Green analytical methods for isosorbide dinitrate determination by UV spectrophotometry and stability indicating HPLC-PDA

Métodos analíticos verdes para determinação de dinitrato de isossorbida por espectrofotometria UV indicativo de estabilidade HPLC-PDA

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ABSTRACT
Two analytical methods, green chemistry based, were developed, validated and compared for quantification of isosorbide dinitrate (ISDN) in sublingual tablets. The UV spectrophotometric method proved to be innovator and was carried out at maximum absorption of 205 nm using water as solvent. The HPLC method was stability indicating type, and ISDN was separated of its degradation products with a mobile phase consisting of methanol: water (50:50, v.v⁻¹), in a flow rate of 1.0 mL.min⁻¹ at room temperature. The forced degradation studies demonstrated the molecule intrinsic stability, which was unstable in hydrolysis and basic photolysis, oxidative medium and heat. In validation, the methods presented correlation coefficients greater than 0.99. The precisions were less than 3%. Both methods were robust for parameters tested and the accuracy values were found suitable. Statistical analysis proved that both methodologies are appropriate for ISDN quantification in quality control routine. The methods were considered simple, accessible, and economical and involved the use of reagents that are harmless to environment, to analysts and to components of the equipment, positive aspects that differentiate these methods from compendia and existing procedures in scientific literature. In addition, this article provides analytical methods both in developed industries and laboratories and in those with a structure still under development, what contributes to the reduction of health risk related to the quality of medicines.

Keywords: isosorbide dinitrate, stability indicating analytical method, high performance liquid chromatography, UV Spectrophotometry, validation.

RESUMO
Dois métodos analíticos, baseados na química verde, foram desenvolvidos, validados e comparados para quantificação de dinitrato de isossorbida (ISDN) em comprimidos sublinguais. O método espectrofotométrico por UV mostrou-se inovador e foi realizado com absorção máxima de 205 nm, utilizando água como solvente. O método HPLC foi do tipo indicador de estabilidade e o ISDN foi separado de seus produtos de degradação com uma fase móvel composta por metanol: água (50:50, v.v⁻¹), com vazão de 1,0 mL.min⁻¹ à temperatura ambiente. Os estudos de degradação forçada demonstraram estabilidade intrínseca da molécula, instável em hidrólise e fotólise básica, meio oxidativo e calor. Na validação, os métodos apresentaram coeficientes de correlação maiores que 0,99. As precisões foram inferiores a 3%. Ambos os métodos foram robustos para os parâmetros testados e os valores de precisão foram considerados adequados. A análise estatística mostrou que ambas as metodologias são apropriadas para a quantificação de DNIS na rotina de controle de qualidade. Os métodos foram considerados simples, acessíveis e econômicos e envolveram o uso de reagentes inofensivos ao meio ambiente, aos analistas e aos componentes do equipamento, aspectos positivos que diferenciam esses métodos dos compêndios e dos procedimentos existentes na literatura científica. Além disso, este artigo fornece métodos analíticos em indústrias e laboratórios desenvolvidos e naquelas com uma estrutura ainda em desenvolvimento, o que contribui para a redução dos riscos à saúde relacionados à qualidade dos medicamentos.

Palavras-chave: dinitrato de isossorbida, método analítico indicativo de estabilidade, cromatografia líquida de alta eficiência, espectrofotometria UV, validação.

1 INTRODUCTION
The life cycle of analytical methods applied to pharmaceutical drug analysis involves the development, validation, and continuous improvement of existing methods (Deidda et al., 2018). Develop new analytical methods and upgrade the existing procedures are very important once, still
nowadays, it is possible to find old drugs and essential medicines, in the literature and official compendiums, with methodologies developed without aligned with laboratories and legislative innovations and tendencies, mainly, as regards the green chemistry principles.

Isosorbide dinitrate (ISDN) is an essential medicine, released in 1970-1980s, that acts as a vasodilator. It is chemically known as 1,4:3,6-dianhydro-D-glucitol-2,5-dinitrate and presents the structure showed in Figure 1. The U.S. Food and Drug Administration (FDA) approved ISDN for angina pectoris treatment and prophylaxis, one of the leading causes of mortality and morbidity due to heart disease in the world (Fung, 1992; Barbero et al., 2016; Micromedex, 2016). The responsible for pharmacologic effect in the drug is a functional group RNO₂, an oxide nitric releaser, which works by relaxing vascular smooth muscles (Fuchs and Wannmacher, 2012).

