

# Tadalafil and Sildenafil illicit association: Stability-indicating HPLC method, photodegradation kinetic and toxicological studies

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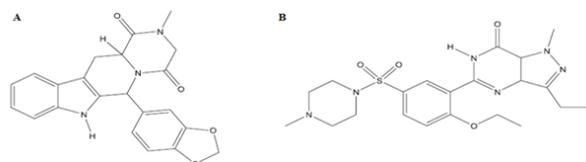
The illicit market of counterfeit medicines containing sildenafil and tadalafil has been causing serious public health problems. Thus, further studies on this illicit association are needed. A stability-indicating HPLC method was developed for simultaneous determination of tadalafil (TAD) and sildenafil (SIL) using a C<sub>18</sub> column (250 x 4.6 mm, 5 μm). Detection was achieved at 284 nm, for TAD, and 292 nm, for SIL. The method was considered to be specific, linear, precise, accurate, robust, and sensitive. In the photodegradation kinetic studies, the drugs showed a first-order reaction rate when isolated, and zero-order when associated. Toxicological assays demonstrated that the photodegraded drugs decreased cell viability in compared to non-degraded drugs, suggesting cytotoxic activity. Additional, mutagenic activity was not observed under the tested conditions. Photodegraded drugs, in association, depicted DNA damage index, suggesting genotoxic effects. The obtained results will be able to support the forensic intelligence laboratories, as well as to alert the population about the risk inherent to consuming counterfeit products.

**Key words:** Drug counterfeiting. Tadalafil. Sildenafil. Stability. Toxicity.

## INTRODUCTION

According to the World Health Organization (WHO), counterfeit medicine is a product which is unduly packaged and labeled in a deliberate manner, having an adulterated identity and/or source. Counterfeiting practices covers both brand-name and generic medicine, commonly resulting in a product of similar composition to their authentic counterparts, but which is partially or entirely lacking their active components, or even supplemented with completely different medicines/drugs instead (Hurtado, Lasmar, 2014).

Medicine counterfeiting spreads to a wide range of therapeutic classes such as antimalarials, antibiotics, antineoplastics, among others. In general, pharmaceutical products with higher rates of falsification are also the ones that are most requested by the population. Among the counterfeit drugs seized in Brazil are anabolics, anorexigenics, cytostatics, and especially, those used to treatment erectile dysfunction (ED) including tadalafil (Cialis<sup>®</sup>) and sildenafil (Viagra<sup>®</sup>), which are represented by their chemical structures in Figure 1 (Ames, Souza, 2012).



**FIGURE 1** - Chemical structures of Tadalafil (A) and Sildenafil (B).

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According to WHO, there are many risks which are inherent to counterfeit medicine, ranging from intoxication to unknown pharmacological activity (WHO, 2017). Once Good Manufacturing Practices (GMP) standards are not followed at any point in the process of making these products, there is lack of origin recognition of the raw materials, lack of quality assurance and inadequate transportation and/or storage. These factors altogether increase the likelihood of health risks to the population. Specifically, the methods used to transport clandestine goods greatly vary and it is highly unlikely that temperature, light and humidity are controlled to ensure these substances' integrity. Thus, the probability of degradation of these formulations' components is high, increasing the concern about the toxicity and safety of counterfeit medicines (Jung *et al.*, 2012).

Different studies carried out by various authors report the simultaneous presence of tadalafil (TAD) and sildenafil (SIL) in falsified samples of Cialis® (Ames, Souza, 2012; Jung *et al.*, 2012; Park, Ahn, 2012). However, the main focus of these studies was the development of quantification methods for their association. Thus, there is room to expand these studies by developing a stability-indicating method for TAD and SIL association.

The International Conference on Harmonization (ICH) guideline requires that stress testing should be carried out to elucidate the inherent stability characteristics of the active substances in pharmaceutical preparations. The light testing is considered a significant factor in drug stability and it's an integral part of stress testing (ICH 1996; ICH 2003).

Studies on the degradation profile of these drugs at different storage conditions, as well as data on their biological safety, are also necessary as the current literature lacks information on the toxicity of these two associated drugs and their degradation products.

The objective of this work was to develop and validate a method for the simultaneous determination of TAD and SIL by Liquid Chromatography (LC) in order to evaluate the photodegradation of both components under ultraviolet radiation, and to carry out preliminary toxicological studies on the biological safety of the drugs in their isolated and associated forms, both not degraded and photodegraded.

## MATERIAL AND METHODS

### Chemical substances

The TAD which was used as the chemical reference substance (RS) was kindly provided by the pharmaceutical industry Multilab (São Jerônimo - Brazil). The SIL which was used as RS was commercially acquired (Galena Química e Farmacêutica Ltda., Brazil). The reagents used were acetonitrile (LC grade, Tedia, USA), o-phosphoric acid (Merck, Germany), triethylamine (Merck, Germany), methyl alcohol (LC grade, Merck, Germany), hydrochloric acid 37% (Neon Comercial Ltda., Brazil), hydrogen peroxide 30% (Fmaia Indústria e Comércio Ltda., Brazil), sodium hydroxide (Dynamics Química Contemporânea Ltda., Brazil) and ultra-pure water obtained by Direct-Q UV 3 Milli-Q® system (Merck Millipore, USA). The other reagents used were all of analytical grade.

