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Near infrared (NIR)-spectroscopy and in-vitro dissolution absorption system 2 (IDAS2) can help detect changes in the quality of generic drugs

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Near infrared (NIR)-spectroscopy and in-vitro dissolution absorption system 2 (IDAS2) can help detect changes in the quality of generic drugs

While Health authorities in Panama strive to increase generic drug use to contain the rising costs of medicines, there is still hesitation to embrace generic drugs. Thus, regulators and drug companies need to ensure the quality, safety and efficacy of generic drugs. One prevailing concern is the absence of control over lot-to-lot changes, which may impact consistent therapeutic performance. The objective of this work was to determine whether near-infrared spectroscopy (NIR) could detect product changes. Calibration models were built using reference (standard) tablets of two products: Virax® (200 mg acyclovir) and Amlopin® (5 mg amlodipine). Then, to assess the sensitivity of NIR to product changes we compared reference versus deliberately-modified formulations of these products. Comparisons were made using principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) of NIR spectra. Several modified lots were different from reference lots, and 3D score plots showed greater discrimination by PLS-DA than PCA. The K^{th} nearest neighbour scores (KNN) of the modified batches were used to classify formulations as identical or not identical versus the reference. In addition, the differences detected by NIR were correlated with different in vitro dissolution and/or permeation in the in vitro dissolution absorption system 2 (IDAS2): NIR and IDAS2 yielded the same difference rank-order of difference for the modified lots tested. This study suggests that NIR and IDAS2 can help detect lots of generic drugs that differ from the reference lots. This strategy may help regulatory agencies in developing countries to safeguard patients against changes in generic products.

Keywords: formulation; dissolution; permeability; near-infrared spectroscopy; IDAS2.

Introduction

Poor access to high-quality medicines in Latin America is a burden that it is yet to be overcome [1]. Despite various initiatives to help solve this problem, the main hurdle remains the lack of a harmonized regulatory policy that can establish improved standards and requirements for drug approval that ensures availability and access to quality drug products within borders in a timely manner [2]. Such a regulatory agency could counter the epidemic of substandard, spurious, falsely labelled, falsified, or counterfeit (SSFFC) medicines that increasingly continues to affect the region as well as to increase the confidence of patients and healthcare professionals in generic pharmaceutical products [3-5]. This problem is particularly accentuated in smaller countries, such as Panama, where many drug products are either imported from insufficiently regulated markets or produced by smaller local/regional companies that do not always comply with acceptable manufacturing standards. To make matters worse, a large proportion of the products purported as 'generics' in this region, are simply copies or 'similars' and have not really demonstrated to be bioequivalent to the innovators; a critical requirement for interchangeability [6]. Against this daunting background, regulatory authorities in Latin American countries still, like their counterparts in developed countries, have the responsibility to ensure the quality, safety and efficacy of drug products used to treat the ailments afflicting their populations. Unfortunately, an increase in regulatory requirements to ensure drug product quality also presents a financial burden for the regional pharmaceutical industry, especially small-medium size manufacturers. One area of particular concern is the potential for (unreported or unintended) changes in formulation or manufacturing processes that may result in inter-lot product variability, as the impact of these changes on safety or efficacy is unknown. Considering the prevailing lack of existence and/or enforcement of

bioequivalence requirements, there is an urgent need to develop/implement rapid and cost-effective strategies to monitor commercial lots of products before they enter the market To this end, we have examined near-infrared (NIR) spectroscopy, a low-cost, versatile technique that has been used in a wide range of applications in the pharmaceutical industry [7], as a potential tool for monitoring possible changes in product characteristics. NIR records the transmittance or reflectance spectra of a drug product in the near-infrared region of the electromagnetic spectrum (780 - 2500 nm), generating an NIR spectra consisting of overtone and combination bands dominated by CH, OH and NH covalent bonds which are interpreted and analyzed quantitatively and qualitatively using multivariate calibration algorithms and chemometrics [7]. Given its speed and non-destructive nature, NIR spectroscopy is used to monitor various steps in the pharmaceutical manufacturing process, such as the identification of raw materials to the quality control of formulations for final release [8,9]. So, we believe that the unique features of NIR could make it a valuable tool to help resource-constrained regulatory authorities in Latin America to monitor the quality of generic drugs in their markets. Since, generally, one lot of each product is subjected to routine quality control tests while the product is being evaluated for registration, it is feasible to obtain NIR data from the same registration lot(s), which could be used as the reference lot for each product. It is safe to assume that if the composition and manufacturing process are not changed, the NIR spectra of new lots should be the same as that of the reference lot; however, conversely, newer lots with different spectra may indicate that some change was introduced in the composition or manufacturing process. Thus, the main objective of this study was to assess whether near-infrared (NIR) spectroscopy is sufficiently sensitive to detect differences between reference lots of Amlopin® (5 mg tablet of amlodipine) or Virax® (400 mg tablet of acyclovir) and deliberately modified lots of

each product. For comparison, calibration models were constructed and validated using as reference tablets of the standard formulation for each product and principal component analysis analysis (PCA) and partial least squares (PLS) discriminant analysis were applied to the NIR spectra of reference and modified tablets. Finally, the K^{th} nearest neighbour score (KNN) of the modified batches was used as an indicator of whether a modified batch was different from the reference batch. To further assess whether the differences in drug formulations detected by NIR spectroscopy/chemometrics were linked to differences in vitro biopharmaceutical properties performance, such as dissolution and permeation, some formulations, from both Virax® and Amlopin® were also evaluated in the in-vitro dissolution absorption system 2 (IDAS2), using the two-stage protocol to mimic transfer from an acidic (i.e. gastric) to near-neutral (i.e. intestinal) environments, and a physiologically relevant dissolution volume.

Materials and Methods

Formulations Evaluation by NIR Spectroscopy

Raw materials

The formulations evaluated contained the following components: Acyclovir from Zhejiang Charioteer Pharmaceutical, China; Amlodipine besylate from Cadila Healthcare, India; Sodium Starch Glycolate (SSG) from Vivastar, Germany; Microcrystalline Cellulose PH 102 (MC) from JRS Pharma, Germany; Sodium Croscarmellose (SC) from Blanver, Brazil; spray-dried Lactose (sdL) from Foremost Farm, USA; Povidone K 30 from BASF, USA; Colloidal Silicon Dioxide (CSD) from Evonik, USA; Magnesium stearate from FACI S.P.A, Carasco, Italy.

Formulations

This study examined two APIs in tablet forms: a high loading API, acyclovir (400 mg tablet, Virax®, Medipan, S.A., Chilibre, Panama), and a low loading API, amlodipine (5 mg tablet, Amlopin®, Medipan, S.A., Chilibre, Panama). For each API, three different lots of tablets of standard formulation and strength (i.e. reference) were obtained commercially. In addition, for each API, tablets were manufactured with: a) modified amount of active, b) unchanged amount of active and varied amounts of excipients and c) unchanged amount of active and substitution of some excipients. All modified lots of tablets were manufactured by Medipan, S.A. (Chilibre, Panama). The modifications of APIs amount and excipients for acyclovir and amlodipine formulations are listed in Tables 1 and 2, respectively. Note that Lot K, the blank lot, occurs in both tables.