Several analytical methods for the quantitative determination of ISDN were described in the literature using different techniques, such as infrared spectrometry (Woo et al., 1973); thin layer chromatography (Carlson and Thompson, 1986); and high performance chromatography liquid (HPLC) (Gelber and Papas, 1983; Mizuno, Shimizo, and Morita, 1983; Carlson, Thompson, and Snell, 1988; Kassey, Pulla, and Prakash, 2014; Neelima and Passad, 2014; Santosh et al., 2014; Madhu et al., 2015; Pola and Gowri, 2015; Priyadarshika, Rao, and Srikanth, 2015). British (BP) and United States (USP) Pharmacopoeias also described HPLC-UV as a method for ISDN sublingual tablets (BP, 2011; USP, 2018). However, it is possible to note that the most of reported methods was tricky or required sophisticated equipment, not easily manageable and was time consuming.

Furthermore, a literature survey did not reveal any simple UV Spectrophotometric method and showed only a few Stability Indicating Analytical Methods (SIAM) for ISDN in pharmaceutical samples (Carlson, Thompson, and Snell, 1988; Kassey, Pulla, and Prakash., 2014; Pola and Gowri,
2015). These SIAMs already described for ISDN have some limitations from the analytical point of view. Such as non-execution of forced degradation studies, absence of validation parameters as limit of detection, which although not mandatory for validation of analytical methods is a very important aspect for SIAM, since it predicts sensitivity of method and guarantee its capacity of detect the analyte under the established experimental conditions with maximum confidence.

In fact, the ISDN SIAM found in the literature has used buffers and toxic reagents in their HPLC methodologies (BP, 2011; USP, 2018). The use of toxic solvents can lead to problems in equipment, and damages to operators and environment in a long term, a fact that has encouraged companies to use environmentally beneficial alternatives in their routine processes to reduce harmful waste (Cue and Zhang, 2009). Therefore, it is highly necessary to develop cost-effective methodologies that also consider the analyst health and environmental impacts.

Considering the indication of ISDN, the sublingual route is one of preferred pathway for medicines administration, as it advantageously leads to rapid absorption of drug into bloodstream (Nibha and Pancholi, 2012). In contrast, impurities, if presents, will be led to blood and can cause harm to consumers health. Thus, methods to this molecule has to be stability indicating to ensure the safety in medicine analysis, which normally is achieved with HPLC techniques. In addition, an UV Spectrophotometry method for ISDN determination in sublingual tablets was developed, to guarantee simple and fast methodologies in quality control (QC) routine. Both techniques were chosen for being commonly part of QC laboratories and for use solvents considered environmental-friendly (Tótoli and Salgado, 2015).

In this context, this work developed, validated and compared two different analytical methods, green analytical chemistry-based, economical and reproducible for quantification of ISDN in sublingual tablets to be used mainly in QC laboratories and pharmaceutical industries. Consequently, its intended to contribute with population in the access to safe and quality medicines.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

ISDN reference standard was obtained from European Pharmacopeia, lot 3.0, declared to contain 40.8% of ISDN and 59.2% of lactose. The marketed formulation (Isordit™, sublingual tablets, containing 5 mg of ISDN, EMS Sigma Pharma LTDA, Brazil) was purchased from local pharmacy. The excipients used in this study and present in the ISDN formulation were lactose, magnesium stearate, croscarmellose sodium, starch, and microcrystalline cellulose. All reagents, namely, sodium hydroxide (NaOH, Neon™, Brazil), hydrogen peroxide (H₂O₂, Alphatec™, Brazil), copper sulphate
(CuSO₄, Reagen™, Brazil), hydrochloric acid (HCl, Alphatec™, Brazil) and methanol (Alphatec™, Brazil) were of analytical grade. The solvents methanol HPLC grade (J.T.Baker™, EUA) and ultra-purified water (Milli-Q™, EUA) were also used.

2.2 HPLC INSTRUMENTATION AND CONDITIONS

Analytes were measured using a high-performance liquid chromatographic system from Shimadzu™ equipped with a PDA detector (SPD-M20A), a binary solvent delivery system (LC-6AD), a manual injector, a communication bus module (CBM-20A), an on-line degasser (DGU-20A) and a LC Solutions™ software.

The mobile phase, consisting in a mixture of methanol and water (50:50, v.v⁻¹), was degassed by ultrasonic bath. Totality solution samples were filtered through 0.45 μm filters prior to analysis and the volume injected was 20 μL. Chromatographic separations were achieved using a C18 (250 mm × 4.6 mm i.d.; 5 μm) column (NTS™), in a flow rate of 1.0 mL.min⁻¹. All analyses were performed at room temperature and monitored at wavelength of 210 nm. Total run time was 16 minutes.

