was (−)-trans-tadalafil, i.e., the 6R,12aS stereoisomer. The results of UV and mass spectrometry analyzes presented by Venhuis *et al.* (30) for 6R,12aS stereoisomer were similar to those obtained in the present study for DP6. Thus, it is likely that DP6 corresponds to the diastereoisomer 6R,12aS, formed via keto-enolic tautomerism according to the mechanisms shown in Figure 6. Also, as described by Jida *et al.* (31), TAD could form the diastereoisomer 6S,12aR under microwave irradiation in acidic conditions by TAD epimerization mechanism. Since 6R,12aS and 6S,12aR are enantiomers, chiral chromatography would be required to separate and identify them. However, it is believed that DP6 is 6R, 12aS, since its formation comes from mild reaction conditions.

The UHPLC-UV method was applied to pharmaceutical analysis (identification, content, uniformity of dosage units and dissolution profile) of genuine and seized medicines. The results showed that, although did not present secondary peaks related to impurities, degradation products or contaminants in the analyzed samples, some batches had contents below the specification or presented values close to the specification lower limit. The presence of API at a correct level is critical for the quality of the medicines, as the administration of incorrect doses may lead to therapeutic ineffectiveness (below specification) or toxicological effects (above specification). The tablets of illegal sample 1, in addition to having SLD content lower than specification, showed a wide variation (AV = 78.3) among units tested, confirming once again the lack of quality control during manufacture of this sample. The results obtained in the uniformity of dosage units is an important parameter to assess the manufacturing process, since an inadequate or non-standard mixture of tablet powder, for example, can result in high content variations among units in the same batch.

Regarding the dissolution test, the units of the sample 1 presented dissolution lower than the specification, which was expected, since, according to the results obtained in the assay and content uniformity, this sample had SLD content much below the specification. In addition to a dissolution below specification, the RSD among the dissolution of units was also high (7.92%), indicating a lack of standardization between units and corroborating the lack of quality of these tablets.

Some genuine and illegal tablets containing TAD did not comply with the Pharmacopeial specification of the dissolution tests and, therefore, do not have the necessary quality parameters.

Dissolution profiles for the genuine and illegal tablets were obtained and compared. The main objective of dissolution profile in routine quality control is to verify the maintenance of quality characteristics and parameters among different batches of a drug, since changes in dissolution profile are indicative of some change in constituents or formulation during manufacturing, which may represent lack of standardization and affect formulation quality.

Conclusion

For the first time, a simple and fast UHPLC-UV method for simultaneous quantification of SLD and TAD in the presence of all their potential degradation products was described, in which eight analytes were separated in <6 min. The developed method was validated and