Preparation of Virax® formulations

The acyclovir formulations were made by wet granulation. In a high-speed mixer, acyclovir, sodium starch glycolate and microcrystalline cellulose PH 102 were mixed for 3 minutes; the mixture was granulated with an aqueous solution of povidone K30. The granules were dried at 50°C in an oven for 12 hours. The dried granules were sieved by 1.0 mm mesh tapered mill at 3000 rpm and mixed with the screened colloidal silicon dioxide and finally the mixture was lubricated with the magnesium stearate for 2 minutes. The mixture was compressed to tablets using flat grooved 11 mm punches in a Ronchi AR 18-23 station tablet press.

Preparation of Amlopin® formulations

The formulations of Amlopin® were manufactured by direct compression. In a V-blender, amlodipine besylate was combined with sodium starch glycolate and microcrystalline cellulose PH 102 for 5 minutes, then lubricated with magnesium

stearate and left mixing for 2 minutes. The mixture was compressed using flat grooved 9 mm punches in the Ronchi AR 18-23 station tablet press.

Data acquisition

Twenty tablets were analyzed from each lot and both sides of each tablet were scanned individually to generate 40 samples per lot. The NIR spectra from each of three standard (reference) lots of Amlopin® and Virax® were acquired and NIR spectra generated from each modified lot were used for comparison with the reference lots. Measurements were performed in the reflectance mode on a Thermo-Nicolet Antaris II FT Near Infrared Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an integrating sphere module. Thirty-two scans per sample at 8 cm^{-1} resolution over the 4000 to 10000 cm^{-1} region were made. Data spacing was 3.85 cm^{-1}. The data files were saved in Thermo-Nicolet Result and saved as Excel csv worksheets to export to other programs for model development.

Data analysis

Data analysis using Thermo-Nicolet TQ Analyst software was limited to inspection of the data and pre-treatment of the spectra to assess data quality. Sub-sets of the data were processed using Unscrambler® from Camo to check feasibility of various Chemometric techniques. Primarily, the NIR data were modelled using Partial Least Squares-Discriminants (PLS-Discriminants), Principal Components Analysis (PCA) and K^{th} Nearest Neighbour (KNN) running under Solo® 8.6 from Eigenvector Research (Manson, Washington, USA). The raw spectra data were assigned to sample class for calibration. The wavenumber region chosen for the model development was dependent on the molecular structure of the active ingredient, the nature of excipients, as well as the signal to noise in the processed spectra. In order to remove scatter and other non-

linearity and to maximize any difference, the raw spectra were pre-processed in the following order [10]: a) Standard normal variate (SNV), b) Savitzky-Golay 2nd Derivative , and c) Mean Centered.

The PLS-discriminant or PCA models were calibrated using three different current lots of either Amlopin® tablets or Virax® tablets that had been obtained commercially. These were the training sets. The algorithm employed for the PLS model was the SIMPLS, which calculates the PLS factors directly as linear combinations of the original variables [11]. The PCA model employed the SVD algorithm [12]. The calibration was cross-validated: venetian blinds w/10 splits and 1 sample per split. The number of leveraged variables or components was chosen to: a) maximize the sensitivity and selectivity of the calibration class standards, b) minimize the root mean standard error of calibration and cross validation, and c) for each molecule, the chemometric analysis was performed with adjusted variables as listed in Tables 3 and 4.

Formulations Evaluation in IDAS2

Materials

C2BBe1 cells were obtained from American Type Culture Collection (Manassas, VA). D-glucose, 2-(N-morpholino)ethanesulfonic acid (MES), Bis(2-hydroxyethyl)amino-tris(hydroxylmethyl)-methane (Bis-Tris), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Simulated intestinal fluid (SIF) powder was purchased from Biorelevant (Croydon, UK). Hanks' balanced salt solution (HBSS, 10X concentrated, Gibco Ref # 14065-056), Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid) (HEPES, 1 M solution), fetal bovine serum (FBS), penicillin-streptomycin mixture, non-essential amino acids

(NEAA), sodium pyruvate, and trypsin were obtained from Thermo Fisher Scientific (Waltham, MA). Rat tail collagen type 1 and Costar® Snapwell plates (6-well format, 1.13 cm^2 insert area, 0.4 μm pore size) were purchased from Corning Life Sciences (Corning, NY). Polytetrafluoroethylene (PTFE) syringe filters (porosity: 0.45 μm, diameter: 13 mm, hydrophilic) were purchased from Scientific Equipment Company (Aston, PA). All drug products and chemicals were stored properly at all times and used before their expiration dates.

C2BBe1 cell culture

C2BBe1 cells were maintained in DMEM containing 10% FBS, 1% NEAA, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator (37°C, 5% CO2). The culture medium was changed three times weekly, and cell growth was observed by light microscopy. When the stock cultures were ~80% confluent, the cells were harvested by trypsinization and seeded at a density of 60,000 cells/cm^2 onto rat tail collagen-coated polycarbonate membrane filters in Snapwell plates to grow and differentiate into polarized cell monolayers for the permeability studies. The culture medium was changed every other day until use (20 to 28 days post seeding). To ensure cell monolayer integrity, prior to experimental use, each batch of cells was subjected to quality control (QC) tests consisting of transport measurements of atenolol (low permeability marker compound), propranolol (high permeability marker compound), digoxin (p-glycoprotein probe substrate) and estrone3-sulfate (BCRP probe substrate), across randomly selected C2BBe1 cell monolayers. Only cell batches that passed QC criteria were selected for subsequent IDAS2 assays.

IDAS2 Experiments

IDAS2 is comprised of a modified USP II dissolution vessel equipped with two permeation chambers. C2BBe1 cell monolayers are mounted at the interface between dissolution medium and the permeation chambers. In this study, the IDAS2 assays were conducted under 2-stage conditions. IDAS2 assays under the 2-stage protocol involved two consecutive steps: 1) the tested drug product initially underwent dissolution in acidic pH 1.6 for 20 minutes to mimic the in vivo gastric condition; 2) the pH was adjusted to pH 6.5 with concomitant increase of SIF concentration to 2.24 mg/ml and dissolution and permeation were monitored for additional 120 minutes in simulated fasted state intestinal environment. The dissolution vessels (n=3) were filled with 400 mL of simulated gastric fluid (SGF) containing 34 mM NaCl and 0.024 N HCl in Millipore deionized water, pH 1.6. The IDAS2 system was set to a temperature of 37°C and a paddle speed of 50 rpm. Once the temperature reached 37°C, an atenolol/minoxidil mixture was added as controls to monitor cell monolayer integrity and inter-assay consistency. Two tablets of either test compound (acyclovir or amlodipine) were then added to start the assay. Donor samples were taken at 10 and 20 minutes. After 20 min of the drug dissolution in SGF, the medium was switched to fasted-state simulated intestinal fluid (FaSSIF) by adding 100 mL of a solution containing 1.12 g of SIF, 40 mL of 8x HBSS and 60 mL of 350 mM Bis-Tris base. Right after verifying that the pH was 6.5 (adjusting it, if necessary), the permeation chambers with C2BBe1 cell monolayers were submerged into the dissolution medium, and 8 mL of permeation medium (HBSS containing 4.5% BSA, pH 7.4) were added into the permeation chambers. The composition of the final FaSSIF medium created by combining 400 mL of SGF and 100 mL of 5X FaSSIF consisted of 3 mM taurocholate, 0.75 mM phospholipids, 175 mM sodium, 133 mM chloride, 29 mM phosphate, and 42