### Equipment and chromatographic conditions

All samples were analyzed in a Shimadzu liquid chromatograph (LC-20AT Prominence), with a quaternary pump system, autosampler, photodiode array detector (DAD), and degasser system (Shimadzu, Kyoto, Japan). Hypersil® C<sub>18</sub> column (250 mm x 4.6 mm, 5 µm) was used for chromatographic separations. The mobile phase was composed of Solution A and Solution B (30:70, v/v), where Solution A comprises acetonitrile:water (80:20, v/v) and solution B comprises methanol:triethylamine 0.3% pH 7.5, adjusted with o-phosphoric acid (57:43, v/v). The elution process was isocratic with a flow rate of 1.0 mL.min<sup>-1</sup> and the injection volume used was 20 µL. Mobile phase components were vacuum filtered through a 47 mm diameter nylon membrane and a 0.45 µm pore (Millipore, Billerica, MA, USA). The temperature of analysis was 25°C, and detection was conducted at 284 nm and 292 nm for TAD and SIL, respectively.

The photodegradation study was conducted in a photostability chamber, with a UVA light source (360 nm 30W/G30 T8, Philips, Amsterdam, Netherlands). The samples were exposed to the light source inside of

disposable cuvettes of a 1 cm optical path, 10 cm from the light source at a controlled temperature.

### Standards and samples working solutions

The working solutions were based on the study by Park and Ahn (2012), where 105 seized samples containing TAD and SIL were analyzed. According to the authors, approximately 25% of the samples had both TAD and SIL, and more than half contained very different amounts than those described in their respective presentations. Based on these results, a linear range for TAD and SIL was determined, as well as the working concentration of 25 and 100  $\mu\text{g}\cdot\text{mL}^{-1}$  for TAD and SIL, respectively.

#### Standard solution preparations

Accurately weighed amounts of TAD and SIL RS, equivalent to 12.5 and 50.0 mg respectively, were transferred to a 50 mL volumetric flask with the aid of 40 mL of methanol. The resulting mixture was sonicated for 20 minutes, and adjusted to the final volume with that same solvent, followed by homogenization. A 1.0 mL aliquot was added to a 10 mL volumetric flask and the volume was adjusted with the same solvent to obtain final concentrations of 25  $\mu\text{g}\cdot\text{mL}^{-1}$  of TAD and 100  $\mu\text{g}\cdot\text{mL}^{-1}$  SIL.

#### Sample solution preparations

The placebo solution (PS) was prepared (an in-house mixture of Viagra<sup>®</sup> and Cialis<sup>®</sup> excipients) out of the pool of excipients described in the commercial products.

In order to prepare the simulated working samples (SWS), the equivalent of 12.5 mg of TAD, 50 mg of SIL and 220 mg PS were weighed and transferred to a 50 mL volumetric flask with the aid of 40 mL of methanol. The solution was then subjected to an ultrasonic bath for 20 minutes, after which the volume was adjusted with that same solvent and homogenized. The resulting solution was filtered on filter paper, and a 1 mL aliquot was removed and transferred to a 10 mL volumetric flask. The volume was adjusted with methanol to obtain a final concentration of 25  $\mu\text{g}\cdot\text{mL}^{-1}$  for TAD and 100  $\mu\text{g}\cdot\text{mL}^{-1}$  for SIL.

### Chromatographic method validation

Validation of the analytical method was performed according to the International Conference on Harmonization (ICH, 2005), using the following parameters: selectivity, linearity, precision, accuracy, robustness, limit of detection and limit of quantification.

#### Specificity

The specificity of the proposed method was determined through the evaluation of possible placebo interference, as well as the likely degradation products formed during forced degradation.

In order to evaluate the LC method stability indicating capability, the TAD RS and SIL RS were stressed under different conditions as part of the forced degradation studies (Bakshi, Singh, 2002).

For the acid hydrolysis assay, 6.25 mg of TAD RS and 25.0 mg of SIL RS were transferred to a 25 mL volumetric flask, aided by 15 mL of methanol. The mixture was sonicated for 20 minutes, and adjusted to a volume of 25 mL with 1 mol L<sup>-1</sup> HCl. After 24 h, an aliquot of 1.0 mL was transferred to a 10 mL volumetric flask, neutralized and diluted with methanol to give a final concentration of 25  $\mu\text{g}\cdot\text{mL}^{-1}$  of TAD and 100  $\mu\text{g}\cdot\text{mL}^{-1}$  of SIL.

For the alkaline hydrolysis, 6.25 mg of TAD and 25.0 mg of SIL were transferred to a 25mL volumetric flask, aided by 15 mL of methanol. The solution was then sonicated for 20 minutes, and adjusted to a volume of 25 mL with 1 mol L<sup>-1</sup> NaOH. An aliquot of 1 mL was transferred to a 10 mL volumetric flask, neutralized and diluted with methanol to give a final concentration of 25  $\mu\text{g}\cdot\text{mL}^{-1}$  of TAD and 100  $\mu\text{g}\cdot\text{mL}^{-1}$  of SIL.