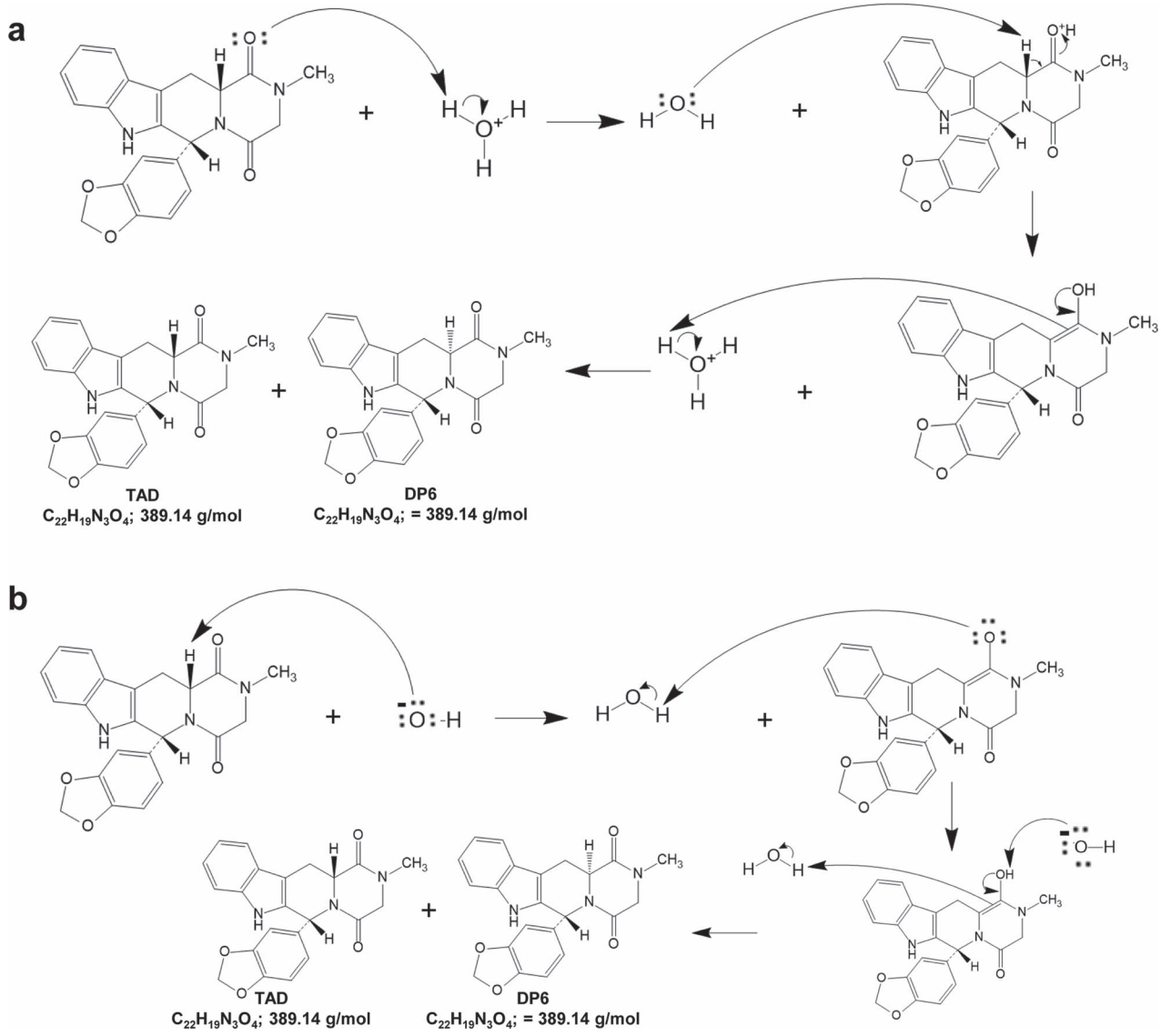

Figure 6. Proposed mechanism of keto-enolic tautomerism of TAD in (a) acidic and (b) alkaline media.

proved to be selective, linear, precise, accurate and robust and can be used both for stability studies and routine quality control analyses.

After a complete forced degradation study, SLD was susceptible to degradation under oxidative conditions (with hydrogen peroxide), whereas TAD degraded in acidic, alkaline and oxidative environments. The chemical structures and mechanisms of the major SLD and TAD degradation products were proposed by means of UHPLC–MS/MS.

The UHPLC-UV method was applied in the evaluation of the quality of genuine and illegal medicines. Some samples did not meet the quality required by Pharmacopeial specifications. Although no degradation products were found in the illegal samples, the main problems observed in the drug products were content below specification and high variation on content between units of the same batch, which may compromise the pharmacological effect.

The results showed that the verification of drug products quality, to which the population is having access, is extremely important. Moreover, the use of illegal medicines can poses as a serious threat to public health, since they are not manufactured following the quality requirements established by regulatory agencies.

Acknowledgements

The authors would like to thank CNPq, CAPES and FAPEMIG for the financial support. The authors also thank the Brazilian Police Authorities for providing the seized medicines.

Funding

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) provided the financial support.

Conflict of interest

The authors have declared no conflict of interest.

References

1. WHO-World Health Organization; Global Surveillance and Monitoring System for substandard and falsified medical products. In *Genebra*. (2017).