2.3 UV SPECTROPHOTOMETER INSTRUMENTATION AND CONDITIONS

UV Spectrophotometric analyses were performed on a Shimadzu UV-Vis spectrophotometer, UV 1800 model, with 10 mm quartz cells. The following solvents were tested to UV method development: water, methanol, 0.1 M NaOH and 0.1 M HCl. ISDN samples solutions (5 μg.mL⁻¹) were scanned from 200 to 400 nm. ISDN presented maximum absorption at 205 nm. The apparent molar absorptivity was also determined.

2.4 PREPARATION OF ISDN STANDARD SOLUTIONS

For preparation of ISDN standard solution (100.0 μg.mL⁻¹) to HPLC-method, 12.2 mg of ISDN reference standard were weighed and transferred to 50 mL volumetric flask. The drug was solubilized in methanol, put in an ultrasonic bath for 30 minutes, and then the final volume was completed with the same solvent. Similarly, ISDN standard solution to UV Spectrophotometric-method was performed, except the solvent used was water. All standard solutions were stored in a refrigerator at 4 °C.

2.5 PREPARATION OF ISDN SAMPLE SOLUTIONS

For preparation of ISDN sample solution, 20 sublingual tablets were weighted and grounded. An equivalent amount of 10.0 mg of ISDN were weighed and transferred to 50 mL volumetric flask.
The same procedure that was performed to ISDN standard solutions was used. Sample solutions concentration were 100 µg.mL\(^{-1}\). All sample solutions were stored in a refrigerator at 4 °C.

2.6 ANALYTICAL METHOD VALIDATION

2.6.1 Forced degradation conditions and selectivity

For drug stability evaluation, with respect to degradation products, intrinsic stability and method selectivity, ISDN reference standard and sublingual tablets were subjected to forced degradation. Sample solutions (1 mg.mL\(^{-1}\)) were exposed during 44 hours and 26 °C to basic hydrolysis (using 0.1 M NaOH as solvent). The sample solutions of ISDN sublingual tablet were also submitted to basic photolysis for 8 hours. Neutral hydrolysis was performed at 60 °C for 24 hours, using water as solvent. To acid hydrolysis (0.1 M HCl) the conditions 72 hours and 60 °C were used. The oxidative condition (3% H\(_2\)O\(_2\)) and metal ions exposition (0.05 M CuSO\(_4\)) were evaluated at 60 °C for 5 hours and 48 hours, and 25 °C and 24 hours, respectively.

Additionally, sample solutions were exposed to the light in an UV camera (UVC – 254 nm), at room temperature, during 48 hours for the acid, basic and neutral photolysis verification. Thermal degradation studies were performed at 80 °C during a period of 6 days.

After the exposition to each degradation condition, the resultant solutions were appropriately diluted and chromatograms were obtained, comparing with control samples (without any degradation exposition). All samples were analyzed in triplicate.

The HPLC method selectivity was evaluated by peak purity of ISDN in forced degradation samples (ICH, 2005; Brazil, 2017).

The selectivity of UV Spectrophotometric method was verified by analyzing solutions containing the sublingual tablets components excepting ISDN (placebo - excipients), ISDN sublingual tablets and ISDN reference standard. The system response was examined for the presence of interference or overlaps with ISDN signal at 205 nm. The standard and sample solutions final concentration was 20 µg.mL\(^{-1}\) (Brazil, 2017).

2.6.2 Linearity

Developed HPLC-method linearity was assessed from analytical curve of main peak areas versus six different concentrations of ISDN standard solutions (1.0 – 6.0 µg.mL\(^{-1}\)). The least squares method was used because it is an efficient strategy for estimating regression parameters and its application is not limited to linear relationships only. In this parameter, the ordinary least squares method was used. Analytical studies were carried out during three consecutive days in the same
concentration range. The evaluation of linear association between the variables was performed by correlation (r) and determination (R²) coefficients. Analytical curve slope and Y-intercept was also obtained. In the slope test, the ANOVA F test was used to assess the significance of the model. Student's t statistic was employed for the intercept test (linear coefficient). In this case, hypotheses H0 were tested, in which the intercept is equal to zero and H1, in which the intercept is different from zero. The normality of residues was evaluated by Anderson-Darling test and homoscedasticity was assessed by the Cochran test, with the following hypothesis test - H0, with the variances of the levels being equal and with H1 representing at least one different variance. All statisticians were performed at a significance level of 5% (ICH, 2005; Brazil, 2017).