For the oxidative reaction, 6.25 mg of TAD and 25.0 mg of SIL were transferred to 25 ml volumetric flask with the aid of 15 ml of methanol. This mixture was sonicated for 20 minutes and the volume was adjusted with 10 % H<sub>2</sub>O<sub>2</sub>. The resulting solution was homogenized and stored for 24 hours at room temperature. Then, an aliquot of 1 mL was transferred to a 10 mL volumetric flask and the volume was adjusted with methanol, obtaining a final concentration of 25  $\mu\text{g}\cdot\text{mL}^{-1}$  for TAD and 100  $\mu\text{g}\cdot\text{mL}^{-1}$  for SIL.

For the photodegradation assay, 6.25 mg of TAD and 25 mg of SIL were transferred to a 25 mL volumetric flask, with the aid of 15 mL of methanol. This mixture was subjected to ultrasonic bath for 20 minutes and its volume was adjusted with the same solvent. An aliquot of 1 mL was then transferred to disposable and sealed cuvettes. The cuvettes were exposed to UVA light for 60 minutes in a photostability chamber at room temperature. The contents of the cuvettes were transferred to a 10 mL volumetric flask and the volume was adjusted with methanol to obtain a final concentration of 25  $\mu\text{g.mL}^{-1}$  for TAD and 100  $\mu\text{g.mL}^{-1}$  for SIL.

#### Linearity

Linearity determination was evaluated by constructing calibration curves with seven concentrations for TAD (2.5-75  $\mu\text{g.mL}^{-1}$ ) and SIL (5.0-200  $\mu\text{g.mL}^{-1}$ ). The linearity was performed by linear regression analysis, which was determined using the least-square regression method.

#### Precision

The precision of the methodology was determined by repeatability (intraday) and intermediate precision (interday) tests. Six replicates of the sample solution, prepared as described in the sub-item "Preparation of the sample solution", were analyzed for repeatability evaluation. Regarding intermediate precision, data was obtained by evaluating two different days, and the results were expressed as a function of the relative standard deviation (RSD%) of all performed measurements.

#### Accuracy

Accuracy was determined at three concentration levels through a RS recovery test. As evidenced in the literature, the simultaneous occurrence of TAD and SIL in falsified formulations occur at various levels of concentration (Park, Ahn, 2012). TAD tablets have alterations with up to or greater than 100% SIL. For this reason, solutions representing these adulterations were prepared, as shown in Table I.

**TABLE I** - Three different levels of concentration for recovery test

Solution*	Concentration levels	TAD – SIL concentration ( $\mu\text{g mL}^{-1}$ )
1	Low – High	10 – 200
2	Medium – Medium	25 – 100
3	High – Low	50 – 10

\*n = 3

#### Robustness

The robustness was determined by analyzing the sample solution, however with deliberate changes to the original method parameters, as described in Table II.

**TABLE II** - Deliberately made changes to the chromatographic conditions following Plackett-Burman factorial design

Factors	Regular chromatographic conditions	(+)*	(-)*
pH	7.5	7.7	7.3
Temperature	25° C	28° C	22° C
Detector	284 – 292 nm	286 – 294 nm	282 – 290 nm
% Acetonitrile	30%	32%	28%
Column	Hypersil C <sub>18</sub>	Hypersil C <sub>18</sub>	Agilent C <sub>18</sub>
Flow rate	1.0 mL	1.05 mL	0.95 mL

\*Higher (+) and lower (-) values as compared to regular conditions. n = 2

Six different factors were selected, and they were evaluated by Plackett-Burman (n = 12) factorial design. Effect (E) of each factor and estimated experimental error (EE) were calculated (Heyden, Nijhuis, Smeyers-Verbeke, 2001).

From the statistical evaluation, a numerical limit value was obtained, which defines if the modification carried out interfered with the quantitative analysis. This value is usually derived from the analytical method test t, according to Equation 1:

Equation 1:

$$t = \frac{|E_x|}{(SE)_e}$$

Where:

$E_x$  = Each factor effect;

$SE_e$  = Estimated experimental error;

The effect was significant if it resulted in a *calculated t* > *critical t*.

*Limit of detection (LOD) and quantification (LOQ)*

The determination of LOD and LOQ was performed based on the signal-to-noise ratio of the analytical run, and verified through the baseline, where the solutions

were prepared at the calculated concentrations and experimentally evaluated.

### Photodegradation kinetics study

The photodegradation kinetics study was performed in the standard solutions of TAD and SIL in their isolated and associated forms. Different solutions were prepared in volumetric flasks by dissolving the drugs in acetonitrile to obtain a final concentration of 250 µg.mL<sup>-1</sup> (TAD), 1000 µg.mL<sup>-1</sup> (SIL) and 250 + 1000 µg.mL<sup>-1</sup> (TAD + SIL). Aliquots of the solutions equivalent to 1 mL were transferred to disposable cuvettes, which were properly sealed, then exposed to UVA radiation in the photostability chamber at the following time intervals: 55, 110, 165, 220, 275 minutes (n = 2). After each period of time, the content of each cuvette was transferred to a 10 mL volumetric flask and its volume was adjusted with methanol and homogenized. The final theoretical concentration was 25 µg.mL<sup>-1</sup> for TAD and 100 µg.mL<sup>-1</sup> for SIL.

