Article

A Mid-Infrared Spectrophotometric Method for Simultaneous Quantification of Naltrexone and Bupropion with Multivariate Calibration

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Naltrexone (NTX) and bupropion (BUP) are used in combination in clinical practice for obesity; however, the existing analytical methods for this drug combination are not sustainable. This work aims to develop an analytical method that is faster, easier and less expensive compared with high performance liquid chromatography (HPLC) and involves green technology without solvents to quantify BUP and NTX in combination. The objective was to validate a method using mid-infrared (MIR) spectroscopy associated with multivariate calibration and chemometrics to simultaneously measure NTX and BUP in a pharmaceutical capsule form. The models were developed using MIR spectroscopy with diffuse reflectance, with interval partial least squares (iPLS) variable selection. The working range selected to optimize the model was from 1885.8 to 1585.4 cm^{-1}. The chosen model was obtained with partial least squares (PLS2) and with data pre-processed by first derivative Savitzky-Golay smoothing followed by mean centering, using four latent variables, providing a root mean square error of prediction of 1.8 mg g^{-1} for NTX and 6.42 mg g^{-1} for BUP. The method was validated according to current international standards. In conclusion, the methods developed in the MIR region provided statistically similar results to the validated chromatographic method for commercial pharmaceutical formulations.

Keywords: obesity, naltrexone, bupropion, mid-infrared, chemometrics

Introduction

Obesity leads to a large number of other chronic diseases and shortens life expectancy. According to the World Health Organization, about 13% of the world's adult population (11% of men and 15% of women) were obese in 2016.[1] Obesity and being overweight are linked to more deaths worldwide than being underweight.[1-3] The available medications to treat obesity often fail and produce serious adverse events. In this context, combinations of two or more drugs have been used in clinical practice; they produce additive or synergistic effects on weight reduction, since they may involve different mechanisms.[4] Recent studies have shown that the combination of naltrexone (NTX) and bupropion (BUP) is effective for weight loss in adults, and this association was approved by the Food and Drug Administration (FDA) in 2014 for the treatment of obesity.[5,6]

There is no pharmacopeial method for the simultaneous quantification of drugs (BUP and NTX) in pharmaceutical forms of industrialized or manipulated products. The majority of methods found in the literature use high performance liquid chromatography (HPLC). In terms of the sample preparation process, infrared (IR) spectroscopy is a faster, easier and less expensive option compared with HPLC. Moreover, chromatographic methods typically require complex pre-treatments, such as interferent removal and/or analyte extraction, which sometimes consume large amounts of organic solvents, engendering organic residues that may cause an environmental impact. This environmental problem is a concern for companies because of the costs associated with the elimination of toxic waste and pollution fines. Qualified professionals are required to conduct HPLC analysis, unlike infrared analysis, which is generally easy to handle. Another factor that often makes the use of these HPLC methods unfeasible in terms of quality control in compounding pharmacies is the high cost of equipment maintenance. IR spectroscopy associated with chemometric tools and multivariate calibration allows

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an investigator to analyze complex samples, such as drug combinations, enabling the use of more than one wavelength in the analysis. It is common to use certain regions of the spectrum, or even the whole spectrum, and this flexibility provides greater selectivity to the method.[7] According to the Brazilian Pharmacopoeia,[8] partial least square (PLS) regression is considered the gold standard chemometric model for drug quantification involving spectrophotometric analyzes (infrared and ultraviolet) because the PLS is a dimensionality reduction model where multivariate data (for example in the mid-infrared (MIR) spectrum, in the region between 4000-400 cm^{-1}, each wavenumber corresponds to a variable, totaling 3600 variables) are projected in a space of smaller coordinates called latent variables (LV), which are independent of each other. This is because PLS is a model where multidimensional and highly correlated (multicolinear) data are reduced to uncorrelated latent variables (LV). The absence of multicollinearity is a mandatory criterion that must be met in all linear regression models.[9-11] Another advantage is that the optimized PLS model can be reduced to a common multiple linear regression, allowing to predict the importance of each independent variable in predicting the concentration of the substance of interest.[12-14] Thus, the aim of this study was to propose a new mid-infrared and chemometric method for the simultaneous quantification of NTX and BUP.

Experimental

Chemicals and reagents

The BUP standard (99.5%) was obtained from Supriya Life Science Ltd. (Mumbai, India). The NTX standard (99.0%) was obtained from Cerilliant (Round Rock, TX, USA; FN 10301502). BUP (100.27%) was obtained from Gemini (Delhi, India) and NTX (98.31%) from Fagron (Milan, Italy). The structure of the drugs are shown in Figure 1. The excipients lactose, microcrystalline cellulose and hydroxypropyl methylcellulose (hypromellose or HPMC) were purchased from Fagron (Milan, Italy).

HPLC grade acetonitrile (ACN) was acquired from Tedia (Fairfield, CT, USA); HPLC grade triethylamine (99.9%), monobasic potassium phosphate (99%) and HPLC grade phosphoric acid (85%) were purchased from Vetec (Rio de Janeiro, Brazil). Commercial capsules with a declared value of 8 mg NTX and 90 mg BUP were obtained from a compounding pharmacy in Curitiba, Brazil. Powder samples were prepared by weighing with an analytical balance (± 0.0001 g), according to the experimental design.
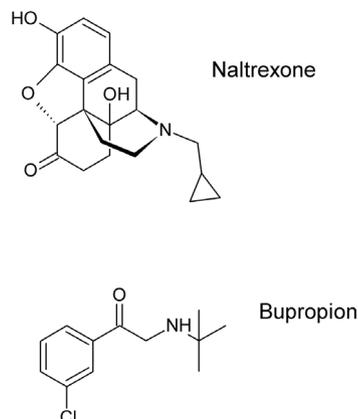

Figure 1. Structure of naltrexone and bupropion.