2. Ames, J., Souza, D.Z.; Falsificação de medicamentos no Brasil; *Revista de Saúde Pública*, (2012); 46: 154–159.
3. Carson, C.C., Noh, C.H.; Distal penile prosthesis extrusion: Treatment with distal corporoplasty or Gortex windsock reinforcement; *International Journal of Impotence Research*, (2002); 14: 81–84.
4. de Veij, M., Deneckere, A., Vandenabeele, P., de Kaste, D., Moens, L.; Detection of counterfeit Viagra® with Raman spectroscopy; *Journal of Pharmaceutical and Biomedical Analysis*, (2008); 46: 303–309.
5. Ortiz, R.S., Mariotti, K., de, C., Romão, W., Eberlin, M.N., Limberger, R.P., *et al*.; Chemical fingerprinting of counterfeits of viagra and cialis tablets and analogues via electrospray ionization mass spectrometry; *American Journal of Analytical Chemistry*, (2011); 2: 919–928.
6. Ortiz, R.S., Mariotti, K.D.C., Schwab, N.V., Sabin, G.P., Rocha, W.F.C., de Castro, E.V.R., *et al*.; Fingerprinting of sildenafil citrate and tadalafil tablets in pharmaceutical formulations via X-ray fluorescence (XRF) spectrometry; *Journal of Pharmaceutical and Biomedical Analysis*, (2012); 58: 7–11.
7. Ortiz, R.S., Mariotti, K.D.C., Limberger, R.P., Mayorga, P.; Physical profile of counterfeit tablets Viagra® and Cialis®; *Brazilian Journal of Pharmaceutical Sciences*, (2012); 48: 487–495.
8. Ortiz, R.S., Mariotti, K.D.C., Fanka, B., Limberger, R.P., Anzanello, M.J., Mayorga, P.; Counterfeit Cialis and Viagra fingerprinting by ATR-FTIR spectroscopy with chemometry: Can the same pharmaceutical powder mixture be used to falsify two medicines? *Forensic Science International*, (2013); 226: 282–289.
9. Coelho Neto, J., Lisboa, F.L.C.; ATR-FTIR characterization of generic brand-named and counterfeit sildenafil- and tadalafil-based tablets found on the Brazilian market; *Science and Justice*, (2017); 57: 283–295.
10. Dinesh, N.D., Vishukumar, B.K., Nagaraja, P., Made Gowda, N.M., Rangappa, K.S.; Stability indicating RP-LC determination of sildenafil citrate (Viagra) in pure form and in pharmaceutical samples; *Journal of Pharmaceutical and Biomedical Analysis*, (2002); 29: 743–748.
11. Abd-Elbary, A., Foda, N.H., El-Gazayerly, O.N.; Stability indicating high performance liquid chromatographic assay for the determination of sildenafil citrate in bulk and in formulations; *Chromatographia*, (2004); 59: 561–566.
12. Tambe, V.S., Deodhar, M.N., Vijayalakshmi, P.; Stability-indicating UPLC-MS/UV method for simultaneous determination of sildenafil citrate and dapoxetine hydrochloride from bulk and formulation; *Indian Journal of Pharmaceutical Sciences*, (2016); 78: 663–672.
13. Rao, D.V.S., Radhakrishnanand, P., Himabindu, V.; Stress degradation studies on tadalafil and development of a validated stability-indicating LC assay for bulk drug and pharmaceutical dosage form; *Chromatographia*, (2008); 67: 183–188.
14. Satheesh, B., Sree Ganesh, K.K., Saravanan, D.; Simultaneous determination of tadalafil and its related compounds in pharmaceutical dosage forms by UPLC; *Journal of Liquid Chromatography & Related Technologies*, (2013); 36: 1451–1465.
15. Patel, J.K., Patel, N.K.; Stability-indicating RP-HPLC method for the determination of Ambrisentan and tadalafil in pharmaceutical dosage form; *Scientia Pharmaceutica*, (2014); 82: 749–763.