The regression coefficients (r) were determined and the best fit observed indicated the reaction order. The degradation rate constant (k), half-life (t<sub>1/2</sub>) and t<sub>90%</sub>, were also calculated.

### Toxicity study

Peripheral blood was collected by venipuncture into sterile vials containing 68 I.U. of sodium heparin (BD Vacutainer®) per mL of blood. The vials were transferred

to the laboratory, and whole blood cultures were established. The blood samples were stored for up to 24 h at 4°C before culturing. This procedure was approved by the Research Ethics Committee of the Federal University of Pampa (Letter of approval nº 27045614000005322).

The leukocyte cultures were prepared with whole-blood samples and immediately transferred to 1 mL of culture medium containing RPMI 1640 supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin, as previously described (Montagner *et al.*, 2010). The cells were then placed in a microaerophilic environment at 37°C for 72 h. The solutions under investigation were added to the blood at 10% concentration. The solutions analyzed included intact and photodegraded (275 min) TAD and SIL (isolated and associated) diluted in phosphate-buffered saline (PBS) at concentrations of 0.18, 0.36 and 0.72  $\mu\text{g mL}^{-1}$  for TAD, and 0.28, 0.55 and 1.1  $\mu\text{g mL}^{-1}$  for SIL. Each group consisted of three culture flasks. Genetic and oxidative parameters were analyzed after 72 h of growth. RPMI 1640 medium was employed as the negative control, and Bleomycin (3  $\mu\text{g mL}^{-1}$ ) as the positive control for all toxicity assessment tests.

Cytotoxicity was assessed by measuring loss of membrane integrity using trypan blue (Burow *et al.*, 1998). Mutagenicity was assessed by micronucleus test, according to the technique described by Schmid (1975) and genotoxicity was measured by comet assay (Singh *et al.*, 1988).

The results were subjected to statistical analysis by one-way ANOVA and Tukey's post hoc test, using a significance level of 5%.

## RESULTS AND DISCUSSION

### Chromatographic method development

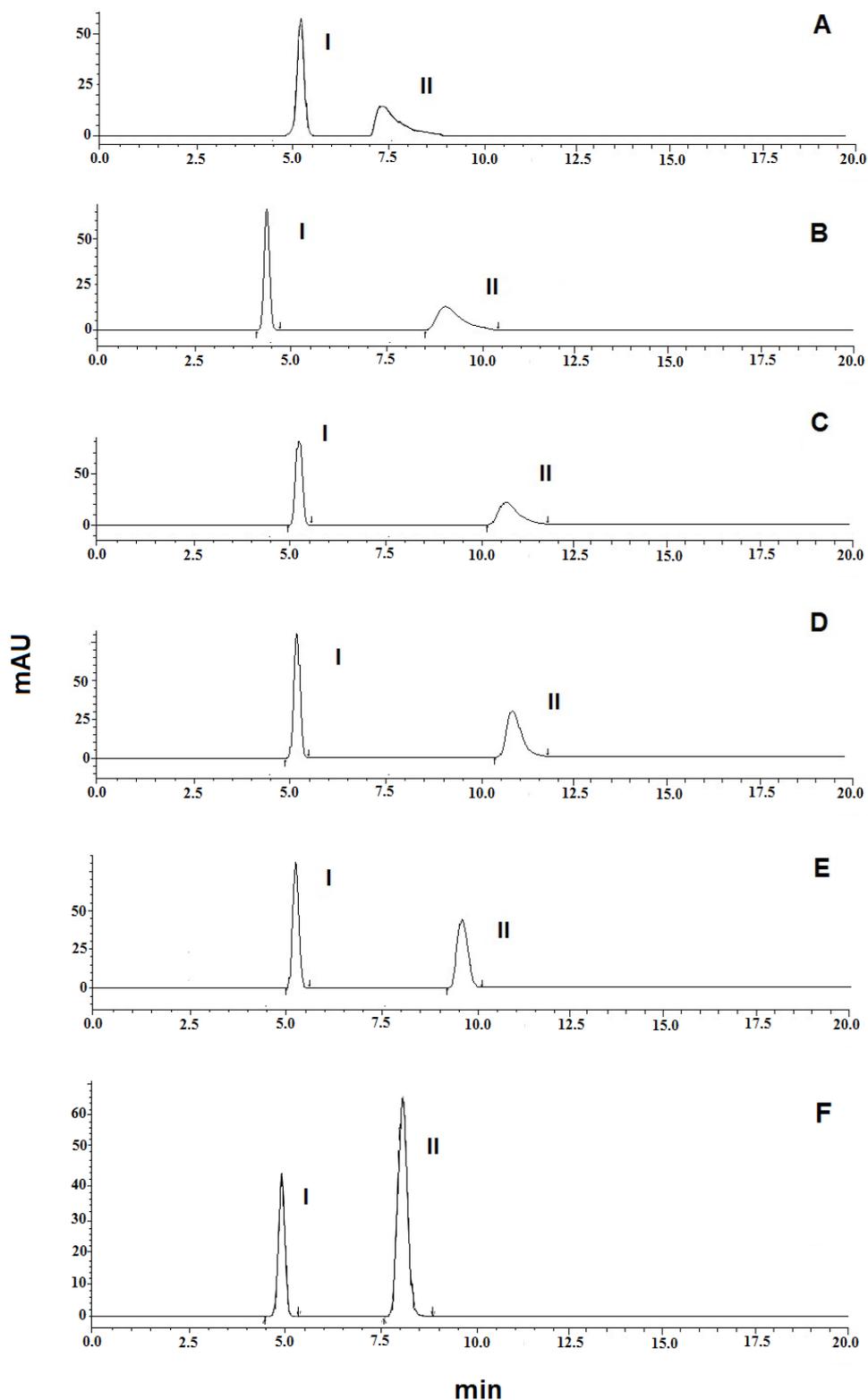
For the initial tests, we consulted previous studies where solvents such as acetonitrile, water and/or methanol were employed (Park, Ahn, 2012; Patel, Patel, 2014). We conducted tests with different compositions and proportions