Apparatus and software and MIR method

The spectral dataset was obtained using a Bruker Alpha Fourier transform infrared (FTIR) spectrometer (Ettlingen, Germain) with a diffuse reflectance infrared Fourier transform (DRIFT) accessory, under controlled temperature (25.0 ± 0.2 °C) and humidity (45-55%). The spectrum was acquired in absorbance mode by using OPUS (version 6.0) for Windows from Brucker Optics (Billerica, MA, USA). The spectra were acquired in the spectral range of 4000-400 cm^{-1}, with 32 scans and a resolution of 4 cm^{-1}. Drugs and excipients were mixed manually, and the spectrum of each one was obtained. Replicate analyses were performed for six samples of the central point to prove repeatability. These samples were also evaluated on another day and by another analyst, and the results were compared to estimate the intermediate precision. A scan of 20 spectra from empty cells was obtained to evaluate the instrumental noise. Prior to the analysis of the samples, the background single channel was performed using the same method used for the analysis of the samples. Data were handled using MATLAB 7.0.1[15] and PLS Toolbox 3.0.[16] Experimental design data were analyzed using Statistica software version 10[17] and Microsoft Excel 2013.[18]

Experimental design

Central composite design (CCD) was used to optimize NTX and BUP concentrations.[19] The CCD[20] was established because it is the only one that allows performing sequential experiments: (*i*) initially, the complete factorial experiment was conducted to evaluate the main and interaction effects of the factors; (*ii*) after, new experiments adding the axial and central points were performed to determine the experimental optimal point, without losing the data of the complete factorial experiment previously performed. The relation between the response variable (the concentration

values of NTX and BUP) and the independent variables (NTX, BUP and excipients) is described by a second-order polynomial equation shown in the equation 1.

$$Y = \beta 0 + \sum_{i=1}^{n} \beta i X i + \left(\sum_{i=1}^{m} \beta i i X i \right)^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n=1} \beta i j X i X j \qquad (1)$$

where Y is the predicted response (dependent variable); $\beta 0$, βi, $\beta i j$ and $\beta i i$ are the model constant, linear, interaction and quadratic term, respectively; Xi and Xj, are the coded values of the experimental parameters. For each variable investigated was used a total of five levels (α, -1, 0, 1, $+\alpha$).

To define the five levels of CCD, was consider the amount of NTX (8 mg), BUP (90 mg) and excipients (152.0 mg) present in the capsule pharmaceutical form, purchased at a compounding pharmacy. The excipients were composed of a mixture of 65% lactose, 15% microcrystalline cellulose and 10% HPMC. Statistica software[17] was used to define the variation of compounds of each sample, interchanging the proportion of excipient, NTX and BUP randomly up to $\pm$ 20% of the standard concentration. Tables S1-S2 and Figure S1 (Supplementary Information (SI) section) show the matrices of the CCD experimental design and the theoretical results of the CCD design, respectively. The software generated 79 samples.

PLS models

The number of samples in the calibration and validation set was determined following the American Society for Testing and Materials (ASTM) guide E1655-05 12,[19] which recommends that the minimum number of samples in the calibration and validation set be determined using the formulas below:

Minimum number of calibration samples =
$$6 \times (\text{number of latent variables} + 1) \quad (2)$$

Minimum number of validation samples =
$$4 \times (\text{number of latent variables}) \quad (3)$$

As the PLS model was optimized with 4 latent variables, the minimum number of training and testing samples was 30 samples (obtained using 6×5) and 16 samples (obtained using 4×4), respectively. Thus, from the 79 samples, 58 samples were used for calibration and 21 samples were used for validation, according to ASTM guide E1655-05 12. The selection of samples from the calibration set and the validation set was performed using the Kennard-Stone algorithm.[21] The PLS calibration model was training using leave-one-out cross-validation.[22] The choice of latent

variable numbers was performed considering lower values of root mean square of calibration (RMSEC) and root mean square error of cross validation (RMSECV). After training the model, the root mean square error of prediction (RMSEP) values were calculated using the prediction dataset. The RMSEC, RMSECV and RMSEP values were calculated using the equation 4.[22,23]

$$RMSE = \sqrt[2]{\frac{\sum_{i=1}^{n} \left(Yi - \hat{Y}i \right)^2}{n}} \qquad (4)$$

where $\hat{Y}i$ and Yi are, respectively, the value predicted by the PLS model and the experimental value for samples i, and n is the sample size.

Accuracy

The accuracy of the PLS model was evaluated considering values of RMSEC, RMSECV and RMSEP, according to the equation 4.[24] The *F* test was used to compare the RMSEP values of models PLS1 and PLS2, in order to identify the best model.[25] Additionally, the evaluation of the prediction of both models was performed considering the bias values (equation 5).

$$Bias = \sum_{i=1}^{nv} \frac{\left(yi - \hat{y}i \right)}{n\upsilon} \qquad (5)$$

where yi: concentration values of reference samples; ŷi: concentration values of predicted samples; nυ: number of samples from the validation set.

Precision

The precision of the PLS model was evaluated following the ASTM E1655 standard,[19] predicting the PLS model calibrated at five concentration levels of the BUP and NTX samples. Six measurements were made for each concentration level. The standard deviation and the relative standard deviation (RSD) of these determinations were calculated according to equations 6 and 7.

$$\delta = \sqrt[2]{\frac{\sum_{j=1}^{ra} \left(Yaj - \hat{Y}a \right)^2}{ra - 1}} \qquad (6)$$

$$RSD = \frac{\sum_{a=1}^{a} \frac{\delta a}{\hat{Y}a} 100}{p} \qquad (7)$$

where ra, a and p are the number of replicates *per* level,

estimated mean value, and the number of evaluated concentration levels, respectively.