mM Bis-Tris in 0.64X HBSS. Donor samples (2 mL each) were taken at 30, 40, 50, 80, 110, and 140 minutes, and immediately filtered through 0.45 μm syringe filters and approximately first mL of filtrate was discarded and the remaining was collected for analysis. Receiver samples were taken at 55, 85, 115, and 145 minutes. For acyclovir, donor samples were analyzed using ultraviolet-visible (UV-vis) spectroscopy at 280nm and receiver samples were analyzed by liquid chromatography-mass spectrometry (LC-MS/MS). For amlodipine, donor samples were analyzed using UV-vis spectroscopy at 230 nm and receiver samples were analyzed by LC-MS/MS.

Results and Discussion

In this study, we propose the implementation of tools to help monitor against inter-lot changes of generic products. This strategy should work well in small Latin American countries because during the registration process (e.g., every 5-7 years) at least one lot of each product is submitted to the regulatory agencies for quality control tests. Thus, for each product, the 'registration' lot could be subjected to additional tests whose results could be used as the baseline or reference against which to measure the performance of future lots of each product.

NIR Analysis

Since many formulation changes would not be detected by routine quality control tests [13], which is not surprising because they are not designed for this purpose, the generation of baseline values based on more sensitive techniques seems necessary. Keeping in mind that the ultimate utility of these measurements will be realized only if they can be performed rapidly and at a reasonable cost, we believe that spectroscopic techniques such as NIR are very attractive because they are quick and non-destructive. To assess the feasibility of using NIR for this application we chose two APIs,

amlodipine 5 mg tablets (Amlopin®) and acyclovir 400 mg tablets (Virax®), widely available in Panama and other countries in the region. Since the first thing we wanted to know was whether NIR could be used to detect changes in the composition of standard formulations of these products, we elaborated 12 batches of each product with pre-determined differences. These products differed in the amount of API and excipients or specific excipients utilized. As a negative control we used a formulation that contained the same amount of excipients as the reference formulation but no active ingredient. All the Virax® tablets with a modified amount (i.e. 115%, 110%, 90% or 85%) of the active ingredient showed clear differences from the reference tablets (containing 100% API) regardless of whether the data was analyzed by PCA or PLS-DA. The range (4,000-10,000 cm^{-1}) of the spectra analyzed in this study showed the most relevant features of the organic substances.

Exploratory analysis based on principal components analysis (PCA)

Since the reference models were constructed from three lots of intact tablets (400 mg acyclovir, Virax® and 5 mg amlodipine, Amlopin®), which by design were substantially different from their respective modified lots, either in the amount of the APIs or in the amount or identity of excipients (Tables 1 and 2), their NIR spectra were expected to be different. However, it was crucial to determine whether indeed the NIR approach was sensitive enough to detect the built-in differences between the reference and modified products. Indeed, the potential utility of this approach to detect inter-lot changes rests on its ability to differentiate between lots that are substantially different from the reference product (i.e. Not-identical) and lots that are not substantially different (i.e. Identical). After using the reference (current) lots of tablets of (Amlopin®) and (Virax®) as training sets to build and validate the calibration models, we compared the reference versus

modified lots of each product using principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and the K^{th} nearest neighbour (KNN) test.

Classification model based on principal component analysis (PCA)

Principal component analysis (PCA) was used to examine the possible clustering in samples and investigate the extent to which NIR features can distinguish among different amounts of the API and excipients in the dosage forms. The PCA model used only the three commercial lots of the drug product as training sets. PCA analysis of Virax® tablets over the 4,000 to 10,000 cm^{-1} range conclusively showed that both, Lot A and Lot B, had a large degree of overlap, with each other but were well-separated from the reference lots (Figure 1A). In addition, PCA analysis also was able to detect a clear separation between modified Lots F, J, and K and the reference lots (Figure 1B), but other modified lots were separated to a lesser degree. Based on the confidence ellipses the API-modified Lot C was similar to the reference lots, and somewhat different from Lot D, but not as much as the difference from A and B. These observations suggest that although differences related to both, higher or lower than normal amounts of API were detected, the spectral properties of the product were more easily distorted by a 10% or greater excess of acyclovir than by a deficit of comparable magnitude. While the 3D score plot for modified excipients unequivocally distinguished modified Lots F, J and K from the reference lots (Figure 1B), the separation for the other excipient-modified lots was not clear (Figure 1B). The greatest distancing of Lot K (placebo) from the reference lot in the 3D plot is not surprising because the reference lot NIR spectrum is dominated by the acyclovir bands.

The 3D scatter plot of the principal components (PCA) for Amlopin® tablets shows that the first three scores on the PCs accounted for almost 73% of the total variation in the NIR spectra (Figure 2). The absence of a clear dispersion among

different lots, together with the overlaps between reference lots and API-modified Lots A-D (Figure 2A), implies that the data structure or relationship may be complex and nonlinear. Similar overlap was observed when comparing the training set reference lots to the excipient-modified Lots E-K (Figure 2B). However, the major dispersion observed for Lot J (replacement of spray-dried lactose for microcrystalline cellulose), suggests that for Amlopin®, spray-dried lactose (sdL) is essential to maintain the spectral integrity of the product. The sensitivity of the product's fingerprint to the amount of sdL could be assessed by evaluating lots containing varying amounts of sdL (instead of the complete substitution used in Lot J).

Calibration model based on partial least squares - discriminant analysis (PLS-DA)

Despite the clear distancing between reference and excipient-modified lots obtained in many cases, PCA was still a poor discrimination model because it functions by reducing dimensionality while preserving much variance in a high dimensional space. PCA, does not consider class labels, and is thus an "unsupervised" classifier [14]. For example, the 3D plot showed clear discrimination only for Lots J and K (placebo). Hence, we also used partial least squares-discriminant analysis (PLS-DA), a pattern recognition method well known for its power of discrimination, to determine whether this strategy would yield greater discrimination than PCA. PLS-DA can be thought of as a "supervised" version of Principal Component Analysis (PCA) in the sense that it achieves dimensionality reduction but with full awareness of the class labels [14]. PLS-DA's supervised nature has been shown to be efficacious in analysis of large, sometimes noisy data sets [15].