of these solvents, such as methanol:water (70:30, v/v) and acetonitrile:water:methanol (30:30:40, v/v), with pH values ranging from 3 to 7, adjusted with o-phosphoric acid. The isocratic system was evaluated, analyzing the flow rate which varied from 0.5 to 1.5  $\text{mL min}^{-1}$ .

From Figure 2, it is possible to observe that by increasing the pH of the aqueous phase, an improvement of the chromatographic peak of SIL (peak II) is induced. The existence of a cationic SIL species at pH values below its pKa (pKa = 5.99) may be related to the way the substance interacts with the stationary phase. The predominance of a neutral species occurs near pH 7, where the best chromatographic peak shape for SIL is observed (Gobry *et al.*, 2000). The addition of a modifier (triethylamine 0.3%) to the solvents was able to correct undesired effects caused by the free silanols at the stationary phase, such as peak asymmetry. This way, the best condition was achieved as described on *Equipment and Chromatographic Conditions* section (Figure 2F). Under the experimental conditions that were developed, the maximum absorption for TAD was 284 nm and for SIL was 292 nm. For this reason, two wavelengths were used in the analysis, increasing the sensitivity of the proposed method for the quantification of SIL and TAD.

The system suitability parameters were met, such as theoretical plate number ( $N_{\text{TAD}} = 4682$  and  $N_{\text{SIL}} = 6983$ ), peak symmetry ( $T_{\text{TAD}} = 1.037$  and  $T_{\text{SIL}} = 1.084$ ) and a retention time of 5.0 minutes for TAD and 8.2 minutes for SIL, with a resolution of 8.5 between the peaks. The system suitability test is an integral part of the analytical method, which confirms the effectiveness and suitability of the operating system.

The mobile phase proposed in this study presents some advantages over those previously described for the quantification of these drugs (Park, Ahn, 2012, Ortiz, Antunes, Linden, 2010). Examples of this are the absence of salts in its composition, an isocratic mode of elution and an easy and fast preparation, thus being able to be routinely used in simultaneous analysis of these formulations.



**FIGURE 2** - Chromatograms obtained for TAD (I) and SIL (II) in method development with different mobile phases, where: (A) methanol:water (70:30, v/v); (B) acetonitrile:water pH 3.0:methanol (30:30:40, v/v); (C) acetonitrile:water pH 4.0:methanol (30:30:40, v/v); (D) acetonitrile:water pH 5.0:methanol (30:30:40, v/v); (E) acetonitrile:water pH 7.0:methanol (30:30:40, v/v); (F) solution A:Solution B (30:70, v/v); Solution A is composed of acetonitrile:water (80:20, v/v) and solution B of methanol:triethylamine 0.3% pH 7.5 (57:43, v/v). Detection was performed at 284 nm.