Intermediate precision

The intermediate precision (RSDpi) of the PLS model for both drugs (BUP and NTX) was evaluated through the standard deviation (SD) of the six replicates of the five concentration levels studied. The analyzes were performed on two consecutive days with two different analysts, using the equation 8.[26]

$$RSDpi = \sqrt{\frac{1}{t(ri-1)}\sum_{i=1}^{t}\sum_{k=1}^{rl}\left(Y_{ik} - \hat{Y}_i\right)^2} \qquad (8)$$

where, ri, t, Y_k are number of measurements *per* level, number of all samples tested and result of the value of k for the i sample, respectively.

Linearity

The linearity of the PLS model of both drugs was evaluated by the correlation between the experimental values and the predicted values of the different concentration levels. Additionally, the analysis of homoscedasticity and normality of residues *versus* reference values was evaluated.

Sensitivity

The sensitivity (SÊN) of the PLS model was calculated using the equation 9; b represents the PLS regression coefficient.[26,27]

$$S\hat{E}N = 1/(\|b_k\|) \qquad (9)$$

Analytical sensitivity

Analytical sensitivity (γ) is the ability of the method to reliably distinguish minimum concentrations, which is determined as the ratio between the slope of the analytical curve and the standard deviation of the analytical signal at a given concentration, taking into account the noise present in the response signals. Analytical sensitivity was calculated using the equation 10.[28,29]

$$\gamma = S\hat{E}N/(\|\delta x\|) \qquad (10)$$

where δx is the estimated experimental noise calculated through the six replicates of the BUP and NTX spectra.

Selectivity

The equation 11 was used to calculate the selectivity.[29,30]

$$S\hat{E}L_{k,I} = n\hat{a}sk,I/(\|x_{ki}\|) \qquad (11)$$

where, **k**, is the instrumental response vector for sample i, whereas nâsk,I is the scalar signal value of liquid analyte for sample i.

Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) values were similarly determined with the traditional univariate calibration method (equations 12 and 13).[30]

$$LOD = 3.3 \times (1/S\hat{E}N) \qquad (12)$$
$$LOQ = 10 \times (1/S\hat{E}N) \qquad (13)$$

Multivariate validation

Selection of excipients and experimental design

The excipient selection for the pharmaceutical formulation was based on the materials used in the industrialized product-Contrave®, which is composed of cysteine chloride, microcrystalline cellulose, hydroxipropilcelulose, magnesium stearate, anhydrous lactose, lactose monohydrate, crospovidone, ethylene-diaminetetraacetic acid (EDTA), colloidal silicon dioxide, carmine indigo aluminum lacquer, polyvinyl alcohol, titanium dioxide, macrogol and talc.[31-33] The proportion of each excipient was the same as that officially used by commercial pharmacies. Lactose is the major excipient, used as a filler and binder, followed by microcrystalline cellulose as a binder, diluent and lubricant, and HPMC as a controlled release agent.[34] Other excipients such as EDTA, silicon dioxide and magnesium stearate are included in very low proportions and would not influence the spectrum of the final mixture (see Figure S2, SI section). Mixtures of excipients were prepared according to an experimental design with three factors, with 5% variation around the expected values (lactose, 65%; microcrystalline cellulose, 20%; and HPMC, 15%).

The medium infrared (MIR) spectra of pure BUP and NTX powder samples are shown in Figure 2. There is similarity between the spectrum of the drugs and the mixture. Excipients are also highly influential on the spectrum, due to their percentage in the mixture.

This study employed a central compound planning experimental design, which defined the number and composition of the samples for the development of the
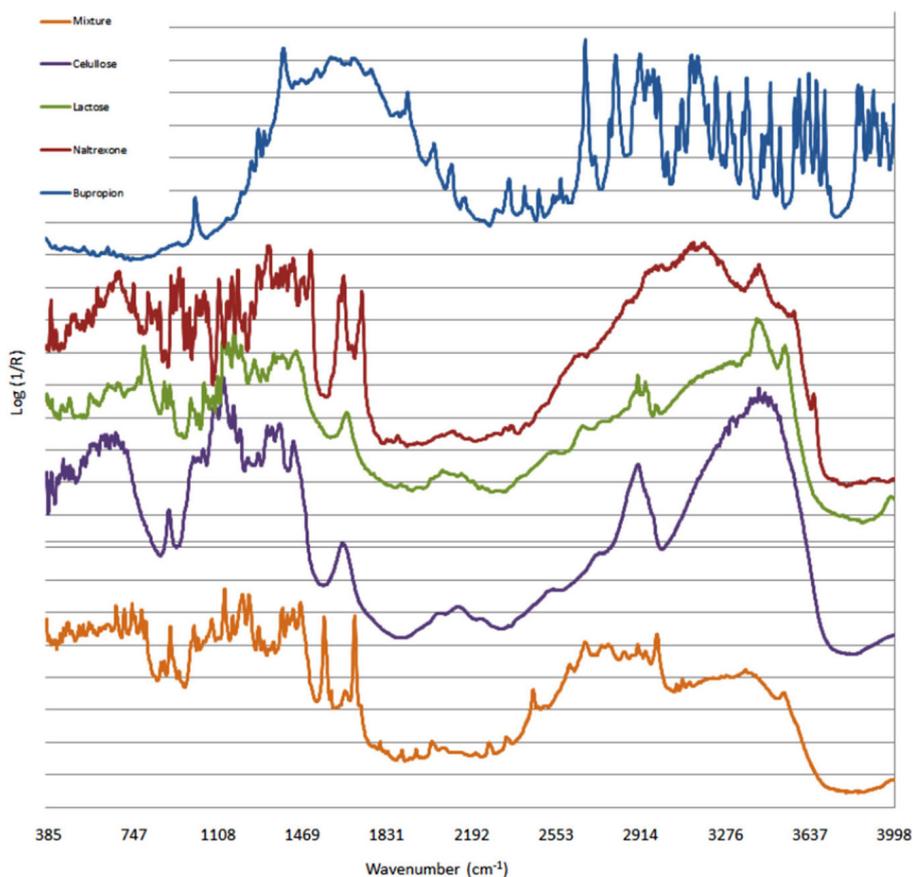
Figure 2. Medium infrared (MIR) spectra (obtained by the DRIFT technique) of drugs and mixture components.

model. Data were analyzed with MATLAB® software,[15] with the wavenumber and absorbance data listed in matrix **X** (composed of 2559 wavenumbers in the columns and 58 samples in the rows) and the reference concentration data for both analytes in the matrix **Y** (composed of 2559 wave numbers in the columns and 58 samples in the rows).