To achieve a better method for classifying the modified lots of Virax® and Amlopin® tablets as identical or non-identical to the unmodified (reference) lots, we

used the supervised pattern classification approach: PLS-DA. The PLS-DA model was constructed with the choice of the optimal number (i.e. 3) of leveraged variables (LVs), which was carried out by a 3-fold cross validation procedure. The plan was to determine if "supervised" PLS-DA would discriminate better than PCA, using identical processing parameters, between the training set lots and the modified lots. Thus, the data for the PLS-DA calibration model was pre-processed in the same manner as that described earlier for PCA (entire wavenumber range, removal of some outliers from calibration, SNV, Savitky-Golay 2nd derivative, and mean centered) using three current (commercial) lots as the training set for each product (Virax® and Amlopin®). The established models for Virax® and Amlopin® tablets were further validated using the same three current lots of each product as validation sets to determine which method was more robust to develop/refine models for general use in the detection of formulation changes. Analysis of the NIR full spectra from the 4,000 – 10,000 cm^{-1} range showed the influence of the number of LVs in the PLS-DA model on the classification of API-modified and excipient-modified lots of Virax®, which indicates that the classification worked well for API-modified lots (Figure 3A), but not as well for excipient-modified batches, except for Lot F (Figure 3B).

PLS-DA models using the partial spectra (4,000 to 5,500 cm^{-1}) performed better than the corresponding full range spectral analysis in differentiating between reference and either API-modified or excipient-modified lots (Figure 4A, B). For example, there was a clear separation between API-modified and the reference lots of Virax®. Note that Lots C and D are separated from the reference lots and, as well as, from each other, although to a lesser extent. In addition, the API-modified Lots A and B were clearly distinguishable from all the other lots. The score plot for the excipient-modified and reference lots showed degrees of separation superior to those attained with PCA or PLS-

DA over the entire wavenumber range. Given the substantial magnitude of the changes incorporated into the modified lots, these results are interpreted as demonstrating a high rate of correct discrimination.

In the case of Amlopin®, compared to the calibration model for PCA, the PLS-DA-derived model using the full spectra was more sensitive using identical wavenumber range and pre-processing, as indicated by a greater separation between the reference lots and the modified lots (A-D) in detecting changes in the formulation (Figure 5A). A similar trend was observed when comparing the reference lots with the excipient-modified Lots E-I (Figure 5B). Despite the wide dispersion shown, the PLS-DA model over the entire wavenumber range did not show clear separation among either API-modified lots or excipient-modified lots (Figure 5A,B). As expected, the excipient-substituted Lot J was the most distant from the reference lots and the other excipient-modified lots. This low dispersion might be explained by the large amount of noise.

PLS-DA results obtained using the 4,000 to 6,000 cm -1 range during model development were very encouraging. The 3D plot of PLS-DA leverage variables for Amlopin® shows separation of reference lots from all the API-modified lots and excipient-modified lots (Figure 6A, B). These results confirm the robustness of these models to discriminate between modified and reference lots.

Classification model based on Kth nearest neighbour (KNN)

K-Nearest Neighbour (KNN) is a linear regression, non-parametric method applied to a set of NIR spectra to predict an unknown spectrum as a function of the K closest spectra available (the K-Nearest Neighbours). A comparison of the KNN values of the prediction (modified) lots with those of the validation (reference) lots allows their classification as identical or not identical. An appropriate value of K has a profound

influence on the discrimination rate of the KNN model, which is determined by cross validation of the reference samples. Generally, the parameter K is an odd number less than 10 and the optimum KNN model is achieved when K = 3. In this study, PCs and LVs input data were used in the KNN classification model. Lots with KNN scores ≤ 1 were classified as identical to their reference lots and those with KNN > 1 as not identical. From the viewpoint of a monitoring program aiming to prevent uncharacterized changes in an approved product, the implication of this approach is that when a given lot is classified as identical, it is reasonable to assume that its composition and manufacturing process have not deviated from those associated with the reference lot. In contrast, it is highly likely that lots classified as non-identical have undergone some change vis-à-vis the reference lots.

The KNN results for the reference lots used as calibration samples (i.e. Lots 1-3) and modified lots used as prediction samples (i.e. Lots A-K) for Virax® are shown in Table 1. When the full range (4,000 – 10,000 cm^{-1}) was used, the lots with higher amounts of acyclovir, API-modified Lot A (115%) and Lot B (110%), showed respective KNN values of 4.15 and 4.72 (Table 5). This classification based on KNN scores is consistent with the observations made with PCA. For example, PCA-derived score plots showed that, the lots containing more acyclovir, A and B, exhibited greater distancing from the reference lots (100%) than the lots containing less acyclovir, C (90%) and D (85%), also had larger KNN scores. That both methods detected differences between all the modified formulations and the reference product suggests that differences equal or greater than 10% in the API amounts of Virax® were sufficient to cause changes in the NIR spectra of the formulations that were quantifiable by both statistical treatments of the data. This observation is reasonable because the magnitude of the modifications dialed into the different formulations (batches) needed to be

sufficient to generate measurable differences in NIR spectra, compared with the reference product, while avoiding hypersensitivity which could translate into an excessive number of false-positives (i.e. over-discrimination).

However, excipient-modified Lot F (50% SSG, completed with MC) and Lot J (0% MC; substituted with sdL) with respective KNN values of 6.05 and 3.10 exhibited the biggest difference when compared to the references lots.

Also, the KNN values calculated with LVs input data (Table 6) agreed with results obtained for PCs input data in that the modified-API Lot A (115%) and Lot B (110%) showed the largest KNN values of 4.84 and 5.57, respectively. In a similar way, the excipient-modified Lot F and Lot J showed the largest KNN values of 8.33 and 3.18, respectively. Together, the different impact of the wavenumber range used for PCA or PLS to generate the PCs and LVs input data for KNN classification indicates that, since the optimal classification model appears to be influenced by multiple factors, classification models need to be optimized for each product.

Although the data for Amlopin® was subjected to the same treatment used with Virax®, the PCs input-derived KNN model for both API and excipient-modified achieved little discrimination in the prediction sets when the full wavenumber range was used (Table 7). Only modified Lots J and K showed discrimination, as indicated by significant KNN values of 8.64 and 1.68. Interestingly, the KNN value of 8.64 determined for Lot J indicates that the associated change in composition (MC was entirely substituted with sdL) had a major significant impact in the NIR data, and thus, this type of change could be readily detected.

Using the LVs input data of full and partial wavenumber ranges to generate the outcome KNN values was successful, with KNN values greater than one for both, API and excipient-modified formulations (Table 8). The modified Lot J showed the highest

difference in both full and partial wavelength regions with KNN values of 25.01 and 35.44, respectively. Also, within the partial-range data there is a clear difference in KNN scores for Lots E and H relative to Lots F, G and I, thus demonstrating the ability of the model not only to distinguish between reference and modified lots, but to distinguish between the excipient modified lots.

Classification model based on K^{th} nearest neighbour (KNN)

To investigate whether the differences in drug formulations detected by NIR spectroscopy/chemometrics could be associated with in vitro performance, such as dissolution and permeation, a few formulations were also evaluated in IDAS2, using the two-stage protocol to mimic transfer from an acidic (i.e. gastric) to near-neutral (i.e. intestinal) environments, and a physiologically relevant dissolution volume.