## Validation

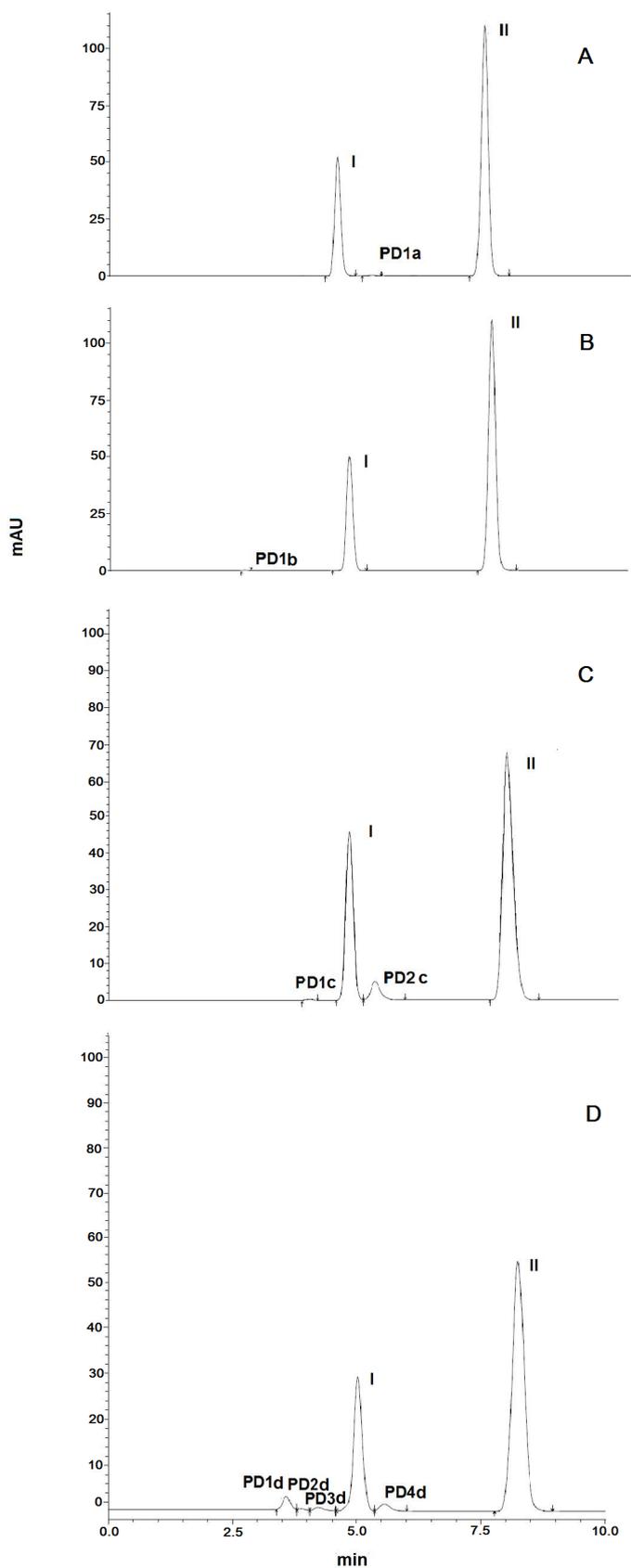
The interference of formulation excipients was evaluated by comparative analysis of the chromatograms obtained with the placebo and RS solutions. According to the chromatograms, an absence of peaks in the same retention time of TAD and SIL is observed, demonstrating that there is no interference from the excipients in the determination of the studied drugs.

The ability to detect the drugs in the presence of their degradation products was evaluated by exposure

of TAD and SIL to the following stress conditions: oxidative, photolytic and hydrolytic (alkaline or acidic). The purpose of degrading the drugs is to induce the formation of possible degradation products and to evaluate the ability of the method to separate them from the analytes (ICH, 2003). Table III presents the tested conditions, extent of drug degradation, and exposure times under both stress conditions, while Figure 3 shows the chromatograms of the forced degradation samples.

**TABLE III** - Forced degradation of associated TAD and SIL

Degrading conditions	Time	Degradation (%)	
		TAD	SIL
Acidic - HCl 1 mol.L <sup>-1</sup>	24 h	9.63	0.76
Alkaline - NaOH 1 mol.L <sup>-1</sup>	24 h	12.80	2.04
Oxidative - H <sub>2</sub> O <sub>2</sub> 10%	24 h	1.88	7.36
Photolic - UVA	1 h	33.50	22.02



**FIGURE 3** - Chromatograms obtained for forced degradation of TAD (I) and SIL (II). **A:** acidic degradation; **B:** alkaline degradation; **C:** oxidative degradation; **D:** photolytic degradation; **PD:** likely degradation products.

Thus, it is possible to observe that the developed chromatographic method is able to determine TAD and SIL in the presence of their degradation products under all tested conditions. In addition, it is suggested that for the chromatographic peak purity tool, the peaks of the drugs under study showed adequate purity (100% pure in all cases), indicating the specificity of the proposed method. The results indicate that the method is stability-indicating, and that the drugs can be quantitatively evaluated in the presence of degradation products and pharmaceutical excipients.

The linearity was determined by constructing calibration curves referring to TAD RS and SIL RS. The method was linear ranging from 2.5 to 75.0  $\mu\text{g.mL}^{-1}$  for TAD and 5 to 200  $\mu\text{g.mL}^{-1}$  for SIL.

The correlation coefficient ( $r$ ) values obtained were 0.9995 and 0.9998 for TAD and SIL, respectively, indicating that the results are directly proportional to drug concentrations. In the ANOVA analysis of the results, it was possible to observe a significant linear regression for TAD ( $F_{\text{calculated}} = 3255 > F_{\text{critical}} = 4.6$ ) and SIL ( $F_{\text{calculated}} = 10236 > F_{\text{critical}} = 4.6$ ), with no significant linearity deviation ( $F_{\text{calculated}} \text{ SIL} = 0.37 < F_{\text{critical}} = 2.96$ ;  $F_{\text{calculated}} \text{ TAD} = 0.56 < F_{\text{critical}} = 2.96$ ) at a significance level of 5%, confirming the linearity of the proposed method.