HPLC-diode array detector (DAD) comparison method

Instrumentation and conditions

In order to compare the results obtained from the MIR method with multivariate calibration, HPLC-DAD analysis was carried out with an Agilent 1100 HPLC system (Wilmington, NC, USA) that consisted of a quaternary G1311A pump, a G1379A degasser, a G1316A column thermostat, a G1329A autosampler manager and a G1315B DAD. The system employed ChemStation® software version A.10.02. Chromatographic separation was conducted using a C18 XBridge (250-4.6 mm, 5 μm) kept at 40 °C. The mobile phase was water with 0.1% triethylamine (pH 6.5 adjusted with orthophosphoric acid) (A) and ACN (B) in the following elution mode: 0.00-5.00 min 20-30% B; 5.00-12.00 min 30-80% B; 12.00-14.00 min 80-20% B; and 14.00-18.50 min 20% B.

The injection volume, wavelength and flow rate were 3 μL, 230 nm and 1 mL min^{-1}, respectively.

Standard stock solutions

BUP and NTX stock solutions were prepared by weighing 10 mg of each drug, which were placed in 10 mL volumetric flasks and the volumes supplemented with ACN, to obtain solutions with a concentration of 1 mg mL^{-1}. The working solution was 16.00 μg mL^{-1} NTX and 180.00 μg mL^{-1} BUP. The diluent was water:ACN (80:20 v/v).

HPLC-DAD method validation

The method was validated according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use.[35] The following parameters were tested: selectivity, linearity, precision, accuracy and robustness. Selectivity was evaluated by comparison of the fortified matrix with the matrix (without analytes). Linearity was measured using five concentrations for each drug, carried out in triplicate (4.80, 6.40, 8.00, 9.60 and 11.20 μg mL^{-1} for NTX and 54.00, 72.00, 90.00, 108.00 and 126.00 μg mL^{-1} for BUP). The calibration curve range

was 60-140% of the working concentration (center point). Precision was assessed by the repeatability and intermediate precision assay, while accuracy was determined by the standard addition method to a placebo. For the robustness test, system variations such as the mobile phase ratio, pH, column temperature, injection volume and different column batches were tested.

Quantification of real samples

The content of 20 capsules containing the combined drugs (8 mg NTX and 90 mg BUP) was homogenized, and ten aliquots of this mixture were weighed and analyzed by DRIFT on a MIR spectrometer. Spectra were obtained from each sample in triplicate. The drug content of the samples (NTX and BUP) was determined using the developed MIR models.

An aliquot of the same sample analyzed with MIR was used for HPLC analysis. Extraction was performed by transferring an amount of the homogeneous powder mixture, equivalent to the amount of NTX and BUP contained in a capsule, to a 100 mL volumetric flask with 50 mL of 0.1 M phosphoric acid solution. This system was mechanically agitated in a Glas-Col mixing apparatus for 30 min, and then the volume was adjusted to 100 mL and filtered through a 0.45 μm pore filter. The extracted solution had a theoretical concentration of 80 μg mL^{-1} of NTX and 900 μg mL^{-1} of BUP. The solution was diluted with 1:10 (v/v) with a water:ACN (80:20, v/v) diluent to obtain a solution at a theoretical concentration of 8 μg mL^{-1} NTX and 90 μg mL^{-1} BUP. Subsequently, the samples were analyzed by HPLC.

Results and Discussion

Figure 3 presents the BUP and NTX spectra; their interpretation helps to highlight the regions that may contribute most towards predictive models. The main bands for NTX are a broad stretching vibration at 2800-100 cm^{-1} (stretching of the OH bond of the carboxyl group) and a deformation vibration at 1200-1500 cm^{-1} (stretching of the C=C bond of the aromatic ring). Other important vibrations relate to stretching of the C=C bond of the benzenic ring; a stretching band at 1400-1600 cm^{-1} and at 1450-1460 cm^{-1} related to CH$_2$ bond scissor deformation; absorption regions at 1700-1800 cm^{-1} indicative of C=O bond stretching; absorption regions at 2800-3100 cm^{-1} representing C–H bond stretching; and absorption regions at 3200-3400 cm^{-1} for O–H bond stretching. There are also absorption regions at 1132 and 1217 cm^{-1} related to cyclopropane C–H and C–C scissor bends, respectively.[36]

The IR absorption spectra for BUP has a band at 1553 cm^{-1} related to an out-of-plane deformation of the amide N–H bond; a peak at 1687 cm^{-1} related to the ketone C=O bond stretching vibration; a peak at 902 cm^{-1} related to the R$_2$NH bond; a peak at 1235 cm^{-1} from C–Cl bond vibration; and a peak at 1457 cm^{-1} indicating C–C bond stretching of the aromatic ring.[37]