For Virax®, although KNN indicated that all formulations were different from the reference lot, regardless of whether the input method used (i.e. PCs or LVs), following the assumption that, in principle, higher KNN values are expected to exhibit greater differences from the reference lot, we selected 3 Virax® formulations (i.e. Lots I, J and F), all classified as Not-identical to the reference lot, but with, respective, KNN values of 1.46/1.66, 3.10/3.18, and 6.05/8.33 (Tables 5 and 6), to determine if IDAS2 could detect dissolution and/or permeation properties consistent with the estimated KNN values. The dissolution results showed that Lot F was the farthest from the standard lot, followed by tablet J, with Lot I being the closest (Figure 7a). This order of separation is similar to that seen in the NIR results. IDAS2 permeation results were consistent with the NIR data in that Lot F also showed the greatest separation from the reference lot. In addition, the intermediate lots, Lot I and Lot J, were almost identical to each other, but different from both, the reference lot and Lot F (Figure 7B). Interestingly, Lot I showed a similar dissolution profile to the reference lot, but lower

permeation than the reference, this result indicates that substitution of sodium starch glycolate with sodium croscarmellose had less effect on acyclovir dissolution but greater impact on its permeation. A conventional dissolution test alone may fail to detect this change in formulation.

In the case of Amlopin®, since formulation classification as Identical or Not-identical based on KNN values depended on the method (i.e. PCA or PLS-DA) used to derive the input variables used in KNN determination, we wanted to know whether IDAS2 could provide another way to experimentally compare formulations. So, for evaluation in IDAS2, we chose one formulation (Lot J) which appeared the farthest from the reference in the 3D Score plot (Figure 5B) as well as several formulations that were much closer to the reference lot, to determine whether they were distinguishable or not using this system. IDAS2 data showed a strong correspondence with the 3D Score plot for the formulations tested because all the lots classified as Not-identical based on the KNN values also behaved differently in IDAS2. Lot J, the most distant from the reference lot, was also different from the intermediate lots (F, G, and H) in dissolution and permeation (Figure 8A, B). Lots F, G, and H, all of which had dissolution and permeation rates clustered together, between the reference lot and Lot J, were consistent with their almost identical KNN values (i.e. 1.58, 1.59 and 1.54 using full rang wavelength rang in Table 8). In addition, Lot J, the formulation with the highest KNN derived from both, PCs input or LVs input, was substantially different from the other 3 formulations tested.

Conclusions

The calibration models derived from NIR spectroscopy were sufficiently sensitive to distinguish between standard (reference) and modified lots of low API loading Amlopin® (containing 5 mg of amlodipine) and high API loading Virax® (containing 400 mg of acyclovir), which demonstrates the potential utility of this technique in monitoring against potential inter-lot product changes. The correlation between NIR/chemometrics and IDAS2 measurements also suggests that the combination of these techniques could facilitate regulatory agencies in developing countries in their efforts to detect changes between different lots products. Specifically, due to the speed, sensitivity, and non-destructive nature of NIR and the dual functions (i.e. dissolution and permeation) of IDAS2, the regulatory authorities could use these tools (during the registration phase) to characterize reference lots and establish suitability criteria that future lots would have to meet to be allowed to enter the market. To capitalize on the potential benefit of this approach, regulatory authorities in Panama, and other countries in the region, would need to modify their current practice of evaluating a single reference product lot during registration, to include at least three reference lots, which appears to be the number of lots necessary to develop suitable NIR models. The evaluation of subsequent lots would be based on a comparison of NIR spectral characteristics of commercial versus registration lots, with identical lots being allowed and not-identical lots requiring additional testing, such as evaluation in IDAS2. This strategy, by making possible the reduction of deviations in product performance, derived from formulation/manufacturing changes, would help ensure the continued quality, safety and efficacy of all future product lots.

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Table 1. Formula composition for standard (current) and modified (API and excipients) lots of Virax®.

Description		Formula composition (% W/W)										
		current	**A**	**B**	**C**	**D**	**F**	**G**	**H**	**I**	**J**	**K**
API: Acyclovir		77.0	85.2	81.5	66.7	63.0	77.0	77.0	77.0	77.0	77.0	0.0
Excipients	MC	16.5	8.3	12.0	26.8	30.5	17.5	14.5	10.5	16.5	0.0	71.7
	SSG	2.0	2.0	2.0	2.0	2.0	1.0	4.0	8.0	0.0	2.0	8.7
	Povidone	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	8.7
	CCsd	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	2.2
	Magnesium stearate	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	8.7
	SC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0
	sdL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	16.5	0.0
Percent total		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Note: MC, microcrystalline cellulose; SSG, sodium stach glycolate; CSD, Colloidal Silicon Dioxide; SC, sodium croscarmellose; sdL, spray dried lactose.

Table 2. Formula composition for standard (current) and modified (API and excipients) lots of Amlopin®.

Description		Formula composition (% W/W)											
		current	A	B	C	D	E	F	G	H	I	J	K
API: Amlodipine besylate		4.2	4.9	4.7	3.8	3.6	4.2	4.2	4.2	4.2	4.2	4.2	0.0
Excipients	MC	90.8	90.1	90.3	91.2	91.4	94.8	92.8	86.8	78.7	90.8	0.0	94.8
	SSG	4.0	4.0	4.0	4.0	4.0	0.0	2.0	8.0	16.1	0.0	4.0	4.2
	Magnesium stearate	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	SC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0	0.0
	sdL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	90.8	0.0
Percent total		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Note: MC, microcrystalline cellulose; SSG, sodium stach glycolate; SC, sodium croscarmellose; sdL, spray dried lactose.

Table 3. Adjustable parameters of chemometric analysis for Virax®.

Chemometric method	Wavenumber region	Number of points in Savitzky-Golay 2nd derivative window	Number of factors or leverage variables
PCA	4,000 - 10,000 cm^{-1}	15	Four Factors
PLS-DA	4,000 - 10,000 cm^{-1}	15	Four Leverage Variables
PLS-DA	4,000 – 5,500 cm^{-1}	15	Four Leverage Variables

Note: Each model was used to predict formulations with criteria for pass or fail as value for K^{th} nearest neighbor distance (KNN) ≤ 1 and for the API and excipient-modified lots to be separated in factor or leverage-variable three-dimensional space from current lots.

Table 4. Adjustable parameters of chemometric analysis for Amlopin®.

Chemometric method	**Wavenumber region**	**Number of Points in Savitzky-Golay 2nd derivative window**	**Number of factors or leverage variables**
PCA	4,000 - 10,000 cm^{-1}	11	Three Factors
PLS-DA	4,000 - 10,000 cm^{-1}	11	Three Leverage Variables
PLS-DA	4,000 - 6,000 cm^{-1}	9	Four Leverage Variables

Note: Each model was used to predict samples with criteria for acceptance or rejection as value for K^{th} nearest neighbour distance (KNN) ≤ 1 and for the modified lot to be separated in factor or leverage-variable three-dimensional space from current lot.

Table 5. KNN scores calculated from PCs input data for tablets of Virax®.