Usually for analysis of commercially available samples there is, for example, information about

excipients and drug concentrations. However, for forensic samples there is no standardization for any of these aspects, so that products with the same source of acquisition may have different components, such as excipients and impurities, as well as variations in their drug concentration. Thus, SWS were prepared as described in the literature for forensic samples (Park, Ahn, 2012; Rowe, Sheskey, Quinn, 2009), in order to reproduce a representative sample containing most of the excipients present in the commercial drugs. In addition, it was possible to simulate the mean concentration of TAD and SIL in agreement with the levels actually found in seized samples, as reported in the literature (Park, Ahn, 2012). The fact that they were prepared in the laboratory allowed us to obtain an sample concentration range, thus not limiting this study to the development of a methodology based on a specific counterfeit product.

The precision was determined from repeatability (intraday) and intermediate precision (interday) by analyzing six sample solutions at the concentration of 25.0  $\mu\text{g.mL}^{-1}$  TAD and 100  $\mu\text{g.mL}^{-1}$  SIL, on the same day and on two different days, respectively.

The results are shown in Table IV and demonstrate that the method was precise, with RSD values  $< 1.5\%$  in all cases.

**TABLE IV** - Values obtained for repeatability and intermediate precision

Drugs	Repeatability*	Intermediate precision**
	Mean content (%) $\pm$ RSD	Mean content (%) $\pm$ RSD
TAD	99.95 $\pm$ 1.07	100.37 $\pm$ 0.59
SIL	100.70 $\pm$ 0.54	101.00 $\pm$ 0.42

\* $n = 6$  \*\* $n = 18$

The accuracy of the method was demonstrated by determining the recovery percentage of known amounts of TAD and SIL RS added to placebo. The recovery percentage of the added amounts ranged from 99.86% to 100.36% for TAD and 100.45% to 101.59% for SIL. The excellent percentage recovery values and low RSD values (< 1.5%) demonstrate the accuracy of the proposed method.

In the robustness test, the determined response was the TAD and SIL percentage in the sample solutions. Drug quantification in the sample solution can be considered robust, as none of the factors that were deliberately changed had a significant effect ( $t_{\text{calculated}} < t_{\text{critical}}$ ,  $\alpha = 0.05$ ). In addition to this, all proposed modifications have not changed the system suitability parameters, i.e., the method still presented optimal peak symmetry, theoretical plate number and satisfactory resolution between peaks.

The LOD determined for TAD was 80 ng mL<sup>-1</sup> and for SIL was 180 ng mL<sup>-1</sup>. Regarding LOQ, the values determined were 120 ng mL<sup>-1</sup> for TAD and 270 ng mL<sup>-1</sup> for SIL, demonstrating the sensitivity of the proposed method.

The method was validated, showing satisfactory data for all of the tested parameters. The validation testifies that the procedure is stability-indicating and suitable for

the analysis of the TAD and SIL illicit association. In this way, the developed method can be employed for forensic analysis of these products and routine quality control of the TAD and SIL commercial products.

### Photodegradation kinetics study

In regards to the degradative conditions that were tested, the photolytic condition induced the highest percentages of degradation in the drugs, and for this reason it was the condition chosen to carry out the kinetic studies, as well as the biological safety assessment. The concentration, log and reciprocal concentration plots of the remaining drugs versus time were determined. For the isolated drugs, degradation followed a first order kinetics. Regarding drug association, degradation followed zero-order kinetics. In Table V, the values obtained from the kinetic parameters for degradation of TAD and SIL are shown, and these were calculated according to their respective reaction order. It is possible to observe that the rate of degradation of both TAD and SIL remained similar when isolated. However, the change in behavior in the rate of degradation when TAD and SIL are associated is characterized by a change in reaction order.

**TABLE V** - Photodegradation kinetics parameters of the drugs

Condition	Drug	k	t <sub>1/2</sub> (min)	t <sub>90%</sub> (min)
Isolated	TAD	0.003620	191.47	29.10
	SIL	0.003645	190.16	28.90
Associated	TAD	0.047741	255.54	51.10
	SIL	0.193826	257.56	51.51

### Toxicity assessment

Although TAD and SIL have been present for a long time in the pharmaceutical market and their toxic potential is adequately elucidated, it is of great importance to evaluate the behavior of both when in association, and additionally, in the presence of their degradation products.

Thus, preliminary *in vitro* studies were carried out to assess the toxicity of these associations.

The concentrations of the samples used in this study were chosen taking into account drug plasma concentrations levels (0.378 µg mL<sup>-1</sup> for TAD and 0.56 µg mL<sup>-1</sup> for SIL) (Shakya *et al.*, 2007; Nichols, Muirhead, Harness, 2002). Thus, we chose intermediate/whole

values closer to the actual plasmatic concentrations (0.36  $\mu\text{g mL}^{-1}$  TAD and 0.55  $\mu\text{g mL}^{-1}$  SIL), a concentration representing twice the plasma concentration (0.72  $\mu\text{g mL}^{-1}$  TAD and 1.1  $\mu\text{g mL}^{-1}$  SIL) and one representing half of the plasma concentration (0.18  $\mu\text{g mL}^{-1}$  TAD and 0.28  $\mu\text{g mL}^{-1}$  SIL).

To evaluate the cytotoxicity of the substances analyzed here, cell viability was measured by employing trypan blue. The number of viable cells in a suspension was determined based on the principle that living cells have intact cell membranes that exclude certain dyes, such as trypan blue. Therefore, the cells were scored as dead or alive based on microscopic analysis where a blue stain was evidence of dead cells (Collins *et al.*, 2008).