To evaluate UV spectrophotometric method linearity, six separate series of ISDN standard solution (5.0 – 30.0 µg.mL⁻¹), in triplicate, were prepared and analyzed. Least square method was executed and all statistic parameters to assessed linearity were performed as done for previous method (ICH, 2005; Brazil, 2017).

2.6.3 Precision

Precision (repeatability and intermediate precision) of HPLC method was assessed by percentage of relative standard deviation (%RSD) determined in a series of six samples measurements, prepared individually, at same concentration (3 µg.mL⁻¹). Similarly, precision of UV-method was evaluated with six replicates at 15 µg.mL⁻¹.

Repeatability was determined in the same day and intermediate precision in three different days (ICH, 2005; Brazil).

The maximum acceptable value for %RSD was that established by Association of Official Analytical Chemists (AOAC, 2016).

2.6.4 Accuracy

Accuracy of each method was tested by the method of standard additions. Samples of ISDN reference standard were added to sublingual tablets solutions (n=3, at each level of 2.4; 3.0 and 3.6 µg.mL⁻¹ to HPLC method and 2.0, 5.0 and 8.0 µg.mL⁻¹ to UV spectrophotometric method). The fortified samples were analyzed and the total amount recovered was calculated in terms of percentage (%R) of ISDN known amount (ICH, 2005; Brazil, 2017). To the concentrations used, the acceptable limit for accuracy was between 80-110% (AOAC, 2016).
2.6.5 Detection and quantification limits (DL and QL)

To HPLC method, DL and QL were calculated using calibration curve as per ICH guideline (ICH, 2005). Three different curves were plotted to obtain the necessary data for the calculation. The curves were constructed with values below the calibration curve concentrations used for linearity.

DL and QL of ISDN in UV Spectrophotometric method were estimated using standard deviation of the blank (solvent) (ICH, 2005; Brazil, 2017).

2.6.6 Robustness

Robustness of methods was evaluated on small and deliberate variations of critical parameters conditions. To HPLC method robustness was determined changing brands of organic solvent and analyst. The parameters were evaluated using Student's test (t).

Robustness of UV Spectrophotometric method was determined by changing wavelength and the micropipettes brands (Bio-Rad™ to Peguepet™).

2.7 SYSTEM SUITABILITY

System suitability for HPLC parameters were determined with six replicate injections of work standard solutions. The chromatographic parameters calculated were retention time, peak area, capacity factor, peak resolution and theoretical plate, all of them in relation of ISDN peak. System suitability was measured based on %RSD (RSD ≤ 2.0) (Food and Drug Administration, 2014).

2.8 METHODS APPLICATION

The applicability of the two developed methods was tested determining ISDN in sublingual tablets. The analyses were carried out on three different days.

In HPLC method, sample solutions of ISDN reference standard and sublingual tablets (concentration of 3 μg.mL⁻¹) were injected in the developed method (n=6). Equivalent methodology was used to evaluate the application of UV spectrophotometric method at concentration of 15 μg.mL⁻¹.

The content (%) mean value was correlated with the specification for ISDN sublingual tablets (90 to 110%) in the United State Pharmacopoeia (USP, 2018). %RSD was also calculated.

2.9 DATA ANALYSIS

Statistical analysis was performed using the software Action Stat™.
3 RESULTS AND DISCUSSION

3.1 ANALYTICAL METHOD DEVELOPMENT

3.1.1 UV Spectrophotometric method

ISDN reference standard has lactose as excipient (diluent), because of its explosive characteristic. Hence, the interference of lactose in the UV absorption of ISDN molecule was evaluated against different solvents.

In UV spectrophotometric method development, methanol and water were the only solvents tested in which there was no UV absorption of lactose on ISDN specific wavelength. Thus, water was chosen as solvent since it presents good solubility, low cost, allows easy preparation of solutions and is a renewable solvent. One of the priority objectives of analytical methods development based on sustainability is the use of non-renewable sources reduction, preferring renewable ones. The UV method proposed, therefore, did not use toxic reagents, it is environmentally friendly and increases safety to operators, key goals to be achieved in greening analytical methods (Gatuzska Migaszwzewski, and Namiesnik, 2013).

In water, UV spectroscopic scanning allowed selecting the maximum wavelength at 205 nm, as the best for ISDN detection in standard and sample solutions.

3.1.2 HPLC Stability Indicating Analytical Method

Differently from UV spectrophotometric methodology development, SIAM development by HPLC requires steps that are more complex, such as decisions about type of SIAM, how conduct stress studies and parameters setting to preliminary tests (i.e. mobile phase, flow rate, pH), as performed in this work.