PCs input data using full range (4,000 – 10,000 cm^{-1})							
Reference Lots		**Modified Lots A-D**			**Modified Lots F-K**		
Lots	KNN Score Distance (k=3)	Lots	KNN Score for k=3	Identical to reference if KNN score ≤ 1	Lots	KNN Score for k=3	Identical to reference if KNN score ≤ 1
1	0.37	A	4.15	Not identical	F	6.05	Not identical
2	0.45	B	4.72	Not identical	G	1.42	Not identical
3	0.33	C	1.19	Not identical	H	1.92	Not identical
		D	1.31	Not identical	I	1.46	Not identical
					J	3.10	Not identical
					K	28.24	Not identical

Table 6. KNN scores calculated from LVs input data for tablets of Virax®.

LVs input data using full range (4,000 – 10,000 cm^{-1})							
Reference Lots		**Modified Lots A-D**			**Modified Lots F-K**		
Lots	KNN Score Distance (k=3)	Lots	KNN Scores for k=3	Identical to reference if KNN score ≤ 1	Lots	KNN Scores for k=3	Identical to reference if KNN score ≤ 1
1	0.38	A	4.84	Not identical	F	8.33	Not identical
2	0.45	B	5.57	Not identical	G	1.76	Not identical
3	0.37	C	1.23	Not identical	H	2.14	Not identical
		D	1.42	Not identical	I	1.66	Not identical
					J	3.18	Not identical
					K	37.68	Not identical

Table 7. KNN scores calculated from PCs input data for tablets of Amlopin®.

PCs input data using full range (4,000 – 10,000 cm^{-1})							
Reference Lots		**Modified Lots A-D**			**Modified Lots E-K**		
Lots	KNN scores	Lots	KNN scores for k =3	Identical to reference if KNN score ≤ 1	Lots	KNN scores for k =3	Identical to reference if KNN score ≤ 1
1	0.34	A	0.35	identical	E	0.38	identical
2	0.38	B	0.41	identical	F	0.40	identical
3	0.30	C	0.39	identical	G	0.46	identical
		D	0.33	identical	H	0.38	identical
					I	0.36	identical
					J	8.64	Not identical
					K	1.68	Not identical

Table 8. KNN scores calculated from LVs input data for tablets of Amlopin®.

LVs input data using full range (4,000 – 10,000 cm^{-1})							
Reference Lots		**Modified Lots A-D**			**Modified Lots E-K**		
Lots	KNN Scores (mean of 40)	Lots	KNN scores for k =3	Identical to reference if KNN score ≤ 1	Lots	KNN scores for k =3	Identical to reference if KNN score ≤ 1
1	0.36	A	1.64	Not identical	E	1.66	Not identical
2	0.47	B	1.62	Not identical	F	1.58	Not identical
3	0.51	C	1.72	Not identical	G	1.59	Not identical
		D	1.57	Not identical	H	1.54	Not identical
					I	1.34	Not identical
					J	25.01	Not identical
					K	4.20	Not identical

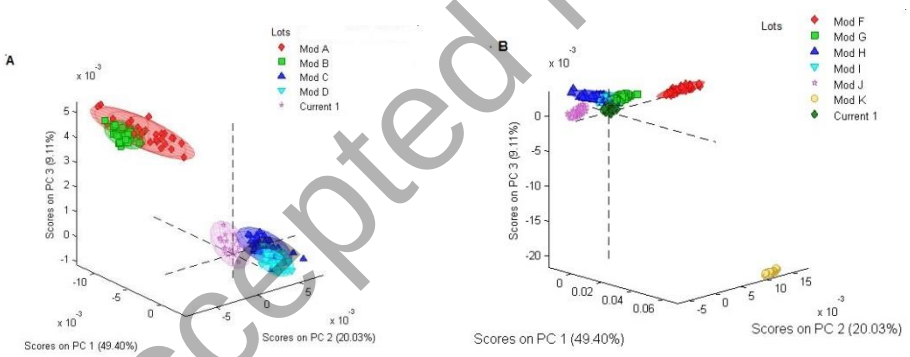

Figure 1. Score plot of PC1 – PC3. The NIR (4,000 – 10,000 cm^{-1}) spectra of Virax® tablets were pre-processed by SNV, Savitzky-Golay 2nd derivative, and mean centered before applying the PCA. (A) Comparison between the reference lots and API-modified lots and (B) Comparison between the reference lots and excipient-modified lots.

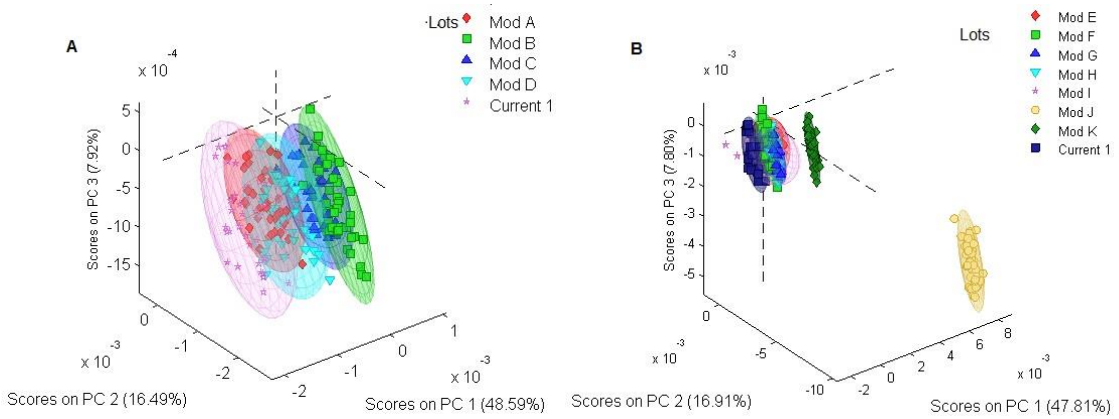

Figure 2. Score plot of PC1 – PC3. The NIR (4,000 – 10,000 cm^{-1}) spectra of the Amlopin® tablets were pre-processed by SNV, Savitzky-Golay 2nd derivative, and mean centered before applying the PCA. (A) Comparison between the reference lots and API-modified lots and (B) Comparison between the reference lots and excipient-modified lots.

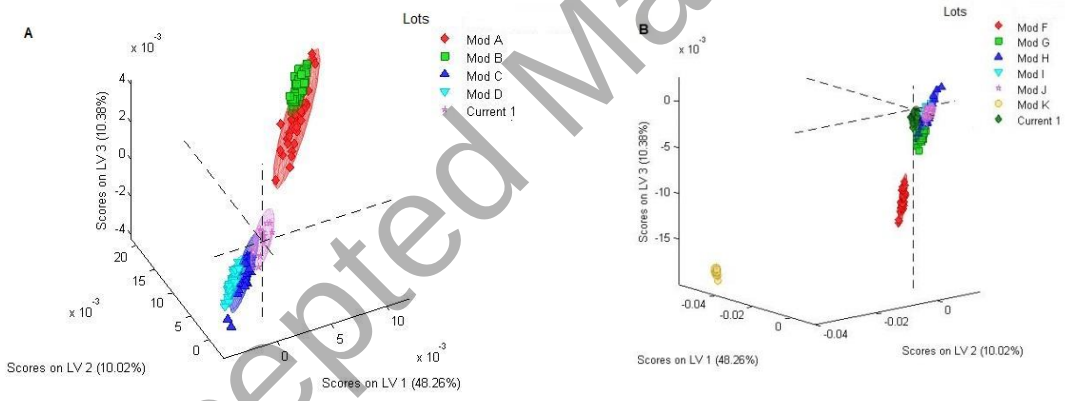

Figure 3. Partial least squares discriminant analysis of full spectra (4,000 – 10,000 cm^{-1}) for Virax® tablets. (A) Comparison between the reference lots and API-modified lots. (B) Comparison between the reference lots and excipient-modified lots.