16. Yang, Y.J., Song, D.M., Jiang, W.M., Xiang, B.R.; Rapid resolution RP-HPLC-DAD method for simultaneous determination of sildenafil, vardenafil, and tadalafil in pharmaceutical preparations and counterfeit drugs; *Analytical Letters*, (2010); 43: 373–380.
17. Fidan, A.K., Bakirdere, S.; Simultaneous determination of sildenafil and tadalafil in legal drugs, illicit/counterfeit drugs, and wastewater samples by high-performance liquid chromatography; *Journal of AOAC International*, (2016); 99: 923–928.
18. Ortiz, R.S., Antunes, M.V., Linden, R.; Determinação de citrato de sildenafila e de tadalafila por cromatografia líquida de ultraeficiência com detecção por arranjo de diodos (CLUE-DAD); *Química Nova*, (2010); 33: 389–393.
19. Rowe, R.C., Sheskey, P.J., Quinn, M.E. (eds); *Handbook of Pharmaceutical Excipients*. Pharmaceutical Press and American Pharmacists Association, Washington, NY, (2009).
20. Agência Nacional de Vigilância Sanitária; Resolução da Diretoria Colegiada – RDC n. 166, de 24 de julho de 2017. In *Dispõe sobre a validação de métodos analíticos e dá outras providências*. Diário Oficial da União, Brasília, DF, (2017).
21. *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Topic Q2(R1): Validation of analytical procedures: text and methodology*, (2005). https://database.ich.org/sites/default/files/Q2_R1__Guideline.pdf (accessed March, 11 2020).
22. Instituto Nacional de Metrologia; Normalização e Qualidade Industrial (INMETRO), DOQ-CGCRE-008. In *Orientações sobre validação de métodos analíticos*, Rio de Janeiro, RJ, (2018).
23. de Souza, S.V.C., Junqueira, R.G.; A procedure to assess linearity by ordinary least squares method; *Analytica Chimica Acta*, (2005); 552: 25–35.
24. The United States Pharmacopeia; *USP 42*, 42nd ed. United States Pharmacopeial Convention, Rockville, (2019).
25. Agência Nacional de Vigilância Sanitária; Resolução da Diretoria Colegiada – RDC n. 31, de 11 de agosto de 2010. In *Dispõe sobre a realização dos estudos de equivalência farmacêutica e de perfil de dissolução comparativo*. Diário Oficial da União, Brasília, DF, (2010).
26. Youden, W.J., Steiner, E.H.; *Statistical Manual of the Association of Official; Analytical Chemists*, 48th ed. Arlington, AOAC, (1975).
27. Hovorka, S.W., Schöneich, C.; Oxidative degradation of pharmaceuticals: Theory, mechanisms and inhibition; *Journal of Pharmaceutical Sciences*, (2001); 90: 253–269.
28. Aceña, J., Pérez, S., Gardinali, P., Abad, J.L., Eichhorn, P., Heuett, N.; Structure elucidation of phototransformation products of unapproved analogs of the erectile dysfunction drug sildenafil in artificial freshwater with UPLC-Q Exactive-MS; *Journal of Mass Spectrometry*, (2014); 49: 1279–1289.
29. Kee, C.L., Chin, L.C., Cheah, N.P., Ge, X., Low, M.Y.; Elucidation of the absolute configuration of a tadalafil analogue found as adulterant in a health supplement by mass spectrometry, chiroptical methods and NMR spectroscopy; *Journal of Pharmaceutical and Biomedical Analysis*, (2019); 173: 47–55.
30. Venhuis, B.J., Zomer, G., Vredenbregt, M.J., de Kaste, D.; The identification of (−)-trans-tadalafil, tadalafil, and sildenafil in counterfeit Cialis® and the optical purity of tadalafil stereoisomers; *Journal of Pharmaceutical and Biomedical Analysis*, (2010); 51: 723–727.
31. Jida, M., Tourwé, D., Ballet, S.; Highly stereoselective one-pot construction of trisubstituted tetrahydro-β-carboline-fused diketopiperazines: A synthetic route towards cialis analogues; *RSC Advances*, (2014); 4: 38159–38163.