MIR multivariate models

Figure S3 (SI section) shows the MIR spectra obtained for 79 samples: plain or separated into validation (green) and calibration (blue). The validation samples cover the main weighing points that would be more likely to occur.
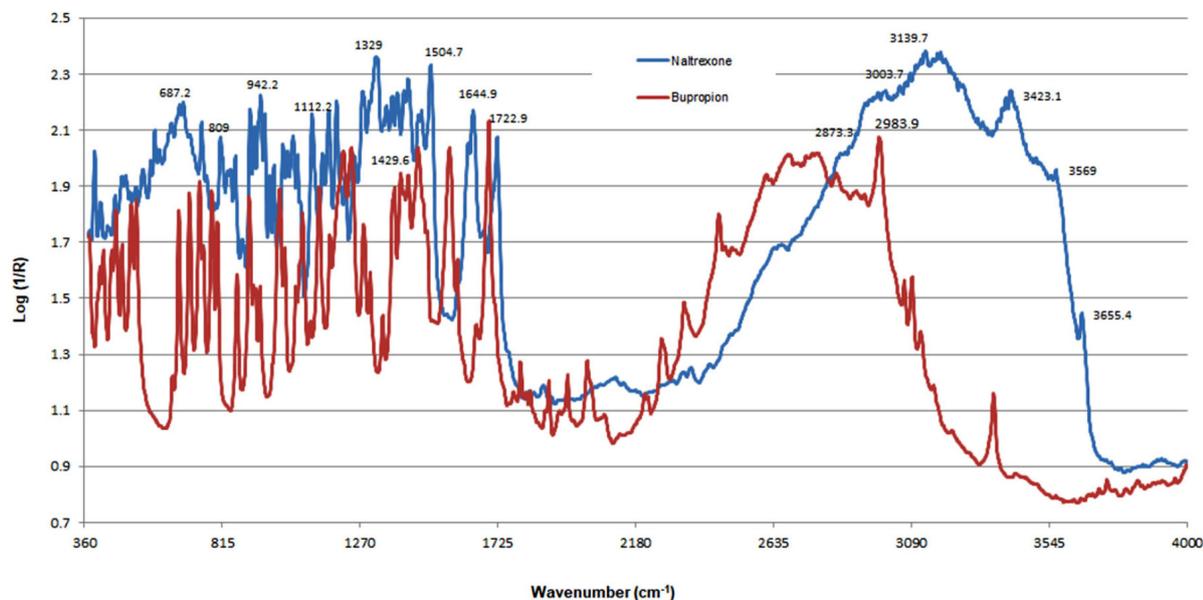

Figure 3. Infrared (obtained by the DRIFT technique) spectrum of bupropion and naltrexone.

Preliminary partial least squares (PLS) models were tested using PLS1 (one important variable *per* model) and PLS2 (two or more simultaneously important variables *per* model). PLS2 had better results for the root mean square error of calibration (RMSEC), root mean square error of calibration with cross validation (RMSECV) and root mean square error of prediction (RMSEP), as well as a lower error of prediction. The chosen model was obtained by pre-processing data from both drugs with first derivative Savitzky-Golay smoothing, followed by mean centering. The chosen spectral range was 1885.8-1585.4 cm^{-1} (Figure S4, SI section), which includes important absorption regions for both drugs. This spectral range was obtained using the iPLS as a variable selection method. In this method, the complete spectra were divided into equal intervals of 213 cm^{-1}.

The spectra for the mixture (drugs and excipients) and each isolated drug in the range selected by iPLS are represented in Figure 4.

In the selected range, NTX presents absorption regions related to the stretching of the benzene ring C=C bond at 1645 cm^{-1} and the stretching of the ketone C=O bond at 1705 cm^{-1}. There is a peak at 1722 cm^{-1} that corresponds to stretching of the carboxylic acid C=O bond, and there is absorption around 1600 cm^{-1}, which also represents stretching of the C=C bond of the aromatic ring.[37]

Figure 5a presents the NTX variable importance in projection (VIP) scores for this model. This factor demonstrates the influence and importance that was given to each wave number in the projection and confirmed the influence of the 1600, 1645, 1705 and 1722 cm^{-1} wavenumbers. BUP shows an absorption peak at 1696 cm^{-1} (stretching of the ketone C=O bond) and another at 1591 cm^{-1} (deformation outside the plane of the N−H amide bond).[18] The absorption at 1653 cm^{-1} refers to the stretching of the benzene ring C=C bond, which is also present in this compound. BUP shows a peak around 1800 cm^{-1} related to an aryl halide (C−Cl). Figure 5b shows the BUP VIP score for this model. This factor confirms the significant influence of the 1600, 1650, 1700 and 1800 cm^{-1} wavenumbers.
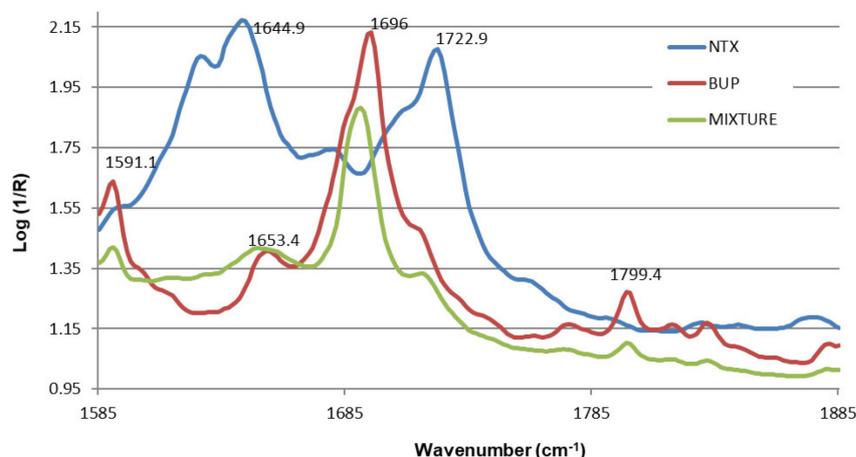