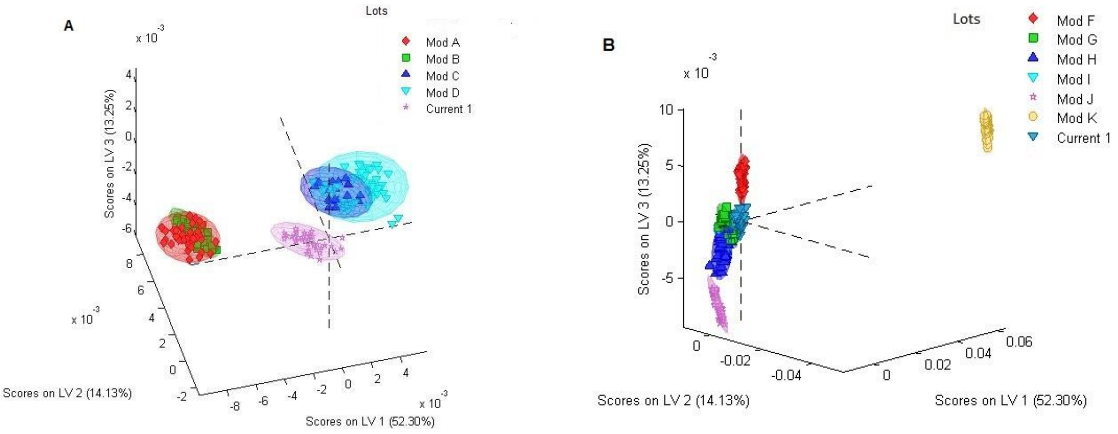

Figure 4. Partial least squares discriminant analysis of partial spectra (4,000 - 5,500 cm^{-1}) for Virax® tablets. (A) Comparison between the reference lots and API-modified lots. (B) Comparison between the reference lots and excipient-modified lots.

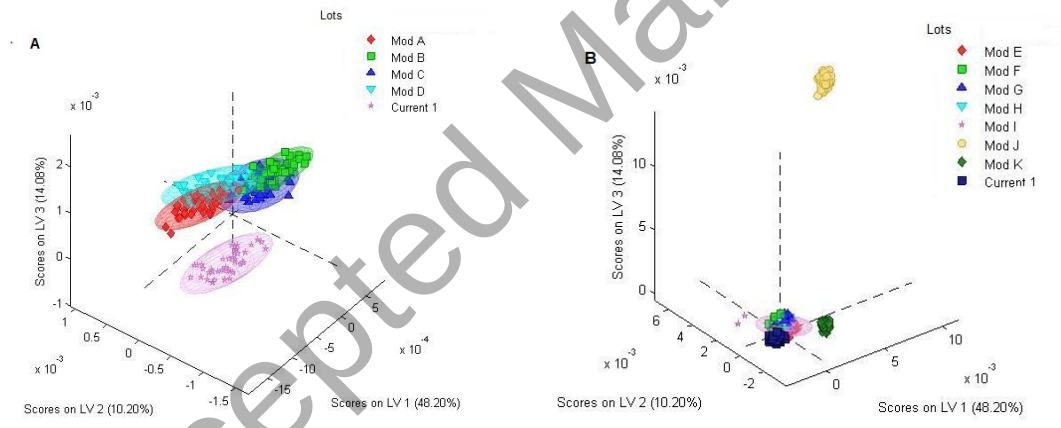

Figure 5. Partial least squares discriminant analysis of full spectra (4,000 – 10,000 cm^{-1}) for Amlopin® tablets. (A) Comparison between the reference lots and the API-modified lots. (B) Comparison between the reference lots and the excipient-modified lots.

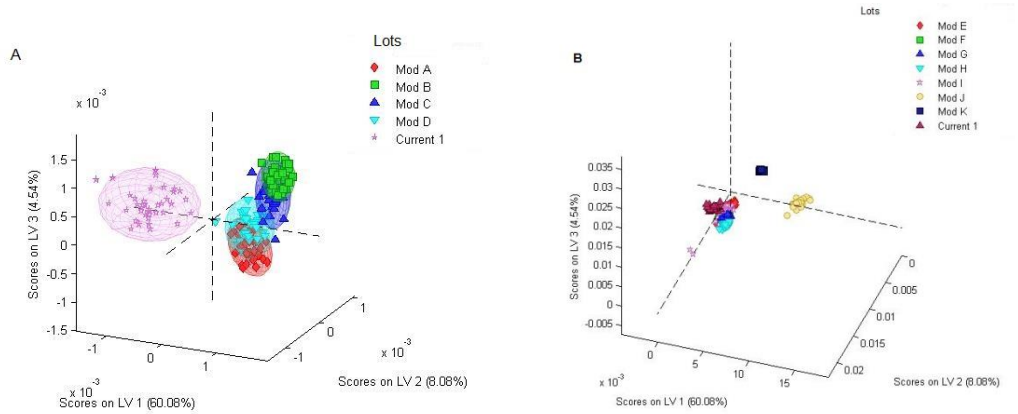

Figure 6. Partial least squares discriminant analysis of partial spectra (4,000 – 6,000 cm^{-1}) for Amlopin® tablets. (A) Comparison between the reference lots and API-modified lots (A-D). (B) Comparison between the reference lots and the excipient-modified lots (E-I).