In HPLC method development, a specific SIAM was settled to ISDN determination for being simple, fast and less costly, essential requirements for pharmaceutical industry. The specific SIAM type is a method that generates a good resolution of the active pharmaceutical ingredients (API) in relation to other substances presents in the chromatogram (Bakshi and Singh, 2002).

Mobile phase solvents selection were based on sensitivity, ease of preparation, cost and compatibility of results in the forced degradation studies, besides ISDN solubility in the mobile phase. The solvents (acetonitrile, methanol and water) were tested in different proportions and distinct flow rates for a chromatographic peak selection with good resolution and symmetry.

Through these tests, it was possible to achieve good chromatographic performance factors increasing water proportion with reduction of flow rate.
The mobile phase defined for HPLC method was methanol and water, 50:50 (v.v⁻¹), in a flow rate 1.0 mL.min⁻¹. This settled mobile phase shows advantage due to used lower proportion of organic solvent, when compared to other conditions tested in this work, and to the method for ISDN described in USP (2018), which are composed of water, pH 4.7 ammonium acetate buffer and methanol (35:10:55 v.v⁻¹). Moreover, the mobile phase selected produces more benefits, once it has reduced the generation of waste that is harmful to the environment. Another advantage of the mobile phase chosen are the solvents characteristics. Water and methanol have both low cost, are easily available, simple to dispose and are not harmful to environment. Still, this mobile phase uses solvents suitable for cleaning and preserving the column, facilitating one of the steps involving the use of HPLC and saving time.

Furthermore, during the method development an evaluation of ISDN molecular behavior, in relation to variations in pH by Chemicalize Beta™ software, demonstrated that the drug’s chemical structure remained 100% in the most stable form in all pH ranges (0 – 14), allowing the development of method without use of buffers to adjust pH. This turn out to be an improvement, since the use of buffer solutions can cause corrosion, abrasion or crystallization in chromatographic columns and systems and encumber the methodology, requiring extensive chromatographic system and column cleaning process. Besides, buffers use is contrary to green chemistry principles since its preparation require a certain amount of time and it has a low shelf-life, making methodologies time consuming and generating high waste volumes (Marco et al., 2019).

ISDN best wavelength for peak detection was obtained at 210 nm, chosen after analysis of chromatograms from the scan with photodiode array detector. The elution mode was isocratic, which means that it is easy to operate throughout the process.

Finally, all these chromatographic conditions lead to a symmetric peak with good separation capacity, as demonstrated by the values of the tail factor (1.86), resolution parameters (2.70) and peak purity (>0.999), assuring that possible degradation products do not coeluted with main peak, promoting a reliable analysis of degraded and in stability samples.

3.2 FORCED DEGRADATION STUDIES

In order to develop a SIAM, it is necessary to carry out forced degradation studies that challenge the worst-case method and point to its stability indicative power (Brazil, 2015). Stress studies were performed to provide some information about the drug stability and to demonstrate the developed method selectivity to measure the changes in concentration of ISDN in sublingual tablets. The results of ISDN reference standard and ISDN sublingual tablets forced-degradation studies were
presented in Table 1. Lactose, the excipient presents in all ISDN samples, did not demonstrate any degradation product.

Table 1. Forced degradation of studies of ISDN

| Stress    | Conditions                        | Degradation ISDN reference standard (%) | Degradation ISDN product (%) |
|-----------|-----------------------------------|----------------------------------------|------------------------------|
| Hydrolysis| 0.1 M HCl; 60°C (±1°C), 72 h, without light | 0                                      | 0                            |
|           | 0.1 M NaOH; 26°C (±1°C), 44 h, without light | 23.12                                  | 19.31                        |
|           | H₂O; 24 h, 60°C (±1°C) without light | 0                                      | 0                            |
| Oxidation | 3% H₂O₂; 60°C (±1°C), 5 h, without light | 6.18                                   | 21.77                        |
|           | 3% H₂O₂; 60°C (±1°C), 48 h, without light | 13.61                                  | 100                          |
| Metal ions| 0.05 M CuSO₄; 25°C (±1°C), 24 h, without light | 0                                      | 0                            |
| Heat      | 80°C, 6 days                      | 18.00                                  | 19.79                        |
| Photolysis| 0.1 M HCl; 25°C (±1°C)48 h, UVC (254 nm) | 0                                      | 0                            |
|           | 0.1 M NaOH, 25°C (±1°C)48 h, UVC (254 nm) | 18.15                                  | 50.66                        |
|           | 0.1 M NaOH; 8h, 25°C (±1°C), UVC (254 nm) | -                                      | 27.68                        |
|           | H₂O; 48 h, 25°C (±1°C), UVC (254 nm) | 0                                      | 0                            |

As can be seen in Table 1, ISDN degraded in basic hydrolysis, basic photolysis, oxidation and heat conditions. The highest proportion of degradation was observed in ISDN sublingual tablets in which ISDN peak area decreased 27.68% in 8 hours over basic photolysis condition.