Figure 4. Medium infrared spectrum (obtained by the DRIFT technique) of drugs and mixture in the range selected by iPLS algorithm 213 cm^{-1}.
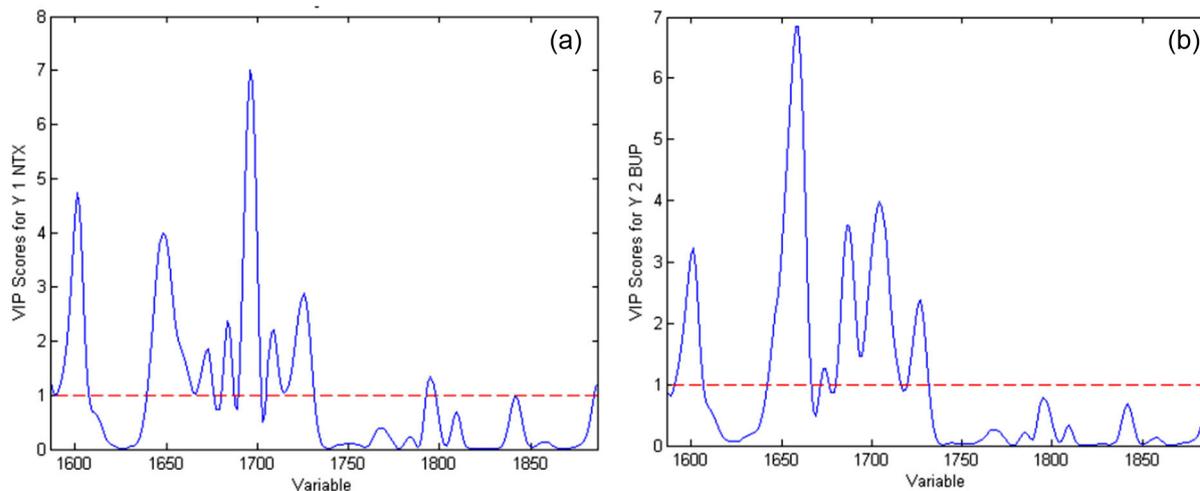

Figure 5. Variable importance in projection (VIP) of naltrexone (a) and bupropion (b).

Table 1. PLS model calibration and validation metrics for NTX and BUP. RMSEC, RMSECV, RMSEP, bias and R^2 values

Figure of merit	NTX / (mg g⁻¹)	BUP / (mg g⁻¹)
RMSEC	1.90	8.99
RMSECV	3.76	10.79
RMSEP	1.47	6.89
Calibration bias	0.00	-5.6843×10^{-14}
Prediction bias	0.40	0.65108
Cross validation bias	-0.62	0.44
R^2	0.71	0.8662

NTX: naltrexone; BUP: bupropion; mg g⁻¹: milligrams of drug *per* gram of the mixture; RMSEC: root mean square error of calibration; RMSECV: root mean square error of calibration with cross validation; RMSEP: root mean square error of prediction.

The PLS2 model explained 99.66% of the variance for block **X** and 90.20% for block **Y**. Table 1 presents the RMSEC, RMSECV and RMSEP values.

The values obtained were calculated relative to the milligrams of drug *per* gram of the total mixture. The RMSEP was lower than the RMSEC, an outcome that is justified mainly because the NTX quantity in the mixture is very low. Possibly the values of RMSECV are higher than RMSEP due to the type of cross validation used, the leave-one-out. This type of validation can overestimate the PLS model in situations where the sample size is greater than 20, which is the same situation in our work.[38,39]

RMSECV generally has larger error values than RMSEP because the RMSECV calculation is based on training data which in general has a larger sample size and greater variability, whereas RMSEP is calculated based on test samples, which in general the sample number is small. In this case, we can conclude that RMSECV and RMSEP values are considered satisfactory.

The LV number was defined based on the results of the graph in Figure S5 (SI section), which represents the RMSECV × LV of each analyte evaluated simultaneously. These data verified that the model does not improve with more than 4 × LV, because the RMSECV increases slightly and the RMSEC increases or does not decrease significantly. The percentage of agreement with the values of the axis did not increase significantly after 4 × LV; therefore, 4 × LV was selected.

Anomalous samples (outliers) were not identified for the drugs in association in the selected model, according to Student *versus* Leverage residues (Figures S6 and S7, SI section), because there were no values ± 2.5 relative to the Student's *t*-test residues and [3 × (LV)]/n, considering 95% confidence limits.

Application of the multivariate model

After the calibration and validation of the PLS model using the samples from the experimental design, the next step was to test the practical application of the PLS model in the quality control of these two drugs. For this, new samples were used consisting of 10 capsules (samples) containing the combination NTX 8 + 90 mg acquired in different commercial pharmacies, aiming to determine the concentration of NTX and BUP and also to determine the metrics of the analytical validation of the method. The multivariate parameters estimated for this validation are listed in Table 2. In general, the PLS method was validated and considered adequate and reliable for its application, because the NTX and BUP concentration values obtained were similar to the values of the quantifications of the same samples using the HPLC method, considered the gold standard method for NTX and BUP quantification (Table 3).

Once a model has been developed, analytical validation is essential for quality control purposes. The multivariate parameters estimated for this validation are listed in Table 2. New samples were submitted to the developed method. In general, the method was validated and considered adequate and reliable for its application.

Table 2. Estimated parameters for development and validation of MIR-PLS2 method

Figure of merit	Parameter	NTX	BUP
Precision / %	RSD (repeatability)	2.9-3.5	3.6-3.7
	RSD (intermediate)	3.2	4.5
Accuracy / (mg g⁻¹)	RMSEC	4.1	9.2
	RMSECV	4.6	10.9
	RMSEP	1.8	6.4
Interval / (mg g⁻¹)		23.1-41.8	296.1-417.3
Selectivity		0.098	0.1262
Analytical sensitivity (c)	γ (mg g⁻¹)	137.97	173.16
	γ (mg g⁻¹)	7.20×10^{-3}	5.8×10^{-3}
Robustness	–	$p > 0.05$	$p > 0.05$
LOD / (mg g⁻¹)	–	0.02	0.02
LOQ / (mg g⁻¹)	–	0.07	0.06

MIR-PLS2: medium infrared-interval partial least squares regression of two drugs; NTX: naltrexone; BUP: bupropion; RSD: relative standard deviation; RMSEC: root mean square error of calibration; RMSECV: root mean square error of calibration with cross validation; RMSEP: root mean square error of prediction; LOD: limit of detection; LOQ: limit of quantification; γ: analytical sentivity.