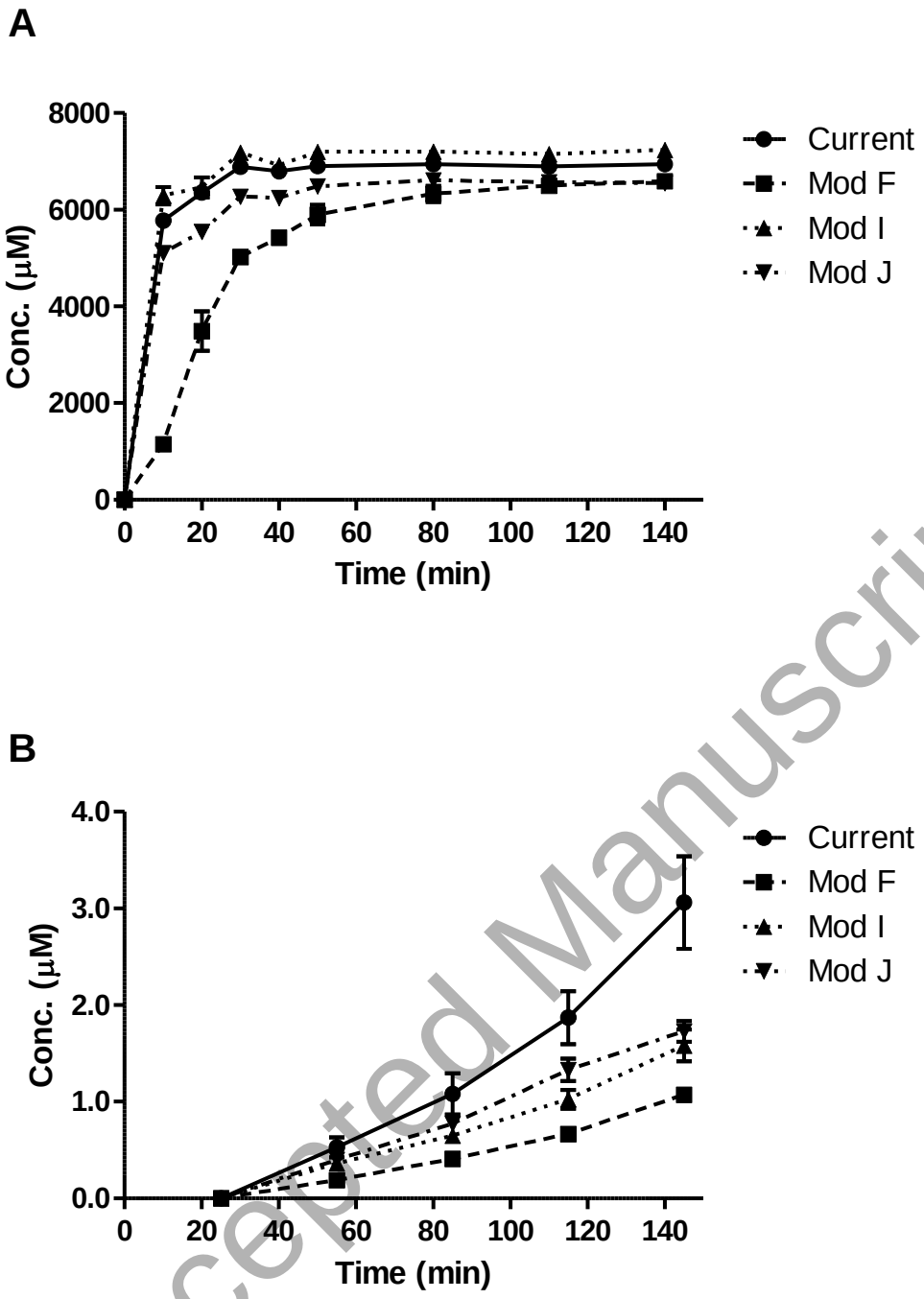

Figure 7. Effect of modifications in acyclovir formulation on in vitro dissolution and permeation. (A) Dissolution profiles of acyclovir formulations in IDAS2 assays. (B) Permeation profiles of acyclovir formulations across C2BBe1 monolayers in IDAS2 assays.

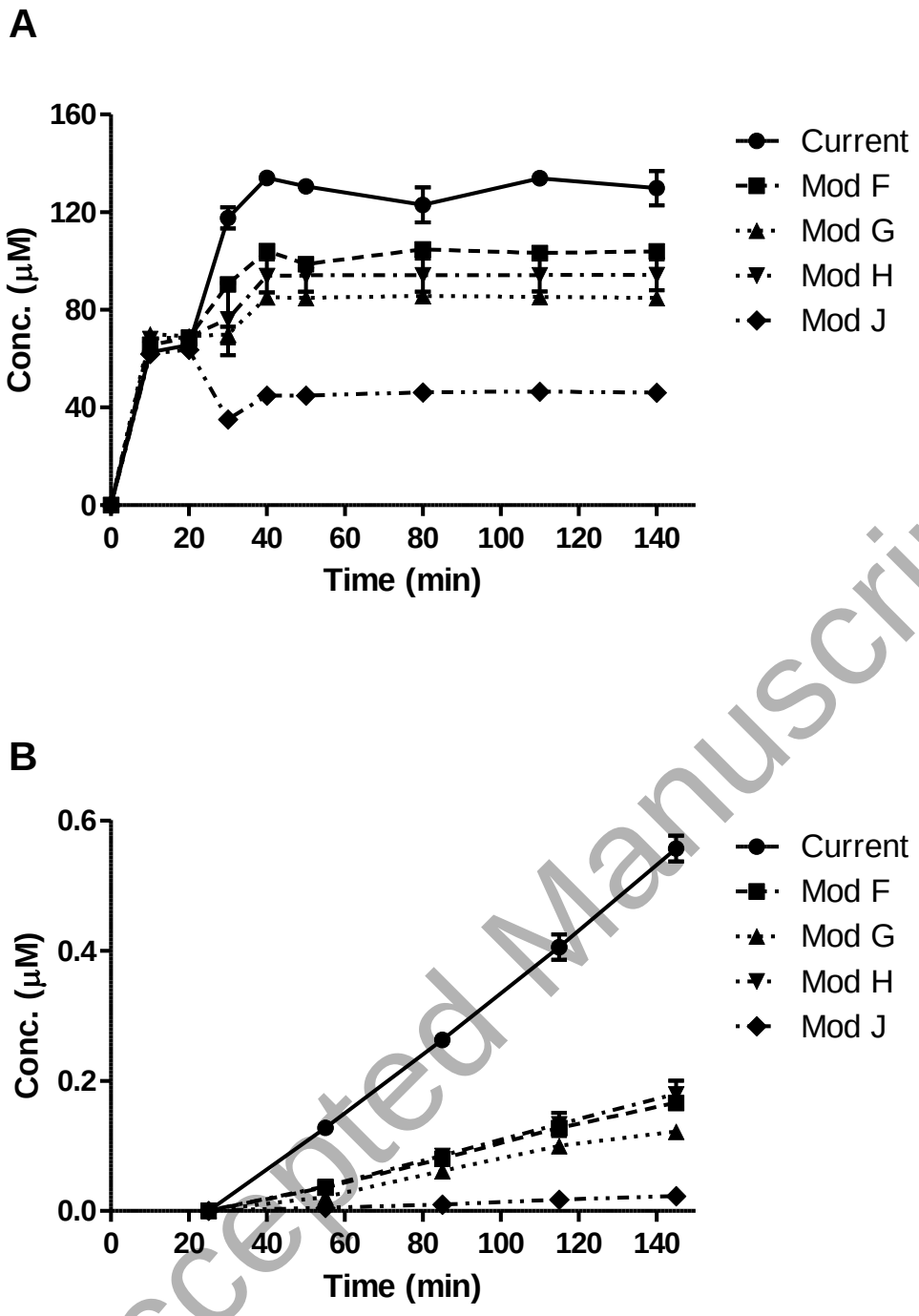

Figure 8. Effect of modifications in amlodipine formulation on in vitro dissolution and permeation. (A) Dissolution profiles of amlodipine formulations in IDAS2 assays. (B) Permeation profiles of amlodipine formulations across C2BBe1 monolayers in IDAS2 assays.