Oxidation was the main ISDN sublingual mechanism of degradation, considering that photodegradation is also a type of oxidation that happens in the presence of light (Aulton, 2005). Further, ISDN sublingual tablets and reference standard has degraded during oxidation with hydrogen peroxide, being the degradation rate for sublingual tablets 3.5 times greater than reference standard, in 5 hours. Basic photolysis followed a similar profile.
On the other hand, ISDN molecule showed a different degradation profile in thermal and basic hydrolysis. It is known that excipients can cause drastic changes in kinetics of degradation, including increasing the speed of the reaction, which may justify the behavior of the active in the formulation (Anvisa, 2013).

Concerning to basic photolysis, a new peak appeared on ISDN sublingual tablets chromatogram evaluation, which was considered a secondary degradation product, possibly due to molecule total degradation after light exposure. UV light can accelerate chemical reactions rates, mainly by oxidation (Marin, 2003). Therefore, this behavior demonstrated ISDN is photosensitivity.

Moreover, the basic hydrolysis and photolysis showed a possible degradation product of ISDN at 5 min, approximately. It has been noted that possible degradations products formed have more polarity than ISDN, because they surged before ISDN in chromatogram. In this way, it is believed that basic medium has been the preponderant factor for molecule degradation. It is likely that the hydroxyl, provided by the medium, has reacted with the nitro functional group and generated an alcohol as a degradation product, as provided in Figure 2. Figure 3 also shows that ISDN peak was successfully separated from possible degradants products in the proposed method.

![Figure 2. Probable mechanism of reaction of the ISDN molecule in basic medium for the production of the possible degradation product formed in the chromatogram](image-url)
Figure 3. Representative chromatogram of ISDN and its degradation product (DP) after basic hydrolysis and basic photolysis.
Significant degradation was also observed in thermal condition stress (18-19.8%); however, it was necessary a long time (6 days) to occur, therefore, it can be indicated that ISDN is probably stable in heat conditions.

In optimized conditions, ISDN and its degradants were well separated, and the chromatographic analysis time was 16 minutes. Typical retention time of ISDN peak was about 13.50 min, excellent for a SIAM approach.

Finally, stress conditions provided the ISDN intrinsic stability besides evidence that the developed analytical method was stability indicating, capable of separate unequivocally the ISDN peak even in the presence of its degradation products.

3.3 ANALYTICAL VALIDATION OF METHODS

3.3.1 UV spectrophotometric method

Regarding selectivity, ISDN UV spectrum did not changed in the presence of common excipients (placebo) used in the formulation of ISDN sublingual tablets. Moreover, absorption spectrum of pure drug sample matched with the marketed formulation sample.

The analytical curve, obtained in the linear range from 5.0 to 30.0 μg.mL⁻¹ in water, demonstrated the strong correlation between the concentrations of ISDN and the absorbance’s obtained, with r and R2 greater than 0.99. The Anderson-Darling test was used to assess the normality of residues. The p-value of 0.0768 attested the normality of the residues at the significance level of 5%. The significance of the slope was verified by F test, from ANOVA and, with a p-value equal to 0, it is understood that it is significantly different from zero. According to Student's t statistic, when evaluating the intercept (linear coefficient), as the p-value (0) of the t-test is less than 0.05, we reject the null hypothesis, in other words, we prove that the intercept is different from zero. Homoscedasticity, with a p-value 0.07, from Cochran Test, did not reject the hypothesis of variances equality at the same level of significance. Linearity data are showed in Table 2.
Table 2: Validation parameters of ISDN analytical methods

| Parameter                  | UV Spectrophotometric | HPLC-PDA          |
|----------------------------|-----------------------|-------------------|
| Linearity                  | 5.0-30.0 μg.mL⁻¹      | 1.0-6.0 μg.mL⁻¹   |
| Regression equation        | y = 0.0262x + 0.0409  | y = 26168x - 6295,8 |
| r                          | 0.9997                | 0.9969            |
| r²                         | 0.9998                | 0.9938            |
| Repeatability (%RSD_max)   | 1.58                  | 1.83              |
| Intermediate precision (%RSD_max) | 1.27                | 2.42              |
| Accuracy (average%)        | 98.25                 | 100.62            |
| Detection Limit (μg.mL⁻¹)  | 0.07                  | 0.0075            |
| Quantification Limit (μg.mL⁻¹) | 0.2                  | 0.025             |

Legend: r: correlation coefficient; R²: determination coefficient; %RSD_max: máximo value of relative standard deviation

Precision was determined studying repeatability and intermediate precision tests (Table 2). Repeatability results showed a RSD of 1.58%, below the maximum value specified (5.3%, AOAC, 2016). In intermediate precision study, %RSD values were not more than 1.27%. These %RSD values are in an acceptable range, indicating that the method have excellent proximity in the measurements results of multiple sampling.