Table 3. Naltrexone and bupropion values obtained in commercial samples by HPLC-DAD and MIR-iPLS2 methods

	Medium concentration *per* capsule		
Drug	Labelled values / (mg g⁻¹ *per* capsule)	MIR-iPLS2	HPLC-DAD
NTX	8.0	6.38 ± 0.92	6.87 ± 0.09
BUP	90.0	90.12 ± 5.50	89.78 ± 3.03

NTX: naltrexone; BUP: bupropion; mg: milligrams of drug; MIR-iPLS2: medium infrared-interval partial least squares regression of two drugs; HPLC-DAD: high performance liquid chromatography with diode array detect.

Accuracy

The average accuracy was evaluated through RMSECV, RMSEC and, mainly, RMSEP. The estimated RMSEP for NTX and BUP were 1.8 and 6.4 mg g⁻¹, respectively. Accuracy can also be evaluated through the relative errors of prediction for each sample. The recovery for each drug was around 100%. The medium value of the relative prediction errors for the validation samples was 4% for NTX and 1.21% for BUP. The *p* values in the *t*-test of theoretical and practical concentrations for NTX and BUP (0.35 and 0.80, respectively) indicated no statistical significance; hence, we concluded that this method is accurate.

Precision

The relative standard deviation (RSD) for repeatability (intra-run precision) for NTX was 2.9-3.5%, while the intermediate precision (inter-run) was approximately 3.2%. For BUP, the RSD for repeatability (intra-run precision) was 3.6-3.7%, while the intermediate precision (inter-run) was approximately 4.5%. These values are consistent with the Brazilian regulations,[8] which prescribe a maximum RSD of 5%. The precision results further confirm that the method is accurate.

Linearity

Figures 6a and 6b show the linearity graph of the PLS2 model for both drugs. The coefficient of determination (R^2) values for NTX and BUP were respectively 0.71 and 0.86, showing satisfactory linearity.
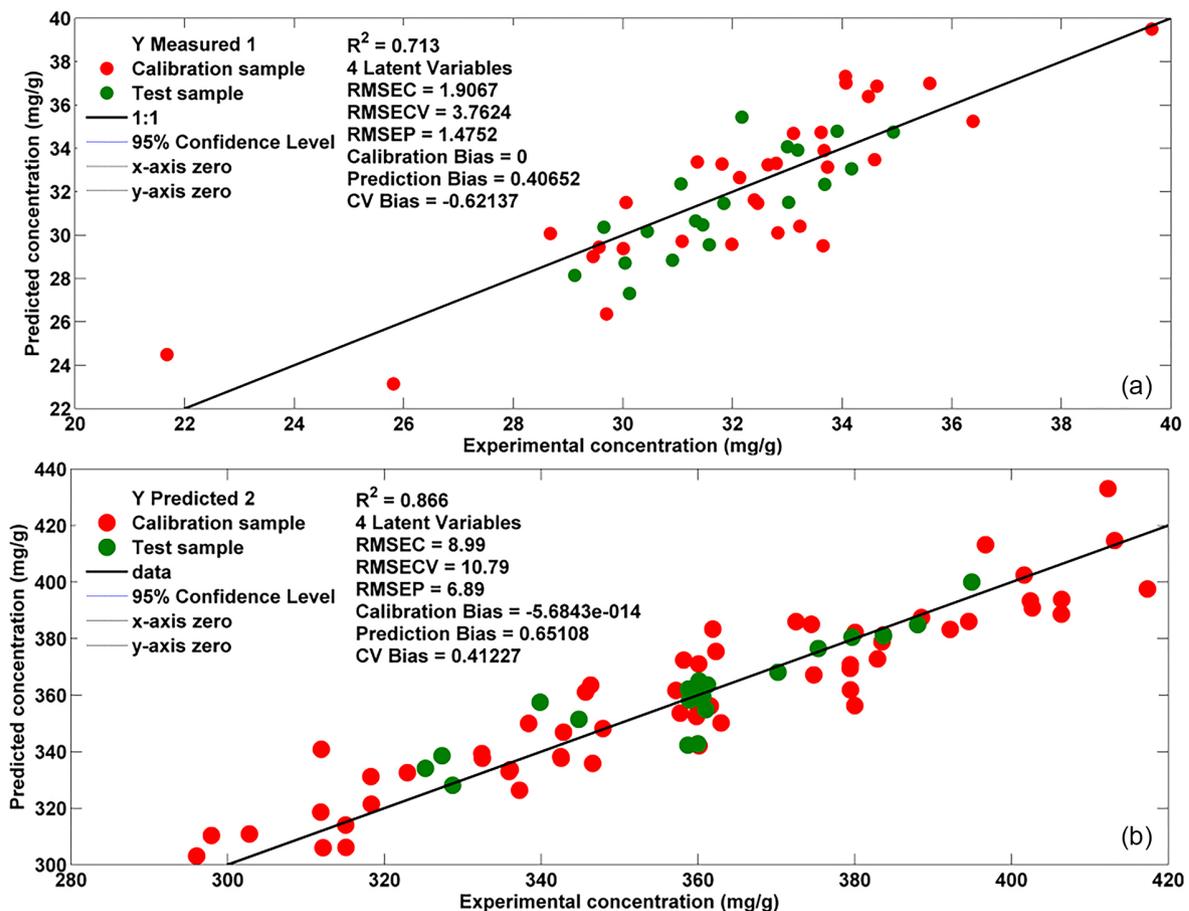

Figure 6. Linear analysis between experimental and predicted concentration of the PLS2 model for quantification of naltrexone (a) and bupropion (b). Outliers were removed from the analysis of the naltrexone model.