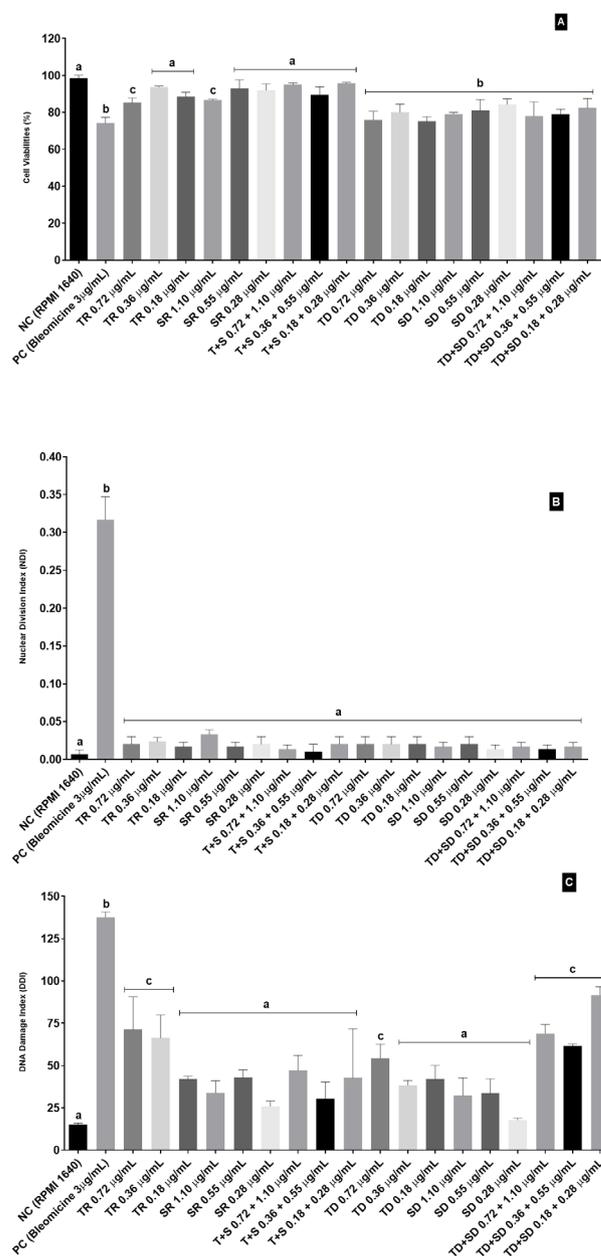
The results obtained in this assay are presented in Figure 4A, where it is possible to observe that the cytotoxicity of both isolated and associated drugs is statistically different compared to the negative control in all the tested concentrations, suggesting cytotoxic activity of photoproducts. The highest concentrations of TAD and SIL, isolated and non-degraded, also presented a statistically significant difference as compared to the negative control.

The micronucleus test evaluates mutagenicity by detecting clastogenic processes as well as defects in the cell spindle. Thus, the presence of micronuclei is evidence of substances that cause chromosomal breaks or that affect spindle components or the centromeric region. The results obtained for the micronucleus test are represented in Figure 4B, where it is observed that all groups were statistically similar to the negative control group. Therefore, this suggests that the drugs analyzed did not present a mutagenic effect under the conditions tested.

The Comet Assay is a sensitive genotoxicological method that evaluates DNA damage in individual cells (Collins *et al.*, 2008). According to the results obtained here, as shown in Figure 4C, all TAD and SIL concentrations, when associated and photodegraded, showed a significant difference in the results of this assay as compared to the negative control group. Therefore, it is suggested that at these concentrations, the samples showed genotoxic potential.

All tests that were presented here were carried out *in vitro*. Further studies should be conducted to try to isolate,

identify, and evaluate the toxicity of these degradation products individually.



**FIGURE 4 - A** – Evaluation of leukocyte viability by Trypan blue exclusion test; **B** – Effects on cell division index; **C** – DNA Damage Index of exposed cells. Evaluation after exposure to TAD and SIL in their isolated, associated, not degrade, and photodegraded forms. Acronyms: Associated and not degraded (T+S); photodegraded TAD (TD), photodegraded SIL (SD), nondegraded TAD (TR), nondegraded SIL (SR), TAD and SIL associated and photodegraded (TD+SD), negative control (NC), positive control (PC). Different letters (a, b, c) indicate a statistically significant difference.

## CONCLUSION

Stability-indicating method was developed and validated for TAD and SIL determination, both isolated or in association, and it can be employed for forensic analysis of these products and also for routine quality control of commercial products.

In regards to the degradative conditions that were tested, the photolytic condition induced the highest percentages of degradation in the drugs. *In vitro* toxicological studies demonstrated that when associated and photodegraded, the drugs showed cytotoxicity and genotoxicity.

The results show that the risk of consuming these counterfeit products is very high, due to probability of degradation of these formulations' components, increasing the concern about the toxicity and safety.

This study might aid in the analysis of these counterfeit medicines at a forensic level, but mainly it produces information available to the population which may help to raise awareness about the risks of consuming products without a certified origin.

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