Accuracy was assessed as recovery rate and was around 100% (95.94%, 97.79% and 101.01%), respecting the range of 80-110% (Table 2) (AOAC, 2016). This result revealed that any small change in the drug concentration in the solutions could be accurately determined by the proposed analytical methods.

In the UV method, DL and QL were found to be 0.07 and 0.2 μg.mL⁻¹, in this order. Thus, the method was considered sensitive, and it can be used for detection and quantification of ISDN over a very wide range of concentrations.

To robustness evaluation, changing wavelengths and micropipette brands did not have any significant effect on absorbance value of ISDN in UV spectrophotometric method. Student’s t-test values cases-variation was not statistically significant (t_cal<t_crit), indicating that the validity of the methods was maintained even with small variations in working conditions.

3.3.2 HPLC Stability Indicating Analytical Method

System suitability is an integrated part of HPLC analytical method that ascertains the effectiveness of operating system. The %RSD value of peak area response was 1.57% (n = 6), considered suitable for the tests. Moreover, chromatographic parameters were evaluated within the specifications (theoretical plate >2000; tailing factor <2.00 and resolution >2.00) (Shabir, 2003). It was concluded the chromatographic system was found to be suitable for use.
In HPLC method, in all stress conditions, ISDN peak purity was greater than or equal to 0.99. This indicates there were no peaks of degradation products/impurities or excipients co-eluting with the major peak. Therefore, it can be inferred that the method was able to measure ISDN among other substances being selective.

Linearity of developed HPLC-method was demonstrated in the interval of 1.0 to 6.0 µg.mL\(^{-1}\). Determination and correlation coefficients were 0.9969 and 0.9938, respectively (Table 2), presenting a good linear association between area and concentration of ISDN. The slope obtained from least squares method showed different from zero (ANOVA F test, p-value 0.0). In the intercept test (linear coefficient), as the p-value (0.0142) of the t test is less than 0.05, we reject the null hypothesis (intercept equal to zero) at the 5% significance level. p-value of 0.5917, calculated by Anderson-Darling test, proved the normality of the residues. At last, Cochran Test, presenting a p-value of 0.5181, confirmed homoscedasticity at a significance level of 5%.

Repeatability and intermediate precision of the method presented good results with values of 1.83% and 2.42%, respectively. Recovery rate were 98.57%, 101.19% and 102.10%, respecting the range of 80-110% (Table 2), ensuring method accuracy (AOAC, 2016). DL result was 7.5 ng.mL\(^{-1}\) and QL value was 25 ng.mL\(^{-1}\). The limits founded were below the limits related to the Reporting, Identification and Qualification of degradation products, stated in ICH Q3B (R2) (ICH, 2006), which signalize an adequate sensitivity for a SIAM.

Robustness parameters of HPLC method were tested by distinct brands of organic phase and the change of analyst to prepare the solution. No significant variations were found (t = 0.25 and 0.74, respectively; n= 6), indicating that developed method was robust.

3.4 METHODS APPLICATION

In UV method, assay average value of ISDN for sublingual tablets was 106.79%, with standard deviation not more than 2.0%.

The result of ISDN quantitative analysis in sublingual tablets with HPLC method was 96.60%, and %RSD was 2.09%.

These results showed both methods were capably to analyze ISDN sublingual tablets, meeting the specifications of USP (90-110%) (USP, 2018).

Analytically, it has been proved by this work that both methods are valid for quantification of ISDN in sublingual pharmaceutical samples. The choice between them, therefore, it will depend on pharmaceutical industry or QC laboratory profile, as well as the objectives of analysis.
UV method described in this work has the advantage to allow DNIS quantification in a few minutes, without generation of pollutants, with simple and affordable equipment, being accessible to small and large QC laboratories, besides to be innovative, since it has not an ISDN UV spectrophotometric method described in literature yet.