NTX's R^2 value was lower than that of BUP. It is important to highlight that according to the literature, infrared calibration models with $R^2 > 0.5$ are considered statistically significant for quantification, as can be seen in the study by Malley et al.,[40] where the infrared quantification of some analytes the values of R^2 were in the range between 0.54-0.69, and in the recent study by Arsego et al.[41] where the R^2 values were in the range between 0.6-0.8, which we can conclude that our results are consistent with the literature. On the other hand, it is important to highlight that a method of quantification of NTX and BUP by HPLC-DAD was developed and validated, which is the analytical method considered the gold standard for quantification of these drugs, concordant with those determined by the PLS method, as can be seen in Table 3 (HPLC data) and Table 2 (PLS model data).

Selectivity and analytical sensitivity

Selectivity is interpreted differently for multivariate models compared to univariate models, and it has no practical interest for quality control purposes. The selectivity definition is only useful within a certain group of samples of similar qualitative composition; it predicts the percentage of the spectrum that was used to determine each drug concentration. For the developed iPLS2-MIR method, an estimated 9.74% of the analytical signal was used to predict NTX, while 12.62% was used for BUP; these outcomes were expected because only one spectrum band was used.

Pure sensitivity cannot be compared with other methods; thus, their values were divided by the estimation of the instrumental noise ($e = 7.3 \times 10^{-4}$) and analytical sensitivity (c). The inverse of c shows the minimum drug concentration that the method was able to discriminate, considering the instrumental noise. The values obtained were satisfactory: 7.20×10^{-3} mg g^{-1} for NTX and 5.8×10^{-3} mg g^{-1} for BUP.

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

These parameters were calculated by software based on the instrumental noise, net analytical signal (NAS).[15,16] The obtained values were considered low (LOD, 0.02 mg g^{-1} for both drugs; LOQ, 0.07 mg g^{-1} for NTX and 0.06 mg g^{-1} for BUP). Of note, there is no method that is used to determine LOQ and LOD for multivariate methods.

Bias

The bias value calculated from the validation samples was zero or close to zero; these data demonstrate the absence of systematic errors in the model MIR-iPLS2.

HPLC-DAD comparison method

To evaluate the MIR results, we employed HPLC-DAD to quantify NTX and BUP. This chromatographic method was selective, because there were no interfering absorption regions in the retention time of the compounds of interest when the exempt matrix was analyzed. In addition, the method was considered linear (r > 0.99), accurate (RSD < 0.40 and recovery > 95%) and robust (RSD < 2%) for the tested conditions. Table S1 (SI section) presents the results of the chromatographic method validation.

Quantification of real samples

Commercial samples were analyzed using the validated HPLC-DAD and MIR-iPLS2 methods (Table 3).

These data demonstrated agreement between the quantification results for the two evaluated methods. According to Student's *t*-tests, there were no significant differences between the predictions of the methods developed for MIR-iPLS2 and HPLC. Hence, the MIR-iPLS2 method can be used to quantify the association of the drugs under study. The *p* values from Student's *t*-tests were 0.06 for NTX and 0.49 for BUP.

The value found for NTX was below that recommended by the European Medicines Agency (EMA)[32] for an isolated drug tablet, which establishes that the content of drugs must be between 90 and 110% of the stated value. This finding highlights the importance of drug quality control, including for manipulated drugs and manual processes, mainly for low dosages, and that the probability of errors is common. For a medicine that is present at a low dose, this issue is crucial. This low dosage can influence the treatment of the patient by reducing the drug's effectiveness.

Conclusions

We developed and validated a DRIFT MIR-iPLS2 spectroscopy method for the simultaneous determination of BUP and NTX in pharmaceutical capsules, according to international guidelines and considering the requirements for multivariate analytical validation. This method along chemometric treatment of the obtained data, proved to be selective, linear and accurate. The use of iPLS2 (fragmented spectrum) improved the model predictions, with the selected spectral range 1885.8-1585.4 cm^{-1}, pre-processing with first derivative Savitzky-Golay smoothing and mean centering, and $4 \times$ LV providing the best model. The method developed and validated by HPLC-DAD met the requirements of analytical validation: it is selective, linear, accurate and precise.

For real samples, the proposed DRIFT MIR-iPLS2 and HPLC-DAD models provided statistically equivalent results. Thus, MIR-iPLS2 with multivariate calibration is a viable alternative to analyze the association of NTX and BUP in a capsule pharmaceutical form, with the advantages of being fast, low cost, having no polluting organic solvents and no residue generation compared with the HPLC-DAD method. With easy access, it may be an option for industrial quality control laboratories and handling pharmacies to control the manufacturing process.

Supplementary Information

Supplementary information (Figures S1-S7 and Tables S1-S3) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

All authors of this article participated directly in the planning, execution and writing of this study. A. C. Novack contributed to the study concept, study design, data collection, data interpretation, development and validation of the economic model, and preparation, writing and editing of the manuscript; C. R. S. Madeira contributed to the study concept, study design, data collection, data interpretation, development of the economic model and manuscript review; T. M Guimarães contributed to the study concept, study design, data collection, data interpretation, development and validation of the economic model, and manuscript review; M. M. Fachi contributed to the study concept, study design, preparation, editing and manuscript review; A. F. Cobre editing and manuscript review; M. S. Piantavini contributed to the study concept, study design and manuscript review; R. Pontarolo contributed to the study concept and manuscript review.

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