The availability of methods with these characteristics is valid to assessed and ensure medicines quality, especially for underdeveloped and developing countries, where advanced instrumental techniques are still not available. However, it is important to point that UV spectrophotometric method is less sensitive than HPLC, since detection and quantification limits and concentration ranges from analytical curves were lower than that for HPLC method (Table 2).

The chromatographic method, although requiring more expensive and complex instrumentation, more robust operators training and spending more time to get the results, compared to UV spectrophotometry, uses a technique widely established in pharmaceutical industry. Besides that, HPLC developed method has the advantage of being more sensitive because it is a SIAM and, so, it is also suitable for samples analysis in stability studies.

At last, despite generating organic residues, the proposed HPLC method was also aligned with green analytical chemistry and could improve processes for pharmaceutical industries, analysts and the environment.

3.5 COMPARISON BETWEEN THE PROPOSED HPLC METHOD AND THE OFFICIAL METHODS

Despite being an old drug, with already established analytical methodologies, ISDN is an essential medicine and therefore it cannot be sold out of the market, requiring continuous analytical methods reviews mainly that applied to quality control. The methods have to follow the updates, mainly in regulatory, technological and sustainable aspects. Still, there are countries that adopt this drug in the list of essential medicines, however do not have monographs in their own pharmacopoeias, alike Brazil. In this way, they reproduce ISDN methodologies described in compendia from other countries, which, so far, are not necessarily cheaper, faster, less polluting or stability indicating when compared to the methods proposed in this work.

USP and British Pharmacopoeias (BP, 2011; USP, 2018) present methods by HPLC for ISDN determination in sublingual tablets. However, as main advantage of developed HPLC method in relation to official procedures, we state our classification as SIAM. This allows the proposed method to be applied in evaluation of stability studies samples as well to known the intrinsic stability of ISDN.
In addition, the HPLC method developed and validated in this work uses solvents less harmful to the environment and the operator.

For instance, British Pharmacopoeia (BP, 2011) method uses as solvent in standard solution preparation the mobile phase composed by ethanol and trimethylpentane, 15:85, v.v\(^{-1}\). Trimethylpentane, which is required largely, is a highly toxic solvent for both human and environment, especially the aquatics (EPA, 2000). Clearly, compendial method procedure constitute a greenless method, because it uses a large proportion of a toxic and bioaccumulable reagent in conjunction with an organic solvent. The new method presented in this work is closer to the trends of green analytical chemistry by proposing a mobile phase with renewable source (water) reagent and an organic solvent recommended for safety, operational health and the environment (methanol) compared to other organic solvents used in chemical analysis (Prat, Hayler, and Wells, 2014).

USP official method has as mobile phase water, 4.7 pH ammonium acetate buffer and methanol (35:10:55, v.v\(^{-1}\)). The use of buffer solution may impair chromatographic columns and systems by inducing corrosion, crystallization and/or abrasion (Collins, Braga, and Bonato, 2006), which generates additional costs to laboratory, such as further solvent expenses, column wash time for each use of the equipment and periodic column replacement. As well, the use of buffer in mobile phase makes the method more time consuming, since it requires a stage to prepare the solvents, which also has a small durability (Marco et al., 2013). This procedure routine is a waste of time, unnecessarily, since it was demonstrated by this work that it is possible to analyze ISDN without adjusting the hydrogen ionic potential, since molecule remains very non-ionized in any pH range.

Thus, the pharmacopoeial methods are interesting, but the proposed method incorporate advantages, such as the fact that it is stability indicating, easy to apply in QC routine and is based on green chemistry, which can improve processes for pharmaceutical industry, analysts and environment. Therefore, it is necessary to consider that the continuous improvement of drugs analytical methods is part of a social conscience allied to a commitment to supply the population with quality medicines.

4 CONCLUSION

In this work, forced degradation studies were carried out for development and validation of a SIAM by HPLC-PDA. An UV spectrophotometric method was also developed, validated and applied for the ISDN in sublingual tablets. The developed methods were validated as per ICH and ANVISA guidelines and all statistical data proves validity of the methods. Forced degradation studies demonstrated molecule intrinsic stability, which presented instability under hydrolysis and basic conditions.
photolysis, oxidative medium and heat. Both methods were simple, economical and involved the use of reagents that are harmless to environment, to analysts and to equipment components, besides being accessible. As indicative of stability, the HPLC-PDA can also be used in stability studies. Such advantages outperformed existing ISDN quantitative determination compendial methods. In addition, it was demonstrated that both methodologies were adequate for the routine analysis in the quality control laboratory.

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CONFLICTS OF INTEREST

There are no conflicts to declare.

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